TBC1D24-TLDc related epilepsy–exercise induced dystonia: rescue by antioxidants in a disease model

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Abstract

Genetic mutations in *TBC1D24* have been associated with multiple phenotypes, with epilepsy being the main clinical manifestation. TBC1D24 consists of the unique association of a Tre2/Bub2/Cdc16 (TBC) and a TBC/Lysin Motif Domain/Catalytic (TLDc) domain. Over than 50 missense and loss of function mutations have been described until now and are spread over the entire protein, but affected individuals always carried bi-allelic TBC domain variants or were compound heterozygous for one TBC and one TLDc domain mutation. Through whole genome/exome sequencing we identified bi-allelic point mutations within the TLDc domain in the index family with the Rolandic-epilepsy exercise induced dyskinesia (RE-EID) phenotype (http://omim.org/entry/608105). A twenty-year long clinical follow-up revealed that epilepsy was self-limited in all three affected patients but exercise-induced dystonia persisted into adulthood in two. We identified *TBC1D24* compound heterozygous mutations, c.1079G>A (p.R360H) and c.1501G>A (p.G501R), in the TLDc domain. We next characterized their functional consequences on *Drosophila's* homologous neuronal function and protein structure. TBC1D24/Skywalker regulates synaptic vesicle trafficking and in a *Drosophila* model neuronally expressing human TBC1D24, we demonstrated that the TBC1D24^{G501R} TLDc mutation causes activity-induced locomotion and synaptic vesicle trafficking defects. Solving the crystal structure of the conserved *Drosophila* TLDc domain predicted destabilizing effects of TBC1D24^{G501R} and to a lesser degree of TBC1D24^{R360H}. The neuronal phenotypes of the TBC1D24^{G501R} mutation are caused by exacerbated oxidative stress sensitivity, which is rescued by treating TLDc mutant animals with antioxidants N-acetylcysteine amide or α-tocopherol as indicated by restored synaptic vesicle trafficking levels and sustained behavioural activity. Our data show that mutations in the TLDc domain of TBC1D24 cause self-limited Rolandic epilepsy and non-remitting exercise-induced dystonia. Humanized TBC1D24^{G501R} *Drosophila* exhibits sustained-activity and vesicle transport defects. We propose that the TBC1D24/Sky TLDc domain is a ROS sensor mediating synaptic vesicle trafficking rates that, when dysfunctional, causes a movement disorder in patients and flies. The TLDc and TBC domain mutations' response to antioxidant treatment we observed in the animal model suggests a potential for combining antioxidant-based therapeutic approaches to TBC1D24-associated disorders with previously described lipid-altering strategies for TBC domain mutations.

Keywords

TBC1D24, rolandic epilepsy, exercise induced dystonia, oxidative stress

Introduction

Over the last several years, more than 50 mutations in *TBC1D24* have been associated with multiple phenotypes with drug-resistant epilepsy and DOORS syndrome being the most frequent (Balestrini *et al.*, 2016). More rare phenotypes include hearing loss, intellectual disability, cerebellar dysfunction, alternating hemiplegia and signs of neurodegeneration (Appavu *et al.*, 2016; Balestrini *et al.*, 2016; Ngoh *et al.*, 2017; Ragona *et al.*, 2017).

TBC1D24 consists of the unique association of a Tre2/Bub2/Cdc16 (TBC) and a TBC/Lysin Motif Domain/Catalytic (TLDc) domain. Missense and loss of function mutations are spread over the entire protein, but most affected individuals are bi-allelic mutant in the TBC domain or compound heterozygous for both the TBC and TLDc domains. Only two patients harboring bi-allelic missense mutations in the TLDc domain have been reported, both homozygous and exhibiting as different phenotypes as DOORS syndrome (Atli *et al.*, 2018) and progressive myoclonus epilepsy (Muona *et al.*, 2015).

TBC1D24 is well conserved across species, including its *Drosophila* counterpart Skywalker (Sky). The TBC domain regulates vesicular membrane trafficking at synapses by acting with Rab-GTPases, such as Arf6 and Rab35, and docks to phosphoinositides in the membrane (Falace *et al.*, 2010; Frasa *et al.*, 2012; Fischer *et al.*, 2016). The function of the TLDc domain is less known, though a function in oxidative stress sensing or resistance has been demonstrated in cell culture (Finelli *et al.*, 2016; Finelli and Oliver, 2017). Loss of TBC1D24/Sky causes synaptic vesicle-associated proteins to excessively traffic to endosomes, where they are sorted and sent to the lysosome for degradation (Uytterhoeven *et al.*, 2011; Fernandes *et al.*, 2014). Hence, TBC1D24/Sky controls protein homeostasis at synapses, and disrupting the function of TBC1D24/Sky or pathogenic mutations in the TBC domain result in severe synaptic defects and epileptic seizures in fruit flies (Fischer *et al.*, 2016) as well as neuronal architectural defects in rodent neurons (Falace *et al.*, 2010, 2014; Milh *et al.*, 2013).

Here we demonstrate that affected individuals of the original Rolandic epilepsy (RE)-writer's cramp-exercise induced dyskinesia (EID) pedigree (Guerrini *et al.*, 1999) (http://omim.org/entry/608105) harbor recessive missense mutations in the TBC1D24 TLDc domain. Through a two-decade follow up we also demonstrate that while epilepsy had a self-limited course, EID persisted into adulthood in two of the three patients. By solving the crystal structure of the TLDc domain we show how the mutations can destabilize the fold of this domain. Using *Drosophila* as a model, we found that the TLDc domain mediates reactive

oxygen species (ROS)-induced defects in synaptic vesicle trafficking. Indeed, TLDc pathogenic mutations cause a molecular phenotype comparable to that of high ROS load. Both cellular and behavioral defects of mutant animals are rescued by antioxidant feeding. We propose here that the TBC1D24/Sky TLDc domain is a direct or indirect ROS sensor mediating synaptic vesicle trafficking rates that, when dysfunctional, causes a movement disorder in patients and flies.

Materials and Methods

Patients

A brother and sister and their first cousin were products of consanguineous marriages between two brothers and two sisters (Fig 1A). They exhibited a syndrome with onset in infancy, featuring focal seizures, often hemifacial, centro-temporal EEG abnormalities, and paroxysmal dystonia precipitated by sustained exercise (Guerrini *et al.*, 1999). They also exhibited forearm dystonia that caused writing to progressively become scribbling and then made it impossible after a few minutes. This manifestation is more akin to EID involving forearm muscles than to classical writer's cramp in which dystonic posturing appears as soon as writing starts. These three patients, aged 11, 22 and 23 years at the time of our original description (Guerrini *et al.*, 1999) have been under clinical follow up for 20 further years.

WES and WGS analysis

All participants had signed an informed consent for research whole exome/genome sequencing (WES/WGS). The study was approved by the Pediatric Ethic Committee of the Tuscany Region in the context of the DESIRE project (EC, Seventh Framework Programme, grant agreement 602531).

WES was performed on selected patients (VI:3-6; VII:2-4) (Fig 1A) as previously described (Poirier *et al.*, 2013). Variants were annotated and filtered with the VarSeq tool (Golden Helix, MT, USA).

We analyzed variants affecting coding regions and essential splice sites and excluded all variants with frequencies higher than 2% in the Genome Aggregation Database (gnomAD). Variants with 5 or more homozygous/hemizygous individuals reported in gnomAD were filtered out. We selected the variants shared among the three affected patients (VII:2, VII:3 and VII:4) according to homozygous recessive and compound heterozygous models.

WGS was performed on patient VII-2 by Illumina HiSeq 3000 sequencing. Reads were aligned with *Novoalign* (http://www.novocraft.com/). We estimated the medium coverage of the read (12) using *SAMtools* (http://samtools.sourceforge.net/) and used the Seattle SNP Annotation tools (http://snp.gs.washington.edu/SeattleSeqAnnotation/) to annotate the SNPs. Validation of candidate variants and segregation testing in additional family individuals (VI:1-3; VI:7-9 and VII:1) were performed by Sanger sequencing.

Bioinformatic impact predictions

We evaluated mutations' pathogenicity for the two *TBC1D24* variants through *in silico* prediction using the dbNSFP database (v3.3a) (Liu *et al.*, 2016) and the scores obtained from Revel (Ioannidis *et al.*, 2016), M-CAP (Jagadeesh *et al.*, 2016) and Eigen (Ionita-Laza *et al.*, 2016), three different tools to evaluate the pathogenicity of rare variants.

To improve the information obtained by the variant-level CADD, Polyphen-2 and SIFT impact predictions, we also used the mutation significance cutoff (MSC) server (http://pec630.rockefeller.edu:8080/MSC/), a quantitative approach that provides gene-level and gene-specific phenotypic impact cutoff values.

Finally, a multiple sequence alignment of the *TBC1D24* orthologous protein sequences was generated by the Jalview software (http://www.jalview.org) with color-coding for physicochemical properties (Zappo color scheme).

Drosophila stocks and genetics

Fly stocks were maintained on standard medium at 21 °C and a 12-hour light/dark cycle. Larva or adult animals were selected for the correct genotype and age-matched flies of other conditions and genotypes were selected for testing. After assigning letters to each experimental group the order of testing was randomized. Neuronal human TBC1D24 was expressed in sky null mutation background (Uytterhoeven *et al.*, 2011). The ROS scavengers, antioxidants N-acetylcysteine amide (AD4, 40 µg/ml) (Jung *et al.*, 2017) or alpha-tocopherol (Vitamin E, 20 µg/ml) (Driver and Georgeou, 2003), were added to the food source in adults 48 hours prior to testing. To optimize solubility, alpha-tocopherol (VitE) was dissolved in water and 0.0005 final volume percent ethanol in two consecutive steps. AD4 was dissolved in pure water. For controls, solvent (H_2O) without antioxidants was added. Transgenic flies were generated (Fischer *et al.*, 2016) from gBlocks (IDT) of the human coding sequences (Supplementary Table 1). pUAST.attB-GFP::TBC1D24^{WT} under a 5xUAS-hsp70 promoter

and three pathogenic alleles were generated by site-directed mutagenesis: G501R and R360H as well as TBC domain mutation R40C (Supplementary Table 2) and verified by sequencing analysis. For crystal structure analysis, the DNA sequence coding for the TLDc domain of Skywalker (residues 401-587) was amplified from *Drosophila sky* (CG9339, isoform A), inserted into plasmid pet28-Sky401-587 and sequencing verified plasmid transformed into E. coli C41(DE3) (Novagen).

Behavior assays

Single-blinded behavior experiments were performed on third-instar larvae and adults. Animals were optically screened for correctly folded wings and healthy walking behavior. Male adults 2–5 d after enclosure were tested by flight and seizure assays (Fischer *et al.*, 2016), with seizure assays startling set to two one minute steps, with one minute rest. Activity scores for paralyzed, slowed and unaffected flies were measured and testing repeated after 5 minutes.

Negative geotaxis assays measured 5 repetitions of sustained climbing activity (Benzer, 1967) in 30 seconds steps, repeated after 5 minutes. Larval light-avoidance determined coordinated movement (Sawin-McCormack *et al.*, 1995).

Protein expression and purification

The DNA sequence coding for the TLDc domain of Skywalker (residues 401-587) was amplified from the *Drosophila sky* gene (CG9339, isoform A), and cloned into the pet28 plasmid (Novagen). Expression and purification of the His-tagged TLDc domain, using a HisTrap column and size-exclusion chromatography, were performed as previously described for the Sky TBC domain (Fischer *et al.*, 2016). Prior to the gel filtration step, the 6xHis tag was removed by overnight incubation with 1% mg/mg thrombin protease followed by a second passage over a HisTrap column to remove uncleaved protein. The protein was concentrated to 10 mg/ml in buffer A, flash-frozen in liquid nitrogen and stored at -80°C.

Crystallization and Structure determination

Crystals of the Sky401-587 protein were obtained at 4°C in 28% PEG 3350,0.1 M Hepes pH 7.5 and 0.6M ammonium citrate. Prior to freezing in liquid nitrogen, the crystals were transferred to cryoprotectant solution consisting of the mother liquor with 20% glycerol.

The Sky401-587 crystals diffracted to 2.05Å resolution. A complete dataset was collected at 100 K on the Proxima 2 beamline (SOLEIL synchrotron, Paris). Data processing and scaling was performed using XDS and XSCALE(Kabsch, 2010). The structure was solved by molecular replacement using the related zebrafish Oxr1 structure as a model (pdb 4ACJ) with Phaser (McCoy *et al.*, 2007). The model was then improved by iterative cycles of refinement with Phenix and manual building in Coot (Emsley *et al.*, 2010). MolProbity was used for structure validation (Chen *et al.*, 2007). X-ray data collection and refinement statistics are listed in Supplementary Table 3. All structural figures were produced with PyMOL (http://www.pymol.org/).

Protein folding free energy prediction

Calculation of the folding stability was performed using PoPMuSiC-2.0 (Dehouck *et al.*, 2009) and Site Directed Mutator (SDM) (Worth *et al.*, 2011).

Imaging

FM1-43 dye uptake - Neuromuscular FM1-43 (4μM)(Invitrogen) dye uptake at larval muscle 6/7 NMJs was performed as previously described (Fischer *et al.*, 2016) to quantify FM1-43 foci from 0.4μm optical slices in single-blinded analysis.

Transmission electron microscopy (TEM) - Third-instar larval NMJ were prepared for TEM and imaged as previously described (Fischer *et al.*, 2016). To analyze double-blinded synaptic bouton data, ultrastructural profile areas and synaptic-vesicle content were quantified in ImageJ as previously described (Fischer *et al.*, 2016).

In vivo fluorescence imaging - Third-instar larvae expressing GFP-tagged TBC1D24 were dissected in HL-3 on Sylgard plates, and the neuromuscular junctions of third-instar larvae was recorded in live-imaging with a Nikon A1R confocal microscope and a 60×, 1.0-NA water immersion lens in stacks of 0.5μm optical sections with standard GFP optics. Images are the maximum-intensity projections of three consecutive slices through the NMJ.

Peroxide stimulation

To induce oxidative stress at the *Drosophila* larval NMJ, we incubated dissected larvae in HL-3 physiological solution containing 50 mM H_2O_2 for 15 minutes (Slabbaert *et al.*, 2016). Before nerve cell stimulation, animals were washed with HL-3 and nerve cords were cut prior

to application of 90- or 60-mM stimulation solution for FM1-43 labeling or for TEM, respectively.

Statistical analysis

Gaussian distribution per genotype and condition was determined with D'Agostino-Pearson normality tests, following respective Welch's or Mann-Whitney t tests. Multiple comparisons with one-way ANOVA utilized Dunnett's or Kruskal-Wallis tests based on the outcome of normality tests. H_2O_2 stimulation effects were determined by two-way ANOVA for significant effects of either genotype, peroxide stimulation or both conditions. Error bars represent the Standard Error of the Mean.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary material. Raw sequencing data of this study are available from the corresponding author on request.

Results

Mutations in the TBC1D24 TLDc domain cause Rolandic epilepsy and exercise-induced dystonia

The three patients are now aged 31, 42 and 43 (Table 1). Focal motor seizures, which manifested infrequently since infancy, never relapsed under carbamazepine or oxcarbazepine treatment after ages 16, 18 and 22. Exercise-induced dystonia was instead still present at last follow-up in two patients, although attacks became much less frequent in adulthood, with both patients reporting they had learned how to limit fatigue or physical exercise by modulating their activities. All three patients still exhibited mild nystagmus and postural tremor of the hands and were treated with trihexyphenidyl as an anti-tremor drug. Treatment with carbidopa/L-dopa and acetazolamide failed to prevent dystonic attacks. Based on the rescuing effects of antioxidants in *Drosophila* we observed, we treated with ubidecarenone Patient VII:4 who reported no benefit and ceased assumption after two months. Brain MRI scan, repeated in adulthood, has remained normal in all three patients.

In our original description, we linked the disease locus to a large homozygous region on chromosome 16 (Guerrini *et al.*, 1999). Sequencing of the critical region (mean depth of 136) for identifying the causative gene under a recessive consanguineous model (multiple loops of consanguinity are present in the pedigree) and, therefore, looking for a consistent homozygous mutation common to the three individuals, failed (Supplementary Table 4). We finally extended the analysis to a recessive model including compound heterozygous mutations.

Only two genes, *ZNF717* and *TBC1D24*, qualified for the compound heterozygous model (Supplementary Table 5). Since the *ZNF717* gene is prone to misinterpretation of pathogenicity (Kwak *et al.*, 2017), the *TBC1D24* gene remained the only candidate, displaying both mutations within the TLDc domain (accession NM_001199107.1), c.1079G>A (p.R360H) and c.1501G>A (p.G501R)(Fig 1B). The c.1501G>A variant was not reported in the gnomAD database whereas the c.1079G>A variant was reported in gnomAD with a low frequency (4/267568 alleles) (Supplementary Table 6A). The two missense substitutions involved evolutionarily conserved residues in orthologues (Fig 1C) and were predicted to be damaging by MSC CADD, Polyphen-2 and SIFT corrected scores (Supplementary Table 6B).

Among the more than fifty patients carrying bi-allelic *TBC1D24* mutations reported so far (Balestrini *et al.*, 2016), only two have homozygous missense mutations in the TLDc domain (Muona *et al.*, 2015; Atli *et al.*, 2018). One patient, homozygous for p.R360L, exhibited progressive myoclonus epilepsy (PME) (Muona *et al.*, 2015). The other patient, homozygous for p.G428R, exhibited DOORS syndrome (Atli *et al.*, 2018). There are no previous reports of patients harboring compound heterozygous missense substitutions both residing within the TLDc domain.

TBC1D24 TLDc domain mutations do not affect protein localization in neurons

To start testing the functional effects of the R360H and G501R mutations in the TLDc domain *in vivo*, we generated fruit flies expressing wildtype and mutant GFP-tagged human TBC1D24 instead of the endogenous fly homolog *skywalker* (*sky*) (Uytterhoeven *et al.*, 2011). *Sky* null mutants die during development, but null mutants expressing wildtype human TBD1C24 in their neurons (nSyb-Gal4) survive. Similarly, *sky* null mutants that express pathogenic mutant GFP-tagged TBC1D24 also live, albeit with behavioral defects (see below). These data indicate that human TBC1D24 is functional in flies and can compensate for the loss of fly *sky*.

GFP-TBC1D24 expressed in neurons of *sky* null mutants is transported to synaptic terminals and localizes to presynaptic boutons of the neuromuscular junction; very little TBC1D24 localizes to the inter-bouton regions or axons, likewise to wildtype *Drosophila Sky* (Uytterhoeven *et al.*, 2011)(Fig 1D). Similarly, pathogenic mutant TBC1D24^{G501R} or TBC1D24^{R360H} concentrate at presynaptic boutons (Fig 1D and not shown). These data indicate that human TBC1D24 expressed in neurons preferentially localizes to synaptic regions, resembling endogenous wildtype fly Sky, and that the pathogenic G501R and R360H in TBC1D24 do not noticeably affect protein abundance or localization.

TLDc domain mutations induce sustained-activity locomotion defects but no seizures

Reported mutations in the TBC domain of TBC1D24 or Sky cause seizures in patients (Balestrini *et al.*, 2016) and in fruit flies (Fischer *et al.*, 2016). To determine if the TLDc mutations we identified cause seizures in fruit flies, we performed seizure assays with the respective genotypes. While *sky* null mutants that express TBC1D24 with mutations in the TBC domain show severe and long-lasting seizure-like activity (Fischer *et al.*, 2016), *sky* null mutants that express TBC1D24^{G501R} or TBC1D24^{R360H} do not and they are very similar to *sky* null mutants that express wildtype human TBC1D24 (Fig 2A). We quantified the activity of these flies by calculating an activity score based on the time it takes the flies to recover after the vortex stimulation. These data indicate that pathogenic mutations in the TLDc domain do not result in obvious seizure-like activity in fruit flies.

Next, we assessed other locomotion-based behavior. We tested sustained activity by assessing if flies sustain climbing activity (negative geotaxis) in five-step trials. We found that *sky* null mutants that express TBC1D24^{G501R} display a significant defect compared to controls (*sky* null mutants that express TBC1D24^{WT}; Fig 2B). S*ky* null mutants that express TBC1D24^{R360H} are very similar to controls, suggesting that the R360H mutation has less severe consequences than G501R (Fig 2B).

Finally, we tested coordinated movements based on larval light-avoidance and determined whether *sky* null mutant larvae that express wildtype or mutant TBC1D24 are able to sense and leave bright areas. We find that the preference indices (PI) to move to a non-lit area of animals expressing TBC1D24^{G501R} or TBC1D24^{R360H} are significantly lower than those of controls (Fig 2C). Our data suggest that when expressed in *sky* null fruit flies, TBC1D24

TLDc mutants cause sustained or coordinated movement defects, and that G501R causes more severe defects than R360H.

The crystal structure of the TLDc domain (Sky 401-587) predicts an effect of the mutations on protein stability

To understand how R360H and G501R might affect TLDc domain integrity, we solved the X-ray crystal structure. The TLDc domain is well conserved across species and the *Drosophila* Sky TLDc domain ($Sky_{401-587}$) was crystallized, however we have not been able to crystallize the human TLDc domain. $Sky_{401-587}$ crystallizes in the $P4_32_12$ space group with two (monomeric) protein molecules in the asymmetric unit and crystals diffracts at 2.05Å. The structure was solved by molecular replacement using Oxr1 from zebrafish as a model (pdb 4ACJ, (Blaise *et al.*, 2012)). Clear electron density is present for most of the proteins except for the residues 401-402 and 517-520 for monomer A; and residues 401-412, 485-489 and 517-519 for monomer B, which are not modelled. The overall fold of $Sky_{401-587}$ is similar to Oxr1 with the protein adopting an α+β fold constituted of 5 α-helices and 11 β-strands (Fig 3A). However, $Sky_{401-587}$ contains two long and flexible loops with additional amino-acids (residues 474-498 and 511-528) when compared to Oxr1. Next, we mapped the pathogenic mutations on the TLDc structure: human G501 corresponds to fly A531 and human R360 corresponds to fly R425 (Fig 3B-C). A531 is buried in the protein and a A531R/G501R mutation would cause steric hindrance and predicts a loss of protein domain stability (Fig 3B). This is supported by calculation of thermal stability using two different predictors (Fig 3D).
The R425H/R360H mutation would disrupt the formation of a surface-exposed salt bridge (Fig 3C) and calculation of thermal stability also suggests an impact of the R425H / R360H mutation on protein stability, although less pronounced than for the A531R/G501R mutation (Fig 3D). These results are consistent with our behavioral studies that indicated more severe phenotypes associated with expression of the G501R mutant. It should be noted that the loss of protein domain stability does not affect the localization of the mutant proteins at synapses (Fig 1D), indicating the defects we observe are not the result of lower protein expression.

Synaptic trafficking defects in TBC1D24-TLDc mutants

Sky/TBC1D24 acts via Rab35-dependent synaptic vesicle transport (Uytterhoeven *et al.*, 2011; Falace *et al.*, 2014; Sheehan and Waites, 2017) and controls protein homeostasis, as *sky* loss promotes protein trafficking to recycling endosomes where these synaptic vesicle

proteins are sorted for degradation (Uytterhoeven *et al.*, 2011). To assess synaptic vesicle trafficking efficiency in *sky* null mutant animals that express TBC1D24^{G501R}, we used FM1-43. FM1-43 is a lipophilic dye that upon nerve stimulation is internalized in newly formed synaptic vesicles and allows us to follow the fate of newly endocytosed vesicles at synaptic terminals. In controls, FM1-43 uniformly labels the synaptic vesicle pool that organizes in a typical doughnut-like shape (Estes *et al.*, 1996), but in *sky* null mutants that express TBC1D24^{G501R}, we find FM1-43 to frequently concentrate in puncta that we previously demonstrated to have endosome identity (Uytterhoeven *et al.*, 2011) (Fig 4A-B, arrowheads). This phenotype is very similar to the vesicle cycling defect we observed in *sky* null mutants that express an inactivating pathogenic TBC domain mutation (TBC1D24^{R40C})(Fig 4A-B) (Fischer *et al.*, 2016). In both controls and mutants, FM1-43 is effectively internalized, indicating endocytosis of synaptic membrane is not affected (Fig 4C). These results indicate that the TLDc mutation causes a loss of Sky function, resulting in synaptic vesicle trafficking defects.

Synaptic trafficking defects in TBC1D24-TLDc mutants are caused by ROS

The TLDc domain of TBC1D24 has been implicated in sensing and/or conferring protection to reactive oxygen species (Finelli *et al.*, 2016). We therefore assessed if ROS affects Sky localization and Sky-dependent synaptic vesicle trafficking. We first exposed synapses that express wildtype and mutant GFP-tagged TBC1D24 to 50mM H_2O_2 for 15 min but did not observe an effect of ROS on Sky protein abundance or localization (Fig 1D, Supplementary Table 7).

Next, we determined the fate of internalized FM1-43 following H_2O_2 exposure. While untreated control synapses (*sky* mutants that express TBC1D24^{WT}) show very few accumulations of FM1-43, H_2O_2-treatment causes a 1.5-fold increase in the number of FM1-43 accumulations following stimulation (Fig 4A,D). This indicates that ROS acutely affects coordinated vesicle trafficking, consistent with previous studies (Wang and Floor, 1998; Afuwape *et al.*, 2017). Next, we tested the effect of pathogenic mutations. The number of FM1-43-labeled accumulations in H_2O_2-treated *sky* mutants with a mutation in the TBC domain (TBC1D24^{R40C}) is also increased 1.5-fold (Fig 4A,D). In contrast, we find that the number of FM1-43 accumulations in H_2O_2-treated *sky* mutants that express the TLDc domain mutation TBC1D24^{G501R} is not increased (Fig 4A,D). This is similar to the effect of H_2O_2 in *sky* null mutants that also do not show an increase in FM1-43 accumulations (Fig 4E). These

results suggest that the vesicle trafficking defect induced by H_2O_2 acts through a functional TLDc domain.

We further investigated the defects in synaptic vesicle trafficking by transmission electron microscopy (TEM) of synaptic boutons (Fig 5A-E). Notably, large cisternal area with a minimum diameter of 80nm is increased in *sky* mutant boutons that express TBC1D24^{G501R}. This is consistent with previous observations in *sky* loss of function mutants that show an increased number of large cisternae (Uytterhoeven *et al.*, 2011; Fischer *et al.*, 2016). This result parallels the increased number of FM1-43 accumulations that we observed in animals expressing TBC1D24^{G501R} (Fig 5A, red arrows). We also treated synapses with H_2O_2 before TEM, resulting in dramatically increased cisternae in control synapses; however, we did not observe such increase in cisternae in H_2O_2-treated *sky* mutants that express TBC1D24^{G501R} (Fig 5B,C). Examination of ultrastructural features are comparable between control synapses (*sky* mutants that express TBC1D24^{WT}) and *sky* mutants that express TBC1D24^{G501R}, including the number of active zones and mitochondria (Fig 5D,E). These data further confirm that ROS-induced vesicle trafficking defects require a functional Sky TLDc domain. Our data are consistent with a model in which pathogenic TLDc domain mutants cause a similar negative effect on TBC1D24 function as high ROS load.

Vitamin E and AD4 rescue defects induced by TLDc mutations

There are two possibilities that explain our results: (1) the TLDc mutations impair TLDc function with a consequent TBC-dependent block of vesicle trafficking under control of TBC1D24; (2) the mutations cause hypersensitivity to ROS with endogenous ROS levels being high enough to inactivate the mutant TLDc domain, again inhibiting TBC1D24-dependent vesicle trafficking. To distinguish between these possibilities, we treated *sky* mutants that express TBC1D24^{G501R} with N-acetylcysteine amide (AD4) or with α-tocopherol (Vitamin E). This caused a significant rescue of the vesicle trafficking defects as gauged by FM1-43 (Fig 6A,B). In addition, AD4 or α-tocopherol also significantly rescued the activity-dependent movement phenotype we observed in *sky* mutants that express TBC1D24^{G501R} (Fig 6C). It should be noted that neither AD4 nor α-tocopherol increase the activity of TBC1D24^{WT} controls. Feeding with AD4 reduced seizure rates in TBC domain mutant flies as indicated by increased activity scores (Fig 6D). These results are consistent with a model where the pathogenic mutations that destabilize the TLDc domain of TBC1D24 (Fig 3D) cause hypersensitivity to ROS (Fig 6).

Discussion

A twenty-year long follow-up of the original RE-EID family we described in 1998 showed that while epilepsy subsided, with patients experiencing 15 to 22-years seizure freedom, EID attacks persisted in two of them. Through sequencing we identified these three patients as the first carriers of compound heterozygous missense mutations within the TLDc domain of TBC1D24 and studied the role of this domain *in vivo*. ROS affects synaptic function (Wang and Floor, 1998; Kaneai *et al.*, 2012) and we show here that the TLDc domain of TBC1D24 is an important ROS sensor that regulates synaptic vesicle trafficking by controlling TBC1D24 function. Based on the solved crystal structure of the TLDc domain and on functional data, we propose that the RE-EID associated mutations in the TLDc domain impact on protein domain stability, hence rendering the protein hypersensitive to physiological level of ROS. We also show that treating animals carrying the RE-EID mutations with antioxidant compounds rescues both clinical (the movement dysfunction) and cellular phenotypes (the synaptic vesicle trafficking defect). Taken together, our work suggests an important role for ROS-load in TBC1D24 pathology.

TLDc domains are found in a variety of proteins and are suggested to function in oxidative stress protection (Finelli *et al.*, 2016). How the TLDc domain senses ROS is not known, but stability of the domain seems to be important, because the targeted mutations in the evolutionarily conserved amino acids involved in ROS protection, as well as the pathogenic mutations we studied here, both destabilize the domain. We surmise that when the TLDc domain is destabilized, ROS gains access more easily to critical regions of the protein domain. In this scenario, destabilizing mutations would be more sensitive to ROS to the degree that endogenous ROS levels may already suffice to affect the TLDc domain. This is consistent with our observations because (1) the effects of the TBC1D24^{G501R} mutant are rescued by antioxidants; (2) the vesicle trafficking defect of TBC1D24^{G501R} is not worsened when synapses are exposed to additional ROS; (3) defects caused by mutations in the TBC domain that are not expected to destabilize the TLDc domain are significantly exacerbated in the presence of ROS.

Skywalker/TBC1D24 regulates synaptic vesicle trafficking, a function encompassed by the TBC domain that interacts with small Rab-GTPases (Frasa *et al.*, 2012). Indeed, pathogenic mutations located in the TBC domain cause synaptic vesicle trafficking defects in fruit flies (Fischer *et al.*, 2016). We show here that mutations in the TLDc domain of human TBC1D24 also result in synaptic vesicle trafficking defects in fruit flies, suggesting a functional

interaction between TLDc and TBC domains. Our attempts to generate full length TBC1D24/Skywalker crystals have not yet been successful. Hence, we do not know the nature of possible physical interactions between TLDc and TBC, but several lines of evidence are pointing to a tight cooperation between the two domains. Indeed, mutations in the TLDc domain of another protein, Oxr1, result in severe loss-of-function of the full-length isoform suggesting that mutating the TLDc domain was sufficient to affect the rest of the protein (Finelli and Oliver, 2017).

Our work suggests a model where a major regulator of synaptic vesicle trafficking, TBC1D24/Sky, is regulated by ROS levels. High ROS load causes damage to biomolecules, temporary neuronal hyperactivity and neuronal loss (Wang and Floor, 1998; Aguiar *et al.*, 2012; Kaneai *et al.*, 2012), but, as we show here, it also targets the TLDc domain of TBC1D24/Sky, causing the protein to become inactive. Our previous work shows that inactive TBC1D24/Sky results in increased trafficking of vesicles to endosomes where dysfunctional proteins are sorted for degradation (Uytterhoeven *et al.*, 2011; Fernandes *et al.*, 2014; Sheehan *et al.*, 2016). The TLDc domain's role as a ROS sensor in TBC1D24/Sky is particularly relevant at synapses. These compartments are often maintained far from the cell body and need to cope with dysfunctional proteins and lipids autonomously from the neuronal cell body (Vijayan and Verstreken, 2017). We propose that the TLDc domain senses high synaptic ROS levels and inactivates the entire TBC1D24, thus promoting vesicle protein trafficking to, and sorting at, endosomes and finally maintaining synaptic protein homeostasis.

Mutations in *TBC1D24* cause a spectrum of phenotypes, with epilepsy being a major manifestation, yet varying in type and severity. Our observation adds another class of phenotypes that encompass an epilepsy-EID syndrome and is caused by compound heterozygous missense mutations within the TLDc domain. In patients with TBC domain mutations, EID has not been reported and epilepsy is usually severe and not self-limited.

In most sporadic patients and dominant families with EID, causative mutations affect the *PRRT2* gene and benign infantile seizures are also observed (Marini *et al.*, 2012). PRRT2 (OMIM*614386) is a proline-rich transmembrane protein. Although its function remains relatively unknown, yeast two-hybrid studies suggest that PRRT2 interacts with synaptosomal-associated protein 25kD (SNAP25) (Stelzl *et al.*, 2005). PRRT2 knockout primary cultures and acute hippocampal slices show slowdown of the kinetics of exocytosis in excitatory neurons with weakened spontaneous and evoked synaptic transmission, while inhibitory neurons show strengthening of basal synaptic transmission and faster depression (Valente *et al.*, 2018).

Exercise-induced dystonia is also observed in patients with mutations of *SLC2A1* (or *GLUT1*) (OMIM*138140), a gene coding for the glucose-transporter-type-1, which assures the energy-independent, facilitative transport of glucose into the brain (Thorens and Mueckler, 2010). A similar phenotype is rarely observed also in carriers of mutations causing early-onset Parkinsonism (such as *PARKIN*, OMIM*602544) or dopa-responsive dystonia (such as *GCH1*, OMIM*602544) (Silveira-Moriyama *et al.*, 2018). The clinical context of the latter conditions, however, does not require differential diagnosis with the *TBC1D24* phenotype we are describing.

Sky mutant fruit flies that express human pathogenic TLDc mutations show motor defects but no apparent seizures, consistent with the idea that "TLDc only" mutations may be at one extreme of the TBC1D24 phenotypic spectrum. However, epilepsy was mild and only expressed in a narrow age range in RE-EID patients and mild seizure-like attacks in flies might have escaped recognition. Descriptions of additional patients carrying "TLDc only" mutations will clarify this notion. We observed vesicle trafficking defects both in flies expressing TBC mutations and in those expressing TLDc mutations; the difference between them being that synapses of flies expressing "TLDc only" mutations lost the ROS-sensitivity to regulate TBC1D24-dependent vesicle trafficking, which might underpin the observed phenotype differences.

We describe a new genetic cause of EID, associated with mild age-related epilepsy, likewise observed with *PRRT2* mutations. We previously reported on the effect of TBC mutations on the molecular structure of TBC domain: a unique positively charged pocket that binds the lipid PI(4,5)P2, or other 5-phosphorylated phospholipids, decreases its affinity for the lipid as a consequence of human mutations (Fischer *et al.*, 2016). This is resolved by pharmacologically enhancing PI(4,5)P2 levels that rescues the clinical defects of the mutant animal model (Fischer *et al.*, 2016). Now we add another tile to a possible therapeutic strategy for TBC1D24-associated diseases that could benefit by combining agents that increase synaptic PI(4,5)P2 levels, targeting TBC dysfunction, together with antioxidants, which target TLDc mutant hypersensitivity to ROS.

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Competing Interests

The authors report no competing interests.

Table 1: Summary of clinical features in the RE-EID patients.

	Patient VII:2	Patient VII:3	Patient VII:4
Age at seizure onset / outset (yrs)	1y / 22y	4y / 16y	3y / 22y
Seizure type	Focal motor, generalized tonic-clonic	Focal motor	Focal motor
Age at onset of exercise induced dystonia (yrs)	3y	4y	2y
Age at follow-up / last brain MRI scan in adulthood	43y / normal	42y / normal	31y / normal
Long term exercise induced dystonia outcome	Last attack at 8y	Still present	Still present
Additional manifestations	Nystagmus, postural hand tremor	Nystagmus, postural hand tremor	Nystagmus, postural hand tremor

Supplementary Material

Supplementary Table 1. DNA oligomers utilized in the construction of TBC1D24 wildtype and G501R plasmids.

eGFP[Linker] gblock

AACTCTGAATAGGGAATTGGGTCTAGAcaccATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCAT
CCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACC
TACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCAC
CCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCA
TGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGT
GAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATC
CTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCA
TCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAA
CACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAA
GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGA
CGAGCTGTACAAAGGTGGGGGTACCGGAGGCGGATCCGATTCCCCGGGATACAATTGC

TBC1D24 WT (Part1)

GATTCCCCGGGATACAATTGCTTCGTTGATAAAGATAAAATGGATGCCGCGATCCAGGATTTGGGACCCAAAGAG
CTCTCCTGTACTGAATTGCAGGAATTGAAACAGTTGGCTCGGCAGGGCTATTGGGCTCAGAGTCATGCATTGCGG
GGCAAGGTGTATCAGCGGCTGATTCGCGATATTCCATGCAGGACTGTCACGCCGGACGCCTCCGTGTACAGCGAT
ATAGTGGGTAAGATCGTTGGAAAGCACTCGTCCTCCTGCCTGCCCCTGCCTGAGTTCGTTGATAACACACAAGTG
CCATCGTATTGCCTGAACGCAAGGGGAGAAGGAGCTGTCAGGAAAATCCTGCTGTGCTTGGCAAATCAGTTTCC
CGATATAAGtTTTTGTCCGGCTCTGCCGGCGGTAGTTGCACTGTTGTTGCATTACTCCATTGACGAAGCTGAATGT
TTCGAGAAAGCCTGCCGGATTCTGGCTTGTAACGACCCGGGTCGTCGTCTGATCGATCAATCGTTCCTGGCTTTC
GAGAGTTCGTGCATGACTTTCGGAGATTTGGTGAATAAGTACTGTCAGGCGGCGCACAAGCTCATGGTCGCCGT
CTCCGAAGATGTTCTCCAAGTGTACGCCGACTGGCAAAGGTGGCTCTTTGGCGAGCTGCCCTTGTGCTATTTCGC
TCGCGTTTTTGATGTCTTTTTGGTTGAAGGCTACAAGGTACTGTATAGGGTAGCATTGGCAATTTTGAAATTCTTT
CATAAGGTGAGGGCAGGCCAACCATTGGAAAGCGATAGTGTAAAACAGGATATCCGAACATTCGTCCGTGATATC
GCGAAGACGGTTAGTCCGGAAAAATTGC

TBC1D24 WT (Part2)

CGGTTAGTCCGGAAAAATTGCTCGAgAAGGCGTTTGCCATTCGGTTGTTCTCGCGAAAAGAGATACAGCTGTTGC
AGATGGCCAACGAAAAAGCGTTGAAACAGAAAGGAATTACGGTAAAACAGAAGAGtGTTAGCTTGAGCAAACG
GCAGTTTGTACATCTGGCGGTCCATGCAGAGAACTTCCGAAGTGAAATAGTCAGCGTGCGGGAGATGCGCGACA
TCTGGTCGTGGGTTCCGGAACGTTTTGCGCTCTGTCAACCACTCTTGCTCTTTAGTAGCCTCCAGCATGGCTATTC
GCTCGCTAGGTTTTATTTTCAATGCGAAGGCCACGAGCCTACGTTGCTCTTGATAAAGACAACCCAAAAGGAAG
TGTGCGGTGCATATCTCTCCACGGACTGGTCGGAACGTAATAAATTCGGCGGAAAGCTCGGATTTTTCGGAACTG
GCGAATGTTTCGTGTTTCGCCTCCAGCCTGAAGTACAACGTTATGAATGGGTCGTGATTAAACACCCCGAACTCA
CGAAGCCACCACCCCTGATGGCTGCCGAACCTACGGCTCCACTGTCGCATAGTGCGTCCTCCGACCCGGCTGAT
CGCTTGAGCCCTTTCTTGGCAGCACGTCATTTCAACCTGCCGTCGAAAACAGAATCCATGTTCATGGCGGGTGGT
AGTGATTGCCTCATTGTTGGCGGAGGAGGTGGTCAGGCTCTCTACATCGACGGCGACCTGAACCGAGGAAGGA
CAAGTCATTGTGATACTTTTAATAACCAACCGCTGTGCAGCGAAAATTTCCTGATAGCTGCGGTCGAGGCGTGGG
GTTTTCAGGACCCAGACACGCAATAGAATTCAAGCTTAGGATCTTTGTGAAGGAACCTT

TBC1D24 G501R

CGGTTAGTCCGGAAAAATTGCTCGAgAAGGCGTTTGCCATTCGGTTGTTCTCGCGAAAAGAGATACAGCTGTTGC
AGATGGCCAACGAAAAAGCGTTGAAACAGAAAGGAATTACGGTAAAACAGAAGAGtGTTAGCTTGAGCAAACG
GCAGTTTGTACATCTGGCGGTCCATGCAGAGAACTTCCGAAGTGAAATAGTCAGCGTGCGGGAGATGCGCGACA
TCTGGTCGTGGGTTCCGGAACGTTTTGCGCTCTGTCAACCACTCTTGCTCTTTAGTAGCCTCCAGCATGGCTATTC
GCTCGCTAGGTTTTATTTTCAATGCGAAGGCCACGAGCCTACGTTGCTCTTGATAAAGACAACCCAAAAGGAAG
TGTGCGGTGCATATCTCTCCACGGACTGGTCGGAACGTAATAAATTCGGCGGAAAGCTCGGATTTTTCGGAACTG
GCGAATGTTTCGTGTTTCGCCTCCAGCCTGAAGTACAACGTTATGAATGGGTCGTGATTAAACACCCCGAACTCA
CGAAGCCACCACCCCTGATGGCTGCCGAACCTACGGCTCCACTGTCGCATAGTGCGTCCTCCGACCCGGCTGAT
CGCTTGAGCCCTTTCTTGGCAGCACGTCATTTCAACCTGCCGTCGAAAACAGAATCCATGTTCATGGCGcGTGGT
AGTGATTGCCTCATTGTTGGCGGAGGAGGTGGTCAGGCTCTCTACATCGACGGCGACCTGAACCGAGGAAGGA
CAAGTCATTGTGATACTTTTAATAACCAACCGCTGTGCAGCGAAAATTTCCTGATAGCTGCGGTCGAGGCGTGGG
GTTTTCAGGACCCAGACACGCAATAGAATTCAAGCTTAGGATCTTTGTGAA

Supplementary Table 2. Primer sequences

FW1	GCTGTACAAAGGTGGGGGTACCGGAGGCGGATCCG
RC1 R360H	ACAGAGCGCAAAAtGTTCCGGAACCCACGAC
FW2 R360H	GTTCCGGAACaTTTTGCGCTCTGTCAACCAC
RC2	TTCACAAAGATCCTAAGCTTGAATTCTATTGCGTGTCTG
FW1 R40C	ACTCTGAATAGGGAATTGGGTCTAGAcaccATGGTGAGCAAGGGCG
RC1 R40C	CAATAGCCCTGgCaAGCCAACTGTTTCAATTC
FW2 R40C	GTTGGCTtGcCAGGGCTATTGGGCTCAGAG
RC2 R40C	TTCACAAAGATCCTAAGCTTGAATTCTATTGCGTGTCTGG

Supplementary Table 3. Crystallization, Data Collection and Refinement Statistics

	Sky401-587
Data statistics	
X-ray source	Proxima 2, SOLEIL
Wavelength (Å)	0.9789
Space group	$P4_3\ 2_1\ 2$
Unit Cell parameters (Å, °)	a = b = 89.48 c = 135.07 α = β = γ = 90.00
Resolution range (Å)	42.38-2.05 (2.17-2.05)
Observed reflections	557217 (87770)
Unique reflections	35238 (5519)
Completeness (%)	99.8 (99.2)
R_{merge} (I)	0.098 (1.455)
Redundancy	15.8 (15.9)
Mean *I/σ(I)*	19.2 (1.9)
Refinement statistics	
R_{work}/R_{free} [a]	0.1948/0.2250
No of protein atoms	2833
No of water	119
B factor (Å^2)	48.9
Rmsd bond (Å)	0.008
Rmsd angle (°)	0.867
Ramachandran favored/outlier (%)	95.3/0.0

Values for the highest-resolution shell are given in parentheses. [a]5% of the reflections are used for the R_{free} calculation.

Supplementary Table 4. Homozygous recessive model data. No shared homozygous recessive variants were observed. Homozygous variants identified in each affected patients are shown.

Patient VII:2

Chr:Pos	Ref/Alt	Identifier	Gene Names	c.DNA effect	Protein effect
1:46489482	G/A	rs200134348	MAST2	NM_001324320.1:c.1631G>A	NP_001311249.1:p.Arg544His
8:52320832	G/C	rs145542518	PXDNL	NM_144651.4:c.3352C>G	NP_653252.3:p.Leu1118Val
14:23790681	-/GGC		BCL2L2-PABPN1,PABPN1	NM_001199864.1:c.433-691_433-689dupGGC,NM_004643.3:c.21_23dupGGC	,NP_004634.1:p.Ala11_Gly12insAla
19:1056109	C/T	rs145232000	ABCA7	NM_019112.3:c.4283C>T	NP_061985.2:p.Ser1428Leu
19:13883034	G/A	rs139306023	MRI1	NM_001031727.3:c.1049G>A	NP_001026897.1:p.Arg350Gln
19:15590167	G/A		PGLYRP2	NM_052890.3:c.16C>T	NP_443122.3:p.Leu6Phe
X:16857989	C/T		TXLNG	NM_018360.2:c.1198C>T	NP_060830.2:p.Arg400Cys
X:122551245	T/A		GRIA3	NM_000828.4:c.1501-8T>A	

Patient VII:3

Chr:Pos	Ref/Alt	Identifier	Gene Names	c.DNA effect	Protein effect
3:42982763	G/A		KRBOX1	NM_001205272.1:c.82G>A	NP_001192201.1:p.Glu28Lys
9:79938036	C/T	rs149694033	VPS13A	NM_033305.2:c.5884C>T	NP_150648.2:p.Arg1962Cys
12:2981314	T/C		FOXM1	NM_202002.2:c.602A>G	NP_973731.1:p.Lys201Arg
20:57768655	G/A	rs61743779	ZNF831	NM_178457.2:c.2581G>A	NP_848552.1:p.Asp861Asn

Patient VII:4

Chr:Pos	Ref/Alt	Identifier	Gene Names	c.DNA effect	Protein effect
2:32476455	C/T	rs113631419	NLRC4	NM_001199138.1:c.478G>A	NP_001186067.1:p.Ala160Thr
9:79898258	T/-		VPS13A	NM_033305.2:c.3119-5delT	
12:2981314	T/C		FOXM1	NM_202002.2:c.602A>G	NP_973731.1:p.Lys201Arg
X:9935597	C/-		CLDN34	NM_001195081.1:c.201delC	NP_001182010.1:p.Thr68Glnfs
X:16857989	C/T		TXLNG	NM_018360.2:c.1198C>T	NP_060830.2:p.Arg400Cys

Chr: chromosome; Pos: position on hg19 human genome build.

Supplementary Table 5. Compound heterozygous model data. Shared variants between the three affected patients are shown.

Patient VII:2 - Patient VII:3 - Patient VII:4 shared variants

Chr:Pos	Ref/Alt	Identifier	Gene Names	c.DNA effect	Protein effect
3:75786765	-/T	rs139062880	ZNF717	NM_001128223.2:c.2009_2010insA	NP_001121695.1:p.Lys672Glufs
3:75790446	G/T	rs79202307	ZNF717	NM_001128223.2:c.258C>A	NP_001121695.1:p.Thr86=
3:75790447	G/A	rs76526276	ZNF717	NM_001128223.2:c.257C>T	NP_001121695.1:p.Thr86Ile
16:2548334	G/A		TBC1D24	NM_001199107.1:c.1079G>A	NP_001186036.1:p.Arg360His
16:2550467	G/A		TBC1D24	NM_001199107.1:c.1501G>A	NP_001186036.1:p.Gly501Arg

Chr: chromosome; Pos: position on hg19 human genome build.

Supplementary Table 6A-B. TBC1D24 mutation nomenclatures, alleles frequencies and *in silico* impact predictions. A) Variant data B) Damage prediction by Mutation Significance Cutoff 99% Confidence Interval.

A) Variant data

Variant	Chr	Start	End	Ref	Alt			
TBC1D24:NM_001199107:exon4:c.1079G>A:p.Arg360His	16	2548334	2548334	G	A			
TBC1D24:NM_001199107:exon7:c.1501G>A:p.Gly501Arg	16	2550467	2550467	G	A			
Func.refGene	Gene.refGene	GeneDetail.refGene	ExonicFunc.refGene	genomicSuperDups	1000g2015aug_all			
exonic	TBC1D24	.	nonsynonymous SNV	.	.			
exonic	TBC1D24	.	nonsynonymous SNV	.	.			
ExAC_ALL	ExAC_AFR	ExAC_AMR	ExAC_EAS	ExAC_FIN	ExAC_NFE	ExAC_OTH	ExAC_SAS	
1.18E-05	0	0	0	0	0	0	7.99E-05	
.	.	.	.	.	.	.	.	
ExAC_ALL2	ExAC_AFR3	ExAC_AMR4	ExAC_EAS5	ExAC_FIN6	ExAC_NFE7	ExAC_OTH8	ExAC_SAS9	
1.53E-05	0	0	0	0	0	0	8.00E-05	
.	.	.	.	.	.	.	.	
gnomAD_exome_ALL	gnomAD_exome_AFR	gnomAD_exome_AMR	gnomAD_exome_ASJ	gnomAD_exome_EAS	gnomAD_exome_FIN	gnomAD_exome_NFE	gnomAD_exome_OTH	gnomAD_exome_SAS
1.27E-05	0	0	0	0	0	1.87E-05	0	3.39E-05
.	.	.	.	.	.	.	.	.
gnomAD_genome_ALL	gnomAD_genome_AFR	gnomAD_genome_AMR	gnomAD_genome_ASJ	gnomAD_genome_EAS	gnomAD_genome_FIN	gnomAD_genome_NFE	gnomAD_genome_OTH	
3.23E-05	0	0	0	0	0	6.67E-05	0	
.	.	.	.	.	.	.	.	
snp138NonFlagged	snp138	avsnp142	avsnp144	clinvar_20150629	SIFT_score	SIFT_pred		
.	.	.	rs765965968	.	0.002	D		
.	.	.	.	.	0.002	D		
Polyphen2_HDIV_score	Polyphen2_HDIV_pred	Polyphen2_HVAR_score	Polyphen2_HVAR_pred	LRT_score	LRT_pred	MutationTaster_score	MutationTaster_pred	
1	D	1	D	0	D	1	D	
1	D	0.999	D	0	D	1	D	
MutationAssessor_score	MutationAssessor_pred	FATHMM_score	FATHMM_pred	PROVEAN_score	PROVEAN_pred	VEST3_score	CADD_raw	CADD_phred
2.91	M	1.42	T	-4.8	D	0.872	6.524	31.0
3.62	H	1	T	-3.59	D	0.954	5.313	25.8
DANN_score	fathmm-MKL_coding_score	fathmm-MKL_coding_pred	GenoCanyon_score	integrated_fitCons_score	integrated_confidence_value	GERP++_RS	phyloP100way_vertebrate	
1	0.988	D	1	0.707	0	5.6	9.934	
0.999	0.997	D	1	0.651	0	5.49	9.51	
phyloP20way_mammalian	phastCons100way_vertebrate	phastCons20way_mammalian	SiPhy_29way_logOdds	Interpro_domain	Interpro_domain10	REVEL	MCAP	Eigen
1.041	1	0.88	18.2	TLDc domain	TLDc domain	0.78	0.1067	0.9255
1.041	1	0.096	18.32	TLDc domain	TLDc domain	0.497	0.1063	0.8787

B) Damage prediction by Mutation Significance Cutoff (MSC) 99% Confidence Interval

Variant	Chromome	Position	ID	Reference_Allele	Alternative_Allele	Gene
TBC1D24:NM_001199107:exon4:c.1079G>A:p.Arg360His	16	2548334	.	G	A	TBC1D24

TBC1D24:NM_001199107:exon 7:c.1501G>A:p.Gly501Arg	16	2550467	.	G	A	TBC1D24

CADD_Score	MSC-CADD_Score	MSC-CADD_Impact_Pred	PolyPhen2_Score	PolyPhen2_Pred	MSC-PolyPhen2_Score	MSC-PolyPhen2_Impact_Pred
31.0	0.002	high	1.000	probably damaging	0.000	high
25.8	0.002	high	0.999	probably damaging	0.000	high

SIFT_Score	SIFT_Pred	MSC-SIFT_Score	MSC-SIFT_Impact_Pred
0.000	D	0.454	high
0.010	D	0.454	high

See Liu et al,. Hum. Mutat. 2016;37:235–241, http://wannovar.wglab.org/ and http://pec630.rockefeller.edu:8080/MSC/ for abbreviations.

Supplementary Table 7. GFP-TBC1D24 Distribution and -Intensity in larval NMJ boutons. Two-way ANOVA of untreated vs peroxide-treated TBC1D24^{WT} vs TBC1D24^{G501R} GFP levels: genotype, ns F(1,55)= 0.4739, p= 0.4941); peroxide, (ns F(1,55)= 0.0363, p= 0.8496); two-way ANOVA of localization as intraboutonicintrabouton coefficient of variance: genotype, ns F(1,55)= 0.0535, p= 0.8180); peroxide, ns F(1,55)= 1.323, p= 0.2551

TBC1D24 Distribution				**TBC1D24 GFP levels**			
WT		**G501R**		**WT**		**G501R**	
Control	**H_2O_2**	**Control**	**H_2O_2**	**Control**	**H_2O_2**	**Control**	**H_2O_2**
0,163967	0,093931	0,054615	0,115731	1616,272	1120,187	719,8249	2345,179
0,135598	0,094485	0,071788	0,199079	1919,501	1327,946	728,3919	2261,625
0,108951	0,096278	0,072198	0,108709	1612,243	1344,808	950,3633	1891,605
0,111424	0,122056	0,084169	0,117689	1648,065	1415,167	963,8909	2193,254
0,100763	0,12964	0,084925	0,20064	2046,061	1454,82	1004,041	2023,287
0,083781	0,138083	0,08614	0,171363	1982,643	1515,332	1021,249	916,746
0,172331	0,141662	0,09876	0,113888	1296,597	1646,494	1046,305	440,8673
0,137678	0,158462	0,09891	0,202441	1328,745	1675,955	1056,252	443,5401
0,122319	0,160333	0,100387	0,178336	1413,71	1677,034	1062,262	465,1995
0,132859	0,165563	0,106709		1195,114	1886,651	1063,707	
	0,167859	0,111894			1942,387	1146,898	
	0,169411	0,113983			2086,212	1351,398	
	0,172133	0,123523			2096,22	1442,092	
	0,214694	0,1308			2118,923	1532,923	
	0,332756	0,132987			2251,865	1577,517	
	0,338495	0,144977			2359,177	1591,405	
		0,156749				1655,252	
		0,159187				2295,706	
		0,160598				2497,523	
		0,181106				2835,608	
		0,221298				2938,707	
		0,275141				2998,008	
		0,298305				3044,016	
		0,504271				3131,89	

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Figure legends

Figure 1: Patients

Sequencing identified a novel pathogenic human TBC1D24 TLDc domain compound heterozygous variant, TBC1D24G^{501R} / TBC1D24R^{360H} in three patients. The phenotype of epilepsy and paroxysmal exercise-induced dystonia (with writer's cramp) was autosomal recessive for all patients. (A) Family pedigree. * = whole exome sequencing performed; *§ = whole exome and genome sequencing performed (B) *TBC1D24* gene and protein structure depicting the location of the two missense substitutions. (C) Multiple sequence alignment between human TBC1D24 and orthologous sequences. Residues were colored according to their physico-chemical properties (Zappo color scheme). Residues affected by the two missense substitutions are indicated. (D) Human GFP::TBC1D24 protein distribution following expression at the *Drosophila* NMJ. Synaptic distribution of the fusion protein compared to antibody-labeled CSP at third-instar NMJ of TBC1D24^{WT} and TBC1D24^{G501R} animals indicates that TBC1D24, like Skywalker, is localized predominantly in the periphery of synaptic boutons. Scale bar: 5 µm.

Figure 2: Behavior

Patient mutations cause activity-related defects in adult and larval *Drosophila* model. (A) Neuronal overexpression of the human TBC1D24^{R360H} or TBC1D24^{G501R} mutations on *sky$^{1/2}$* deficiency background. Adult fly seizure assays indicated no difference in the activity scores of either mutation in comparison to wild-type *w^{1118}* and to wild-type TBC1D24 controls. (B) In contrast, sustained activity of TBC1D24^{G501R} animals is significantly impaired compared to TBC1D24^{WT}. n= 30-60 animals in 2 technical replicates (negative geotaxis), unpaired t test TBC1D24^{WT} vs TBC1D24^{G501R}: * p= 0.032, ns: not significant. TBC1D24^{G501R} third-instar larvae exhibit deficits in coordinated movement. (C) In TBC1D24^{G501R} animals, the light-avoidance reaction is absent in comparison to TBC1D24^{WT} and TBC1D24^{R360H}. Adult assays, tested in two technical replicates per group of 10 animals. Dunnett's test (seizures): *w^{1118}* vs TBC1D24^{WT} ns p= 0.1180, vs TBC1D24^{G501R}: ns p= 0.1603, vs TBC1D24^{R360H}: ns p= 0.8450. n= 30-80 animals. Dunnett's test (geotaxis): *w^{1118}* vs TBC1D24^{WT} ns p= 0.0810, vs TBC1D24^{G501R}: *** p= 0.0005, vs TBC1D24^{R360H}: ns p= 0.9979. n= 30-60 animals. Larval assays, tested in two technical replicates per group of 10 animals: Dunnett's test: *w^{1118}* vs TBC1D24^{WT} ns p= 0.0838, vs TBC1D24^{G501R}: **** p= 0.0001, vs TBC1D24^{R360H}: ** p= 0.0042. n= 30-40 animals.

Figure 3: X-ray Structure

Crystal structure of the TLDc domain of the fly TBC1D24 orthologue (Sky) and mapping of the residues affected by pathological mutations. (A) Cartoon representation of the TLDc domain (residues 413 to 587) of the fly TBC1D24 orthologue. β–strands, α-helices and loops are colored magenta, cyan and pink respectively. A flexible loop region (residues 517 to 520) that could not be modeled in the structure is indicated by a dotted curved line. Residues R425 and A531, corresponding to R360 and G501 in huTBC1D24, are shown as sticks with carbon atoms colored green. (B) Zoom in on the region around A531. A531 is completely buried. Distances to closest neighbors are indicated by orange dotted lines. (C) Zoom in on the region around R425. R425 makes a salt bridge with E497 (yellow dotted lines). (D) Prediction of the effect of the mutations R425H and A531R (corresponding to R360H and G501R in huTBC1D24) on the stability of the Sky TLDc domain using the predictors SDM and PoPMuSiC-2.0. In this table, negative values of ΔΔG indicate a destabilization: the larger the destabilizing effect the more negative the ΔΔG value.

Figure 4: FM1-43

The TBC1D24^{G501R} mutation or oxidative stress increase endocytic vesicle cycling, indicated by accumulations of FM1-43 dye inside the NMJ. These accumulations occur significantly less often in TBC1D24^{WT} controls. (A,B) Stimulated TBC1D24^{G501R} NMJ harbor an average of 0.03 puncta/μm^2 compared to a density of approximately 0.01 puncta/μm^2 in TBC1D24^{WT} NMJ. (B) Bar graph of synaptic vesicle accumulation density. (C) Endocytic vesicle uptake rates are unchanged by TLDc mutations. FM1-43 uptake measured as MFI of dye retention in synaptic boutons showed comparable uptake rates for mutant and wildtype TBC1D24 variants. Dunnett's test, TBC1D24^{WT} vs TBC1D24^{R360H}: ns p=0.9682, vs TBC1D24^{G501R}: ns p= 0.8347. n= 3-9 animals. (D) Oxidative stress at the NMJ increases the FM1-43 accumulation density in stimulated boutons of wildtype and TBC domain mutant animals. The R40C mutation in another domain, the TBC domain, results in accumulation numbers comparable to those in TBC1D24^{G501R} in absence of oxidative stress. ROS induced via application of 50mM H_2O_2 increased the density of endocytosed accumulations in TBC1D24^{WT} and TBC domain mutation TBC1D24^{R40C} 1.6-fold, whereas in the TLDc mutation TBC1D24^{G501R} accumulation density was unchanged by ROS, indicating that the TLDc domain mutation makes TBC1D24 insensitive to oxidative stress. One-way ANOVA

Kruskal-Wallis test for HL-3-treated TBC1D24^{WT} vs TBC1D24^{R40C}, TBC1D24^{G501R}: ** p= 0.0071, ** p= 0.0011. Two-way ANOVA: F(2, 109)= 3.93, * p= 0.0225. Sidak's multiple comparison test for HL-3 vs H_2O_2: TBC1D24^{WT} * p= 0.0285, TBC1D24^{R40C} * p= 0.0157, TBC1D24^{G501R} ns p= 0.9066. (E) The density of endocytic vesicle accumulations in *sky*$^{2/d0}$ mutants without overexpression of human TBC1D24 protein is very high even compared to a patient mutation background. These mutants however are not significantly affected by peroxide stimulation. Student's t test: ns p= 0.3135. n= 3-5 animals.

Figure 5: TEM

Transmission electron microscopy of stimulated synaptic boutons treated with control buffer or peroxide. (A) TBC1D24^{G501R} overexpression on *sky*$^{1/2}$ deficiency background results in high levels of synaptic vesicles larger than 80 nm. Red arrows point at enlarged synaptic vesicles (SV). Following H_2O_2 treatment delivering oxidative stress to the synapse, stimulated boutons contain a larger fraction of SV in TBC1D24^{WT} compared to the non-treated condition. (B) In contrast, TLDc domain mutant TBC1D24^{G501R} animals show no significant peroxide-induced change in large SV areas, while a strong increase is detected in TBC1D24^{WT} following peroxide treatment. (C) Quantification of large SV density in peroxide-treated versus non-treated condition. One-way ANOVA, Dunnett's test (cisternal/bouton area): TBC1D24^{WT} vs TBC1D24^{G501R}, TBC1D24^{WT} +H_2O_2, TBC1D24^{G501R} +H_2O_2: * p= 0.0441, * p= 0.0124, * p= 0.0284. Two-way ANOVA, F(1, 40)= 4.306, * p= 0.0445. Sidak's multiple comparison test for HL-3 vs H2O2: TBC1D24WT ** p= 0.0090, TBC1D24^{G501R} ns p= 0.9995. (D) NMJ synaptic vesicle release sites and (E) mitochondria density are unchanged by the G501R mutation at presynaptic terminals. Dunnett's test (T bar): TBC1D24^{WT} vs TBC1D24^{G501R} ns p= 0.8691, vs TBC1D24^{R40C} ns p= 0.4534. Dunnett's test (Mitochondria): TBC1D24^{WT} vs TBC1D24^{G501R} ns p= 0.4202, vs TBC1D24^{R40C} ns p= 0.7951.

Figure 6: ROS protection rescues TBC1D24$^{(G501R)}$ phenotype

(A,B) ROS scavenger efficiently rescued endosomal trafficking defects, indicated by a significant decrease in FM1-43 dye accumulation density in stimulated boutons. Animals were fed with antioxidant N-acetylcysteine amide (AD4) or with α-tocopherol (VitE) and incubated in physiological HL-3 ringer solution. Compared to animals raised on the same food without supplements, FM1-43 accumulation density was significantly lower in the AD4-

treated condition. One-way ANOVA: FM1-43, Dunnett's test: TBC1D24^{G501R}(without supplement) vs TBC1D24^{G501R} (AD4): ** p= 0.0065, TBC1D24^{G501R} (w/o supplement) vs TBC1D24^{G501R} (VitE): ns p= 0.1107. n= 5-6 animals. (C) Negative geotaxis performance was significantly higher in AD4-treated TBC1D24^{G501R} animals compared to wildtype controls. Dunnett's test: TBC1D24^{WT}(w/o supplement) vs TBC1D24^{WT}(AD4) ns p=0.9998, vs TBC1D24^{WT}(VitE) ns p=0.3751, vs TBC1D24^{G501R} (w/o supplement) *** p= 0.0004, vs TBC1D24^{G501R}(AD4) ns p= 0.3457, vs TBC1D24^{G501R}(VitE) ns p= 0.3751; TBC1D24^{G501R} (w/o treatment) vs TBC1D24^{G501R} (AD4) * p= 0.0227, vs TBC1D24^{G501R}(VitE) ns p= 0.3135. n= 100-130 animals. (D) Seizures in the TBC domain mutant TBC1D24^{R40C} are significantly rescued by treatment with antioxidant AD4. Unpaired t test: TBC1D24^{R40C} vs. TBC1D24^{R40C}(AD4) * p= 0.0322. n= 70 animals.