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# Loss of the Chr16p11.2 ASD candidate gene *QPRT* leads to aberrant neuronal differentiation in the SH-SY5Y neuronal cell model

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## Abstract

**Background:** Altered neuronal development is discussed as the underlying pathogenic mechanism of autism spectrum disorders (ASD). Copy number variations of 16p11.2 have recurrently been identified in individuals with ASD. Of the 29 genes within this region, *quinolinate phosphoribosyltransferase (QPRT)* showed the strongest regulation during neuronal differentiation of SH-SY5Y neuroblastoma cells. We hypothesized a causal relation between this tryptophan metabolism-related enzyme and neuronal differentiation. We thus analyzed the effect of *QPRT* on the differentiation of SH-SY5Y and specifically focused on neuronal morphology, metabolites of the tryptophan pathway, and the neurodevelopmental transcriptome.

**Methods:** The gene dosage-dependent change of *QPRT* expression following Chr16p11.2 deletion was investigated in a lymphoblastoid cell line (LCL) of a deletion carrier and compared to his non-carrier parents. Expression of *QPRT* was tested for correlation with neuromorphology in SH-SY5Y cells. *QPRT* function was inhibited in SH-SY5Y neuroblastoma cells using (i) siRNA knockdown (KD), (ii) chemical mimicking of loss of *QPRT*, and (iii) complete CRISPR/Cas9-mediated knock out (KO). *QPRT-KD* cells underwent morphological analysis. Chemically inhibited and *QPRT-KO* cells were characterized using viability assays. Additionally, *QPRT-KO* cells underwent metabolite and whole transcriptome analyses. Genes differentially expressed upon KO of *QPRT* were tested for enrichment in biological processes and co-regulated gene-networks of the human brain.

**Results:** *QPRT* expression was reduced in the LCL of the deletion carrier and significantly correlated with the neuritic complexity of SH-SY5Y. The reduction of *QPRT* altered neuronal morphology of differentiated SH-SY5Y cells. Chemical inhibition as well as complete KO of the gene were lethal upon induction of neuronal differentiation, but not proliferation. The *QPRT*-associated tryptophan pathway was not affected by KO. At the transcriptome level, genes linked to neurodevelopmental processes and synaptic structures were affected. Differentially regulated genes were enriched for ASD candidates, and co-regulated gene networks were implicated in the development of the dorsolateral prefrontal cortex, the hippocampus, and the amygdala.

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**Conclusions:** In this study, *QPRT* was causally related to in vitro neuronal differentiation of SH-SY5Y cells and affected the regulation of genes and gene networks previously implicated in ASD. Thus, our data suggest that *QPRT* may play an important role in the pathogenesis of ASD in Chr16p11.2 deletion carriers.

**Keywords:** Autism, 16p11.2, Quinolinate phosphoribosyltransferase, Quinolinic acid, Kynurenine, CRISPR/Cas9, Sholl analysis

## Background

Altered neuronal development is suggested to be one of the major drivers in the etiology of autism spectrum disorders (ASD). Neuropathological studies based on postmortem brains of ASD patients reported aberrant neuronal development including reduced dendritic branching in the hippocampus [1], smaller pyramidal neurons in the language associated Broca's area [2], abnormal minicolumnar organization in the cerebral cortex leading to decreased inter-areal connectivity [3], and disorganized layers of the cortical areas [4]. The underlying etiology of ASD is mainly based on different genetic findings including de novo copy number variations (CNVs). These CNVs, in particular deletions, have been recurrently shown to alter genetic regions in ASD individuals [5], specifically affecting neurodevelopmental genes [6].

One of the most recurrent CNVs in ASD resides within Chr16p11.2 spanning ~600 kb. Overall, duplications and deletions of 16p11.2 can be identified in 0.8% of ASD cases [7]. A deletion of this region is associated with a nine times higher likelihood of developing ASD, and the duplication is associated with a nine times higher risk of both ASD and schizophrenia [8]. While developmental delay or intellectual disability can occur in some cases of 16p11.2 duplication carriers, they are more common in deletions [9, 10].

The 16p11.2 CNV region spans 29 genes which showed gene dosage-dependent expression in lymphoblastoid cell lines (LCLs) of CNV carriers, leading to a differential expression of genes implicated in biological processes such as synaptic function or chromatin modification [11].

A study in zebrafish showed that the majority of the human Chr16p11.2 homologous genes are involved in nervous system development: loss of function of these genes led to an altered brain morphology for 21 of 22 tested genes [12]. Double heterozygous knockouts of the Chr16p11.2 homologs *double C2 domain alpha* (*DOC2A*) and *family with sequence similarity 57, member Ba* (*FAM57BA*) induced hyperactivity, increased seizure susceptibility and increased body length and head size in zebrafish [13]. In mice, CNVs of the homologous 16p11.2 region induced differing phenotypes. Two studies reported the deletion to result in a reduction of the skull [14] or brain size [15], accompanied by

gene dosage-sensitive changes of behavior and synaptic plasticity [14] as well as altered cortical cytoarchitecture, and reduction of downstream extracellular signaling-related kinase (ERK/MAPK) effectors [15]. Comparing individual brain regions in mice with deletions to wild-type animals, Horev and associates identified six regions with an increased volume, which were not altered in duplication carriers (see Dataset S04 in the original publication [16]). In another study, mice carrying a heterozygous microduplication of the region showed increased dendritic arborization of cortical pyramidal neurons [17]. Via network analysis of protein-protein-interaction, the authors identified the gene coding for mitogen-activated protein kinase 3 (MAPK3) as hub gene. MAPK3 plays a role in signaling cascades involved in proliferation and differentiation. A recent study focused on different effects of 16p11.2 deletions in male and female mice and reported impairments of reward-directed learning in male mice accompanied by male-specific overexpression of *dopamine receptor D2* (*DRD2*) and *adenosine receptor 2a* (*ADORA2A*) in the striatum [18]. Both genes have been discussed in the context of ASD [19, 20].

While the functional validation of the entire CNV models the genomic status of the patients, investigating gene dosage effects of single genes located in Chr16p11.2 is useful to understand their individual contribution to the complex and diverse pathologies of ASD. In zebrafish, the suppression of *potassium channel tetramerization domain containing 13* (*KCTD13*) was associated with macrocephaly whereas overexpression led to microcephaly [21]. In mice, the same study showed a reduction of *KCTD13* to result in increased proliferation of neuronal progenitors, which is also suggested to result in macrocephaly. Further, a heterozygous deletion of the gene coding for major vault protein (*MVP*) induced a reduction of functional synapses in mice [22]. *TAO kinase 2* (*TAOK2*), also located in 16p11.2, was found to be essential for the development of basal dendrites and axonal projections in cortical pyramidal neurons of mice [23]. Chr16p11.2 genes *DOC2A*, *KIF22*, and *T-box 6* (*TBX6*) are required for the development of neuronal polarity in mouse hippocampal cultures [24].

In ASD patients, multiple brain measures such as the thalamic or total brain volume were reported to be

increased in 16p11.2 deletion carriers and reduced in duplication carriers [25, 26]. Another study integrated physical interactions of 16p11.2 proteins with spatiotemporal gene expression of the human brain. The authors identified the KCTD13-Cul3-RhoA pathway as being crucial for controlling brain size and connectivity [27]. Still, only few genes of the Chr16p11.2 region have been investigated for their specific role in neuronal differentiation in human models.

Here, we investigated the SH-SY5Y neuroblastoma cell model as a well-studied and feasible model for neuronal differentiation in vitro. Previously, we reported that a continuous application of brain-derived neurotrophic factor (BDNF) and retinoic acid (RA) leads to neuronal cells of most likely cortical identity with a transcriptomic signature reminiscent of that of neocortical brain tissue developed for 16–19 weeks post-conception [28]. In addition, we showed expressed genes in the differentiated SH-SY5Y model to be co-regulated within modules, several of which were associated with neurodevelopmental disorders such as the orange module. We then implemented three complementary statistical methods to identify genes that were (i) differentially regulated upon differentiation, (ii) significantly involved in the independent processes active during differentiation and/or (iii) that were significantly changed over time. Finally, we described a list of 299 robustly regulated genes that appeared to be significant in all three analyses [28].

We here report that of the 29 genes located within Chr16p11.2, a total of 10 genes were identified by at least one of the three implemented statistical approaches. However, only the gene coding for *quinolinate phosphoribosyltransferase* (*QPRT*) was identified by all three analyses. In addition, *QPRT* was one of the most highly expressed genes of the Chr16p11.2 region and showed the highest regulatory fold change (FC) after induction of neuronal differentiation. Also, *QPRT* was co-regulated with an early upregulated gene module (MEorange) which showed significant enrichment for ASD candidate genes [28].

*QPRT* codes for an enzyme of the kynurenine pathway, the primary route for tryptophan catabolism, which results in the production of nicotinamide adenine dinucleotide (NAD<sup>+</sup>). In addition, it is the only enzyme catabolizing quinolinic acid (QUIN), a potent excitotoxin acting as N-methyl-D-aspartate receptor (NMDA-R) agonist. QUIN is also linked to astroglial activation and cell death as originally identified in the context of Alzheimer's disease [29]. *QPRT-KO* mice showed increased QUIN levels in the brain [30] and increased excretion of QUIN in urine [31]. A significant increase of QUIN was observed in blood plasma of children with ASD when compared to their age-matched healthy control siblings [32]. Furthermore, *QPRT* was identified as an interaction

partner of the ASD candidate neuroligin 3 (NLGN3; [33]), suggesting an involvement of *QPRT* in the formation of the postsynaptic density.

Here, we hypothesized that *QPRT* is implicated in neuronal differentiation and that reduced *QPRT* expression following its deletion results in alterations of neuro-morphological development. We first tested the gene dosage-dependent expression of *QPRT* in a patient-specific LCL of one Chr16p11.2 deletion carrier. We then analyzed the expression of *QPRT* and its co-regulated gene set for correlation with the development of neuronal morphology in SH-SY5Y wild-type (WT) cells. To study the effects on neuronal morphology, we inhibited *QPRT* function in SH-SY5Y cells using (i) siRNA knock-down (KD), (ii) chemical mimicking of loss of *QPRT*, and (iii) complete CRISPR/Cas9-mediated knock out (KO). *QPRT-KD* cells underwent morphological analysis. Chemically inhibited and *QPRT-KO* cells were characterized using viability assays. To understand the effects of *QPRT* loss on the kynurenine pathway and QUIN levels, we additionally performed a metabolite analysis of the generated *QPRT-KO* cells. To explore the systems-wide interaction network of *QPRT*, we investigated the transcriptomic signature of *QPRT-KO* cells. Finally, to understand the role of *QPRT* in neural development, we tested the genes associated with *QPRT-KO* for enrichment among gene-networks implicated in human brain development [34].

## Methods

### Cellular models

#### *Patient-specific lymphoblastoid cell lines (LCLs)*

Lymphoblastoid cell lines (LCLs) were generated as previously published [35, 36]. We investigated LCLs generated from one Chr16p11.2 heterozygous deletion carrier and his non-carrier parents. The child was diagnosed with autism (ICD10: F84.0) based on both the Autism Diagnostic Interview-Revised (ADI-R; [37, 38]) and the Autism Observation Schedule (ADOS; [39]). The patient presented with a severe impairment of social interaction, hyperactive and aggressive behavior as well as language delay, and an average non-verbal IQ = 90. The deletion was identified in a screen of 710 children with ASD and their parents using the Illumina Human OmniExpress Microarray and validated via real-time PCR (unpublished data). To investigate *QPRT* gene expression at transcriptional level in exponentially growing cell cultures of the Chr16p11.2 deletion carrier compared to his non-carrier parents,  $1 \times 10^6$  viable cells were inoculated into 5 ml RPMI medium supplied with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 1X GlutaMAX (all from GIBCO®, Life Technologies, Paisley, UK). Volume was doubled when cultures reached a density of  $1 \times 10^6$  cells/ml. Finally, cells were

harvested with a total volume of 20 ml at a density of again  $1 \times 10^6$  cells/ml. Whole RNA was extracted, including DNase treatment, and reversely transcribed using the GeneJet RNA Purification Kit and the RevertAid H Minus first strand cDNA synthesis kit (both from Thermo Fisher Scientific) following the manufacturer's protocols. Real-time RT-PCR was performed using the Universal Probe Library (UPL; Roche) and a StepOne Plus device (Applied Biosystems) and normalized to *glucuronidase beta* (*GUSB*; see Additional file 1: Supplementary Methods and Table S1).

### **Neuroblastoma cell line SH-SY5Y**

Out of possible cell types to investigate neuronal differentiation, SH-SY5Y is well studied, feasible, and with an optimized protocol shows reproducible differentiation of cells into cortical-like neurons [28]. For proliferation, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% sodium pyruvate (all from Life Technologies), and 1% penicillin/streptomycin (PAA). As described in our previous study [28], SH-SY5Y cells were differentiated using a continuous application of retinoic acid (RA) and brain-derived neurotrophic factor (BDNF). Differentiation media consisted of Neurobasal<sup>®</sup>-A medium supplemented with 1x GlutaMAX, 1x B-27 supplement (all from Life Technologies), 10  $\mu$ M RA, 2 mM cAMP (both from Sigma-Aldrich), 50 ng/ml hBDNF (Immunotools), 1% penicillin/streptomycin (PAA), and 20 mM KCl. Cells were differentiated for 11 days changing the medium every other day. The time points for extraction of mRNA or protein and imaging were set 24 h after media changes (0/undifferentiated cells, 1, 3, 5, 7, 9, and 11 days of in vitro differentiation).

To study the morphological development of WT SH-SY5Y cells during neuronal differentiation, we transfected proliferating cells with pmaxGFP (Lonza) using Metafectene Pro (Biontex) according to the manufacturer's protocol. One day after transfection, cells were seeded 1:2 in co-cultures with untransfected cells with a density of  $1 \times 10^4$  cells/cm<sup>2</sup> to allow imaging of individual transfected cells. Cultures were imaged using a Motic AE31 fluorescence microscope (Motic). Images were analyzed using custom macros in ImageJ [40] available on request. In short, all images were equalized, despeckled, and the background was subtracted using the rolling ball method prior to binarization (Auto threshold "Otsu-dark"). Sholl analysis [41], a concentric circle method, was performed using the respective ImageJ plugin with manual selection of the cells' center and with fitting polynomial regression of the fifth degree, as suggested by the manual [42]. The following morphological parameters were assessed: maximum intersections (maximum number of neuritic intersections for one radius), sum of intersections (sum of all intersections of one

cell), enclosing radius (the outer radius intersecting the cell, describing the longest distance between soma and neurites), intersecting radii (number of radii intersecting a cell, also describing the distance between soma and neurites), average number of intersections (the number of intersections analyzed divided by the number of intersecting radii; a measure describing neuritic complexity) and maximum intersections radius (the distance from the soma where most neurites are present, i.e., the site of the highest branching density; also reflecting neuritic complexity). Morphological parameters have been assessed in parallel during the previous transcriptomic analysis [28].

We made use of the previously published whole transcriptome data of differentiating SH-SY5Y cells (gene expression omnibus repository (GEO) under the accession number GSE69838 [28]) and analyzed *QPRT* expression as well as regulation of modules of co-expressed genes for correlation with the simultaneously assessed morphological parameters of SH-SY5Y neuronal differentiation (see also the "Statistical analysis" section below). Furthermore, *QPRT* expression during neuronal differentiation was validated in the same mRNA extracts using real-time RT-PCR. RNA extraction, cDNA generation, and real-time RT-PCR were performed as described above and in Additional file 1: Supplementary Methods. Expression values were normalized to *GUSB* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

### **Reduction of QPRT in neuroblastoma cell line SH-SY5Y siRNA-mediated knockdown (KD) of QPRT**

*QPRT* expression in SH-SY5Y was reduced using three different siRNAs (siQ1–siQ3) and compared to a non-targeting control siRNA (siCtrl) using the Ambion Silencer Select siRNAs (Thermo Fisher; Additional file 1: Supplementary Methods). siRNA-generated KD of *QPRT* after 11 days of differentiation was proven in parallel to the morphological analysis on protein level via Western Blot as described in Additional file 1: Supplementary Methods. In short, *QPRT* (mouse anti-*QPRT*, Abcam) and control  $\beta$ -Actin (ACTB; mouse anti- $\beta$ -Actin, Sigma) were detected using the secondary antibody anti-mouse IgG HRP-conjugated (Santa Cruz), the ECL Prime Western Blot Detection Reagent, and the Amersham Hyperfilm ECL (both from GE Healthcare). To allow imaging of fluorescent single cells in order to study morphological effects of *QPRT* knockdown, we co-transfected SH-SY5Y cells with mCherry (Plasmid #30125; Addgene). In a 96-well format,  $5.2 \times 10^4$  cells/cm<sup>2</sup> were reversely transfected with 120 ng of mCherry and 5 nM siRNA using Lipofectamine RNAi Max and OptiMEM (Thermo Fisher).

### **CRISPR/Cas9 mediated knock out (KO) of QPRT**

CRISPR/Cas9 gene editing was performed as described elsewhere [43]. In short, sgRNAs were designed [44] and

oligos were ordered from Sigma-Aldrich and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Plasmid #62988; Addgene). Two sgRNAs targeting different sequences of *QPRT* were designed to generate homozygous knock out in addition to the non-targeting empty control vector (see Additional file 1: Supplementary Methods and Supplementary Results). Validated plasmids (Sanger sequencing) were transfected into SH-SY5Y cells using Metafectene Pro (Biontix) according to the manufacturer's protocol with subsequent puromycin (Sigma-Aldrich) selection using 750 ng/ml for 7 to 14 days. After low-density seeding, single clones were isolated using 5  $\mu$ l of Trypsin-EDTA (Thermo Fisher Scientific) and expanded in 96-well plates. Overall, we sequenced at least ten clones per construct and confirmed homozygous single cell clones at least twice by Sanger sequencing including the empty control vector. Clones were named as follows: del268T (*QPRT* NM\_014298 del268T; Ex2.1), ins395A (*QPRT* NM\_014298 ins395A; Ex2.2), eCtrl (empty control vector). Both indels induced premature stop codons as validated in silico and by Sanger sequencing. KO of *QPRT* was further shown on RNA and protein level using real-time RT-PCR and Western Blot, respectively. RNA extraction, cDNA generation, and real-time RT-PCR as well as protein extraction and Western Blots were performed as described above and in Additional file 1: Supplementary Methods. RNA expression values were normalized to *GUSB* and *GAPDH*, and protein expression was descriptively compared to *GAPDH* expression.

#### Functional analyses in neuroblastoma cell line SH-SY5Y *siRNA-mediated KD of QPRT*

The day after transfection (see above), media were changed from proliferation to differentiation media. After changing the media every other day for a time course of 11 days, cells were imaged using an ImageXpress Micro XLS (Molecular Devices). Images were pre-processed in MetaXpress and analyzed in ImageJ using Sholl analysis (as described above).

#### Chemical mimicking of *QPRT* loss

To measure the effect of chemical *QPRT* inhibition, we seeded WT SH-SY5Y cells with a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. After cells have attached overnight, media were changed to either fresh proliferation or differentiation media containing 0 (reference), 5, or 10 mM of the *QPRT* inhibitor phthalic acid (PA; Sigma; directly diluted in the respective media; [45]) and incubated for 3 days followed by propidium iodide (PI) viability assays. PI and Hoechst33342 (both from Sigma) were pre-diluted in DPBS (Life Technologies) and used with final concentrations of 1  $\mu$ g/ml and 10  $\mu$ g/ml for the assay, respectively. Prior to analysis, supernatant including detached and

dead cells was removed and attached cells were washed with DPBS. Next, attached cells were incubated with PI and Hoechst33342 for 5 min at 37 °C and imaged using the ImageXpress Micro XLS microscope (Molecular Devices). Images were acquired through DAPI and TRITC channels and analyzed using the MetaXpress macro "Cell Scoring". Percentage of dead cells after application of 5 and 10 mM PA was compared to the 0 mM reference for proliferating and differentiating cells, respectively.

To test if the observed cell death upon chemical inhibition of *QPRT* during differentiation is driven by an accumulation of the *QPRT* substrate quinolinic acid (QUIN; Sigma), we exposed WT cells to this neurotoxin. Cells were seeded with a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup> and media were changed after 24 h to either fresh proliferation or differentiation media containing 0 (reference; vehicle H<sub>2</sub>O only), 5, or 250  $\mu$ M of QUIN [46] and incubated for 3 days followed by PI viability-assays (as described above). Percentage of dead cells after application of 5 and 250  $\mu$ M QUIN was compared to the 0  $\mu$ M reference for proliferating and differentiating cells, respectively.

#### CRISPR/Cas9-mediated KO of *QPRT*

**Viability assay** To measure the effect of *QPRT-KO* on the viability of differentiating SH-SY5Y, KO and eCtrl cells were seeded at a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. The following day, media were changed to differentiation media. After 3 days, PI viability-assays were performed (as described above).

**Rescue experiments of *QPRT-KO* cells** We further aimed to rescue the effects of a potential *QPRT-KO*-driven increase of QUIN by inhibiting downstream pathways. Cells were seeded with a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. After 24 h, media were changed to differentiation media containing (i) 0 (reference; vehicle H<sub>2</sub>O only), 6, and 12  $\mu$ M of the NMDA-R antagonist MK801 [47], (ii) 0 (reference; vehicle H<sub>2</sub>O only), 0.5, and 1 mM of nitric oxide synthase 1 (NOS1) inhibitor L-NAME [48], and (iii) 0 (reference; vehicle H<sub>2</sub>O only), 5, and 10 mM of NAD<sup>+</sup> [49]. PI viability assays were performed after an incubation of 3 days (as described above). All rescue experiments were performed with differentiating *QPRT-KO* cell lines compared to the eCtrl.

**Metabolite analysis** To characterize *QPRT-KO* at the level of the kynurenine pathway, i.e., tryptophan catabolism, we analyzed its metabolites in cell culture supernatants using ultra-high-performance liquid chromatography (UHPLC; for tryptophan (TRP), kynurenine (KYN), 3-hydroxykynurenine (3HK), 3-hydroxyanthranilic acid (3HAA), anthranilic acid (AA) and kynurenic acid (KA)),

and gas chromatography-mass spectrometry (GC/MS; for picolinic acid (PIC) and QUIN). Cell lines were seeded with a density of  $2 \times 10^4$  cells/cm<sup>2</sup> and grown in proliferation or differentiation media for 3 days as described above. Supernatants of replicates were harvested and stored at  $-80^\circ\text{C}$  until further proceedings. UHPLC and GC/MS were performed as described previously [32, 50] (see also Additional file 1: Supplementary Methods).

**Massive analysis of cDNA ends (MACE)** To investigate the transcriptomic changes induced by *QPRT-KO*, we performed a whole transcriptome analysis using the RNA-Seq approach MACE. MACE sequencing in contrast to RNA sequencing reads cDNA ends only rather than whole transcripts, i.e., poly-A tails. The output values of MACE-Seq are absolute counts per cDNA end. This approach is more sensitive compared to classical RNA-Seq. This method allows the detection of low abundant transcripts and differentiation between alternative 3'UTRs with high accuracy [51, 52]. However, it does not allow to identify alternative exon usage.

Cell lines (three biological replicates of each WT, eCtrl, and KO cells) were seeded with a density of  $2 \times 10^4$  cells/cm<sup>2</sup> in proliferation medium. After 3 days, media were changed to differentiation and cells were differentiated for 3 days without media changes. RNA was prepared as described above. RNA integrity number (RIN) was analyzed using the LabChip GX system and only samples with RINs above 9.7 underwent further analysis. MACE analysis including quality control was outsourced to GenXPro (Frankfurt) [53]. Reads were mapped to the human genome version hg38 using the Bowtie2 algorithm implementing the "--sensitive-local" parameter and standard settings as published [54].

A total of 12 genes found to be significantly differentially expressed in KO cells with an absolute log<sub>2</sub> fold change (FC) above 2.5 were chosen for validation via real-time RT-PCR using the UPL system as described above with *GAPDH*, *GUSB*, and *proteasome 26S subunit, non-ATPase 7 (PSMD7)* as housekeeping genes. Additionally, the GOIs *QPRT*, *nicotinamide nucleotide adenyltransferase 2 (NMNAT2)*; only downstream enzyme of *QPRT* differentially regulated upon KO of *QPRT*, and *NLGN3* were included for validation (Additional file 1: Table S1).

#### Statistical analysis

If not stated otherwise, statistical analyses were performed using R version 3.2.3.

#### Group differences

Group differences between mRNA expression in LCLs of a deletion carrier compared to his non-carrier parents were tested using *t* test. Group differences of morphological

parameters were tested using ANOVA and pairwise ANOVA (type III error) with Tukey's honest significant difference (HSD) correcting for multiple testing; Tukey's FDRs were considered significant below a threshold of  $\text{FDR} \leq 0.05$ . Group differences between treated and untreated samples (e.g., PI assays) were tested using *t* test; uncorrected *p* values are reported. All samples were compared to their respective control (e.g., non-targeting siRNA) or reference (e.g., 0 mM of inhibitor).

#### Correlations

Correlation between morphological parameters and gene expression or eigengene expression was tested based on the Pearson's product moment correlation coefficient following a t-distribution. Reported *p* values are Bonferroni corrected for 126 tests (6 morphological parameters, 20 gene modules, 1 gene expression).

#### Targeted mRNA expression

Data of real-time RT-PCR experiments were analyzed using the  $2^{\Delta\Delta\text{Ct}}$  method [55]. Group differences were tested using *t* test. For validation of RNA-Seq data, real-time RT-PCR data and RNA-Seq (MACE) normalized reads were tested for correlation as described above using Pearson's correlation tests.

#### Transcriptome analysis

Overall 32,739 transcripts were targeted by MACE analysis and defined as the gene universe (reference gene panel). Differentially expressed genes were identified using the "DESeq2" pipeline [56] with the gene expression as the dependent variable and the respective groups as the independent variable. No additional covariates have been included since the samples did not differ with respect to RIN, number of total counts, or batch. Hierarchical cluster analysis using the top 2000 genes based on variance was performed to exclude any technical outlier samples (for detailed quality checks see Additional file 1: Figure S6). Four different cell lines underwent MACE analysis: the untreated wild-type (WT), the empty control vector (eCtrl), the KO cell line *QPRT*<sup>-/-</sup> del268T, and the KO cell line *QPRT*<sup>-/-</sup> ins395A. Three technical replicates were sequenced for each cell line. Differential gene expression induced by *QPRT-KO* was assessed by comparing (i) WT vs eCtrl, (ii) eCtrl vs KO del268T as well as (iii) eCtrl vs KO ins395A independently. A gene was considered to be significantly associated with *QPRT-KO* if (i) no significant change was identified between the WT and the eCtrl cell line ( $\text{FDR} > 0.1$ ), (ii) if a significant ( $\text{FDR} < 0.05$ ) difference was observed between both the eCtrl and KO del268T and between eCtrl and KO ins395A, and (iii) the direction of the effect was the same in both of the comparisons eCtrl vs KO del268T and eCtrl vs KO

ins395A, respectively. For the differential expression analysis between two groups, raw counts of the respective replicates were loaded into DESeq2 using the “DESeqDataSetFromMatrix” function. Differential expression was estimated using the function “DESeq” with the option “fitType = ‘local’”. Significantly up and downregulated genes underwent GO term analysis (Additional file 1: Supplementary Methods). Genes surviving quality check (more than 10 reads per gene in at least 6 of the 12 samples) were subjected to weighted gene co-expression network analysis (WGCNA; Additional file 1: Supplementary Methods).

#### **Gene list enrichment test in gene networks of brain development**

To gain a deeper insight into the brain-specific effects of up and downregulated genes from MACE analysis, we used part of a framework proposed by Yousaf et al. [57]. In short, this framework uses the Allen Brain Atlas dataset of Kang and colleagues, who have identified 29 co-regulated gene sets using the spatiotemporal transcriptome of the human brain from early embryonal development to late adulthood [34]. Each module corresponds to specific biological processes involved in brain development and aging. We tested these modules for enrichment with the sets of genes up or downregulated upon *QPRT-KO* in SH-SY5Y using Fisher’s exact tests. Expression patterns of the enriched modules were then visualized using heatmaps of the eigenvalues of the respective modules for each human brain region over time.

## **Results**

### **Patient-specific lymphoblastoid cell lines (LCLs)**

To replicate previous reports [11] of altered *QPRT* expression in 16p11.2 CNV carriers, we compared *QPRT* expression in a patient-specific LCL of a deletion carrier and his unaffected parents. We confirmed a dosage dependent expression of *QPRT* at mRNA level (16p11.2 deletion carrier vs. non-carrier parents; logFC = -0.68,  $p = 0.014$ ; Additional file 1: Figure S1a).

### **Correlation of QPRT and neuritic complexity in SH-SY5Y wild-type cells**

RNA expression of *QPRT* during SH-SY5Y differentiation significantly correlated between microarray [28] and real-time RT-PCR ( $\rho = 0.88$ ;  $p = 0.0098$ ; Additional file 1: Figure S1b). Further, we used the expression data of *QPRT* as well as of the modules of genes co-regulated during neuronal differentiation and tested them for their correlation with morphological parameters using Sholl analysis during 11 days of neuronal differentiation (Additional file 1: Figure S1c and d). Both *QPRT* expression and the eigenvalue of its associated module (MEorange) significantly

correlated with the average number of intersections (*QPRT* microarray:  $\rho = 0.86$ , FDR =  $8.16E-05$ ; *QPRT* RT-PCR:  $\rho = 0.54$ , FDR = 0.020; MEorange:  $\rho = 0.93$ , FDR =  $1.71E-07$ ; Additional file 1: Figure S1d).

In summary, we report a correlative association between *QPRT* expression and a measure for the development of neurite complexity during in vitro neuronal differentiation of wild-type SH-SY5Y cells. To investigate if the correlation of *QPRT* expression with neuritic complexity is causal or secondary, i.e., due to the progressive neurite growth over time, we performed functional inhibition analysis of *QPRT*.

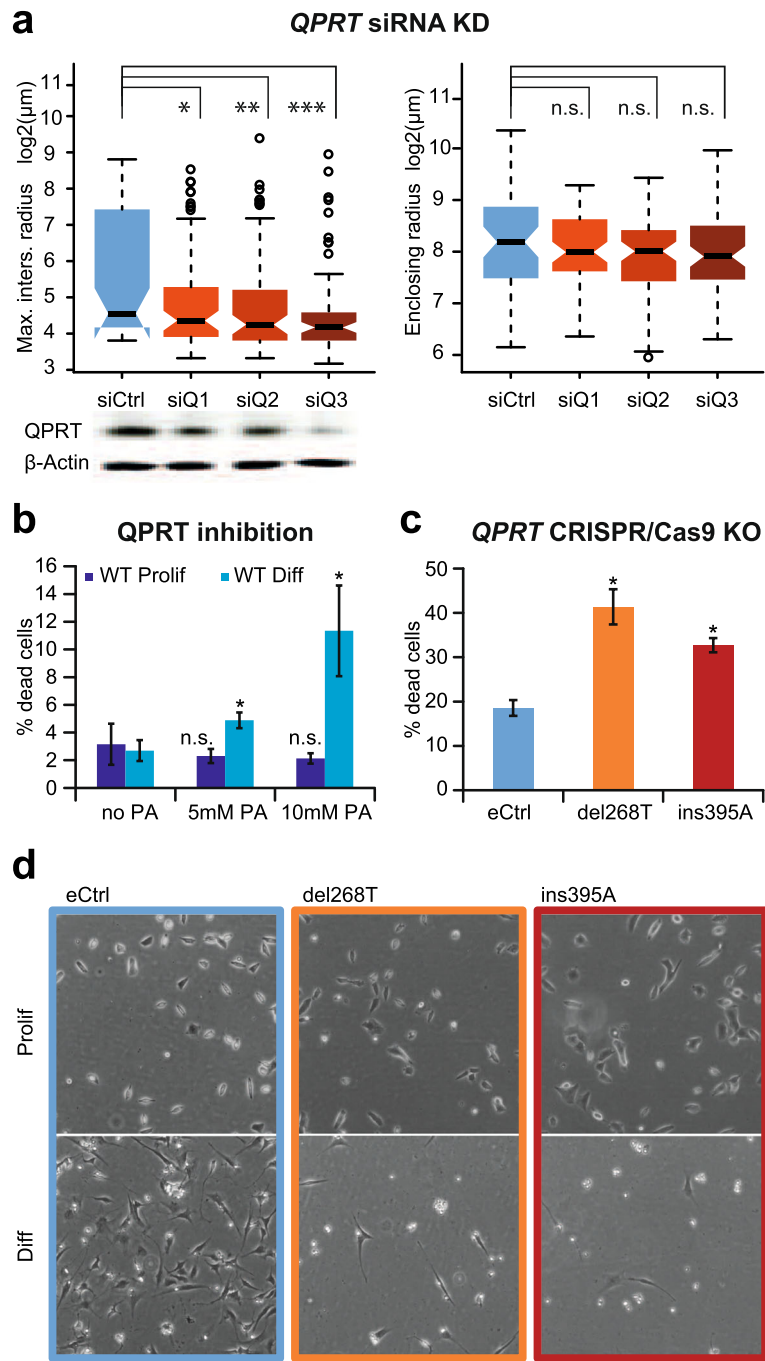
### **Functional validation in neuroblastoma cell line SH-SY5Y siRNA mediated knockdown (KD) of QPRT**

All three siRNAs targeting three different sites of *QPRT* (named here siQ1–siQ3) induced a decrease in *QPRT* protein after 11 days of differentiation as confirmed by Western Blot (Fig. 1a). At neuromorphological level, KD cell lines compared to control cell lines (siCtrl) showed a significant decrease of the maximum intersections radius, altering neuritic complexity in that the site of highest branching density was shifted closer towards the soma ( $p$  siQ1 0.027, siQ2 0.001, siQ3  $3.8E-04$ ; means [SD] siCtrl 103.98[115.54]; siQ1 56.50[80.53]; siQ2 56.29[97.17]; siQ3 41.51[76.70]). The overall cell size or the enclosing radius, did not change upon KD of *QPRT* (all  $p > 0.6$ ; means [SD] siCtrl 349.60[223.68]; siQ1 286.98[125.05]; siQ2 270.06 [135.62]; siQ3 293.81[176.86]; Fig. 1a; Additional file 1: Figure S2).

### **Mimicking of QPRT loss**

Cells exposed to an inhibitor of *QPRT*, the chemical phthalic acid (PA), showed an increased cell death upon induction of differentiation, and thus no end-point morphological analysis was performed. Application of PA for 3 days led to a dosage-dependent increase of cell death during differentiation (5 mM PA: FC = 1.81,  $p = 0.019$ ; 10 mM PA: FC = 4.21,  $p = 0.039$ ) but not during proliferation (5 mM PA: FC = 0.73,  $p = 0.432$ ; 10 mM PA: FC = 0.68,  $p = 0.355$ ; Fig. 1b).

We further hypothesized that inhibition of *QPRT* might lead to increased levels of its neurotoxic substrate quinolinic acid (QUIN). Thus, we exposed wild-type cells to elevated QUIN levels. However, we did neither observe an increase in cell death during proliferation (all  $p > 0.3$  for 5  $\mu$ M and 250  $\mu$ M QUIN) nor during differentiation (all  $p > 0.3$  for 5  $\mu$ M and 250  $\mu$ M QUIN; Additional file 1: Figure S5a) compared to vehicle only (0  $\mu$ M). Since the findings from chemical inhibition and mimicking of *QPRT* inhibition suggested that *QPRT* is causally linked to differentiation deficits independent of its substrate QUIN, we aimed at elucidating the



**Fig. 1** Functional analysis of *QPRT* in SH-SY5Y cells. **a** siRNA-mediated knockdown (KD) of *QPRT*. Cells were transfected with a non-targeting siRNA (siCtrl) and three different siRNAs targeting *QPRT* (siQ1–siQ3). Transfected cells were differentiated for 11 days followed by morphological analysis. Knockdown of *QPRT* was confirmed at protein level. Upon *QPRT*-KD, cells showed a significant decrease of the maximum intersections radius when compared to the non-targeting control, i.e., the maximum complexity of neurites was significantly closer to the cell soma. None of the three KDs differed with respect to the enclosing radius when compared to the non-target control, i.e., the length of the neurites was not different (Additional file 1: Figure S2). Maximum intersections radius: radial distance of the maximum number of intersections from the cell body. Enclosing radius: outer radius intersecting the cell. All *p* values were corrected for multiple testing using Tukey's HSD correction. **b** Chemical inhibition of *QPRT*. Application of the *QPRT* inhibitor phthalic acid (PA) for 3 days led to a dose-dependent significant increase of cell death in differentiating wild-type SH-SY5Y cells. In proliferating cells, *QPRT* inhibition did not change the rate of cell death. **c** Viability assays of CRISPR/Cas9 mediated *QPRT*-knock out (KO) cells. Percentage of cell death was assessed performing viability assays after 3 days of differentiation showing a significant increase of cell death in both generated *QPRT*-KO cell lines. **d** Representative images of *QPRT*-KO cells after differentiation. KO of *QPRT* led to observable cell death during 9 days of differentiation but not during proliferation



underlying processes in SH-SY5Y cells with a stable loss of *QPRT*.

#### CRISPR/Cas9 mediated knock out (KO) of *QPRT*

**Generation and viability** Using two separate sgRNA sequences (see Additional file 1: Supplementary Methods and Supplementary Results), we generated two homozygous *QPRT-KO* cell lines, del268T and ins395A (NM\_014298), both of which showed a nearly complete loss of expression of *QPRT* mRNA expression (proliferating cell lines: del268T: below detection limits, ins395A: FC = 0.17,  $p = 0.046$ ; 2 days of differentiation: del268T: FC = 0.13,  $p = 0.003$ ; ins395A: FC = 0.15,  $p = 0.003$ ; all compared to the proliferating empty control vector (eCtrl); Additional file 1: Figures S3 and S4). For proliferating cells, *QPRT-KO* was furthermore descriptively confirmed at protein level (Additional file 1: Figure S4). While the eCtrl and both *QPRT-KO* cell lines were growing comparably during proliferation, both KO cell lines died upon differentiation. After 3 days of differentiation, we observed a significant increase of cell death in the KO cell lines when compared to eCtrl cells harboring the empty control vector (eCtrl to del268T: FC = 2.23,  $p = 8.2E-04$ ; eCtrl to ins395A: FC = 1.76,  $p = 5.2E-04$ ; Fig. 1c), and after 9 days of differentiation, merely no differentiating KO cells were detected under the microscope (Fig. 1d).

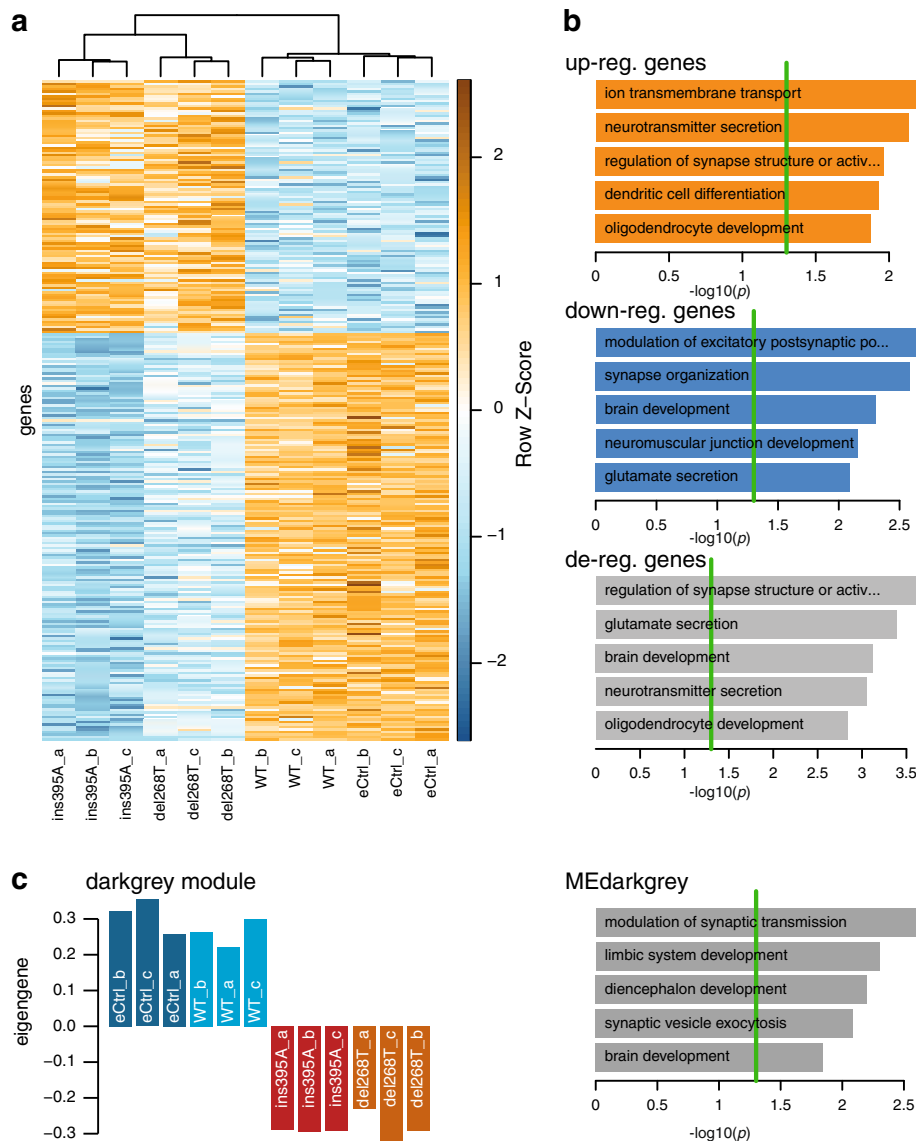
**Rescue experiments using small compounds** We tried to rescue the *QPRT-KO* effect by inhibiting the effector pathways downstream of QUIN, i.e., inhibit a potentially induced neurotoxicity by hyperactivation of NMDA-R as well as replenish  $NAD^+$ , which is the downstream metabolite of *QPRT* as well as the final outcome of the kynurenine pathway. Neither the NMDA-R antagonist MK801 (both *QPRT-KO* compared to eCtrl: FC > 1.7,  $p < 3.9E-03$  for 0  $\mu$ M; FC > 1.6,  $p < 0.04$  for 6  $\mu$ M; FC > 1.6,  $p < 1.5E-03$  for 12  $\mu$ M) nor the inhibition of the NMDA-R downstream enzyme NOS1 by L-NAME (both *QPRT-KO* compared to eCtrl: FC > 1.3,  $p < 0.05$  for 0 mM; FC > 1.3,  $p < 0.02$  for 0.5 mM; FC > 1.5,  $p < 0.01$  for 1 mM) nor supplying  $NAD^+$ , the downstream product of tryptophan catabolism (both *QPRT-KO* compared to eCtrl: FC > 1.3,  $p < 9E-03$  for 0 mM; FC > 1.3,  $p < 1.6E-03$  for 5 mM; del268T compared to eCtrl: FC = 1.20,  $p = 0.1$ ; ins395A compared to eCtrl: FC = 1.54,  $p = 0.009$  for 10 mM), resulted in a significant reduction of cell death for both of the KO cell lines upon differentiation (Additional file 1: Figure S5b).

**Metabolite analysis** To test if the loss of *QPRT* results in an altered metabolite profile of the kynurenine pathway, we implemented UHPLC and GC/MS analysis. We

were able to detect tryptophan (TRP), kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3HK), anthranilic acid (AA), and picolinic acid (PA). QUIN could not be detected in any of the samples, while 3-hydroxyanthranilic acid (3HAA) could only be detected in the differentiated (eCtrl and both of the KO) cell lines only. However, no significant changes could be observed in any of the metabolites when comparing both of the *QPRT-KO* cell lines to the control cell lines (Additional file 1: Figure S5c).

**MACE transcriptome analysis of *QPRT-KO*** Since in our cell model the effect of *QPRT-KO* on neuronal differentiation was not related to changes in the kynurenine pathway or the neurotoxic effects of QUIN, we aimed to further elucidate the effect of the KO on differentiating cells implementing a transcriptome-wide analysis. Overall, we were able to measure the expression of 32,739 transcripts (Additional file 2: Table S2). Statistical analysis identified 269 differentially regulated genes (103 upregulated and 166 downregulated; Fig. 2a; Additional file 2: Table S2) expressed in all three replicates of both KO cell lines, all with an FDR  $\leq 0.05$ . The 12 genes (Table 1) with an absolute  $\log_2FC > 2.5$  were technically validated using real-time RT-PCR with an average correlation between RNA sequencing data and real-time RT-PCR of  $\rho = 0.91$  (SD = 0.10). *QPRT-KO* was also confirmed (eCtrl vs del268T:  $\log_2FC = -2.44$ , FDR =  $1.07E-207$ ; eCtrl vs ins395A:  $\log_2FC = -3.17$ , FDR =  $9.88E-291$ ). Of the genes coding for components of the kynurenine pathway, only *NMNAT2* was significantly downregulated upon *QPRT-KO* (eCtrl vs del268T:  $\log_2FC = -0.70$ , FDR =  $2.10E-07$ ; eCtrl vs ins395A:  $\log_2FC = -1.51$ , FDR =  $6.70E-28$ ). The ASD-implicated protein *NLGN3* was differentially downregulated in the del268T only (eCtrl vs del268T:  $\log_2FC = -0.43$ , FDR =  $6.58E-02$ ; eCtrl vs ins395A:  $\log_2FC = -0.09$ , FDR =  $9.15E-01$ ) and was thus not considered to be regulated upon KO of *QPRT*.

The genes upregulated upon KO of *QPRT* were enriched (all  $p$  values  $< 0.05$ , Fig. 2b; Additional file 3: Table S3) for GO annotated biological processes involved in neurotransmitter secretion, regulation of synapse structure, or activation (Fig. 2b) but also negative regulation of cell growth and negative regulation of cytoskeleton organization among others (Additional file 3: Table S3). In addition, upregulated genes were enriched for the GO term apoptotic process involved in morphogenesis via the genes *BCL2 antagonist/killer 1 (BAK1)* and *scribbled planar cell polarity protein (SCRIB)*. Genes downregulated in KO cell lines showed enrichment for GO terms including synapse organization, modulation of excitatory postsynaptic potentials, or glutamate secretion (Fig. 2b) or positive regulation of neuron differentiation, positive regulation of dendritic spine development as



**Fig. 2** Whole transcriptome analysis of *QPRT-KO* cells and control cell lines. **a** Heatmap of differentially expressed genes. Upon KO of *QPRT*, 269 genes were significantly differentially expressed between both of the KO and the eCtrl cell line ( $FDR \leq 0.05$ ) but not between the controls (wild-type and eCtrl,  $FDR > 0.1$ ). Overall, ins395A descriptively shows stronger effects than del268T. **b** GO term enrichment for differentially regulated genes. Upregulated genes were associated with GO terms including neurotransmitter secretion, negative regulation of cell growth and negative regulation of cytoskeleton organization (all  $p < 0.05$ ; Additional file 3: Table S3). Genes downregulated upon *QPRT-KO* were enriched for GO terms involved in processes of neuronal development (positive regulation of neuron differentiation, positive regulation of dendritic spine development, and synapse organization (all  $p < 0.04$ )) and neurotransmitter transport (potassium transport, as well as glutamatergic processes like glutamate secretion and regulation of glutamate receptor signaling pathway (all  $p < 0.05$ )). Deregulated genes were enriched for processes like neurotransmitter secretion and brain development (all  $p < 0.05$ ; Additional file 3: Table S3). All  $p$  values account for the hierarchical structure of the gene ontology (GO) and can thus be considered as corrected for multiple testing. **c** Regulation of the dark grey gene set. This module was identified as *QPRT-KO* associated module harboring genes downregulated upon *QPRT-KO* when comparing both KO cell lines to wild-type and eCtrl cells (all  $p < 8E-07$ ). GO term enrichment analysis of this module again revealed processes involved in brain development and synaptic transmission and plasticity ( $p < 0.05$ ), confirming the association of *QPRT-KO* with neuronal development

well as ion transmembrane transport (Fig. 2b; Additional file 3: Table S3). We also observed significant enrichment for ASD genes among highly differentially regulated candidates. Of the 269 differentially regulated genes, 15 were listed in the SFARI gene [58] with an

evidence score of 4 or better ( $p = 9.2E-04$ , odds ratio OR [95% confidence interval] = 2.68 [1.47–4.54]), for example *gamma-aminobutyric acid type A receptor beta 3 subunit (GABRB3)*, *potassium voltage-gated channel subfamily Q member 3 (KCNQ3)*, *syntrophin*

**Table 1** Differentially expressed genes upon *QPRT-KO* with  $|\log_2FC| > 2.5$

Gene	Chr	del268T vs eCtrl		ins395A vs eCtrl		WT vs eCtrl		SFARI score	ASD literature
		FDR	log <sub>2</sub> FC	FDR	log <sub>2</sub> FC	FDR	log <sub>2</sub> FC		
<i>COX17</i>	3q13.33	3.33E-138	-5.94	1.78E-154	-5.63	0.30	-0.13	/	/
<i>GUCA1A</i>	6p21.1	4.56E-41	-5.38	3.97E-46	-5.88	0.20	0.26	/	/
<i>COX17P1</i>	13q14.13	1.09E-84	-5.19	5.82E-93	-5.28	0.59	-0.11	/	/
<i>VSTM2A</i>	7p11.2	1.81E-10	-5.00	6.36E-12	-5.28	0.61	-0.27	/	/
<i>KCNQ3</i>	8q24.22	5.77E-04	-4.76	1.25E03	-3.04	0.46	0.49	3	Role for <i>KCNQ3</i> in epilepsy and autism [75]
<i>CCK</i>	3p22.1	2.56E-04	-3.18	5.97E-05	-3.13	0.17	0.68	/	Candidate gene for Asperger's in a microdeletion case study [76]
<i>GABRB3</i>	15q12	3.04E-43	-3.00	9.43E-54	-3.58	0.80	0.04	2	CNV Chr15q11-13 implicated in ASD; polymorphisms associated with ASD [62, 77]
<i>BRINP1</i>	9q33.1	8.93E-09	-2.92	2.09E-09	-2.85	0.20	-0.53	/	-/- mice: autism-like behavior including reduced sociability and altered vocalization [78]
<i>LINC01760</i>	1p21.3	1.02E-02	2.74	4.93E-03	2.62	0.95	-0.30	/	/
<i>SNTG2</i>	2p25.3	7.04E-04	-2.71	5.06E-07	-5.58	0.72	0.22	4	Region linked with ID [64], associated with ASD [65]; interaction partner of neuroligins, interaction altered by ASD associated mutations [66]
<i>ARHGAP20</i>	11q23.1	1.63E-09	-2.65	4.08E-15	-3.93	0.45	-0.30	/	/
<i>SRRM4</i>	12q24.23	1.29E-18	-2.61	2.25E-26	-3.40	0.54	-0.20	/	-/+ mice: multiple autistic-like features [79]

Chr chromosomal region, *del268T* CRISPR/Cas9 induced mutation (deletion of one nucleotide) in exon 2 of *QPRT*, *ins395A* CRISPR/Cas9-induced mutation (insertion of one nucleotide) in exon 2 of *QPRT*, *eCtrl* control cell line with empty CRISPR/Cas9 control vector, *WT* wild-type SH-SY5Y cell line untreated, *FDR* False discovery rate, *log<sub>2</sub> FC* log<sub>2</sub> fold change, *SFARI score* score in the SFARI database [58] (a smaller score means higher evidence), *ASD references* Pubmed was searched for "gene and autism" and "gene and ASD"

*gamma 2 (SNTG2)*, or *contactin associated protein-like 2 (CNTNAP2)*.

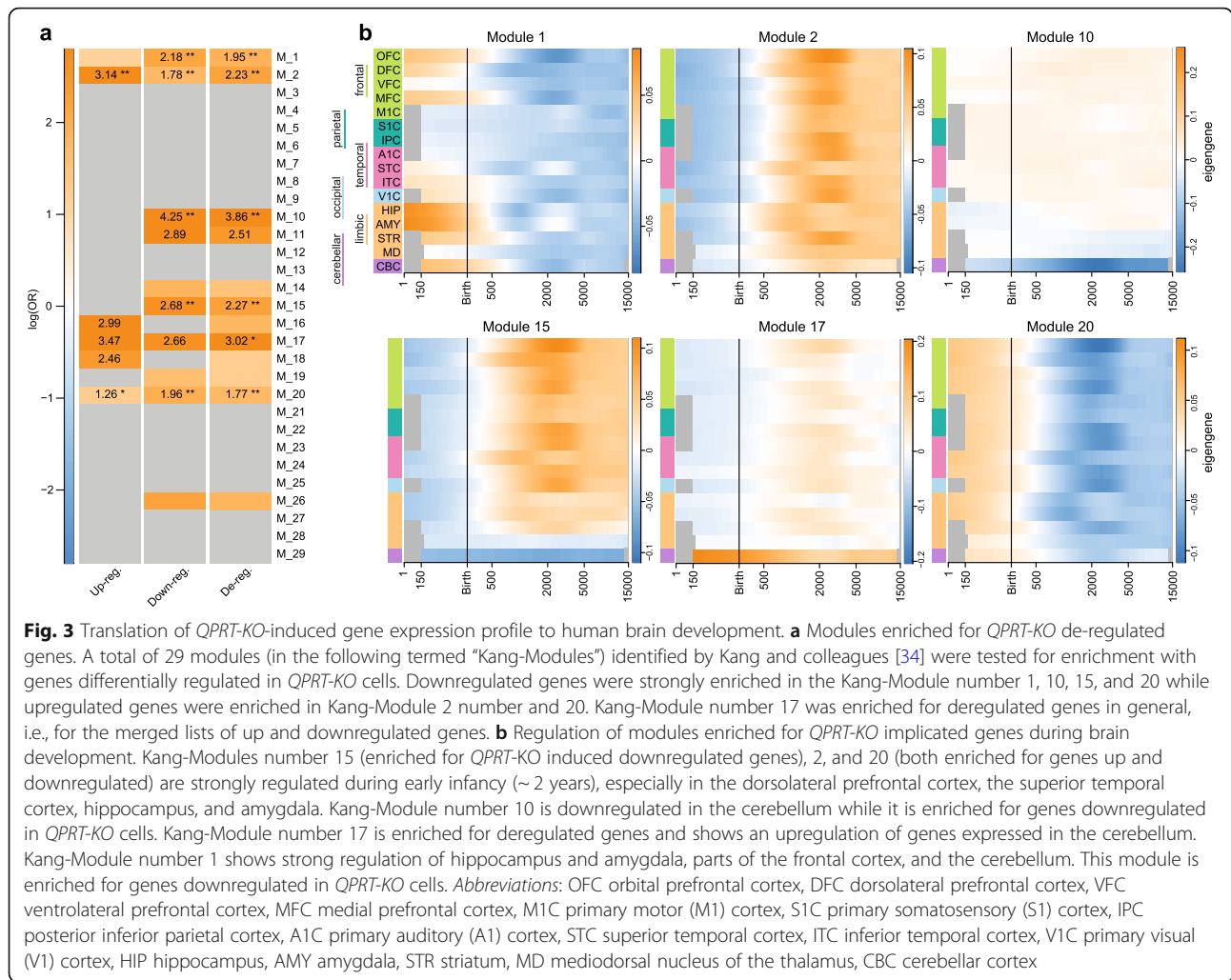
A total of eleven out of 166 downregulated genes and one gene of 103 upregulated genes showed a  $|\log_2FC| > 2.5$  (Table 1). Literature search for these twelve highly regulated genes revealed that six had been published in the context of ASD before (Table 1).

**Gene network analysis** At gene network level, we identified 20 co-regulated modules upon KO. Here, *QPRT* was co-regulated within a module associated with modulation of synaptic transmission, synaptic vesicle exocytosis, or limbic system and diencephalon development (Fig. 2c) as well as neurotransmitter secretion or negative regulation of neurogenesis ( $p < 0.04$ ). Overall, the eigenvalue of this module was not different between controls and KOs. We identified one co-regulated gene set (dark grey, Fig. 2c) to be associated with *QPRT-KO* as it was significantly downregulated in both KO cell lines when compared to the control cell lines (KOs versus eCtrl or WT, all  $p < 8E-07$ ). GO term enrichment analysis of this module again revealed processes involved in brain development and synaptic transmission and plasticity ( $p < 0.05$ ; Fig. 2c; Additional file 3: Table S3), confirming the association of *QPRT-KO* with neuronal development.

**Translation to developmental brain expression data**

Finally, we aimed to translate the effects of *QPRT-KO* onto the spatial and temporal gene expression network

of the brain using previously published data [34]. In this previous work, the authors report 29 gene modules (termed "Kang-Module" in the following) that are co-regulated during the development of human brain regions. We observe that the *QPRT-KO*-induced downregulated genes were strongly enriched in the Kang-Modules number 1 (odds ratio OR [95% confidence interval] = 8.86 [3.12–20.45],  $p = 5.76E-04$ ), number 2 (OR = 5.96 [3.55–9.75],  $p = 2.21E09$ ), number 10 (OR = 69.92 [20.70–188.44],  $p = 2.64E-07$ ), number 15 (OR = 14.58 [6.00–30.72],  $p = 1.57E-06$ ) and number 20 (OR = 7.13 [4.40–11.40],  $p = 7.74E-13$ ) while the *QPRT-KO* upregulated genes were enriched in Kang-Modules number 2 (OR = 23.20 [11.25–49.97],  $p = 1.07E-16$ ) and number 20 (OR = 3.54 [1.46–7.76],  $p = 4.18E-02$ ). Kang-Module 17 was enriched for *QPRT-KO*-induced deregulated genes in general, i.e., for the merged lists of up and downregulated genes (OR = 20.41 [2.33–82.57],  $p = 2.45E-02$ ; Fig. 3a). By plotting the eigengene value of the Kang-Module over time for each region, we observed that number 1 shows strong early upregulation in the hippocampus (HIP) and the amygdala (AMY) during embryonal development while it is downregulated in all tested brain regions after birth (Fig. 3b). Kang-Module 1 is also slightly upregulated in embryonal development in parts of the frontal cortex (orbitofrontal cortex (OFC), dorsolateral prefrontal cortex (DFC) and medial prefrontal cortex (MFC)), the striatum (STR), and the cerebellar cortex (CBC). Kang-Module 2 is overall



downregulated prenatally and shows upregulation after birth, with maximum expression between the ages of 6 and 14, i.e., during middle and late childhood in OFC, MFC, posterior inferior parietal cortex (IPC), primary auditory (A1) cortex (A1C), inferior temporal cortex (ITC), primary visual (V1) cortex (V1C), and STR. Kang-Module 10 is only expressed in the CBC with a peak around the age of 6 years, while Kang-Module 15 is strongly downregulated during embryonal development in the CBC. The low expression of Kang-Module 15 in the CBC is stable across the tested time course, and it is slightly up-regulated in other brain regions, showing peaks between the years 6 and 14 in OFC, DFC, ventrolateral prefrontal cortex (VFC), MFC, IPC, A1C, ITC, and V1C. Kang-Module 17 shows a constant active expression in the CBC over time with a peak during early development in the CBC. Kang-Module 20 is an overall early upregulated gene network downregulated after the age of ~ 2 years, showing the

strongest downregulation between the years 6 and 14 in OFC, MFC, IPC, A1C, ITC, V1C, and STR.

### Discussion

Based on our differential analysis, we report a causal relation between *QPRT*, located in the ASD-associated CNV region Chr16p11.2 and neuronal differentiation of SH-SY5Y cells. A gene dosage reduction or inhibition of *QPRT* affects morphological parameters during neuronal differentiation as well as the regulation of genes and gene networks that were previously implicated in ASD. This includes processes like synapse organization or brain development. In summary, our findings suggest a neurodevelopmental role for *QPRT* in the etiology of ASD in 16p11.2 deletion carriers.

As expected from previous findings [11], in one deletion carrier with ASD, we confirmed that *QPRT* was expressed in a gene dosage-dependent manner strengthening the role of *QPRT* as a potential risk gene in the pathology of Chr16p11.2 deletion syndrome. By

comparing our previously published transcriptome data of SH-SY5Y wild-type cells [28] to the morphological changes during neuronal differentiation described in the present study, we found *QPRT* to be correlated with the development of the neuritic complexity. These results suggest a potential regulatory link between *QPRT* and neuronal maturation. Our findings of the KD and KO cell models further underline this interpretation: the KD of *QPRT* led to subtle changes of SH-SY5Y neuronal complexity in line with previous reports using mouse models of Chr16p11.2 [17] as well as iPS cells generated from 16p11.2 CNV carriers [59] and post-mortem studies of ASD individuals [1]. Inhibition of *QPRT* activity as well as genetic KO both led to cell death of differentiating but not proliferating cells. These findings suggest that enough protein is left for the survival of differentiating cells upon KD of *QPRT* while the loss of *QPRT* is lethal for differentiating SH-SY5Y cells. Interestingly, the administration of the *QPRT* substrate QUIN, which is a potent excitotoxin, did not show any effect on neither proliferating nor differentiating cells. Previous findings suggested that a reduction of *QPRT*, the only enzyme catabolizing quinolinic acid (QUIN), leads to an accumulation of QUIN, which in turn may induce neuronal cell death by over-activating NMDA-R and increase nitric oxide (NO) production [46]. Altered QUIN levels could also lead to a change in NAD<sup>+</sup> production which in turn could change poly (ADP-ribose) polymerase (PARP) activity [60]. In *QPRT-KO* mice, the striatum showed an accumulation of QUIN leading to neurodegeneration [30] as well as altered expression of enzymes of the kynurenine pathway and of NMDA-receptors. No ASD-like behaviors were studied in these mice, but the animals showed no growth or developmental abnormalities. As the kynurenine pathway was associated with Parkinson's disease [61], the authors performed a behavioral test measuring the stride length of WT and KO mice. Indeed, they reported shorter stride lengths in aged but not middle-aged *QPRT-KO* mice as usually seen in mouse models of Parkinson's disease [30]. In another study, no differences of histological features of the cerebrum of *QPRT-KO* mice were observed [31]. Although we were not able to mimic *QPRT* loss in WT cells by application of increased QUIN levels, we tried to rescue differentiating *QPRT-KO* cells from cell death during differentiation by modulation of QUIN-related metabolism and signaling. Inhibition of NMDA-receptors was expected to protect *QPRT-KO* cells from the possible QUIN-induced excitotoxicity. However, the application of different concentrations of an NMDA-R inhibitor did not prevent cell death. Similarly, inhibition of NOS1 with the aim to reduce the production of NO did not affect viability. Finally, we tried to increase the viability of

differentiating cells via the application of NAD<sup>+</sup> to rescue PARP activity. Again, *QPRT-KO* cells could not be rescued from cell death during differentiation. In accordance with these observations, *QPRT-KO* cells did not show changes in the expression levels of any genes coding for PARP enzymes. In addition, QUIN could not be detected in any of our samples, and none of the metabolites of tryptophan catabolism was significantly changed upon KO of *QPRT* in the performed metabolite assays. Taken together, we conclude that the detrimental effect of *QPRT* loss on viability during SH-SY5Y differentiation is independent of QUIN levels as well as of QUIN-induced metabolic changes or the kynurenine pathway in general.

To elucidate the underlying mechanisms of *QPRT-KO*-induced cell death during SH-SY5Y neuronal differentiation, we performed a hypothesis-free transcriptomic approach. These findings suggest that loss of *QPRT* may lead to an increased negative regulation of cytoskeleton organization and to an inhibition of neuronal differentiation and dendritic spine development. In our KD model, we observed an alteration of neuritic complexity, which strongly supports the functional role of *QPRT* in these processes.

The functional association of *QPRT* with ASD is supported by the following results of our study: *QPRT-KO* led to inhibition of *GABRB3*, which has been well established as an ASD risk gene [62]. *GABRB3* codes for a subunit of an inhibitory GABA receptor. It is located on Chr15q11-13, a region strongly implicated in ASD [63]. Similarly, the gene *SNTG2* also downregulated in *QPRT-KO* cells, codes for a synaptic scaffolding protein involved in actin and PDZ domain binding. *SNTG2* is located in 2p25.3, a region linked to intellectual disability [64] and associated with ASD [65]. Furthermore, *SNTG2* protein interacts with *neuroligins* (*NLGN*), and this interaction is altered by ASD associated mutations in the *NLGN* genes [66]. *QPRT* was also found to physically interact with *NLGN3* (neuroligin 3; [33]). Although the function of this interaction is still unclear, it is likely that *QPRT* is involved in the formation of the postsynaptic density of GABAergic neurons. *KCNQ3*, another ASD candidate gene, downregulated upon *QPRT-KO*, encodes a protein regulating neuronal excitability. Variants of this gene were found to be implicated in the development of ASD and epilepsies [67]. Finally, we also found *CNTNAP2*, a well-replicated risk gene for ASD [68] located in 7q22-q36, to be downregulated in *QPRT-KO* cells. In a previous study, we reported *CNTNAP2* promoter variants reducing transcription to be risk factors for ASD [69].

Besides ASD-associated differentially regulated genes, we report six novel candidates (i.e., *COX17*, *GUCA1A*, *COX17P1*, *VSTM2A*, *LINC01760*, and *ARHGAP20*). At

this stage, we cannot find any link to neuronal development for the photoreceptor-associated *guanylate cyclase activator 1A GUCA1A*, the preadipocyte development implicated *V-set and transmembrane domain containing 2A VSTM2A* gene, and the long intergenic non-coding RNA *LINC01760*. However, the *cytochrome c oxidase copper chaperone COX17* (and its pseudogene *COX17P1*) and the *Rho GTPase-activating protein 20 ARHGAP20* are functionally related to neuronal development: *COX17* is part of the terminal component of the mitochondrial respiratory chain, catalyzing the electron transfer from reduced cytochrome c to oxygen, and might thus be involved in the regulation of oxidative stress and energy metabolism, both processes that have previously been associated with ASD [36]. The *ARHGAP20* enzyme is implicated in neurite outgrowth and thus potentially associated with the here observed neuromorphological phenotypes [70].

Translating the in vitro transcriptomic effect of *QPRT-KO* in SH-SY5Y cells to the gene networks active during human brain development [34], we observed differentially regulated genes to be enriched in modules previously associated with cell cycle regulation (Kang-Module 1), transcription factors regulating progenitor cell fate (Kang Module 20), neuronal development (Kang-Module 10 and 20), morphogenesis (Kang Modules 17 and 2), and synaptic transmission (Kang-Module 2 and 15). In addition, the Kang-Modules identified to be affected by *QPRT-KO* were also reported to be highly co-expressed ( $\rho \geq 0.68$ ) with markers for glutamatergic (Kang-Module 10) and GABAergic neurons (Kang-Modules 2 and 15) or astrocytes (Kang-Module 2). In addition, Kang-Module 1 is implicated in the early development of the hippocampus and amygdala, two regions significantly associated with ASD after meta-analysis [71, 72]. These associations identified in the translational approach suggest that *QPRT* loss might trigger alterations in the development of excitatory/inhibitory neuronal networks, a pathomechanism postulated to underlie the etiology of ASD [73, 74].

In summary, our findings allow us to conclude that in the neuroblastoma model SH-SY5Y a loss of *QPRT* impairs neuronal development in vitro by changing genetic networks previously implicated in the etiology of ASD. To confirm these results and the role of *QPRT* in the etiology of ASD in general, further studies in other neuronal or animal models are needed. In particular, analyzing the above described *QPRT-KO* mouse model [30, 31] could elucidate the effect of *QPRT* loss at systems level.

## Conclusions

Here, we report *QPRT*, a gene within the ASD-associated 16p11.2 CNV region, to be essential for and causally related to SH-SY5Y neuronal differentiation in vitro. This corroborates postmortem findings of a disturbed

neuronal development in ASD. The functional mechanism is still elusive; however, based on our results, we can exclude alterations of QUIN levels or other products of the kynurenine pathway in the here described SH-SY5Y model. Further, the transcriptomic approach suggests that a reduced availability of *QPRT* impacts on genes and networks associated with ASD, such as neuron differentiation, synapse organization, or the development of excitatory/inhibitory neurons. Overall this study suggests an alteration of *QPRT* expression or of *QPRT* related genes to be underlying the etiology of ASD in 16p11.2 CNV carriers. Further studies are needed to confirm our findings in more mature neuronal systems or animal models.

## Additional files

**Additional file 1: Supplementary information:** Supplementary methods, supplementary results, supplementary table S1, supplementary figures S1-S6. (DOCX 555 kb)

**Additional file 2: Supplementary table S2.** Results from DESeq2 analysis and count data from RNA-Seq (MACE analysis). (XLSX 5308 kb)

**Additional file 3: Supplementary table S3.** Results from GO term enrichment analysis. (XLSX 36 kb)

## Abbreviations

3HAA: 3-Hydroxyanthranilic acid; 3HK: 3-Hydroxykynurenine; A1: Primary auditory; A1C: Primary auditory (A1) cortex; AA: Anthranilic acid; ACTB:  $\beta$ -Actin; ADI-R: Autism Diagnostic Interview-Revised; ADORA2A: Adenosine receptor 2A; ADOS: Autism Observation Schedule; AMY: Amygdala; ARHGAP20: Rho GTPase-activating protein 20; ASD: Autism spectrum disorder; BAK1: BCL2-antagonist/killer 1; BDNF: Brain-derived neurotrophic factor; BRINP1: BMP/retinoic acid-inducible neural specific 1; CBC: Cerebellar cortex; CCK: Cholecystokinin; CNTNAP2: Contactin-associated protein-like 2; CNV: Copy number variation; COX17: COX17, cytochrome c oxidase copper chaperone; COX17P1: COX17, cytochrome c oxidase copper chaperone pseudogene 1; del268T: *QPRT* NM\_014298 del268T; Ex2.1; DFC: Dorsolateral prefrontal cortex; DMEM: Dulbecco's modified Eagle medium; DOC2A: Double C2 domain alpha; DPBS: Dulbecco's phosphate buffered saline; DRD2: Dopamine receptor D2; eCtrl: Empty control vector; ERK/MAPK: Extracellular signaling-related kinase; FAM57BA: Family with sequence similarity 57, member B; FBS: Fetal bovine serum; FC: Fold change; GABRB3: Gamma-aminobutyric acid type A receptor beta3 subunit; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GC/MS: Gas chromatography-mass spectrometry; GUCA1A: Guanylate cyclase activator 1A; GUSB: Glucuronidase beta; HIP: Hippocampus; HSD: Honest significance difference; ins395A: *QPRT* NM\_014298 ins395A; Ex2.2; IPC: Posterior inferior parietal cortex; ITC: Inferior temporal cortex; KA: Kynurenine acid; KCNQ3: Potassium voltage-gated channel subfamily Q member 3; KCTD13: Potassium channel tetramerization domain containing 13; KD: Knockdown; KIF22: Kinesin family member 22; KO: Knock out; KYN: Kynurenine; LCL: Lymphoblastoid cell line; LINC01760: Long intergenic non-protein coding RNA 1760; MACE: Massive analysis of cDNA ends; MAPK3: Mitogen-activated protein kinase 3; MFC: Medial prefrontal cortex; NAD: Nicotinamide adenine dinucleotide; NLGN: Neuroligins; NLGN3: Neuroligin 3; NMDA-R: N-methyl-D-aspartate receptor; NMNAT2: Nicotinamide nucleotide adenyltransferase 2; NO: Nitric oxide; NOS1: Nitric oxide synthase 1; OFC: Orbitofrontal cortex; PA: Phthalic acid; PARP: Poly (ADP-ribose) polymerase; PI: Propidium iodide; PIC: Picolinic acid; PSM7: Proteasome 26S subunit, non-ATPase 7; *QPRT*: Quinolinate phosphoribosyltransferase; QUIN: Quinolinic acid; RA: Retinoic acid; RIN: RNA integrity number; SCRIB: Scribbled planar cell polarity protein; siCtrl: Non-targeting siRNA control; siQ1-siQ3: *QPRT* targeting siRNA1-3; SNTG2: Syntrophin gamma 2; SRRM4: Serine/arginine repetitive matrix 4;

STR: Striatum; TAOK2: TAO kinase 2; TBX6: T-box 6; TRP: Tryptophan; UHPLC: Ultra-high-performance liquid chromatography; UPL: Universal probe library; V1: Primary visual; V1C: Primary visual (V1) cortex; VFC: Ventrolateral prefrontal cortex; VSTM2A: V-set and transmembrane domain containing 2A; WGCNA: Weighted gene co-expression network analysis; WT: Wild type

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#### Availability of data and materials

The RNA-sequencing dataset supporting the conclusions of this article is available at the GEO repository, GEO accession number: GSE113734 at <https://www.ncbi.nlm.nih.gov/geo/>. Additional files are provided with the manuscript.

#### Authors' contributions

AGC and DH designed the study and wrote the manuscript. DH, SL, and IS performed the experiments. CMF, RW, and AY supported the manuscript preparation. AGC, DH, NK, BR, and AY performed the statistical analyses. TA, BKG, and MMT shared their experience and protocols for CRISPR/Cas9 experiments. GJG and CKL shared expertise on tryptophan metabolism and performed the metabolite analysis. SF and IS helped in designing and performing siRNA experiments, as well as imaging of transfected cells in a high throughput manner, and shared their expertise and equipment. AGC, CMF, and AAP hosted fruitful discussions for project planning and strongly supported the project. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All study participants have given written informed consent, and the genetic study has been positively reviewed by the ethic's committee Frankfurt (No 267/09).

#### Consent for publication

All authors agree to publish the presented work.

#### Competing interests

All authors declare that they have no competing interests.

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