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## Olfactory stem cells reveal MOCOS as a new player in autism spectrum disorders

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## Abstract

With an onset under the age of three, autism spectrum disorders (ASD) are now understood as diseases arising from pre- and/or early post-natal brain developmental anomalies and/or early brain insults. In order to unveil the molecular mechanisms at play during the misshaping of the developing brain, we chose to study cells that are representative of the very early stages of ontogenesis, namely stem cells. Here, we report on MOCOS, an enzyme involved in purine metabolism (Mendel and Kruse, 2012), as a newly identified player in ASD. We found in adult nasal olfactory stem cells of 11 adults with ASD that *MOCOS* is downregulated in most of them when compared to 11 age- and gender-matched control adults having no neuropsychiatric disorders. Genetic approaches using *in vivo* and *in vitro* engineered models converge to indicate that altered expression of *MOCOS* results in neurotransmission and synaptic defects. Furthermore, we found that *MOCOS* misexpression induces increased oxidative stress sensitivity. Our results demonstrate that altered MOCOS expression is likely to impact on neurodevelopment and neurotransmission, and may explain comorbid conditions, including gastro-intestinal disorders. We anticipate our discovery to be a fresh starting point for the study on the roles of MOCOS in brain development and its functional implications in ASD clinical symptoms. Moreover, our study suggests the possible development of new diagnostic tests based on MOCOS expression, and paves the way for drug screening targeting MOCOS and/or the purine metabolism to ultimately develop novel treatments in ASD.

## **Introduction**

Autism spectrum disorders (ASD) are complex neurodevelopmental diseases arising from multifactorial genetic, epigenetic and environmental origins (Gepner and Feron, 2009; Lai et al., 2014; Levy et al., 2009; Weintraub, 2011). Systems-level connectivity features (Rubenstein, 2010) and plausible neuroanatomical (Travers et al., 2012), cellular (Kern and Jones, 2006; Zeidan-Chulia et al., 2014), and molecular (Geschwind, 2011) underpinnings of ASD have been highlighted. Although hundreds of susceptibility genes have been identified, being generally involved in neurobiological functions such as neurogenesis, synaptogenesis and neurotransmission (Berg and Geschwind, 2012), collectively they account for 10-20% of ASD cases at most (Abrahams and Geschwind, 2008). Accordingly, discovering common genetic traits being more representative of ASD population becomes a priority for early diagnoses (Walsh et al., 2011), investigation of ASD physiopathology and identification of new therapeutic targets.

ASD-associated profiles are underpinned by atypical neural development often accompanied by epilepsy, gastrointestinal disorders and other comorbid disorders (Lai et al., 2014). Despite numerous studies, the etiopathology as well as the physiopathogeny of these disorders remain largely elusive. Up to date, genetic studies on ASD have mainly used cellular material that, even though being sufficient for mutagenic studies, might be irrelevant to identify gene misexpression during development. In order to seek for novel candidates at play in ASD, we chose to study cells that are representative of early stages of ontogenesis. To this end, we decided to use human nasal olfactory stem cells (OSCs) displaying multipotent properties (Murrell et al., 2005) (Delorme et al., 2010) and proved to be useful for transcriptomic analyses in the context of brain disorders (Boone et al., 2010; Matigian et al., 2010).

Our study is based on a relatively homogeneous cohort of nine adults with severe autism and low to very low developmental abilities, plus two adults with mild or moderate autism and no or mild cognitive abilities (Asperger syndrome or high-functioning autism), paired with 11 age- and gender-matched control individuals. We first chose a non hypothesis-driven approach and looked for transcriptome anomalies, using pangenomic cDNA microarrays. We observed a dysregulated expression of genes already associated to ASD. However, for the very first time, we identified a new candidate gene, the *MOLybdenum COfactor Sulfurase (Mocos)*. Since next to nothing was known about this gene, we decided to assess its roles during the development of the nervous system, via *C. elegans*, an animal model widely used in studies focused on genetic and molecular anomalies in the developing brain. We found that the genetic ablation of *Mocos* induced abnormal morphologies in some neuronal populations and an alteration of the response to various stresses.. Then, we moved back to human cells and looked at their capacity to respond to a stressful situation. We also generated human induced pluripotent cells (iPS) in which the gene *Mocos* was partially knocked down and assessed their capacity to create synapses when differentiated into neurons.

## **Materials and methods**

### ***Participants***

A complete written and oral information on the goal and procedure of this research was given to the participants or their legal tutors, and a signed informed consent was obtained from all of them prior to their involvement in the study. All procedures were approved by the local ethical committee (Comité de Protection des Personnes, files #205016 and #205017) of Marseille.

Patients with ASD (n=11) were recruited in three public hospital units (Valvert Hospital, Marseille; Montperrin Hospital, Aix-en-Provence; Edouard Toulouse Hospital, Marseille), in two socio-medical private units (Foyer d'accueil médicalisé, Pélissanne; Service d'accompagnement medico-social pour adultes autistes, Salon de Provence), and through a specialized ASD diagnosis consultation (Montperrin Hospital, Aix-en-Provence). The patients were diagnosed according to ICD-10 (World Health Organization, 1993) and DSM-5 (American psychiatric association, 2013) criteria for pervasive developmental disorders and autism spectrum disorders, respectively. Control patients were recruited to match the age and gender of each ASD patient that was endorsed in the cohort. None of the healthy controls was neither presenting a neuropsychiatric disorder nor taking medication. Demographics of the cohort is summarised in supplementary Tables 1 and 2. Details on, risk factors, comorbidity, medication and MRI data of ASD patients of the cohort are given in supplementary Tables 3-5.

### ***Nasal biopsies***

Nasal biopsies were performed by an ENT surgeon as we previously described (Girard et al., 2011). For ASD patients, the tissue was biopsied right before a brain magnetic resonance imaging (MRI) examination, requiring a general anesthesia in all of them. Control participants were biopsied during an ENT surgery.

### ***Cell culture***

For nasal olfactory stem cells (OSCs) culture, fresh biopsies were immediately placed in growth medium containing DMEM/Ham F12 supplemented with 10% Fetal Bovine Serum (FBS) and 100 units/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen). OSCs were purified from the *lamina propria* and cultivated as described before (Delorme et al., 2010; Girard et al., 2011). All experiments using OSCs were performed with cells at a low passage number (P1 to P5). When the two cohorts were compared, the same passage number was used.

For induced pluripotent stem cells (iPSCs) culture, we used previously established and characterized iPSC lines (Xia et al., 2013) derived from human fibroblasts by using an episomal-based reprogramming strategy. In feeder-free conditions, human iPSCs were cultured in chemically defined growth media, mTeSR (StemCell technologies), on growth-factor-reduced Matrigel (BD Biosciences)-coated plates. Briefly, 70–80% confluent human ESCs/iPSCs were treated with dispase (Invitrogen) for 7 min at 37°C and the colonies were dispersed to small clusters and lifted carefully using a 5 ml glass pipette, at a ratio of ~1:4. Prior to the generation of *MOCOS*<sup>+/-</sup> iPSCs, iPSCs were transferred onto irradiated neomycin-resistant MEFs and cultured in WiCell media composed of DMEM/F12 (Invitrogen), 20% Knockout serum replacement, 10 ng/ml bFGF, 1 mM GlutaMax, 0.1 mM non-essential amino acids and 55 µM β-mercaptoethanol. After 2 to 3 days of culture in these conditions, MEFs-conditioned medium was used instead of fresh WiCell.

Mytomycin-C treated Mouse Embryonic Fibroblasts (MEFs, Chemicon) and irradiated neomycin-resistant MEFs (Applied Stem Cell) were plated onto gelatin (0.1%)-coated plate in a medium containing DMEM supplemented with 10% FBS and 1% non-essential amino acids. For our experimental conditions, MEFs were plated at 200,000 cells/well of a 6-well plate or 1x10<sup>6</sup> cells/100mm dish. MEFs-conditioned medium was prepared by replacing the



MEF medium of Mytomycin-C treated MEFs with WiCell media, 24hours after MEFs thawing. The conditioned media was collected and filtered (0.2  $\mu\text{m}$ ) every 24hours for up to 5 days of MEFs culture. All cell lines were maintained in an incubator (37°C, 5% CO<sub>2</sub>) with media changes every day (iPSCs, MEFs) or every second day (OSCs).

Primary cultures of cortical neurons were prepared from C57/Bl6 mice embryos according to the guidelines of the Ethics Committee of the Medical Faculty of Marseille and conform to National and European regulations (EU directive Nu 86/609). Pregnant mice were deeply anesthetised with sodium pentobarbital (Ceva Santé Animale) prior to remove E16 embryos from their placenta. Embryos were quickly removed and placed into cold Hank's balanced salt solution (HBSS, Life Technologies) containing 5 U/ml penicillin/streptomycin (Life Technologies). Embryos were decapitated and cerebral cortices were dissected prior to be enzymatically dissociated for 10 min at 37°C in HBSS containing 0.1% trypsin and 10 mg/ml DNase I (Sigma-Aldrich, Saint Quentin Fallavier, France). The enzymatic reaction was stopped with DMEM containing 10% Fetal calf serum (FCS, Life Technologies) and 5 U/ml penicillin/streptomycin (Life Technologies). Further mechanical dissociation was carried out by trituration through a Pasteur pipette. After centrifugation at 1,250 rpm during 5 min at room temperature, cell pellets were resuspended in plating medium containing DMEM supplemented with 10% FCS and 5 U/ml penicillin/streptomycin. Cell suspension was plated at a seeding density of  $1.5 \times 10^5$  cells/well onto 12 mm diameter glass coverslips precoated with 1 mg/ml poly-L-Lysine (Sigma-Aldrich) in filtered-borate buffer pH 8.5, and were incubated at 37°C in a humidified chamber containing 5% CO<sub>2</sub>. After 1 hour, the plating medium was replaced by a serum free defined medium containing Neurobasal (Life Technologies) supplemented with 2% B27 supplement (Life Technologies), 5 U/ml penicillin/ streptomycin, 2.5 mM Glutamine (Life Technologies). Neurons were allowed to grow for 4 days (37°C, 5% CO<sub>2</sub>). Then, cells were fixed for 20 min with Antigenfix solution

(Diapath) at room temperature. After washing in PBS 1X, cells were stocked at 4°C until being processed for immunostaining.

### ***Hybridization on pangenomic cDNA arrays***

In order to limit biases, OSCs from the 22 individuals were collected by the same operator, under the same conditions: i.e. identical culture medium, level of confluency (100%) and equal passaging (P3). In addition, in order to avoid any circadian rhythm-related variations in gene expression, cells were collected at the same time of the day. Total RNA was then isolated using RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration was determined using a nanodrop 2000 spectrophotometer (Thermo Scientific) and RNA integrity assessed on an Agilent 2100 Bioanalyzer. Sample amplification, labeling, and hybridization were performed in line with the Agilent two-color microarray-base analysis (low input quick amp labeling) protocol (Agilent Technologies). In brief, total RNA was reverse transcribed into cDNA using the T7 promoter primer. The reaction intending to synthesize cyanine-3 labeled cRNA from cDNA was performed in a solution containing dNTP mix, T7 RNA polymerase and cyanine 3-dCTP and then incubated at 40°C for 2 hours. Labeled cRNA was purified and fragmented before hybridization on Agilent whole human genome Expression Arrays, containing 62,975 oligonucleotide probes, at 65°C for 17 hours. Raw microarray signals were scanned and extracted using Agilent Feature Extraction Software (Agilent Technologies). AgiND R package was used for quality control and normalization. Quantile methods and a background correction were applied for data normalization. Microarray data have been deposited in NCBI-GEO under the accession number GSE63524.

### ***Microarray data analysis***

Biological interpretation of the data was performed using two bioinformatic softwares: Ingenuity Pathway Analysis (IPA, Ingenuity Systems; <http://www.ingenuity.com/>) and MeV

(MultiExperiment Viewer Version 4.3; <http://mev.tm4.org>). The first program was used to identify biological functions and upstream regulators enriched in the lists of differentially expressed genes associated to the psychiatric condition. Right-tailed Fisher's exact test was used to calculate a p-value determining the top statistically significant biological functions assigned to the data set. The second program allowed us to draw a volcano plot representation, enabling a visual identification of genes that display statistically significant changes. For both analyses, we considered only genes with an absolute fold change of 1.5 and a p value <0.05.

### ***RNA isolation and Real-time PCR analysis***

RNA samples extracted from OSCs were tested with qRT-PCR in order to quantify the expression of *MOCOS*. Total RNA (1 µg) was subjected to reverse transcription reaction to synthesize cDNA using oligo dT, RNase Out and M-MLV RT enzyme (Invitrogen, ThermoFisher Scientific, Villebon sur Yvette, France) according to the manufacturer's instructions. Real time qPCR experiments were carried out with the 7500 Fast real-Time PCR system (Applied Biosystems, ThermoFisher Scientific, Villebon sur Yvette, France), using TaqMan® Fast Universal PCR Master Mix (2X) and two TaqMan® Gene Expression Assays (*MOCOS*: Hs00215742\_m1; *TBP*: Hs00920495\_m1). Experiments used 7.5 ng of previously prepared cDNA and samples were run in triplicates. Relative expression levels were determined according to the  $\Delta\Delta C_t$  method where the expression level of the mRNA of interest is given by  $2^{-\Delta\Delta C_t}$  where  $\Delta\Delta C_t = \Delta C_t \text{ target mRNA} - \Delta C_t \text{ reference mRNA}$  (*TBP*) in the same sample.

### ***Western blot analysis***

OSCs were lysed and homogenized in RIPA buffer (Sigma-Aldrich) containing a cocktail of protease inhibitors (1/100, Calbiochem). Protein concentrations were determined by the DC protein assay (Biorad, Hercule, CA) according to the manufacturer's protocol. For all

conditions, Western blot were performed from 20 µg of proteins, using SDS-PAGE 10% polyacrylamide resolving gels (MiniBlot system, Bio-Rad) and then transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). Before blocking, the blots were stained with ponceau red to visualize transfer efficiency. Protein-containing membrane was blocked using PBS-5% skim milk for 2 h at room temperature and then incubated overnight at 4°C with a rabbit anti-MOCOS antibody (Novus biologicals NBP2-14243, 95kDa, 1/100) and a mouse anti-GAPDH antibody (Millipore MAB374, 37k Da, 1/5000) in PBS-5% skim milk. After incubation with primary antibodies, blots were washed three times for 10 min with a solution containing PBS and 0.2% Tween-20 (PBS-T), incubated with goat anti-rabbit-HRP (1/1000, Jackson Immunoresearch) or goat anti-mouse-HRP (1/5000, Jackson ImmunoResearch) in PBS-5% skim milk for 2 hours at RT, and washed three times for 10 min each with PBS-T. Finally, proteins were detected using a chemiluminescence kit (Amersham RPN2209). Chemiluminescent signals were projected on X-ray film and digitalized, and the signals were quantified using NIH ImageJ software (version 1.43).

### ***Animals***

*C. elegans* strains were maintained at 20°C under standard growth conditions. Strains used in these studies include: N2 (wild type), *mocs-1(tm3708)*, *mocs-1(ok3439)*, *ric-3(md158)*, *eri-1(mg366)*, *mev-1(kn1)*, *arls37 [Pmyo-3::ssGFP]*, *nuIs183 [unc-29-nlp-21::YFP]*(Sieburth et al., 2007) and VAP1::RFP (generous gift of L. Bianchi, University of Miami). The modified WRM0612B\_E02 fosmid was obtained from the TransgeneOme project. In this vector, the GFP coding sequence was fused to the 3' end of *mocs-1*(Sarov et al., 2012). Wild type transgenic animals were selected after bombardment of *unc-119(ed3)* mutants. The *tm3708* mutant contains a 621 base pair deletion that removes intron 1 and part of exon 2 (See Fig. 3a). The *tm3708* deletion was predicted to represent a null allele because the mutation is

generating a premature amber stop codon after only 10% of the coding region. The *ok3439* deletion removes part of exon 2 and all subsequent exons up to exon 5 (See Fig. 3a). This mutant displays an in frame deletion of the L-cysteine desulfurase domain: *ok3439* is predicted to be devoid of 163 amino acids in a region known to bind the PLP cofactor and mobilize sulfur from L-cysteine. Thus *ok3439* deletion mutants are supposed to be free of their cysteine desulfurase activity while the MOSC domain remains intact.

C57/Bl6 mice (8-week old) were used to evaluate the expression of MOCOS in various organs as well as to establish primary cultures of neurons. All murine experiments were conducted according to the guidelines of the Ethics Committee of the Medical Faculty of Marseille and conform to National and European regulations (EU directive Nu 86/609). Animals were housed in air-flow racks on a restricted access area and maintained on a 12 hour light/dark cycle at a constant temperature ( $22 \pm 1^\circ\text{C}$ ).

#### ***Paraquat assay***

For paraquat sensitivity during development, adult animals were placed on plates containing increasing concentrations of paraquat (0.15, 0.22, 0.44, 0.66 and 4 mM), allowed to lay eggs for 6 hours and then removed. Over a 4-day period, the proportion of animals at every development stage (L1-L4) progeny was scored. The total number of worms that survived after exposure to 4 mM of paraquat was assessed. To test the sensitivity of adult worms to oxidative stress, young adults (day1) were transferred to fresh plates containing 4 mM of paraquat. The total number of worms that survived was counted after 24, 48 and 72 hours.

#### ***RNAi procedures***

Bacteria expressing dsRNA directed against R03A10.3 and L4440 were part of a *C. elegans* RNAi library expressed in *E. coli* (Geneservice, Cambridge, UK). RNAi was delivered by feeding as previously described (Kamath et al., 2001). In brief, L4 hermaphrodites were placed on RNAi feeding plates. After 4 days, F1 progeny were transferred either to plates

filled with nematode growth medium (NGM) agar supplemented with 20 mM paraquat or to control unsupplemented NGM plates.

### ***ROS assay in OSCs***

Generation of intracellular reactive oxygen species (ROS) in human OSCs was measured by staining with CellROX® Green Reagent (Molecular Probes) according to the manufacturer's instructions. In brief, OSCs from ASD and control patients were seeded in 96-well plates and maintained in DMEM/HAM culture medium supplemented with insulin, transferrin, selenium (ITS-X, 1%; Invitrogen) for 24h. CellROX® Green Reagent (5  $\mu$ M) was added to the culture medium for 30 minutes at 37°C, either at baseline or one hour after addition of staurosporine (1  $\mu$ M) in the culture medium. After washing, fluorescence was measured with a Beckman Coulter plate reader. Experiments were performed in triplicate.

### ***Aldicarb and levamisole-induced paralysis assays***

Acute sensitivities to aldicarb and levamisole were assayed in paralysis assays as previously described (Mahoney et al., 2006). For the aldicarb assay, Thirty to forty young adult animals were transferred to NGM agar plates containing 0.25 mM aldicarb and day1 adult worms were scored every 30 min (moving ratio). For the levamisole-induced paralysis assay, NGM agar plates containing various concentrations of levamisole (0.1, 0.2, 0.5, 0.8 and 1 mM) were prepared 1 day prior to the assay and adult worms were tested for complete paralysis after 60 min of drug exposure. Animals were considered paralyzed if they failed to move when prodded with a platinum wire.

### ***Fluorescence imaging and quantitative analysis of NLP-21 and GFP containing vesicles***

Live animals were immobilized with 50 mM Na-azide in M9 buffer and NLP21::YFP or GFP distribution was evaluated in cholinergic motor neurons (soma and dorsal cord axons) and/or coelomocytes. Stacks of confocal images were obtained on an inverted microscope

(LSM780; ZEISS) using a 63X NA 1.4 oil immersion objective. All quantitative analysis were performed using ImageJ software (National Institutes of Health) as described previously (Sieburth et al., 2007). Results are expressed as values normalized to wild type. For the dorsal nerve cords analysis, mean grey levels intensities were measured on maximal intensity projections of stacks of 5 consecutive optical slices. Individual coelomocytes and nerve cord neurons were imaged by 3D-stacks using the same final magnification. Mean vesicular diameters and mean intensities were measured on the optical slice passing through the largest part of each individual vesicle using a 3D-threshold and particle detection.

### ***Construction of MOCOS gene correction TALENs and donor vectors***

We decided to design two pairs of TALENs. TALEN target sites 1 and 2 were identified using the TAL effector-Nucleotide Targeter (TALE-NT) program (Christian et al., 2010). Chosen targets had a T at the -1 position and showed the best score. TALENs recognizing the target sites were constructed using the Golden Gate Assembly method with the TALE Toolbox kit from Addgene (cat#1000000019) (Zhang et al., 2011). The constructed TALEN pairs targeting exon 3 and intron 3 of the *MOCOS* gene were named as MOCOS1-5'/MOCOS1-3' and MOCOS2-5'/MOCOS2-3', respectively. The *MOCOS*-donor vector was constructed by In-Fusion HD (Clontech). In brief, an *FRT*-PGK-EM7-neo-bpA-*FRT* fragment was subcloned into the EcoRI (New England Biolabs) and BamHI (New England Biolabs) sites of the pUC19 vector (New England Biolabs) together with 0.9 kb of 5' homology and 0.8 kb of 3' homology arms, which were amplified by PCR of genomic DNA from integration-free iPSCs (Xia et al., 2013) with the following primers (5' homology arm; 5'- GACGGCCAGTGAATTCTGTCATCAGTGGCATTATTTGCAGCACAAC-3' and 5'- TTTTGCTCCCTTCCTGGTGTCCCT-3', 3' homology arm; 5'- TTACCTCACCGACAGCCACACCTCCGTA-3' and 5'- CGACTCTAGAGGATCCGGCCTGGTAGCTCACCTCCTTACCC) using Q5 Hot Start

High-Fidelity DNA Polymerase (New England Biolabs) following manufacturer's instructions.

### ***Surveyor assay***

To examine the efficacy of the generated TALENs, we performed Surveyor assay in HEK293 cells as previously described(Sanjana et al., 2012).

### ***Generation of the MOCOS gene-knockout human iPSCs.***

To disrupt *MOCOS* gene, integration-free iPSCs were chosen as host cells(Xia et al., 2013). For generation of the heterozygous *MOCOS* knockout iPSCs,  $1.5 \times 10^7$  feeder-free cultured iPSCs were dissociated by AccuMax (Innovative Cell Technologies) and resuspended in 1ml of Mouse embryonic fibroblasts (MEF)-conditioned medium containing 10 mM ROCK inhibitor Y-27632 (Biomol Inc.). Cells were electroporated with 8 mg of TALEN expression vectors and 32 mg of donor vectors and were plated onto 100 mm dishes precoated with  $1 \times 10^6$  irradiated neomycin-resistant MEFs (Applied Stem Cell). Two days after electroporation, G418 (50 mg/ml; Invitrogen) was added to the medium to start positive selection. After 18 days, G418-resistant clones were transferred to 96-well plates and expanded for further characterization. Gene-targeted clones were determined by PCR of genomic DNA from drug-resistant clones with the following primers (P1: 5'-TGGCTCAGGGACAGACACAGGTTACTACCAG-3' and P4: 5'-AGCCACCATTTTCAGCCTTGACACACTCGAC-3;) using PrimeSTAR GXL DNA Polymerase (TAKARA) following manufacture's protocol (see Fig. 4g and Supplementary Fig. 3). To remove the neomycin-resistance cassette, we generated a pCAG-Flpo-2A-puro vector, which, under control of a CAG promoter, expresses the genes for Flpo recombinase(Raymond and Soriano, 2007), and puromycin N-acetyltransferase (puro). *MOCOS* heterozygous knockout iPSCs cultured on Matrigel were transfected with pCAG-



Flpo-2A-puro vector using FuGENE HD (Promega). Two days after transfection, puromycin (1 µg/ml; Invitrogen) was added to the medium to enrich Flpo recombinase expressing cells. Two days later, puromycin was withdrawn, and after about 10 days, cells were individualized and plated onto MEF feeder cells at a density of 300-3000 cells / 75 cm<sup>2</sup> in the presence of 10 µM Y-27632 (Biomol Inc.). After 2 weeks, the emerging colonies were hand picked and expanded. Removal of the neomycin-resistance cassette was verified by PCR with the same primers (5'-TGGCTCAGGGACAGACACAGGTTACTACCAG-3' and 5'-AGCCACCATTTTCAGCCTTGACACACTCGAC-3) using PrimeSTAR GXL DNA Polymerase following manufacture's protocol (see Supplementary Fig. 3). The determined heterozygous knockout clones (*MOCOS*<sup>+/-</sup>) were used in another round of gene knockout to try to generate homozygous knockout cells. For this, we repeated the same steps. However, no biallelic *MOCOS* knockout clones (*MOCOS*<sup>-/-</sup>) were ever generated after three attempts.

### ***Neuronal differentiation from hiPSCs***

First we derived neural stem cells (NSCs) from iPSC (*MOCOS*<sup>+/+</sup> and *MOCOS*<sup>+/-</sup>) as described elsewhere (Liu et al., 2012) with slight modifications. In brief, feeder-free culture of human iPSCs were passaged onto matrigel-coated plate at about 20% confluence. Culture medium was then switched to Neural Induction Medium 1 (NIM-1: 50% Advanced DMEM/F12 (Invitrogen), 50% Neurobasal (Invitrogen), 1x N2 (Invitrogen), 1x B27 (Invitrogen), 2 mM GlutaMAX (Invitrogen) and 10 ng/mL hLIF (Prospec), 4 µM CHIR99021 (Reagents Direct), 3 µM SB431542 (Stemgent), 2 µM Dorsomorphin (Sigma), and 0.1 µM Compound E (EMD Chemicals Inc.)). Cells were treated with NIM-1 for 2 days, and then switched to Neural Induction Medium 2 (NIM-2: 50% Advanced DMEM/F12, 50% Neurobasal, 1x N2, 1x B27, 2 mM GlutaMAX, 10 ng/mL hLIF, 4 µM CHIR99021, 3 µM SB431542 and 0.1 µM Compound E) for another 5 days. The cultures were then split onto matrigel-coated plates with Accumax (Innovative Cell Technologies) and cultured in Neural

Stem cell Maintenance Medium (NSMM) containing 50% Advanced DMEM/F12, 50% Neurobasal, 1x N2, 1x B27, 2 mM GlutaMAX, 10 ng/mL hLIF, 3  $\mu$ M CHIR99021, and 2  $\mu$ M SB431542. NSC culture. NSCs were maintained on Matrigel in NSMM. NSCs were passaged once reaching 80% confluency using Accumax and seeded at about 20% confluency (Cytoone). Medium was changed every day. For the initial 6 passages, NSCs were treated with 10  $\mu$ M Y-27632 (Biomol Inc.) during splitting. From passage 7, NSCs were induced to differentiate into neurons.

For neuronal differentiation, human NSCs were plated onto poly-ornithine (Sigma) and laminin coated dishes in NSMM. After 2 to 3 days, medium was switched to Neurobasal medium (Invitrogen) supplemented with N2 (0.5X), B27 (1X) and FGF2 (10 ng/ml, Preprotech). Four days later, FGF2 was withdrawn from the medium, and after another 4 days, medium was switched to Neurobasal media supplemented with B27 (1X) and brain derived neurotrophic factor (BDNF, 20 ng/ml, R&D Systems). Differentiated cells were maintained up to 4 weeks after FGF2 withdrawal prior to perform immunocytological analyses.

### ***Immunocytochemistry***

For mouse tissues staining, adult mice (n=3) were transcardially perfused with 20 ml ice-cold saline (0.9% NaCl), followed by 50 ml ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.2, and their brain, kidneys, liver and gut were dissected and post-fixed for 2 h in 4% Paraformaldehyde. Then, each organ was rinsed and cryoprotected in 0.32M sucrose prior to be processed for cryosectioning. Each organ was embedded with cryostat mounting medium (Cryomount), cryo-sectioned at 20  $\mu$ m thickness (Leica CM30505), thaw-mounted on pre-coated slides (Superfrost Plus, Thermo Scientific) and stored at  $-80^{\circ}\text{C}$  until use. Sections were air-dried for one hour before being processed for immunohistochemistry.

For immunocytochemistry, cells to be stained were prepared on glass cover slips (coated with the appropriate substrate when necessary). Cells were fixed in 4% paraformaldehyde for 12 minutes at room temperature and washed at least 3 times in PBS prior to be processed for immunocytochemistry.

Tissue sections/cells were first incubated for 1 hour at room temperature (RT) with a blocking buffer containing 3% BSA, 5% appropriate serum and 0.1% Triton X-100 in PBS. Then, tissue sections/cells were incubated for 1 hour at RT (or overnight at 4°C) with the following primary antibodies diluted in blocking solution: rabbit polyclonal anti-MOCOS (1:100 for cells and 1:20 for tissue sections; Novus biologicals NBP2-14243), chicken polyclonal anti-MAP2 (1:300; Abcam, Ab5392), mouse monoclonal anti-TUJ-1 (1:500; COVANCE MMS-435P); rabbit polyclonal anti-SYNAPSIN (1:250; Millipore AB1543P ). After three washes in PBS, tissue sections/cells were incubated with either Alexa 488 or Alexa 594 or Alexa 568 fluorescent secondary antibodies (Molecular Probes) diluted at 1:500 in PBS for 1 hour at RT; and counterstained with DAPI prior to be washed and mounted. Tissue sections/cells were examined using a Zeiss LSM 710 Laser Scanning Confocal Microscope (Zeiss).

### ***Statistical evaluation***

Statistical analyses were performed by using standard unpaired Student t-tests (two-tailed, 95% confidence intervals) or one-way ANOVA, and were carried out using Prism Software (GraphPad) or Excel software (Microsoft). For microarray data analyses, Right-tailed Fisher's exact test was used to calculate a p-value determining the top statistically significant biological functions assigned to the data set. All data are presented as mean $\pm$ s.d. and represent a minimum of three independent experiments with at least three technical replicates except when otherwise indicated. A value of  $P < 0.05$  was considered significant.



## Results

We first established the transcriptome profiles of OSCs isolated from nasal biopsies (Girard et al., 2011) of 11 patients diagnosed with ASD and 11 age- and gender-matched control individuals (Supplementary Tables 1-5). Gene microarray analysis identified 156 genes that were differentially expressed in at least one ASD patient (Supplementary Table 6), of which 31 were dysregulated in more than a third of the cohort (Supplementary Table 6, in red). Notably, 9 out of these 156 genes – *ADAM23*, *CADMI*, *FOS*, *FOSB*, *JAG1*, *MEST*, *OXTR*, *SFRP1* and *XIST* – have been previously associated to ASD (Parker et al., 2014). Ingenuity Pathway Analysis (IPA) on the pool of differentially regulated genes identified developmental disorders and gastrointestinal diseases as two of the most represented categories associated with these genes (Supplementary Table 7). Furthermore, IPA predictive analysis highlighted that, among the main upstream regulators of the differentially expressed genes, three – *TNF*, *IFNG* and *IL1B* – are associated with inflammation (Supplementary Table 7), a functional process widely associated with ASD (Chez et al., 2007; Depino, 2013). In addition, gene cluster analysis revealed that all patients were affected by a partial dysregulation of the TGF- $\beta$ /Wnt pathways (Fig. 1a and Supplementary Table 8), known to play an important role in ASD (Ashwood et al., 2008; Kalkman, 2012). Second in line among the most affected pathways, purine metabolism appeared largely disturbed with the misexpression of at least one of two enzyme-coding genes, *MOCOS* and *AOXI*, in 10 out of 11 patients (Supplementary Table 8). Whereas *AOXI* was found differentially expressed in 3 out of 11 patients, its pattern of expression was heterogeneous when compared to the subtle but robust *MOCOS* downregulation displayed by ~80% (8/11) of the patients composing our cohort (Fig. 1b,c), which is amazing when considering that hundreds of susceptibility genes account for 10-20% of ASD cases at most (Abrahams and Geschwind, 2008). Importantly, *MOCOS* downregulation in ASD patients'

OSCs was confirmed at both the RNA and protein levels (Fig. 1c-f). Interestingly, metabolic disorders of purine metabolism have been described to affect the nervous system and able to induce autistic features (Manzi et al., 2008; Page and Coleman, 2000). Accordingly, we decided to focus on MOCOS, an enzyme acting upstream in the purine catabolism pathway, and investigate its putative role in ASD.

The fact that MOCOS adds a sulfur on the molybdenum cofactor and turn XDH into its active form to catalyse the oxidation of hypoxanthine to xanthine and, further down, the oxidation of xanthine to uric acid, led us to retrospectively investigate whether our patients suffered from xanthinuria (Mendel and Kruse, 2012; Yamamoto et al., 2003). In addition, and because creatinine and ferritin are often associated with uric acid production, we also assessed their expression in the blood and the urine. Except in one patient that displayed a high level of xanthine, our analyses did not reveal any direct correlation between these three measurements (Supplementary Table 9). These results led us to propose that MOCOS misexpression might affect other functions and we decided to investigate whether it could play a role in brain functions.

We first sought to look at the dynamic expression of MOCOS in the brain. Using the Allen Brain Atlas tool, we found that *MOCOS* is expressed in numerous areas of the developing and adult human brain. Confirming the expression pattern suspected by transcriptomic analyses in human, adult mouse brain tissue analysis confirmed the presence of MOCOS, at the protein level, in multiple brain areas such as the hippocampus, the cortex, the cerebellum and the brainstem (Fig. 2a and Supplementary Fig. 1a). Furthermore, MOCOS was readily detected in MAP2<sup>+</sup> murine neurons *in vitro* and *in vivo* (Fig. 2b-d and Supplementary Fig. 1a). In parallel, we found that MOCOS is expressed in other tissues such as the intestine, the kidney and the liver (Fig. 2e,f and Supplementary Fig. 1b). Since *MOCOS* is a gene highly conserved across species, we additionally analysed its expression in

*Caenorhabditis elegans* by using a transgenic strain in which the GFP coding sequence was fused to the 3' end of the orthologous gene *mocs-1* (Sarov et al., 2012). We observed GFP expression as early as the 8-cell stage of the intestine and throughout the larval and adult life of the animal in the cytoplasm of cells in the intestine as well as in the head (Fig. 2g). Regarding the latter, *mocs-1* reporter expression was detected in sheath and socket glial cells (Fig. 2g and Supplementary Fig. 1c). Altogether, these data suggest that throughout evolution, MOCOS plays an important role in development and adult life. Importantly, the multicellular/multiorgan distribution of MOCOS fits with the search of a pleiotropic gene that could explain complex disorders such as ASD.

In order to investigate whether altered expression of *MOCOS* correlates with ASD-associated phenotypes, we used two independent *C.elegans* strains mutant for *mocs-1* and bearing a nonsense mutation (*tm3708*) or a frameshift mutation (*ok3439*) (Fig. 3a). Several studies having reported a greater oxidative stress in children with autism (James et al., 2006; McGinnis, 2004), we first assessed the sensitivity of *mocs-1* mutants to paraquat, an oxygen radical-generating agent. Whereas development was slowed down in all worms exposed to 0.22 mM of paraquat, 82% and 52% of *tm3708* and *ok3439* mutants, respectively, reached the L4 larval stage when compared with wild type animals (Fig. 3b), demonstrating an increased sensitivity to oxidative stress. Interestingly, the observed effects were dose-dependent as larval development was blocked at the L2/L3 stages in *mocs-1* mutants treated with higher concentrations ( $\geq 0.44$  mM), and led to a significantly lower survival rate in *tm3708* (12%) and *ok3439* (0%) mutants comparatively to wild type (98.5%) animals exposed at 4 mM of paraquat (Fig. 3b,c). Confirming the role and specificity of *mocs-1* in response to paraquat, similar results were obtained with *eri-1* animals fed with an RNAi against R03A10.3, the *mocs-1* predicted gene (Fig. 3d). Similarly, we observed that exposure to paraquat at an adult stage strongly decreases the viability of *mocs-1* mutants as well as *eri-*

*1* animals fed with R03A10.3 RNAi when compared with their respective controls (Fig. 3e,f). Then, we evaluated whether a similar feature could be observed in patients' OSCs displaying *MOCOS* downregulation. Validating further our initial observations in worms, those cells revealed a higher production of reactive oxygen species (ROS), at baseline and one hour after staurosporine treatment, when compared with their respective controls (Fig. 3g). Notably, this feature is in line with the fact that *MOCOS* adds a sulfur atom on the molybdenum cofactor of AOX1, an enzyme involved in the regulation of ROS homeostasis. Altogether, these data demonstrate that altered expression of *MOCOS* may increase sensitivity to oxidative stress, a feature that has been widely reported to contribute to the development and clinical manifestations of autism (James et al., 2006; McGinnis, 2004).

To date, the physiopathology of ASD is commonly accepted to be partly due to a defective regulation of neurotransmission (Gepner and Feron, 2009; Sudhof, 2008). Accordingly, we assessed synaptic function in *mocs-1* mutants. To this end, we evaluated the synaptic transmission at the neuromuscular junction by monitoring the sensitivity of *mocs-1* mutants to the paralyzing effects of aldicarb, an acetylcholinesterase inhibitor (Mahoney et al., 2006). We found that *tm3708* and *ok3439* animals were resistant to aldicarb when compared with N2 wild type worms (Fig. 4a). Notably, we observed similar paralytic and lethal effects in wild type and *mocs-1* mutant worms exposed to increasing concentrations of levamisole, ruling out an alteration of the postsynaptic receptor function (Supplementary Fig. 2a). Next, we reasoned that aldicarb resistance in *mocs-1* mutants could be the consequence of a decreased neuropeptide secretion. Accordingly, we analyzed the secretion of the YFP-tagged neuropeptide NLP-21 in cholinergic motor neurons. As shown in Figure 4, NLP-21 puncta fluorescence intensity was significantly reduced in the dorsal cord axons of *tm3708* hypomorph mutants ( $23.5\% \pm 3$ ) when compared to wild type animals and a similar decrease was observed in coelomocytes ( $29.2\% \pm 8.5\%$ ) of both groups of animals (Fig. 4b,c).



Together, these data suggested a reduced number of dense-core vesicles (DCVs) in the axons as well as a quantitatively reduced but functionally normal neuropeptide secretion. Further investigations on *tm3708* mutants showed a significant accumulation of NLP-21 in the soma of dorsal cord neurons and demonstrated that NLP-21 was localized into larger vesicles ( $\geq 0.2 \mu\text{m}^2$ ) when compared to control animals, suggesting that defects in neuropeptide packaging account for the observed phenotype (Fig. 4d-f). Of note, the observed phenotypes were confirmed in *tm3708* mutants harboring an integrated *pmyo-3::ssGFP* transgene (Supplementary Fig. 2b-d). Interestingly, similar analyses on *ok3439* neomorph mutants revealed no difference of NLP-21 distribution in dorsal cord neurons but a significant decrease number within coelomocytes (Fig. 4b-e), highlighting that *ok3439* mutants have decreased neuropeptide secretion when compared with wild type animals. In summary, although the two *mocs-1* mutant strains were found differentially affected, probably due to the nature of the mutation, our results indicate that deregulation of MOCS-1 expression may affect the neuropeptide secretory system.

This finding prompted us to use induced pluripotent stem cells (iPSC) for validating the role of MOCOS in human neuronal cells. Using TALEN technology, we successfully derived four heterozygous knockout (*MOCOS*<sup>+/-</sup>) iPSCs lines (Fig. 4g and Supplementary Fig. 3). Notably, several attempts at establishing homozygous knockout (*MOCOS*<sup>-/-</sup>) iPSC lines failed, suggesting an important role of MOCOS in human cell homeostasis, at least during early development. To investigate whether MOCOS can play a role in neurotransmission, *MOCOS*<sup>+/-</sup> iPSCs and their respective isogenic *MOCOS*<sup>+/+</sup> control lines were differentiated into neurons (Fig. 4h). The synaptic compartment being an important element for neurotransmission, we analysed the number of synaptic buttons in iPSCs-derived neurons (Fig. 4h). Of outmost importance, and even though we did not observe difference in their capacity to generate TUJ1-expressing neurons, those derived from *MOCOS*<sup>+/-</sup> iPSCs

displayed a lower number of SYNAPSIN+ synaptic buttons (Fig. 4i,j). These results confirm that MOCOS is a newly identified molecule at play during neurodevelopment and neurotransmission.

## **Discussion**

Together, our results highlight MOCOS as a new candidate molecule implicated in ASD pathogenesis and unveil novel biological functions associated with MOCOS, including roles in stress response and neurotransmission. Bearing in mind that *MOCOS* was found downregulated in most ASD patients of our cohort, the phenotypes observed in our cellular and animal models are of the most relevance to establish a causal link between MOCOS misregulation and ASD-associated clinical symptoms. Of interest, metabolic defects have been associated with autistic symptoms with a prevalence higher than that found in the general population (Manzi et al., 2008), including purine metabolism disorders (Page and Coleman, 2000). Furthermore, xanthines and more generally purine derivatives were found to have psychostimulant properties through their possible interaction with multiple receptors (Shi and Daly, 1999). Additionally, this is the first time that MOCOS is described as a putative modulator of other developmental and adult functions.

Among body metabolism biomarkers in ASD, and as shown in our results, oxidative stress indicators stand out. It has been observed that urinary antioxidants are found in smaller amounts in ASD patients and that their levels of production are correlated with symptom severity (Damodaran and Arumugam, 2011). Misexpressed molecules include, for example, glutathione, advanced glycation end products (AGE), superoxide dismutase and metal-binding proteins such as transferrin (iron) and ceruloplasmin (copper) (for a recent review, Goldani et al, 2014). Lately, a diminished seric expression of glutathione, glutathione peroxidase, methionine, and cysteine has been highlighted in a meta-analysis from 29 studies on ASD subjects (Frustaci et al, 2012). Along this line, purines and purine-associated

enzymes are recognised markers of oxidative stress. Reactive oxygen species (ROS) are generated during the production of uric acid, catalysed by xanthine oxidase and xanthine dehydrogenase (Schulz et al, 2011). Conversely, uric acid is nowadays recognized as a protective factor acting as a ROS scavenger (Waring, 2002; Nabipour et al, 2011). Interestingly, allopurinol, a xanthine oxidase inhibitor, was found efficient in reducing symptoms, especially epileptic seizures, in ASD patients displaying high levels of uric acid (Coleman et al, 1974). However, in our cohort, only 3 out of 10 patients exhibited an abnormal uric acid secretion. It can therefore be postulated that still unknown other MOCOS-associated mechanisms play a role in the unbalanced stress response observed in ASD olfactory stem cells.

According to our results, MOCOS is also associated to neurotransmission abnormalities, that have previously been proved to be strongly affected in ASD (Gepner, Feron, 2009). It has not escaped our notice that the enzymatic nature of our candidate molecule fits well with the wide-ranging and unspecific neurotransmitter imbalance observed in ASD. As a molecule involved in the formation of dense core vesicles (DCV) and, further down, neurotransmitter secretion, MOCOS seems to act on the container rather than the content, on the vehicle rather than one of the transported components. Interestingly, other studies have highlighted the potential role of DCV-associated proteins in ASD (Sadakata et al, 2013; Paemka et al, 2013; Zhao et al, 2014).

In addition, as a key element of purine metabolic pathway, MOCOS may indirectly be involved in an extensive stimulation of neurotransmitter machinery. Indeed, it is established that xanthines and more generally purine derivatives display psychostimulant properties, acting at the A1 and A2A subtypes of the adenosine receptors (Lorist and Tops, 2003) but also other central receptors as  $\beta$ 1/ $\beta$ 2-Adrenergic, 5-HT1 serotonin, 5-HT2 serotonin,

muscarinic, nicotinic, GABA A, Ca<sup>2+</sup> channel, in the cortex and the cerebellum (Shi and Daly, 1999).

Furthermore, an abnormal expression of MOCOS may alter the production and functioning of gephyrin, a post-synaptic protein that anchor and cluster GABAA and glycine receptors at the membrane (Tyagarajan and Fritschy, 2014). Indeed, in addition to its scaffolding role, gephyrin is involved in molybdenum cofactor (MOCO) biosynthesis(Fritschy et al, 2008). It can therefore be surmised that a MOCOS-associated fluctuation of MOCO alter the synaptic function of gephyrin. Conversely, as recent studies have stressed gephyrin as a candidate gene in ASD (for a review, Chen et al, 2014), we may postulate that genetic mutations disrupt MOCO synthesis and consequently MOCOS expression. It should however be pointed out that no dysregulated transcript expression of gephyrin was ever observed in our ASD cohort.

Identifying and manipulating downstream effectors of *MOCOS* will be the next critical step to better understand its mechanisms of action. In parallel, we plan to ascertain some of its upstream regulators. For example, it is known that the promoter region of *MOCOS* include binding sites for several transcription factors, including GATA3, NRF2 or SIRT1, three genes that have been associated to ASD, fragile X syndrome, epilepsy and/or oxidative stress (Rout and Clausen, 2009; Napoli et al, 2014; Furnari et al, 2014; Kumari and Usdin, 2014). In conclusion, our study opens an unexplored new avenue for the study of MOCOS in ASD, and could set bases for the development of new diagnostic tools as well as the search of new therapeutics.

### **Conflict of interest**

The authors declare no conflict of interest

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## FIGURE LEGENDS

Figure 1. **Human nasal olfactory stem cells reveal a subtle but robust downregulation of *MOCOS* in ASD patients.**

**a)** Network analysis of differentially expressed genes, in at least 4 ASD patients of the cohort, using Ingenuity Pathways Analysis (IPA). Note that *TGFBI/WNT catenin* are the most affected signaling pathways. Solid and dashed lines indicate direct and indirect interactions, respectively. Consistently overexpressed or underexpressed genes are in red or blue boxes, respectively. Up and down misexpressed genes are in green boxes. **b)** Volcano plot distribution of the microarray data. The blue and red vertical bars indicate magnitude fold-changes (FC) over [1.5] and the dashed black-line shows the  $p = 0.05$  threshold, with points above the line having  $p < 0.05$ . Note that the average for all *MOCOS* probes (10 in total on the microarray) display the same pattern of expression. **c)** Graph depicting *MOCOS* gene expression fold changes in ASD patients, relative to control patients, as detected by microarray and RT-qPCR. Note that highly similar results were obtained with both



techniques. **d)** Representative pictures showing MOCOS immunostaining of nasal olfactory stem cells (OSCs) isolated from one ASD patient (right panel) and his matched healthy individual (left panel). Nuclei were counterstained with DAPI. **e)** Representative Western Blot for the detection of MOCOS and GAPDH expression in control and ASD patients. **f)** Quantification of MOCOS expression, expressed as a ratio to GAPDH, in ASD and control OSCs using the Western blot technique. Data are represented as mean  $\pm$  SD. \*  $p < 0.05$ . Scale bars: 10  $\mu$ m (d).

**Figure 2. MOCOS is expressed in various *C. elegans* and mouse organs including the brain.**

**a)** Sagittal section of an adult mouse brain showing the very high expression of MOCOS in the cortex (CTX), the hippocampus (HIP), the outer layers of the cerebellum (CB) and the dorsal area of the olfactory bulb (OB). **b)** Representative picture of MAP2<sup>+</sup> neurons (red) in the CA1 layer of the hippocampus (dashed white box in a) immunostained for MOCOS (green) and counterstained with DAPI. On the right panel, high magnification view. **c,d)** Representative pictures at low (c) and higher magnification (d) of cultivated murine neurons immunostained with the indicated antibodies. **e,f)** Mouse gut sections displaying an intense MOCOS staining in ciliated cells (e) and glands (f). **g)** MOCS1 expression in the developing *C.elegans* was monitored using a *mocs-1::GFP* transgenic strain. Representative pictures showing that MOCS1 was found highly expressed in the intestine and the head in the embryo (left panel at the gastrula stage, DIC: differential interference contrast), at larval stages (L1 on the picture) and maintained throughout adult life. Arrows indicate sheath (white) and socket (yellow) glial-like cells, as well as intestinal (red) cells. Scale bars: 100  $\mu$ m (e and f,

left panels), 20  $\mu\text{m}$  (g, middle panels), 10  $\mu\text{m}$  (b,c,d; e and f, right panels; g, left panels and right panel),

Figure 3. **MOCOS expression alterations induce increased stress sensitivity.**

**a)** Cartoon depicting the main structural characteristics of the gene encoding the Molybdenum cofactor sulfurase in plants (*A.thaliana*), humans and *C.elegans*. Note the sequence homology between the latter two. The nonsense *tm3708* mutant lacks intron 1 and part of exon 2 while the frameshift *ok3439* mutant lacks part of exon 2 and all subsequent exons up to exon 5. **b)** Larval development is slowed down, in a dose-dependent manner, in *mocs-1* mutants exposed to paraquat. N2 animals were used as wild type controls. *mev-1* worms, a strain hypersensitive to paraquat, was used as an experimental control for the paraquat assay. **c)** Bar chart depicting the reduced survival rate of *mocs-1* larval mutants exposed to high level of paraquat (4mM). **d)** Disturbed larval development in *eri-1* animals fed with an RNAi directed against *mocs-1* (R03A10.3) when compared to control *eri-1* worms fed with RNAi against the empty vector L4440. **e)** Time course survival rate of *mocs-1* adult mutants exposed to high level of paraquat (4 mM). N2 and *mev-1* animals were used as controls as defined in b). **f)** Bar chart demonstrating the reduced survival rate of *eri-1* adult animals fed with an RNAi directed against *mocs-1* (R03A10.3) comparatively to their control (fed with RNAi against L4440), after exposure to high level of paraquat (4 mM). **g)** ASD patients' nasal olfactory stem cells (OSCs) produce higher levels of reactive oxygen species (ROS), at baseline and one hour after staurosporine treatment. Data are represented as mean  $\pm$  SD. \*  $p < 0.05$ . n= 80 to 100 animals per group. Experiments were run in triplicate.

Figure 4. **MOCOS expression alterations result in neurotransmission and synaptic defects.**

**a)** Paralyzing effects of aldicarb (a cholinesterase inhibitor) in wild type (N2) worms and *mocs-1* mutants (*tm3708*, *ok3439*). *ric-3*, a strain resistant to aldicarb, was used as an experimental control for the aldicarb assay. Note that *mocs-1* mutants exhibit a resistance to the paralytic effect of aldicarb (n=50-70 animals per group). **b,c)** Representative pictures (b) and quantitative analysis of the mean fluorescence intensity (c) of the YFP-tagged neuropeptide NLP-21 in the dorsal cord axons of cholinergic motor neurons for each of the indicated *C.elegans* strains. **d)** Representative pictures showing the NLP-21 loading in neuronal soma (upper row) and coelomocytes (lower row) for each of the indicated strains. **e)** Quantitative analysis of the NLP-21 loading in neuronal soma (upper panel) and coelomocytes (lower panel), as measured by mean fluorescent intensity relative to N2 animals. **f)** Bar charts depicting the mean area of NLP-21-loaded dense core vesicles (DCVs) present in neuronal soma (upper panel) and coelomocytes (lower panel), in *mocs-1* mutants relative to wild type N2 animals. **g)** Schematic representation of the TALEN-based strategy used to monoallelically invalidate *MOCOS* in human iPSCs. Half arrows indicate primer sites for PCR (P1, P2, P3 and P4). Red triangles indicate the FLPo recognition target (*FRT*) site. **h)** iPSC-derived neural stem cells were differentiated into neurons and immunostained with anti-TUJ1 and anti-SYNAPSIN antibodies. Nuclei were counterstained with DAPI. **i,j)** Representative pictures (i) and quantification (j) of synaptic density in *MOCOS*<sup>+/-</sup> NSC-derived neurons compared to those derived from isogenic control lines (*MOCOS*<sup>+/+</sup>). Data are represented as mean ± SD. \* p < 0.05. Scale bars: 100 μm (h, left panel); 10 μm (b,i); 4 μm (h, right panels) and 2 μm (d).