

ORIGINAL ARTICLE

# Heterozygous *KIDINS220*/*ARMS* nonsense variants cause spastic paraplegia, intellectual disability, nystagmus, and obesity

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## Abstract

We identified *de novo* nonsense variants in *KIDINS220*/*ARMS* in three unrelated patients with spastic paraplegia, intellectual disability, nystagmus, and obesity (SINO). *KIDINS220* is an essential scaffold protein coordinating neurotrophin signal pathways in neurites and is spatially and temporally regulated in the brain. Molecular analysis of patients' variants confirmed expression and translation of truncated transcripts similar to recently characterized alternative terminal exon

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splice isoforms of *KIDINS220*. *KIDINS220* undergoes extensive alternative splicing in specific neuronal populations and developmental time points, reflecting its complex role in neuronal maturation. In mice and humans, *KIDINS220* is alternatively spliced in the middle region as well as in the last exon. These full-length and *KIDINS220* splice variants occur at precise moments in cortical, hippocampal, and motor neuron development, with splice variants similar to the variants seen in our patients and lacking the last exon of *KIDINS220* occurring in adult rather than in embryonic brain. We conducted tissue-specific expression studies in zebrafish that resulted in spasms, confirming a functional link with disruption of the *KIDINS220* levels in developing neurites. This work reveals a crucial physiological role of *KIDINS220* in development and provides insight into how perturbation of the complex interplay of *KIDINS220* isoforms and their relative expression can affect neuron control and human metabolism. Altogether, we here show that *de novo* protein-truncating *KIDINS220* variants cause a new syndrome, SINO. This is the first report of *KIDINS220* variants causing a human disease.

## Introduction

Next Generation Sequencing (NGS) techniques provide a time and cost-efficient method to identify novel genes. We used this approach to investigate two patients presenting with an apparently unique combination of features, including obesity, spastic paraplegia, intellectual disability, nystagmus and macrocephaly and identified different, *de novo*, heterozygous, nonsense variants in *KIDINS220* in each patient. A third patient with similar features was ascertained through DECIPHER (decipher.sanger.ac.uk) after the DDD study shared a *de novo* candidate variant identified by trio exome analysis (1,2).

*KIDINS220* (Kinase D interacting substrate of 220 kDa), also known as ARMS (Ankyrin Repeat-rich Membrane Spanning), is a conserved scaffold protein that controls axonal and dendritic maturation (3,4). The spatiotemporal expression of different *KIDINS220* isoforms is finely tuned in the mammalian brain, and knock-out *Kidins220* animals show developmental central nervous system anomalies (5–7). Recently, it was shown that distinctive neuronal populations express different *KIDINS220* splice isoforms at specific times during development, including newly characterized alternative terminal exon (ATE) splice isoforms (8). The expression of the complete range of these isoforms, with the corresponding domains for thus far poorly characterized interaction partners, may be crucial for proper neuronal development. We show here that our patients' variants result in truncated isoforms of *KIDINS220* similar to an ATE splice isoform expressed mainly during adulthood. Our patients' neurological phenotype may be caused by disruption of full-length *KIDINS220* splice isoform repertoire expression and lack of essential interaction domains that are necessary during embryonic development of the neural network. We further show that perturbation of *KIDINS220* levels causes a spasticity phenotype in zebrafish. We therefore suggest that *KIDINS220* variants cause a new syndrome characterized by spastic paraplegia, intellectual disability, nystagmus and obesity (SINO syndrome).

## Results

### *De novo KIDINS220* variants are implicated in patients with a unique phenotype of SINO

We performed NGS analysis in three unrelated patients with SINO (Fig. 1 and Table 1, Supplementary Materials).

Two patients were sequenced for a targeted gene panel of 582 genes to investigate the suspected genetic cause of syndromic and non-syndromic obesity (OBESITOME, UMC Utrecht, The Netherlands) (Supplementary Material, Table S1). We identified heterozygous *KIDINS220* variants, NM\_020738.2:c.4050G >

A, p.(Trp1350\*) and NM\_020738.2:c.4096C > T, p.(Gln1366\*), in patient 1 and 2, respectively. Both variants result in a premature stop codon. Their *de novo* occurrence was confirmed by Sanger sequencing of both patients and their parents. There were no further *de novo* variants in other shared genes on the OBESITOME panel. Independently, whole exome sequencing on patient 3 revealed a heterozygous *de novo* insertion of an A nucleotide, NM\_020738.2:c.4520dup, resulting in a frameshift and predicted premature truncation of the protein p.(Leu1507Phefs\*4) (1,2) (Fig. 2A and B).

### Variant *KIDINS220* RNA expression results in truncated *KIDINS220* in patient fibroblasts and HEK293T cells

All variants identified here fall within the last two exons of the gene (Fig. 2A and B). Sanger sequencing on fibroblast cDNA from patient 1 showed that variant c.4050G > A resulted in proper mRNA expression (Fig. 2C). This result was confirmed by Quantitative PCR, with the relative expression of *KIDINS220* before and after the variant c.4050G > A equal in patient fibroblast cDNA compared with control fibroblast cDNA, indicating that the shortened transcript escaped nonsense-mediated decay (NMD) (data not shown). The mammalian NMD pathway is unable to recognize premature termination codons in the last exon and the last ~55 bp of the penultimate exon (9). Shortened mRNA transcripts that escape NMD are translated into truncated protein products possibly lacking important C-terminal domains. Variants in the penultimate exon resulting in premature stop codons that escape NMD are reported in other syndromes, e.g. autosomal dominant Robinow syndrome (10). Furthermore, we used cDNA sequencing to assess known alternative splicing of exon 25 and exon 26 in the middle region of *KIDINS220*, which has not yet been determined in fibroblast cells. Patient and control cDNA sequencing of the region flanking and containing exons 25 and 26 revealed that fibroblasts from both our patient 1 and control express *KIDINS220* with exon 26 removed by alternative splicing (GenBank accession no. KJ812120), indicating that exon 26 is removed in all fibroblast cells (data not shown).

We analyzed total protein extract of fibroblasts of patient 1 by Western blot analysis and detected a 150 kDa band in addition to the 220 kDa wild-type *KIDINS220* band, confirming expression of the truncated, Trp1350\* protein (Fig. 2D). The variants of patient 1 and 2 were also overexpressed separately in HEK293T cells to visualize the size of the truncated protein products, using human isoform containing both exons 25 and 26 (GenBank accession no. KJ812119) (8). The presence of a 150 kDa protein coinciding with the p.(Trp1350\*) variant of patient 1 and a 167 kDa protein coinciding with the p.(Gln1366\*) variant of patient 2 were

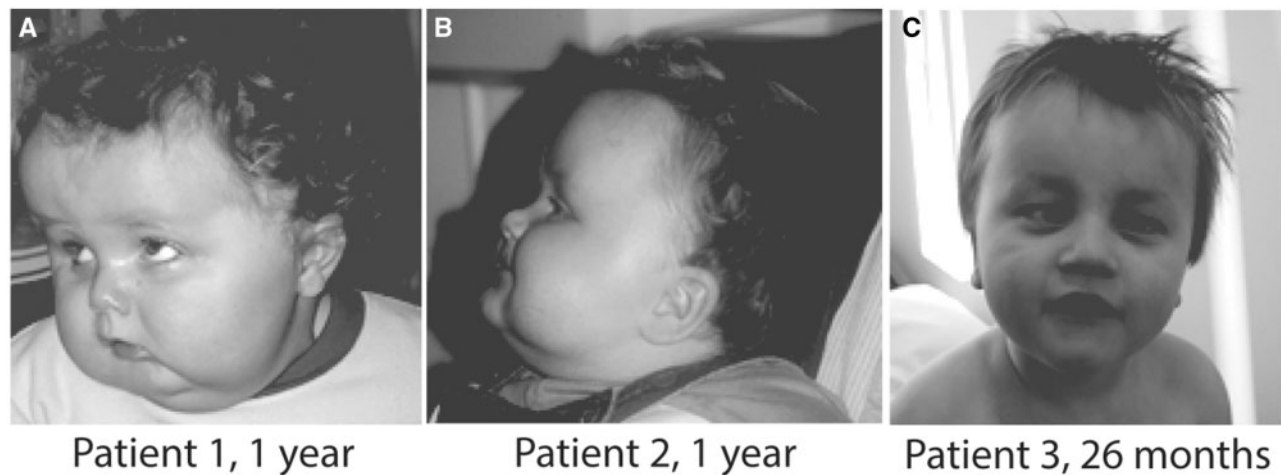


Figure 1. Clinical features of patients. Note obesity, macrocephaly, brachycephaly and eye abnormalities.

Table 1. Clinical phenotype of the three patients with *KIDINS220* *de novo* variants

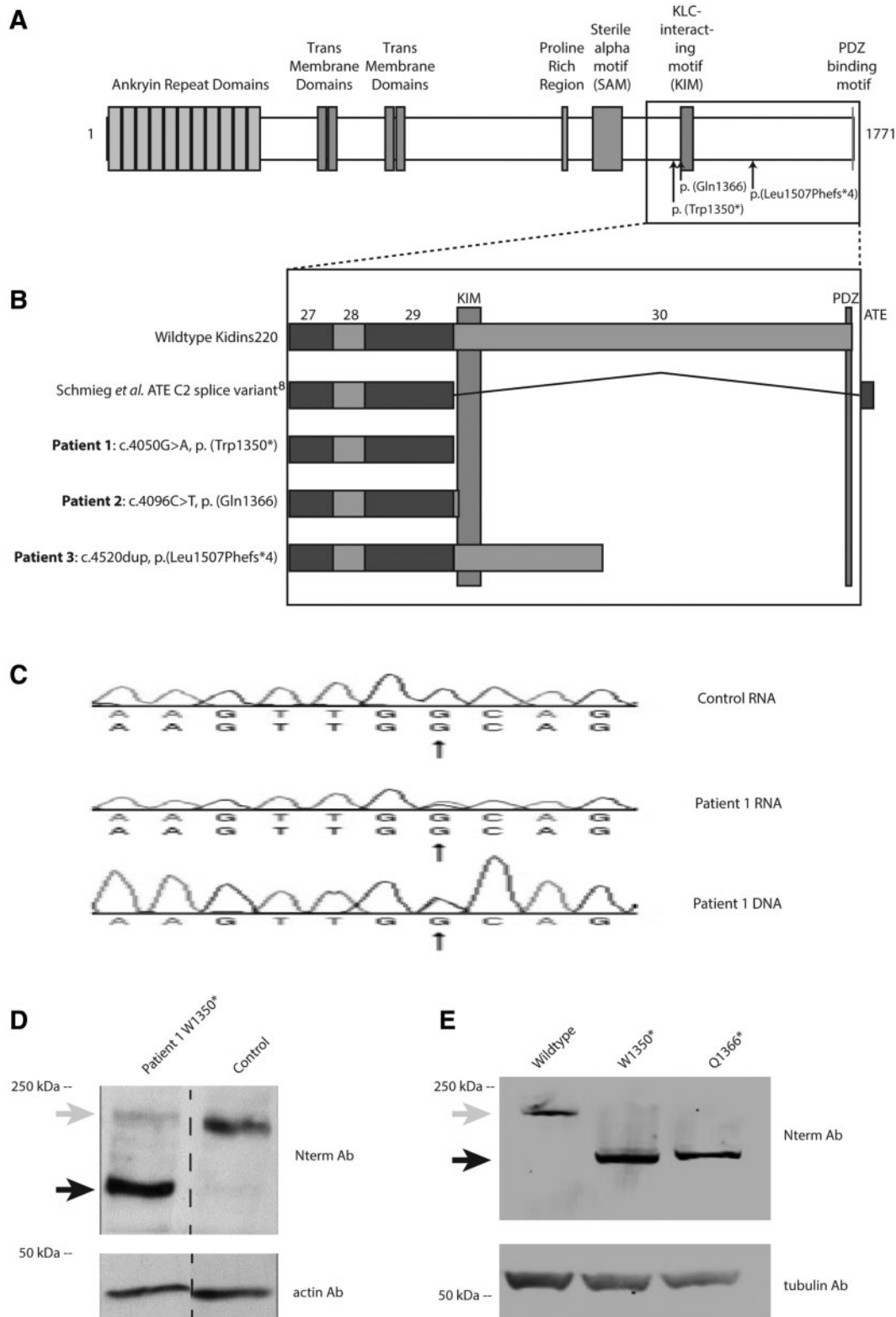
	Patient 1	Patient 2	Patient 3
Prenatal Scan	Dilatation of lateral ventricles	Dilatation of lateral ventricles	Dilatation of lateral ventricles
Polyhydramnios	–	+	?
Gestational age (weeks)	38	40	41
Weight (birth)	2.26 kg (second centile)	3.65 kg (75th centile)	3.28 (50th centile)
Weight (infancy)	>99.6th centile	99.6th centile	98th centile
Weight (puberty)	>99.6th centile	>99.6th centile	Overweight
Height (infancy)	91–98th centile	91st centile	75th centile
Height (puberty)	91st centile	91st centile	?
OFC (birth)	25–50th centile	?	?
OFC (infancy)	>>99.6th centile	99.6th centile	98th centile
OFC (puberty)	99.6th centile	>99.6th centile	25–50th centile
Feeding pattern	Nl	Nl	Nl
Dysmorphic features	Brachyplagiocephaly Bossed forehead Deep set eyes	Brachyplagiocephaly Prominent forehead Deep-set eyes Crowded teeth	Brachyplagiocephaly Prominent forehead
Vision	Reduced acuity Hypermetropia Astigmatism	Squint Hypermetropia Astigmatism	Squint Hypermetropia Esotropia
Nystagmus	+	+	+
ERG	Nl	?	?
VEP	Post retinal dysfunction	?	?
Development	Delayed	Delayed	Delayed
Neurology	Axial hypotonia Spastic paraplegia	Spastic paraplegia	Spastic paraplegia
Brain MRI	Dilated lateral and third ventricles	Dilated third and lateral ventricles	Dilated lateral ventricles
	Normal fourth ventricle Reduced white matter bulk Mild delay in myelination	Normal fourth ventricle Reduced white matter bulk	High riding third ventricle Partial agenesis of corpus callosum
	Mild generalized atrophy	Mild generalized atrophy	
Bone age	?	Slightly delayed	?
Eating habits	Nl	Nl	Nl
Development	Moderate global delay	Moderate global delay	Moderate global delay

Nl, normal, +, present, –, absent, ?, not reported; OFC, occipito frontal circumference; ERG, electroretinogram; VEP, visual evoked potential.

detected by Western blot. Only wild-type 220 kDa protein was identified in HEK293T extracts overexpressing the wild-type *KIDINS220* (Fig. 2E). The *KIDINS220* protein product of these variants is strikingly similar to that produced by ATE splicing in mouse and humans (ATE C2) (Fig. 2B) (8).

#### Motor neuron specific expression of *KIDINS220* variants induces spasms in zebrafish

Zebrafish is a powerful, versatile vertebrate model to study human motor neuron diseases (11,12). By using the Gal4/UAS approach (13,14) we achieved motor neuron-specific expression of



**Figure 2.** De novo dominant nonsense variants in *KIDINS220*. (A) Variants p.(Trp1350\*) and p.(Gln1366\*) result in truncated protein without the full KIM domain, and p.(Leu1507Phefs\*4) results in a slightly longer protein containing the full KIM domain. (B) Comparison of the wild-type *KIDINS220* exon structure versus an alternate terminal ending splice variant described by Schmieg *et al.* (8) and the three patients described in this study (C) *KIDINS220* cDNA sequencing verifies that there is no mRNA NMD in patient 1. Western blot analysis of (D) total extracts from patient 1 versus a control fibroblast and (E) total HEK293T extracts expressing wild-type and variant (Trp1350\* and Gln1366\*) forms of *KIDINS220*. Expression of *KIDINS220* was tested with antibodies against the N-terminal part of *KIDINS220* (Nterm Ab). The truncated protein products caused by *KIDINS220* variants are indicated with a black arrow and the expected full-length wild-type protein product by a grey arrow. Anti-actin was used as a loading control for the fibroblasts and anti-tubulin for the HEK293T cells.



human *KIDINS220* variants in zebrafish embryos and analyzed their effect *in vivo*. Transgenic tg(Isl1BAC:GalFF) fish used here constitutively express Gal4 in motor neurons; when DNA constructs containing an upstream activation sequence (UAS) driving transcription are supplemented by microinjection, mosaic larvae are obtained in which Gal4 binds to the UAS sequence to express *KIDINS220* solely in motor neurons. Embryos tg(Isl1BAC:GalFF) microinjected with UAS:*KIDINS220* wild-type or variant p.(Trp1350\*) displayed spasms in the trunk at 5 days post fertilization (dpf), confirming a functional link between spasticity and disruption of *KIDINS220* levels and/or function (Fig. 3). This was slightly more evident in embryos expressing the mutant *KIDINS220*. Representative recordings of the spasm phenotypes can be found in the Supplementary Material online.

### Kidins220 variants do not result in different cellular localizations in Neuro-2A cells

During differentiation, *KIDINS220* is transported to the neurite tips of PC12 neuronal cells, a process that is altered in *KIDINS220* isoforms with an ATE (4,8). We examined the cellular location of wild-type and truncated *KIDINS220* in Neuro-2A cells, a cell line where *KIDINS220* is endogenously expressed, albeit at lower levels than in PC12 cells (4). Following cotransfection of HA-tagged wild-type or p.(Gln1366\*) *KIDINS220* as well as a mCherry reporter, Neuro-2A cells were differentiated with retinoic acid for a total of 5 days. Fixation was performed at day 5 and immunofluorescence performed with an anti-HA antibody. Differentiated cells expressing wild-type or p.(Gln1366\*) exhibited localization of *KIDINS220* throughout the entire cell body, as well as in the neurite tips. There was no difference in the cellular localization of the wild-type or variants (Fig. 4).

### Discussion

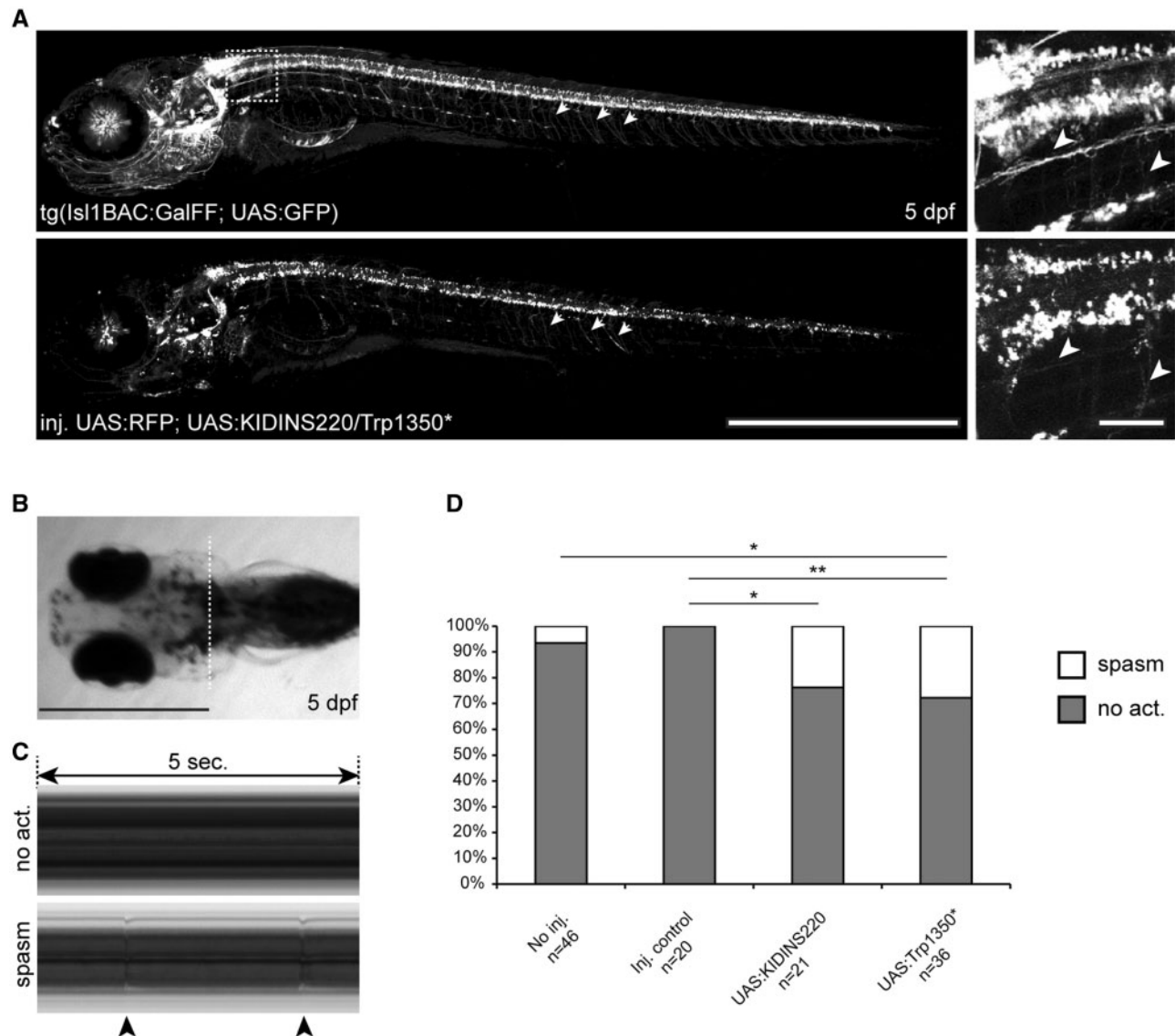
*KIDINS220* controls axonal and dendritic maturation in the developing mammalian brain, and is integral to the branching, stabilization and complexity of cortical and hippocampal dendrites (3,4,7). The cytoplasmic N-terminus contains 11 ankyrin repeats. There are four transmembrane segments in the central part of the molecule (15). The C-terminal tail, also exposed to the cytoplasm, contains several protein–protein interacting domains: a proline-rich domain, Sterile Alpha Motif (SAM), Kinesin Light Chain (KLC)—Interactive Motif (KIM) and PDZ-binding motif (3,16). These domains enable the recruitment of cell-specific adaptors and effectors to activated Tropomyosin receptor Kinases (Trk) and are involved in functional interactions with other receptors such as the Glutamate, Ephrin and Vascular Endothelial Growth Factor receptors (6,17). The KIM domain mediates intracellular trafficking of *KIDINS220* through direct binding to KLC-1 and -2 and is important for a correct response to neurotrophic stimuli (16,18). Expression of *KIDINS220* is developmentally regulated and differential splicing occurs in specific tissues such as the brain, heart, and skeletal muscle. The alternative splicing is complex and developmental occurrence of *KIDINS220* splice isoforms with the middle region and/or last exon removed varies in cortical, hippocampal and motor neurons (8). Schmieg *et al.* (8) demonstrated in mice that *KIDINS220* with exon 30 included is expressed in embryonic tissue; in contrast, a similar *KIDINS220* splice variant lacking exon 30 is present solely in adult tissue. Motor neurons exhibited the highest variety of alternative splicing in the middle region, but did not exhibit any ATE splicing at any time during

development. Our patients' variants result in isoforms similar to *KIDINS220* splice variants with ATE splicing. The constitutive expression of truncated isoforms similar to ATE splice isoforms that are not usually expressed in the developing embryo or present in motor neurons, may perturb the complex splice regulation necessary for proper neurite development and result in the phenotype seen in our patients.

In this manuscript we show that *KIDINS220* nonsense variants are causal for a characteristic phenotype most likely due to disruption of the repertoire of splice isoforms available for developing neuronal populations. Interference in the delicate balance of *KIDINS220* isoform expression in the neurite tips will impede the various Neurotrophin, Glutamate, Ephrin and Vascular Endothelial Growth Factor receptor associations required for correct neuronal network development. The C-terminal region of *KIDINS220* contains the KIM and PDZ domains that are required for protein interactions and transport. Lack of the KIM domain and its interaction with KLC-1 subunit KIF5A (Kinesin Family Member 5A) impairs protein transport to newly formed neurite tips (16). Similarly, absence of a PDZ domain will interfere with the interaction of *KIDINS220* with SNX27 (Sorting Nexin Family Member 27), an important protein for transport to the plasma membrane (19). We here show in *KIDINS220* transfected Neuro-2a cells, both wild-type and *KIDINS220* variant p.(Gln1366\*) successfully localized to the differentiating neurite tips, indicating an alternate as-of-yet undetermined molecular pathology due to the variants seen in our patients.

Studies of *Kidins220* during zebrafish and *Xenopus laevis* embryogenesis showed dynamic expression in the nervous system including the hypothalamus, eye, branchial arches, heart and somites, partly correlating with the anomalies observed in our patients (20,21). Mice completely lacking *Kidins220* are not viable (5–7). The extensive neuronal cell death in the brain of mutant embryos results in abnormal ventricle enlargement. This observation appears concordant with the pre and post natal ventriculomegaly observed in our patients. Heterozygous mice display defects in dendritic growth and branching, indicating the role of *Kidins220* in Brain-Derived Neurotrophic Factor (BDNF)-induced dendritic development (6,7,22). Although the heterozygous and wild-type mice have, at several age time points, comparable total *Kidins220* protein levels in the brain, it seems likely that the temporal balance between *Kidins220* isoforms in the brain is crucial for correct neural development (5). Structural cardiac anomalies and defects of the vascular and immune system were also observed in *Kidins220*  $-/-$  embryos (5,6), but are not seen in any of our patients, possibly due to different isoforms being required for normal heart and brain development (8).

Zebrafish is an excellent model for human motor neuron disorders (11). Motor neuron-specific expression in zebrafish using the wild-type or variant p.(Trp1350\*) *KIDINS220* resulted in spasms. The observed difference in the induction of spasms in zebrafish embryos expressing the wild-type or p.(Trp1350\*) truncated *KIDINS220* may reflect the complex regulation of *KIDINS220* tissue-specific splicing and expression. More conclusively, disruption of *KIDINS220* expression levels in motor neurons functionally links *KIDINS220* deregulation to nervous system impairment. The specific features in our zebrafish model, in particular the presence of spasms that are not observed in affected humans, confirm the effects of *KIDINS220* perturbation on neuronal function but are also likely to reflect anatomical differences in the central nervous system between zebrafish and humans. Specifically, the absence of corticospinal and rubrospinal tracts in zebrafish results in the replication of human lower motor neuron disorders more faithfully than



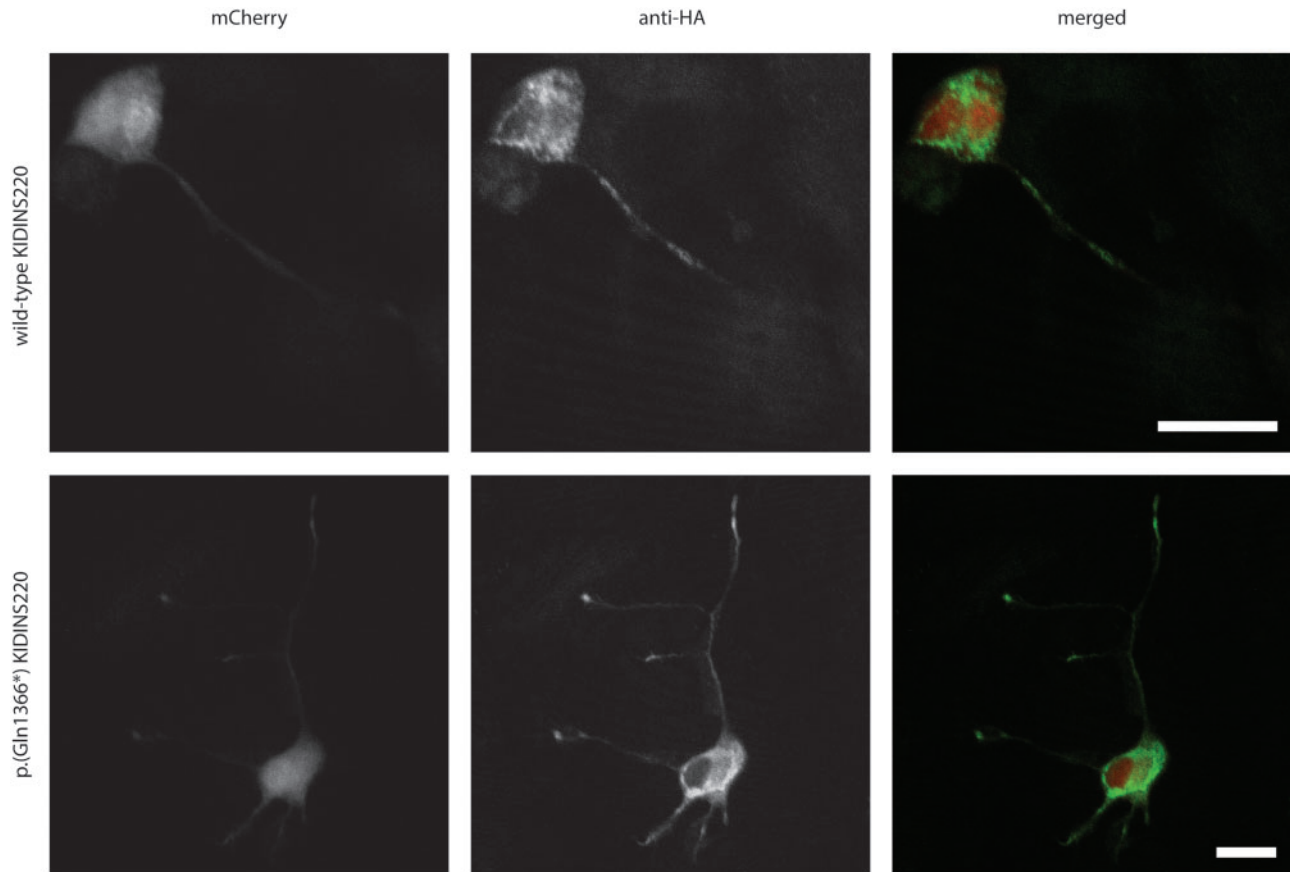
**Figure 3.** Effect of KIDINS220 and Trp1350\* expression in zebrafish embryo motor neurons. (A) Tissue-specific expression of KIDINS220 variants in 5 dpf zebrafish embryos was achieved by microinjection of UAS:KIDINS220 and UAS:Trp1350\* DNA in tg[Isl1BAC:GalFF; UAS:GFP] embryos (upper panel). A DNA UAS:RFP construct was co-injected (lower panel) to evaluate the efficiency of the microinjections. White arrowheads: axonic extensions of the motor neurons. (B) 5 dpf zebrafish embryo (ventral view; anterior is left). White dotted line: position of kymographs presented in C. (C) Visualization of spastic activity on thoracic kymographs. Representative kymographs of embryos showing no spastic activity (upper panel, no act.) and spastic activity (lower panel, spasm). Black arrowheads point at spastic events as visualized on the kymograph. Representative recordings are available as supplementary Material files. (D) Quantification of spastic events in the different conditions of the study. Expression of KIDINS220 wt and Trp1350\* in motor neurons results in increased spastic activity. No act., No spastic activity. \* $P < 0.05$ ; \*\* $P < 0.01$ ; Fisher's exact test. Scale bars: 1 mm in A (left panels), B; 50 microns in A (right panels).

human upper motor neuron disorders on the phenotypical level. KIDINS220 is expressed in cortical and hippocampal neurons, encompassing the upper motor neuron system, and as such this dysfunction can be classified as an upper motor neuron disorder. Nonetheless, zebrafish have provided a suitable partial model for insights into the molecular and genetic mechanisms of other upper motor neuron disorders, such as hereditary spastic paraplegias (24–26).

The pathophysiology of obesity is difficult to explain. KIDINS220 is known to act as a downstream substrate for protein kinase D and mediates multiple receptors signaling including BDNF and other neurotrophins. Since neurotrophins have been implicated in the molecular and cellular processes underlying body weight regulation, disruption of this finely tuned Trk

pathway could possibly explain the obesity phenotype in these patients (27,28). Increased expression of KIDINS220 has been linked with perturbation of TrkA/p75 neurotrophin receptor complex association and overexpression of the differently spliced KIDINS220 ATE C2 leads to a substantial increase in TrkA expression (8,23). Further research is needed to provide insight into this interaction.

Not every loss-of-function variant in the last exon of KIDINS220 causes the phenotype observed in our patients as illustrated by the presence of three KIDINS220 variants that are present heterozygously in five healthy individuals in the Exome Aggregation Consortium (ExAC) database (ExAC, Cambridge, MA (URL: <http://exac.broadinstitute.org>, last accessed September 2015). These variants, resulting in p.Glu1530Ter, p.Arg1736Ter and



**Figure 4.** Kidins220 truncated variant is able to localize to neurite tips in Neuro-2A cells. Representative image of transfected KIDINS220 wild-type and variant p.(Gln1366\*) in differentiated Neuro-2A cells. Immunofluorescence of cotransfected mCherry (red), anti-HA-tagged wild-type or p.(Gln1366\*) KIDINS220 (green) show that the truncated KIDINS220 variant is transported to developing neurite tips. Scale bar = 20 microns

p.Ser1740Ter, are shortly after the variant in our patient 3, pointing to a specific effect of the variants presented in this study.

Our patients manifest similarities with MOMO Syndrome (Macrosomia, Obesity, Macrocephaly and Ocular abnormalities; OMIM 157980); however, prenatal ventricular dilatation and spastic paraplegia are not features of this condition. KIDINS220 Sanger sequencing of 10 MOMO patients did not yield any variants. We therefore suggest that our patients have a distinct syndrome characterized by SINO.

In conclusion, we show here that *de novo* heterozygous non-sense KIDINS220 variants cause SINO syndrome. This is the first report of KIDINS220 variants causing a human disease. Future research might provide more insights in the role of KIDINS220 in the pathogenesis of spastic paraplegia and obesity.

## Materials and Methods

### Clinical Features

The clinical features of the three patients are summarized in Figure 1 and Table 1 and case reports can be found in Supplementary Materials.

### NGS and analytical pipeline

NGS on patients 1 and 2 was performed with fragment libraries prepared and enriched for the genomic regions of interest using a 1M custom OBESITOME microarray (Agilent Technologies, Santa

Clara, CA, USA) as previously described in (29,30). The OBESITOME contained 582 obesity-related genes, with the genes selected on presumed obesity relevance from several databases, pathway sources, and research articles (Supplementary Material, Table S1). The OBESITOME consisted of 55 samples that were pooled and run as a full slide on the SOLiD 5500XL (Life Technologies, Carlsbad, CA). Color space reads were mapped and aligned against GRCh37/hg19 reference genome using a custom pipeline based on the BWA software and annotated as described previously (31,32). Mean sample coverage for the entire run of 55 samples on the OBESITOME was 100×, median coverage was 87× and 85% of genomic positions were covered by more than 20 reads. The mean coverage for patient 1 and 2 was 161× and 170×, respectively. The criteria for variant detection coverage were set at 10 unique reads, and a non-stringent cut-off for heterozygote allele calls was set at 25–75%. Variants were filtered against the reference frequencies from the datasets of NCBI dbSNP Build 137 for Human (<https://www.ncbi.nlm.nih.gov/SNP>, last accessed July 2014), Exome Variant Server (EVS) (<http://evs.gs.washington.edu/EVS>, last accessed July 2014), 1000Genomes (<http://www.1000genomes.org>, last accessed July 2014), Genome of the Netherlands (<http://www.nlgenome.nl>, last accessed July 2014) or our in-house dataset, with novel variants and rare variants less than a 0.5% minor allele frequency threshold retained for further analysis. For each patient, a final variant list was compiled including the location in the genomic sequence of the variant, the amino acid change and prioritized on the conservation score and predicted effect on protein function using prediction programs



(Genomic Evolutionary Rate Profiling, Polyphen2, and Sorting Tolerant From Intolerant). Confirmation and segregation analysis in the family of the selected single nucleotide variants or small insertions and deletions was performed by Sanger sequencing.

Whole exome sequencing and analysis for patient 3 was performed as described by the Deciphering Developmental Disorders (DDD) study (1,2). DNA of patient 3 was extracted from saliva samples and exome capture performed using Agilent Sureselect 55MB Exome Plus (Agilent Technologies, Santa Clara, CA, USA). Exomes were sequenced on the Illumina HiSeq (Illumina, San Diego, CA, USA) and *de novo* analysis performed using DeNovoGear (33). *De novo* variants were validated using Sanger sequencing.

All variants have been submitted to the Leiden Open Variation Database located at <http://databases.lovd.nl/shared/genes/KIDINS220>, last accessed April 2016.

### KIDINS220 Sanger sequencing in a MOMO cohort and patient cDNA

To determine if variants within *KIDINS220* were causal for MOMO syndrome, the full coding regions and exon-intron boundaries of *KIDINS220* were analyzed in a cohort of 10 MOMO patients by Sanger sequencing.

To evaluate the impact of *KIDINS220* variants on RNA expression on our patients, total RNA was isolated from fibroblasts of patient 1 using TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA) and converted to cDNA with the High-Capacity cDNA Archive Kit (Life Technologies, Carlsbad, CA, USA). Material from patients 2 and 3 was not available. Sanger sequencing on the full cDNA of patient 1 was performed. Furthermore, Quantitative PCR was performed on cDNA of both patient and control fibroblasts using primers before and after the variant c.4050G > A to evaluate if expression of the mRNA was reduced compared with the control. Primer sequences: 5'-CCTGAAGACCCACGTTTCC-3'/5'-AAGCTGAAGTTGAGTGTAGG-3' and 5'-GCTCAGATGTCCAGTTAGAAG-3'/5'-CTGATGAAC TCTGACCATGTAATA-3'.

### Fibroblast and HEK293 protein preparation and immunoblotting

Fibroblasts of patient 1 and control samples were cultured, lysed, and Western blot analysis performed on the total protein using an antibody recognizing the N-terminus of *KIDINS220* (34). Fibroblasts of patient 1 and control samples were cultured according to standard conditions. Frozen cell pellets were lysed at 4°C in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton, 1% Na-deoxycholate, 0.1% SDS and 2 mM EDTA pH8.0 in the presence of protease inhibitors (cOmplete, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosSTOP, Roche, Basel, Switzerland) for 30 min, and sonicated for 10 s. Samples were spun for 5 min at 13,000 rpm at 4°C.

Western blot analysis was then performed on the total protein extract of the patient fibroblasts using an antibody recognizing the N-terminus of *KIDINS220* (34). The supernatant was diluted in 2× Sample buffer (8% SDS/25% Glycerol/0.05M Tris pH 6.8/200 mM DTT/Bromophenol Blue/H<sub>2</sub>O) and separated on 6 or 8% SDS-PAGE gels, followed by blotting onto polyvinylidene difluoride membranes (Biorad, Veenendaal, The Netherlands). Blots were blocked with either 2% bovine serum albumin (BSA)/0.05% Tween/phosphate buffered saline (PBS) and incubated overnight with the *KIDINS220* antibody. Blots were washed with 0.05% Tween/PBS for three times 10 min each at room temperature and incubated with

the appropriate secondary antibody. After washing three times in 0.05% Tween/PBS and once in PBS, blots were developed with Pierce Enhanced Chemiluminescent Western Blotting Substrate (Life Technologies, Carlsbad, CA, USA).

To visualize the molecular weight of the truncated proteins of patients 1 and 2, we introduced the identified point variants in HEK293T cells and performed Western blot analysis on the total protein as described above. HEK293T cells were grown at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM): Ham's F10 nutrient medium (HAMF10) (1:1) medium containing 10% FBS and 1% penicillin/streptomycin. The wild-type *KIDINS220* sequence corresponding to the long isoform of human *KIDINS220* (GenBank accession no. KJ812119) was obtained from an expression construct by using full-length *KIDINS220* primers (primers available upon request) (16). Wild-type *KIDINS220* was ligated into the TOPO entry plasmid pCR8/GW/TOPO (Life Technologies BV, Bleiswijk, The Netherlands) and the mutations encoding for Trp1350\* and Gln1366\* were engineered into the *KIDINS220* expression construct using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and custom designed primers. The presence of the introduced mutations was confirmed by Sanger sequencing. HEK293T cells were transiently transfected with wild-type and variant DNA constructs using Polyethylenimine (Polyscience, Eppelheim, Germany), grown for 24 h after transfection and prepared and analyzed as described earlier.

### Zebrafish expression construct and microinjections in zebrafish embryos

The wild-type *KIDINS220* cDNA sequence or the patient variant Trp1350\* were introduced into pCR8/GW/TOPO. The *KIDINS220* middle entry vectors were then used in a multi-site Gateway reaction (ThermoFisher Scientific, Waltham, MA, USA) together with p5E-UAS, p3E-IRES-EGFPpA and as destination vector pDestTol2pA3 (35) to obtain UAS:*KIDINS220* expression vectors. Circular plasmid DNA of UAS:*KIDINS220* constructs was injected at 30 ng/μl in the presence of 25 ng/μl Tol2 mRNA into tg(Isl1BAC:GalFF; UAS:GFP) (14) embryos at the 1-cell stage together with 10 ng/μl pT2ZUASRFP as a fluorescent reporter (36). Healthy embryos displaying robust Isl1-specific RFP fluorescence at 5 dpf was used for spastic activity recordings.

### Zebrafish high speed imaging

Embryos at 5 dpf were mounted in 3% methylcellulose (Sigma-Aldrich, Zwijndrecht, The Netherlands) prepared in E3 embryonic medium prior to imaging for 20–60 s with a Hamamatsu C9300-221 high speed CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) at 82–165 fps mounted on a Leica DM IRBE inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at room temperature using Hokawo 2.1 imaging software (Hamamatsu Photonics GmbH, Herrsching am Ammersee, Germany). Image analysis was subsequently carried out with ImageJ (<http://rsbweb.nih.gov/ij/>, last accessed June 2015). Fischer's exact test was employed to determine significance between Zebrafish groups with different construct injections.

### Transfection and immunofluorescence of transfected Neuro2A cells

GW1-MCS expression constructs with a N-terminal HA tag were created with wild-type or p.(Gln1366\*) *KIDINS220* inserts. Neuro-2A



cells were seeded at 10 000 cells/cm<sup>2</sup> on poly-L-lysine coated 10mm coverslips and grown for 24 h in DMEM supplemented in 10% FCS and penicillin/streptomycin. The following day, cells were transfected in DMEM only using Lipofectamine2000 (Thermo Fischer Scientific, Waltham, MA, USA) with N-terminal HA-tagged wild-type or p.(Gln1366\*) KIDINS220 in the GW1-MCS expression vector and cotransfected with a pcDNA/FRT/TO/mCherry expression construct. Cells were incubated for 4 h at 37°C and 5% CO<sub>2</sub> and medium removed, washed with PBS and replaced with differential medium (DMEM, 1% FCS, 5 uM retinoic acid). Cells were differentiated for 5 days, and differential medium was replaced after 2 and 4 days.

For fixation, medium was removed, the cells were washed once with PBS, and the coverslip treated with 4% PFA (paraformaldehyde) for 15 min at room temperature before being washed again with PBS. Cells were then permeabilized with 0.1% TritonX100 in PBS for 20 min, washed with PBS, blocked in 3% BSA/PBS for 20 min, and washed again. The coverslip was then incubated with 2 ug/ml rabbit Anti-HA-tag antibody Chip Grade in 3% BSA/PBS (ab9110; Abcam, Cambridge, UK) overnight at 4°C. Coverslips were washed three times in PBS and probed with 1:1000 polyclonal goat anti-rabbit CY5 (Jackson ImmunoResearch, West Grove, PA, USA) in 3% BSA/PBS for 1 h at room temperature. Cells were washed three times in PBS and then stained in 1:5000 DAPI (Thermo Fisher Scientific, Waltham, MA, USA), in 3% BSA/PBS for 15 min, washed three times in PBS, and mounted using Vectashield Antifade Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Coverslips were visualized using a TCS SP8 Confocal Laser Scanning Platform (Leica, Wetzlar, Germany).

### Study Approval

Written informed consent from the parents of the all three patients was received prior to inclusion in this study.

### Supplementary Material

Supplementary Material is available at HMG online.

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