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The *MID1* gene product in physiology and disease

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Abstract

MID1 is an E3 ubiquitin ligase of the Tripartite Motif (TRIM) subfamily of RING-containing proteins, hence also known as TRIM18. MID1 is a microtubule-binding protein found in complex with the catalytic subunit of PP2A (PP2Ac) and its regulatory subunit alpha 4 ($\alpha 4$). To date, several substrates and interactors of MID1 have been described, providing evidence for the involvement of MID1 in a plethora of essential biological processes, especially during embryonic development. Mutations in the *MID1* gene are responsible of the X-linked form of Opitz syndrome (XLOS), a multiple congenital disease characterised by defects in the development of midline structures during embryogenesis. Here, we review MID1-related physiological mechanisms as well as the pathological implication of the *MID1* gene in XLOS and in other clinical conditions.

1. Introduction

The *MID1* gene encodes an E3 ubiquitin ligase that belongs to the Tripartite Motif (TRIM) family, hence also the name *TRIM18* for this gene (Reymond et al., 2001). The human *MID1* gene is located on the short arm of the X chromosome very close to the pseudoautosomal boundary (Van den Veyver et al., 1998) and the study on its peculiar genomic location was concomitant with its identification as being responsible for a rare genetic disease, Opitz G/BBB Syndrome (OS) (Quaderi et al., 1997). OS is a congenital disorder characterised by defects in the development of embryonic midline structures and *MID1*, stemming from *Midline-1*, is reported mutated in patients with this disease (Opitz, 1987; Quaderi et al., 1997).

The human *MID1* gene is located in the distal end of the X-specific region close to the pseudoautosomal region (PAR) and spans approximately 400 kb of the genome (Perry et al., 1998; Quaderi et al., 1997; Van den Veyver et al., 1998). Interestingly, its mouse orthologue, *Mid1*, is located on the same chromosome but spans the pseudoautosomal boundary with the first exons present only in the X-specific region while the 3' exons contained in the PAR, hence both on the X and on the Y chromosome (Palmer et al., 1997). In both humans and rodents the gene is composed of 9 coding exons and upstream to the first coding exon, the

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MID1 gene presents alternative 5' untranslated exons and its transcription is driven by at least five alternative promoters (Landry and Mager, 2002). This results in several *MID1* transcript isoforms that involve not only the 5' of the gene and several polyadenylation signals but also the coding region (Landry and Mager, 2002; Winter et al., 2004, 2007). The main features of the *MID1* gene are summarised in Table 1. *MID1* expression is complex and its regulation also includes the action of microRNAs. To date, the following have been found to regulate *MID1* translation: miR-19, miR-340, miR-374 and miR-542 that bind the 3'-UTR in HEK293 cells (Unterbruner et al., 2018) and miR-135b in a lymphoblastoid cell line (Arigoni et al., 2013). We will also address *MID1* regulation in specific context throughout the review.

Despite more than 20 years of research on *MID1* function, the pathogenesis of OS still remains unresolved. In addition, the involvement of *MID1* in clinically relevant conditions also in adulthood has further highlighted the need of better understanding its biological function. Here, we will summarise the findings reported on *MID1* referring to recent reviews for specific issues.

2. The *MID1* gene product

The TRIM family members are characterised by the presence of an N-terminal module composed of 3 domains: a RING domain, one or two B-box domains and a coiled-coil region (Reymond et al., 2001). The family members are further classified with respect to their C-terminal domain composition (Short and Cox, 2006). In the case of *MID1* and its close paralogues, the tripartite motif carries 2 B-boxes and is followed by a COS domain, a Fibronectin type III repeat (FN3), and a PRY-SPRY domain (Fig. 1) (Quaderi et al., 1997; Reymond et al., 2001; Short and Cox, 2006).

Despite the growing roles of the TRIM family members in several biological processes and medical conditions (Di Rienzo et al., 2020; Hatakeyama, 2017; van Gent et al., 2018), the biophysical structure of the TRIM proteins is still lacking, likely as a consequence of purification difficulties and propensity to aggregate. *MID1* is no exception, although interesting structural details obtained by NMR spectroscopy are available for the TRIM proteins characteristic B-box domains. The first domain to be characterised was the B-box 1 with the discovery that its structure closely resembles the folds of the RING domain through the coordination of 2 zinc atoms (Massiah et al., 2006). Interestingly, even though there is minimal primary sequence similarity between B-box 1 and B-box 2 domains, the latter consists of a short α -helix and a structured loop of anti-parallel β -strands, which adopts a structure similar to *MID1* B-box 1 and other RING structures (Massiah et al., 2007). In addition, when solved together, the two B-box domains pack against each other in a stable interaction reminiscent of the interaction of RING dimers (Tao et al., 2008). The other *MID1* domain structurally characterised is the COS domain (Short and Cox, 2006) that follows the Coiled-coil region and that adopts a helix-loop-helix structure in which the N- and C-terminal ends are in close proximity (Wright et al., 2016). Although we are still far from a complete structure of the *MID1* protein, these structural data have been helpful to define and interpret the localisation and biochemical activity of the *MID1* protein as described in the following sections.

2.1. Association with the microtubules

MID1 is a microtubule-associated protein (Cainarca et al., 1999; Schweiger et al., 1999). This association occurs throughout the cell cycle phases, not only with interphase microtubules but also with the mitotic spindle and midbodies during cytokinesis (Cainarca et al., 1999; Gholkar et al., 2016; Zanchetta et al., 2017; Zanchetta and Meroni, 2019). Short and colleagues discovered a new domain, mentioned above, involved in microtubule binding and located between the coiled-coil and the FN3 domains: the COS-box that is to date a signature for microtubule-associated TRIM proteins (Fig. 1) (Short and Cox, 2006). More recently, structural data on the COS domain and further biochemical findings have revealed that both the coiled-coil and the adjacent COS domain are necessary for microtubule binding (Wright et al., 2016). MID1 is a phosphoprotein and its phosphorylation status can modulate microtubule binding (Liu et al., 2001). Initial findings showed that phosphorylation of MID1 promotes its association with microtubules; however, later studies using mutants in the putative serine residue (Ser96) undergoing phosphorylation indicated that MID1 phosphorylation reduces its association with microtubules (Aranda-Orgilles et al., 2008a). Whether this difference is due to diverse experimental conditions or to several phosphorylatable residues is presently unknown and this issue deserves further studies. MID1 phosphorylation status also regulates its own bi-directional transport on microtubules: both phosphatase inhibition and the simulation of a permanent phosphorylation of MID1 can block transport of MID1 on microtubules in a kinesin- and dynein-dependent process (Aranda-Orgilles et al., 2008a).

It is still not clear if MID1 association with microtubules affects their dynamics and stability. When MID1 microtubule localisation is impaired, e.g. when MID1 harbours mutations found in OS patients, microtubules distribution and dynamics are not affected (Cainarca et al., 1999; Schweiger et al., 1999). On the contrary, in some instances, expression of exogenous MID1 results in the protection of microtubules from depolymerising drugs (Schweiger et al., 1999). In addition, the cooperation of MID1 with one of its partners, MIG12 (MID1 interacting G-12 like protein, or MID1IP1), results in microtubule stabilisation (Berti et al., 2004). MIG12 is detected on microtubules if co-transfected together with MID1 and this interaction protects microtubules from depolymerisation. Moreover, antibodies against acetylated tubulin, marking stable tubulin, decorate the MID1-MIG12 bundles, suggesting that the complex is able to stabilise microtubules (Berti et al., 2004). The experiments above have been performed with overexpressed MID1 in Cos-7 cells, therefore the stabilising effect might be exacerbated by its sustained and strong expression. Physiologically, it is possible that microtubule stabilisation might occur only when needed, finely tuned and regulated. Interestingly, MID1-deprived HeLa cells display division defects, including cytokinetic arrest and delayed or aborted abscission, which induce cell binucleation or death (Gholkar et al., 2016). Moreover, in the same cell type a recently discovered partner of MID1, BRAF35 (BRCA2-Associated Factor 35), is involved in cytokinesis and both are localised at the midbody where stable microtubules are present (Zanchetta and Meroni, 2019; Zanchetta et al., 2017). These recent data suggest that MID1 can regulate microtubule dynamics in defined cell cycle phases. The MID1-dependent control of microtubule dynamics may also regulate other cellular processes, such as cell

adhesion and cell migration, in which microtubules are involved (Demir et al., 2014; Latta and Golding, 2012).

2.2. E3 ubiquitin ligase activity

Ubiquitination is a post-translational modification that mainly targets a substrate for proteasome-dependent degradation, but can also modify its cellular localisation, modulate its activity or alter interaction with other proteins (Ciechanover, 2015; Hershko and Ciechanover, 1998; Schulman and Harper, 2009). Ubiquitin contains 7 lysines (K6, K11, K27, K29, K33, K48, K63) that can be employed to build chains with different topology. In addition, the N-terminal residue (Met1) can also be employed to generate linear chains (Huang and Zhang, 2020). Mono- or poly-ubiquitination determines the fate of the substrates (Kulathu and Komander, 2012). Linkage through K48 leads to proteasome-mediated degradation; K63 is involved in DNA repair, endocytosis and Nuclear Factor κ -light-chain-enhancer of activated B cells (NF- κ B) signalling, whereas much less is known about the functioning of the other lysine linkages. Ubiquitin conjugation is catalysed by an enzymatic cascade, involving the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. E3 ubiquitin ligases are responsible for substrate recognition thus favouring ubiquitin transfer to it (Komander and Rape, 2012). On the basis of domain structures, E3 ubiquitin ligases can be classified into two main families: the homologous to E6-AP COOH Terminus (HECT) family and the RING-containing protein family (Huibregtse et al., 1995; Joazeiro and Weissman, 2000; Buetow and Huang, 2016).

MID1, like the other members of the TRIM family, belongs to the class of RING-containing E3 ubiquitin ligases (Meroni and Diez-Roux, 2005). Although the indication that MID1 is an E3 ubiquitin ligase was proposed by several authors for years (see below), only in 2011 MID1 enzymatic activity was biochemically characterised (Han et al., 2011; Napolitano et al., 2011). Bacterially produced recombinant MID1 shows auto-E3 ubiquitin ligase activity *in vitro* and, as expected, the RING domain is essential for this activity. MID1 is able to interact with several E2 conjugating enzymes thus possibly promoting several types of ubiquitin chains (Han et al., 2011; Napolitano et al., 2011). Although this is still a matter of debate in the TRIM family field, the B-boxes within the tripartite motif appear to have a role in the MID1 E3 ubiquitin ligase activity (Han et al., 2011). Indeed, some RING-less TRIM members, such as TRIM29 and TRIM16, have been shown to possess E3 ligase activity likely through their B-box domains (Bell et al., 2012; Dou et al., 2019). Acting as an E3 ubiquitin ligase, MID1 controls the stability and/or the activity of substrates, some of which are summarised in the next paragraphs.

2.3. MID1/ α 4/PP2A complex

One of the first discovered MID1 interactors was Alpha4 (α 4), the mammalian homologue of the yeast protein TAP42 and a non-canonical subunit of protein phosphatase 2A (PP2A) (Di Como and Arndt, 1996; LeNoue-Newton et al., 2016). This interaction is mediated by the C-terminal domain of α 4 (Liu et al., 2001) and the B-box1 domain of MID1 (Troockenbacher et al., 2001). α 4 is diffusely distributed in both nucleus and cytoplasm, but it assumes a typical microtubular pattern in MID1-overexpressing Cos-7 cells (Liu et al., 2001). Indeed, Short and colleagues have proposed that MID1 tethers α 4 to microtubules

(Short et al., 2002). Conversely, high levels of $\alpha 4$ displace MID1 from microtubules, without affecting microtubular structure and dynamics (Liu et al., 2001). Studies performed *in vitro* demonstrated that a RING-B-box1 fragment is able to build poly-ubiquitin chains on $\alpha 4$ and that a form of this fragment harbouring the OS P151L mutation within B-box 1 cannot poly-ubiquitinate $\alpha 4$, although it is not clear if this is due to a lost interaction between MID1 and $\alpha 4$ (Wright et al., 2017). Nevertheless, no enrichment of ubiquitinated $\alpha 4$ and no changes in its level were observed upon MID1 overexpression in cells, leading to a first conclusion that $\alpha 4$ was not a substrate of MID1 E3 ubiquitin ligase activity. Instead, in presence of exogenous MID1 the level of the catalytic subunit of PP2A (PP2Ac) was decreased (Troddenbacher et al., 2001). Consistently, OS patients' fibroblasts carrying *MID1* mutations, show increased levels of PP2Ac, leading to a general hypo-phosphorylation of microtubule-associated proteins targeted by this phosphatase (Troddenbacher et al., 2001). Therefore, it was concluded that $\alpha 4$ serves as an adaptor protein promoting the formation of a ternary MID1/ $\alpha 4$ /PP2Ac complex. However, more detailed analyses in HEK293T cells have shown that $\alpha 4$ has one ubiquitin interacting motif (UIM) that permits $\alpha 4$ mono-ubiquitination, preventing the building of poly-ubiquitin chains on PP2Ac (LeNoue-Newton et al., 2011; McConnell et al., 2010).

Therefore, on one hand, some findings suggest that $\alpha 4$ promotes poly-ubiquitination of PP2Ac by scaffolding PP2Ac to MID1 (Troddenbacher et al., 2001); on the other hand, further studies show $\alpha 4$ to be a protective factor for PP2Ac degradation, through its UIM mono-ubiquitination (LeNoue-Newton et al., 2011; McConnell et al., 2010). The protective effect of $\alpha 4$ is not due to the fact that it can inhibit MID1 function or sequester MID1 from PP2Ac, since it is known that both MID1- and PP2Ac-binding domains are needed for $\alpha 4$ protective function. So, how can MID1 build poly-ubiquitin chains on PP2Ac? Several works show that MID1 mono-ubiquitinates $\alpha 4$, leading to its calpain-mediated cleavage, switching $\alpha 4$ activity from protective to degradative. In particular, the cleavage involves the C-terminal domain of $\alpha 4$, the one implicated in MID1 interaction (Watkins et al., 2012). Indeed, Han and colleagues show that the RING domain of MID1 mono-ubiquitinates a 45-amino acid polypeptide derived from the C-terminus of $\alpha 4$ (Han et al., 2011), further confirmed in Watkins analyses (Watkins et al., 2012). Taken together, all these results indicate that MID1 loss-of-function leads to a reduction of calpain-mediated cleavage of $\alpha 4$, increasing the protection of PP2Ac from degradation, explaining the hypo-phosphorylation of several Microtubule Associated Proteins (MAPs) as seen in OS fibroblasts (Troddenbacher et al., 2001). In this scenario, poly-ubiquitination of PP2Ac might be performed by (an)other E3 ubiquitin ligase(s), since the cleavage of $\alpha 4$ makes MID1 detach from MID1/ $\alpha 4$ /PP2Ac complex, leading to the hypothesis that cleaved $\alpha 4$ might redirect PP2Ac localisation promoting its polyubiquitination. In this way, only the microtubule pool of PP2Ac is subjected to proteasomal degradation.

Given PP2A involvement in the regulation of several biological processes (Sontag, 2001), the MID1/ $\alpha 4$ /PP2Ac complex participates in the modulation of these activities mainly on microtubules. For instance, this complex has been shown to regulate mammalian Target Of Rapamycin Complex 1 (mTORC1) signalling in tumour cells (U2-OS, MCF-7, HCT-116) and in Mouse Embryonic Fibroblasts: increased PP2A levels, resulting from depletion of MID1 or $\alpha 4$, lead to disruption of the mTOR/Raptor complex and downregulation of

mTORC1 signalling. In the same way, cells derived from OS patients show decreased mTORC1 formation, S6K1 phosphorylation, cell size, and cap-dependent translation, all of which is rescued by re-expression of wild-type *MID1* (Liu et al., 2011). Further, MID1/ α 4/PP2A-dependent dysregulation of mTORC1 could influence additional pathways, especially those subjected to feedback inhibition by mTORC1, such as PI3K/AKT and Ras/ERK (Carracedo et al., 2008). mTORC1 signalling is implicated in cytoskeletal dynamics and intracellular transport as well as in cell migration (Huang and Fingar, 2014; Liu et al., 2011), so it is tempting to speculate that dysfunctions in this signalling pathway in OS might contribute to patients' phenotype. Moreover, mTORC1 signalling is involved in processes like autophagy, protein synthesis, cell metabolism and cell growth and proliferation (Jhanwar-Uniyal et al., 2019) and given the role of MID1/ α 4/PP2Ac ternary complex in regulating mTORC1 complex association (Liu et al., 2011), MID1 absence might have an effect also in these processes (Fig. 2). Further, it was reported that MID1/ α 4/PP2Ac ternary complex is involved in TFEB (Transcriptional Factor EB) activity through FGF21 activation. FGF21, a fasting-induced hormone, induces calcium release from the ER, in fasting or starvation conditions, promoting DREAM (Downstream Regulatory Element Antagonist Modulator) shuttling into the nucleus. DREAM is a repressor of *MID1* gene expression (see also "Role in embryonic development") and, as consequence, PP2Ac accumulates, dephosphorylates TFEB allowing its translocation into the nucleus, where it activates target genes involved in lysosome biogenesis, autophagy and lipid metabolism (Chen et al., 2017).

2.4. Association with mRNAs and translational control

MID1 acts not only on proteic substrates but is also part of ribonucleoprotein (RNP) complexes (Aranda-Orgilles et al., 2008b). In fact, the MID1/ α 4 complex is able to bind several mRNAs, specifically carrying purine-rich region and forming hairpin structures, also named MID1 association sequence (MIDAS) and whose consensus is [AT]GG\w(1,4) [AT]GG\w(1,4) [AT]GG (Aranda-Orgilles et al., 2011, 2008b; Hettich et al., 2014). This RNA-protein interaction brings the transcripts in close proximity to ribosome- and translation-associated factors, namely elongation factor 1 α (EF-1 α -, receptor of activated protein kinase C1 (RACK1), Nucleophosmin (NPM), Annexin A2 (ANXA2) and several 40S ribosomal proteins, thus promoting protein translation (Aranda-Orgilles et al., 2008b; Hettich et al., 2014; Kohler et al., 2014; Krauss et al., 2013; Matthes et al., 2018b). The disruption of the MID1/ α 4 binding using an α 4 mutated peptide is sufficient to abrogate the enhancing effect on the translation of mRNAs that are bound to the mRNP complex (Monteiro et al., 2018).

Among the MIDAS-harboured transcripts, the 3-phosphoinositide dependent protein kinase-1 (*PDPK-1*) has been identified in association with the MID1-containing complex. PDPK-1 is a serine/threonine kinase involved in many cellular processes that plays a pivotal role, among others, in the mTOR signalling (Carneiro et al., 2015). Mutations in the *MID1* gene have been demonstrated to reduce PDPK-1 protein translation efficiency, since a functional *MID1* gene is able to rescue its synthesis (Aranda-Orgilles et al., 2011). Another example is represented by the β -secretase 1 (*BACE1*) sequence that encompasses several sites that fold like a MIDAS motif enabling the *BACE1* transcript to bind the MID1-complex promoting its translation (Aranda-Orgilles et al., 2011).

However, neither the MID1 protein nor the other components of the multiprotein complex seem to contain any known RNA-binding domain (Aranda-Orgilles et al., 2008b), although newly identified RNA-interacting regions are expanding the number of putative RNA-binding proteins. Several members of the TRIM protein family, indeed, have recently emerged as direct RNA binding partners through the C-terminal domains (i.e. NHL or PRY/SPRY domains) (Williams et al., 2019). However, no evidence of a direct association between MID1 protein alone and RNA has been yet provided.

2.5. MID1 and the Sonic hedgehog pathway

The hedgehog family, comprising Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) is a group of signalling molecules involved in several biological processes ranging from cell proliferation, differentiation and survival to the establishment of the vertebrate body plan (Ingham and McMahon, 2001). Once produced, these signalling molecules bind to the membrane protein Patched (Ptch) thus activating another membrane protein, Smoothed (Smo). The signal is then transduced into the nucleus by Gli transcription factors, whose activity is modified by kinesin-like protein KIF7 and the suppressor of fused (SuFu) (Sasai et al., 2019).

The findings above suggest that a tight link exists between MID1 and the mTORC1 signalling. Among the several networks in which mTORC1 is involved, it is observed that inhibition of mTORC1 activity upon rapamycin treatment reduces the translocation of the Sonic Hedgehog (Shh) pathway transcription factor GLI3 from the cytoplasm to the nucleus (Krauss et al., 2008). Likely, this involves the shuttling of the activator form of GLI3 (GLI3A), as the effect of this reduction in the nucleus leads to a decreased transcription of a Shh pathway target gene Cyclin D1. In HeLa cells, activation of PP2A via inhibition of its catalytic subunit degradation, mediated by MID1/ α 4 complex, leads to cytosolic retention of GLI3 and, as a consequence, a reduction of its function as a transcription activator (Krauss et al., 2008, 2009). In contrast, α 4 overexpression significantly shifts GLI3 to the nucleus (Krauss et al., 2008). In the effort to explain how MID1/ α 4/PP2A axis regulates GLI3 localisation, it has been discovered that MID1 could interact with Fu, a component of the Shh pathway, promoting its ubiquitination (Schweiger et al., 2014). This ubiquitination involves K6-, K48- and K63 linkages but if this is a direct consequence of MID1 E3 ubiquitin ligase activity and if this modification leads to a proteasome-mediated cleavage of Fu producing a 90 kDa N-terminal fragment is presently unclear. Though, a mimic of the putative N-terminal fragment increases GLI3 nuclear localisation. If the cleavage of Fu is reduced, e.g. due to MID1 silencing, nuclear translocation of GLI3 decreases leading to a consequent reduction of the expression of SHH target genes (Schweiger et al., 2014) (Fig. 3).

Indeed, several studies indicate that MID1 is involved in a reciprocal crosstalk with the Shh pathway. Although these data have been studied in cancer cell lines, the crosstalk between MID1 and the Shh pathway can be relevant also in development given the strong implication of the Shh pathway in ventral midline definition as also observed in the phenotypic signs of OS. Findings in *Xenopus* during development showed that ectopic *Shh* induces *mid1* expression in the entire developing optic vesicle, but also in the prospective forebrain

(Pfirrmann et al., 2016) (see also below) (Fig. 3). However, Shh can also suppress *mid1* expression and *mid1* can act upstream of Shh, due to the induction of *bmp4* expression in the chicken Hensen's node (Granata and Quaderi, 2003) (see also below). In this way, a feedback loop may exist, regulating the balance between *mid1* expression with medium levels of Shh inducing *mid1* expression, and lower levels suppressing *mid1* expression (Fig. 3).

3. The role of MID1 in embryonic development

3.1. The MID1 gene and Opitz G/BBB syndrome

OS is a malformative disease characterised by defects in the development of embryonic midline structures and the *MID1* gene is mutated in patients with the X-linked form of the disease, XLOS (Quaderi et al., 1997). Up to now, approximately one hundred mutations have been reported in the *MID1* gene in association with OS (Cox et al., 2000; Fontanella et al., 2008; Li et al., 2016; Quaderi et al., 1997; Winter et al., 2016). They are distributed along the entire length of the gene and are represented by missense and nonsense mutations, splice site mutations, and indels, especially involving the 3' of the gene. In some patients the *MID1* gene is completely or partially deleted (Ferrentino et al., 2007) (Fig. 1). The presence of different kind of mutations involving the entire gene suggests loss-of-function (LOF) as the pathogenetic mechanism underlying this disease (Cox et al., 2000). It is interesting though that no point mutations have been found to date in the RING domain, suggesting possible different consequences of such alterations, if occurring.

XLOS, like several genetic developmental syndromes, is characterised by high variability of the clinical signs. In *MID1*-mutated patients, not only some of the signs might be absent but also if present their severity is variable. Variability is not dependent (or not completely dependent) on the type of mutations present as even affected members of the same family may show different clinical manifestations (Fontanella et al., 2008). The most frequently observed signs are: dysmorphic features, mainly represented by hypertelorism, often associated with other craniofacial features, such as cleft of lip and palate, frontal bossing, large nasal bridge, low-set ears, etc.; laryngo-tracheoesophageal abnormalities, ranging from tracheomalacia to esophageal clefts; and external genitalia abnormalities that, being an X-linked disease affecting mainly males, are predominantly represented by various-degree-hypospadias (Robin et al., 1996). In addition to the above clinical manifestations, XLOS *MID1*-mutated patients can present with cardiac abnormalities, mainly atrial-septal defects; anal defects, principally imperforate or ectopic anus; as well as other less frequent signs (Quaderi et al., 1997). XLOS also shows a neurological component presented as anatomical cerebellar vermis hypoplasia and agenesis or hypoplasia of the corpus callosum as well as cognitive and developmental delays (Fontanella et al., 2008). The prevalence of X-linked Opitz G/BBB syndrome ranges from 1:50,000 to 1:100,000 in males. The females are usually carriers of the mutation on one allele and they present minor manifestations, mainly ocular hypertelorism, likely as effect of X-inactivation mosaicism, although this issue would merit further investigations.

3.2. Expression during embryonic development

Being implicated in a congenital malformation disorder, *MIDI* is highly expressed during embryonic development where it exerts its main function. Embryogenesis is a long and complex process in which a series of remodelling events takes place in a sequential manner. The analysis of the physiological role and expression of *MIDI* in human tissues during development is an extremely difficult and controversial aspect, as such scientific knowledge regarding this issue is fairly restricted. Several model organisms recapitulate the same steps of organogenesis as in humans, thus representing the ideal alternative to human embryos for these studies providing also the experimental models to study the mechanisms. In general, the steps of organogenesis are conserved among different species. Several staging systems have been developed in order to describe the sequence of events that occurs during embryonic development: Hamburger-Hamilton (HH) for chick, Nieuwkoop-Faber (NF) for *Xenopus*, embryonic days (E) for mouse/rat. The Carnegie System (CS) provides a unified and standardised chronology of embryonic stages of the vertebrate embryos, based on the progressive morphological development. In line with that, the physiological expression profile and role of the orthologue genes of *MIDI* during embryogenesis have been explored at different time points during development in multiple animal models: mouse (*Mid1*) (Dal Zotto et al., 1998; Lancioni et al., 2010; Lu et al., 2013), chicken (*cMid1*) (Granata and Quaderi, 2003; Latta and Golding, 2012; Richman et al., 2002) and *Xenopus* (*xMid1*) (Pfirrmann et al., 2016; Suzuki et al., 2010), thus covering approximately all embryonic developmental phases (Fig. 4).

The earliest analysis of *MIDI* expression has been carried out in chicken embryos starting from gastrulation (Granata and Quaderi, 2003). At Carnegie stage (CS) 3 a weak expression of *cMid1* in the anterior portion of the horseshoe-shaped domain is detected, which becomes stronger at CS4. Starting from that stage, *cMid1* expression, which is first symmetrically detected in the ectoderm of Hensen's node, is gradually restricted to the mesendoderm of the right side of the node. During neurulation, by CS8 *cMid1* displays a weak bilateral symmetric pattern in the node and a strong signal is observed in the developing neural folds (Granata and Quaderi, 2003). The neural plate exhibits *cMid1* expression especially in the cranial region at CS9 (Latta and Golding, 2012; Richman et al., 2002). Specific signal is detected in the neural epithelium of the forebrain, as well as in the head mesenchyme adjacent to midbrain and hindbrain, in the forming rhombomeres and in the presomitic mesoderm (Richman et al., 2002). In mouse and in *Xenopus*, at CS9 *Mid1* is uniformly expressed at high level virtually in all embryonic tissues, except for the developing murine heart (Dal Zotto et al., 1998; Quaderi et al., 1997; Suzuki et al., 2010). Starting from stage CS10, in the late phase of neurulation, *cMid1* mRNA shows a strong appearance in rhombomeres 2/3 in chicken embryos (Latta and Golding, 2012; Richman et al., 2002). At this stage *xMid1* is expressed along the midline in the *Xenopus* neural tube, in the otic and optic vesicles and in the anlage of the cement gland (Suzuki et al., 2010). Within the prospective Central Nervous System (CNS), nearly the entire ventral neuroepithelium exhibits sensible expression of *cMid1* transcript in chicken embryos at CS12, with notably strong levels within the diencephalic vesicle (Richman et al., 2002). Moreover, relatively high transcription of *cMid1* at the base of the allantois is also observed at this stage (Richman et al., 2002).

At CS14 the strongest expression of *Mid1* in murine developing CNS is described in the proliferating compartment of dorsal midbrain and hindbrain (Lancioni et al., 2010). Similar expression profile is found in human embryos at CS14, stage in which *MIDI* is transcribed particularly in the three primary brain vesicles of the developing CNS (forebrain, midbrain and hindbrain) (Pinson et al., 2004), but also in chicken embryos that display at this stage strong *cMid1* expression in the craniofacial region, comprising frontal process of maxilla, the growing mandible and nasal mesenchyme (Richman et al., 2002). Furthermore, other developing structures present *MIDI* expression in different species between CS14 and CS15: the otic vesicle, the forming organs of the digestive system, the branchial arches, the mesonephros, the dorsal root ganglia, sclerotomes, anlagen of limbs, the midgut (which will give rise to the tracheal region), the urogenital tract, the developing eyes and heart (Pinson et al., 2004; Richman et al., 2002). Up to CS17, murine and chicken embryos show a similar craniofacial expression as in the previous stages of development, with increasing level of *Mid1* mRNA in the rostral part of the rising CNS, with particular regard to the proliferating telencephalic epithelium (Dal Zotto et al., 1998; Li et al., 2016; Richman et al., 2002).

As embryogenesis proceeds, human *MIDI* expression in the diencephalon is progressively reduced, while at CS18 a strong signal is detected in the telencephalic vesicles (Pinson et al., 2004). In addition, epithelia and mesenchyme of the respiratory and digestive developing organs (i.e the trachea and oesophagus), as well as the vertebra and the forming heart, even at lower extent, show appreciable expression of the transcript both in human and in chicken embryos (Pinson et al., 2004; Richman et al., 2002). Pattern of expression of human *MIDI* at CS19 is similar as at CS18, with even greater increase of signal in telencephalon and in metencephalon (especially in the cerebellar primordium), while it decreases in the mesencephalon (Pinson et al., 2004). High level of transcript is also observed in the epithelia of nasal and oral cavities, in the forming oesophagus and larynx, in the medial part of the tongue, in the spinal cord, in the dorsal root ganglia, in the neurosensory retina, in the metanephros and in the anal fold (Pinson et al., 2004). As in human, from the tailbud of *Xenopus* and E14.5 embryonic mouse (both corresponding to CS20) to the final embryonic stage (CS23), high expression level of *Mid1* depicts the hindbrain and the midbrain, the developing kidney, the forming organs of the respiratory and the digestive system (e.g. lungs and stomach), the whole eye vesicle (mainly proliferating neurons of the neural retina and undifferentiated cells of the lens express *Mid1*) and the urethral epithelium (Dal Zotto et al., 1998; Pfirrmann et al., 2016; Suzuki et al., 2010); while only the interventricular septum shows transcription of *MIDI* gene within the defining human heart (Pinson et al., 2004).

The developmental expression of *MIDI* among different models is very similar, although not completely overlapping. For instance, while the chicken presents expression of the *cMid1* mRNA in the whole developing heart (Richman et al., 2002), no heart expression is observed in murine embryos (Dal Zotto et al., 1998) and restriction of the transcript in the interventricular septum is detected in the forming human heart (Pinson et al., 2004). These observations could be due to biological variability in the species-specific expression of the gene, in accordance also with the fact that the *MIDI* gene maps within the X chromosome in a region characterised by high genomic instability, which is diversely regulated in distinct species (i.e. murine *Mid1* gene spans the pseudoautosomal boundary, escaping X-inactivation; while in human *MIDI* is fully X-specific and subjected to X-inactivation), thus

explaining divergence in gene expression (Dal Zotto et al., 1998). However, it noticeably emerges that the expression of *MIDI* transcript during embryogenesis correlates with structures and organs whose development is affected in OS.

3.3. Role in embryonic development

Even though the expression of *MIDI* transcript has been clearly detected in the structures and organs that are affected in OS patients, the first evidence of a direct involvement of *MIDI* gene in development has been provided through analysis in chicken embryos at early developing stages, assessing the implication of this E3 ubiquitin ligase in establishing the physiological/molecular asymmetry at the Hensen's node (Granata and Quaderi, 2003). In fact, both ectopic overexpression of *cMid1* in the physiologically *Mid1*-devoid left side and morpholino-induced knock-down of its expression on the right side of the node alter the proper asymmetrical expression of genes that are implicated in the definition of laterality early in chicken development (Granata and Quaderi, 2003). Importantly, *cMid1* is able to modulate the levels of Shh in the perinodal region, thus assuming a pivotal role in controlling midline-lateral developmental progression with implication in the pathogenesis of OS (Granata and Quaderi, 2003) (Fig. 3C).

Furthermore, one of the features of the OS patients is the occurrence of neurological signs. Several studies conducted in mouse, *Xenopus* and chicken embryos highlight *Mid1* physiological contribution to different neurodevelopmental events, starting from early migration of the Cranial Neural Crest (CNC) cells (Latta and Golding, 2012), through neural tube closure (Suzuki et al., 2010), to visual system and cerebellar development (Dierssen et al., 2012; Lancioni et al., 2010; Nakamura et al., 2017; Pfirrmann et al., 2016). Indeed, some of these processes are known to be defective in OS patients, in particular the morphogenesis of the cerebellum. According to a mouse model of the disease, depletion of *Mid1* in the embryos causes anatomical brain abnormalities in the dorsal midbrain and cerebellar regions, i.e. hypoplasia of the anterior cerebellar vermis, the medial cerebellar region, caused by rostralisation of the midbrain/cerebellum boundary and downregulation of *Fgf17*, likely due to molecular defects occurring during midgestation (Fig. 5) (Lancioni et al., 2010). This defect has been shown to be associated with motor learning impairment (Lancioni et al., 2010). Similarly, another transgenic mouse line showing reduced level of *Mid1* in cerebellum and hippocampus as consequence of the expression of a dominant active Downstream Regulatory Element Antagonistic Modulator (DREAM) mutant has been reported to exhibit a critical shortening of the rostro-caudal axis of the cerebellum, along with a severe delay in neuromotor development (Dierssen et al., 2012). Likewise, transgenic mice depleted for *Rac1* and *Rac3*, that belong to the Rho family of small GTPases and are known to play important roles during embryogenesis show downregulation of *Mid1* (Nakamura et al., 2017). Interestingly, axonogenesis, dendritogenesis, tangential and radial migrations of immature neurons are impaired in these transgenic mice, resulting in hypoplasia of the medial internal granule layer of the cerebellum. All these findings indicate a role of *MIDI* E3 ubiquitin ligase in contributing to cerebellar morphogenesis and function. In addition, the analysis of an independent *Mid1* knock-out mouse line has revealed its essential role in axon development and precise neuronal projection pattern establishment in corpus callosum during embryogenesis (Lu et al., 2013). Indeed, *Mid1* silencing in cultured

neurons, isolated from cerebral cortex and hippocampus, accelerates axon growth and branch formation (Lu et al., 2013). This study suggests Mid1 as a negative regulator of axon growth that ensures axons precise patterning, which is crucial for proper circuit development.

Evidence of Mid1 involvement in additional embryonic processes, albeit not directly associated with the occurrence of OS defects yet, has been provided. Following gastrulation, neurulation occurs; the neural plate bends and elevates, converging towards the dorsal midline and giving rise to the neural tube. To achieve a correct closure, this first rudiment of the CNS requires dynamic morphological changes of the neuroepithelial cells (De Pascalis and Etienne-Manneville, 2017; Leonard and Taneyhill, 2019). In *Xenopus*, the simultaneous depletion of *xMid1* and of its close paralogue, *xMid2*, disrupts the proper microtubule organisation of midline neuroepithelial cells causing defective neural tube closure (Fig. 5) (Suzuki et al., 2010). Concomitant with the neural tube closure, a primary event is the migration of the CNC cells, destined among other fates to populate the branchial arches and the frontonasal process (Abramyan and Richman, 2018). Disruption of cMid1 function in chicken embryos results in reduction of CNC speed and subsequent deficit in cranial ganglia formation, especially the trigeminal one, through PP2Ac regulation (Fig. 5) (Latta and Golding, 2012).

In addition, silencing of *xMid1* in *Xenopus* results in the enlargement of the eyes at tadpole stage, with aberrant retinal folds and loss of sharp boundaries of retinal layers (Pfirrmann et al., 2016). This is due to the lack of proteasomal degradation and consequential sustained level of Pax6, a transcription factor necessary for the specification of the eye prospective retinal field that later must be removed for the proper formation of the eyestalk and the differentiation of retinal precursor cells (Ashery-Padan et al., 2000; Pfirrmann et al., 2016; Plaza et al., 1995; Shaham et al., 2012).

In model organisms, the misregulation of Mid1 results in abnormalities comparable to those observed in OS patients. However, the phenotype seen in humans shows a wider range of defects that are often absent in other organisms. Nevertheless, when Mid1 functionality is compromised, distinct models commonly share the impairment in midline structures and laterality determination, corroborating the involvement of Mid1 in such developing processes.

4. The role of MID1 in adulthood

4.1. MID1 promotes allergic asthma

Following the first interest on the role of the *MID1* gene in development driven by its involvement in XLOS, several subsequent findings indicate possible roles of the *MID1* gene product also in adult life. In particular, mice sensitised and challenged with house dust mite (HDM) or rhinovirus infection, showing pathological signs of allergic asthma with airway hyper-reactivity and mucus production, upregulate *Mid1* in bronchial cells at both mRNA and protein levels (Collison et al., 2013). The observed upregulation is Toll-Like Receptor 4 (TLR4)-dependent and can be recapitulated by administering recombinant Tumour necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) (Collison et al., 2013). This

upregulation is associated with decreased PP2A activity and PP2Ac protein level and, conversely, silencing of *Mid1* attenuates HDM-induced asthma signs. Therefore, Mid1 promotes allergic airway disease by limiting PP2A-mediated deactivation of NF- κ B and p38 Mitogen-Activated Protein kinase (p38MAPK) among others (Collison et al., 2013; Foster et al., 2017). These results were confirmed by the observation that MID1 activates proinflammatory signalling in bronchial epithelial cells from human subjects. A similar mechanism is likely involved in a model of eosinophilic esophagitis and pulmonary fibrosis (Collison et al., 2019, 2015).

By considering the immune response, other evidence suggests that MID1 can control lytic granule exocytosis and polarisation as well as migration in mouse T cells. It is well known that microtubule cytoskeleton plays an important role in the secretion of lytic granules and thereby in cytotoxic lymphocytes (CTLs)-mediated target cell killing (Huse et al., 2008). Mid1 is strongly upregulated in murine CTLs, controlling T-cell receptor signalling, centrosome trafficking and exocytosis of lytic granules (Boding et al., 2014a). Another aspect described in murine T cells concerns Mid1 involvement in cell migration. Indeed, Mid1 localises to the uropod of migrating CTLs, modulating polarisation and migration (Boding et al., 2014b), probably by affecting microtubule dynamics known to be crucial in the *in vivo* migration of T-cells (Dong et al., 2013). Whether this function is present also in other cell types that use different migration mechanisms is an issue that deserves further studies.

4.2. Neurodegeneration and cancer

MID1 plays a role also in two highly relevant pathological areas, considering their social and clinical burden: neurodegeneration and cancer. We will briefly mention these issues as thoroughly reviewed elsewhere (Griesche et al., 2016; Winter et al., 2016).

Concerning the first area, neurodegeneration, among the transcripts that share the MIDAS motif is the family of CAG repeats-containing mRNAs. The trinucleotide CAG, which encodes for glutamine, represents an unstable region that could vary in length, expand the number of the repetition of the codon within the gene and thus cause the synthesis of a toxic polyglutamine protein. Structurally, the RNAs with expanded CAG triplet can form hairpin structures, the stability of which increases with repeat-numbers (Gatchel and Zoghbi, 2005; Mirkin, 2007). Some of the CAG repeat mRNAs have been tested for their ability to associate with the MID1-complex. In particular, Huntingtin (*HTT*), Ataxin2 (*ATXN2*), Ataxin3 (*ATXN3*) and Ataxin7 (*ATXN7*) transcripts are all part of the MID1-containing mRNPs. Interestingly, both the strength of the interaction and the increase of protein translation are dependent on the CAG repeat size (Griesche et al., 2016; Krauss et al., 2013). Reducing MID1 protein level or masking RNA-MID1 association (i.e. by using the small molecule furamide able to inhibit the recruitment of MID1 to mutant *HTTRNA*) are sufficient to abrogate the interaction of those mRNAs with the MID1-containing complex and significantly reduce the protein levels, strongly corroborating the idea that MID1, within the mRNP complex, plays a crucial role in mediating the binding and the efficiency of the translation machinery with possible implications in neurodegeneration (Krauss et al., 2013; Matthes et al., 2018b). Interestingly, the mRNA of both APP, the amyloid precursor protein,

and BACE1, the secretase responsible for the formation of the neurotoxic amyloid- β ($A\beta$) peptide, have been detected in association with MID1-containing complex (Matthes et al., 2018a). This interaction induces *BACE1* and *APP* translation while, conversely, by interfering with the assembly of the mRNP complex both protein levels are reduced (Hettich et al., 2014; Matthes et al., 2018a). Interestingly, MID1 is overexpressed in post-mortem tissues from Alzheimer disease patients presenting with amyloid- β plaques and hyperphosphorylated Tau (Schweiger et al., 2017).

As true for several genes involved in developmental processes, some of the MID1-related networks are tightly controlled in somatic adult cells and when dysregulated can promote tumorigenesis. In the case of MID1, activation of the Shh pathway, as observed in some cell types, can be relevant in some cancer types, e.g. medulloblastoma (Kumar et al., 2019; Tamayo-Orrego and Charron, 2019). Another field where the role of MID1 might be relevant is prostate cancer. Indeed, MID1 can bind Androgen Receptor (*AR*) mRNA to induce its translation; *AR* transcript harbours a purine-rich region characterised by two trinucleotide repeats: the polyCAG and the polyGGY repeats, thereby depicting an additional type of mRNAs capable of interacting with MID1 complex (Demir et al., 2014; Kohler et al., 2014). Reciprocally, *AR* can control MID1 transcription. This crosstalk between MID1 and *AR* is important to regulate prostate cancer cells proliferation and consistently MID1 is over-expressed in prostate cancer cell lines showing aggressive phenotype (Kohler et al., 2014). Despite several reports on the role of MID1 in cancer, the precise role and types of tumours showing MID1 involvement still need to be further studied to evaluate the possibility of considering MID1 as a therapeutic target.

5. Conclusions and future perspectives

MID1 is found within the cells in a ternary complex together with $\alpha 4$ and PP2Ac. This seems to be the central core of MID1 cellular function with implication in the regulation of the mTORC1 signalling pathway and of the Shh network. However, the MID1- $\alpha 4$ -PP2Ac complex stoichiometry and dynamics still need to be further investigated. As explained above, only a restricted pool of PP2Ac is regulated by MID1 and, in turn, also the involved signalling pathways can be regulated in confined space and time within the cell. Moreover, it is still unclear if the translational control exerted by MID1 on selected mRNAs is dependent on $\alpha 4$ and PP2Ac and in such case, through which mechanism. The most important issue to unravel, however, is how MID1 alterations are pathogenetic during embryonic development leading to XLOS. Of the networks above, the role of the Shh pathway in the establishment of the midline during development has been long known and in fact mutations in the *SHH* gene cause holoprosencephaly, a complex human brain malformation associated with facial anomalies due to abnormal definition of the ventral midline (Dubourg et al., 2007). Additionally, mutations found in the *GLI3* gene, another component of the Shh pathway, cause a range of phenotypes including Greig and Pallister-Hall syndromes, characterised by hypertelorism and polydactyly (Johnston et al., 2010; Vortkamp et al., 1991). Therefore, the existence of a crosstalk between Shh and MID1 is not surprising. MID1 loss-of-function mutations cause a phenotype that is somewhat opposite to holoprosencephaly suggesting that the reciprocal regulation between these two factors might be impaired in OS patients. Further, what is still unclear is whether the crosstalk between Shh and MID1 involves also

mTORC1 signalling, since several works confirm the interrelationship between Shh pathway and mTORC1 signalling (Nanta et al., 2019). One can speculate the existence of a triple interplay involving MID1- α 4-PP2Ac complex, mTORC1 and Shh pathway, in which mutations and/or alterations may interfere with the correct midline establishment during embryonic development.

In addition a feature not addressed in this review is MID1 ability to homo- and hetero-dimerise. Indeed, through its coiled-coil domain, MID1 is able to dimerise like all the members of the TRIM family (Cainarca et al., 1999; Short et al., 2002). As mentioned at the beginning, the structure of the entire MID1 protein is not solved, therefore at present, we cannot fully understand the conformation of MID1 homodimer and we do not appreciate the extent of homodimerisation necessity for microtubule association and binding to substrates (Short and Cox, 2006; Short et al., 2002). Within this topic, another issue that deserves further investigation is the functional relationship between MID1 and its close and interacting paralogue, MID2/TRIM1 (Buchner et al., 1999). Both bind α 4 but whether they both participate in PP2A control and in the mTORC1 and Shh signalling is still unknown (Short et al., 2002). Functional redundancy of the two proteins has been shown in early chicken development (Granata et al., 2005) but other studies indicate that they can have overlapping but not coincident functions. For example, MID1 and MID2 interact with Astrin that is ubiquitinated only by MID2 (Gholkar et al., 2016; Zanchetta and Meroni, 2019); on the same line, despite several analyses, MID2 mutations are not associated to OS or similar pathologies but to an X-linked mental retardation syndrome characterised by a milder phenotype (Geetha et al., 2014). It would be interesting to investigate possible modulatory (and reciprocal?) functions of the two paralogues in the variable expressivity of these pathogenetic conditions in presence of different combination of mutated alleles.

In conclusion, there are still several open questions on the physiological functions of MID1 and further experimental research is required to get insights into MID1-related pathogenesis of developmental and adulthood clinical conditions.

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The corresponding Gene Wiki entry for this review can be found here: <https://en.wikipedia.org/wiki/MID1>.

Abbreviations:

ANXA2	Annexin A2
APP	amyloid precursor protein
AR	Androgen Receptor

BACE1	β -secretase 1
BRAF35	BRCA2-Associated Factor 35
CNC	Cranial Neural Crest cells
CNS	Central Nervous System
COS	C-terminal subgroup One Signature
CS	Carnegie System
CTLs	cytotoxic lymphocytes
DREAM	Downstream Regulatory Element Antagonist Regulator
E	Embryonic days
EF-1α	Elongation Factor 1 α
FGF21	Fibroblast Growth Factor 21
FN3	Fibronectin type III repeat
HECT	E6-AP COOH Terminus
HH	Hamburger- Hamilton
LOF	Loss-of-Function
MAP	Microtubule Associated Protein
MIDAS	MID1 Association Sequence
MIG12	MID1 interacting G-12 like protein
mTORC1	mammalian Target Of Rapamycin Complex 1
NF-κB	Nuclear Factor κ -light-chain enhancer of activated B cells
NF	Nieuwkoop- Faber
NMR	Nuclear Magnetic Resonance
NPM	Nucleophosmin
OS	Opitz Syndrome
p38MAPK	p38 Mitogen-Activated Protein kinase
PAR	Pseudoautosomal region
PPDK-1	3-Phosphoinositide Dependent Protein Kinase
PP2A	Protein Phosphatase 2A
PTCH	Patched

RACK1	Receptor of Activated protein kinase C1
RING	Really Interesting New Gene
RNP	ribonucleoprotein
SHH	Sonic Hedgehog
SMO	Smoothened
SPRY	SPLa and the RYanodine receptor
SUFU	Suppressor of Fused
TFEB	Transcriptional Factor EB
TLR4	Toll-Like Receptor 4
TRAIL	Tumour necrosis factor–Related Apoptosis-Inducing Ligand
TRIM	TRipartite Motif
UIM	ubiquitin interacting motif
XL0S	X-linked Opitz G/BBB Syndrome

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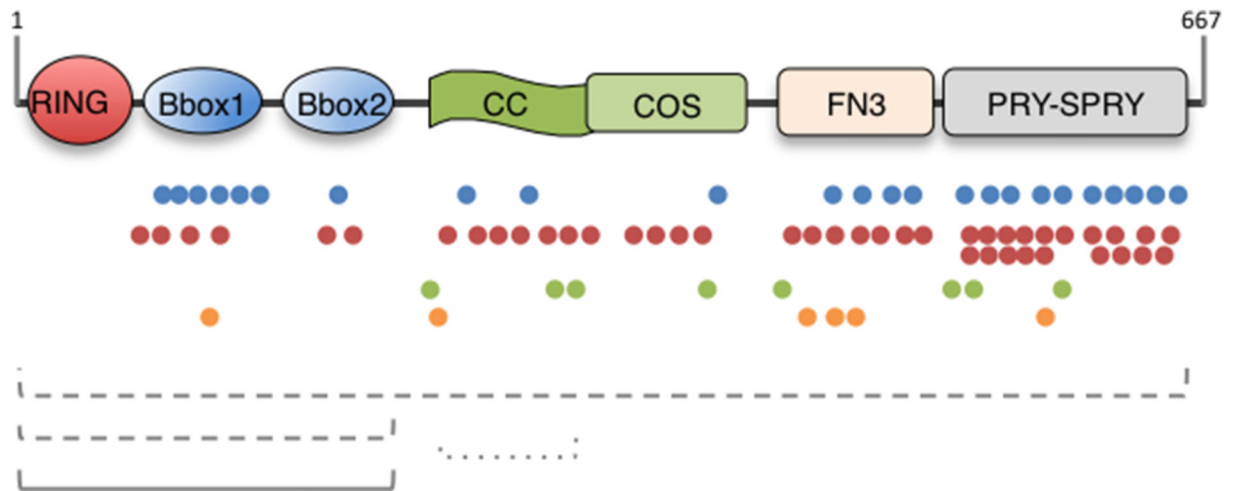


Fig. 1. MID1 protein domain structure and OS-associated mutations.

The domain composition of the MID1/TRIM18 protein is depicted. The MID1 protein is 667 residue-long and the limits of the single domains are following in brackets: RING (10–59), Really Interesting New Gene domain; B-Box (B1, 114–164; B2, 170–212), B-Box domain; CC (219–319), Coiled-coil; COS (320–380), C-terminal subgroup one signature; FN3 (382–472), Fibronectin type III repeat; PRY (483–528), domain associated with SPRY domains; SPRY (538–657), SPIa and the RYanodine Receptor. Below the scheme, colour dots represent the different mutations reported so far in OS patients: blue dots, missense mutations; red dots, nonsense and truncating mutations; green dots, splice site mutations; orange dots, inframe indels. The dashed lines represent deletions and rearrangements; the continuous line represents duplications. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

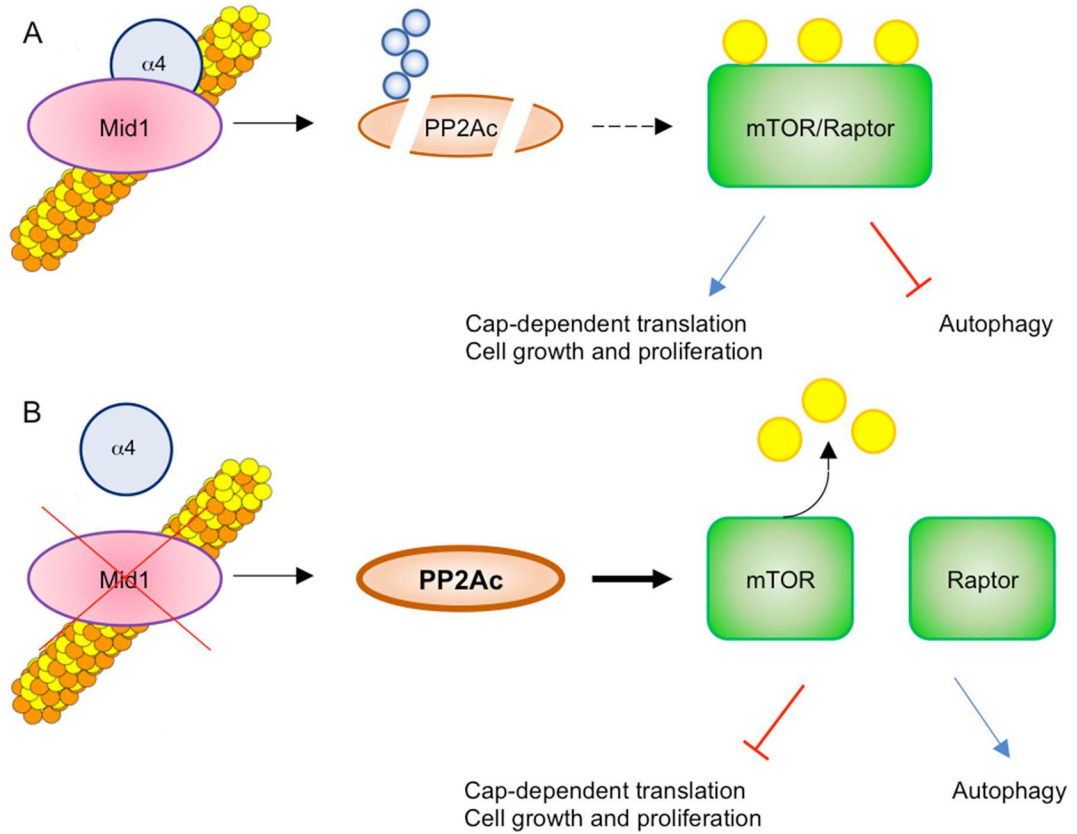


Fig. 2. MID1 regulates mTORC1 signalling through PP2A.

A) The microtubular pool of PP2Ac is the target of MID1 activity, leading to its proteasomal-dependent degradation upon poly-ubiquitination (Blue circles). Since mTOR/Raptor association is dependent on PP2A, the degradation of the latter leads to an increased formation of the mTOR/Raptor complex, activating its signalling pathway (phosphorylated form, yellow circles). B) MID1 loss-of-function reduces PP2Ac degradation, leading to de-phosphorylation and decreased association of mTOR and Raptor, causing a drop of active mTORC1 complex formation and signalling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

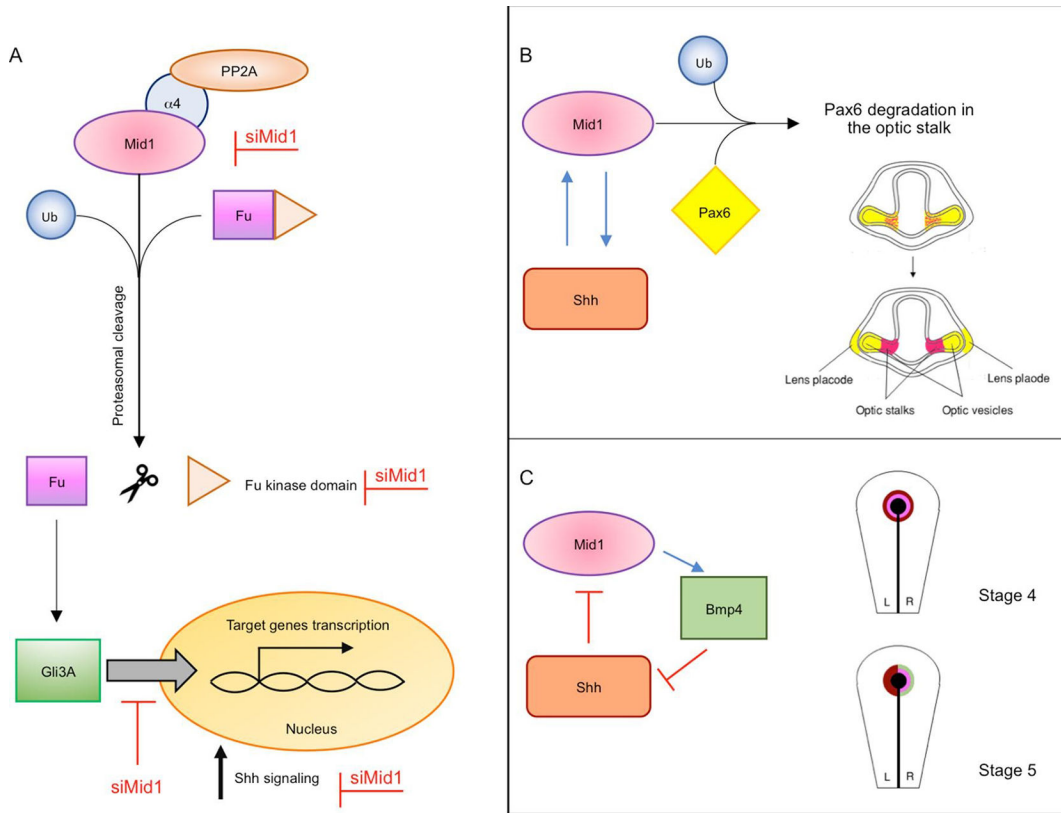


Fig. 3. Model of Shh pathway regulated by MID1.

A) MID1 is involved in the proteasome-dependent cleavage of the Fu kinase domain, leading to GLI3A (activator) translocation into the nucleus. This process activates Shh target genes expression increasing Shh signalling. Silencing of MID1 (red writings) impairs Fu cleavage thus reducing Shh signalling. B) In *Xenopus*, an overlapping expression of *mid1* (in pink) and *pax6* (in yellow) is observed in the optic stalk, in early stages during the development of the visual system. *mid1* is expressed within the forming optic stalk under the control of Shh. Here, *mid1* regulates the ubiquitination and proteasomal degradation of Pax6 protein that is cleared from the optic stalk region, setting the border between the optic stalk and the retina via Mid1. C) In chicken development, both *Shh* (dark red) and *cMid1* (pink) are initially expressed bilaterally in the Hensen's node (black) until stage 5. *cMid1* then induces the expression of *Bmp4* (green) on the right side of the node. *Bmp4* represses right-sided *Shh* expression, thus restricting Shh to the left side of the node. Left-sided Shh represses *cMid1* expression on the left, restricting it to the right side of the node, together with *Bmp4*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

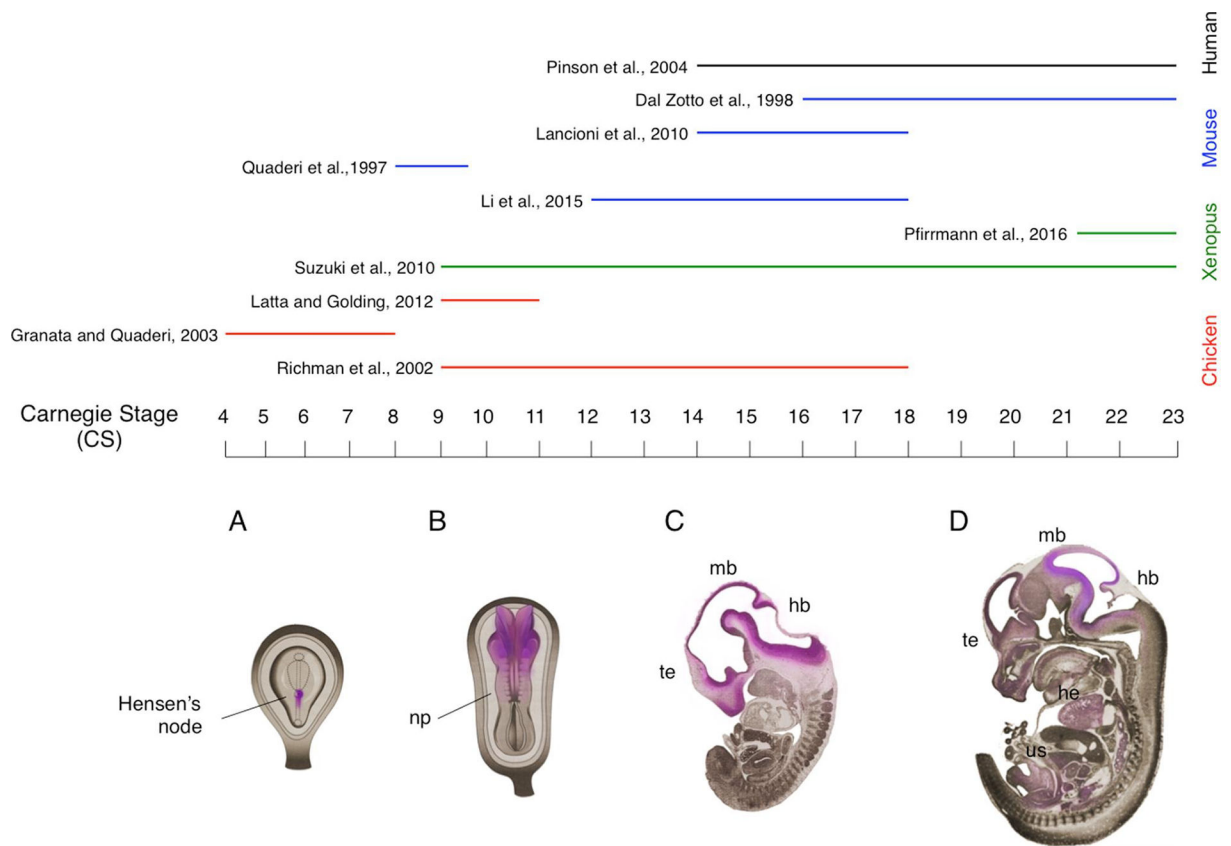


Fig. 4. Distribution of *MID1* during embryonic development.

In the upper part, expression of *MID1* in specific developmental stages is summarised. The coloured lines indicate the models used in the studies as indicated on the right-hand side. In the bottom part a schematic representation of *Mid1* distribution at different stages of embryonic development is shown (pink shading); Carnegie stages (CS) are indicated. A) *Mid1* distribution is restricted to the right side of the Hensen's node; B) the cranial region of the neural plate (np) displays the strongest expression of *Mid1* at CS9; C) *Mid1* is mainly transcribed in the proliferating compartments of telencephalic vesicle (te), dorsal midbrain (mb) and hindbrain (hb); D) at late embryonic stages, high levels of *Mid1* transcript are particularly described in the developing hindbrain and midbrain. *Mid1* mRNA is also present in the heart (he) and in several organs of the urogenital system (us). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

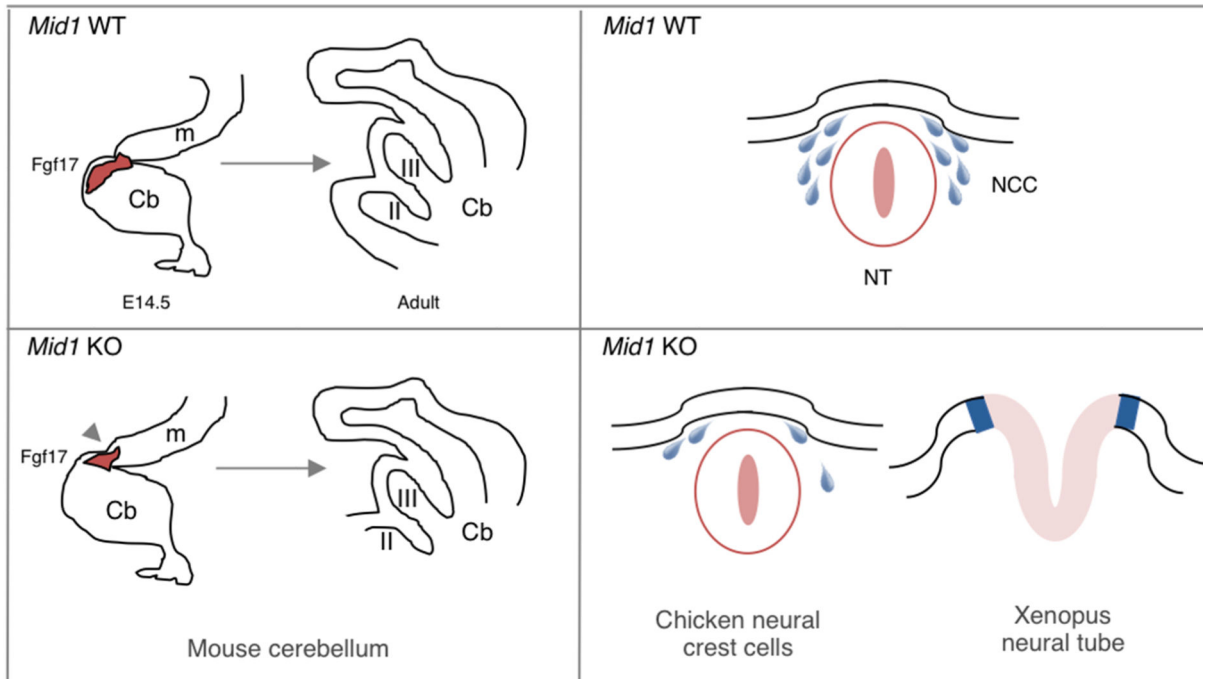


Fig. 5. Mutations in *Mid1* determine neuroanatomical defects during embryonic development. Left-hand side, mouse *Mid1* knock-out causes a defective morphogenesis of the cerebellum (cb) and anterior midbrain (m) during development, including the rostralisation of the midbrain/hindbrain boundary (arrowhead) during midgestation (embryonic day 14.5, E14.5) and the consequent malformation of adult cerebellar most anterior lobes (lobes II and III). Right-hand side, *Mid1* is implicated in proper neurulation event during embryonic development: defects in cranial neural crest cells (NCC) migration observed in chicken and in neural tube (NT) closure in *Xenopus* are associated with silencing of *Mid1*.

Table 1

Characteristics of the main isoform of the *MIDI* gene, transcript and protein in *Homo sapiens*, *Mus musculus* and *Gallus gallus*.

	Homo sapiens ^(a)	Mus musculus ^(b)	Gallus gallus ^(c)
Gene symbol	MIDI	Mid1	MIDI
NCBI Gene ID	4281	17,318	373,920
HGN/CGI/CGNC	7095	1,100,537	48,957
OMIM	300,552		
Chromosomal location	Xp22.2	X F5 (79.19 cM)	1
Genome coordinates	chrX:10445309–10833682	ChrX:169685199–169990798	Chr1: 125930064–126095986
GeneBank (gene)	NC_000023.11	NC_000086.7	NC_006088.5
Ensembl transcript ID	ENSG00000101871	ENSMUSG000000035299	ENSGALG00000038504.2
RefSeq (mRNA)	NM_000381.4	NM_010797.3	NM_204129.1
RefSeq (protein)	NP_000372.1	NP_034927.2	NP_989460.1
Gene length (kbp)	665.9	305.6	185.92
mRNA length (bp)	6463	3974	2420
Number of exons	10	10	10
Number of coding exons	9	9	9
CCDS code	CCDS14138.1	CCDS41215.1	
UniProt name	E3 ubiquitin-protein ligase Midline-1	E3 ubiquitin-protein ligase Midline-1	Midline 1
UniProtKB/Swiss-Prot ID	O15344-1	O70583-1	Q71R46-1
Length of protein in AA	667	680	667
Mass (kDa)	75.2	76.1	75.4

Data sources for *Homo sapiens*: <https://www.ncbi.nlm.nih.gov/gene/4281>; <https://www.genecards.org/cgi-bin/carddisp.pl?gene=MIDI>; https://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000101871;r=X:10445310-10833654; <https://omim.org/entry/300552?search=MIDI&highlight=mid1>; <https://www.ncbi.nlm.nih.gov/CCDS/CcidsBrowse.cgi?REQUEST=CCDS&DATA=CCDS14138>; <https://www.uniprot.org/uniprot/O15344>.

Data sources for *Mus musculus*: <https://www.ncbi.nlm.nih.gov/gene/17318>; https://www.ensembl.org/Mus_musculus/Gene/Summary?g=ENSMUSG000000035299;r=X:169685199-170005736; <https://www.ncbi.nlm.nih.gov/CCDS/CcidsBrowse.cgi?REQUEST=CCDS&DATA=CCDS41215>; <https://www.uniprot.org/uniprot/O70583>.

Data sources for *Gallus gallus*: <https://www.ncbi.nlm.nih.gov/gene/373920>; <http://birdgenenames.org/egnc/GeneReport?id=48957>; <https://www.uniprot.org/uniprot/Q71R46#sequences>

(a) Genomic coordinates based on GRCh38.p13

(b) Genomic coordinates based on GRCm38.p6

(c) Genomic coordinates based on GRCg6a