



Lab Resource: Stem Cell Line

Copy number variations in healthy subjects. Case study: iPSC line CSSi005-A (3544) production from an individual with variation in 15q13.3 chromosome duplicating gene *CHRNA7*

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ABSTRACT

CHRNA7, encoding the neuronal alpha7 nicotinic acetylcholine receptor (α7nAChR), is highly expressed in the brain, particularly in the hippocampus. It is situated in the 15q13.3 chromosome region, frequently associated with a Copy Number Variation (CNV), which causes its duplication or deletion. The clinical significance of *CHRNA7* duplications is unknown so far, but there are several research data suggesting that they may be pathogenic, with reduced penetrance. We have produced an iPSC cell line from a single healthy donor's fibroblasts carrying a 15q13.3 CNV, including *CHRNA7* in order to study the exact role of this CNV during the neurodevelopment.

Resource table	Clonality	Clonal
Unique stem cell line identifier	Method of reprogramming	<i>Non integrating episomal vectors</i>
Alternative name(s) of stem cell line	Genetic Modification	<i>NO</i>
Institution	Type of Modification	<i>N/A</i>
Contact information of distributor	Associated disease	<i>N/A</i>
Type of cell line	Gene/locus	<i>15q11q13 CHRNA7</i>
Origin	Method of modification	<i>N/A</i>
Additional origin info	Name of transgene or resistance	<i>N/A</i>
Cell Source	Inducible/constitutive system	<i>N/A</i>
	Date archived/stock date	<i>April 2017</i>
	Cell line repository/bank	<i>N/A</i>
	Ethical approval	<i>Casa Sollievo della Sofferenza Ethical Committee, approval number: 75/CE</i>

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1panel A
Phenotype	Qualitative analysis immunofluorescence	Staining of pluripotency markers: OCT4 (green) TRA-1-60 (red)	Fig. 1panel B
	Quantitative analysis: qPCR	Expression of pluripotency markers: LIN28, SOX2, L-MYC, OCT4	Fig. 1panel C
Genotype Identity	Karyotype (G-banding) and resolution	46XX, Resolution 450–500	Fig. 1panel H
	Microsatellite PCR (mPCR) OR STR analysis	N/A 19 loci tested; all matched	N/A Data available with the authors
Mutation analysis (IF APPLICABLE)	qPCR	Increased copy number	Fig. 1panel I
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by N-Garde Mycoplasma PCR kit (EuroClone): Negative	Supplementary Figure S1
Differentiation potential	<i>In vitro</i> : embryoid body formation	Embryoid bodies. Expression of genes in embryoid bodies: NESTIN, PAX6, SOX1, T, EOMES, FOXA2, GATA4, SOX 17.	Fig. 1panel E Fig. 1panel F
	<i>In vivo</i> : teratoma formation	Proof of three germ layers formation: Ectoderm, Mesoderm, Endoderm	Fig. 1panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Resource utility

The generation of 15q13.3 CNV iPSC, including *CHRNA7*, may be useful in both scientific and clinical fields, because it allows us, on the one hand, to study the role of *CHRNA7* duplication on the development of the nervous system, and on the other hand, to eventually develop targeted therapeutics.

Resource details

The region 15q13.3, including *CHRNA7* gene, is a chromosome region particularly prone to rearrangements, such as microdeletion/microduplication (CNVs), due to an enrichment of low copy repeat (LCR) elements (De Jaco et al., 2017). Microdeletion of 15q13.3 has been found to cause neurological phenotypes with high penetrance, while gains encompassing *CHRNA7* is even more enigmatic, making the genetic counselling problematic because experimental and epidemiological data are still controversial (Gillentine et al., 2017a, 2017b). The influence of *CHRNA7* duplication can be studied directly on patients' cells through induced pluripotent stem cell (iPSCs) technology, facilitating the comprehension of its influence on brain development. Our donor is a subject with a normal working and family life, who has no dysmorphic features or other physical abnormalities, and no form of hyperactivity, distractibility, or social impairment. The sole cytogenetic feature of this donor is the presence of CNV, inherited from her father, affecting the cytogenetic region 15q13.3. The study was carried out, with the patient's consent, utilizing cells from a skin biopsy. We reprogrammed the fibroblasts into iPSCs, using non-integrative episomal vectors containing the reprogramming factors OCT4, SOX2, L-MYC, KLF4, LIN28, shp53. The clones with Embryonic Stem-like morphology (Fig. 1A) were characterized in indirect immunofluorescence (IIF) microscopy using specific antibodies against the surface marker TRA-1-60 and the transcription factor OCT4, confirming their expression: TRA-1-60 protein is present in cell surfaces and OCT4 protein is evident in the nuclear compartment of the iPS cells (Fig. 1B). Using qPCR we verified and confirmed the presence in the clones of endogenous expression of the pluripotency markers, *LIN28*, *SOX2*, *OCT4* and *L-MYC* using, as negative control, the wild-type fibroblasts (Fig. 1C). After more than ten passages, we confirmed the suppression of exogenous reprogramming factor expression to negligible levels by qPCR using, as positive control, the nucleofected fibroblasts (Fig. 1D). Subsequently, pluripotency capacity of the iPSCs was tested, differentiating the iPSC both *in vitro*

through embryoid bodies assay (Fig. 1E) and *in vivo* through a teratoma assay (on nude immune-deficient mice; NOD/SCID). The expression level of the markers of each germ layer was demonstrated, through qPCR, in the embryoid bodies using, as negative control, the iPSCs (Fig. 1F). Also in the iPSC-derived teratoma, hematoxylin-eosin images showed the presence of ectodermal derivatives: neuroepithelial structure with characteristic melanin deposits, mesodermal derivatives: adipose tissue, endodermal derivatives: secreting (dashed line) and batiprismatic lining (arrowheads) epithelia (Fig. 1G). Moreover, we confirmed that, after more than twenty passages, the iPSC line displayed a normal karyotype (46, XX) (Fig. 1H). Short tandem repeat analysis (STR) showed that the DNA profile of the donor's fibroblasts was identical to the derived iPSCs (data available from the authors). qPCR on genomic DNA verified that the *CHRNA7* CNV was retained in the iPSCs (Fig. 1I). All cells tested negative for mycoplasma contamination (Supplementary File S1). These data demonstrate that we have successfully generated a stable patient specific iPSC line, useful for studying 15q13.3 interstitial duplication encompassing *CHRNA7* at the molecular level (See Table 1).

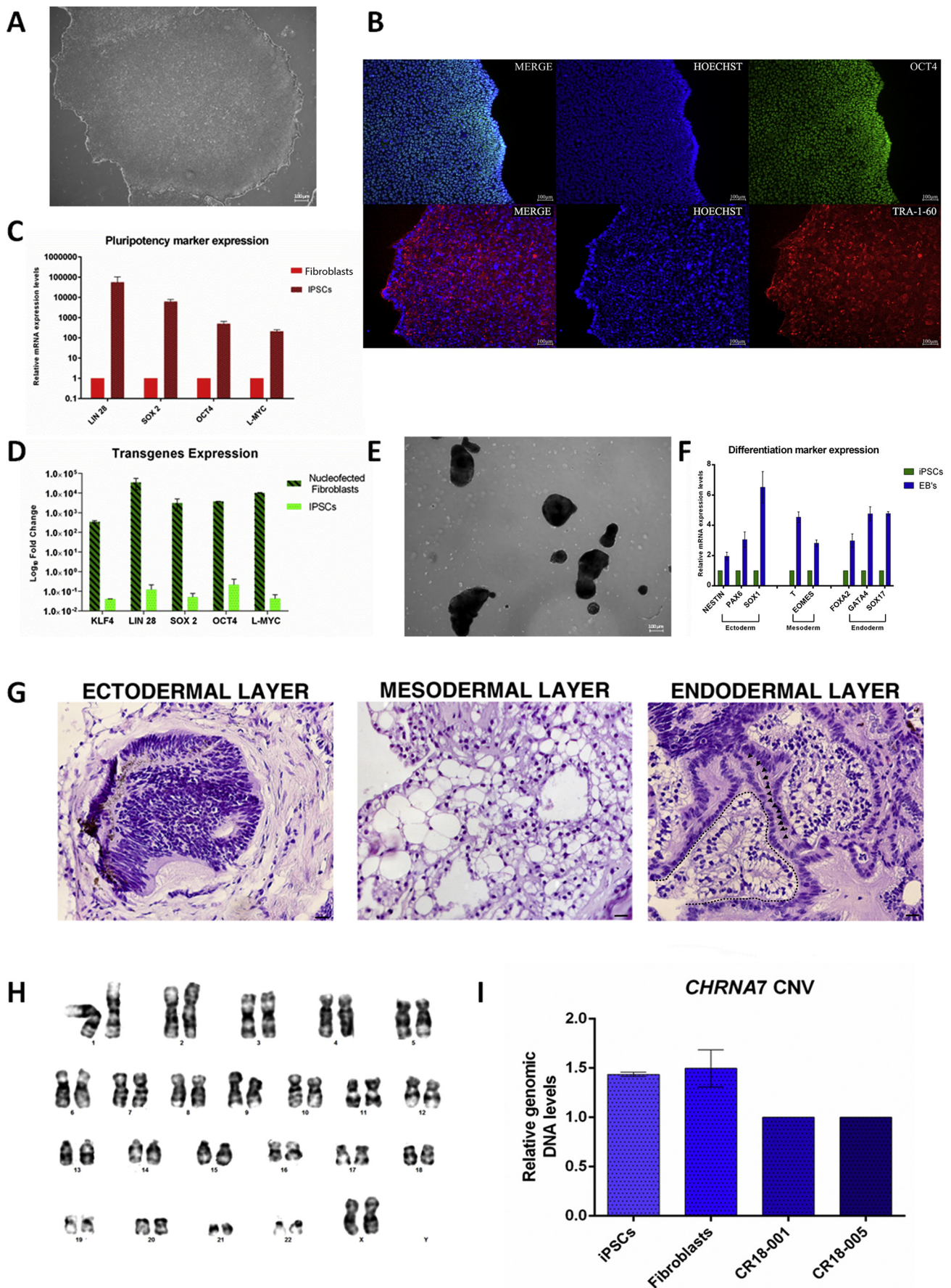
Materials and methods

Primary skin fibroblast culture

Skin fragments were cut and plated on a 35 mm tissue-culture dish with Dulbecco's Modified Eagle Medium (DMEM) High Glucose supplemented with 20% FBS, 2 mM L-Glutamine, 100 U/ml Penicillin-Streptomycin and 1 × Non-Essential Amino Acids (Sigma Aldrich) and cultured for 30 days to allow fibroblasts to grow out. Fibroblasts were split by Trypsin once a week, seeding 2 × 10⁵ cells into a 100 mm dish.

Cellular reprogramming

Approximately 1 × 10⁵ fibroblasts were nucleofected with 4D-Nucleofector™ X unit (Lonza), using P2 primary cell nucleofector kit and FF113 program. A mix of three pCXLE-based episomal vectors were used: pCXLE-hOCT4-shp53, pCXLE-hUL and pCXLE-hSK (Addgene). Cells were cultured in fibroblasts medium for 6 days. On day 7, cells were plated on Matrigel (Corning) coated dishes (BD Biosciences) in NutristemXF medium (Biological Industries). The emerging iPS colonies were selected according to their hESC-like colony morphology and expanded under feeder-free conditions. Both fibroblasts and iPSCs were



(caption on next page)

Fig. 1. Characterization of COL03 iPSC.

A. COL03 iPSC colonies morphology **B.** Immunofluorescence staining shows expression of pluripotency markers: OCT4 (green), TRA-1-60 (red), cell nuclei with HOECHST (blue). **C.** qPCR analysis for pluripotency markers verified the expression levels of endogenous transcripts in COL03 iPSC. This experiment was repeated twice in triplicate using independently prepared cDNAs. **D.** qPCR analysis for transgenes transcripts, after 10 passage, in COL03 iPSC compared to nucleofected fibroblasts. This experiment was repeated twice in triplicate using independently prepared cDNA. **E.** COL03 EBs in floating condition. **F.** qPCR analysis of all three germ layer markers (Ectoderm, Mesoderm and Endoderm) from COL03 Embryoid Bodies. Data denote actin-normalized fold changes relative to undifferentiated parental iPSC. **G.** Histological sections of all three germ layer (Ectoderm, Mesoderm and Endoderm), derived from teratoma stained with hematoxylin and eosin. **H.** iPSC karyogram shows normal ploidy of COL03 female subject. **I.** qPCR analysis on genomic DNA verified that the CHRNA7 CNV was retained in the iPSCs.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	Life technologies (A13998)
	Mouse anti-TRA-1-60	1:100	Life technologies (411000)
Differentiation Markers	N/A	N/A	N/A
Secondary antibodies	anti-Rabbit AlexaFluor 488	1:10000	Invitrogen (A11034)
	anti-Mouse AlexaFluor 555	1:10000	Invitrogen (A21422)
Primers			
Sybr Green Primers used for qPCR	Target	Forward/Reverse sequence (5'-3')	
Exogenous Episomal genes	eOCT4	Fwd: CAT TCA AAC TGA GGT AAG GG Rev: TAG CGT AAA AGG AGC AAC ATA G	
	eKLF4	Fwd: CCA CCT CGC CTT ACA CAT GAA GA Rev: TAG CGT AAA AGG AGC AAC ATA G	
	eLIN28	Fwd: AGC CAT ATG GTA GCC TGA TGT CCG C Rev: TAG CGT AAA AGG AGC AAC ATA G	
	eL-MYC	Fwd: GGC TGA GAA GAG GAT GGC TAC Rev: TTT GTT TGA CAG GAG CGA CAA T	
	eSOX2	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TTT GTT TGA CAG GAG CGA CAA T	
Pluripotency Markers (qPCR)	OCT4	Fwd: CCC CAG GGC CCC ATT TTG GTA CC Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC	
	LIN28	Fwd: AGC CAT ATG GTA GCC TGA TGT CCG C Rev: TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG	
	L-MYC	Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC Rev: CAG GGG GTC TGC TCG CAC CGT GAT G	
	SOX2	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TCA CAT GTG TGA GAG GGG CAG TGT GC	
House-Keeping Genes (qPCR)	β-ACTIN	Fwd: GGC ATC CTC ACC CTG AAG TA Rev: GGG GTG TTG AAG GTC TCA AA	
Genotyping			
Targeted mutation analysis	CHRNA7	Fwd: GCAGGAAGTGCTGGCCTATG Rev: GGTCCAAACGTGGGCTCC	
TaqMan primers used for qPCR	Target	Forward/Reverse sequence (5'-3')	
Differentiation markers	SOX1	Hs01057642_s1	
	NESTIN	Hs04187831_g1	
	PAX6	Hs00240871_m1	
	T	Hs00610080_m1	
	EOMES	Hs00172872_m1	
	GATA4	Hs00171403_m1	
	FOXA2	Hs00232764_m1	
House-Keeping Gene	SOX17	Hs00751752_s1	
	βActin	Hs 99999903_m1	

cultured in 5% CO₂ at 37 °C. The iPSC colonies were mechanically-detached and amplified once a week, the split ratio of iPSCs was usually 1:6. N-Garde Mycoplasma PCR kit (EuroClone) was used to verify the absence of mycoplasma contamination.

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde for 20 min at room temperature and blocked in PBS containing 20% Normal Goat Serum. 0.1% Triton X-100 was used for 30 min for only OCT4 staining. Next, primary antibodies, listed in Table 2, diluted in 5% BSA, were added and incubated O/N at 4 °C. After washing, Alexa-Fluor-conjugated secondary antibodies were added for 1 h at room temperature. Cellular

nuclei were counterstained with Hoechst. Microphotographs were taken using a Nikon C2 fluorescence microscope.

RNA extraction and reverse-transcription

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real-time PCR (qPCR)

qPCR was performed on cDNA using SYBR Green primers for pluripotency markers and TaqMan primers for differentiation markers

(Table 2) in a 7900HT Fast Real-Time PCR system (Applied Biosystem). Each reaction was performed in triplicate with β -ACTIN gene as reference. $2^{-\Delta\Delta CT}$ method was adopted to calculate the relative expression levels.

The same method was used to confirm the presence of *CHRNA7* duplication on DNA extracted from iPSC line using column-based extraction kit (DNeasy Blood & Tissue kit; QIAGEN, Hilden, Germany). DNAs extracted from blood and previously tested negative by SNP-array were used as controls. *TERT* gene was used as a reference. Data analysis was performed as described in Carbone et al. (2008).

Teratoma formation

Approximately 3×10^6 cells were detached using Dispase (Gibco). Cellular suspension was collected at 800 rpm for 5 min and then resuspended in 100 μ l of Matrigel. Cells were injected into the right flank of nude immune-deficient mice (NOD/SCID) following ethical guidelines. Teratomas formed in 4–8 weeks. Upon removal, tumors were histologically analyzed to check for their *in vivo* differentiation capacity into derivatives of all three germ layers: ectodermal, mesodermal and endodermal.

Embryoid body *in vitro* differentiation

Mechanically-detached iPSCs were plated in a Petri dish in floating condition. Nutristem-XF medium was then gradually switched with DMEM F-12, 20% Knock-out serum replacement (Gibco), 0.1 mM β -mercaptoethanol, $1 \times$ NEAA, 50 U/ml Penicillin-Streptomycin, 2 mM L-glutamine in 3 days. EBs were cultured in suspension for 14 days, changing the medium every two days, then pelleted and RNAs were extracted for qRT-PCR analysis.

Karyotype analysis

Cells were cultured in chamber slides (Thermo Fisher Scientific) and treated with a 0.1 μ g/ml COLCEMID solution (Thermo Fisher Scientific) for 60 min at 37 °C. Metaphases were obtained by adding 30 mM KCl in 10% FBS at 37 °C for 6 min and by fixation using cold fresh-made 3:1 ethanol:acetic acid solution. Karyotype analysis was carried out on GTG-banded metaphases. Fifteen metaphases were counted and three

karyotypes analyzed (450–500 resolution). Only clonal aberrations were considered, following the ISCN recommendations.

STR analysis

Fibroblasts and iPSCs DNA were extracted by Dneasy blood and tissue kit (QIAGEN) following manufacturer's suggestions. PCR amplification of 19 distinct STRs (D13S252, D13S305, D13S634, D13S800, D13S628, D18S819, D18S535, D18S978, D18S386, D18S390, D21S11, D21S1437, D21S1409, D21S1442, D21S1435, D21S1446, DXS6803, XHPRT, DXS1187) was carried out using the QST⁺Rplusv2 kit (Elucigene Diagnostics), PCR products were separated on an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper version 4.0 (Applied Biosystems).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.09.002>.

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References

- Carbone, A., Bernardini, L., Valenzano, F., Bottillo, I., De Simone, C., Capizzi, R., Capalbo, A., Romano, F., Novelli, A., Dallapiccola, B., Amerio, P., 2008 Dec. Array-based comparative genomic hybridization in early-stage mycosis fungoides: recurrent deletion of tumor suppressor genes BCL7A, SMAC/DIABLO, and RHOF. *Genes Chromosom. Cancer* 47 (12), 1067–1075.
- De Jaco, A., Bernardini, L., Rosati, J., Tata, A.M., 2017. Alpha-7 nicotinic receptors in nervous system disorders: from function to therapeutic perspectives. *Cent. Nerv. Syst. Agents Med. Chem.* 17 (2), 100–108.
- Gillentine, M.A., Berry, L.N., Goin-Kochel, R.P., Ali, M.A., Ge, J., Guffey, D., Rosenfeld, J.A., Hannig, V., Bader, P., Proud, M., Shinawi, M., Graham, B.H., Lin, A., Lalani, S.R., Reynolds, J., Chen, M., Grebe, T., Minard, C.G., Stankiewicz, P., Beaudet, A.L., Schaaf, C.P., 2017. The cognitive and behavioral phenotypes of individuals with *CHRNA7* duplications. *J. Autism Dev. Disord.* 47 (3), 549–562. <https://doi.org/10.1007/s10803-016-2961-8>.
- Gillentine, M.A., Yin, J., Bajic, A., Zhang, P., Cummock, S., Kim, J.J., Schaaf, C.P., 2017 Dec 7b. Functional consequences of *CHRNA7* copy-number alterations in induced pluripotent stem cells and neural progenitor cells. *Am. J. Hum. Genet.* 101 (6), 874–887. <https://doi.org/10.1016/j.ajhg.2017.09.024>.