4 Discussion

The RBCC protein MID1, which is involved in ventral midline development, has been shown to form a macromolecular cellular complex, whose components were, up to now, mainly unknown. Several domains of the MID1 protein are putative protein–protein interaction domains that potentially contribute to the formation of this complex. However, apart from their participation in protein binding, no other function could be assigned to them so far. In order to shed light on the functions exerted by some of the domains of MID1, both Bboxes were characterised during this thesis. These studies allowed the identification of a novel pathomechanism for the development of OS.

In addition, after identifying the MID1 complex partners via affinity chromatography and mass spectrometry (MS), the MID1 protein was characterised as the core of a novel translation unit that associates with microtubules. Therefore a new panel of functions for the MID1 protein was introduced.

4.1 A novel pathomechanism for OS caused by mutations in the Bboxes domains of MID1

In a broader context, Bboxes have been shown to play an important role for the correct positioning of RBCC proteins within the cell and to participate in protein-protein interactions (Cao et al., 1997; Reymond et al., 2001). Determining the solution structure of the Bbox of XNF7 in *Xenopus* revealed a novel zinc-binding fold; however, no functional clues could be deduced from this structure. In this thesis, the basic functions Bbox1 and Bbox2 of the RBCC protein MID1 were elucidated and, in addition, the dependence of Bbox1 function on an intact Bbox2 *in vivo* was described.

Recently, we showed that the microtubule-associated MID1 protein targets the pool of microtubule-associated PP2A towards ubiquitin specific modification and degradation. This can only be achieved after bringing the catalytic subunit of PP2A into close proximity to the MID1 RING finger domain via the $\alpha 4$ protein as adapter. MID1 mutations causing OS are mostly clustered in the C-terminal end of the protein and lead to disruption of microtubule-association of MID1, which then forms clumps in the cytosol and, therefore, leads to accumulation of PP2Ac at the microtubules. We could further show that the binding of $\alpha 4$ takes place through the Bbox1 of MID1 and that the cytoplasmic clumps consisting of C-terminally mutant MID1 harbour, in addition, $\alpha 4$ tightly bound to Bbox1 (Schweiger and Schneider, 2003; Trockenbacher et al., 2001).

In this thesis, two novel mutations in the Bbox1 region of MID1, namely A130T and C145S, both causing full-blown OS were described. Several lines of evidence indicate that these mutations specifically compromise the protein-protein interaction interface between MID1 and α4 without producing major changes in the overall structure of MID1. Mutations in the Bbox1 domain of MID1 seem not to disturb the structure of the entire protein, which can still perfectly bind to microtubules. In addition, circular dichroism (CD) spectra of intact and A130T mutated Bbox1 have shown no significant differences in their overall structure (R. Schneider, unpublished data). However, when examining the interaction with α4, it becomes obvious that mutations in this domain generate a MID1 form that is unable to bind α4. Hence, while Bbox1 mutated MID1 associates to microtubules, co-expressed α4 remains evenly distributed in the cell, can not be pulled down by MID1-immunoprecipitation and does not interact with MID1 in yeast two-hybrid experiments. Homology modelling of the MID1 Bbox1 structure based on the XNF7 structure shows that the respective leucine corresponding to A130 (Figure 4.1, underlined) is on the surface of Bbox1, intriguingly located at the bottom of a hydrophobic pocket (Borden et al., 1995b). This suggests that α4 might bind MID1 via this pocket, and that the exchange of the small hydrophobic alanine to the more bulky and slightly hydrophilic threonine could severely interfere with correct high binding affinity between α4 and MID1. On the other hand, using the same XNF7 model, residue C145 would participate in zinc binding. Since the ability to bind zinc is critical to maintain a native 3D structure of a protein, an exchange of the excellent metal ligand cysteine into a serine, would hinder the interaction between α4 and MID1. This change probably interferes with the correct folding of the domain and presentation of the hydrophobic core to α4, despite not having major apparent effects on the structure of the entire MID1, as it appears intact in immunofluorescence experiments.

C^{119}QF**C**^{122}DQDPAQDAVTK**C**^{137}VT**C**EVSY**C**^{142}DE**C**^{145}LKAT **H**^{150}PNKKPFTG**H**^{158} **MID1-Bbox1**

C^{6} SE**H**^{9} DERLKLY**C**^{17} KD**D**GTLS**C**^{25} VI **C**^{28} RDSL **H**^{34} AS**H**^{37} **XN F7-Bbox**

C^{175}LE**H**^{178} EDEKVNMY**C**^{187}VT**D**DQLI **C**^{195}AL**C**^{198}KLVGR**H**^{205} RD**H**^{208} **MID1-Bbox2**

Figure 4.1. Homology with XNF7 Bbox
Comparison of Bbox1- and Bbox2-MID1 sequences with Bbox-XNF7 sequence. Conserved cysteines and histidines including their positions are indicated in bold. A130 in Bbox1 is underlined. Zinc binding residues are indicated in red (Borden et al., 1995b). C139 and corresponding aspartic acid are indicated in blue.

Although yeast two-hybrid experiments with MID1 deletion constructs clearly indicated that exclusively Bbox1 is responsible for the binding to α4 (Trockenbacher et al., 2001), further analysis of Bbox2 mutations revealed that there is a clear regulatory influence of the Bbox2 on the interaction between α4 and MID1 *in vivo*. During this thesis, the study of a MID1-Bbox2 mutation (C195F), which has been found in an OS patient and affects a conserved residue (Figure 4.1), by immunofluorescence showed loss of MID1 colocalisation with microtubules and complete failure to bind α4 *in vivo*. However, immunoprecipitation and yeast two-hybrid experiments showed that α4-binding *in vitro* remains intact, which points at specific intracellular conditions that are mediated by the Bbox2 and are disturbed by this mutation.

Interestingly, when the study was extended to different engineered mutations, it was observed that the influence of Bbox2 mutations on *in vivo* binding abilities of MID1 change with the character of the particular mutation. While mutations disturbing conserved amino acids (Figure 4.1, amino acids in bold) hamper MID1 association with microtubules and α4, mutations affecting non-conserved amino acids only disrupt MID1 interaction with α4. These differences can probably be explained by more severe effects that some amino acid exchanges have on the global structure of the protein.

Most of the conserved residues included in this study, apart from being conserved, were supposed to participate in zinc binding, as predicted by comparative analysis with the XNF7 Bbox (C175, H178 and C198, marked in red in Figure 4.1). In C175A, H178Y or C198A mutated MID1, the unfair trade of a metal ligand residue, such as cysteine or histidine, for a residue unable to bind metal atoms, such as alanine or tyrosine, is likely to affect the correct stability and folding of the domain. Consequently, the disturbance of the overall structure of MID1 due to mutations in these residues denotes that intact zinc-binding plays a highly important role in the *in vivo* function of the Bbox2, as it appears not only to regulate α4-MID1 interaction, but also to influence the correct folding and positioning of the entire protein.

Nevertheless, the C195F mutation, despite not affecting a known zinc-binding site in Bbox2 residue, had similar drastic consequences as the above described mutations. It has been previously suggested that the conserved region between this cysteine and C187, which forms a flexible loop, composes another divalent metal binding site that involves interaction with another domain (Borden, 1998; Borden et al., 1995b). Given that the Bbox1 also contains some unligated putative metal ligands, such as C134, C139 or C142, it could well be that both domains cooperate to bind a zinc ion (Figure 4.1). Such an arrangement would also explain the strong influence that mutations in the Bbox2 domain exert on Bbox1 protein binding.

Mutations affecting non conserved residues on the other hand, such as Q192R or V183T, only affect the interaction of MID1 with α4 but not with the microtubules, which is another conspicuous indication of the close relation of the two Bboxes, and evidences the high

importance of an intact Bbox2 for the proper functioning of Bbox1, even when the protein is correctly folded and positioned.

Interestingly, mutations in a zinc-binding ligand in Bbox1, such as C145S, seem not to have similar drastic consequences on the global structure of the entire MID1. This might be due to an extra cysteine at position 139 of Bbox1 that has also been proposed to participate in zinc binding (Borden et al., 1995b; Reymond et al., 2001). This cysteine is commonly conserved in Bbox1 domains along RBCC proteins, while the Bbox2 and the XNF7-Bbox have a conserved aspartic acid at that position (Figure 4.1, blue residues). Therefore, this residue could further stabilise the structure of this domain, and of the entire protein (Borden et al., 1995b), and would keep effects of a C145 mutation mild.

In summary, during this thesis it could be shown that the Bbox2 domain acts as a flexible regulatory junction that provides the different domains of MID1 with their proper relative disposition, thus linking different components necessary for the MID1-ubiquitin ligase function. This would explain why *in vitro* experiments, which do not present the steric effects encountered in an *in vivo* system, allow the interaction of MID1 constructs having an intact α4-binding interface in Bbox1, despite having mutations in Bbox2. Further, they describe a novel

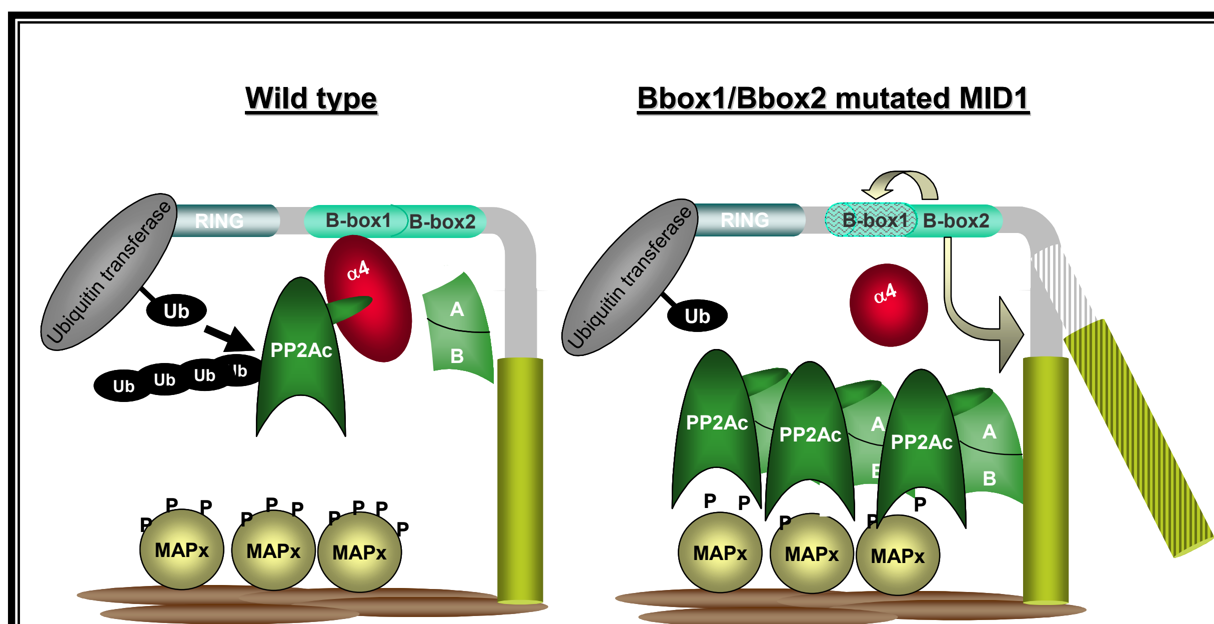

Figure 4.2. Pathomechanism model

After displacement of A and B subunits, microtubule-associated PP2Ac binds to the B-Box1 of MID1 via the α4 linker protein. In this conformation, microtubule-associated PP2Ac becomes ubiquitinated through MID1-E3 ubiquitin-ligase function and is degraded by the proteasome (left model-wild-type). Mutations in either the Bbox1 or the Bbox2 lead to a loss of MID1/α4/ PP2A interaction (right drawing- Bbox1/Bbox2 mutated MID1). Ubiquitination and degradation of PP2Ac is no longer possible and PP2Ac enriches at the microtbules. Two arrows indicate the parts of MID1 influenced by Bbox2 (adapted from Schweiger and Schneider, 2003).

pathomechanism for the development of Opitz BBB/G syndrome in patients with mutations in either the Bbox1 or the Bbox2 (Figure 4.2). In both cases, loss of interaction with α4 seems to be crucial, fact that once again underlines the importance of this protein as a linker between PP2Ac and the ubiquitin ligase MID1 (Schweiger and Schneider, 2003; Trockenbacher et al., 2001).

4.2 The MID1 multiprotein complex

4.2.1 MID1 forms part of a ribonucleoprotein complex

In addition to its previously identified association with tubulin (Schweiger et al., 1999), during this thesis it was found by affinity chromatography and MS, and subsequently verified by immunoprecipitation, that the MID1 complex includes several ribosomal proteins of the small subunit (S3, S8). Several other proteins that are also closely related to ribosome function namely EF-1α, NPM, RACK1 and p40, were found as well (Filipenko et al., 2004; Ford et al., 1999; Nilsson et al., 2004; Okuwaki et al., 2002; Tarapore et al., 2006; Vedeler and Hollas, 2000) in the protein complex. ANXA2 was also identified within the MID1 complex, fact that further supports a role for the MID1 complex at the ribosome (Filipenko et al., 2004; Vedeler and Hollas, 2000). Moreover, several heat shock proteins, Hsp90, Hsc70 and Hsc60 were identified in the complex, together with another multifunctional chaperone protein, p32 (Storz et al., 2000). Among these heat shock proteins, special attention should be drawn to Hsp90. Besides assisting protein folding, and in contrast to other heat shock proteins, Hsp90 is known for having many different substrates or “clients” and facilitating their functions (Richter and Buchner, 2001). Interestingly, it has been shown this year that Hsp90 also regulates ribosomal function by protecting several ribosomal proteins including S3 (novel component of the MID1 complex) and S6 (a downstream factor of the TOR pathway, to which the MID1 complex is related (Schweiger and Schneider, 2003)), from ubiquitination and proteasome dependent degradation, thereby regulating their stability and activity (Kim et al., 2006).

Association of MID1 complex with the big ribosomal subunit, 60S, was confirmed by immunoprecipitation experiments. Following, MID1 complex association with the entire ribosome was confirmed and characterised by discontinuous sucrose gradients. Different experimental conditions demonstrated it to be a stable association, which was only disrupted by high concentrations of salt or by substances that cause ribosomal disruption, such as EDTA or puromycin. Some other members of the complex such as RACK1 or NPM (Nilsson et al., 2004; Okuwaki et al., 2002), which are known to be more closely related to ribosomes, remained bound under all conditions tested. However, although an interaction of the MID1 complex with ribosomes could be demonstrated, it remains unclear whether MID1 itself associates directly or through any of the previously reported ribosome-related proteins.

Similar to other well-characterised proteins forming RNPs, such as FMRP (Tamanini et al., 1996), RNase A treatment prior to gradient loading demonstrated that the association of the MID1 complex with ribosomes is RNA-dependent. In addition, association of the complex to poly-rG ribopolymers and G-quartet like RNA structures in Eph receptor and ephrin mRNAs could be proven in this thesis. Since direct association of MID1 through its zinc-binding domains with RNA could not be confirmed and MID1 does not contain any of the known RNA binding domain, MID1 most probably incorporates RNA into the complex through any of the RNA-binding proteins of the complex or through an RNA helicase that has been found previously to interact with MID1 in a yeast two-hybrid screen (R. Schneider, unpublished data).

4.2.2 The components of the MID1 complex associate with microtubules

In agreement with MID1 association with microtubules (Schweiger et al., 1999), most of the novel members of the MID1 complex could be found in microtubule fractions, pointing to an important function of the complex exerted at the microtubules. Supporting this hypothesis, the majority of the proteins identified in the MID1 complex have previously been reported to associate with microtubules. Thus, EF-1α, one of the most abundant proteins, has been reported to participate in the compartmentalisation of protein translation at the cytoskeleton (Condeelis, 1995). In addition, it has been shown to be a microtubule-associated protein that binds, stabilises and promotes assembly of microtubules *in vitro*, independently of its role in protein translation (Moore and Cyr, 2000; Moore et al., 1998; Ohta et al., 1990; Shiina et al., 1994).

Hsp90, another abundant cytosolic protein, and several Hsc70 homologues have also been found to localise at microtubules (Czar et al., 1996; Liang and MacRae, 1997). Since some of its clients need to be transported in the cell as a prerequisite to carry out their function, Hsp90 has been proposed to participate in microtubule-based movements (Craig et al., 1994). Moreover, it has been shown to be a component of the main microtubule organizing centre (MOTC), the centrosome, ensuring its correct functioning, including microtubules nucleation and centrosome duplication (de Carcer, 2004; de Carcer et al., 2001; Doxsey, 2001; Lange et al., 2000). Similarly, NPM has been found to participate in centrosome duplication upon phosphorylation by CDK2/cyclin E on Thr^{199} (Okuda, 2002; Tokuyama et al., 2001). In addition, it participates in a variety of mitotic processes, upon phosphorylation of Ser^{4} through polo-like kinase1 (Plk1), the major mitotic regulator kinase (de Carcer, 2004; Zhang et al., 2004).

ANXA2 and RACK1 have also been shown to exert some of their functions in collaboration with the cytoskeleton. ANXA2 is the most abundant protein in fractions containing cytoskeleton-bound polysomes (Vedeler and Hollas, 2000), and RACK1 was found to reside in cytoskeleton fractions from unstimulated fibroblasts and epithelial cells (Hermanto et al., 2002).

The fact that many of the proteins identified in the MID1 complex have previously been localized to microtubules, in addition to their ribosomal function, appears to be of outstanding significance for the putative function of the complex. This implies that the MID1 complex, including PP2A and α4, could well be the core of a microtubule-associated translation unit that carries active polysomes and RNA.

4.2.3 The MID1 complex and its role in translation at the microtubules

RNPs are often described as functional venues that carry pre-mRNAs and mRNAs in the cell. Proteins participating in these complexes mostly exercise functions on mRNA export, localisation, translation and stability. At the same time, contained mRNAs need to have mRNA localisation signals, which are commonly situated in the 3´UTR, where they are the least likely to interfere with any other function. In addition, RNPs are highly dynamic complexes, in which their components, usually fulfilling a large variety of functions, come and go, responding to the necessities of the complex at a given moment (reviewed in Dreyfuss et al., 2002; Mohr and Richter, 2001; St Johnston, 2005). A central function of RNPs is the active transport along the cytoskeleton in order to ensure asymmetric mRNA localisation in the cell. Some of the best-studied systems in which asymmetric RNA localisation has been reported to be fundamental are oocyte polarisation, embryonic axis formation (Bashirullah et al., 1998; Driever and Nusslein-Volhard, 1988; Lasko, 1999; Lehmann and Nusslein-Volhard, 1986), and local protein synthesis in dendrites and growth cones (Steward and Schuman, 2003; Steward and Worley, 2001).

FMRP, mutated in patients with Fragile X syndrome (FXS, one of the most frequent causes of mental retardation), is a well-established example of an RNP in charge of translation and mRNA transport along the cytoskeleton in neurons (Antar et al., 2004; Bagni and Greenough, 2005; Zalfa and Bagni, 2005). A subset of brain mRNAs, including EF-1α (Sung et al., 2003), has been identified to associate with FMRP-containing RNPs (Brown et al., 1998; Sung et al., 2000; Zalfa et al., 2003). In addition, its association with polyribosomes and subsequent link with translation has also repeatedly been reported (Ceman et al., 1999; Feng et al., 1997; Tamanini et al., 1996; Zalfa and Bagni, 2005).

Another RNA-binding protein, Staufen, also forms an RNP complex with transport-cargo properties along the cytoskeleton. It has been in-depth studied for its role in mRNA trafficking and translation in *Drosophila*, where it plays an essential role in the localisation of *oskar* and *bicoid* mRNAs in the oocyte to the posterior and anterior poles respectively (Li et al., 1997; St Johnston, 2005). In mammals, the two homologues of Staufen, have also been shown to form RNPs, to associate with polyribosomes, and to participate in microtubule-dependent transport of RNA in neurons (Kanai et al., 2004; Kiebler et al., 1999; Tang et al., 2001b; Thomas et al., 2005). Interestingly, it has been recently reported to co-immunoprecipitate with FMRP, EF-1α,

nucleolin, tubulin, protein phosphatase 1, HuR and RNA helicase A (Thomas et al., 2005; Villace et al., 2004), showing that mammalian Staufen is closely related to the previously described FMRP-RNP.

After having characterised the MID1 complex, it is striking noticeable its properties resemble many of the characteristics required to be a RNP in charge of transport along microtubules, supporting a novel model for the function of the MID1 complex (Figure 4.3). Similar to FMRP and Staufen, the MID1 complex associates with polyribosomes, the translation related protein EF-1α, several multifunctional proteins, microtubules and RNA. Moreover, PP2A and its regulatory subunit, α4, are also main components of the complex, which integrate the MID1 complex into the TOR pathway, one of the main translation regulatory pathways (Schweiger and Schneider, 2003). However, MID1 has never been found in the nucleus, where the target mRNAs to be transported should be recruited, which suggests that any of the other members of the complex would bring the mRNAs from the nucleus to the microtubules. An attractive candidate would be NPM, which binds RNA, is involved in ribosome biogenesis, and has been shown to shuttle between the nucleus and the cytosol.

A required motor protein, which would allow the movement of the MID1 complex along the microtubules remains to be identified.

4.2.4 The MID1 complex and translational repression

For the successful completion of mRNA localisation and compartmentalisation of protein production in the cell, the translation of the transported mRNAs needs to be repressed during transport and until their protein functions are required. Although little is known about the mechanisms governing translational repression during transport, some processes have been already described (St Johnston, 2005).

For example, in *Drosophila* oocytes and early embryos, translation of the anteriorly localised *bicoid* mRNA transcripts is regulated by delayed cytoplasmic polyadenylation of the respective mRNAs, which formerly carry a very short poly(A) tail incompatible with efficient protein translation. A more complicated process that has been described for mRNAs localised to the posterior pole, such as *oskar*, involves the binding of the translation repressor Bruno to *cis*-elements in its 3´UTR while the transcript is being transported to the pole and, once there, Bruno falls off and translation is derepressed. However, in recent years, the role of RNA silencing in translational repression as a novel mechanism for translational repression has gained attention. At least two genes that encode components of the RNA silencing pathway, namely *aubergine* and *armitage* (involved in microtubule polarisation), have been implicated in the localisation of *oskar* transcripts (Kavi et al., 2005; Kloc and Etkin, 2005; Wilhelm and Smibert, 2005). Similar mechanisms for the regulation of translation have been attributed to the FMRP protein, which has been shown to associate with non-coding RNAs (ncRNA) and

microRNAs (miRNA) that contain sequences complementary to FMRP target mRNAs and thereby, represses their translation (Jin et al., 2004a; Jin et al., 2004b; Zalfa et al., 2005).

During this thesis, it was described an interaction of a microtubule-associated translation unit with PP2A and its negative regulators α4 and MID1, all essential players of the mTOR pathway, that regulates the translation of selected capped and 5´TOP mRNAs (Duvel and Broach, 2004; Fingar and Blenis, 2004; Peterson et al., 1999). Selective up-regulation of PP2A activity at low MID1/α4 levels or activities during transport could therefore be another mechanism of translational inhibition in mRNPs (Figure 4.3). Interestingly, last year, it was reported that RACK1 mRNA contains a TOP motif and that its translation depends on a rapamycin-sensitive pathway, such as the mTOR pathway (Loreni et al., 2005). Since RACK1 is one of the complex components, this would suggest self-regulatory properties of the complex.

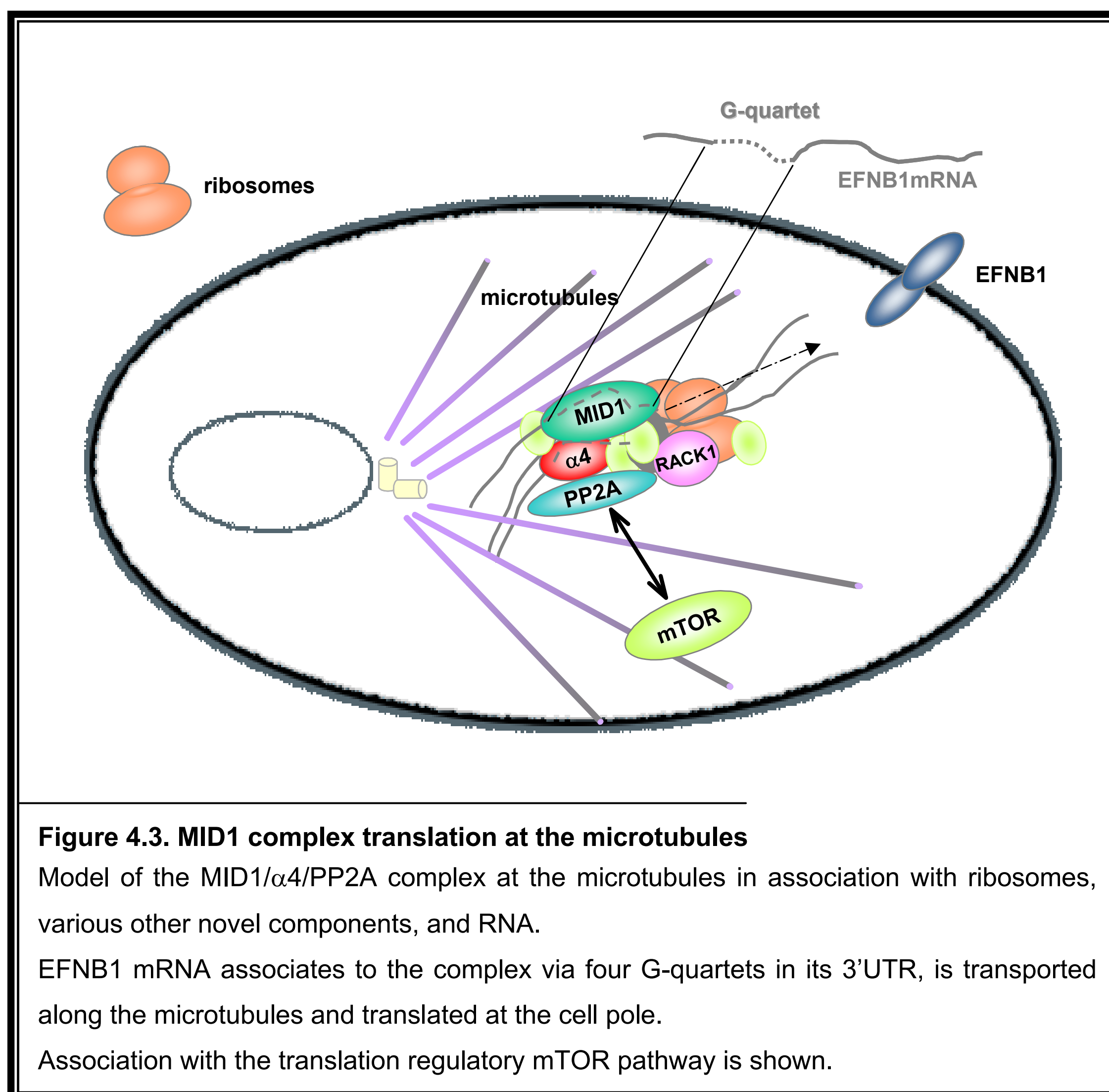

Figure 4.3. MID1 complex translation at the microtubules
Model of the MID1/α4/PP2A complex at the microtubules in association with ribosomes, various other novel components, and RNA.
EFNB1 mRNA associates to the complex via four G-quartets in its 3'UTR, is transported along the microtubules and translated at the cell pole.
Association with the translation regulatory mTOR pathway is shown.

Nevertheless, it would also be interesting to know whether the MID1 complex carries any miRNAs or ncRNA that could assist the complex in regulating the translation of transported

mRNAs. In addition, the identification of mRNAs carried by the complex will also shed light on how the MID1 complex regulates the translation of its target mRNAs.

4.2.5 MID1 complex and development of the ventral midline development: implications in cell migration

As mentioned previously, improper function of the MID1 complex leads to X-linked OS, which is characterised by defective fusion of ventral midline structures, whose establishment heavily rely on polarised cells, including asymmetric distribution of signalling molecules and reorganization of the cytoskeleton. Two fundamental processes during midline development governed by this mechanism are NCC migration and EMT of epithelial cells (Jones and Trainor, 2005; Roessler and Muenke, 2001; Schweiger and Schneider, 2003). Dysfunctional transport of mRNA along the microtubule in defective MID1-containing RNPs would, as suggested in this thesis, inhibit asymmetric protein production in cells involved in these processes and therefore, would form the molecular basis for an attractive model for the development of OS.

Cell polarisation, adhesion and migration are fundamentally related processes that require the asymmetric concentration of cellular activities mediated by the cytoskeleton. Cell migration involves the formation of protrusions such as lammellipodia and filopodia at the leading edge of the cell, followed by nuclear translocation and retraction of the cell rear (Small and Kaverina, 2005). During migration, adhesion to the extracellular matrix, commonly mediated by integrins, needs to be carefully regulated for the cell to move properly. The family of small Rho GTPases has an essential role in the regulation of adhesion formation and cytoskeleton reorganization. Although cell migration has often been described in terms of actin cytoskeleton reorganization, already in 1970 it was shown that also the disruption of microtubules in fibroblasts leads to disruption of cell polarity and arrest of direct locomotion (Vasiliev et al., 1970). However, how actin and microtubule cytoskeletons collaborate to provide correct migration is only starting to be understood (Kole et al., 2005).

Microtubules are often discussed as cellular highways that transport signalling molecules to modulate the tension of the actin cytoskeleton and the disassembly of cell adhesions. They have been shown to meet actin at focal adhesions formed at the cell front and rear during migration and negatively regulate them by promoting their turnover or impeding their growth (Kaverina et al., 1999; Krylyshkina et al., 2003; Palazzo and Gundersen, 2002). In addition, active transport along the microtubules has been shown to be essential for cell migration; kinesin inhibition mimics the changes in cell polarisation and adhesion found during microtubule disruption with nocodazole (Kaverina et al., 1997; Krylyshkina et al., 2002). In addition, focal adhesions have microtubule-capturing and -stabilizing abilities that could help to prolong the communication of the microtubules with focal adhesions and thereby, allow the delivery of more signals (Kaverina et al., 1998).

Recently, novel adhesion structures have been defined, namely spreading initiation centres (SICs), which only appear in early stages of cell migration. Despite being highly similar to focal adhesions, they have been shown to also contain RNA, RNA-binding proteins and RACK1, one of the MID1/α4 interaction partners, supporting that asymmetric mRNA transport and compartmentalised protein translation of proteins is required for the formation of focal adhesions (de Hoog et al., 2004). In line, it has previously been shown that cytoskeleton dependent recruitment of mRNA and ribosomes to focal adhesions provides local synthesis of proteins in response to integrin-mediated signalling from the extracellular matrix and mechanical tension (Chicurel et al., 1998). According to these results, it has been proposed that not only molecular trafficking, but also translation, might be required in the early establishment of focal adhesions (Nilsson et al., 2004).

Knowing that the MID1 protein bundles and stabilize microtubules, a critical step for the organization of the leading edge (Schweiger et al., 1999), and that the MID1 complex, which contains RNA-binding proteins, ribosomes, RNA and RACK1, participates in mRNA localisation via microtubules, it is very appealing to think that it could also participate in the transport of mRNAs required for the formation and regulation of early focal adhesions or SICs. Moreover, the MID1 complex contains some proteins, such as RACK1 or ANXA2, that also associate with the actin cytoskeleton; therefore, the complex might as well provide a link for the cross-talk communication between actin and microtubules.

4.2.6 The MID1 complex components and their involvement in cell migration, polarisation and adhesion

Apart from associating with SICs, RACK1 has previously been shown to be a scaffolding protein that participates in cell spreading, establishment of early focal adhesions and cell-cell contacts. To fulfil these functions, it recruits Src, STATs and PKCs, among others, and links them to integrin receptors upon activation by insulin-like growth factor I receptor (IGF-IR) signalling (Cox et al., 2003; Hermanto et al., 2002; Meares et al., 2004; Miller et al., 2004; Sklan et al., 2006; Zhang et al., 2006). As mentioned before, RACK1 has also been proposed to be involved in the localisation of translation by the recruitment of RNA and ribosomes to focal adhesions (Nilsson et al., 2004). In addition to RACK1, p32, another interaction partner of the MID1/α4 complex, bind atypical PKC isozymes (Sklan et al., 2006; Storz et al., 2000), which respond to integrin signalling and are central players in the regulation of cell spreading and focal adhesion assembly (Disatnik et al., 2002; Henrique and Schweisguth, 2003). Independent studies have also shown that activation of p32, in collaboration with integrins, induces cell adhesion and spreading in endothelial cells (Feng et al., 2002; Ghebrehiwet et al., 2003). Interestingly, p32 (also referred as to Hyaluronan binding protein 1, HABP1) interacts with hyaluronan (HA), which is known to form a pericellular matrix concomitant to detachment during

mitosis or cell migration and, in combination with HA, p32 has been shown involved in the regulation of adhesion and de-adhesion (Sengupta et al., 2005).

Another member of the complex that has been closely related to cell adhesion is p40, a conserved receptor of laminin. Laminin is a very abundant extracellular molecule in basal laminae synthesized in very early embryos or in epithelial cells (Ford et al., 1999). Curiously, p40 has always attracted attention for its dual function in cell adhesion and ribosomal assembly and maintenance. Recently, it has been found that midkine also binds to p40 thereby competing with laminin, and that this interaction leads to enhanced protein translation (Kazmin et al., 2003). Interestingly, coming back to the mechanisms of ventral midline development, midkine is highly expressed in mouse NCC during development and plays an important role in cell migration (Mitsiadis et al., 2003; Mitsiadis et al., 1995; Qi et al., 2001).

Also ANXA2, another complex partner, has been shown to participate in cell adhesion and migration. Thus, it rapidly localises to cell-cell contacts after IGF-RI stimulation (Meares et al., 2004; Zhao et al., 2003), and has been suggested to be responsible for the initial recruitment of GTPase activity, that is essential for the formation of filopodia at the leading edge of migrating fibroblasts and epithelial cells (Balch and Dedman, 1997; Hansen et al., 2002; Nobes and Hall, 1995).

The importance of the microtubule-organizing centre, the centrosome, in cell polarisation and migration should also not be forgotten. The centrosome determines the direction of migration by placing itself in front of the nucleus, and projecting microtubules to the leading edge (Badano et al., 2005). After generation of the leading edge, and for the successful progression of migration, the nucleus must be translocated towards the front, a process that is highly governed by the centrosome. Alterations of this process have been linked to monogenic disorders. For instance, some mutations in lissencephaly gene 1 (LIS1) and doublecortin gene (DCX), the protein products of which localise to centrosomes, lead to defects in neural migration and microtubule dynamics. A connection between these proteins and the MID1 complex has been suggested previously since their phosphorylation status is regulated by microtubule-associated PP2A activity (Schweiger and Schneider, 2003; Trockenbacher et al., 2001). Interestingly, two novel members of the MID1 complex, namely NPM and Hsp90, have been shown to also play key roles in centrosome function.

In our group, we have observed that overexpression of $\alpha4$ in HeLa or COS-7 cells results in the formation of filopodia to which $\alpha4$ locate, indicating that it might play a role in the formation or regulation of filopodia. These protrusions are no longer observed when MID1 recruits $\alpha4$ to microtubules in non-polarised cells (Schweiger et al, unpublished data). Given that filopodia formation in the leading edge is essential for cell migration, further studies would be necessary to investigate whether MID1 could be involved in bringing $\alpha4$ to the locations

where protrusions need to arise in polarised cells, and whether α4 indeed plays a role in filopodia formation or regulation.

Last but not least, ephrin molecules (ligands and receptors), and specially ephrin-B1, the mRNAs of which were shown during this thesis to be associated to the MID1 complex, are also involved in cell migration and, in particular, in the migration of NCC. Apart from being known for having Rho family GTPases as the major downstream targets, which points at a central involvement in the formation of focal adhesions (see above), it has been shown that EphB-ephrin-B engagement is a critical determinant of integrin-mediated responses (Huynh-Do et al., 1999; Pasquale, 2005). In line, it has been shown that ephrin-B1 and EphB2 regulate the cytoarchitecture and spatial organization of kidney cells through Rho family GTPases, and that EphB activation promotes cell adhesion and induces focal adhesion enlargement (Ogawa et al., 2006).

In summary, most of the proteins that compose the MID1 complex have been reported to participate in cell adhesion, migration and local protein synthesis. Therefore, a concerted organization of their functions in the MID1 complex is an attractive model for the regulation of proper cell migration, polarisation, and adhesion, all essential processes for the correct development of the ventral midline.

4.2.7 EphB and ephrin-B mRNAs can be integrated in the MID1 complex

As earlier outlined, Eph receptors and ephrins have key roles in the regulation of cell migration, polarisation and adhesion during development. Their functions, often described in the context of NCC pathfinding, heavily rely on the establishment of asymmetric gradients of their proteins in polarised cells (Pasquale, 2005; Poliakov et al., 2004; Robinson et al., 1997). This makes them particularly attractive mRNA candidates to be positioned in the cell via a mechanism involving mRNA transport along the microtubules.

Within the frame of this study, it was demonstrated that the MID1 complex associates to G-quartet like structures mainly in the 3´UTRs of EphB receptor and ephrin-B mRNAs. G-quartets were identified in two ephrins (B1 and B2) and five Eph receptor mRNAs (B1, B2, B3, B4, B6), indicating that probably only those ephrin molecules participate in functions exerted by the MID1 complex. However, it is not possible to exclude a role for the others in the MID1 complex, which might contain another not-yet-targeted sequence in their mRNAs that also associates with the MID1 complex. Interestingly, it was possible to shown that the number of identified G-quartets varies among the ephrin molecules with up to four motifs identified in the *EFNB1* mRNA, mutations of which lead to craniofrontonasal dysplasia (Wieland et al., 2004). While G-quartets were present in 22% of all genes available from the ESEMBL database, only 1,33% of those had more than 3 G-quartets. In RNA-protein pull-down experiments, it was

further showed that increasing numbers of G-quartets in EFNB1 lead to an increase in protein binding affinity, which is not dependent on the length of the transcript. Consequently, the additive effect of the protein binding affinity seen with the *EFNB1* mRNA combined with a systematic displacement of lower affinity binding mRNAs might be an effective mechanism for a highly specific inclusion of G-quartet containing mRNAs in the described mRNPs.

As mentioned previously, the phenotypes of craniofrontonasal dysplasia syndrome and OS present striking overlaps, both of them being characterised by malformations of the facial ventral midline, such as hypertelorism/telecanthus or broad nasal bridge. This kind of malformation is most probably caused by defective cell migration and/or polarisation, and most likely affects NCC-migration and -orientation (Schweiger and Schneider, 2003; Wieland et al., 2004). Interestingly, recently, it has been reported that cardiac and cranial NCCs, but not trunk NCCs, require EFNB1 to migrate properly during development in mice (Davy et al., 2004). Moreover, these NCCs require EFNB1 autonomously and non-autonomously to properly complete craniofacial development, especially during palate elevation and fusion, an important process disrupted in many patients with OS and craniofrontonasal dysplasia. Furthermore, it has been shown that ephrin-B1, in addition to other EphB receptors and B-class ligands, participates in regulating the formation of the corpus callosum (Mendes et al., 2006), which also presents with defects in some OS patients. Given that EFNB1 is localised at chromosomal position Xq12-q13.1, it was also suggested as a candidate gene for FG syndrome, another ventral midline malformation syndrome, which has been mapped to Xq12-q21.3.

Therefore, the findings of this thesis, which involve the MID1 complex assisted transport of Eph receptor and ephrin mRNAs to the required places of the migrating cell in order to establish a protein gradient, are an attractive explanation for the conspicuous phenotypic overlap between OS and craniofrontonasal dysplasia and other midline malformation syndromes.