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## Juberg-Marsidi syndrome and Brooks syndrome are allelic X-linked intellectual disability syndromes due to a single mutation (p.G4310R) in HUWE1

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**Juberg-Marsidi syndrome and Brooks syndrome are allelic X-linked intellectual disability syndromes due to a single mutation (p.G4310R) in *HUWE1***

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## ABSTRACT

**Background.** X-linked intellectual disability (XLID) syndromes account for a substantial number of males with ID. Much progress has been made in identifying the genetic cause in many of the syndromes described 20-40 years ago. Next generation sequencing (NGS) has contributed to the rapid discovery of XLID genes and identifying novel mutations in known XLID genes for many of these syndromes.

**Methods.** Two NGS approaches were employed to identify mutations in X-linked genes in families with XLID disorders. One involved exome sequencing of genes on the X chromosome using the Agilent SureSelect Human X Chromosome Kit. The second approach was to conduct targeted NGS sequencing of 90 known XLID genes.

**Results.** We identified the same mutation, a c.12928 G>C transversion in the *HUWE1* gene, which gives rise to a p.G4310R missense mutation in two XLID disorders: Juberg-Marsidi syndrome (JMS) and Brooks syndrome. Although the original families with these disorders were considered separate entities, they indeed overlap clinically. A third family was also found to have a novel *HUWE1* mutation.

**Conclusions.** As we identified a *HUWE1* mutation in an affected male from the original family reported by Juberg and Marsidi, it is evident the syndrome does not result from a mutation in *ATRX* as reported in the literature. Additionally, our data indicate that JMS and Brooks syndromes are allelic having the same *HUWE1* mutation.

### Strengths and limitations of the study

1. Using the power of Next Generation Sequencing we have linked Juberg Marsidi syndrome (JMS) and Brooks syndrome as allelic conditions
2. This study provides better organization to the field of X-linked disorders by providing evidence that JMS is not caused by mutation in the *ATRX* gene

- 3. We provide clinical descriptions and comparisons to support our conclusions
- 4. The reported missense mutations have detrimental effects on HUWE1 functional Activity
- 5. Based on the limited numbers of patients/families we are unable to make specific genotype-phenotype correlations at this time

Data Sharing

Raw data or clarification of reported results can be obtained by emailing Michael Friez (friez@ggc.org).

## INTRODUCTION

In the two decades since the discovery of the trinucleotide repeat expansion in *FMR1* responsible for Fragile X syndrome, substantial progress has been made in defining the other X-linked Intellectual Disability syndromes at the molecular level.[1-3] Mutations in over 100 X-chromosome genes have been assigned at least provisional roles in these syndromes.[2, 4] Some syndromes, initially believed to be separate conditions, have now been lumped together while others that appeared to be the same have been separated on the basis of molecular findings.[2, 3, 5, 6]

Juberg-Marsidi syndrome (JMS) has heretofore been considered by some observers to be allelic to Alpha-Thalassemia Intellectual Disability (ATRX syndrome) while Brooks syndrome (aka Brooks-Wisniewski-Brown syndrome) has been considered to be distinct from JMS.[7-10] A missense mutation in *ATRX* has been previously reported in a family considered to have JMS.[8] Upon clinical reevaluation of the original family reported by Juberg and Marsidi (1980), we considered the findings in the sole affected male still alive to be inconsistent with Alpha-Thalassemia Intellectual Disability. While there are some similarities between JMS and ATRX syndrome, there are distinctive differences.[7] Specifically the surviving male in the Juberg-Marsidi family had bifrontal narrowing, hypotelorism, prominent nose with overhanging columella, and thin lips, all facial manifestations very different than those of ATRX syndrome. Subsequently we identified in this male, a novel missense alteration in *HUWE1*, a gene located at Xp11.23 that encodes an E3 ubiquitin ligase that regulates numerous proteins, such as key cellular factors p53 and Mcl1.[11, 12] The identical missense alteration in *HUWE1* was also identified and shown to properly segregate in another

XLID syndrome reported by Brooks et al. (1994).[10] The clinical phenotypes in the two conditions previously considered to be unrelated show close similarity. Herein, we update the clinical and molecular findings in these two families and report an additional family with another missense mutations in *HUWE1*.

**METHODS**

**Informed Consent**

The authors note that we have obtained proper informed consent from the participants (or parental consent for participants under 18 years of age) for use of their personal information, including photos, for publication purposes. Copies of these consents have been shared with the office of the Editor.

**Families**

Clinical Update: Family 1 (Juberg-Marsidi Syndrome, K9149)[7]

Clinical findings in four affected males in 3 generations included generalized undergrowth, severe intellectual disability, tall forehead, epicanthus, short palpebral fissures, pale retina, flat nasal bridge, large or cupped ears, small penis with undescended testes, and deafness.[7] The two brothers in generation II died in infancy or childhood.

The pedigree and clinical findings were updated in 2006. The nephew (III-2 in Figure 1A), age 27 years, was found to have moderate hearing loss of unknown type but believed to be related to recurrent otitis media, severe growth impairment (all measurements below the third centile) and intellectual disability (IQ < 20). Craniofacial findings included facial asymmetry, bifrontal narrowing, furrowing of the forehead,



prominent brow giving the eyes and midface a sunken appearance, hypotelorism, upslanting palpebral fissures, prominent nose with overhanging columella, long philtrum, and thin lips with a beak at the midpoint of the upper lip (Figure 1). Additional features were brachydactyly, decreased musculature of the distal legs, foot deformation (right foot everted, left foot with midfoot varus), scoliosis, flexion of the hips, genu vara, soft loose skin over the hands and hyperextensible joints. Sexual hair and penis appeared normal but the testes were not palpable. He had no speech, but uttered sounds when under stress and used gestures to indicate his desires (Table 1).

Table 1. Comparison of Findings in Families with Missense Mutations and Duplications of *HUWE1*\*

	Juberg and Marsidi <sup>7</sup>	Brooks et al. <sup>10</sup>	Family 3	Froyen et al. <sup>22</sup>				Isrie et al. <sup>23</sup>	Mattei et al. <sup>9</sup>
	K9149	K9223		A323	UK444	UK106	Dup HUWE1		ATR Mutation
Affected individuals	4M	3M	2M	4M	3M	3M	24M	2M	7M
Mutation	p.G4310R	p.G4310R	p.R4063Q	p.R4013W	p.R4187C	p.R2981H	Dup	p.R4013W	p.R1272Q
Low birth weight (<3c)	4/4	3/3	2/2	0/1	-	-	0/12	-	2/2
Low birth length (<3c)	4/4	-	2/2	-	-	-	0/1	-	1/2
Short stature (<3c)	4/4	3/3	2/2	-	1/1	-	0/19	0/2	-
Microcephaly (<3c)	4/4	3/3	2/2	0/4	0/1	0/1	1/19	0/2	1/2
Macrocephaly (>97c)	0/4	0/3	0/2	2/4	0/1	0/1	0/19	0/2	0/2
Strabismus	2/3	2/3	-	-	1/1	-	1/11	0/2	-
Epicanthus	2/2	3/3	-	-	-	-	3/8	0/2	1/1
Deep-set eyes	1/1	3/3	2/2	-	-	-	0/8	1/2	-
Blepharophimosis/small pf	4/4	3/3	-	-	-	-	3/8	0/2	1/1
Cupped ears	1/3	2/3	2/2	0/4	-	1/2	3/8	0/2	0/1
Prominent nose	1/3	3/3	2/2	0/3	-	-	3/8	0/2	0/1
Thin lip(s)	2/3	3/3	2/2	1/3	-	-	0/9	-	0/1
Undescended	4/4	-	0/2	0/1	-	-	0/8	-	2/3

testes									
Deafness	4/4	1/3	0/2	1/4	-	-	0/20	-	1/3
Seizures	1/4	0/3	0/2	0/4	-	-	0/8	-	-
Hypotonia	1/4	0/3	0/2	1/1	-	-	5/11	-	1/1
Severe ID (IQ <50)	4/4	3/3	2/2	4/4	-	-	12/17	2/2	3/3
Absent/limited speech	3/3	3/3	1/2	1/3	0/1	2/2	5/8	-	-
Other findings	Bifrontal narrowing, Hypotelorism, Contractures	Bifrontal narrowing, Short philtrum, Contractures, Clumsiness	Contractures	Tapered fingers	Clumsiness	Contractures		Hypertelorism Downsloping pf	Nonattentive gaze, GI disturbances

<3c = less than 3<sup>rd</sup> centile; >97c = greater than 97<sup>th</sup> centile; pf = palpebral fissures; GI = gastrointestinal; dash (-) indicates information not available.

\* The *de novo* p.V950A mutation in one of two brothers with autism reported by Nava et al. [25] is not included because of lack of clinical details.

All females, including two obligate and four nonobligate carriers identified by gene testing, had normal growth, craniofacial appearance and intellectual function. Marked skewing (>90:10) of X chromosome inactivation in all carrier females provided a plausible reason for absence of clinical manifestations. One carrier subsequently had an affected son. He was undergrown when delivered at 38 weeks with a weight of 1510 g, head circumference of 30 cm, and length of 42 cm, all measurements less than the third centile for 38 weeks. He had hypotelorism, small appearing eyes, flat nasal bridge, right cleft lip and palate, supraaortic stenosis, small hands and feet, externally rotated feet and undescended testes. Ultrasound examination of the brain showed a small cyst (1.0 x 1.6 cm) within each lateral ventricle. He failed the newborn hearing test.

Clinical Update: Family 2 (Brooks Syndrome, K9223)[10]

Clinical findings in two affected brothers and a nephew were generalized intrauterine and postnatal undergrowth, severe intellectual disability, triangular shaped face, low posterior hairline, bifrontal narrowness, malar flatness, blepharophimosis, optic atrophy, esotropia, nystagmus, bulbous nose, low-set and cupped ears, short philtrum, thin tented upper lip, and pectus excavatum (Figure 1B).[10] They were poorly coordinated and became self-abusive, hyperactive, and spastic with large joint contractures.

The pedigree and clinical findings were updated in 2010. The affected males were ages 23, 45, and 52 years. All growth parameters remained well below the third centile. Affected males became ambulatory by about age three years, but remained nonverbal or had very limited speech (Table 1). Hearing loss was believed to be related to recurrent otitis media. Cranial imaging showed enlarged ventricles and small brain.

Three carrier females had normal intellectual function and showed no phenotypic abnormalities. X-inactivation was markedly skewed (>90:10) in the two carriers who were informative at the *AR* locus.

### Clinical Report: Family 3.

Family 3 consists of two brothers, now aged 10 and 13 years (II-5 and II-4 in Figure 1C), who have severe intellectual disability, microcephaly and postnatal growth failure. The brothers have three sisters, one of whom has minor literacy problems. Their mother also has a minor learning difficulty, not present in her 3 brothers and sister. X-inactivation testing in the mother and the daughter with literacy problems was uninformative.

The two brothers were born at term gestation with weights and lengths at term at or below the 3<sup>rd</sup> centile, being 2.52 kg and 2.67 kg and 44 cm and 47 cm, respectively.

Neither was microcephalic at birth but have been <3<sup>rd</sup> centile since early childhood. Early motor milestones were delayed with both boys not walking independently until after age two years. The oldest brother had tight Achilles tendons and required serial casting and ankle-foot orthoses. His gait was broad based and ataxic, with reduced extension of both hips and knees. The younger brother had better language skills, with over 100 single words, and could follow single step commands; the older brother only had a few single words.

Both brothers had early onset growth failure, with significant short stature. Their heights were more than -4.0 SD from the mean by age three years. Dynamic testing excluded growth hormone deficiency (peak growth hormone levels 54.3 mIU/L and 13.9 mIU/L to glucagon stimulation). Alternative causes of short stature were not identified. Both boys were treated with and responded to growth hormone, with their heights now approaching the 3<sup>rd</sup> centile. Neither boy had clinical or biochemical evidence of other endocrine abnormalities. Their genitalia were normal and the older brother had normal pubertal development. The youngest brother has astigmatism, but otherwise vision and hearing in both boys is normal.

Both brothers are now markedly microbrachycephalic, with head circumference of 47cm (<3<sup>rd</sup> centile) and 48cm (<3<sup>rd</sup> centile), respectively. They have sparse scalp hair, which was more obvious in early infancy. Craniofacial features include deep-set eyes, broad nasal bridge and nasal tip, a wide mouth, a thin upper lip and prognathism (Figure 1C and Table 1). The older brother's ears are cupped, his eyebrows are thick and his face is now coarse, resembling the pictures of the patient with Juberg-Marsidi syndrome (Figure 1A).

Skeletal surveys in both boys demonstrated only brachycephaly and delayed bone age (prior to treatment with growth hormone). Neuroimaging showed some increased extra-axial space and ventriculomegaly, white matter loss in the parietal, occipital and periventricular regions, a normal pituitary gland and a posterior pituitary bright spot.

### Marker Analysis

As the clinical evaluation of the original Juberg-Marsidi family was not consistent with ATRX syndrome and sequencing of *ATRX* failed to detect a mutation, marker analysis using 28 X-chromosome markers was conducted.[13]

### Sequencing

Genomic DNA from an affected male in the original family with JMS (Family 1) was enriched for exons of 718 genes on the X chromosome using the Agilent SureSelect Human X Chromosome Kit and subjected to next generation sequencing as previously described.[14] DNA from an affected male in family 2 (Brooks syndrome) was enriched for 90 XLID genes using RainDance microdroplet technology (RainDance Technologies, Lexington, MA). Sequencing of these 90 genes was performed on an Applied Biosystems SOLiD 3 Plus Sequencer.

### Preparation of Whole Cell Extracts

Lymphoblastoid cells were collected and washed twice with ice cold PBS, followed by flash freezing of the cell pellet in liquid N<sub>2</sub>. Subsequently, the thawed cells were

resuspended in one packed cell volume (PCV) of lysis buffer I (10 mM Tris-HCl pH 7.8, 200 mM KCl, 1 mM NEM, 0.1 mM MG-132, 1 µM PMSF, 2 µM leupeptin, 3 µM bestatin, 1.5 µM pepstatin) and two PCVs of lysis buffer II (10 mM Tris-HCl pH 7.8, 600 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% NP-40, 1 mM NEM, 0.1 mM MG-132, 1 µM PMSF, 2 µM leupeptin, 3 µM bestatin, 1.5 µM pepstatin). The samples were further incubated for 30 minutes at 4°C, sonicated and centrifuged. The supernatants were either stored at -80°C or used directly for immunoblot analysis.

**Immunoblotting**

High molecular weight proteins present in the whole cell extract were separated on a 7% Tris-Acetate polyacrylamide (PA) gel, while lower molecular weight proteins (p53 and Mcl-1) were separated by a 10% Tris-Glycine PA gel. After separation, proteins were transferred to an Immobilon-FL membrane (Millipore), followed by subsequent immunoblotting. Primary antibodies against HUWE1 (Bethyl Laboratories), p53 (Santa Cruz) and Mcl-1 (BD Pharmingen) were detected using infrared (IR) Dye-conjugated secondary antibodies (Rockland). The signal was visualized using direct IR fluorescence via the Odyssey Scanner (LI-COR Biosciences).

**shRNA knockdown**

Cells from male control were lentivirally transduced with plasmids carrying the non-specific control shRNA (pGIPZ-Ns, Open Biosystems) or the Mule-targeting shRNA (GIPZ Human HUWE1 shRNA, Clone-ID V3LHS\_353153, Open Biosystems). Stable

clones were subsequently selected with puromycin, while the selection-efficiency was monitored by the percentage of GFP-positive cells.

## RESULTS

Segregation analysis of 28 markers spaced along the X chromosome indicated 4 markers from DX51055 (Xp11.2) to AR (Xq12) were linked to the JMS phenotype (data not shown). The region did not include the *ATRX* gene confirming the negative gene sequencing and clinical impression that JMS was not allelic to *ATRX*. But as the region contained many genes, next generation sequencing of exons on the X chromosome was initiated.

For family K9149 with JMS, NGS of genes on the X chromosome identified only one unique variant within the “linked” interval, a G>C transversion at nucleotide 12928 (c.12928G>C) in *HUWE1*. This change results in a missense mutation, p.G4310R. The finding was confirmed by Sanger sequencing. The alteration segregated with the phenotype (Figure 1A) as expected since *HUWE1* is within the “linked” interval.

Next generation sequencing of an affected male in family K9223 with Brooks syndrome for 90 XLID genes identified the same missense *HUWE1* mutation as the only unique change after other SNPs were removed from further consideration based on available database and in-house frequency information. Sanger sequencing confirmed the NGS finding and the alteration segregated properly in the family (Figure 1B). The Sorting Intolerant From Tolerant (SIFT), PolyPhen-2 and Mutation Taster prediction algorithms indicated that replacing a nonpolar amino acid with one that is



charged is not expected to be tolerated, consistent with a damaging effect on the protein (Supplementary Table 1).

The affected brothers in Family 3 were shown to have a mutation in *HUWE1* (c.12188G>A; p. R4063Q), which alters a highly conserved amino acid in the HECT domain. The mutation arose as a *de novo* event in the mother and is also present in two of her daughters (Figure 1C). Neither this alteration or the shared variant for families 1 and 2 have been reported in the available databases, including the Exome Variant Server database that includes exome data for approximately 6500 individuals.

In order to determine if the p.G4310R mutation affected the HUWE1 function, protein levels were compared between the cells derived from an affected male (Figure 1A, III-2) from the original Juberg-Marsidi syndrome family (K9149) and the cells from a healthy male family member (Figure 1A, IV-1). As shown in Figure 3, the amount of HUWE1 in cells from the affected male was significantly reduced to approximately 39% of the HUWE1 level in the cells from the healthy relative (Figure 1A, IV-1). The levels of two important HUWE1 substrates Mcl1 and p53 were significantly increased in the patient's cells (Figure 4). To confirm that reduced HUWE1 levels directly cause the upregulation in p53 and Mcl1 levels, we performed HUWE1 knock-down experiments in cells from healthy individuals. Upon HUWE1 knock-down, we observed an accumulation in Mcl1 and p53, thus indicating that the change in HUWE1 status is directly correlated with changes in p53 and Mcl1 levels (Supplementary Figure 1). Taken together these findings suggest that p. G4310R mutation identified in the affected male from the Juberg-Marsidi syndrome family (K9149) leads to reduced E3 ligase levels and consequent accumulation of downstream targets p53 and Mcl1.



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3 In family 3, levels of the HUWE1 p.R4063Q protein were increased, and  
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5 consequently the levels of p53 substrate were reduced in the two affected brothers  
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7 compared with their normal father (Figure 5). Taken together, altered levels of mutant  
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9 HUWE1 proteins and as a consequence the levels of its target substrates suggest that  
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11 missense changes potentially affect HUWE1 function.  
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## 15 16 17 18 DISCUSSION

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20 HUWE1 plays a role in various essential physiological processes by regulating  
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22 the stability of multiple proteins. Initially HUWE1 was shown to bind the tumor  
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24 suppressor p53, thus inhibiting transactivation.[11, 15] Further HUWE1 was shown  
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26 through ubiquitylation to contribute to regulation of the anti-apoptotic protein Mcl1.[12]  
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28 Additional substrates of HUWE1 include proteins such as histones,[16] transcription  
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30 factor Miz1,[17] DNA polymerases beta and lambda,[18, 19] topoisomerase  
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32 TopBP1,[20] and others.  
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36 Among the 200 named X-linked intellectual disability syndromes, a number of  
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38 entities have been found to be allelic using molecular technologies.[2, 3, 5, 6] Prominent  
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40 among these are *PQBP1*-associated XLID (Renpenning, Sutherland-Haan, Hamel  
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42 Cerebro-Palato-Cardiac, Golabi-Ito-Hall and Porteous syndromes), *ATRX*-associated  
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44 XLID (Alpha-Thalassemia Intellectual Disability, Carpenter-Waziri, Holmes-Gang,  
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46 Chudley-Lowry, and XLID-Hypotonia-Arch Fingerprints syndromes), and *ARX*-  
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48 associated XLID (Partington, West, Lissencephaly and Abnormal Genitalia, and  
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50 Hydranencephaly with Abnormal Genitalia syndromes).  
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In 1983, Mattei et al. reported an XLID family with seven affected males which they considered to have Juberg-Marsidi syndrome.[9] Villard et al. (1996) found a missense mutation in the helicase domain of *ATRX* in the family, and since that time Juberg-Marsidi syndrome has been called an *ATRX*-associated XLID syndrome in much of the literature.[8] Our clinical re-evaluation of the surviving male in the original Juberg-Marsidi syndrome family showed findings that were not consistent with *ATRX*-associated XLID, specifically bifrontal narrowing, hypotelorism, prominent nose and thin lips, prompting us to question their conclusion. Sequencing of the *ATRX* gene in this male failed to find a pathogenic alteration (data not shown). Further, review of the males reported by Mattei et al. (1983) shows them to have clinical findings very different from Juberg-Marsidi syndrome and more consistent with *ATRX*-associated XLID (Table 1).

The availability of Next Generation Sequencing provided an opportunity to sequence 718 X-chromosome genes simultaneously in the surviving affected member of the Juberg-Marsidi family. This effort identified a missense mutation in *HUWE1* (c.12928G>C; p.G4310R) which was considered to be likely disease-causing (Mutation Taster), not tolerated (SIFT) and probably damaging (PolyPhen-2) (Figure 2 and Supplementary Table 1). Functional studies performed on a cell line from the surviving affected male in the original family with Juberg-Marsidi syndrome showed significantly decreased HUWE1 protein levels and consequently increased levels of two well-known substrates, p53 and Mcl-1 as compared to a normal family member (Figures 3 and 4).

This molecular finding clearly establishes JMS as an XLID disorder caused by a mutation in *HUWE1* rather than in *ATRX*. The same mutation was also found in the family reported by Brooks et al. (1994) using a 90-gene XLID panel (Supplementary

table).[10] Comparisons of the phenotype of Juberg-Marsidi and Brooks syndromes convinced us that they were examples of the same syndrome clinically. We have been unable to link the two kindreds through ancestry or common area of residence. A third family was also found to have a slightly more proximal missense mutation (p.R4063Q) which altered a highly conserved arginine in the HECT domain, which was likewise predicted to be deleterious (Supplementary Table 1). HUWE1 p.R4063Q protein had increased stability and p53 levels reduced in the lymphoblast cell lines derived from the affected males in this family. Vandewalle et al.[21] recently reported that an extra dose of HUWE1 affects axon branching of dorsal cluster neurons of *D. melanogaster* through the Wnt/3-catenin pathway.

Four other families with different missense mutations in *HUWE1* have been reported by Froyen et al. (2008) and Isrie et al (2013).[22, 23] In family A323 reported by Froyen et al., four affected males had moderate ID and were macrocephalic but otherwise nondysmorphic.[22, 24] One male was reported to have elbow contractures. The phenotype in this family appears highly variable with a subsequent affected male having severe intellectual disability. Carrier females were also macrocephalic and had learning difficulties. The second family (UK444) had three affected males in two generations. One male had short stature, normal head circumference, long face, strabismus, pointed chin, clumsiness, and some articulation problems. He was ambulatory, verbal, and worked in a sheltered workshop. The third family (UK106) had three affected males. These males had severe ID, normal head circumference, no language, ambulatory difficulties because of knee contractures, shyness or gaze avoidance, and constipation. Published photographs show small-appearing eyes and

cupped ears. Of these three families, family UK106 appears most like Juberg-Marsidi-Brooks syndrome.

Froyen et al. (2008) also reported variable length microduplications in Xp11.22 (400-800 kb) in six families with nonsyndromal XLID, including MRX17 and MRX31.[21] Isrie et al. also reported a p.R4013W mutation in HUWE1 in 2 brothers with severe intellectual disability, relatively large head, hypertelorism, and downslanting palpebral fissures.[23] Nava et al. (2012) reported a *de novo* mutation, p.V950A in the more severely affected of 2 brothers with autism.[25] No further details were provided. Gene analysis has not been reported in the family considered by Morava et al. (1996) to have Brooks syndrome. [26]

It appears that the phenotypic spectrum resulting from *HUWE1* mutations is quite broad (Froyen et al. 2008, Isrie et al. 2013, this report).[22, 23] The Juberg-Marsidi-Brooks phenotype appears most severe with marked growth impairment, somatic manifestations, absent or limited speech and delayed ambulation. Two other families (families A323 and UK444 of Froyen et al. 2008) are less severely affected, having normal or large head circumference, minimal facial dysmorphism, and milder intellectual disability.[22, 24] It is of interest that carrier females in families with less severe phenotypic expression may have mild expression, not being protected by skewed inactivation of their affected X chromosome. The location of the mutations in the reported cases does not suggest an obvious explanation for the phenotypic variability. The mutation in Juberg-Marsidi-Brooks syndrome is located in the HECT domain (the catalytic domain of the HUWE1 ubiquitin protein ligase) as are the mutations in the third family reported here and in the more mildly affected UK444 family.[22] The other two

missense mutations in *HUWE1*, the p.R2981H mutation in a severely affected family and the p.R4013W mutation in a family with a less severe phenotype, are located between the WWE and HECT domains.[22] Further cases with *HUWE1* mutations will be necessary to determine if a genotype-phenotype correlation can be made.

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FIGURE LEGENDS

**Figure 1.** Pedigrees of families with *HUWE1* mutations. A. Pedigree of K9149. Photographs show microcephaly, cupped ears and thin upper lip in infant (II-6); and 27-year-old nephew (III-2) from original family reported by Juberg and Marsidi <sup>7</sup>. B. Pedigree of family K9223. Photographs show microcephaly, cupped ears, blepharophimosis, deeply set eyes, depressed nasal bridge, and bulbous nasal tip in affected male (III-1) from original family reported by Brooks et al. <sup>10</sup> at age 4 months and 4 years. C. Pedigree of family 3. The affected boys' mother (I-2) and one sister (II-2) have mild learning disabilities. Photographs show microcephaly, frontal hair upsweep, deep set eyes, broad nasal tip, wide mouth, thin upper lip, and prominent jaw in brothers, ages 10 (II-5) and 13 years (II-4). The older brother also has cupped ears, thick eyebrows and coarse face. + = presence of mutation, - = normal, ↗ = proband, ■ = affected, ⊙ = carrier.

**Figure 2.** Domains and missense mutations found in *HUWE1* with the relative boundaries of each domain indicated. The known missense alterations are shown below the DUF4414 and HECT domains. The p.G4310R mutation reported in this paper is in red. The WWE domain is named for its conserved residues and is predicted to mediate specific protein-protein interactions in ubiquitin and ADP-ribose conjugation systems. The C-terminal catalytic HECT belongs to a subclass of ubiquitin-protein E3 ligases. DUF: Domains of Unknown Function. DUF913 belongs to a family found in various ubiquitin protein ligases and DUF4414 is in a family of domains frequently found in DNA-binding proteins of the URE-B1 type.

**Figure 3.** E3 ubiquitin ligase HUWE1 protein level is decreased in the affected male from the Juberg-Marsidi syndrome family (K9149). (A) Immunoblot analysis of the HUWE1 protein level a lymphoblastoid cell line from the affected male (Figure 1A, III-2) and in a normal male in the family (Figure 1A, IV-1). The star indicates unspecific bands. (B) Quantification of three independent experiments as the one presented in (A); error bars indicate mean  $\pm$  SD; \*\*  $p < 0.01$ .

**Figure 4.** Protein levels of well-known HUWE1 substrates are upregulated in the affected male from the Juberg-Marsidi syndrome family (K9149). Immunoblot analysis of Mcl-1 (A) and p53 (C) protein levels in a normal male in the family (Figure 1A, IV-1) and a lymphoblastoid cell line from the affected male (Family 1A, III-2). (B) and (D) Quantification of three independent experiments as the ones presented in (A) and (C), respectively; error bars indicate mean  $\pm$  SD; \* $p < 0.05$ .

**Figure 5.** Functional assessment of the p.R4063Q HUWE1 mutation in family 3. HUWE1 protein levels are increased in p.R4063Q lymphoblastoid cell lines (LCLs) derived from the affected brothers and results in moderate reduction in the levels of the p53 protein. Unaffected father (lane 1), affected sons (lanes 2 and 3). Total LCL protein lysates were western blotted and probed for HUWE1, p53 and B-tubulin (loading control).

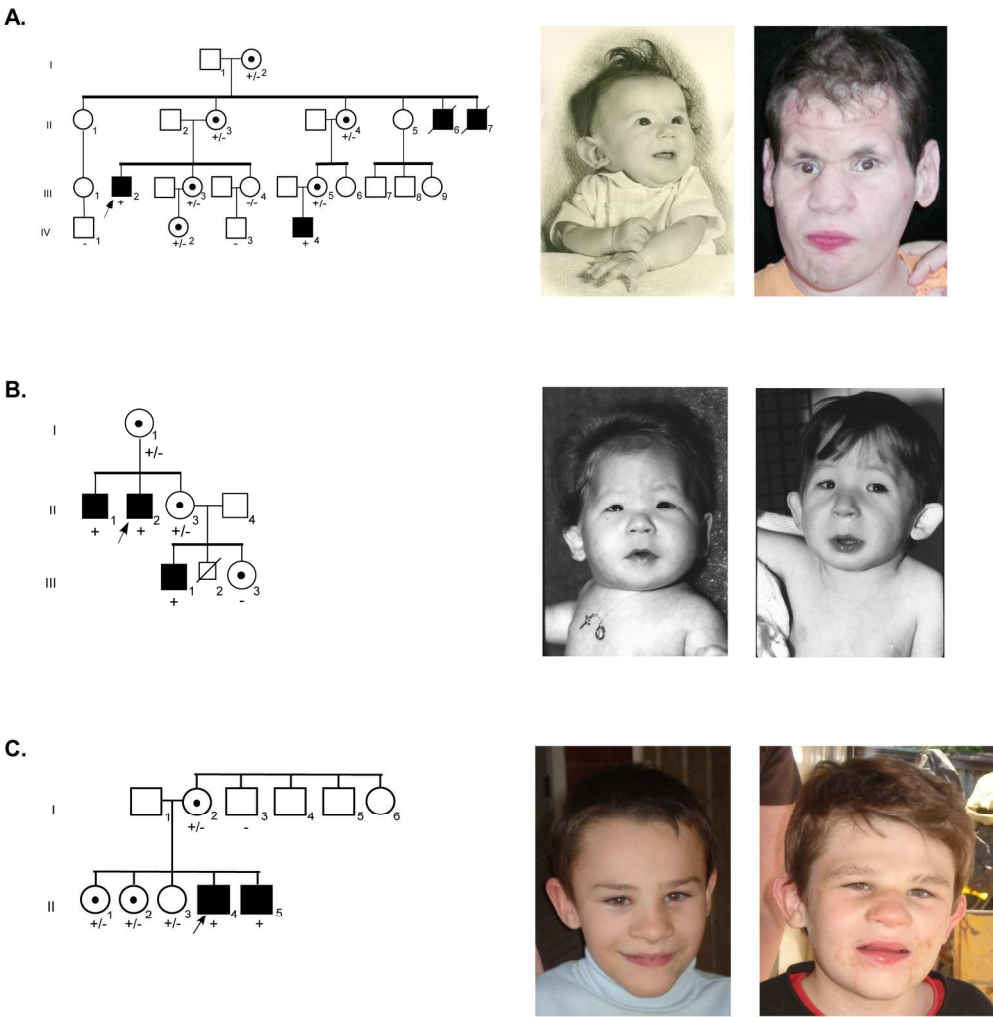


Figure 1.  
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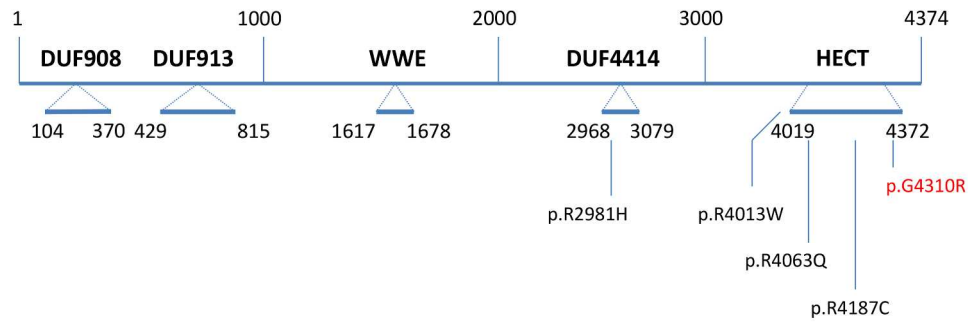


Figure 2.  
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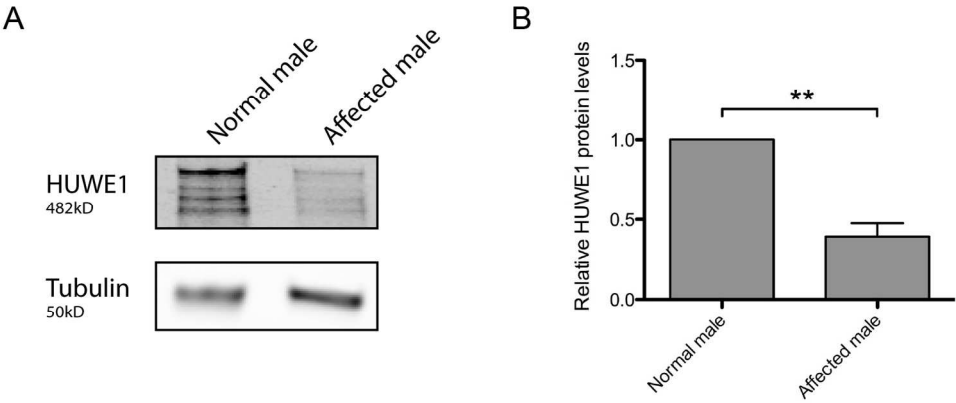


Figure 3.  
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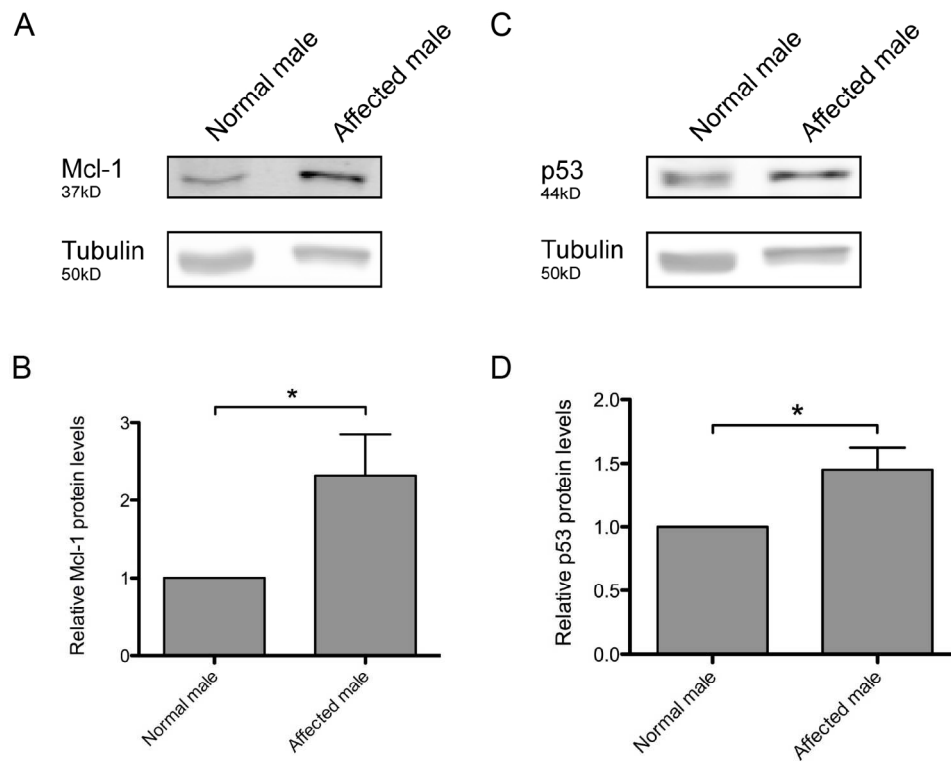


Figure 4.  
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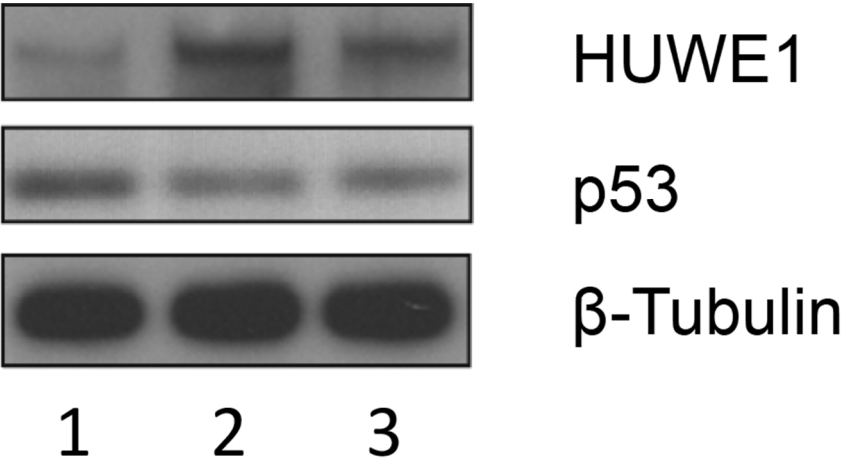
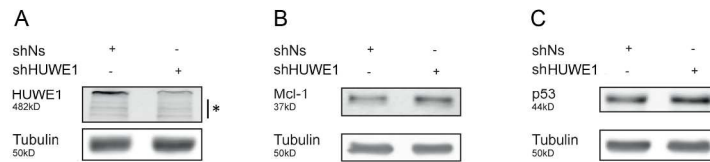


Figure 5.  
86x42mm (300 x 300 DPI)





**Supplementary Figure 1.** Protein levels of the well-known Mule (*HUWE1*) substrates Mcl-1 and p53 are stabilized upon Mule knockdown. Immunoblot analysis of Mule (A), Mcl-1 (B) and p53 (C) protein levels in a healthy individual lymphoblastoid cell line expressing non-specific (Ns) or Mule-targeting shRNA. Star in (A) indicates unspecific bands.

224x103mm (300 x 300 DPI)

Supplementary Table 1. Predicted Effects of the Alterations in *HUWE1* on Protein Function

Alteration		Mutation Taster	SIFT	PolyPhen-2
p.R4063Q	c.12188G>A	Disease-causing	NT*	Probably Damaging (.999)
p.G4310R	c.12928G>C	Disease-causing	NT	Probably Damaging (1.000)
p.R4013W**	c.12037C>T	Disease-causing	NT	Probably Damaging (.999)
p.R4187L**	c.12559C>T	Disease-causing	NT	Probably Damaging (1.000)
p.R2981H**	c.8942G>A	Disease-causing	NT	Probably Damaging (.999)

\*NT- Not Tolerated

\*\*Three missense mutations reported by Froyen et al.[22] and Isrie et al.[23]

182x57mm (300 x 300 DPI)

# BMJ Open

## HUWE1 mutations in Juberg-Marsidi and Brooks syndromes: The results of an X-chromosome exome sequencing study

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# HUWE1 mutations in Juberg-Marsidi and Brooks syndromes: The results of an X-chromosome exome sequencing study

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## ABSTRACT

**Background.** X-linked intellectual disability (XLID) syndromes account for a substantial number of males with ID. Much progress has been made in identifying the genetic cause in many of the syndromes described 20-40 years ago. Next generation sequencing (NGS) has contributed to the rapid discovery of XLID genes and identifying novel mutations in known XLID genes for many of these syndromes.

**Methods.** Two NGS approaches were employed to identify mutations in X-linked genes in families with XLID disorders. One involved exome sequencing of genes on the X chromosome using the Agilent SureSelect Human X Chromosome Kit. The second approach was to conduct targeted NGS sequencing of 90 known XLID genes.

**Results.** We identified the same mutation, a c.12928 G>C transversion in the *HUWE1* gene, which gives rise to a p.G4310R missense mutation in two XLID disorders: Juberg-Marsidi syndrome (JMS) and Brooks syndrome. Although the original families with these disorders were considered separate entities, they indeed overlap clinically. A third family was also found to have a novel *HUWE1* mutation.

**Conclusions.** As we identified a *HUWE1* mutation in an affected male from the original family reported by Juberg and Marsidi, it is evident the syndrome does not result from a mutation in *ATRX* as reported in the literature. Additionally, our data indicate that JMS and Brooks syndromes are allelic having the same *HUWE1* mutation.

**Strengths and limitations of the study**

1. Using the power of Next Generation Sequencing we have linked Juberg Marsidi syndrome (JMS) and Brooks syndrome as allelic conditions
2. This study provides better organization to the field of X-linked disorders by providing evidence that JMS is not caused by mutation in the *ATRX* gene
3. We provide clinical descriptions and comparisons to support our conclusions
4. The reported missense mutations have detrimental effects on HUWE1 functional Activity
5. Based on the limited numbers of patients/families we are unable to make specific genotype-phenotype correlations at this time



## INTRODUCTION

In the two decades since the discovery of the trinucleotide repeat expansion in *FMR1* responsible for Fragile X syndrome, substantial progress has been made in defining the other X-linked Intellectual Disability syndromes at the molecular level.[1-3] Mutations in over 100 X-chromosome genes have been assigned at least provisional roles in these syndromes.[2, 4] Some syndromes, initially believed to be separate conditions, have now been lumped together while others that appeared to be the same have been separated on the basis of molecular findings.[2, 3, 5, 6]

Juberg-Marsidi syndrome (JMS) has heretofore been considered by some observers to be allelic to Alpha-Thalassemia Intellectual Disability (ATRX syndrome) while Brooks syndrome (aka Brooks-Wisniewski-Brown syndrome) has been considered to be distinct from JMS.[7-10] A missense mutation in *ATRX* has been previously reported in a family considered to have JMS.[8] Upon clinical reevaluation of the original family reported by Juberg and Marsidi (1980), we considered the findings in the sole affected male still alive to be inconsistent with Alpha-Thalassemia Intellectual Disability. While there are some similarities between JMS and ATRX syndrome, there are distinctive differences.[7] Specifically the surviving male in the Juberg-Marsidi family had bifrontal narrowing, hypotelorism, prominent nose with overhanging columella, and thin lips, all facial manifestations very different than those of ATRX syndrome. Subsequently we identified in this male, a novel missense alteration in *HUWE1*, a gene located at Xp11.23 that encodes an E3 ubiquitin ligase that regulates numerous proteins, such as key cellular factors p53 and Mcl1.[11, 12] The identical missense alteration in *HUWE1* was also identified and shown to properly segregate in another

XLID syndrome reported by Brooks et al. (1994).[10] The clinical phenotypes in the two conditions previously considered to be unrelated show close similarity. Herein, we update the clinical and molecular findings in these two families and report an additional family with another missense mutations in *HUWE1*.

**METHODS**

**Informed Consent**

The authors note that we have obtained proper informed consent from the participants (or parental consent for participants under 18 years of age) for use of their personal information, including photos, for publication purposes. Copies of these consents have been shared with the office of the Editor.

**Families**

Clinical Update: K9149 (original Juberg-Marsidi Syndrome family) [7]  
Clinical findings in four affected males in 3 generations included generalized growth retardation, severe intellectual disability, tall forehead, epicanthus, short palpebral fissures, pale retina, flat nasal bridge, large or cupped ears, small penis with undescended testes, and deafness.[7] The two brothers in generation II died in infancy or childhood.

The pedigree and clinical findings were updated in 2006. The nephew (III-2 in Figure 1A), age 27 years, was found to have moderate hearing loss of unknown type but believed to be related to recurrent otitis media, severe growth impairment (all measurements below the third centile) and intellectual disability (IQ < 20). Craniofacial findings included facial asymmetry, bifrontal narrowing, furrowing of the forehead,

1  
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3 prominent brow giving the eyes and midface a sunken appearance, hypotelorism,  
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6 upslanting palpebral fissures, prominent nose with overhanging columella, long  
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9 philtrum, and thin lips with a beak at the midpoint of the upper lip (Figure 1). Additional  
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11 features were brachydactyly, decreased musculature of the distal legs, foot deformation  
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13 (right foot everted, left foot with midfoot varus), scoliosis, flexion of the hips, genu vara,  
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15 soft loose skin over the hands and hyperextensible joints. Sexual hair and penis  
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17 appeared normal but the testes were not palpable. He had no speech, but uttered  
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19 sounds when under stress and used gestures to indicate his desires (Table 1).  
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25 All females, including two obligate and four nonobligate carriers identified by  
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27 gene testing, had normal growth, craniofacial appearance and intellectual function.  
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29 Marked skewing (>90:10) of X chromosome inactivation in all carrier females provided a  
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31 plausible reason for absence of clinical manifestations. One carrier subsequently had an  
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33 affected son. He had growth retardation when delivered at 38 weeks with a weight of  
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35 1510 g, head circumference of 30 cm, and length of 42 cm, all measurements less than  
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37 the third centile for 38 weeks. He had hypotelorism, small appearing eyes, flat nasal  
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39 bridge, right cleft lip and palate, supralvalvar aortic stenosis, small hands and feet,  
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41 externally rotated feet and undescended testes. Ultrasound examination of the brain  
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43 showed a small cyst (1.0 x 1.6 cm) within each lateral ventricle. He failed the newborn  
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45 hearing test.  
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53 Clinical Update: K9223 (original Brooks Syndrome family) [10]  
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Clinical findings in two affected brothers and a nephew were generalized intrauterine and postnatal growth retardation , severe intellectual disability, triangular shaped face, low posterior hairline, bifrontal narrowness, malar flatness, blepharophimosis, optic atrophy, esotropia, nystagmus, bulbous nose, low-set and cupped ears, short philtrum, thin tented upper lip, and pectus excavatum (Figure 1B).[10] They were poorly coordinated and became self-abusive, hyperactive, and spastic with large joint contractures.

The pedigree and clinical findings were updated in 2010. The affected males were ages 23, 45, and 52 years. All growth parameters remained well below the third centile. Affected males became ambulatory by about age three years, but remained nonverbal or had very limited speech (Table 1). Hearing loss was believed to be related to recurrent otitis media. Cranial imaging showed enlarged ventricles and small brain.

Three carrier females had normal intellectual function and showed no phenotypic abnormalities. X-inactivation was markedly skewed (>90:10) in the two carriers who were informative at the *AR* locus.

Clinical Report: Family 3.

Family 3 consists of two brothers, now aged 10 and 13 years (II-5 and II-4 in Figure 1C), who have severe intellectual disability, microcephaly and postnatal growth failure. The brothers have three sisters, one of whom has minor literacy problems. Their mother also has a minor learning difficulty, not present in her 3 brothers and sister. X-inactivation testing in the mother and the daughter with literacy problems was uninformative.

The two brothers were born at term gestation with weights and lengths at term at or below the 3<sup>rd</sup> centile, being 2.52 kg and 2.67 kg and 44 cm and 47 cm, respectively. Neither was microcephalic at birth but have been <3<sup>rd</sup> centile since early childhood. Early motor milestones were delayed with both boys not walking independently until after age two years. The oldest brother had tight Achilles tendons and required serial casting and ankle-foot orthoses. His gait was broad based and ataxic, with reduced extension of both hips and knees. The younger brother had better language skills, with over 100 single words, and could follow single step commands; the older brother only had a few single words.

Both brothers had early onset growth failure, with significant short stature. Their heights were more than -4.0 SD from the mean by age three years. Dynamic testing excluded growth hormone deficiency (peak growth hormone levels 54.3 mIU/L and 13.9 mIU/L to glucagon stimulation). Alternative causes of short stature were not identified. Both boys were treated with and responded to growth hormone, with their heights now approaching the 3<sup>rd</sup> centile. Neither boy had clinical or biochemical evidence of other endocrine abnormalities. Their genitalia were normal and the older brother had normal pubertal development. The youngest brother has astigmatism, but otherwise vision and hearing in both boys is normal.

Both brothers are now markedly microbrachycephalic, with head circumference of 47cm (<3<sup>rd</sup> centile) and 48cm (<3<sup>rd</sup> centile), respectively. They have sparse scalp hair, which was more obvious in early infancy. Craniofacial features include deep-set eyes, broad nasal bridge and nasal tip, a wide mouth, a thin upper lip and prognathism (Figure 1C and Table 1). The older brother's ears are cupped, his eyebrows are thick

and his face is now coarse, resembling the pictures of the patient with Juberg-Marsidi syndrome (Figure 1A).

Skeletal surveys in both boys demonstrated only brachycephaly and delayed bone age (prior to treatment with growth hormone). Neuroimaging showed some increased extra-axial space and ventriculomegaly, white matter loss in the parietal, occipital and periventricular regions, a normal pituitary gland and a posterior pituitary bright spot.

**Marker Analysis**

As the clinical evaluation of K9149, the original Juberg-Marsidi family, was not consistent with ATRX syndrome and sequencing of *ATRX* conducted at the Greenwood Genetic Center failed to detect a mutation, marker analysis using 28 X-chromosome markers was done.[13]

**Sequencing**

Genomic DNA from affected male, III-2, in family K9149 was enriched for exons of 718 genes on the X chromosome using the Agilent SureSelect Human X Chromosome Kit and subjected to next generation sequencing as previously described.[14] DNA from affected male, III-1, was enriched for 90 XLID genes using RainDance microdroplet technology (RainDance Technologies, Lexington, MA). Sequencing of these 90 genes was performed on an Applied Biosystems SOLiD 3 Plus Sequencer.

**Preparation of Whole Cell Extracts**

Lymphoblastoid cells were collected and washed twice with ice cold PBS, followed by flash freezing of the cell pellet in liquid N<sub>2</sub>. Subsequently, the thawed cells were resuspended in one packed cell volume (PCV) of lysis buffer I (10 mM Tris-HCl pH 7.8, 200 mM KCl, 1 mM NEM, 0.1 mM MG-132, 1 µM PMSF, 2 µM leupeptin, 3 µM bestatin, 1.5 µM pepstatin) and two PCVs of lysis buffer II (10 mM Tris-HCl pH 7.8, 600 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% NP-40, 1 mM NEM, 0.1 mM MG-132, 1 µM PMSF, 2 µM leupeptin, 3 µM bestatin, 1.5 µM pepstatin). The samples were further incubated for 30 minutes at 4°C, sonicated and centrifuged. The supernatants were either stored at -80°C or used directly for immunoblot analysis.

### Immunoblotting

High molecular weight proteins present in the whole cell extract were separated on a 7% Tris-Acetate polyacrylamide (PA) gel, while lower molecular weight proteins (p53 and Mcl-1) were separated by a 10% Tris-Glycine PA gel. After separation, proteins were transferred to an Immobilon-FL membrane (Millipore), followed by subsequent immunoblotting. Primary antibodies against HUWE1 (Bethyl Laboratories), p53 (Santa Cruz) and Mcl-1 (BD Pharmingen) were detected using infrared (IR) Dye-conjugated secondary antibodies (Rockland). The signal was visualized using direct IR fluorescence via the Odyssey Scanner (LI-COR Biosciences).

### shRNA knockdown

Cells from male control were lentivirally transduced with plasmids carrying the non-specific control shRNA (pGIPZ-Ns, Open Biosystems) or the Mule-targeting shRNA



(GIPZ Human HUWE1 shRNA, Clone-ID V3LHS\_353153, Open Biosystems). Stable clones were subsequently selected with puromycin, while the selection-efficiency was monitored by the percentage of GFP-positive cells.

RESULTS

Segregation analysis in K9149 of 28 markers spaced along the X chromosome indicated 4 markers from DX51055 (Xp11.2) to AR (Xq12) were linked to JMS (data not shown). The region did not include the *ATRX* gene confirming the negative gene sequencing and clinical impression that JMS was not allelic to *ATRX*. But as the region contained many genes, next generation sequencing of exons on the X chromosome was initiated.

NGS of genes on the X chromosome in individual III-2 identified only one unique variant within the “linked” interval, a G>C transversion at nucleotide 12928 (c.12928G>C) in *HUWE1*. This change results in a missense mutation, p.G4310R. The finding was confirmed by Sanger sequencing. The alteration segregated with the phenotype (Figure 1A) as expected since *HUWE1* is within the “linked” interval.

Next generation sequencing of individual III-1 in family K9223 for 90 XLID genes identified the same missense *HUWE1* mutation as the only unique change after other SNPs were removed from further consideration based on available database and in-house frequency information. Sanger sequencing confirmed the NGS finding and the alteration segregated properly in the family (Figure 1B). The Sorting Intolerant From Tolerant (SIFT), PolyPhen-2 and Mutation Taster prediction algorithms indicated that



replacing a nonpolar amino acid with one that is charged is not expected to be tolerated, consistent with a damaging effect on the protein (Supplementary Table 1).

The older brother, II-4, in Family 3 was found to have a mutation in *HUWE1* (c.12188G>A; p. R4063Q) by an x exome analysis. This mutation alters a highly conserved amino acid in the HECT domain. The mutation arose as a *de novo* event in the mother and is also present in her other affected son as well as two of her daughters (Figure 1C). Neither this alteration or the shared variant for families 1 and 2 have been reported in the available databases, including the Exome Variant Server database that includes exome data for approximately 6500 individuals.

In order to determine if the p.G4310R mutation affected the HUWE1 function, protein levels were compared between the cells derived from an affected male (Figure 1A, III-2) from the original Juberg-Marsidi syndrome family (K9149) and the cells from a healthy male family member (Figure 1A, IV-1). As shown in Figure 2, the amount of HUWE1 in cells from the affected male was significantly reduced to approximately 39% of the HUWE1 level in the cells from the healthy relative (Figure 1A, IV-1). The levels of two important HUWE1 substrates Mcl1 and p53 were significantly increased in the patient's cells (Figure 3). To confirm that reduced HUWE1 levels directly cause the upregulation in p53 and Mcl1 levels, we performed HUWE1 knock-down experiments in cells from healthy individuals. Upon HUWE1 knock-down, we observed an accumulation in Mcl1 and p53, thus indicating that the change in HUWE1 status is directly correlated with changes in p53 and Mcl1 levels (Supplementary Figure 1). Taken together these findings suggest that p. G4310R mutation identified in the affected male from the

Juberg-Marsidi syndrome family (K9149) leads to reduced E3 ligase levels and consequent accumulation of downstream targets p53 and Mcl1.

In family 3, levels of the HUWE1 p.R4063Q protein were increased, and consequently the levels of p53 substrate were reduced in the two affected brothers compared with their normal father (Figure 4). Taken together, altered levels of mutant HUWE1 proteins and as a consequence the levels of its target substrates suggest that missense changes potentially affect HUWE1 function.

DISCUSSION

HUWE1 plays a role in various essential physiological processes by regulating the stability of multiple proteins. Initially HUWE1 was shown to bind the tumor suppressor p53, thus inhibiting transactivation.[11, 15] Further HUWE1 was shown through ubiquitylation to contribute to regulation of the anti-apoptotic protein Mcl1.[12] Additional substrates of HUWE1 include proteins such as histones,[16] transcription factor Miz1,[17] DNA polymerases beta and lambda,[18, 19] topoisomerase TopBP1,[20] and others.

Among the 200 named X-linked intellectual disability syndromes, a number of entities have been found to be allelic using molecular technologies.[2, 3, 5, 6] Prominent among these are *PQBP1*-associated XLID (Renpenning, Sutherland-Haan, Hamel Cerebro-Palato-Cardiac, Golabi-Ito-Hall and Porteous syndromes), *ATRX*-associated XLID (Alpha-Thalassemia Intellectual Disability, Carpenter-Waziri, Holmes-Gang, Chudley-Lowry, and XLID-Hypotonia-Arch Fingerprints syndromes), and *ARX*-

associated XLID (Partington, West, Lissencephaly and Abnormal Genitalia, and Hydranencephaly with Abnormal Genitalia syndromes).

In 1983, Mattei et al. reported an XLID family with seven affected males which they considered to have Juberg-Marsidi syndrome.[9] Villard et al. (1996) found a missense mutation in the helicase domain of *ATRX* in the family, and since that time Juberg-Marsidi syndrome has been called an *ATRX*-associated XLID syndrome in much of the literature.[8] Our clinical re-evaluation of the surviving male in the original Juberg-Marsidi syndrome family showed findings that were not consistent with *ATRX*-associated XLID, specifically bifrontal narrowing, hypotelorism, prominent nose and thin lips, prompting us to question their conclusion. Sequencing of the *ATRX* gene in this male failed to find a pathogenic alteration (data not shown). Further, review of the males reported by Mattei et al. (1983) shows them to have clinical findings very different from Juberg-Marsidi syndrome and more consistent with *ATRX*-associated XLID (Table 1).

The availability of Next Generation Sequencing provided an opportunity to sequence 718 X-chromosome genes simultaneously in the surviving affected member of the Juberg-Marsidi family. This effort identified a missense mutation in *HUWE1* (c.12928G>C; p.G4310R) which was considered to be likely disease-causing (Mutation Taster), not tolerated (SIFT) and probably damaging (PolyPhen-2) (Figure 5 and Supplementary Table 1). Functional studies performed on a cell line from the surviving affected male in the original family with Juberg-Marsidi syndrome showed significantly decreased HUWE1 protein levels and consequently increased levels of two well-known substrates, p53 and Mcl-1 as compared to a normal family member (Figures 3 and 4).

This molecular finding clearly establishes JMS as an XLID disorder caused by a mutation in *HUWE1* rather than in *ATRX*. The same mutation was also found in the family reported by Brooks et al. (1994) using a 90-gene XLID panel (Supplementary table).[10] Comparisons of the phenotype of Juberg-Marsidi and Brooks syndromes convinced us that they were examples of the same syndrome clinically. We have been unable to link the two kindreds through ancestry or common area of residence. A third family was also found to have a slightly more proximal missense mutation (p.R4063Q) which altered a highly conserved arginine in the HECT domain, which was likewise predicted to be deleterious (Supplementary Table 1). *HUWE1* p.R4063Q protein had increased stability and p53 levels reduced in the lymphoblast cell lines derived from the affected males in this family. Vandewalle et al.[21] recently reported that an extra dose of *HUWE1* affects axon branching of dorsal cluster neurons of *D. melanogaster* through the Wnt/3-catenin pathway.

It appears that the phenotypic spectrum resulting from *HUWE1* mutations is quite broad Table 1. The Juberg-Marsidi-Brooks phenotype appears most severe with marked growth impairment, somatic manifestations, absent or limited speech and delayed ambulation. Two other families (families A323 and UK444 of Froyen et al. 2008) are less severely affected, having normal or large head circumference, minimal facial dysmorphism, and milder intellectual disability.[22, 23] It is of interest that carrier females in families with less severe phenotypic expression may have mild expression, not being protected by skewed inactivation of their affected X chromosome. The location of the mutations in the reported cases does not suggest an obvious explanation for the phenotypic variability. The mutation in Juberg-Marsidi-Brooks syndrome is

located in the HECT domain (the catalytic domain of the HUWE1 ubiquitin protein ligase) as are the mutations in the third family reported here and in the more mildly affected UK444 family.[22] The other two missense mutations in *HUWE1*, the p.R2981H mutation in a severely affected family and the p.R4013W mutation in a family with a less severe phenotype, are located between the WWE and HECT domains.[22] Further cases with *HUWE1* mutations will be necessary to determine if a genotype-phenotype correlation can be made.

**Acknowledgement:** We would like to thank the families who were patient while we searched for the genetic cause over many years. Dedicated to the memory of Ethan Francis Schwartz, 1996-1998.

**Contributions:** MJF, MJB, SS were responsible for sequencing family 2. SSB and CoS contributed clinical information and material for family 2. RES reviewed clinical material for all families and summarized them. MF, MEC LCA, MAS, AG, LM contributed family 3. MB, BVL conducted the functional HUWE1 studies. SMcG conducted bioinformatic analysis. CS coordinated sample collection for all families. MC, RK conducted analysis of family 3. RJS evaluated family. PST and FA conducted sequencing of family 1. JG and YYY analyzed data for family 3. CES coordinated the study of all families. MF, RES, CES wrote the manuscript.

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**Competing Interests:** None declared.

**Data Sharing:** Raw data or clarification of reported results can be obtained by emailing Michael Friez ([friez@ggc.org](mailto:friez@ggc.org)).

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FIGURE LEGENDS

**Figure 1.** Pedigrees of families with *HUWE1* mutations. A. Pedigree of K9149. Photographs show microcephaly, cupped ears and thin upper lip in infant (II-6); and 27-year-old nephew (III-2) from original family reported by Juberg and Marsidi <sup>7</sup>. B. Pedigree of family K9223. Photographs show microcephaly, cupped ears, blepharophimosis, deeply set eyes, depressed nasal bridge, and bulbous nasal tip in affected male (III-1) from original family reported by Brooks et al. <sup>10</sup> at age 4 months and 4 years. C. Pedigree of family 3. The affected boys' mother (I-2) and one sister (II-2) have mild learning disabilities. Photographs show microcephaly, frontal hair upsweep, deep set eyes, broad nasal tip, wide mouth, thin upper lip, and prominent jaw in brothers, ages 10 (II-5) and 13 years (II-4). The older brother also has cupped ears, thick eyebrows and coarse face. + = presence of mutation, - = normal, ↗ = proband, ■ = affected, ⊙ = carrier.

**Figure 2.** E3 ubiquitin ligase *HUWE1* protein level is decreased in the affected male, III-2, from family K9149. (A) Immunoblot analysis of the *HUWE1* protein level a lymphoblastoid cell line from the affected male (Figure 1A, III-2) and in a normal male in the family (Figure 1A, IV-1). The star indicates unspecific bands. (B) Quantification of three independent experiments as the one presented in (A); error bars indicate mean ± SD; \*\* p < 0.01.

**Figure 3.** Protein levels of well-known *HUWE1* substrates are upregulated in the affected male III-2, from family K9149. Immunoblot analysis of Mcl-1 (A) and p53 (C) protein levels in a normal male in the family (Figure 1A, IV-1) and a lymphoblastoid cell line from the affected male (Family 1A, III-2). (B) and (D) Quantification of three

independent experiments as the ones presented in (A) and (C), respectively; error bars indicate mean  $\pm$  SD; \* $p < 0.05$ .

**Figure 4.** Functional assessment of the p.R4063Q HUWE1 mutation in family 3.

HUWE1 protein levels are increased in p.R4063Q lymphoblastoid cell lines (LCLs) derived from the affected brothers and results in moderate reduction in the levels of the p53 protein. Unaffected father I-1 (lane 1), affected sons, II-3 and II-4 (lanes 2 and 3). Total LCL protein lysates were western blotted and probed for HUWE1, p53 and B-tubulin (loading control).

**Figure 5.** Domains and missense mutations found in HUWE1 with the relative boundaries of each domain indicated. The known missense alterations are shown below the DUF4414 and HECT domains. The p.G4310R mutation reported in this paper is in red. The WWE domain is named for its conserved residues and is predicted to mediate specific protein-protein interactions in ubiquitin and ADP-ribose conjugation systems. The C-terminal catalytic HECT belongs to a subclass of ubiquitin-protein E3 ligases. DUF: Domains of Unknown Function. DUF913 belongs to a family found in various ubiquitin protein ligases and DUF4414 is in a family of domains frequently found in DNA-binding proteins of the URE-B1 type.

Table 1. Comparison of Findings in Families with Missense Mutations and Duplications of *HUWE1*\*

	Juberg and Marsidi[7]	Brooks et al.[10]	Family 3	Froyen et al.[22]			Isrie et al.[24]	Mattei et al.[9]
	K9149	K9223		A323	UK444	UK106	Dup <i>HUWE1</i> 24M	<i>ATRX</i> Mutation 7M
Affected individuals	4M	3M	2M	4M	3M	3M		
Mutation	p.G4310R	p.G4310R	p.R4063Q	p.R4013 W	p.R4187C	p.R2981H	Dup	p.R1272Q
Low birth weight (<3c)	4/4	3/3	2/2	0/1	-	-	0/12	2/2
Low birth length (<3c)	4/4	-	2/2	-	-	-	0/1	1/2
Short stature (<3c)	4/4	3/3	2/2	-	1/1	-	0/19	-
Microcephaly (<3c)	4/4	3/3	2/2	0/4	0/1	0/1	1/19	1/2
Macrocephaly (>97c)	0/4	0/3	0/2	2/4	0/1	0/1	0/19	0/2
Strabismus	2/3	2/3	-	-	1/1	-	1/11	-
Epicanthus	2/2	3/3	-	-	-	-	3/8	1/1
Deep-set eyes	1/1	3/3	2/2	-	-	-	0/8	-
Blepharophimosis/ small pf	4/4	3/3	-	-	-	-	3/8	1/1
Cupped ears	1/3	2/3	2/2	0/4	-	1/2	3/8	0/1
Prominent nose	1/3	3/3	2/2	0/3	-	-	3/8	0/1
Thin lip(s)	2/3	3/3	2/2	1/3	-	-	0/9	0/1
Undescended testes	4/4	-	0/2	0/1	-	-	0/8	2/3
Deafness	4/4	1/3	0/2	1/4	-	-	0/20	1/3
Seizures	1/4	0/3	0/2	0/4	-	-	0/8	-
Hypotonia	1/4	0/3	0/2	1/1	-	-	5/11	1/1
Severe ID (IQ <50)	4/4	3/3	2/2	4/4	-	-	12/17	3/3
Absent/limited speech	3/3	3/3	1/2	1/3	0/1	2/2	5/8	-
Other findings	Bifrontal narrowing, Hypotelorism, Contractures	Bifrontal narrowing, Short philtrum, Contractures, Clumsiness	Contractures	Tapered fingers	Clumsiness	Contractures	Hypertelorism Downslanting palpebral fissures	Nonattentive gaze, GI disturbances

<3c = less than 3<sup>rd</sup> centile; >97c = greater than 97<sup>th</sup> centile; pf = palpebral fissures; GI = gastrointestinal; dash (-) indicates information not available.

\* The *de novo* p.V950A mutation in one of two brothers with autism reported by Nava et al.[25] is not included because of lack of clinical details.

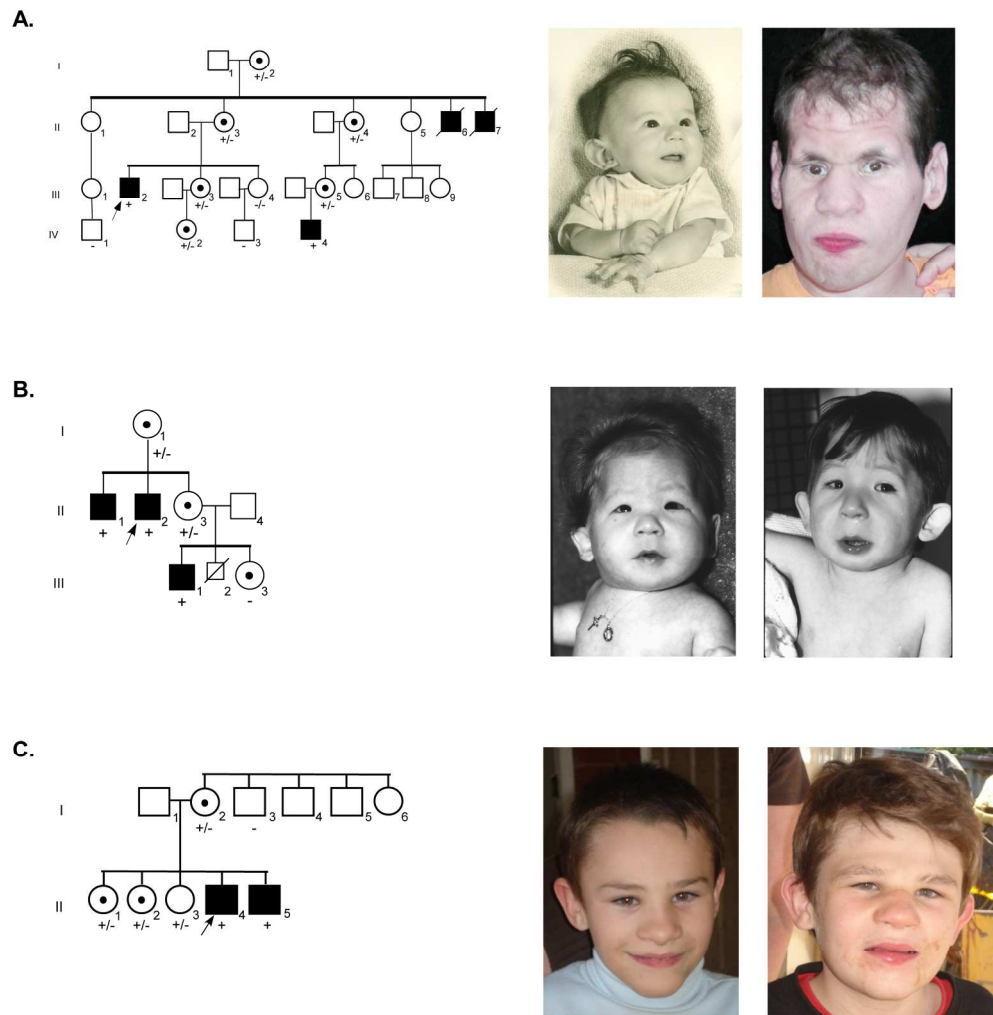


Figure 1  
188x203mm (300 x 300 DPI)



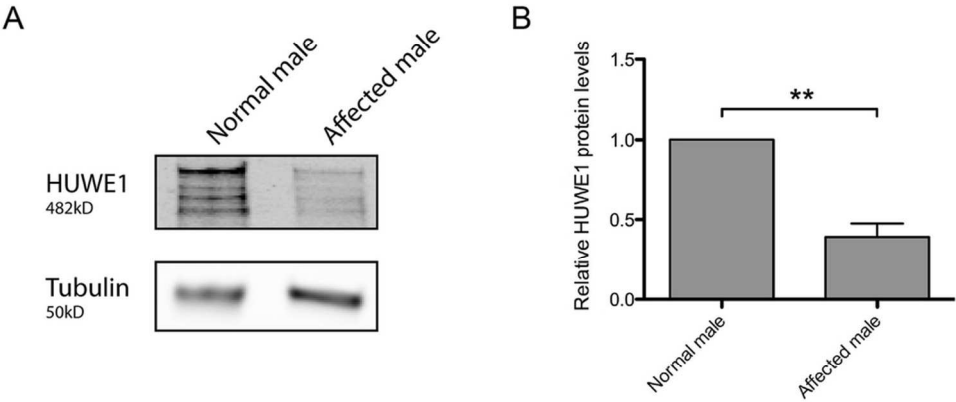


Figure 2  
89x48mm (300 x 300 DPI)

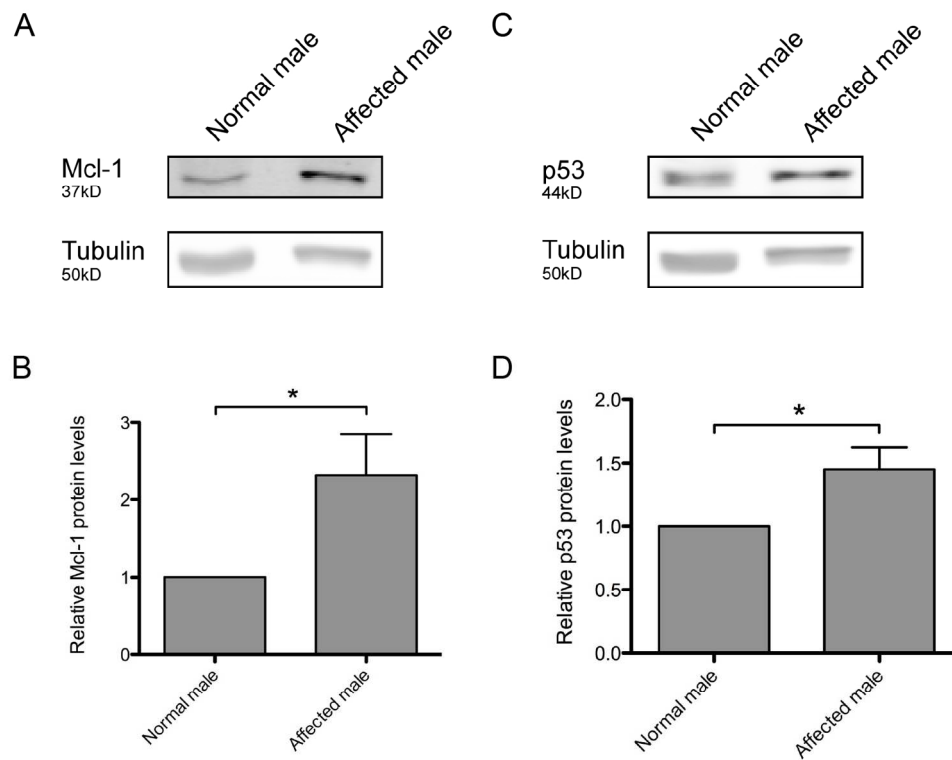


Figure 3  
156x127mm (300 x 300 DPI)

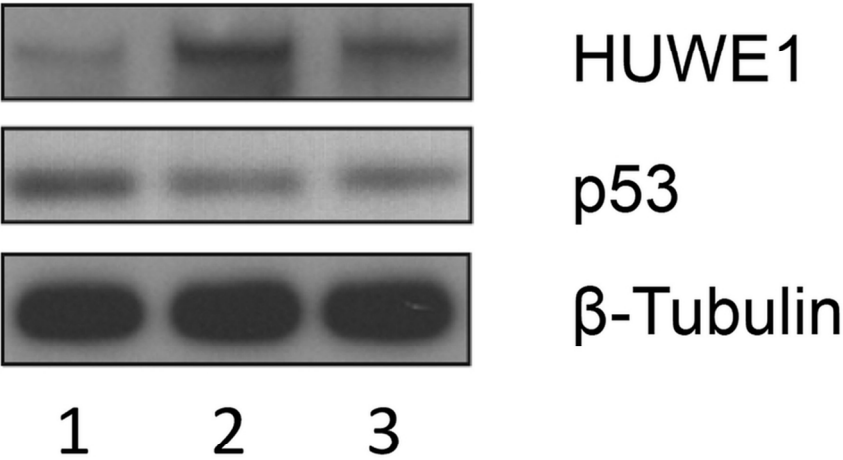


Figure 4  
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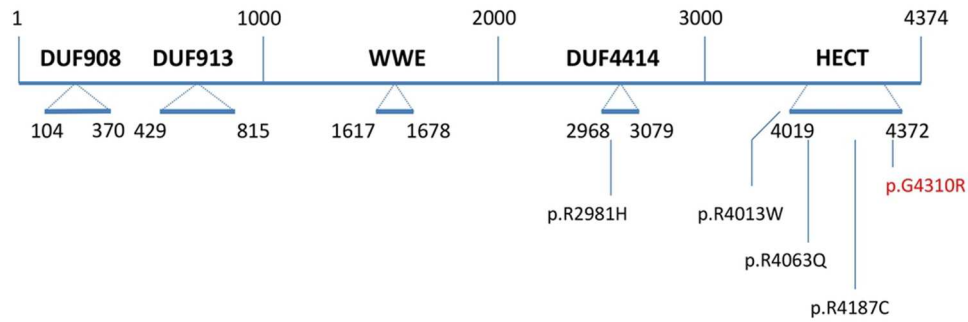
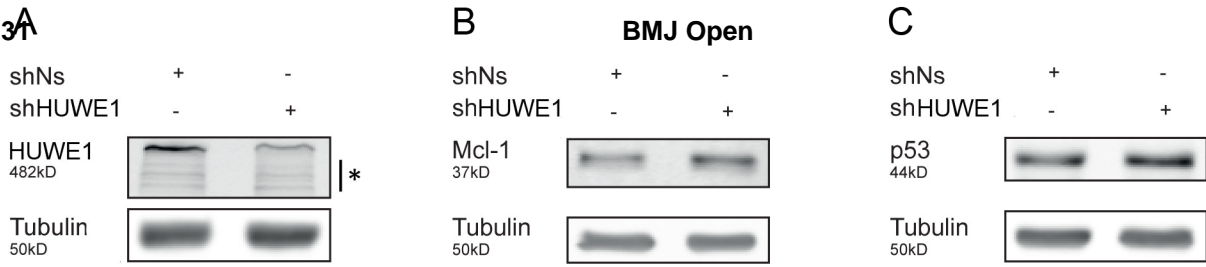


Figure 5  
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**Supplementary Figure 1.** Protein levels of the well-known Mule (*HUWE1*) substrates Mcl-1 and p53 are stabilized upon Mule knockdown. Immunoblot analysis of Mule (A), Mcl-1 (B) and p53 (C) protein levels in a healthy individual lymphoblastoid cell line expressing non-specific (Ns) or Mule-targeting shRNA. Star in (A) indicates unspecific bands.

	Alteration		Mutation Taster	SIFT	PolyPhen-2
1	p.R4063Q	c.12188G>A	Disease-causing	NT*	Probably Damaging (.999)
2	p.G4310R	c.12928G>C	Disease-causing	NT	Probably Damaging (1.000)
3	p.R4013W**	c.12037C>T	Disease-causing	NT	Probably Damaging (.999)
4	p.R4187L**	c.12559C>T	Disease-causing	NT	Probably Damaging (1.000)
5	p.R2981H**	c.8942G>A	Disease-causing	NT	Probably Damaging (.999)
6					
7					

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\*NT- Not Tolerated

\*\*Three missense mutations reported by Froyen et al.[22] and Isrie et al.[23]