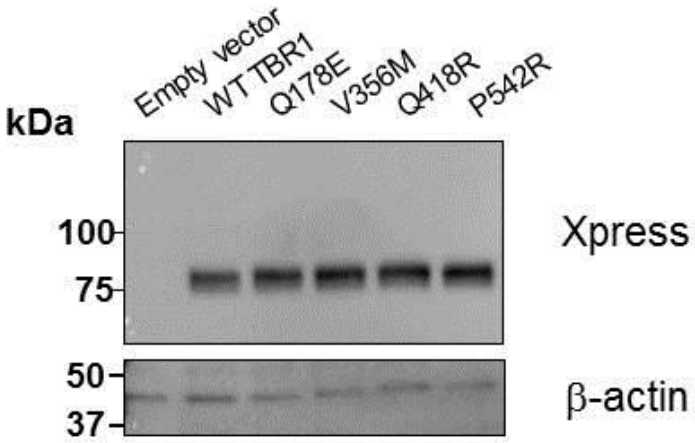
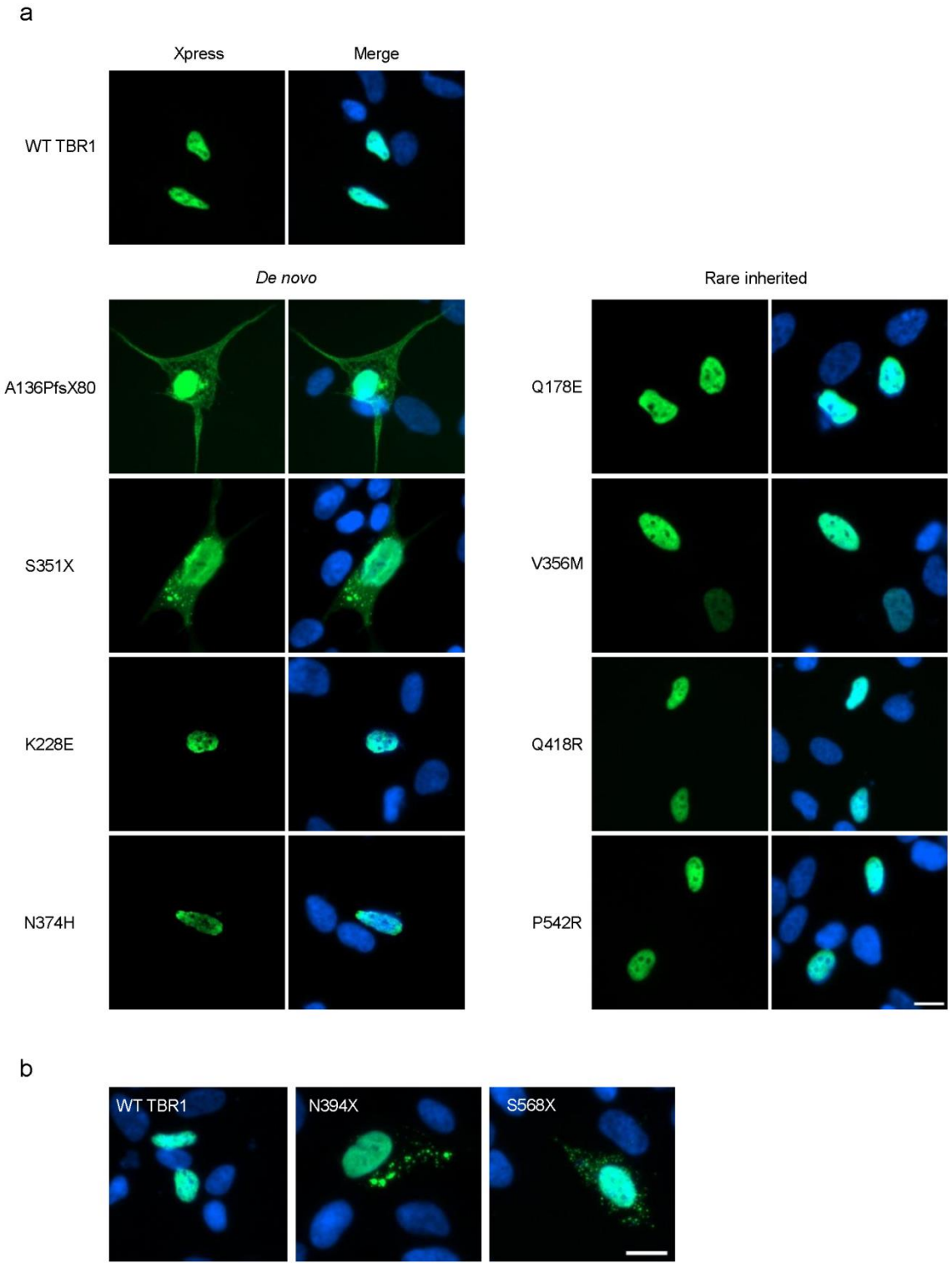
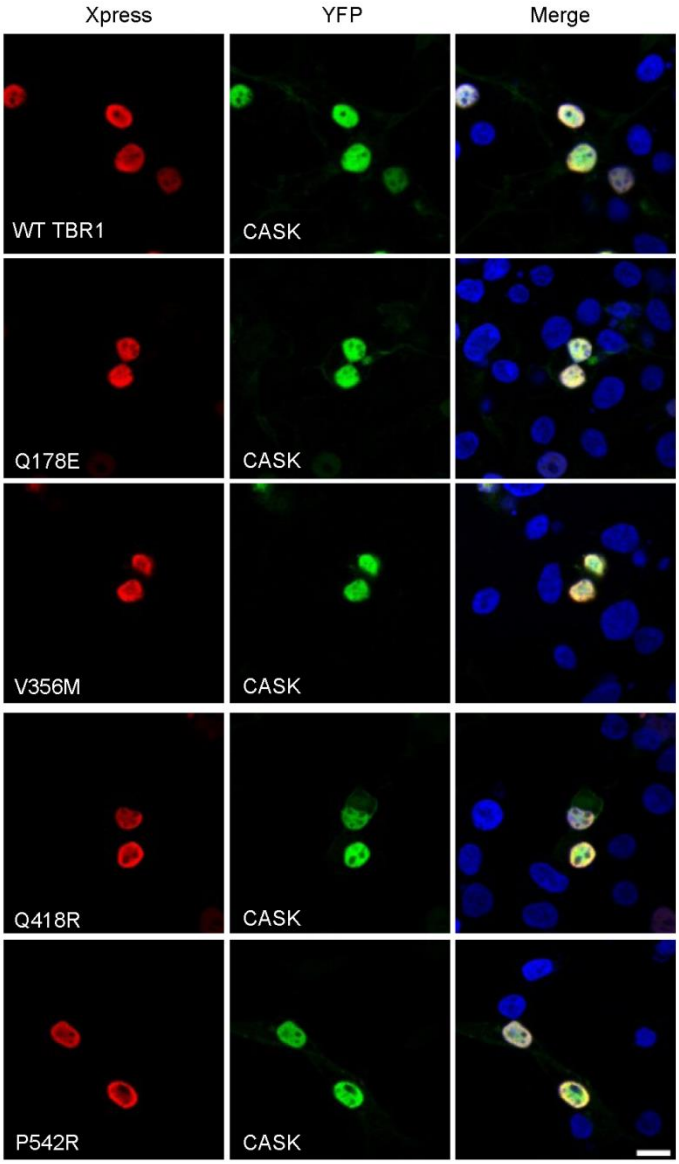



**Supplementary Figure 1**. TBR1 variants arising from inherited mutations are expressed in similar levels to WT protein. Whole cell lysates from HEK293 cells transfected with TBR1 variants in pcDNA4.HisMax (at equimolar concentrations) were immunoblotted using anti-Xpress antibody. The expected molecular weights for the TBR1 proteins are: ~78 kDa. Equal protein loading was confirmed by stripping the blot and reprobing using an anti-β-actin antibody.

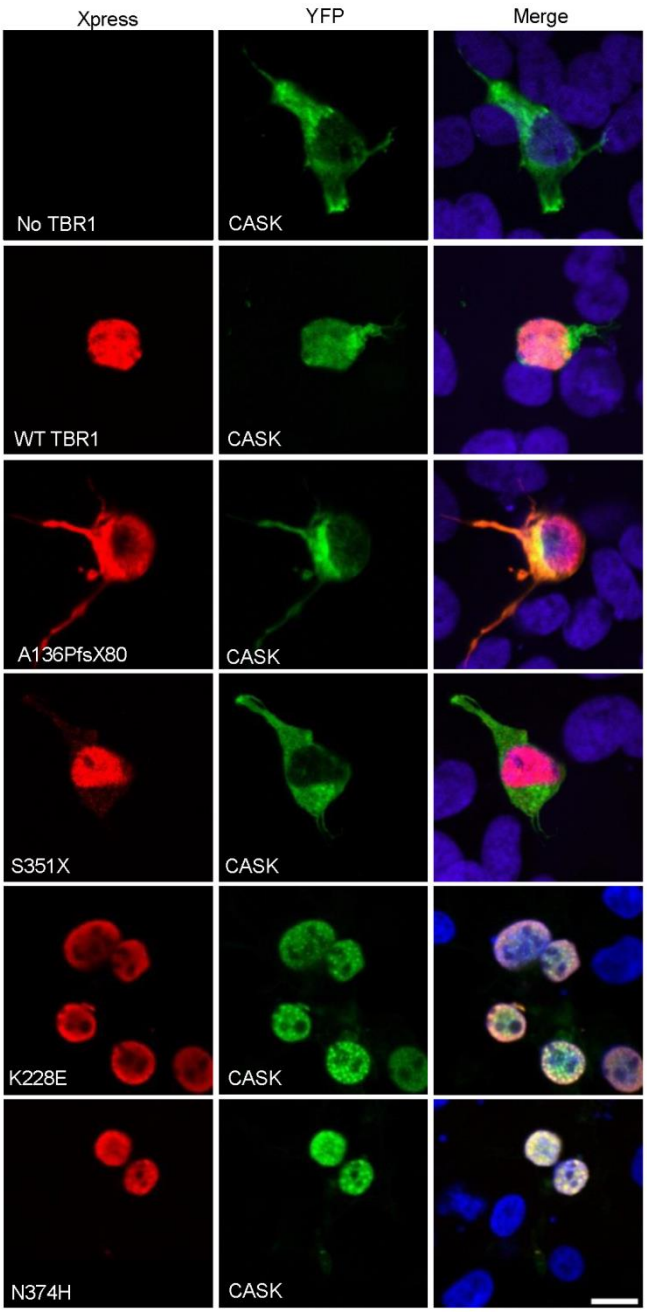



**Supplementary Figure 2**. *De novo* mutations in *TBR1* disrupt subcellular localization in SHSY5Y cells. (**a**) Fluorescence micrographs of cells transfected with TBR1 variants. Xpress-tagged TBR1 proteins are shown in green. (**b**) Fluorescence micrographs of cells transfected with synthetic TBR1 variants fused to YFP (green).

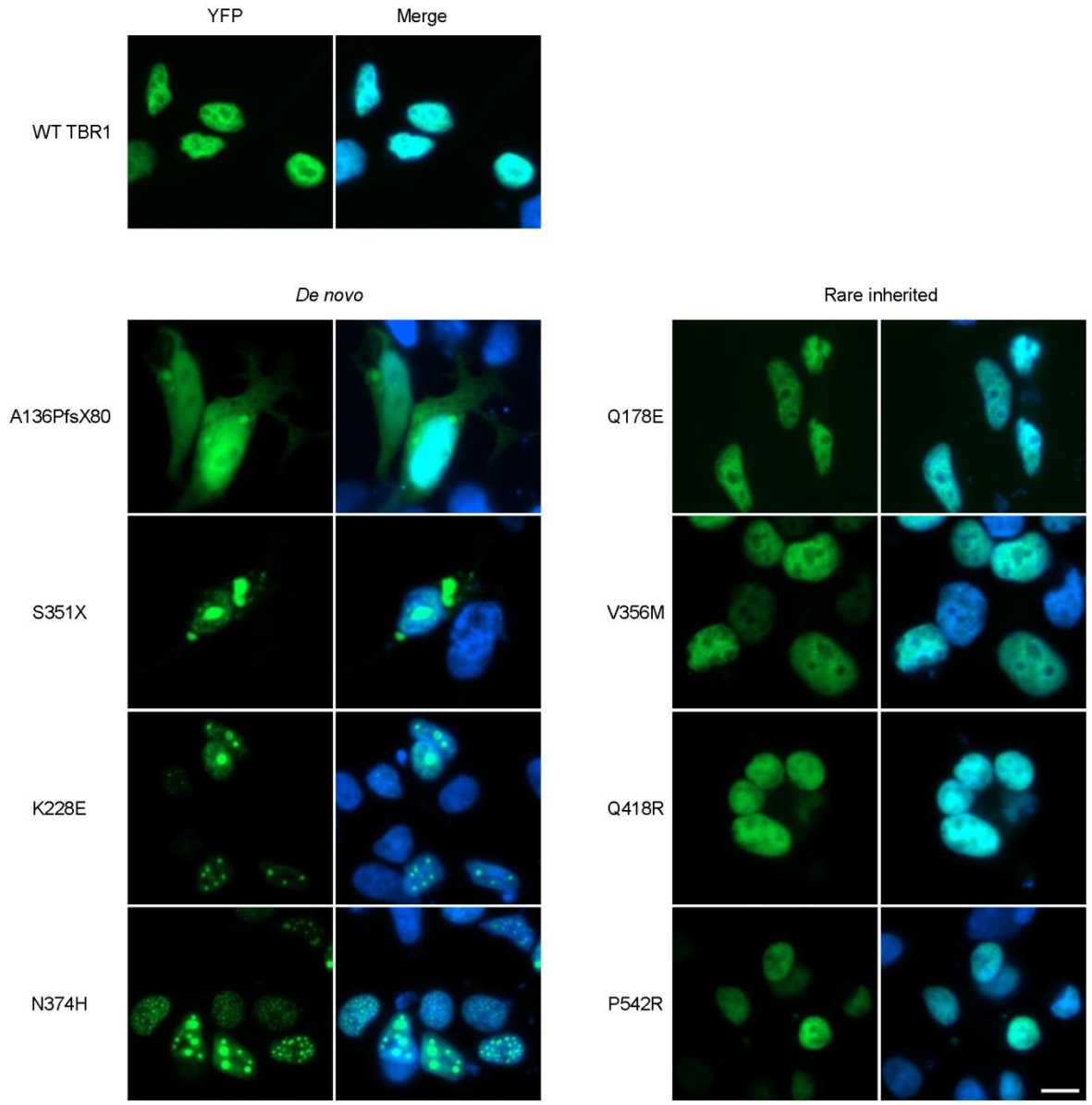
In both (a) and (b), Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 μm.



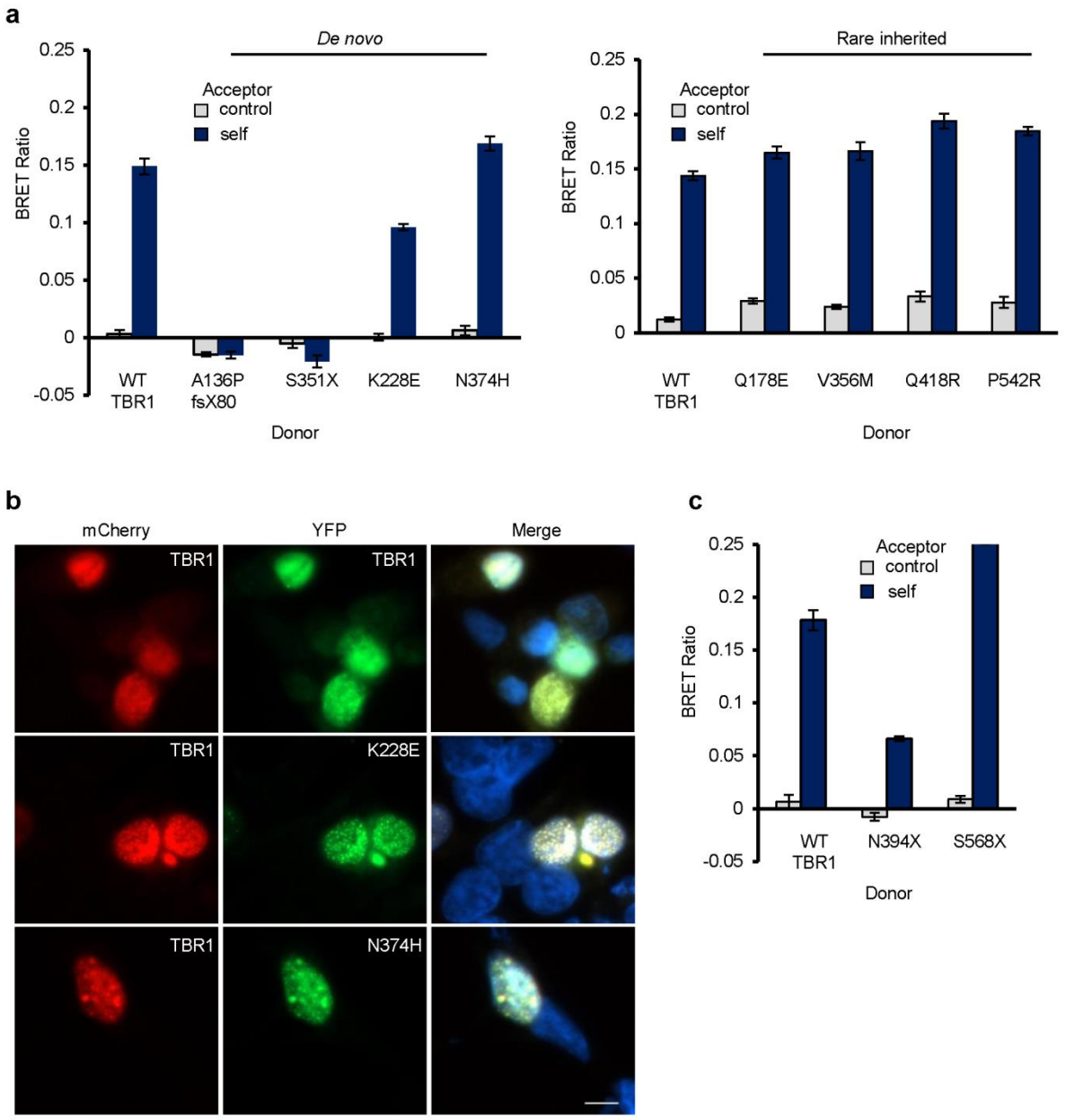

**Supplementary Figure 3**. Inherited missense mutations in *TBR1* do not disrupt interactions with CASK. Fluorescence micrographs of HEK293 cells co-transfected with CASK and TBR1 variants. Xpress-tagged TBR1 proteins are shown in red (left hand side), whereas CASK fused to YFP is shown in green (middle). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 µm.




**Supplementary Figure 4**. *De novo* truncating mutations in *TBR1* disrupt interactions with CASK. Fluorescence micrographs of SHSY5Y cells co-transfected with TBR1 variants and CASK. Xpress-tagged TBR1 proteins are shown in red (left hand side), whereas CASK fused to YFP is shown in green (middle). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 5 μm.

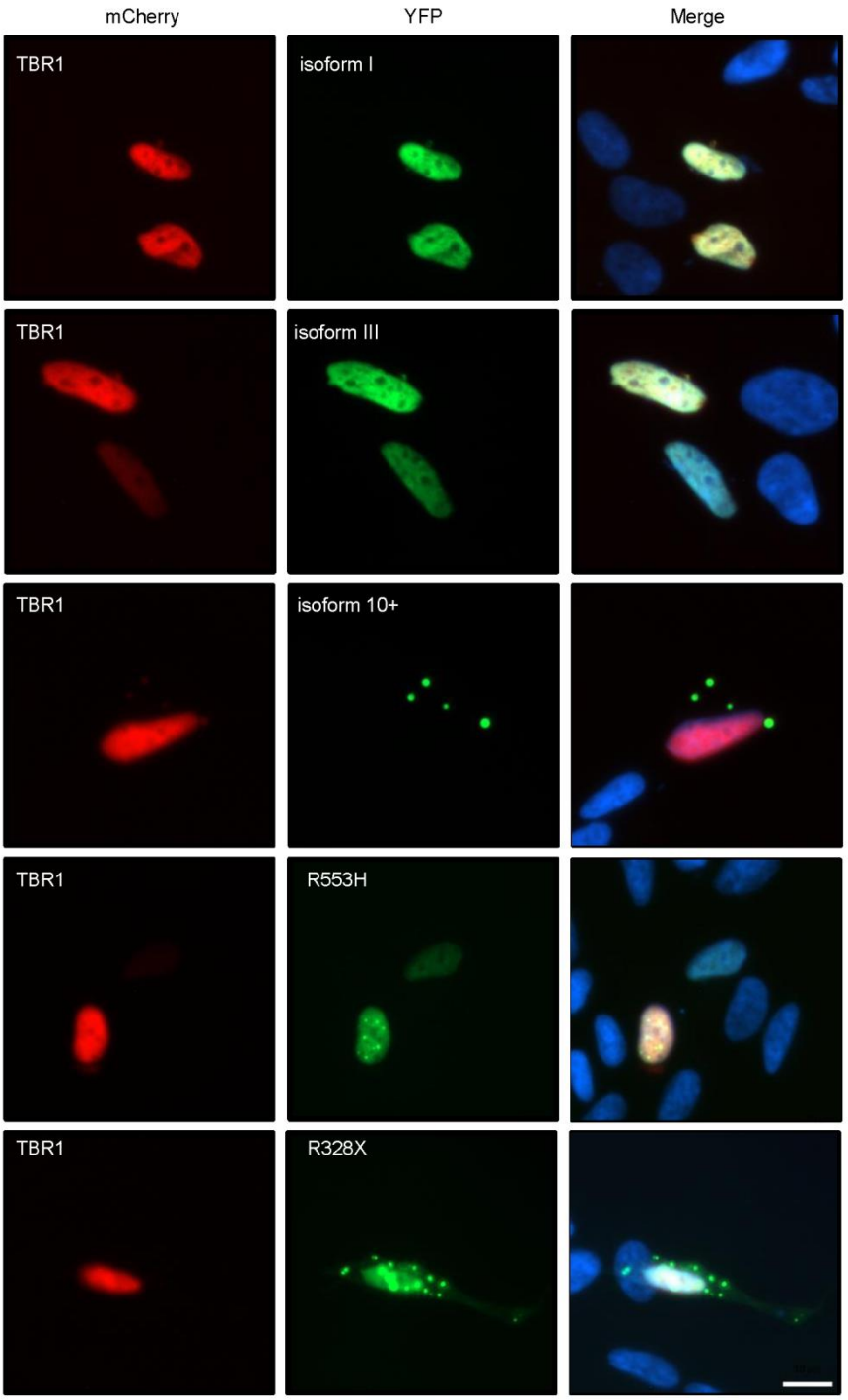



**Supplementary Figure 5**. Subcellular localization of YFP-TBR1 fusion proteins. Fluorescence micrographs of HEK293 cells transfected with TBR1 variants fused to YFP (green). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 µm.

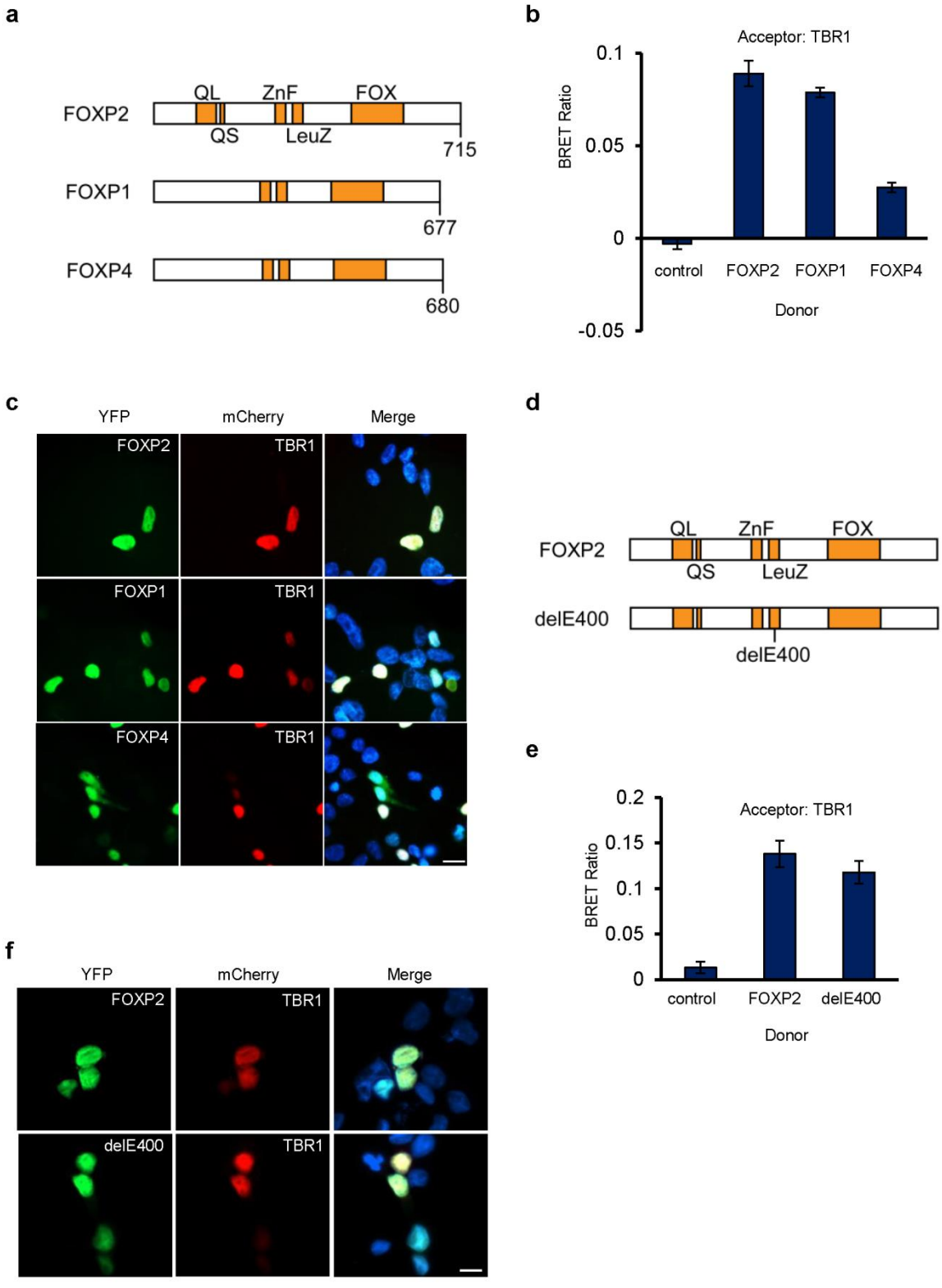



**Supplementary Figure 6**. Homodimerization of TBR1 variants found in ASD cases and of synthetic TBR1 truncations. (**a**) BRET assays for homodimerization of TBR1 variants found in ASD probands. (**b**) Fluorescence micrographs of HEK293 cells co-transfected with WT, K228E or N374H TBR1 variants and WT TBR1. TBR1 protein fused to m-Cherry is shown in red (left hand side), whereas TBR1 protein variants fused to YFP are shown in green (middle). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 µm. (**c**) BRET assays for homodimerization of

synthetic TBR1 proteins fused to YFP. In a and c, bars represent the corrected mean BRET ratios ±SEM of one experiment performed in triplicate. BRET assays were performed in HEK293 cells.




**Supplementary Figure 7**. Fluorescence images of SHSY5Y cells co-transfected with TBR1 and FOXP2 variants. WT TBR1 protein fused to m-Cherry is shown in red (left hand side), whereas FOXP2 variants fused to YFP are shown in green (middle). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 µm.




**Supplementary Figure 8**. TBR1 interacts with FOXP transcription factors. (**a**) Schematic representation of FOXP family proteins: FOXP2, FOXP1 and FOXP4. All three proteins share functional domains, such as a zinc finger (ZnF), a leucine zipper

(LeuZ) and a FOX DNA-binding domain (FOX). FOXP2 has long (QL) and short (QS) polyglutamine tracts which are absent in FOXP1 and FOXP4. (**b**) BRET assay for interaction between WT TBR1 and FOXP family members. (**c**) Fluorescence images of cells co-transfected with WT TBR1 and FOXP proteins. TBR1 co-localizes with FOXP1 and FOXP2 in the nucleus. FOXP4 is predominantly cytoplasmic, but is also found in the nucleus where it co-localizes with TBR1. FOXP proteins fused to YFP are shown in green (left hand side), whereas WT TBR1 protein fused to m-Cherry is shown in red (middle). Scale bar, 10 µm. (**d**) Schematic representation of a FOXP2 dimerization domain variant (delE400). (**e**) BRET assay for interaction between WT TBR1 and FOXP2 dimerization domain variants. (**f**) Fluorescence images of cells co-transfected with WT TBR1 and FOXP2 dimerization domain variants. FOXP2 and delE400 co-localize with TBR1 in the nucleus. FOXP2 protein variants are shown in green (left hand side), whereas TBR1 protein fused to m-Cherry is shown in red (middle). Hoechst counterstain (blue) indicates the location of nuclei. Scale bar, 10 µm. In B and E, bars represent the corrected mean BRET ratios ±SEM of one experiment performed in triplicate. All experiments were performed in HEK293 cells.

**Supplementary Table 1.** Brief description of phenotypic presentation of 7 patients with *TBR1* mutations.

| **Patient** | 11480.p1 | 13814.p1 | 13796.p1 | 12994.p1 | 14332.p1 | 13702.p1 | 13060.p1 |
|---|---|---|---|---|---|---|---|
| **Event Type** | *de novo* | *de novo* | *de novo* | *inherited* | *inherited* | *inherited* | *inherited* |
| **HGVS** | p.A136PfsX80 | p.K228E | p.S351X | Q178E | Q178E | Q418R | p.P542R |
| **Sex** | Male | Male | Female | Male | Male | Male | Male |
| **Age at Testing (years)** | 7 | 7 | 8 | 11 | 4 | 5 | 6 |
| **Primary Diagnosis** | Autism Spectrum Disorder | Autism Spectrum Disorder | Autism Spectrum Disorder | Autism Spectrum Disorder | Autism Spectrum Disorder | Autism Spectrum Disorder | Autism Spectrum Disorder |
| **Additional Psychiatric Diagnoses** | Mixed expressive-receptive language disorder; specific learning disability (reading) | Developmental Coordination Disorder | None | None | None | None | None |
| **Cognitive Functioning** | Intellectual Disability | Intellectual Disability | Intellectual Disability | Average Range | Intellectual Disability | Borderline Intellectual Functioning | Average Range |
| **Verbal IQ** | 24 | 75 | 69 | 96 | 28 | 58 | 98 |
| **NonVerbal IQ** | 41 | 78 | 63 | 99 | 39 | 86 | 81 |
| **Current Language Level** | Nonverbal | Verbally fluent | Phrase speech | Verbally fluent | Nonverbal | Verbally fluent | Verbally fluent |
| **Language Delay** | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Language Regression** | Yes | No | No | No | No | No | Possible |
| **Other Behavioral Concerns** | None | Elevated externalizing symptoms | Elevated externalizing symptoms | Elevated internalizing/ externalizing symptoms | Elevated internalizing symptoms | Elevated internalizing symptoms | None |

Note: Language Delay = single words > 24 months or phrase speech > 36 months; ASD diagnosis confirmed with ADOS, ADI, clinical judgment. HGVS: Human Genome Variation Society nomenclature.

**Supplementary Table 2.** Summary of primer sequences used to create TBR1 and FOXP2 constructs using site-directed mutagenesis.

| Gene | Protein Variant | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---|---|---|---|
| *TBR1* | S351X | GCACGGAGGACACCTAGCCAGCCCG | CGGGCTGGCTAGGTGTCCTCCGTGC |
| | K228E | CGGAGATGATCATCACCGAGCAGGGAAGGCGCATGTT | AACATGCGCCTTCCCTGCTCGGTGATGATCATCTCCG |
| | N374H | CGTCACCGCCTACCAGCACACGGATATTACACA | TGTGTAATATCCGTGTGCTGGTAGGCGGTGACG |
| | Q178E | CGGCTACCCCTACCCAGAGCAGTACGG | CCGTACTGCTCTGGGTAGGGGTAGCCG |
| | V356M | CAGCCCGGCCGCATGCAGACGTTCA | TGAACGTCTGCATGCGGCCGGGCTG |
| | Q418R | GCCGCGCTCGCGGATCGTGCCCG | CGGGCACGATCCGCGAGCGCGGC |
| | P542R | CTACGCCGACCGGTCGGGCTGGG | CCCAGCCCGACCGGTCGGCGTAG |
| *FOXP2* | | | |
| | R553H | CTTGGAAGAATGCAGTACATCATAATCTTAGCCTGCAC | GTGCAGGCTAAGATTATGATGTACTGCATTCTTCCAAG |
| | delE400 | CAGCTTTCTAAAGAACGCCGTCTTCAAGCAATGATG | CATCATTGCTTGAAGACGGCGTTCTTTAGAAAGCTG |

**Supplementary Table 3**. Summary of primer sequences used to create TBR1 and FOXP2 constructs. Restriction sites are underlined.

| Gene | Protein Variant | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---|---|---|---|
| *TBR1* | N394X | GAATTCATGCAGCTGGAGCACTGCCTT | TCTAGATTAATCCCGAAATCCTTTTGC |
| | S568X | GAATTCATGCAGCTGGAGCACTGCCTT | TCTAGATTAGTTGGGCCAGCAGGGCAG |
| *FOXP2* | isoform I | AGGGATCCAGGAATCTGCGACAGAG | AGTCTAGATTATTCCAGATCTTCAGATAAAGG |
| | isoform III | GAGGATCCTGACTCCCCAGGTGATCACCCCTC | AGTCTAGATTATTCCAGATCTTCAGATAAAGG |
| | isoform 10+ | AGGGATCCAGGAATCTGCGACAGAG | GTCTAGATCATTTACTGTTTATAAAGCAATATGC |
| | R328X | AGGGATCCAGGAATCTGCGACAGAG | CTCTAGATTATCTTGCACTTAGAACTGAAG |
| | delC1 | AGGGATCCAGGAATCTGCGACAGAG | GTCTAGACATGGGAATGTTGTATTTGTC |
| | delC2 | AGGGATCCAGGAATCTGCGACAGAG | GTCTAGAAGGTTTGGGAGATGGTTTGGG |
| | delC3 | AGGGATCCAGGAATCTGCGACAGAG | GTCTAGAGTCTCGTCTTGCACTTAGAAC |
| | delC4 | AGGGATCCAGGAATCTGCGACAGAG | GTCTAGATTGAGGCAGCGATTGGACAGG |
| *FOXP1* | | AGGGATCCAAGAATCTGGGACTGAGACA | AGTCTAGATTACTCCATGTCCTCGTTTACT |
| *FOXP4* | | AGGATCCTGGTGGAATCTGCCTCGGAGAC | CTCTAGATTAGGACAGTTCTTCTCCCGGCA |