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ARTICLE

De novo loss-of-function mutations in *WAC* cause a recognizable intellectual disability syndrome and learning deficits in *Drosophila*

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Recently *WAC* was reported as a candidate gene for intellectual disability (ID) based on the identification of a *de novo* mutation in an individual with severe ID. WAC regulates transcription-coupled histone H2B ubiquitination and has previously been implicated in the 10p12p11 contiguous gene deletion syndrome. In this study, we report on 10 individuals with *de novo* *WAC* mutations which we identified through routine (diagnostic) exome sequencing and targeted resequencing of *WAC* in 2326 individuals with unexplained ID. All but one mutation was expected to lead to a loss-of-function of WAC. Clinical evaluation of all individuals revealed phenotypic overlap for mild ID, hypotonia, behavioral problems and distinctive facial dysmorphisms, including a square-shaped face, deep set eyes, long palpebral fissures, and a broad mouth and chin. These clinical features were also previously reported in individuals with 10p12p11 microdeletion syndrome. To investigate the role of WAC in ID, we studied the importance of the *Drosophila* WAC orthologue *(CG8949)* in habituation, a non-associative learning paradigm. Neuronal knockdown of *Drosophila CG8949* resulted in impaired learning, suggesting that WAC is required in neurons for normal cognitive performance. In conclusion, we defined a clinically recognizable ID syndrome, caused by *de novo* loss-of-function mutations in *WAC*. Independent functional evidence in *Drosophila* further supported the role of *WAC* in ID. On the basis of our data *WAC* can be added to the list of ID genes with a role in transcription regulation through histone modification.

INTRODUCTION

Intellectual disability (ID) is a heterogeneous disorder, both clinically and genetically. To date, >650 genes have been associated with ID and novel genes are still being identified. The introduction of trio-based whole-exome sequencing (WES) in individuals with ID has proven to be a valuable approach for the identification of novel ID genes, especially for those individuals who do not show a clinical recognizable syndrome.[1,2] In addition to the identification of mutations in known disease genes, WES has facilitated the identification of candidate ID genes. To establish the pathogenicity of mutations in such candidate ID genes, it is essential to identify additional individuals with an overlapping phenotype and a mutation in the same

gene.[3–5] With increasing availability of WES in routine diagnostics[6] as well as technological advances facilitating targeted resequencing of candidate ID genes in larger cohorts of samples,[7] chances of finding such additional individuals are increasing. Furthermore, supporting evidence and insights into underlying mechanisms can be obtained from functional studies in cell or animal models.[8,9]

Previously, we and others separately reported on an individual with a *de novo* mutation in the 'WW domain-containing adapter with coiled-coil' (*WAC*) gene using trio-based exome sequencing.[1,10] The mutations were reported as potential cause of disease, based on mutation severity, protein function, its expression in fetal stages and high expression in adult brain.[1,10,11] *WAC* encodes a

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protein-regulating transcription-coupled histone H2B ubiquitination and contains two evolutionary conserved domains, including an N-terminal WW domain interacting with RNA polymerase II and a C-terminal coiled-coil domain promoting the RNF20/RNF40's E3 ligase activity for ubiquitination at active transcription sites.[12,13] Furthermore, the RNF20/40/WAC complex may have a role in cell cycle checkpoint activation upon genotoxic stress.[12]

In addition, WAC has previously been implicated in ID based on the finding that deletions of chromosome 10p12p11 result in a contiguous gene deletion syndrome, for which the shortest deleted region contains two genes, WAC and BAMBI. All individuals with a deletion of at least these two genes were reported to have a similar phenotype including ID, behavior problems and dysmorphic features, supporting a disease cause of WAC heterozygous loss-of-function.[14–17]

Although it may well be hypothesized that WAC haploinsuffiency may explain the ID phenotype observed in the 10p12p11 contiguous gene syndrome, and ID in individuals with mutations in this gene, detailed evidence to support this hypothesis is lacking. In the present study we aimed to identify additional individuals with de novo mutations in WAC by using different sequencing strategies to define the clinical spectrum associated with WAC haploinsufficiency. Finally, to address the role of WAC in cognition, we investigated the role of the Drosophila WAC orthologue in habituation, a form of non-associative learning.

MATERIALS AND METHODS

Diagnostic exome sequencing

Individuals 1, 3, 4, 5 and 6 were ascertained through family-based WES in a diagnostic setting using techniques as described before.[1] All clinically relevant candidate de novo mutations were validated using Sanger sequencing, and subsequently tested for absence in parental DNA samples. Individual 1 was previously reported as part of a large study assessing the clinical utility of WES, in which she was also identified to have a second de novo mutation in MIB1.[1] Individuals 7 and 8 were identified in a large multicenter study to establish the contribution of de novo coding mutations to autism spectrum disorder.[18,19] Apart from the de novo mutation in WAC these two patients were not reported to have additional de novo mutations.[19]

Database searches for copy number variations disrupting WAC

We systematically searched for individuals with small deletions including WAC in our in-house database and international databases such as the database of the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA) and the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER).[20,21]

Targeted resequencing of WAC

Upon identification of the de novo mutation in Individual 1, targeted resequencing was performed on a cohort of 2326 patients with unexplained ID using molecular inversion probes (MIPs) as described previously.[7] This cohort was selected from the in-house collection of the Department of Human Genetics of Radboud University Medical Center (Nijmegen, The Netherlands) containing patients with unexplained ID. Candidate loss-of-function mutations as well as highly conserved missense mutations (PhyloP > 5) were validated by standard Sanger sequencing approaches on DNA extracted from peripheral blood. For assessing the de novo occurrence of validated mutations, DNA from the parents was tested. This study was approved by the institutional review board 'Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen'.

Deposition of genotypes and phenotypes in a locus-specific database

All mutations and phenotypes reported in this publication are deposited in the locus-specific database for WAC, under the realm of the Leiden Open Variation Database (LOVD; http://databases.lovd.nl/shared/genes/WAC). Variant information and phenotypes can be retrieved using the following submission entries: Individual 1: #00054835; Individual 2: #00054836; Individual 3: #00054837; Individual 4: 00054838; Individual 5: #00054839; Individual 6: #00054848; Individual 7: #00054849; Individual 8: #00054850; Individual 9: #00054851; and Individual 10: #00054852.

Drosophila lines and maintenance

Fly stocks were kept on standard Drosophila diet (cornmeal/sugar/yeast) at 25 °C and 45–60% humidity at 12 h:12 h light/dark cycle. Inducible RNAi lines targeting the Drosophila WAC orthologue CG8949 (vdrc48307, vdrc107328) and corresponding genetic background control lines (vdrc60000, vdrc60100) were obtained from the Vienna Drosophila RNAi Center.[22] The s19 value, which refers to the specificity of the dsRNA hairpin construct,[23] is 1.00 for vdrc48307 with no off-target and 0.99 for vdrc107328 (two possible off-targets, CG11122 and CG11354).

RNAi was induced using the UAS-Gal4 system and the panneuronal driver lines: elav-Gal4 w1118; 2xGMR-wIR; elav-Gal4, UAS-Dicer-2 or nSyb-Gal4 w1118, UAS-Dicer-2; nSyb-Gal4..[9,24] Flies were reared and tested at 25 °C (elav-Gal4) and 28 °C (nSyb-Gal4) and 70% humidity. The ubiquitous actin-Gal4 driver w1118; P(w[+mC] = Act5c-Gal4)/CyO obtained from Bloomington Drosophila Stock Center,[25] was used to generate RNAi-mediated knockdown for quantitative PCR (qPCR).

Analysis of CG8949 mRNA levels from larval brains by qPCR

RNA isolations (three biological replicates) from third instar larvae brains were performed using Arcturus Picopure RNA Isolation Kit (Life Technologies, Bleiswijk, The Netherlands). RNA was treated with DNase (DNAfree Kit, Ambion, Bleiswijk, The Netherlands). First-strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Biorad, Veenendaal, The Netherlands). Gene expression was analysed by real-time PCR (7900HT Fast Real-Time PCR system, Applied Biosystems, Bleiswijk, The Netherlands). PCR reactions were performed in a volume of 25 μl containing 150 nM primers and GoTag Green Mastermix (Promega, Leiden, The Netherlands). Primer sequences used for amplification of CG8949: 5′-TGGAATTACGACAACGATGG-3′ and 5′-TAACTGGCTTCCGAGGTAGG-3′. BetaCop was used as reference gene, primer sequences: 5′-AACTACAACACCCTGGAGAAGG-3′, 5′-ACATCTTCTCCCAATTCCAAAG-3′.

Light-off jump reflex habituation

The light-off jump reflex habituation assay was performed as previously described.[26] Habituation of the startle jump response towards repeated light-off stimuli of 3–7-day-old individual male flies was tested in two independent 16-unit light-off jump habituation systems. A total of 32 flies (16 flies/system) were simultaneously exposed to series of 100 short (15 ms) light-off pulses with a 1 s inter-pulse interval. The noise amplitude of wing vibrations following every jump response was recorded for 500 ms after the start of light-off pulse and an appropriate threshold was applied to filter out the background noise. Data were collected and analysed by a custom-made Labview Software (National Instruments). High initial jumping responses to light-off pulse decreased with the growing number of trials and flies were considered habituated when they failed to jump in five consecutive trials (non-jump criterion). Habituation was scored as the mean number of trials required to reach the non-jump criterion (trials to criterion, TTC). Main effects of genotype (mutant vs control), day and test system on log-transformed TTC values were tested using linear model regression analysis (lm) in R statistical software (R version 3.0.0 (2013-04-03)).[27]

RESULTS

Identification of individuals with de novo CNVs affecting WAC

After the identification of the de novo mutation c.139C > T (NM_016628.3) leading to nonsense mutation p.(Arg47*) in Individual 1 (as reported before[1]), we set out to find additional individuals with mutations affecting WAC to obtain more evidence for its involvement in ID. Systematic analysis of DECIPHER and ECARUCA,
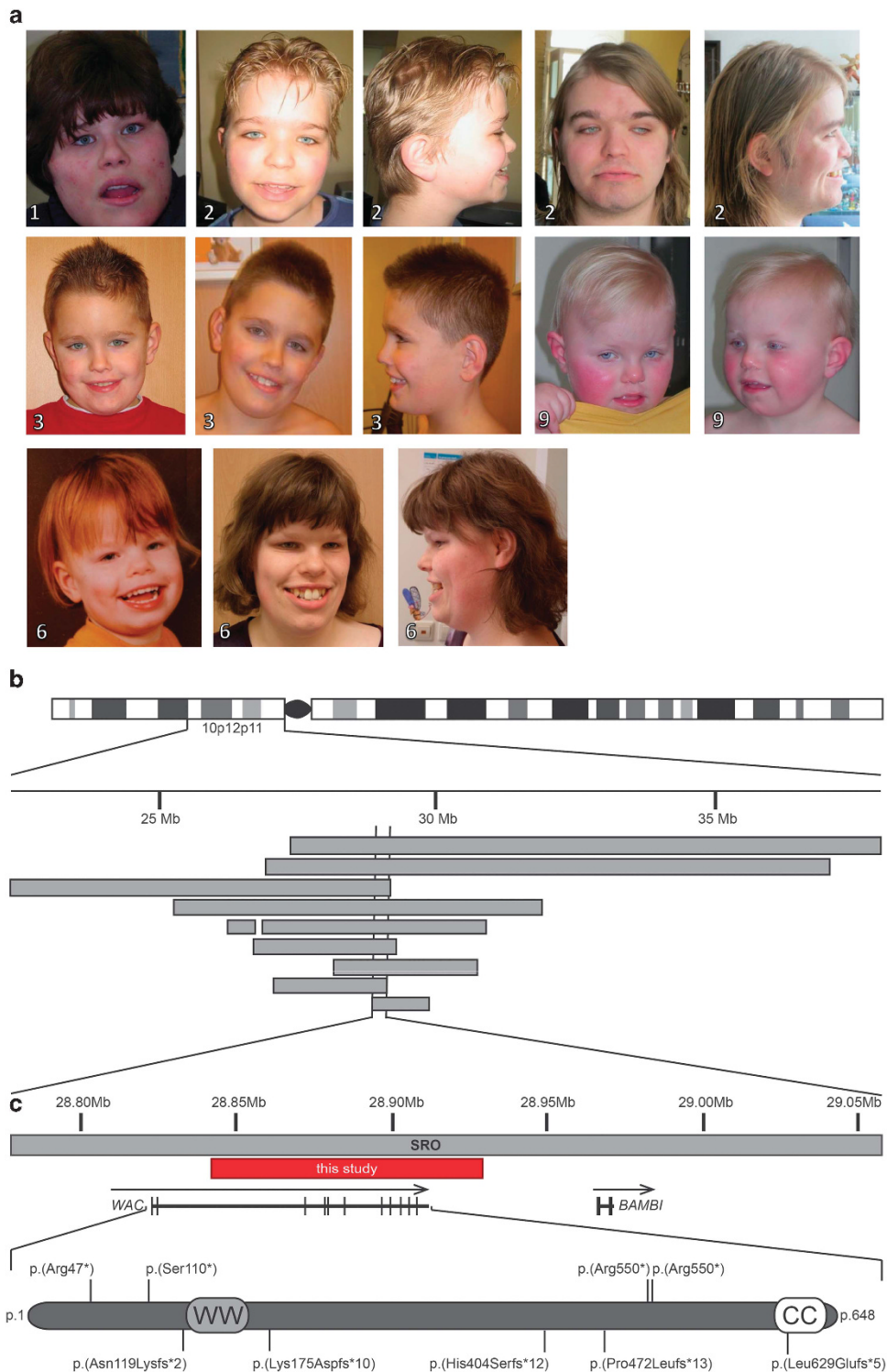
Figure 1 Individuals with WAC mutations. (**a**) Frontal and lateral photographs of individuals with *de novo* mutations in *WAC*. All individuals shared overlapping facial dysmorphisms including a square-shaped face, long palpebral fissures, broad mouth and broad chin. Additional features included deep set eyes, epicanthal folds and short philtrum in Individual 1 (photograph at the age of 19 years); low posterior hairline, broad forehead, simple ears, hypertelorism, deep set eyes, low-set full eyebrows, synophrys, deep nasal bridge, flat nose, bifid tongue and broad gums in Individual 2 (photographs at the age of 12 years and 23 years); brachycephaly, posterior ear creases, broad forehead, prominent antihelix, low-set full eyebrows, synophrys and prominent teeth in Individual 3 (photographs at the age of 4 years and 9 years); prominent antihelix, frontal bossing, dental crowding, broad teeth and high palate in Individual 6 (photographs at the age of 3 years and 20 years) and prominent antihelix and deep set eyes in Individual 9 (photographs at the age of 3 years). Photographs were published with consent. (**b**) The genomic region involved in the 10p12p11 contiguous gene deletion region with the previously published microdeletions, represented by gray horizontal bars.[14–16] (**c**) Detailed view of the smallest region of overlap (SRO) and the deletion described in this study, represented by a red horizontal bar. In addition, *de novo* mutations in *WAC* (NM_016628.3) reported in this study are shown according to their relative position at protein level.

two databases collecting clinically relevant copy number variants (CNVs), for CNVs affecting WAC yielded one small deletion (Individual 2; Figure 1). This deletion, hg19 chr10:g.(?_288422777)_ (28929097_?)del, disrupted the coding sequence of *WAC* by deletion of exons 5-14 (NM_016628.3). The shortest region of deletion overlap of the chromosome 10p12p11 contiguous gene deletion syndrome was previously determined by nine deletions ranging in size between 0.99 and 10.66 Mb.[14–17] Comparison of the deletion in Individual 2 to the shortest region of deletion overlap indicates *WAC* as a only remaining candidate gene for the ID phenotype (Figure 1b).

Diagnostic exome sequencing in individuals with neurodevelopmental disorders to identify *de novo* point mutations in *WAC*

In routine diagnostic trio-based exome sequencing for individuals with unexplained ID, performed as described before,[1] we identified four additional individuals with *de novo* loss-of-function mutations in *WAC* (NM_016628.3): c.329C > A, p.(Ser110*) in Individual 3; c.1885_1886del, p.(Leu629fs) in Individual 4; c.356dup, p.(Asn119fs) in Individual 5 and c.1648C > T, p.(Arg550*) in Individual 6 (Figure 1c). In addition, two more individuals with *de novo* mutations were identified by trio-based exome sequencing of a large cohort of individuals with autism spectrum disorder:[18,19] c.523_524del, p.(Lys175fs) in Individual 7 and c.1209_1212del, p.(His404fs) in Individual 8. Whereas exome sequencing had identified a second *de novo* mutation in Individual 1 (MIB1; NM_020774.2:c.521G > A; p.(Arg174His)),[1] no further clinically relevant *de novo* mutations were identified in Individuals 3–8, leaving *WAC* haploinsufficiency as the most likely candidate to explain disease.

Targeted resequencing of *WAC* in an ID cohort identifies additional *de novo* mutations

On the identification of multiple *de novo* loss-of-function mutations in *WAC*, we performed targeted resequencing of this gene in a cohort of over 2300 individuals with unexplained ID using MIPs as described before.[7] This cohort was selected from the in-house collection of the Department of Human Genetics of Radboud University Medical Center containing individuals with unexplained ID. All candidate loss-of-function mutations as well as highly conserved missense mutations (PhyloP > 5) were validated by standard Sanger sequencing approaches. For assessing the *de novo* occurrence of validated mutations, DNA from the parents was tested. This targeted screen identified two additional *de novo* truncating mutations: c.1415del, p.(Pro472fs) in Individual 9 and c.1648C > T, p.(Arg550*) in Individual 10 (Figure 1c).

WAC mutation spectrum in control individuals

Of all ten *de novo* mutations identified, nine are predicted to directly result in nonsense-mediated decay of the RNA transcripts; the *de novo* frameshift in Individual 4 is located in the last exon, suggesting it may skip nonsense-mediated RNA decay. None of the *de novo* mutations are reported in our in-house variant database containing exome sequencing variants detected in 5031 individuals, nor in ExAC, a large database collecting NGS variants in over 60 000 exomes as proxy for variant allele frequencies in the general population.[28] The latter, however, does contain three other, presumable loss-of-function, variants (by insertion–deletion events), each observed only once in ~ 100 000 alleles. Whereas these three variants have not been validated by Sanger sequencing, thereby possible being sequencing artefacts

rather than true mutations, this observation may also reflect the very mild end of the ID spectrum in the general population.

Clinical spectrum associated with *WAC* haploinsuffiency

Clinical evaluation of all individuals with *de novo* loss-of-function mutations in WAC showed distinct phenotypic overlap (Table 1; Supplementary Information (clinical descriptions); and Supplementary Table S1). All, but one individual, had ID. The range of ID observed ranged from mild-to-severe and was accompanied by language and motor delay. In addition, individuals showed a variety of neurological problems including hypotonia (6/9), with remarkable manifestation in the oral region resulting in dysarthria, and behavioral problems (10/10). The latter recurrently included autism (4/9), anxiety (3/10), concentration disorder (4/10) and/or sleep disturbance (6/10). Other overlapping features consisted of unexplained reduced vision (3/9) and respiratory problems (7/9) with recurrent respiratory infections reported most often (5/7). Notably, all individuals had overlapping facial dysmorphisms consisting of a square shape of the face, deep set eyes, long palpebral fissures, broad mouth and broad chin (Figure 1a).

Panneuronal knockdown of the *Drosophila* WAC orthologue results in learning deficit

To obtain independent evidence for the involvement of *WAC* in the ID phenotype of the described individuals, we decided to study the functional consequences of WAC knockdown using *Drosophila* as a model. The *Drosophila* genome contains a previously uncharacterized *WAC* orthologue named *CG8949*, not be confused with the unrelated *Drosophila WAC* (wee Augmin) gene. *CG8949* codes for several protein isoforms, the longest one consisting of 876 amino acids, and shows the highest expression in adult ovaries and the larval central nervous system.[29,30] WAC is 26% identical and 39% conserved over the C-terminal 588 amino acids of the fly protein, with sequence similarity distributed over the whole protein, further characterized by clusters of short sequences of high conservation for the important functional motifs of the protein.[13]

We investigated the role of the *Drosophila* WAC orthologue in light-off jump reflex habituation paradigm. Habituation is a simple form of non-associative learning, in which an initial strong behavioral response towards a repeated, non-threatening stimulus gradually wanes. It provides a filtering mechanism, which is an important prerequisite for higher cognitive functioning.[31,32] Using the light-off jump reflex habituation, we have previously identified learning deficits in number of *Drosophila* ID models.[9,24,26] Two independent inducible RNAi lines targeting the *Drosophila* WAC orthologue *CG8949* (vdrc48307 and vdrc107328) and their corresponding genetic background control lines (vdrc60000 and vdrc60100) were obtained (Vienna *Drosophila* RNAi Center[22]) and fly stocks were kept under standard conditions. Expression of *CG8949* was specifically downregulated in neurons using the UAS-Gal4 system.

The efficiency of ubiquitous RNAi knockdown was measured using qPCR on RNA isolated from *Drosophila* brains of third instar larvae, representing the tissue and developmental stage with the highest expression of *CG8949* allowing for an efficient detection of expression differences. There was no significant *CG8949*[vdrc107328]-mediated knockdown on *CG8949* expression levels (88% remaining gene expression; $P = 0.16$, student's t-test; Supplementary Table S2). In contrast, *CG8949*[vdrc48307]-mediated RNAi reduced levels of *CG8949* to 58% remaining gene expression ($P < 0.01$, Student's t-test; Supplementary Table S2).

Table 1 Clinical details of individuals with WAC mutations

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 7	Individual 8	Individual 9	Individual 10
Gender	Female	Male	Male	Male	Female	Female	Female	Female	Female	Male
Age at last visit	22 years	12.5 years	9 years	17 months	3 years	20 years	6 years	9 years	12 years	7 years
Mutation WAC (NM_016628.3)										
cDNA change	c.139C>T	Partial gene Deletion	c.329C>A	c.1885_1886del	c.356dup	c.1648C>T	c.523_524del	c.1209_1212del	c.1415del	c.1648C>T
Amino acid change	p.(Arg47*)	Deletion	p.(Ser110*)	p.(Leu629fs)	p.(Asn119fs)	p.(Arg550*)	p.(Lys175fs)	p.(His404fs)	p.(Pro472fs)	p.(Arg550*)
Chromosome position (Hg19)	Chr10:28824551	chr10.g.(?_28842277?)_(28929097_?)del	Chr10:28872382	Chr10:28908476_28908477	Chr10:28872409	Chr10:28905193	Chr10:28879674_28879675	Chr10:28899671_28899674	Chr10:28900827	Chr10:28905193
Growth										
Birth weight (g)	NR	3600 (>+2 SD)	3600 (0 SD)	NR	2920 (0 SD)	3070 (–1 SD)	NR	NR	3380 (0 SD)	4500 (+2.5 SD)
Height (cm)	NR	149.7 (–1.1 SD)	131.3 (–1.5 SD)	75.4 (–2 SD)	87 (–3 SD)	156.5 (–2.5 SD)	+0.86 SD	+ 2.88 SD	161.0 (+0.75 SD)	114.8 (–1.75 SD)
Weight (kg)	NR	48.8 (+2.1 SD)	31.5 (+0.75 SD)	10.0 (+2 SD)	13 (+0.7 SD)	75.5 (+3.5 SD)	+1.7 SD	–2.9 SD	61.2 (+2 SD)	26.2 (+3 SD)
Head circumference (cm)	NR	56.3 (+1.1 SD)	52.0 (–0.5 SD)	48.5 (0 SD)	50.4 (–0.6 SD)	54 (–1 SD)	+2.9 SD	+ 0.53 SD	56.0 (+1.5 SD)	54.9 (+2 SD)
Development										
Intellectual disability	Severe	Mild (IQ 58)	Mild (IQ 65)	Not assessed owing to young age	Mild	Moderate (IQ 44)	No (IQ 89)	Mild (IQ 61)	Mild (IQ 65)	Mild (IQ 61)
Adaptive ability	+	+	+	+	+	+	70 (–2 SD)	69 (–2 SD)	+	+
Speech delay	No speech	+ Dysarthria	+ 2.5 years: first words Dysarthria	+ 1.5 years: no words, only babbles	+ Dysarthria	+ 2 years: first words Dysarthria	– 20 months: first words	+ 5 years: first words	+ 5 years: first words	+ 1.5 years: first words
Motor delay	+ 34 months: first steps	+ 24 months: first steps	+ 24 months: first steps	+ 1.5 years: walks with support	+ 36 months: walks unsupported	+ 2 years: first steps	+	NR	+ 22 months: first steps	+ 27 months: first steps
Behaviour										
Anxiety	–	+	–	–	–	+	–	–	+	–
Concentration problems/hyperactivity	–	–	+	–	+	+	–	–	+	+
Sleep disturbances	+	+	–	+	+	+	–	–	+	–
Autism	–	+	–	–	NR	+	+	+	–	–
Other	Self-mutilation	–		Age 1 year: lack of eye contact		–	–	–	–	–
Neurological										
Epilepsy	+	–	–	–	–	–	–	–	–	–
Oral hypotonia	NR	+	+	+	+	+	–	–	+	+
Hypotonia	NR	+	+	+	+	–	–	–	+	+
Toe walking	+	–	+	–	NR	–	NR	NR	–	+
Ocular										
Vision	Normal	Normal	Unexplained reduced vision	To further establish: lack of eye contact and staring	Hypermetropia (+3.75 Dpt)	Unexplained reduced vision	Normal	Hyper/hypometropia	CVI	Normal
Strabismus	–	–	+	–	–	+	–	–	–	+

Table 1 (Continued)

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 7	Individual 8	Individual 9	Individual 10
Pulmonal										
Abnormal breathing pattern	+	+	−	−	NR	−	NR	±[a]	−	−
Recurrent respiratory infections	NR	+	+	+	−	+	NR	±[a]	+	−
Asthma	−	+	+	−	−	−	NR	±[a]	−	−
Extremities										
Brachydactyly fingers	NR	−	+	−	−	+	NR	NR	+	−
Pedes plano valgi	NR	−	+	+	−	+	NR	NR	+	+
Fetal finger pads	NR	−	+	−	−	+	NR	NR	+	+
Other	−	Broad first digits	Sandal gap Simian crease	−	Short hands and feet	Tapering fingers	−	−	Hip dysplasia Simian crease	−
Other										
Neonatal feeding difficulties	+	−	+	−	+	−	−	−	+	−
Other clinical features	Hernia diafragma-tica, pubertas tarda	Laryngomalacy Hoarse voice	Fever convulsions, fatigue, phimosis, hypertrichosis back	Large frontal fontanel	Low IgG class 2 and 3	Fatigue, paroxysmal atonia	−	Kidney problems	−	Inverted nipples
Additional investigations										
MRI/CT	Normal	Normal	Megacisterna magna	Normal	Normal	Normal	NR	Abnormal (no details available)	Mild asymmetrical right frontotemporal hemisphere	Mild enlarged ventricles
Muscle biopsy	−	−	Low mitochondrial activity	−	−	Normal	NR	NR	−	−
Genetic analysis	250k SNP array:−; MECP2: −; RAI1:−; TCF4: −; Angelman methylation: −	FMR1:−; RAI1: −	Subtelomeric regions: −; 250k SNP array: −; OPA1: −; POLG: −; RYR1:−	Cytoscan HD array: 9p22.1 gain and Xp22.31 gain, maternally inherited	FMR1: −; RAI1: −; EHMT1:− Cytoscan HD array: 6 Mb region of homozygosity chromosome 6q14.2q15	Angelman methylation: −; FMR1: −; CACNA1A:−; array CGH:−	NR	NR	FMR1: −; Subtelomeric regions: −	250k SNP array: −
Other (clinically relevant) de novo variant(s) by exome sequencing	MIB1 (NM_020774.2) c.521G>A; p.(Arg174His)	Not tested	None	None	None	None	None	None	Not tested	Not tested

Abbreviations: CVI, cerebral visual impairment; Dpt, diopter; IQ, intelligence quotient; NR, not reported.
[a] ± indicates that Individual 8 is reported to have pulmonal problems, but this is not further specified.
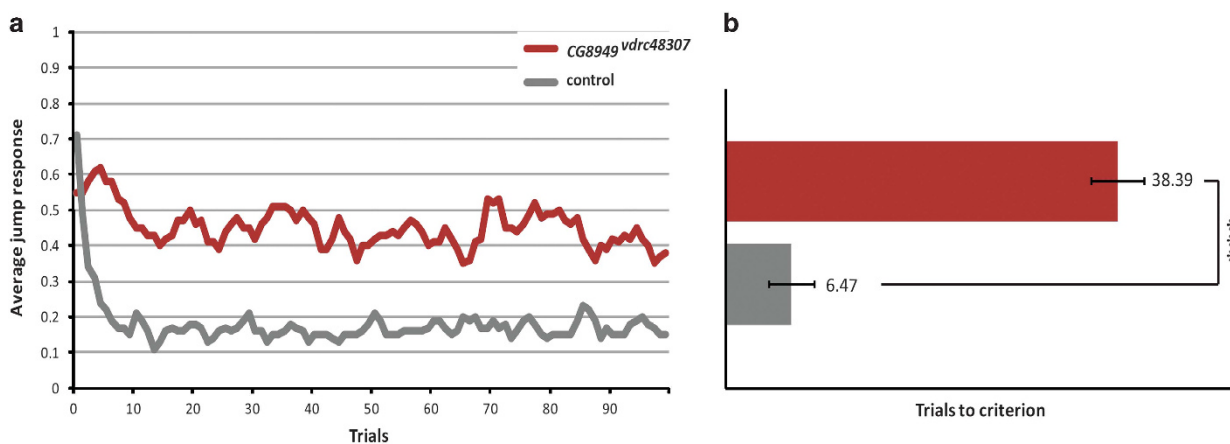
Figure 2 Knockdown of the *Drosophila* WAC orthologue CG8949 affects non-associative learning in the light-off jump reflex habituation paradigm. Jump responses of 3–7-day-old individual male flies were induced by repeated light-off pulses (100 trials) with a 1 s inter-trial interval. *CG8949* knockdown flies (*CG8949*^{vdrc48307}; genotype: 2xGMR-wIR/+; UAS-*CG8949*^{vdrc48307}/elav-Gal4, UAS-Dicer-2) are plotted in red and genetic background control flies are plotted in dark gray. Habituation was scored as the mean number of trials required to reach the non-jump criterion (TTC). Main effects of genotype (mutant *vs* control), day and test system on log-transformed TTC values were tested using linear model regression analysis.[27] (**a**) Average jump response across 100 light-off trials. (**b**) Mean TTC of *CG8949*^{vdrc48307} (TTC = 38.39, *n* = 54) *vs* mean TTC of control flies (TTC = 6.47, *n* = 49). Quantification of average jump responses revealed that flies with pan-neuronally induced *CG8949* knockdown habituated significantly slower (***$P < 0.001$, linear model regression analysis).

Flies were exposed to series of 100 short (15 ms) light-off stimuli with 1 s interval between stimuli. Both control and *CG8949*^{vdrc48307} knock-down flies showed good initial jump response; there was no significant difference between the initial startle response of *CG8949*^{vdrc48307} and control flies (*t*-test, $P = 0.469$). Whereas, control flies quickly habituated to the repeated light-off stimuli, *CG8949*^{vdrc48307} knock-down animals failed to adapt their behavioral response and retained high average jump response throughout the whole experiment (Figure 2). This defect was statistically significant (fold-change = 5.93; $P = 5.21 \times 10^{-12}$). No habitation defects were seen in the *CG8949*^{vdrc107328} knock-down flies (data not shown), as was to be expected based on the insufficient mRNA knockdown.

DISCUSSION

Here, we report the identification of a novel clinically recognizable syndrome caused by haploinsufficiency of *WAC*. All but one patient showed mild ID, with speech and motor delay, whereas one had an overall more severe ID phenotype, epilepsy and an absence, rather than delay, of speech. All patients had neurological problems including hypotonia and a variety of behavioral problems including autism, anxiety, concentration problems, sleep disturbance and/or self-mutilation. Notably, all patients had overlapping facial dysmorphisms consisting of square shape of the face, deep set eyes, long palpebral fissures, broad mouth and broad chin. Complementary experimental evidence in *Drosophila* showed a role of the evolutionarily conserved WAC proteins in cognitive processes and a role for the *Drosophila* WAC orthologue in non-associative learning.

Previously, another individual was reported with a truncating mutation in *WAC*[10] who shows a phenotype similar to the individuals reported in this study (Supplementary Table S1). Also, a large-scale study aiming at the identification of genetic causes underlying developmental disorders recently reported the identification of one *de novo* nonsense mutation in *WAC* but further clinical details of this individual were lacking, hampering detailed phenotypic comparison.[33] Interestingly, three of our patients were negative tested for *RAI1*, known to cause Smith–Magenis syndrome.[34] The coarse facial appearance as well as the ID with variable behavior problems of the individuals with *WAC* mutations have similarities with individuals reported with Smith–Magenis syndrome.[35]

Interstitial deletions including *WAC* were previously described and associated with ID.[14–17] Wentzel *et al.*[14] presented six individuals with an interstitial deletion at 10p12p11, all sharing a region of overlap including two genes: *BAMBI* and *WAC*. All individuals were reported to have developmental delay, abnormal behavior and facial dysmorphic features including a bulbous nasal tip, deep set eyes, synophrys/thick eyebrows and full cheeks. This phenotype is highly similar to the phenotype observed in the current individuals and consistent with our finding that loss of *WAC* causes ID and the characteristic facial dysmorphisms in 10p12p11 microdeletion syndrome (Supplementary Table S1). In this 10p12p11 microdeletion syndrome, cardiac abnormalities have frequently been reported (7/9 individuals) and heterozygosity of two other genes, *LYZL1* and *SVIL*, has been suggested to contribute to the development of these cardiac abnormalities.[14] This is in line with the fact that in none of our individuals cardiac abnormalities were present. Epilepsy has been reported in two out of nine individuals with deletion of 10p12p11 and is present in only one of our individuals (Individual 1). This more severely affected individual carried also a *de novo MIB1* mutation. This variant has been reported twice in ExAC, containing NGS variants in healthy controls of several ethnicities.[28] Moreover, one missense and one nonsense mutations in *MIB1* were identified previously and segregated each in two large dominant families with affected individuals with cardiomyopathy, but without ID.[36] Therefore, a contribution of the second mutation in *MIB1* as potential modifier of the more severe phenotype is unlikely. The more severe phenotype may be caused by other yet unknown potential genetic modifier(s) or reflects the severe end of the clinical spectrum caused by *WAC* haploinsufficiency.

Human *WAC* encodes a protein containing a WW domain-containing adapter and a coiled-coil region. WAC is an evolutionary conserved protein, but its exact function is still unknown. Protein–protein interaction studies suggest a role in the regulation of histone H2B ubiquitination and gene transcription.[12] Interaction of WAC via the coiled-coil domains with RNF20 and RNF40 activates UBE2A-mediated H2B ubiquitination. On the basis of this interaction, RNF20 and RNF40 could be considered as candidate genes for ID. Interestingly, *de novo* mutations in both genes have been described in individuals with autism and unrelated unaffected siblings.[19] A clear relation to a clinical phenotype, if any, remains to be established. RNA polymerase II recruits WAC to active transcription sites by binding to the WW domain. On the basis of sequence homology it has also been suggested that WAC is involved in RNA processing or transcription.[13] Besides co-localization of WAC with splicing factor SC35 in nuclear speckles, there is, however, currently no further evidence for a role in RNA splicing. The localization of WAC in the nucleus supports its suggested function in gene transcription via UBE2A histone H2B ubiquitination. Although WAC and UBE2A lack a direct interaction, they are both likely to function within a protein complex important for histone H2B ubiquitination and transcription regulation.[12] Interestingly, for *UBE2A*, microdeletions and point mutations have been associated in males with an X-linked inherited clinical syndrome characterized by ID, seizures, absent speech, urogenital and skin anomalies. Recent work on *Drosophila* has uncovered a novel role of UBE2A in clearance of defective mitochondria from the synaptic compartment and in synaptic plasticity.[37] On the basis of the suggested interaction of WAC and UBE2A being part of the same complex, and their clinical phenotypes both including ID, we experimentally addressed whether *CG8949*, like fly UBE2A, is required for synaptic vesicle cycling, mitochondrial functioning and morphology.[37] We found all these processes unperturbed in our *Drosophila* model (Supplementary Figure S1). Despite the unavailability of the second *CG8949* knockdown fly as an independent confirmation of the habituation phenotype, our data supports the role of WAC in cognition by the deficit in non-associative learning.

In summary, we describe a clinically recognizable syndrome owing to loss-of-function mutations in *WAC*, which is characterized by mild ID, hypotonia, behavioral problems and facial dysmorphisms consisting of square shape of the face, deep set eyes, long palpebral fissures, broad mouth and broad chin. Complementary experimental evidence in *Drosophila* showed a role of the evolutionarily conserved WAC proteins in cognitive processes and a role for the *Drosophila WAC* orthologue in non-associative learning. WAC is a component of the evolutionary conserved histone H2B ubiquitination complex, regulating gene transcription. On the basis of our results, *WAC* can be added to the growing list of genes involved the H2B ubiquitination complex leading to cognitive defects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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