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The MID1 protein is a central player during development and in disease

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TABLE OF CONTENTS

1. ABSTRACT

Loss-of-function mutations in the *MID1* gene cause a rare monogenic disorder, Opitz BBB/G syndrome (OS), which is characterized by malformations of the ventral midline. The *MID1* gene encodes the MID1 protein, which assembles a large microtubule-associated protein complex. Intensive research over the past several years has shed light on the function of the MID1 protein as a ubiquitin ligase and regulator of mTOR signalling and translational activator. As a central player in the cell MID1 has been implicated in the pathogenesis of various other disorders in addition to OS including cancer and neurodegenerative diseases. Influencing the activity of the MID1 protein complex is a promising new strategy for the treatment of these diseases. In this review we will summarize the current knowledge about MID1, its involvement in the pathogenesis of OS and other diseases and possible strategies for therapy development.

2. INTRODUCTION

In 1997 the *MID1* gene attracted attention of human geneticists, because it was found that mutations cause a rare monogenic disorder, Opitz BBB/G syndrome (OS) (1). Patients with OS suffer from malformations of the ventral midline including, but not limited to, hypertelorism, hypospadias, heart defects, structural brain abnormalities and cleft lip and palate. In addition, about 30 percent of patients present with intellectual disability and/or developmental delay. Because several of the malformations seen in OS are indicative of abnormalities in neural crest cell development OS is considered to be a neurocristopathy. However, the specific role of MID1 in human neural crest cells (NCCs) is still awaiting discovery. Concerning the function of MID1 numerous studies from our lab and others have discovered that MID1 is a central player in the cell and is critically involved in several important cellular processes. As a ubiquitin ligase the microtubule-associated MID1 protein activates the degradation of

the mammalian target of Rapamycin (mTOR) antagonist PP2A and thereby acts as a mTOR activator (2, 3). Furthermore, it assembles a large protein complex that binds to target mRNAs and regulates their translation. mTOR is one of the most important signalling pathways in the cell regulating cell growth and proliferation as well as transcription and local translation. Several studies have highlighted the role of MID1 in the development and progression of cancer. In cancer cell lines the MID1-PP2A complex together with its interaction partner mTOR controls the subcellular localization and transcriptional activity of the sonic hedgehog effector GLI3 (4). In addition, MID1 induces the translation of the mTOR activator PDPK1 (5). Inhibition of PDPK1 stops tumour growth in several tumours (6, 7). In prostate cancer cells MID1 binds to the androgen receptor (AR) mRNA and induces its translation. Also, MID1 is over expressed in a subtype of prostate cancer cells with a very aggressive phenotype (8) suggesting that MID1 is a tumour and metastasis-promoting factor.

In addition to tumourigenesis, MID1 has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's (AD) and Huntington's disease (HD). Concerning AD, MID1 binds to and regulates the translation of the BACE1 mRNA (9) implicated in the generation of amyloid plaques and enhances the phosphorylation of Tau (10), a pathological component of neurofibrillary tangles. In HD, MID1 specifically binds to and enhances translation of the mutant Huntingtin mRNA carrying an expanded CAG repeat (11).

Targeting MID1 is a promising new concept for treatment of diseases like AD, HD, or cancer. Indeed, the first substances that disassemble the MID1 protein complex have been identified and results from treating cells with these substances are encouraging (9, 10, 12). This review will discuss the current knowledge about the *MID1* gene and protein, about its involvement in OS, other tumourigenic and neurodegenerative diseases and about possible therapeutic interventions.

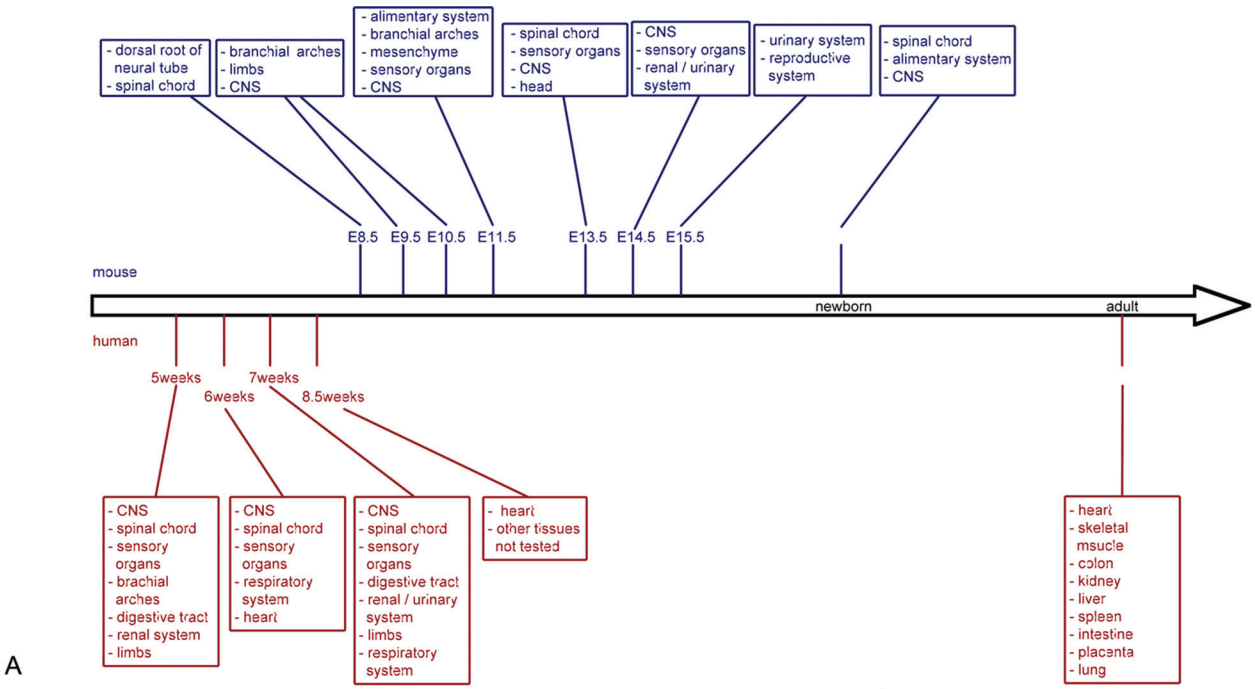
3. THE *MID1* GENE

The *MID1* gene is located on human chromosome Xp22.2. and spans approximately 400 kb of genomic sequence. In 1997, *MID1* was identified and found to be disrupted in patients with OS (1). Because of its causative role in OS *MID1* has rapidly moved into the field of scientific interest. The *MID1* gene consists of nine constitutive coding exons and several alternatively spliced exons (13). Most of the alternative exons have a regulatory role towards *MID1* expression as they introduce premature stop codons into the *MID1* message that mediate mRNA degradation by the nonsense-mediated mRNA decay (NMD) pathway. At the transcriptional level, *MID1* expression is regulated by at least five promoter regions with unique transcription initiation sites. These promoter regions are dispersed over 250 kb of genomic sequence and generate *MID1* transcripts with alternative 5'untranslated regions (5'UTRs). The alternative *MID1* promoters act in concert with alternative polyadenylation signals to drive tissue-specific expression of *MID1* (Figure 1) (14).

4. THE MID1 PROTEIN

The *MID1* gene encodes the MID1 protein, a member of the RING (Really Interesting New Gene) finger family (1). MID1 comprises 6 conserved domains (Figure 2), four of which belong to the RBCC motif common to RING finger proteins: The RING finger, followed by two B-Boxes and a coiled-coil domain. The N-terminal RING finger is characterized by the Cys-His motif Cys-X_2-Cys-X_{11}-Cys-X-His-X_2-Cys-X_2-Cys-X_{22}-Cys-X_2-Cys, with X representing any amino acid. The two B-Boxes are also characterized by a Cys-His motif: Cys-X_2-His-X_7-Cys-X_7-Cys-X_2-Cys-X_5-His-X_2-His. Both RING finger domains and B-Boxes play a role in protein-protein interactions. Following the B-Boxes MID1 contains a coiled-coil domain. This domain is responsible for homodimerization of MID1 as well as heterodimerization of MID1 with its sister protein MID2 (15). The C-terminus MID1 contains a fibronectin type III domain (FNIII) and a B30.2. domain, two protein-protein interaction domains. Via its C-terminal end MID1 associates with microtubules (16). This association is not static but allows anterograde and retrograde transport of MID1 along the microtubules (17).

Ubiquitination of a target protein involves three steps: binding between ubiquitin and an ubiquitin-activating enzyme (E1), transfer of ubiquitin to an ubiquitin conjugating enzyme (E2), and transfer of ubiquitin from the E2 enzyme to its target protein, which is catalysed by an E3 enzyme. Ubiquitin modification can either target proteins for degradation by the proteasome or mediate other functional changes. Several RING finger proteins have an E3 ubiquitin ligase activity (18). An E3 ubiquitin ligase activity of MID1 was first described for its target protein PP2A: MID1 binds to the alpha4 regulatory subunit of PP2A with its B-Box1 domain (2, 19). Via this binding MID1 catalyses the polyubiquitination and proteasomal degradation of the catalytic subunit of PP2A (PP2Ac) (2). Furthermore, MID1 mediates the ubiquitination of alpha4 in two distinct ways: either catalysing polyubiquitination, which targets alpha4 for degradation via the proteasome (20), or catalysing monoubiquitination, which promotes calpain cleavage (21). Another substrate of the MID1 E3 ubiquitin ligase is the kinase Fused (Fu), which is involved in the sonic hedgehog-signalling (SHH) pathway. Here, MID1 catalyses the formation of a ubiquitin chain that initiates a proteasome dependent cleavage event (22). This aspect of MID1 function will be discussed in detail in section 6.



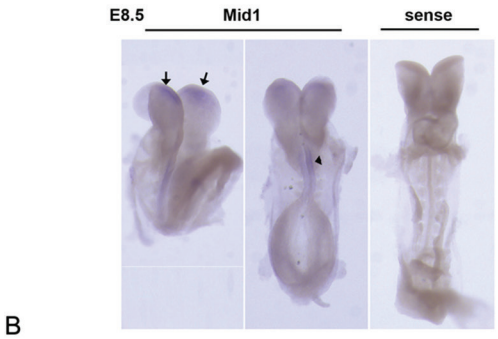


Figure 1. Tissue and developmental stage specific expression of MID1. (A) Schematic summarizing current knowledge about MID1 expression patterns in human and mouse. (B) *In situ* hybridization of an E8.5 mouse embryo with a Mid1 specific probe and a control sense probe. At this stage Mid1 expression is restricted to the dorsal root of the neural tube (arrow) and the spinal cord (arrowhead) (35-41).

By inhibiting PP2A activity MID1 enhances the activity of another central enzyme: the mTOR/TORC1 complex. The TORC1 complex consists of the mTOR kinase, mLST8, and raptor, which are involved in recruiting substrates for phosphorylation by the kinase domain of mTOR. mTORC1 regulates diverse cellular functions, including translation induction. Here, mTOR phosphorylates its two substrates 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and S6K (40S ribosomal S6 kinase), both of which control translation initiation (reviewed in (23)). Perturbations of the MID1-PP2A complex affect mTORC1 signalling at the level of mTOR-Raptor complex formation: depletion of MID1 increases PP2A levels, which in turn leads to the disruption of the mTOR-Raptor complex. As a consequence, mTORC1 signalling is down regulated (3) (Figure 3).

Besides regulating the activity of the translational regulators PP2A and mTOR, MID1 assembles a large microtubule-associated protein complex that contains several proteins involved in mRNA transport and translation, such as elongation factor 1a (EF-1a), RACK1, Annexin A2, Nucleophosmin and ribosomal proteins. This protein complex specifically binds to G-rich and to a lesser extend to U-rich RNAs (24). These data suggest that one cellular function of MID1 is directing this translational regulation machinery to the cytoskeleton. In sections 6, 7, and 8 we will give more detailed information about already identified target mRNAs that are regulated by MID1.

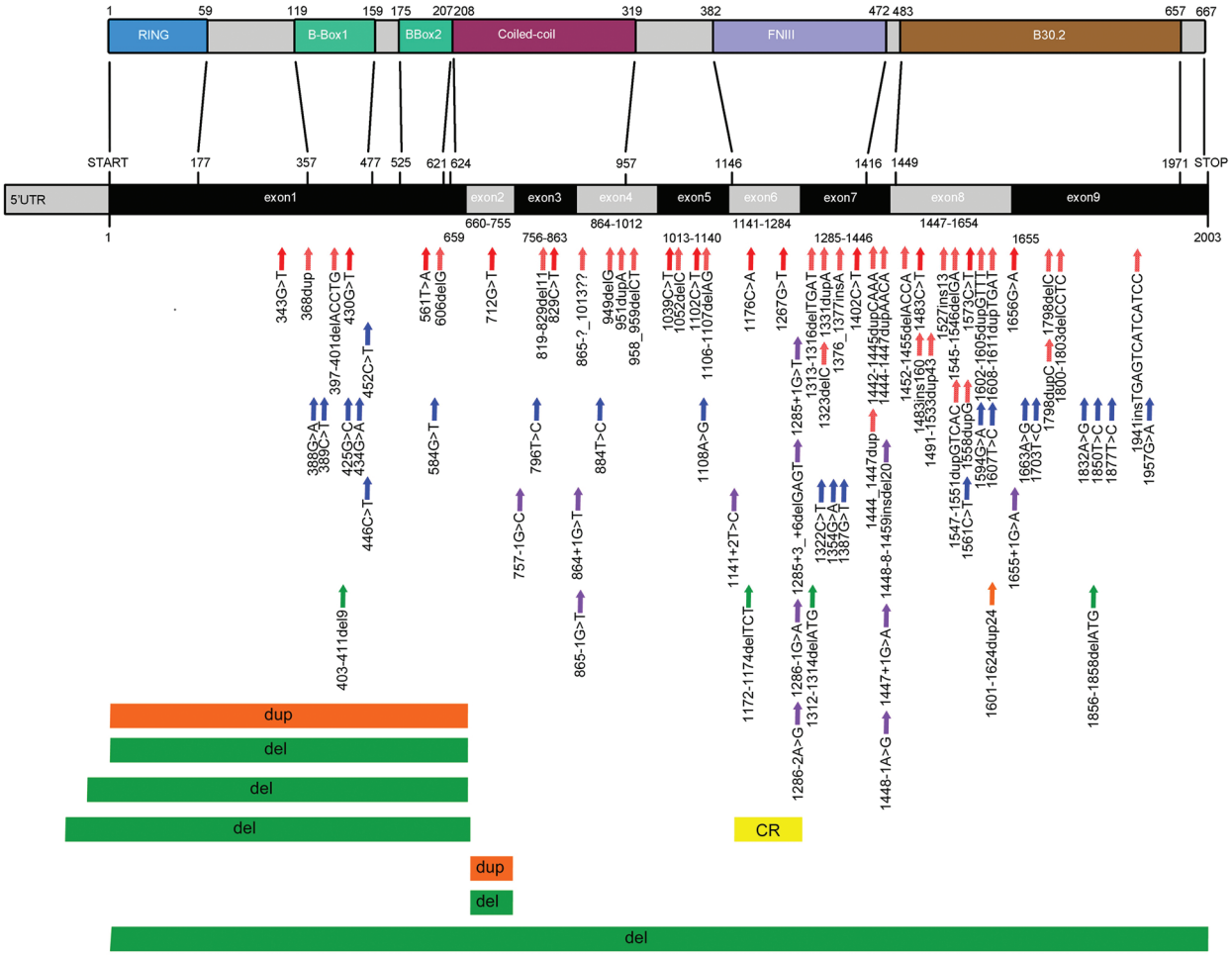


Figure 2. The MID1 gene product. Schematic showing the MID1 protein and the MID1 mRNA. Mutations that have been reported in OS patients are depicted. The nomenclature of the mutations in the MID1 cDNA follows the rules suggested by (103), with 1 corresponding to the A of the ATG initiation translation codon. Mutations are labelled as follows: red arrows show mutations that lead to premature stop (plain: point mutations, patterned: small deletions or insertions). Blue arrows indicate point mutations leading amino acid exchanges. Orange bars or arrows visualize deletions. Green bars or arrows visualize insertions. Violet arrows show mutations affecting the RNA (splice sites). The yellow bar indicates a complex rearrangement that includes 4 breakpoints and consists of a 5571 bp deletion, a 362 bp inversion, a 15 bp deletion and a 3 bp insertion (42, 104-109).

5. MID1 IS INVOLVED IN MIDLINE MALFORMATION SYNDROMES

5.1. Opitz BBB/G syndrome

Opitz BBB/G syndrome (OS) is a ventral midline malformation disorder that was first described by John Opitz in 1969 as two distinct entities, the BBB syndrome with cleft lip/palate and intellectual disability (25) and the G syndrome with gastrointestinal malformations (26). Based on the phenotypic overlap it was later realized that BBB and G syndrome indeed represent one syndrome, which was then reclassified as Opitz BBB/G syndrome (27, 28).

OS is genetically heterogeneous with one locus on chromosome Xp22 and a second locus on chromosome 22q11.2. (29). Clinically, the X-linked and the autosomal dominant form of OS cannot be distinguished. In contrast to Xp22 where the causative gene, *MID1*, was identified in 1997 (1), the gene on chromosome 22q11.2. causing autosomal dominant OS remained unclear for a long time. Recently, missense mutations in the *SPECC1L* gene on 22q11.2. that segregated with the phenotype were identified in patients with autosomal dominant OS (30). SPECC1L is a cytoskeletal crosslinking protein involved in microtubule stability and actin cytoskeletal reorganization and plays a role in cell adhesion and migration (31). Since *SPECC1L* mutations could not be found in another family that had been linked to chromosome 22q11.2. by Robin *et al*. (29) and also not in the other 17 probands with suspected autosomal dominant OS, it is well possible that *SPECC1L* is not the only causative gene for OS in this chromosomal region. This is further supported by the fact that patients with the OS phenotype and 22q11.2. deletions, which do not contain *SPECCL1*, have been reported (32-34).

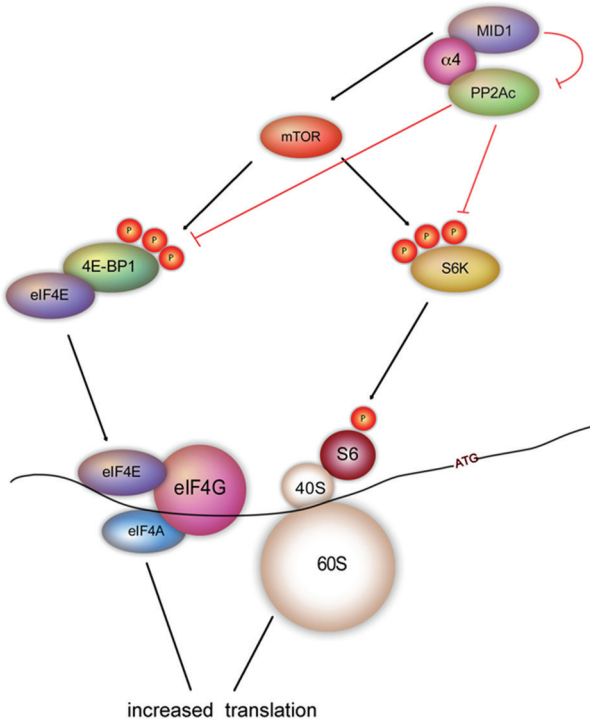


Figure 3. MID1 regulates mTOR/PP2A-dependent translation. Schematic showing MID1's effect on S6K-dependent translation. MID1 is a negative regulator of PP2A and a positive regulator of mTOR. PP2A and mTOR control the phosphorylation status and activity of their downstream targets S6K and 4E-BP1. 4E-BP1 regulates the composition of the eukaryotic translation initiation factor (eIF) complex that contains the proteins eIF4A, eIF4E, and eIF4G. Phospho-activated S6K phosphorylates and enhances the activity of its targets eIF4B and ribosomal protein S6, which in turn unwind and linearize the 5'UTRs of the bound mRNAs and promote ribosome binding and translation initiation.

5.2. MID1 mutations in OS

Since its discovery as the causative gene for X-linked OS in 1997, numerous additional mutations have been identified in the *MID1* gene in OS patients. These include missense and nonsense mutations as well as frameshift and splice site mutations. In addition, small intragenic deletions and duplications as well as whole gene deletions have been identified (35-41). Initially, *MID1* mutations were thought to result in loss-of-function of the MID1 protein as seen in OS patients with whole gene deletions. There is, however, evidence that a gain-of-function mechanism exists in addition, as some OS patients with brain anomalies carry C-terminal truncating mutations in the *MID1* gene that could generate truncated MID1 proteins (42) (Figure 2). In agreement, over expression of a C-terminal truncated Mid1 protein in the developing neocortex of Mid1 knockout (KO) mice led to a neuronal migration defect that was not observed upon over expression of wild type Mid1 (43). Although a MID1 gain-of-function mechanism as the cause of brain anomalies in OS patients is an attractive hypothesis, there are several lines of evidence that argue against such a mechanism. Firstly, Mid1 KO mice present with brain anomalies that are also seen in OS patients, namely hypoplasia of the cerebellar vermis (44). Due to the lack of Mid1 expression, this defect can only be caused by a loss-of-function of Mid1 in the KO mice. Secondly, MRI examination of the brain of a subset of OS patients revealed no neuronal migration disorders in these patients (42). Future studies of specific MID1 mutants in cellular and animal models will show the effects of these mutations on MID1 expression and function in detail.

5.3. OS is a midline malformation disorder

OS patients present with a variety of ventral midline defects. The phenotype is highly variable even among siblings carrying the same mutations. Hypertelorism (wide distance of the eyes) and hypospadias (urogenital abnormality in males) have been described in 98 and 85 percent, respectively, of OS patients with MID1 mutations and are the most prominent features in OS. In addition, OS patients may present with further ventral midline defects. Laryngo-tracheo-esophageal defects, e.g. laryngo-esophageal clefts, dysphagia and swallowing difficulties occur in approximately 60 percent of OS patients with MID1 mutations. Additional defects observed in OS patients include cleft lip and palate, heart defects, anal defects, brain anomalies, including agenesis or hypoplasia of the cerebellar vermis, agenesis or hypoplasia of the corpus callosum, large cisterna magna and Dandy Walker malformations, and developmental delay (motor delay and/or mild to severe intellectual disability) (42). Some of the malformations seen in OS such as hypertelorism are indicative of neural crest cell abnormalities. Others, e.g. hypospadias, may be caused by other mechanisms. In the following section we will discuss possible pathomechanisms involved in the development of these specific OS malformations.

5.3.1. OS - a neurocristopathy

Several of the malformations that are typical for OS, i.e. hypertelorism, broad nasal bridge, cleft lip and palate and heart defects occur in organs or tissues that are derived from the neural crest. Neural crest cells (NCCs) are pluripotent stem cells that give rise to almost all cell types of ectodermal and mesodermal origin. During development NCCs are induced from the border that separates the neural from the non-neural ectoderm just before neural tube closure. Subsequently, they undergo an epithelial-mesenchymal transition (EMT) and migrate away from the neural plate border, meanwhile quickly separating into discrete streams. NCCs of the head, the cephalic neural crest, separate into three subpopulations, one surrounding the eye and two, which are generated around the otic vesicle. Cephalic NCCs that differentiate into neurons and glia of the cranial ganglia stop dorsally whereas cephalic NCCs that form the bones and cartilages of the face migrate further ventrally into the branchial arches (summarized in (45)) (Figure 4A).

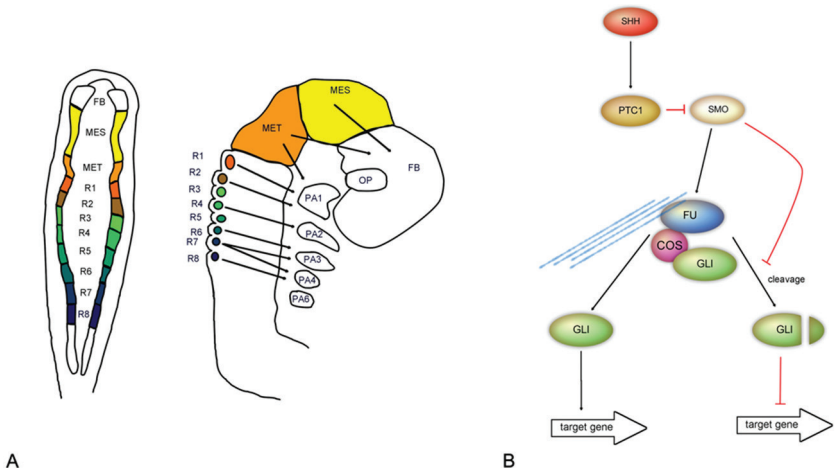


Figure 4. (A) Neural crest cell migration. Left: schematic showing the embryo neural tube before neural crest migration. Forebrain (FB), mesencephalon (MES), metencephalon (MET), and rhombomeres (R1-8) are indicated. Right: sagittal view of an embryo. Routes of neural crest migration are indicated (pharyngeal arches (PA1-6), optic vesicle (OV)). (B) The SHH signalling pathway. SHH ligand binds to and inhibits its receptor PTC1. Upon SHH binding PTC1 releases its suppressive effect on its target protein Smo. Smo in turn activates a number of downstream signalling events, finally regulating the activity of the SHH target transcription factors GLI1, GLI2, and GLI3. Depending on the presence or absence of SHH, the GLI proteins undergo a cleavage process: in presence of SHH GLI proteins act as a full-length transcriptional activator, while in absence of SHH GLI proteins can get cleaved into transcriptional repressors.

Craniofacial dysmorphisms can occur as a consequence of defective cephalic NCC formation, migration, proliferation or differentiation. It is poorly understood, which of these processes involve MID1 function. In contrast to patients with MID1 mutations, the Mid1 KO mouse does not show any neural crest-specific defects (43, 44). This is surprising in light of the conserved expression pattern of MID1 in NCCs and NCC-derived tissues between human, mouse, and chicken. In all three species MID1 is expressed at high levels in the first and second branchial arches during development, which correlates with the patterning defects of craniofacial skeletal elements seen in OS patients (1, 46, 47). One explanation for the lack of NCC-specific defects in the Mid1 KO mouse could be compensation by Mid2 for the loss of Mid1 in this model. Data obtained from a study in chicken indicate that cMid1 is involved in regulating the speed of migration of a subset of cranial NCCs (48). At early stages cMid1 is expressed in a subset of migrating rhombomere 2 (r2) NCCs but not in migrating r4 or r6 NCCs. Expression of a dominant-negative cMid1 construct in r2 resulted in an inhibition of NCC migration. Expression of a wt cMid1 construct in r4, on the other hand, increased NCC migration speed. Although it is still not known whether this function of cMid1 is conserved in mammalian species these experiments suggest that a decrease in NCC migration speed caused by mutations in MID1 contributes to the craniofacial dysmorphisms seen in OS patients. In addition, MID1 may also be involved in other NCC-related processes. For example, in chicken embryos cMid1 positively regulates the expression of Snail, a transcription factor that induces EMT, suggesting that cMid1 regulates the delamination of cranial NCCs from the neural tube (49). Because MID1 is also expressed in the branchial arches and frontonasal processes at later stages it may also regulate NCC proliferation and/or differentiation. Taken together, the facial dysmorphic features seen in OS could be caused by MID1 loss-of-function in any single or in combinations of the aforementioned steps of NCC development. Neurocristopathies that are due to defective NCC migration, proliferation, and/or differentiation show remarkable craniofacial phenotypic overlap with OS (Table 1). In contrast, defects in NCC formation typically result in a pathologically reduced number of NCCs migrating towards the face and consequently to a hypoplasia of the facial bones and cartilages (summarized in (45)). The lack of hypoplasia of the facial bones and cartilages in OS suggests that NCC are formed, in principle, in OS patients. Together these observations suggest that the OS-specific craniofacial dysmorphisms are caused by defective NCC migration, proliferation and/or differentiation rather than by defects in NCC formation, pointing at shared molecular pathways underlying OS and neurochristopathies.

A prime example for such a shared underlying molecular mechanisms is misregulation of *ephrin-B1* signalling. Mutations in *ephrin-B1* (*EFNB1*) cause craniofrontonasal dysplasia (CFNS) (50, 51), which is characterized by hypertelorism, bifid nasal tip, cranium bifidum occultum, craniosynostosis, cleft lip and palate and facial asymmetry. *Efnb1* KO mice share some but not all of the phenotypic features of CFNS, namely a delay in the ossification of calvarial bones leading to a frontal foramen similar to cranium bifidum occultum in humans

Table 1. Craniofacial phenotypic overlap between OS and other neurocristopathies

Phenotype	OMIM	Craniofacial phenotype overlapping with OS	Genetic cause (mutations in)	Defects in NCC
Opitz BBB/G syndrome (X-linked)	300000	Hypertelorism, prominent forehead, broad nasal bridge, grooved nasal tip, cleft lip/palate, craniosynostosis, microcephaly	*MID1*	Unknown
Craniofrontonasal dysplasia	304110	Hypertelorism, bifid nasal tip, craniosynostosis and facial asymmetry	*EFNB1*	Migration, differentiation, proliferation
Mowat-Wilson syndrome	235730	Hypertelorism, wide nasal bridge, prominent nasal tip, cleft palate, microcephaly	*ZEB2*	Migration, EMT
Noonan syndrome	163950	Hypertelorism	*PTPN11*	Differentiation
LEOPARD syndrome	151100	Hypertelorism, broad, flat nose, cleft palate	*PTPN11*	Migration, differentiation
Greig syndrome	175700	Hypertelorism	*GLI3*	Differentiation

and a cleft palate. In the mouse ephrin-B1 regulates the migration of NCCs. Initially it was found that both the global and NCC-restricted loss of ephrin-B1 in KO mouse models changes the migration pattern of NCCs from the stereotypical pattern of migration in discrete streams observed in wt mice to a wandering behaviour in mutant mice (52). However, this change in migration behaviour does not seem to be the sole cause of craniofacial anomalies found in the mutant mice. Thus, follow-up studies identified important functions of ephrin-B1 during the differentiation and proliferation of NCCs (53, 54). Whereas a delayed NCC differentiation is responsible for the development of a frontal foramen, reduced proliferation in the anterior palatal shelf mesenchyme causes the cleft palate phenotype in ephrin-B1 mutant mice. Theoretically, the craniofacial dysmorphisms seen in OS could be caused by similar mechanisms. Although a clear connection between MID1 and Eph/Ephrin signalling is not evident, we have found that the MID1 complex binds to EFNB1 mRNA *in vitro* (55). Future studies should analyze possible consequences of this interaction for EFNB1 expression and NCC development *in vivo*.

Another shared molecular pathway underlying OS and other neurochristopathies is the SHH pathway. The SHH ligand binds to its receptor PTC1, which activates a number of downstream signalling events, finally regulating the activity of the SHH target transcription factors GLI1, GLI2, and GLI3. Transduction of the SHH signal has been best studied in *Drosophila*, in which a cytosolic protein complex containing the proteins Fused (Fu), Costal2 (Cos2), and Suppressor of Fused (SuFu) controls activity of the GLI homologue cubitus interruptus (Ci) by regulating a cleavage process: in presence of the hedgehog (Hh) signal Ci maturates into a full-length transcriptional activator, while in absence of Hh Ci gets cleaved into a transcriptional repressor. While in *Drosophila* only one Hh ligand exists, three Hh orthologues are present in mammals: indian hedgehog, desert hedgehog, and SHH. Many aspects of Hh signalling are conserved between species, but the detailed molecular mechanisms in human SHH signalling are less well understood (reviewed in (56)) (Figure 4B).

It has been proposed recently that the face is a barometer of Hh pathway activity (57). Insufficient Hh signalling leads to facial contraction and consequently to hypotelorism or even cyclopia whereas excessive Hh signalling causes an expansion of the face with hypertelorism and broad nasal bridge up to facial duplications. In humans, mutations in the SHH effector *GLI3* cause Greig cephalopolysyndactyly syndrome (GCPS; OMIM 175700), which involves hypertelorism and polydactyly (58, 59). The phenotype of GCPS suggests excessive SHH signalling to be disease causing. GLI3 can act either as a transcriptional activator or as a repressor. The GLI3 repressor antagonizes SHH signalling. A loss of this repressor function of GLI3 may result in excessive SHH signalling and cause the facial widening observed in GCPS patients.

As is outlined below (section 6) the MID1-PP2A complex controls the subcellular localization and transcriptional activity of GLI3 in cancer cell lines (4). In addition, point mutations in GLI3 inhibit the MID1-dependent activation of GLI3, suggesting a shared underlying mechanism for OS and gliopathies (60). In OS patients with MID1 mutations altered GLI3 transcriptional activity may contribute to the development of craniofacial dysmorphisms. Therefore, future studies aimed at investigating the functional relationship between MID1 and SHH/GLI3 in NCCs would be useful.

5.3.2. Structural brain defects in OS patients

Brain malformations have been described in 36 percent of OS index cases with detected *MID1* mutations. The most frequent of these malformations is cerebellar vermis agenesis or hypoplasia (summarized in (42)), a defect that is recapitulated in the *Mid1*$^{-/Y}$ mouse (44).

The cerebellum is a part of the hindbrain that also includes the pons and medulla. During embryonic

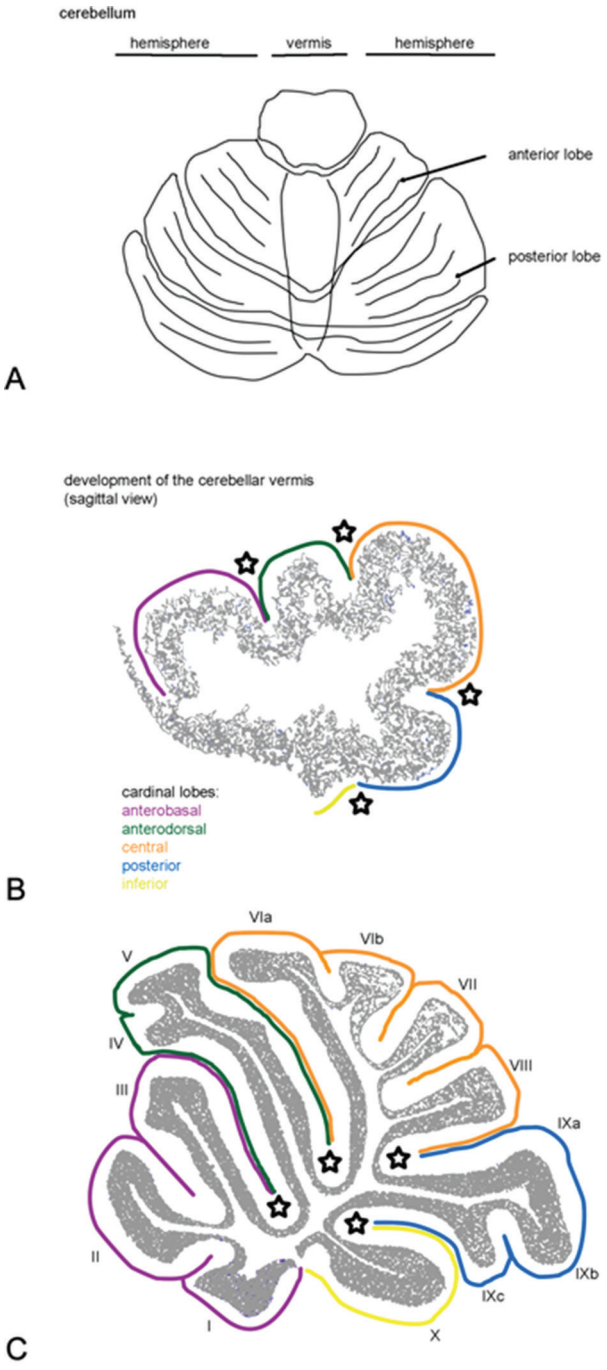


Figure 5. Morphology of the cerebellum. (A) Schematic of the cerebellum showing the localization of the cerebellar vermis and the two hemispheres. (B,C) Schematic showing cerebellar foliation. (B) Sagittal view of the cerebellar vermis. The five cardinal lobes (anterobasal (violet), anterodorsal (green), central (orange), posterior (blue) and inferior (yellow)), and the fissures (stars) as they appear at E18.5. are shown. (C) The adult lobules (I-X) develop from the cardinal lobes; stars indicate principal fissures.

development the hindbrain or rhombencephalon can be subdivided into eight segments termed rhombomeres. From anterior to posterior they are called r0-r7. The cerebellum originates from r0 (also called the isthmus) and r1. The isthmus is the topographical boundary between the midbrain and the hindbrain. Simultaneously, it acts as a signalling centre that controls the patterning of the cerebellum and maintains the midbrain/hindbrain boundary (summarized in (61)). In the adult mammalian brain the cerebellum is composed of the two cerebellar hemispheres separated by the cerebellar vermis (Figure 5A). The cerebellum has a striking morphology, with folia separated by fissures of different lengths. The five cardinal lobes are designated from anterior to posterior anterobasal, anterodorsal, central, posterior, and inferior lobes and give rise to the ten definitive lobes of the adult cerebellum (Figure 5B). In this context the anterobasal cardinal lobe develops into the definitive lobes I, II and III (Figure 5C). In the *Mid1*$^{-/Y}$ mice cerebella formation of the anterobasal cardinal lobe is abnormal and consequently in the adult brain the vermis of these mice shows hypoplasia and abnormalities of lobes I, II, and III (44). These abnormalities are not due to postnatal foliation defects but are evident at prenatal stages. Hence, at E14.5 the lower region of the inferior colliculus, a midbrain structure that adjoins the isthmus, is shortened and the dorsal isthmus is rostralized in *Mid1*$^{-/Y}$ mice. This suggests that the anterior vermis hypoplasia seen in these mice is most probably caused by a defective definition of the midbrain hindbrain boundary.

One molecular network underlying MID1-dependent changes in midbrain structure is Fgf signalling. At midgestation *Fgf8* is expressed at high levels in the isthmus and separates the *Otx2*-positive midbrain neuroepithelium from the *Gbx2*-positive hindbrain neuroepithelium (summarized in (61)). Another member of the fibroblast growth factor family, *Fgf17*, is also expressed in the isthmus and, in contrast to *Fgf8*, *Fgf17* expression in the isthmus extends until E14.5 (62). In agreement, Fgf8 is important during early stages of midbrain hindbrain boundary development whereas Fgf17 is important during later stages.

In chicken Mid1 positively regulates the expression of *Fgf8* in Hensen's node (49). Interestingly, expression of *Fgf8* was not altered in the isthmus of *Mid1*$^{-/Y}$ mice but instead expression of *Fgf17* was reduced at E13.5 and E14.5 *Fgf17*$^{-/-}$ mice phenotypically overlap with *Mid1*$^{-/Y}$ mice (62). However, there are also phenotypic differences between *Fgf17*$^{-/-}$ and *Mid1*$^{-/Y}$ mice. Although *Fgf17*$^{-/-}$ mice present with malformations of the anterior vermis, the phenotype seems to be milder than in the *Mid1*$^{-/Y}$ mice. This suggests that in addition to a reduced *Fgf17* expression other mechanisms might be involved in the development of a vermis hypoplasia in *Mid1*$^{-/Y}$ mice. On the other hand *Fgf17*$^{-/-}$ mice have a hypoplastic inferior colliculus, a phenotype that is more severe than the shortening of the inferior colliculus seen in the *Mid1*$^{-/Y}$ mice. A reason for this discrepancy might be that expression of *Fgf17* is not completely lost

in *Mid1*$^{-/Y}$ mice. In *Fgf17*$^{-/-}$ mice the complete absence of *Fgf17* leads to proliferation defects in the cerebellar precursor cells (62). In contrast, no proliferation defects were detected in the *Mid1*$^{-/Y}$ mice (44).

The mechanism of dorsal isthmus rostralization, *Fgf17* downregulation and malformation of the cerebellar lobes I, II and III upon the loss of Mid1 is still unknown. As outlined above MID1 regulates the activity of the SHH transcription factor GLI3 in cancer cell lines (4). In the mouse Gli3 regulates the development of the cerebellum at multiple steps (63). An increase in Gli3 repressor levels causes the dorsal midbrain/r1 defects in Shh signalling mutants (64). A series of *Gli3* mutants further demonstrated that the Gli3 repressor is an important regulator of growth and patterning of the dorsal midbrain/r1 but not of the ventral midbrain/r1 (63). The Gli3 repressor is essential to form a normal inferior colliculus, isthmus and anterior cerebellum. In addition Gli3 is crucial to attenuate the growth of r1, midbrain and isthmus through enhancing cell death and reducing cell proliferation. It is also involved in establishing a normal foliation pattern. The inactivation of Gli3 results in an expanded expression domain of *Fgf8* and *Fgf17*. Hence, Gli3 is necessary to restrict the expression of *Fgf8* and *Fgf17* to the isthmus. In comparison to the cerebellar phenotype of *Mid1*$^{-/Y}$ mice the inactivation of *Gli3* leads to much more severe defects in cerebellar development. If Mid1 regulates the activity of Gli3 during cerebellar development this regulation might be spatially and temporally restricted. Future studies should analyze such a regulatory role of Mid1 towards Gli3 activity in the *Mid1*$^{-/Y}$ mice.

A detailed analysis of Mid1 function in mouse cortical neurons and *in vivo* in the Mid1 KO mouse has highlighted an essential role for Mid1 during axon growth and branching (43). The depletion of Mid1 in cortical neurons promoted axon growth and branch formation. An *in vivo* transfection of shRNAs targeting Mid1 into a subpopulation of cortical neural progenitor cells (NPCs) at E15 caused an increased axon length and a mis-positioning of the callosal axons originating from the transfected neurons. Thus while the axons of control neurons had crossed the midline and axon bundles were restricted to the corpus callosum the axon terminals of Mid1 knockdown neurons were positioned further away from the midline, a phenotype that was also observed in the Mid1 KO mouse and was due to increased PP2Ac levels. Whether axon development is commonly disrupted in OS patients is not known. Interestingly, a recent study combining whole exome sequencing with diffusion tensor imaging (DTI) has identified a female patient with intellectual and development disability and hypertelorism who carried a missense mutation in the *MID1* gene (65). DTI examination revealed an underdeveloped arcuate fasciculus in this patient. The arcuate fasciculus is a brain white matter pathway connecting Wernicke's and Broca's area and is important for language and cognitive development.

5.3.3. Possible causes of urogenital anomalies seen in OS

OS patients can present with a range of urogenital abnormalities. The most common among these is hypospadias, which occurs in 85 percent of male OS cases with MID1 mutations (42). Other urogenital abnormalities include cryptorchidism, ambiguous genitalia, bifid scrotum, hypoplastic scrotum, micropenis, umbilical and inguinal hernia, incomplete prepuce, vesicoureteric reflux and hydronephrosis. The pathomechanism leading to urogenital abnormalities including hypospadias in OS is poorly understood.

Hypospadias affects 1 in 125 males and is therefore the second most common human birth defect (66). Most cases of hypospadias are idiopathic but lees than 10 percent are genetically caused (67). One of the causative genes is the *androgen receptor* (*AR*) gene. The AR is important for penile and urethral development and mutations in the *AR* gene cause partial androgen insensitivity syndrome with perineoscrotal hypospadias and micropenis (68). In embryonic development, the genital tubercle (GT) gives rise to the male and female external genitalia. In male mice, proper mesenchymal expression of AR is necessary for GT masculinisation. Thus, mesenchymal AR KO male mice exhibit a female type development of their GT (69).

Recently MID1 was found to up regulate AR protein levels in several prostate cancer cell lines and its expression itself was negatively regulated by androgen signalling (see also section 6) (8). Like the *AR* gene MID1 is expressed in the GT in human embryos (46). However, if MID1 is necessary for establishing correct AR protein levels during GT development remains elusive. If MID1 loss-of-function mutations as seen in OS led to decreased AR protein levels in the GT, this could explain the urogenital anomalies seen in OS.

SHH signalling is essential for external genitalia development (70). Shh homozygous KO mice are characterized by an agenesis of their external genitalia and Shh inhibition after the induction of GT outgrowth leads to a retarded GT outgrowth and to defects in the differentiation of the ventral side of the GT. External genitalia development in humans in general and the involvement of the SHH pathway in particular is poorly understood. In humans GLI3 seems to be important for external genitalia development, since mutations in the GLI3 gene cause Pallister Hall syndrome (PHS), which is characterized by urogenital abnormalities including hypospadias and micropenis. As described in section 6 the MID1-PP2A complex interacts with GLI3 (4), and point mutations in GLI3, as observed in PHS, inhibit the MID1-dependent activation of GLI3, suggesting a shared

underlying mechanism for OS and gliopathies (60). In OS patients with MID1 mutations altered GLI3 activity may thus represent one possible molecular mechanism underlying hypospadias.

6. THE MID1 COMPLEX IS INVOLVED IN CARCINOGENESIS

The MID1 complex interacts with and regulates diverse signalling pathways that are associated with carcinogenesis. In this section we will discuss how the MID1 protein complex promotes tumour growth by interacting with three different tumourigenic pathways: SHH, AR, and PDPK1 signalling.

Similar to the MID1-PP2A complex, the SHH signalling pathway not only plays an important role during embryogenesis, but is also involved in carcinogenesis (71-75). In this context was shown that the MID1-PP2A complex, together with mTOR, controls the subcellular localization and transcriptional activity of the GLI3 transcriptional activator in cancer cell lines with autonomously activated SHH signalling. Inhibition of the MID1 complex and mTOR activity results in cytosolic retention and decreased transcriptional activity of GLI3 towards its transcriptional target gene *Cyclin D1*, while inhibition of PP2A has the opposite effect (4). The regulatory effect of the MID1-PP2A complex on GLI3 is, however, not caused by a direct modification of GLI3 by the MID1 complex, but is instead mediated by interaction of MID1 with GLI3-interacting protein Fu. In detail, MID1 catalyzes the ubiquitination of Fu, which leads to proteasome-dependent cleavage of Fu. By this cleavage, the N-terminal kinase domain of the Fu kinase is removed and the C-terminal fragment of Fu stimulates the GLI3 nuclear localization and transcriptional activity. Thereby MID1 indirectly controls the transcriptional activity of GLI3 in cancer cell lines (22). Increased SHH signalling is associated with growth/proliferation of diverse tumours (71-75). Our data suggest that MID1-dependent activation of GLI3 is one mechanism underlying autonomously activated SHH signalling in some cancer types. Since the GLI3 target gene *Cyclin D1* promotes cell growth, an inhibition of the MID1 complex could therefore be useful to stop growths of SHH-dependent cancer types.

The androgen receptor (AR) is a ligand-activated transcription factor found in both benign and malignant prostate epithelial cells. Upon androgen binding, several intracellular processes are activated resulting in the nuclear localization and transcriptional activity of the AR. Depletion of androgens, either surgically by castration or medically, is a standard treatment for prostate cancer. However, this does not fully deplete androgens within the tumour itself, nor does it affect adrenal androgen synthesis. Therefore, after a period in which the tumour does not grow, AR reactivation occurs and tumour proliferation resumes. This castration-resistant phenotype of prostate cancers is associated with up regulation of AR expression (reviewed in (76)). In this context it has been shown that the MID1 protein complex binds to two sites of the AR mRNA: a CAG repeat region and a GGY repeat region. Binding of the MID1 complex to the AR mRNA induces AR translation (8). Consequently, the knockdown of MID1 or its complex partners in prostate cancer cells leads to reduced AR levels. At the same time AR regulates MID1 transcription: the MID1 gene contains several AR binding sites (8). In the presence of Androgen, AR suppresses MID1 transcription, whereas androgen withdrawal increases MID1 and concomitantly AR-protein levels (8). In line with these data MID1 is over expressed in a subtype of prostate cancer cells with a more aggressive phenotype (8), suggesting that MID1 is a tumour and metastasis promoting factor. In line, disruption of the MID1 complex by siRNA inhibits prostate cancer cell growth and reduces AR protein levels (12). Similarly treatment of prostate cancer cells with the anti-diabetic drug metformin, which led to an inhibition of the binding between the AR mRNA and the MID1 complex, reduced AR protein levels and inhibited growth and migration of prostate cancer cells (12). Together, these findings suggest that MID1 is an important regulator of tumour cell proliferation in prostate cancer and an inhibition of the MID1 complex could therefore be useful to stop tumour growth.

Finally MID1 also promotes another tumourigenic pathway, PDPK1 signalling. PDPK1 has several cellular functions including regulation of cell proliferation (77). PDPK1 induces the activity of mTOR and by stimulating the mTOR pathway induces protein translation and cell proliferation (78, 79). Inhibition of PDPK1 stops tumour growth in tumours like head and neck squamous cell carcinoma (6, 7). The MID1 complex binds to a specific motif within the PDPK1 mRNA, the so-called MIDAS (MID1 associates sequence) (5). Binding of MID1 to the PDPK1 mRNA leads to an increased translation of PDPK1 (5), which in turn induces proliferation. Therefore, inhibition of the MID1 complex could be useful to stop the growth of PDPK1-dependent cancer types.

All these data show that MID1 stimulates cancer growth and suggest that reducing MID1 protein levels should stop proliferation. Expression of miRNAs targeting MID1 should therefore stop cell growths in tumours, which are MID1-dependent. However, not all tumour types seem to be regulated by MID1 in a similar fashion. In some kinds of tumours there seems to be an opposite trend. For example during progression of mammary carcinogenesis silencing of MID1 by miRNA 153b promotes tumour progression (80).

Taken together, all these findings suggest that MID1 is a key player in distinct types of cancers. Therefore a subset of tumours, proliferation of which

is regulated by MID1, would be treatable with a MID1-targeting compound.

7. THE MID1 COMPLEX REGULATES THE TWO PATHOLOGICAL HALLMARKS OF ALZHEIMER'S DISEASE (AD): HYPERPHOSPHORYLATED TAU AND PRODUCTION OF AMYLOID PLAQUES

The MID1 protein is involved in the pathogenesis of AD. AD is the most common form of dementia in the elderly (81). The disease is characterized by two pathological hallmarks: extracellular amyloid plaques, which contain Abeta, and intracellular neurofibrillary tangles, which are composed of hyperphosphorylated Tau (82). The Abeta peptides are generated by sequential cleavage of the Amyloid Precursor Protein (APP). First, full-length APP is cleaved by the beta-secretase BACE1. Then the resulting C-terminal fragments (CTF) get cleaved by the gamma-secretase protein complex generating Abeta (83). Multiple lines of evidence have shown that overproduction of Abeta is linked to neuronal dysfunction and neuronal death (84). The second pathological hallmark of AD - neurofibrillary tangles - are mainly composed of hyperphosphorylated Tau. Tau is a microtubule-associated protein that stabilizes microtubules. In AD patients' brains Tau phosphorylation is increased by three to four fold. Upon phosphorylation, Tau dissociates from microtubules, resulting in microtubule destabilization (85, 86). PP2A is the main Tau phosphatase and dephosphorylates Tau at AD-relevant phospho-sites (87). Both decreased PP2A activity and expression occur in AD brains (88-92). In addition, the toxic properties of AD-Tau seem to be linked to its hyperphosphorylation, since dephosphorylating AD-Tau converts it back into a protein with properties indistinguishable from that found in non-AD brains (93). Therefore, pharmacological induction of PP2A is a promising approach to reduce AD-related Tau pathology.

We have shown that the biguanide metformin disassembles the MID1-PP2A complex *in vitro*, which in turn leads to an increased PP2A activity. Treatment of primary cortical neurons with metformin therefore leads to a decreased phosphorylation of Tau at AD-specific phospho-sites. This effect can be blocked by PP2A inhibitors, proving that it is PP2A-dependent (10). Besides regulating Tau-phosphorylation, the MID1-PP2A complex has been also linked to Abeta generation. Here, the MID1 complex binds to and regulates the translation of the BACE1 mRNA. Metformin, by disassembling the MID1-PP2A complex, reduces BACE1 protein translation thereby reducing BACE1 activity (9). Altogether, these studies suggest that disassembly of the MID1-PP2A complex by metformin may target both pathological hallmarks of AD, namely Abeta containing extracellular amyloid plaques and intracellular neurofibrillary tangles, which are composed of hyperphosphorylated Tau. Therefore future studies should investigate the potential therapeutic value of MID1 complex inhibition for treating and/or preventing AD.

Despite these promising results, the use of metformin for treating AD has been discussed controversially in the literature: while, in line with the above mentioned beneficial effects, metformin has been shown to reduce hyperphosphorylated Tau and Aβ in mouse models for diabetes and tissue culture models for insulin resistance (94, 95), other studies showed an opposite trend, namely an increased BACE1 expression upon metformin treatment (96). Furthermore, population studies comparing groups of diabetic patients that were treated with different anti-diabetic drugs and control groups of non-diabetic individuals did not show a preventive effect of metformin with respect to the risk of developing AD (97). Of note, one challenge in such studies is that the diagnosis of AD in the early disease stages is difficult.

Taken together, targeting the MID1-PP2A complex may be of therapeutic value for treating and/or preventing AD. However, since metformin has numerous pleiotropic effects, a compound that is more specific for the MID1-PP2A complex may be more suitable.

8. THE MID1 COMPLEX INTERACTS WITH AND REGULATES TRANSLATION OF MUTANT HUNTINGTIN (HTT): IMPLICATIONS INTO HUNTINGTON'S DISEASE

In this section we will discuss how the MID1 protein complex is implicated in HD. HD is the most common polyglutamine (polyQ) disease and is caused by a CAG repeat expansion within exon1 of the *HTT* gene. The CAG repeat motif encodes a polyQ stretch in the HTT protein, which is pathologically expanded in HD patients leading to decreased solubility of the protein and aggregate formation. Aggregates of mutant HTT protein in the brains of HD patient are the pathological hallmark of the disease (98-102).

We have shown that the MID1 complex binds to the CAG repeat motif of the HTT mRNA and induces its translation. This binding is repeat length-dependent with the mutant CAG repeat binding substantially more MID1 protein. The MID1 protein recruits its interaction partners PP2A and S6K, a target of mTOR and PP2A, to the mutant HTT mRNA (11). While being a negative regulator of PP2A, MID1 also stimulates the activity of the PP2A opponent mTOR (2, 3). PP2A and mTOR control the phosphorylation and the phospho-dependent activity of several translation regulators, including S6K. Phospho-activated S6K phosphorylates and activates its targets, eIF4B (eukaryotic translation initiation factor 4B) and ribosomal protein S6. Upon phosphorylation these S6K targets bind to and modify the 5'UTR of their target mRNAs,

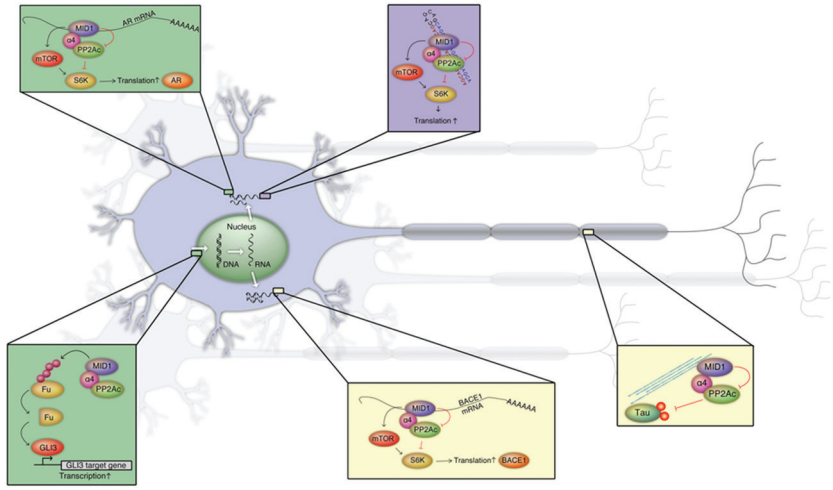


Figure 6. Schematic showing the MID1-function with respect to different diseases. Boxes coloured in green show how MID1 influences cancerogenesis: on one hand MID1 regulates the acitivity of the transcription factor GLI3 in tumour cells with autonomously activated SHH signalling. Here, MID1 ubiquitinates the GLI3-activating protein Fu, inducing a cleavage process that triggers Fu-activity towards GLI3. On the other hand MID1 binds to and induces the translation of the AR mRNA, which is involved in progression of prostate cancer. Boxes coloured in yellow show how MID1 is involved in regulation of the pathological hallmarks of AD. Here, MID1 regulates the translation and consequently the activity of BACE1, which is a crucial enzyme in Abeta production. Furthermore, MID1 regulates the activity of PP2A, thereby influencing hyperphosphorylation of Tau. The violet box shows how MID1 is involved in HD: MID1 binds to and stimulates the translation of mutant HTT mRNA.

thereby promoting ribosome assembly and translation initiation. MID1 stimulates translation of the mutant HTT protein by recruiting S6K to the mutant HTT mRNA and concomitantly inhibiting PP2A and inducing mTOR (11). Therefore, pharmacological targeting of MID1 complex should block translation of the disease-causing mutant HTT protein and thus could be useful as HD therapy.

9. CONCLUSION

MID1 is a central enzyme involved in many different processes in the cell (Figure 6). While essential during development, MID1 seems to be negligible in adults, because individuals with non-functional MID1 suffer from malformations, that were established during embryogenesis, but do not show any further age-related phenotypes. Therefore targeting MID1 as a therapeutic approach for treatment of diseases like AD, HD, or cancer has most likely no side effects.

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