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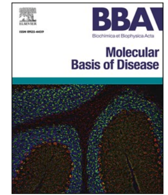

A unique missense mutation in the RING domain impairs MID1 E3 ubiquitin ligase activity and localisation and is associated with uncommon Opitz Syndrome-like signs

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A R T I C L E I N F O

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A B S T R A C T

- MID1/TRIM18 is a member of the RING-containing Tripartite Motif family of E3 ubiquitin ligases.
- *MID1* mutations cause X-linked Opitz Syndrome (XLOS), a neurodevelopmental genetic disease.
- We detected a Cys56Arg substitution in a family with history of midline developmental defects as the first variant identified in MID1 catalytic RING domain.
- This variant affects MID1 ubiquitin E3 activity and alters MID1 subcellular localisation and microtubule dynamics in a unique manner if compared to the other XLOS-associated mutations.
- Our data suggest that the relationship between MID1 activity and its cellular distribution is a crucial issue to fully understand MID1 physio-pathological role.

MID1, also known as TRIM18, belongs to the Tripartite Motif (TRIM) family of RING-containing E3 ubiquitin ligases [1,2]. As such, MID1 is able to mediate substrate ubiquitination and the best-characterised target to date is Phosphatase 2A catalytic subunit (PP2Ac) [3–5]. Mutations in the *MID1* gene cause X-linked Opitz Syndrome (XLOS; OMIM: 300000), a rare genetic disease characterised by incorrect development of the embryonic midline, although the physio-pathological role of MID1 is still unclear [1]. XLOS-affected male patients primarily present with craniofacial dysmorphisms, such as ocular hypertelorism and cleft lip/palate, hypospadias and other developmental defects [6]. Loss-of-function mutations detected in the *MID1* gene consist of missense, nonsense, and frameshift variants, as well as partial or complete gene deletions and duplications. So far, approximately 90 different pathogenic variants have been reported affecting any protein regions with the exception of the RING catalytic domain [6].

Genetic analyses performed on an 11-year-old male presenting with atypical neurodevelopmental signs, only partially overlapping with a classical XLOS synopsis, detected a hemizygous change at position c.166 T > C of *MID1* (NM_000381; chrX:10,567,382 hg38). Segregation analysis confirmed that the mother of the patient and her two sisters are carriers of the variant inherited from their father (Fig. 1A). The variant results in a missense mutation, p.Cys56Arg, within the RING catalytic domain of the gene product. The RING domain is characterised by a regular pattern of 8 cysteine and histidine residues that coordinate 2 zinc atoms thus allowing proper domain folding [2]. One of these cysteine residues is mutated into an arginine residue in the reported

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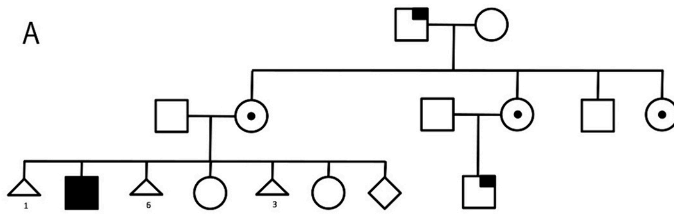
A



B

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MID1_C56R     ------METLESELTCPICLELFEDPLLLPCAHSLCFNCAHRILVSHCATNESVESITAFQRPTCRHVITLSQR
                          1  2          3 4  5  6                      7  8
TRIM18/MID1   ------METLESELTCPICLELFEDPLLLPCAHSLCFNCAHRILVSHCATNESVESITAFQCPTCRHVITLSQR
mTrim18       ------METLESELTCPICLELFEDPLLLPCAHSLCFNCAHRILVSHCATNESVESITAFQCPTCRHVITLSQR
cTrim18       ------METLESELTCPICLELFEDPLLLPCAHSLCFNCAHRILVSHCASNEPVESITAFQCPTCRYVITLSQR
zTrim18       ------METLESELTCPICLELFEDPLLLPCAHSLCFNCAHRILVSHCSSTKPLESISAFQCPTCRYVITLNQR

TRIM1         ------METLESELTCPICLKLFEDPLLLPCAHSLCFSCAHRILVSSCSSGESIEPITAFQCPTCRYVISLNHR
TRIM13        ------MELLEEDLTCPICCSLFDDPRVLPCSHNFCKKCLEGILEGSV--RNSLWRPAPFKCPTCRKETSATGI
TRIM10        MASAASVTSLADEVNCPICQGTLREPVTIDCGHNFCRACLTRYCEIPGPD-----LEESPTCPLCKEPFRPGSF
TRIM21        MASAARLTMMWEEVTCPICLDPFVEPVSIECGHSFCQECISQVG-----------KGGGSVCPVCRQRFLLKNL
TRIM4         ----MEAEDIQEELTCPICLDYFQDPVSIECGHNFCRGCLHRNWAPG---------GGPFPCPECRHPSAPAAL
TRIM11        MAAPDLSTNLQEEATCAICLDYFTDPVMTDCGHNFCRECIRRCWGQP---------EGPYACPECRELSPQRNL
TRIM17        MEAVELARKLQEEATCSICLDYFTDPVMTTCGHNFCRACIQLSWEKARGKKGRRKRKGSFPCPECREMSPQRNL
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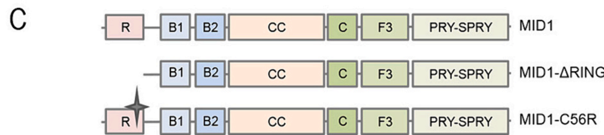
C



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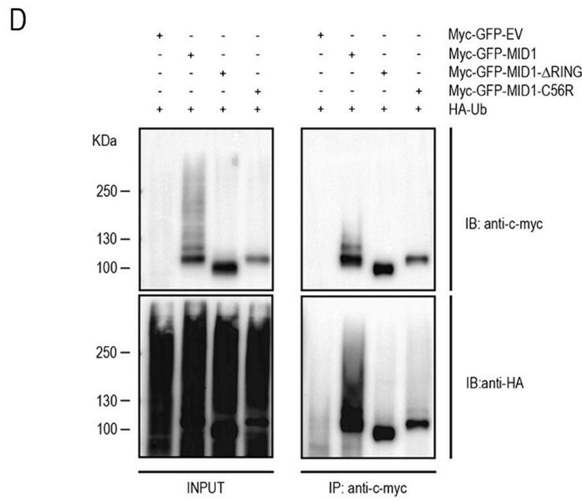

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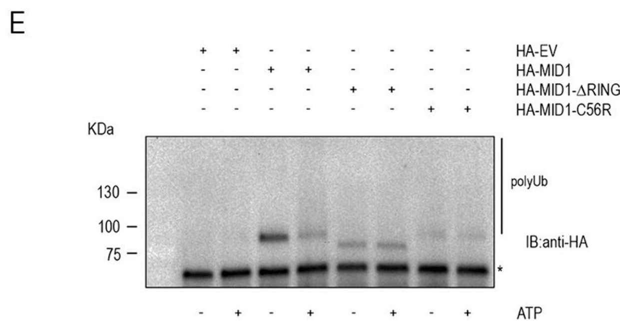

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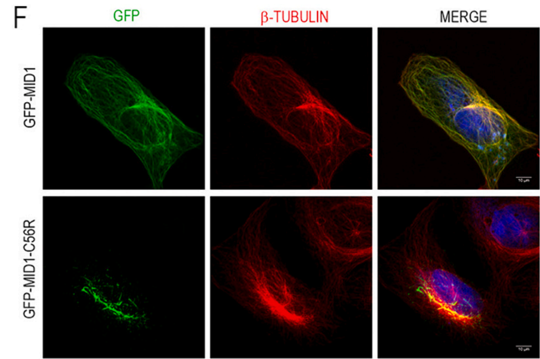

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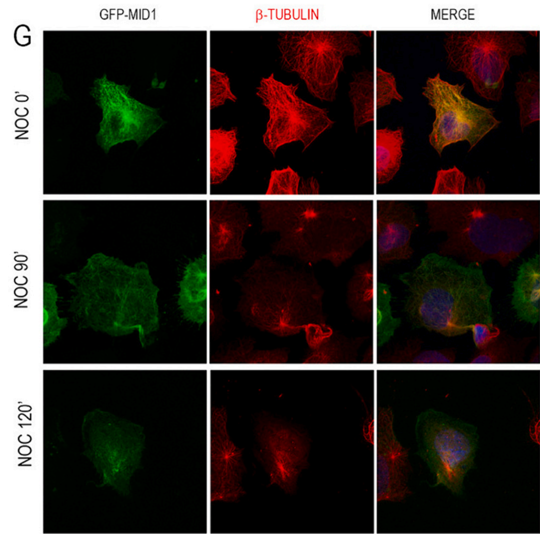

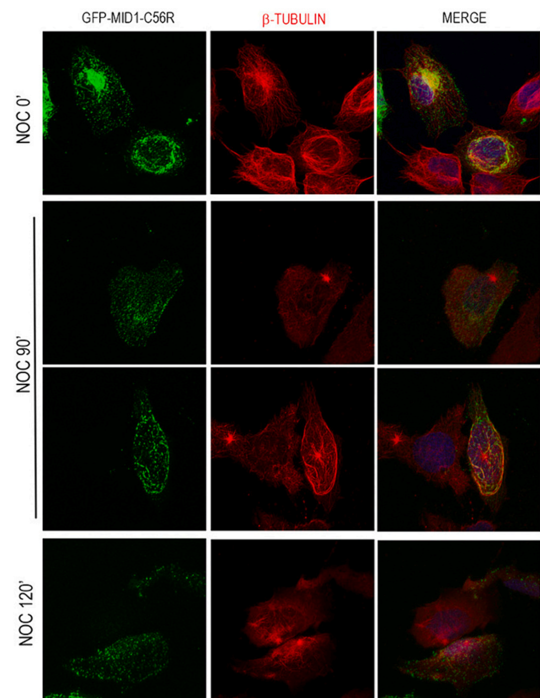

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Fig. 1. Identification and characterisation of MID1 Cys56Arg mutation. A) Pedigree of the family in which the variant was detected. The proband is indicated with full square symbol; partially full square symbols indicate the presence of hypospadias only; dotted circles indicate carrier females. B) MID1 RING domain alignment. Primary amino acid sequence of the RING domain of MID1 (TRIM18/MID1) compared to the C56R mutant sequence where the substitution is highlighted in red. The 8 residues involved in zinc atoms binding are numbered 1–8 and bold/green. RING multiple alignment with the *Mus musculus* (mTrim18), *Gallus gallus* (cTrim18) and *Danio rerio* (zTrim18) orthologues and with other TRIM family members is shown. C) Scheme of full-length protein (MID1), of the catalytically inactive ΔRING construct (MID1-ΔRING), and of the Cys56Arg mutant (MID1-C56R). R, RING; B1, B-box 1; B2, B-box 2; CC, coiled-coil region; C, COS; F3, Fibronectin type 3 repeat; PRY-SPRY, PRY-SPRY domains. D) Cellular ubiquitination assay performed upon either Myc-GFP-MID1, domain-deleted E3 incompetent MID1 mutant (ΔRING) or Cys56Arg mutant (MID1-C56R) overexpression together with HA-Ubiquitin (HA-Ub) in U2OS cells. Immunoprecipitation with anti-c-Myc antibody was performed and eluted proteins were analysed by immunoblot with the indicated antibodies to detect either the MID1 forms or the ubiquitinated proteins. As control, cells were transfected with a Myc-GFP empty vector (EV). E) *In vitro* ubiquitination assay in the presence of the indicated MID1 forms as putative E3 ligases in presence (+) or absence (−) of ATP. The MID1 form is detected with anti-HA antibody. Poly Ub, poly-ubiquitin smear; * indicates IgG. F) Confocal immunofluorescence images of U2OS cells transfected with either MID1 or MID1-C56R GFP constructs as indicated and immunostained with anti-β tubulin followed by Cy3-conjugated anti-mouse antibody. Nuclei are stained with DAPI (scale bar: 10 μm). G) Confocal immunofluorescence images of U2OS cells transfected with either GFP-MID1 (upper panels) or GFP-MID1-C56R (lower panels) constructs and treated with nocodazole for 90 min (NOC 90′) and 120 min (NOC 120′); NOC 0′ indicates untreated cells. The microtubular apparatus is detected with anti-β tubulin antibody followed by Cy3-conjugated anti-mouse antibody. Nuclei are stained with DAPI.

family (Fig. 1B). Protein sequence alignment of the RING domain of MID1 orthologues and of other TRIM family members reveals that the MID1 cysteine 56 is indeed highly conserved (Fig. 1B). Consistently, this variant is not a reported polymorphism and *in silico* pathogenicity analysis predicts this variant to be damaging and strongly pathogenic. As pathogenic OS *MID1* mutations in the RING domain of the protein have never been reported to date, we sought to better investigate the functional effect of this newly identified variant.

Through the RING, TRIM proteins act as E3 ubiquitin ligases by bringing together the ubiquitin-charged E2 conjugating enzyme and the specific substrate, thus promoting the covalent ubiquitin modification of the latter, as well as mediating their own ubiquitination (auto-ubiquitination) [7,8]. As the detected variant occurs in a crucial residue within the catalytic domain, we sought to test the ability of the MID1 Cys56Arg mutant to promote ubiquitination. We introduced the base substitution detected in the patient into tagged-MID1 mammalian vectors (from here on, C56R constructs) to test against wild-type MID1 and the MID1-ΔRING form, which carries complete deletion of the RING domain and serves as catalytically dead control (Fig. 1C). We performed an auto-ubiquitination assay by transiently expressing each of the 3 Myc-GFP-tagged constructs mentioned above together with HA-ubiquitin. The transiently expressed MID1 forms were immunoprecipitated with the antibody against their tag (anti-c-Myc) and the subsequent immunoblot was revealed with either anti-c-Myc or anti-HA (ubiquitin) antibodies (Fig. 1D). High Molecular Weight (HMW) smears were detected in correspondence to wild-type MID1 but not, or highly reduced, with the Cys56Arg form in both the total lysate and in the immunoprecipitate, most likely representing MID1 ubiquitinated forms. *Re*-blot with anti-HA confirmed that this smear does indeed represent ubiquitinated species, resulting from MID1 auto-ubiquitination. A reduced but still visible band, likely corresponding to mono-ubiquitination, suggests that a possible residual functional interaction with a 'primer' E2 conjugating enzyme or a sub-optimal interaction with other E2s might still occur in presence of the Cys56Arg variant. However, the mutant appears to be severely impaired in its ability to produce long poly-ubiquitin chains. As expected, MID1-ΔRING is completely unable to promote ubiquitination, consistently with the absence of the catalytic domain (Fig. 1D). Analogous assays, either reciprocally immunoprecipitating ubiquitinated cellular proteins and revealing with an anti-MID1 tag or using HA-tagged wild-type and mutated forms of MID1 co-expressed with His-tagged ubiquitin, were consistently showing comparable results (Fig. S1). As a further confirmation that the Cys56Arg mutation drastically reduces the ability of MID1 to promote auto-ubiquitination, we immunopurified the 3 HA-tagged MID1 forms from transiently transfected HEK293T cells and used them to perform an *in vitro* ubiquitination assay in the presence of each component of the ubiquitination cascade. The ubiquitination reaction was carried out in presence of ATP, or without it as control. In the presence of ATP, MID1 is predominantly present as ubiquitinated HMW species compared to the reaction devoid of ATP. Conversely, this pattern is not observed when the Cys56Arg variant is used for the *in vitro* ubiquitination reaction, similar to the ΔRING form, further indicating a markedly reduced E3 ligase activity of this variant (Fig. 1E). Together, these results indicate a drastic impairment of ubiquitin E3 ligase activity of the Cys56Arg MID1 mutant, consistent with the crucial position of this substitution within the RING domain. Whether this impairment is relevant for the ubiquitination of the specific targets and if it is related to different ubiquitin chains modification is an issue that needs further investigations.

MID1 is a cytoskeletal protein associated with the microtubular apparatus through the COS-box [9–11]. Several XLOS-associated mutations reported to date cause the protein to dissociate from microtubules and concentrate in cytoplasmic bodies [9,10,12]. We investigated the effect of this RING substitution on MID1 subcellular localisation upon transient transfection of GFP-tagged constructs and co-staining with an anti-β-tubulin antibody to decorate microtubules. While wild-type MID1 shows full co-localisation with microtubules as reported (Fig. 1F, upper panels), the MID1-C56R mutant behaves differently and its 'fragmented' localisation overlaps with microtubules only in minimal part, often in the aster region (Fig. 1F, lower panels). Consistent results are observed in a different cell line and using HA-tagged MID1 forms (Fig. S2). No co-localisation was observed for both forms with other cytoskeletal structures, *e.g.* actin and intermediate filaments (Fig. S2). These results suggest that change of a RING domain residue implicated in zinc atom coordination alters MID1 subcellular localisation, displacing it into peri-nuclear structures whose nature is undetermined but clearly different from the cytoplasmic bodies defined by classical XLOS-associated variants, consistent with this mutation being so unique in its position.

Despite the marginal co-localisation with the microtubules, we often observed the Cys56Arg variant in fragmented filamentous structures close to the microtubule organising perinuclear region. We thus transiently transfected U2OS cells with either wild-type or C56R constructs and treated them with nocodazole, an agent that promotes tubulin depolymerisation. Cells begin to depolymerise the microtubule apparatus as early as 90 min upon nocodazole treatment and this is seen in both non-transfected and MID1 transfected cells. Depolymerisation of microtubules is then completed within 120 min of nocodazole treatment, again independently on the presence or absence of exogenous MID1, which is accordingly dispersed in the cytoplasm upon treatment, as previously reported [9] (Fig. 1G, GFP-MID1). Also in cells transfected with MID1-C56R we observed complete depolymerisation of microtubules after 2 h of treatment but, differently from the wild-type-overexpressing cells, at 90 min we can observe cells with completely depolymerised microtubules (Fig. 1G, GFP-MID1-C56R, NOC 90′ upper images) as well as cells in which microtubules are still partially polymerised and filamentous (Fig. 1G, GFP-MID1-C56R, NOC 90′ lower images). Thus, despite the lack of full association with the microtubules, Cys56Arg presence in the aster region can provide partial protection from depolymerisation. Interestingly, in the cells showing partially depolymerised microtubules, its localisation pattern follows the spared

microtubular fragments, consistent with a protective effect. Further, the Cys56Arg variant was not diffused upon full depolymerisation but maintained a defined fragmented distribution, though in more peripheral cellular regions (Fig. 1G, GFP-MID1-C56R, NOC 120′). The perinuclear localisation and the observed partial protection from induced depolymerisation may suggest interference with microtubule nucleation and/or dynamics [13].

In summary, we demonstrated that this firstly reported RING variant presents unique features if compared to classic XLOS-associated *MID1* mutants reported to date, consistently with an atypical clinical presentation. In the classic XLOS mutations, MID1 E3 ligase activity is displaced from the microtubules. With the Cys56Arg mutation instead, we are facing a lack of activity while the protein is residually retained on the microtubules. Why this mutated form, which preserves intact microtubule binding domains, does not fully co-localise with the microtubules raises the question of the relationship between MID1 activity, correct folding and dimerization, and its microtubular localisation, an issue worth further investigation to understand the function of the MID1 protein and its role in the pathogenesis of midline developmental defects.

The absence to date of XLOS mutations in the RING domain led to hypothesise a possible different clinical outcome of such mutations, if any. The phenotype of the patient reported here is compatible with XLOS clinical synopsis, however, the proband shows additional signs that are not commonly observed in classic *MID1*-associated XLOS patients, such as malformation of cortical development and pachygyria, and a higher variable expressivity in this family than usual. The peculiar cellular and biochemical features of the Cys56Arg reported in this work might explain the observed phenotype difference, although we cannot exclude that still undefined variants within the MID1 non-coding regions, in its paralogue MID2 or in other modifier genes participating in the same pathway(s) could also influence the phenotype and the variable presentation within the family. Nevertheless, our observations are compatible with a very recent report showing aberrant brain development of engineered RING-less MID1 organoids that is not observed in full knock-out ones and murine line [14,15].

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CRediT authorship contribution statement

Martina Mascaro: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Luigi D'Ambrosio:** Writing – review & editing, Investigation. **Elisa Lazzari:** Writing – review & editing, Writing – original draft, Investigation. **Berta Almoguera:** Writing – review & editing, Visualization, Resources, Investigation. **Saoud Tahsin Swafiri:** Writing – review & editing, Conceptualization. **Melania Eva Zanchetta:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Germana Meroni:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2024.167126.

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