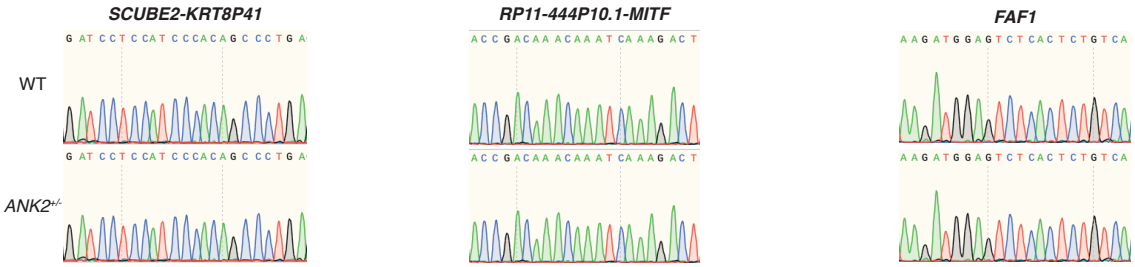
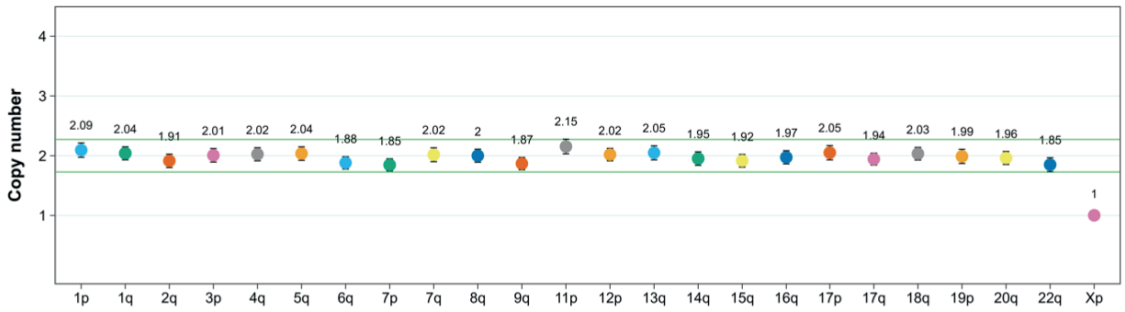
A

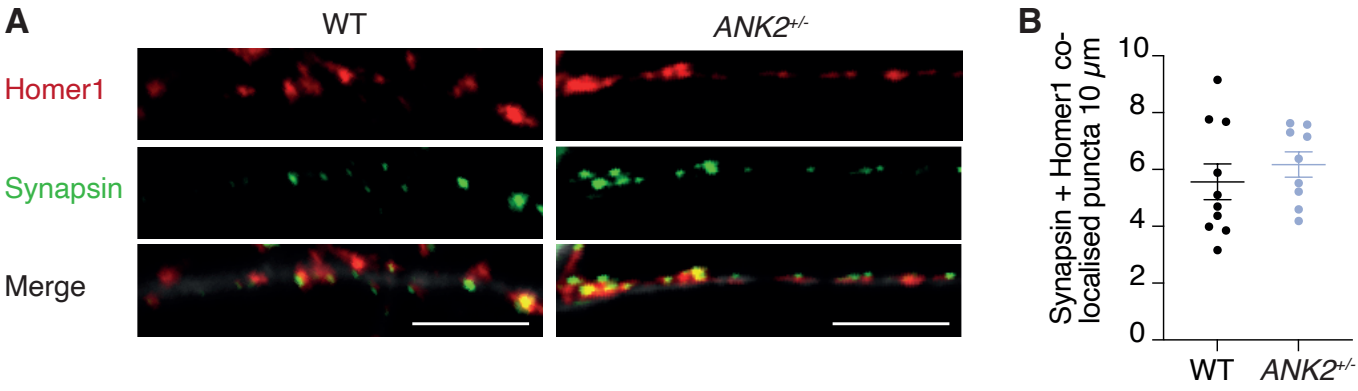
Gene	Chr	Position	CFD	On target	Primers
SCUBE2-KRT8P41	11	9113950	0.56	False	Fw: TCGTCGGCAGCGTCGGGAGTGGAAGGAATTGGGG Rv: GTCTCGTGGGCTCGGTTGTGCATGAGCTAGGTGGG
RP11-444P10.1-MITF	3	69769316	0.54	False	Fw: TCGTCGGCAGCGTCGGATCACTGGGTGACACAGG Rv: GTCTCGTGGGCTCGGATGGCTGTCTAGGGTCCACT
FAF1	1	51264348	0.39	False	Fw: TCGTCGGCAGCGTCGCATCAGCTCTCCAGTTATACA Rv: GTCTCGTGGGCTCGGTCTTCAAGATCAGTCAGTGAGGT



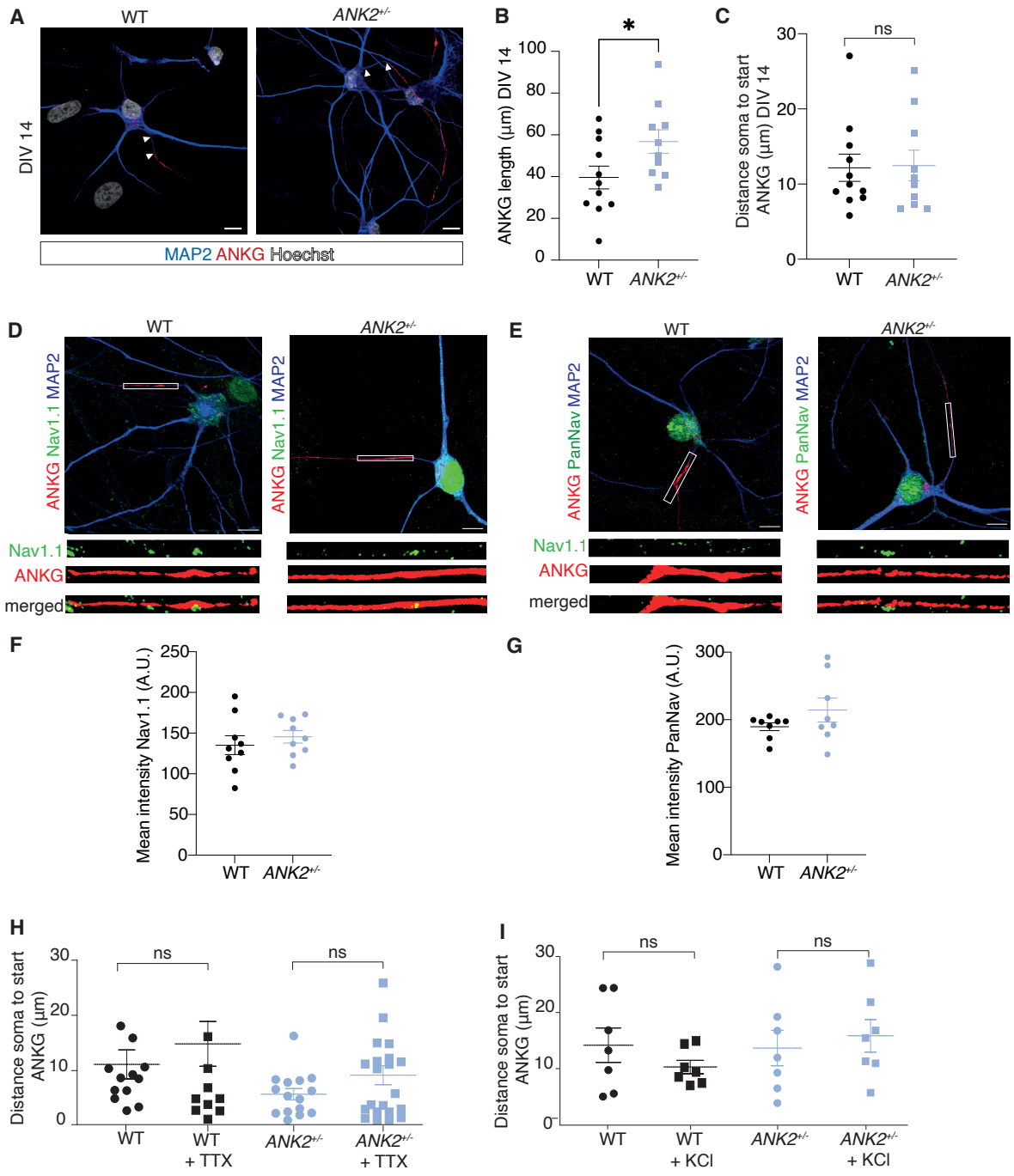
B



Supplementary Fig 1. Characterization of *ANK2*$^{+/-}$ iPSCs. Related to Figure 2. (A) Top three potential off-target sites have been sequenced and no mutations were detected in *ANK2*$^{+/-}$ iPSCs. **(B)** *ANK2*$^{+/-}$ iPSCs showed no genetic abnormalities after genomic editing.



Supplementary Fig 2. Quantification of functional synapses. Related to Figure 3. (A) Representative images of glutamatergic synapses. Homer1 in red as a postsynaptic marker and synapsin in green as a presynaptic marker. Scale bare, 5 µm. **(B)** Quantification of co-localized Homer1/Synapsin (number per 10 µm dendrite). *n* = 9-10 cells/group at DIV 28.



Supplementary Fig 3. Distance soma to start AIS in WT and *ANK2*$^{+/-}$ iNeurons. Related to Figure 4. (A) Representative images of WT and *ANK2*$^{+/-}$ iNeurons immunostained for MAP2 (blue) and ANKG (red) at DIV 14 (scale bar 10 µm). Arrowheads indicate the start and end of the AIS. **(B)** Quantification of ANKG length by immunostaining in WT and *ANK2*$^{+/-}$ iNeurons at DIV 14, n = 10-11 cells/group. **(C)** Quantification of the distance between soma and proximal end of ANKG staining by immunostaining in WT and *ANK2*$^{+/-}$ iNeurons at DIV 14, *n* = 10-11 cells/group. **(D)** Representative images of WT and *ANK2*$^{+/-}$ iNeurons

immunostained for ANKG (red) Nav1.1 (green) and MAP2 (blue) at DIV 28 (scale bar 10 μm). Boxes indicate a zoom-in of the AIS co-expressed with Nav1.1. **(E)** Representative images of WT and *ANK2*$^{+/-}$ iNeurons immunostained for ANKG (red) PanNav (green) and MAP2 (blue) at DIV 28 (scale bar 10 μm). Boxes indicate a zoom-in of the AIS co-expressed with PanNav **(F)** Quantification of mean intensity of Nav1.1 in the AIS at DIV 28, n = 8-9 cells/group. **(F)** Quantification of mean intensity of PanNav in the AIS at DIV 28, n = 8 cells/group. **(H)** Quantification of distance soma to start ANKG shown by immunostaining in WT and *ANK2*$^{+/-}$ iNeurons under basal condition or treated with 1 μM TTX, n = 9-21 cells/group. Analyzed by one-way ANOVA and Tukey's post hoc analysis. **(I)** Quantification of distance soma to start ANKG shown by immunostaining in WT and *ANK2*$^{+/-}$ iNeurons under basal condition or treated with 10 mM KCl, n = 7 cells/group. Analyzed by one-way ANOVA and Tukey's post hoc analysis.

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 7	Individual 8	Individual 9	Individual 10	Individual 11	Individual 12
Age (year)	17	13	15	12	10	18	9	12	9	1.75	1.6	7
Sex	Male	Female	Male	Female	Male	Male	Female	Female	Male	Male	Female	Male
Genomic location (GRCH37)	g.114257872_114257873del	g.114239672G>A	g.114170950C>T	g.113682175_114039872del	g.114209543G>A	g.114251520C>T	g.113905001_114223000del	g.114177059_114177060del	g.114277134_114277135delTCinsA	g.114179468G>A	g.114284505G>T	g.114163336C>T
cDNA location	c.3632_3633del	c.2797-1G>A	c.922C>T	c.-288710_85-55700del	c.2179-1G>A	c.3019C>T	c.-65884_247692 3del	c.1159_1160del	c.7360_7361delTCinsA	c.1288-1G>A	c.10768G>T	c.862C>T
Protein change	p.Ser1244Met(fs*5)	p.(?);-	p.Arg308*	N/a	p.(?);-	p.Arg1007*	N/a	p.Lys387Glu(fs*10)	p.Ser2454Ile(fs*7)	p.(?);-	p.Glu3590*	p.Arg288*
Mutation type	Deletion leading to frameshift	Probable leading to frame shift	Nonsense	Deletion	Splice site variant	Nonsense	Deletion	Deletion leading to frameshift	Indel leading to frameshift	Splice site variant	Nonsense	Nonsense
Inheritance	*De novo*	*De novo*	*De novo*	Unknown	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*
ID / DD	Mild	Mild	Mild	Mild	Mild *	Severe	None	Moderate	Mild	None	None	Mild
Speech development	Delayed	Delayed	Delayed	Unknown	Unknown	Severely delayed (non-verbal)	Unknown	Delayed with regression	Severely delayed	Normal (speaks words at 19 moths)	Babbles and uses syllables, no words (14 months)	Delayed
Motor development	Unknown	Unknown	Delayed	Unknown	unknown	Yes	Unknown	Normal	Normal	Normal	Normal	Delayed (walking 23 months)
Autism spectrum disorder	Yes	Yes	Probable	Yes	Unknown	Yes	Yes	No	No	N/a	N/a	Yes
Other behavioral problems	Gilles de la Tourette, ADHD, DCD	Attention difficulties,	ADD	Unknown	Unknown	Agitation, sleep disturbances	Unknown	ADHD, agitation and aggression	ADHD, aggressiveness	-	-	-
Medication	Dex-amphetamine, risperidone	-	Sertraline, methylphenidate	-	Dexmethyl-phenidate	-	-	Risperidone, melatonin	-	-	-	-
Congenital anomalies	None	Hyperlaxity	None	Congenital hip dysplasia	Generalized overgrowth	-	Unknown	Severe laryngomalacia (day 8)	-	-	-	-
Hypotonia	Possible	Yes	Yes	Unknown	Yes	No	Unknown	No	Yes	Yes	No	-
Brain MRI	-	Normal	Normal	-	Unknown	Normal	-	Abnormal (hyperintensities and atrophy)	-(normal CT)	Normal	Normal	-
ECG	Intraventricular conduction delay	Normal	Intraventricular conduction delay	Normal	Normal	None	Unknown	Normal	Normal	Unknown	Unknown	Not performed
Structural abnormalities	Small atrial septal defect and patent foramen ovale	None	Small atrial septal defect type II	None	None	None	Unknown	None	Unknown	Unknown	Unknown	-
Epilepsy	No	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Yes	-

Other	NRXN1 variant, maternally inherited	KNCQ3 variant, paternally inherited (paternal grandmother also carrier) and 18p11.32 duplication, maternally inherited. Mother was diagnosed with classical autism, but is highly educated		Severe feeding difficulties for years (necessating peg tube feeding)	Maternally inherited c.1555G>A (p.E519K) VUS in SCN9A; paternally inherited c.8120G>A (p.R2707Q) VUS in TRIO. ALTE at 11 months	Missense variant ALG13, maternally inherited		Sleep apnea	-		Mild increase of muscle tone during infancy with mild coordination deficits	-

VNS: vagal nerve stimulator. ADHD: attention deficit hyperactivity disorder. ADD: attention deficit disorder. DCD: developmental coordination disorder. ALTE: apparent life threatening event. VUS: variant of unknown significance.

Comments table 1: * IQ not tested; estimated mild ID, patient has individualized education plan. ID = intellectual disability. DD = developmental delay. IS= infantile spasms. LGS= lennox-gastaut syndrome. ALTE= apparent life threatening event, GTCS= generalized tonic-clonic seizures.

Supplementary Table 1: General characteristics and variants of *ANK2*-related patients associated with neurodevelopmental disorder

g.DNA	c.DNA (NM_001148.5)	Protein change	Protein effect	SampleID	Reference article	Reported phenotype
4:g.114155754G>A	c.483+2339G>A	p.?	Intronic variant	n/a	Kong 2012	ASD
4:g.114163415C>G	c.891+50C>G	p.?	Intronic variant	SSC03315	Satterstrom 2020	ASD
4:g.114170950C>T	c.922C>T	p.(Arg308Ter)	Nonsense	Patient 3		NDD, epilepsy
4:g.114177051T>G	c.1151T>G	p.(Leu384Arg)	Missense	08C74775	Satterstrom 2020	NDD
4:g.114177060del	c.1160del	p.(Lys387ArgfsTer10)	Frameshift	Patient 8		NDD, epilepsy
4:g.114179468G>T	c.1288-1G>T	p.?	Splice acceptor variant	Patient 10		Epilepsy
4:g.114186083C>T	c.1417C>T	p.(Arg473Ter)	Nonsense	127b	Ji 2019	NDD, epilepsy
4:g.114203820G>A	c.1882-11G>A	p.?	Intronic variant	13882	Iossifov 2014	ASD
4:g.114203886C>T	c.1937C>T	p.(Ser646Phe)	Missense	n/a	Swayne 2017	Cardiac arrhytmia, epilepsy
4:g.114209543G>A	c.2179-1G>A	p.?	Splice acceptor variant	Patient 5		NDD, epilepsy
4:g.114214640C>G	c.2421C>G	p.(Ile807Met)	Missense	UK10K_SKUSE5080174	De_Rubeis_Nature_2014	ASD

4:g.114232545C>T	c.2683C>T	p.(Arg895Ter)	Nonsense	12645	Iossifov 2014	ASD
4:g.114239672G>A	c.2797-1G>A	p.?	Splice acceptor variant	Patient 2	Lelieveld 2016	NDD
4:g.114251469C>T	c.2968C>T	p.(Arg990Ter)	Nonsense	14256	Iossifov 2014	ASD
4:g.114251470G>A	c.2969G>A	p.(Arg990Gln)	Missense	n/a	Smith 2015	Cardiac arrhytmia
4:g.114251520C>T	c.3019C>T	p.(Arg1007Ter)	Nonsense	Patient 6		NDD, epilepsy
4:g.114254247C>T	c.3262C>T	p.(Arg1088Ter)	Nonsense	DEASD_0140_001	Rubeis 2014	ASD
4:g.114254305A>G	c.3320A>G	p.(Glu1107Gly)	Missense	DDD4K.04291	DDD 2017	NDD
4:g.114257872del	c.3731del	p.(Ser1244MetfsTer5)	Frameshift	Patient 1		NDD
4:g.114264189C>G	c.4139C>G	p.(Pro1380Arg)	Missense	10C105731	Rubeis 2014	ASD
4:g.114267127G>A	c.4320G>A	p.(Leu1440=)	Synonymous	236-15-DR	Satterstrom 2020	ASD
4:g.114269433A>G	c.4373A>G	p.(Glu1458Gly)	Missense	n/a	Mohler 2003	Cardiac arrhytmia
4:g.114274438T>A	c.4664T>A	p.(Val1555Asp)	Missense	n/a	Mohler 2007	Cardiac arrhytmia
4:g.114275304C>T	c.5530C>T	p.(Pro1844Ser)	Missense	14130	Iossifov 2014	ASD
4:g.114275804C>A	c.6030C>A	p.(Leu2010=)	Synonymous	14367.s1	Krumm 2015	ASD
4:g.114276678_114276691del	c.6904_6917del	p.(Thr2302PhefsTer16)	Frameshift	676-05-104737	Satterstrom 2020	ASD
4:g.114277602del	c.7828del	p.(Asp2610ThrfsTer23)	Frameshift	13768	Iossifov 2014	ASD
4:g.114277134_114277135delinsA	c.7360_7361delinsA	p.(Ser2454IlefsTer7)	Frameshift	Patient 9		NDD

4:g.114278455del	c.8681del	p.(Asp2894AlafsTer20)	Frame shift	Patient 4		NDD
4:g.114279628T>C	c.9854T>C	p.(Ile3285Thr)	Missense	n/a	Krogh Broendberg 2015	Cardiac arrhytmia
4:g.114280060A>T	c.10286A>T	p.(Glu3429Val)	Missense	1-0234_004	Yuen 2015	ASD
4:g.114284505G>T	c.10768G>T	p.(Glu3590Ter)	Nonsense	Patient 11		Epilepsy
4:g.114288907C>A	c.11218C>A	p.(Leu3740Ile)	Missense	n/a	Mohler 2004	Cardiac arrhytmia
4:g.114288920C>A	c.11231C>A	p.(Thr3744Asn)	Missense	n/a	Mohler 2004	Cardiac arrhytmia
4:g.114294462C>T	c.11716C>T	p.(Arg3906Trp)	Missense	n/a	Mohler 2004	Cardiac arrhytmia
4:g.114294537G>A	c.11791G>A	p.(Glu3931Lys)	Missense	n/a	Mohler 2004	Cardiac arrhytmia
4:g.114302677A>G	c.*50A>G	p.?		DDD4K.01859	DDD 2017	NDD
4:g.114302686G>A	c.*59G>A	p.?		212-21046-1	Stessman	NDD

Supplementary Table 2: Variants in *ANK2* associated with neurodevelopmental disorder and epilepsy of this cohort and previously reported in literature using NM_001148.5 (GRCh37)