# 5 Discussion

## 5.1 The RuvBL1 structure exhibits a typical nucleotide-binding pocket and a novel domain important for its cellular functions

The crystal structure of RuvBL1 showed the formation of a hexamer in which each monomer appeared complexed with one ADP molecule. It displayed all the structural motifs for ATP binding and hydrolysis, and exhibited some similarities to the bacterial ATPase and helicase RuvB. Both proteins fold into three domains. Two of the three domains of RuvBL1, namely DI and DIII, contained the conserved motifs needed for ATP binding and hydrolysis, and resembled RuvB domains. DI of RuvBL1 and DI of RuvB are similar, both being a triangle-shaped nucleotide-binding domain with a Rossmann-like α/β/α fold composed of five parallel β-strands with two flanking α-helices on each side. The smaller third domain of RuvBL1, DIII, was composed of five α-helices and resembled DII of RuvB. This domain formed a bundle located near the Walker A motif and was also important for ATP binding. The core five-stranded β-sheet of the Rossmann-like α/β/α fold in domain I was similar to the $AAA^+$ module of other family members, such as RuvB (branch migration) (Putnam et al. 2001), NSF-D2 (membrane fusion) (Lenzen et al. 1998), SV40 large tumour antigen (replication of viral DNA) (Enemark and Joshua-Tor 2006; Gai et al. 2004; Li et al. 2003), the $AAA^+$ domain of PspF (transcription activation) (Rappas et al. 2006) and the hexameric ATPase P4 of dsRNA bacteriophage ϕ12 (RNA packaging inside the virus capsid) (Mancini et al. 2004). However, there was a striking difference between RuvBL1, RuvB and the other $AAA^+$ proteins. The domain I of RuvBL1 contained an insertion of 174 amino acids constituting a novel domain which appeared to be unique to RuvBL by database searches (Putnam et al. 2001). This insertion is not present in RuvB and other $AAA^+$ proteins and may explain why the RuvB structure could not be used as a model for the Molecular Replacement method in order to solve the three-dimensional structure of RuvBL1. For that reason, the selenomethionine-substituted RuvBL1 was used for structure determination with the Single-Wavelength Anomalous Dispersion method.

The spatial arrangement of the seven β-strands in the novel domain II of RuvBL1 resembled that of the DNA-binding domains of different proteins involved in DNA metabolism, such as replication protein A (Bochkarev et al. 1997). The nucleic acid-binding studies I performed with the purified domain confirmed that DII represents a new functional domain of RuvBL1 important for DNA/RNA binding.

Other groups have previously localised RuvBL1 and RuvBL2 regions responsible for interaction with proteins such as c-Myc and β-catenin (Bauer et al. 2000; Weiske and Huber 2005; Wood et al. 2000). Interestingly, the crystal structure of RuvBL1 showed that these regions belong to domain II, which is ideally situated to interact with other proteins as it protrudes out of the hexameric ring. Diverse studies demonstrate that RuvBL1 and RuvBL2 have opposite effects on transcriptional regulation. RuvBL1 mostly activates transcription, whereas RuvBL2 limits the activities of diverse transcription factors (Bauer et al. 2000; Cho et al. 2001; Wood et al. 2000). For example, both proteins have antagonistic effects on gene expression regulated by β-catenin (Bauer et al. 2000; Rottbauer et al. 2002). It was shown that the binding site for β-catenin in RuvBL1 and RuvBL2 is located in the region encompassing amino acids 183-210 and 187–210, respectively (Bauer et al. 2000). The crystal structure of RuvBL1 demonstrated that this region is situated in domain II. It is conceivable that two different proteins arose from bacterial RuvB in the course of evolution in order to create an activating and a repressing protein for the control of important gene regulatory processes.

RuvBL1 and RuvBL2 also interact with c-Myc, a transcription factor involved in oncogenic transformation processes and often mutated in human cancers. The c-Myc-binding site in RuvBL1 and RuvBL2 was mapped to amino acids 136-187 (Wood et al. 2000) which belong to the accessible domain II. The N-terminal portion of c-Myc contains the MbII domain necessary for all c-Myc biological activities, including oncogenic transformation, apoptosis and the ability to block differentiation and stimulate cell proliferation (Evan et al. 1992; Li et al. 1994; Penn et al. 1990; Stone et al. 1987). This MbII domain was shown to bind to domain II in RuvBL1 and RuvBL2 (Wood et al. 2000). A missense mutation in the RuvBL1 ATPase motif acts as a dominant inhibitor of c-Myc oncogenic activity but does not inhibit normal cell growth (Wood et al. 2000). It can be speculated that the association with c-Myc stimulates the ATPase activity of RuvBL1, which is in turn necessary for the activation of target genes. While RuvBL1 is an essential mediator of c-Myc oncogenic transformation (Dugan et al. 2002; Wood et al. 2000), no role has been determined for RuvBL2 in this pathway, although it also binds to c-Myc.

Recently it has been shown that Hint1, a co-regulator in TCF-β-catenin-mediated transcription, affects oligomerisation of RuvBL1 and RuvBL2 by directly binding to both proteins (Weiske and Huber 2005). The Hint1-binding region in RuvBL1 and RuvBL2 was localised to amino acid residues 214-295 and 218-289, respectively. The authors suggested that this region serves for multimerisation, because binding to Hint1 disrupts formation of

RuvBL1/RuvBL1, RuvBL2/RuvBL2 and RuvBL1/RuvBL2 complexes. The crystal structure of the RuvBL1/RuvBL2 complex supports the assumption that Hint1 disrupts RuvBL1/RuvBL2 complex formation, because the mentioned residues are located in DII and are involved in the interaction between the two hexameric rings. In contrast, the three-dimensional structure of the RuvBL1 hexamer showed that residues 214-295 of domain II are not important for oligomerisation, because the interface between monomers within the hexamer is made up entirely by DI and DIII. However, it is possible that association of Hint1 with a part of DII close to the hexameric ring sterically disturbs the interaction between the monomers. Based on these findings, it is a very attractive theory that certain cofactors could recruit RuvBL1 or RuvBL2 to specialised functions by disturbing RuvBL1/RuvBL2 complex formation. One example for this is the finding that RuvBL2 binds to ATF2 and affects its transcriptional activities, whereas RuvBL1 does not (Cho et al. 2001). An additional example is the observation that RuvBL1 is a key modulator of the apoptotic activity for E2F1 by virtue of its binding to the transactivation domain (Dugan et al. 2002), while RuvBL2 does not seem to play a role in this pathway. Also in mitosis, RuvBL1 and RuvBL2 could play separate roles in different functional complexes as both proteins show distinct localisations during cell division (Sigala et al. 2005).

The crystal structure of RuvBL1 in combination with the interaction studies previously conducted by other groups highlight that RuvBL1 and RuvBL2 interact with transcription factors and other proteins mainly through their novel domain II, which is not present in RuvB and other $AAA^+$ family members. These findings suggest that the insertion of DII in domain I of RuvBL1 and RuvBL2 occurred during evolution in order to extend the functions of these eukaryotic proteins beyond that of bacterial RuvB.

## 5.2 RuvBL1 and RuvBL2 not only function separately but also assemble into a dodecameric complex and act together

In agreement with other published data (Ikura et al. 2000; Puri et al. 2007), my experiments showed that RuvBL1 and RuvBL2 formed an equimolar complex *in vitro*, with a molecular mass of about 600 kDa corresponding to a dodecamer. Indeed, the three-dimensional structure of the RuvBL1/RuvBL2 complex revealed a dodecamer with a central channel consisting of two hexameric rings which interact with each other via the domains II. The hexameric ring shaped structures of RuvBL1 and the dodecameric RuvBL1/RuvBL2 complex correlate well with data on other $AAA^+$ proteins which have also been shown to form hexamers or double

hexamers *in vitro*. Examples include RuvB (Putnam et al. 2001), SV40 (Li et al. 2003) and MCM (minichromosome maintenance) (Pape et al. 2003). The composition of the two hexameric rings could not be determined in this investigation, because the structures of RuvBL1 and RuvBL2 are too similar to allow distinction between both proteins at 4 Å resolution. The quality of the complex crystals needs to be improved in order to obtain higher resolution data. Clear electron density of the amino acid side chains is required to distinguish between RuvBL1 and RuvBL2 monomers in the complex. The relatively low resolution of the R1-DII/R2-DII structure may be due to the presence of 10 % hexamers beside the dodecamers in solution, which may prevent the best diffracting crystals to grow thicker. An adequate gel filtration material separating complexes in the high molecular weight range above 200 kDa may allow the isolation of the dodecameric complex from the remaining 10 % hexamers, provided that the existence of hexamers is not due to equilibrium of association and dissociation of the dodecameric complex.

However, the equimolar ratio of RuvBL1 to RuvBL2 could represent either two homohexamers or mixed hexamers containing equimolar amounts of each protein. Analysis of the interacting regions between both hexamers revealed a particular helix located in domain II. In RuvBL1 this helix contained two positively charged Lys residues that would repel each other in case of helix-interaction between two RuvBL1 monomers in opposite hexamers. These findings probably favour a model consisting of two homohexamers formed by RuvBL1 and RuvBL2, respectively. The electron microscopy structure of the wild-type RuvBL1/RuvBL2 complex previously examined at 20 Å resolution also supports this hypothesis (Puri et al. 2007). In agreement with the crystal structure of RuvBL1/RuvBL2, the results show two stacked hexameric rings forming a dodecamer with flat surfaces at the top and the bottom. The electron microscopy structure of the complex solution shows clear differences between the top and bottom rings (Puri et al. 2007), whereas one hexameric ring closely resembles the RuvBL1 hexamer determined by x-ray crystallography. In contrast with this, both hexamers looked very similar within the dodecameric crystal structure of the truncated RuvBL1/RuvBL2 complex. It is possible that the hexameric rings in the wild-type complex are more flexible and have slightly different conformations leading to differences between both rings. Unfortunately crystallization trials with the wild-type complex were never successful.

I also attempted to solve the structure of RuvBL2. Since the crystals of wild-type RuvBL2 did not diffract, I cloned different constructs, mainly surface mutants and truncated versions of RuvBL2. One construct with a truncated domain II, namely RuvBL2-DII (RuvBL2ΔE134-

E237) crystallized. Although the crystals diffracted to 2.3 Å resolution, the diffraction pattern of RuvBL2 exhibited a high mosaicity and could not be used for structure determination. Mosaicity was either due to the long c-axis of the unit cell or to a partial internal disorder within the crystal. An appropriate additive stabilising the protein and increasing the internal order of the crystal was not identified yet, but could probably improve the diffraction pattern of the RuvBL2-DII crystals.

The proportions of hexamers and dodecamers in the RuvBL1/RuvBL2 complex solutions were determined with SAXS and gave interesting results. Compared to the complexes with a truncated domain II, the RuvBL1wt/RuvBL2wt complex had the highest amount of hexamers (28 %) beside dodecamers (72 %) in solution. In contrast to this, the RuvBL1wt/RuvBL2-DII complex consisted almost completely of dodecamers (99 %), while the complex of RuvBL1-DII and RuvBL2-DII was composed mainly of dodecamers (90 %) and only 10 % hexamers. These findings suggest that domain II, which was very flexible in the hexameric structure of RuvBL1, might be more stabilised in the truncated complexes (R1wt/R2-DII and R1-DII/R2-DII) compared to the wild-type complex (R1wt/R2wt), thus improving the interaction of hexamers and favouring the formation of stable dodecamers. It is possible that the dodecameric wild-type complex of RuvBL1 and RuvBL2 containing the full-length flexible domain II fluctuates more within the cell and only associates when it is needed for the cellular function, for example during chromatin remodelling. It can probably dissociate into hexameric rings afterwards so that RuvBL1 and RuvBL2 can work separately. As discussed above, it is conceivable that other proteins recruit RuvBL1 and RuvBL2 to specialised functions by interacting with their domain II. Since this domain was responsible for association of the two hexameric rings, the interaction with other proteins might disturb the dodecameric structure. It is important to note that dodecameric complexes of RuvBL1 and RuvBL2 have never been isolated in *in vivo* studies. It is therefore unknown whether the association of two hexameric rings forming a dodecamer only happens *in vitro*. Although they are found together as part of larger complexes in a 1:1 molar ratio, it is possible that within the complexes RuvBL1 and RuvBL2 are bound to other proteins that disturb the interaction between hexamers.

## 5.3 The functions of RuvBL1 and RuvBL2 require binding to nucleic acids

The involvement of RuvBL1 and RuvBL2 in chromatin remodelling, DNA repair and transcription (Bauer et al. 2000; Fuchs et al. 2001; Ikura et al. 2000; Jonsson et al. 2004; Shen

et al. 2000) implies that they can bind to ds and ssDNA. In addition, RNA binding could be important during transcription and small nucleolar ribonucleoprotein assembly (Watkins et al. 2004). The diameter and the electrostatic potential of the central channel of RuvBL1 are indications that it may bind single-stranded nucleic acids. The channel diameter of about 18 Å is comparable to the values determined for other hexameric $AAA^+$ proteins, such as replicative helicase RepA (Niedenzu et al. 2001) (PDB 1GY8) or the E1 replicative helicase from Papillomavirus (PDB 2GXA). The diameter is in all cases too small for dsDNA to pass through. Several hexameric $AAA^+$ proteins are known to interact with nucleic acids, with the central channel of the hexamer as the most likely main interaction site. It is believed that this interaction is mediated by one or more loops that extend into the central channel. In the case of the hexameric gene 4D ring helicase from bacteriophage T7 (T7 gp4D), three charged loops facing the central channel of the ring are implicated in binding to ssDNA (Singleton et al. 2000). In the E1 replicative helicase from Papillomavirus (Enemark and Joshua-Tor 2006), the ssDNA interacts via its phosphates or sugar moieties with residues from two hairpin loops, forming hydrogen bonds or van der Waals interactions. In RuvBL1, two such loops were also present; the first (residues 101-108) includes the positively charged Lys107 and lies at the bottom of the central ring channel whereas the second (residues 334-351) contains the negatively charged Glu342 and is located near the top of the central ring channel.

The largely negative electrostatic potential of the inner surface of the RuvBL1 channel is similar to that in the hexameric ATPase P4 of dsRNA bacteriophage Φ12 (Mancini et al. 2004) which binds to ssRNA, and also to that in the helicase RepA (Niedenzu et al. 2001). In contrast, channels shown to bind to dsDNA are wider and positively charged (Fletcher et al. 2003; Li et al. 2003) to be able to accommodate the negatively charged dsDNA sugar-phosphate backbone. This is clearly the case for the SV40 large tumour antigen helicase (Gai et al. 2004; Li et al. 2003) which has a positively charged central channel wide enough to accommodate strand separation and forked DNA unwinding.

Indeed, I have shown that RuvBL1 binds to dsDNA, ssDNA, and ssRNA in a nonsequence-specific fashion, in line with an implication in processes that require binding to nucleic acids. The crystal structure of RuvBL1 revealed the existence of a new domain resembling the DNA-binding domains of different proteins involved in nucleic acid metabolism. EMSA experiments with the purified domain II of RuvBL1 confirmed that this domain was involved in nucleic acid binding. In addition, RuvBL1 with a truncated domain II was tested and found to bind to all nucleic acid substrates in a sequence-independent fashion. In this truncated construct the central channel of RuvBL1 could be involved in ssDNA/RNA binding. In

contrast to RuvBL1, RuvBL2 did not bind to the tested nucleic acid substrates. One reason might be the striking difference between the central channels of RuvBL1 and RuvBL2. As mentioned above, RuvBL1 had two putative DNA-binding loops facing its channel, one containing the positively charged Lys107 and the other one including the negatively charged Glu342. Both residues are substituted by serines in the RuvBL2 structure. The amino acid serine has only a polar hydroxyl-group, but is not charged. For that reason the central channel of RuvBL2 might have a much lower affinity for nucleic acids. However, based on my EMSA experiments I cannot entirely exclude that RuvBL2 interacts with nucleic acids. It may have a preference for specific sequences that I did not test in my experiments. In addition it is known that RuvBL1 and RuvBL2 can be recruited by the RNA polymerase II holoenzyme complex and by diverse transcription factors (discussed in 5.1), like c-Myc and β-catenin (Bauer et al. 2000; Feng et al. 2003; Wood et al. 2000). These proteins may be required for guiding RuvBL1 and RuvBL2 to the correct target DNA.

Interestingly, it has been shown that association of ATF2 with RuvBL2 is required for its ability to mediate DNA repair of double-strand breaks, since the inhibition of this association impairs double-strand break repair after infrared radiation (Cho et al. 2001). These results suggest that RuvBL2 interacts with DNA and mediates DNA repair when the transcription factor ATF2 is guiding it to the damaged DNA. In line with these findings, it is possible that RuvBL2 also interacts with RNA when it is bound to the appropriate complex, for example during snoRNP assembly. In contrast to RuvBL2, RuvBL1 bound to RNA under my assay conditions. This could be important for its function in snoRNP assembly. A different line of investigation has shown that the association of RuvBL1 with snoRNPs correlates with a restructuring event which leads to the stabilisation of the snoRNP complex before it enters the nucleolus (Watkins et al. 2004). It was speculated that RuvBL1 promotes this restructuring event, acting as molecular motor and regulating protein-protein or protein-RNA interactions within the snoRNP.

In agreement with the observed differences in nucleic acid binding between RuvBL1 and RuvBL2, their complex showed significantly weaker DNA binding compared to RuvBL1. The results of my experiments suggest that only RuvBL1 is responsible for DNA binding within the RuvBL1/RuvBL2 complex, since purified RuvBL2 did not bind to the tested DNA substrates. In the central channel of the complex only RuvBL1 monomers contribute DNA binding loops. Since RuvBL2 lacks such loops, but makes up half of the dodecameric ring, DNA binding of the complex should be significantly decreased. In addition the complex structure revealed that interaction between both hexamers is mediated by domain II, which is

certainly involved in DNA binding of RuvBL1. Given that this domain is concealed due to the interaction with the adjacent monomer in the opposite hexameric ring, DNA binding of the RuvBL1/RuvBL2 complex may be strongly affected.

## 5.4 Domain II of RuvBL1 and RuvBL2 is involved in regulation of ATP consumption

RuvBL1 and RuvBL2 are highly conserved $AAA^+$ proteins and it has been shown that their *in vivo* ATPase activities are needed for chromatin remodelling and transcription (Feng et al. 2003; Jonsson et al. 2001; Wood et al. 2000). In agreement with the results of Ikura *et al.* (Ikura et al. 2000) and Qiu *et al.* (Qiu et al. 1998), I could only detect a weak ATPase activity of purified human RuvBL1 and RuvBL2. In higher eukaryotes important cellular processes, such as those dealing with DNA metabolism are often regulated by large multiprotein complexes and it is therefore likely that strong RuvBL1 or RuvBL2 enzymatic activity can only be seen in such an environment. It is for instance known that many hexameric helicases interact with accessory proteins that stimulate their enzymatic activities (Allen and Kornberg 1991; Ayora et al. 1999; Ustav et al. 1991). Conversely, viral genomes are much smaller and helicases such as my positive control SV40 large tumour antigen are therefore able to act on their own, without the need for partners to activate their function. The three-dimensional structure of RuvBL1 revealed that an ADP molecule was tightly bound between DI and DIII and that access to the ATPase active site was additionally blocked by hexamerisation, thereby limiting the exchange between ADP and ATP. These data suggest that conformational changes are required to open up the nucleotide-binding pocket, thus enabling the enzymatic function of RuvBL1 and RuvBL2.

Importantly, the RuvBL1/RuvBL2 complex exhibited a 3-4 fold increase in ATP consumption compared to the single proteins. Assembly into hexameric and dodecameric structures has been found to stimulate the ATPase activity of most $AAA^+$ proteins. The MCM proteins from *Saccharomyces cerevisiae* show little or no ATPase activity on their own, but in the complex containing all six subunits this activity is significantly stimulated (Davey et al. 2003). A similar observation was made with the replication factor C (RFC) clamp loader proteins (Yao et al. 2003). In both cases, complex formation is essential for activity, since one subunit provides the ATP-binding function through the Walker A motif (P-loop) and the other subunit provides the arginine finger, which is important for catalysis (Hishida et al. 2004; Ogura and Wilkinson 2001; Schwacha and Bell 2001). ATP hydrolysis sites are found at the subunit

interface of many $AAA^+$ proteins and explain why the active form of many $AAA^+$ proteins is hexameric. The RuvBL1 hexameric structure revealed that the conserved Arg357 corresponded to Arg170 in RuvB which was shown to function as an Arg finger (Putnam et al. 2001), allowing the efficient hydrolysis of ATP by binding to the γ-phosphate group. In the RuvBL1 hexamer, Arg357 from the adjacent monomer contributed to the active site and was in sufficiently close proximity to the nucleotide-binding pocket to be able to act as an Arg finger. For this reason hexamerisation of RuvBL1 could be a requirement for efficient ATP hydrolysis. The results presented here reveal differences between the abilities of RuvBL1 and RuvBL2 to form stable oligomers. Diverse experiments showed that purified RuvBL1 was predominantly a monomer in solution with concentrations below 6 mg/ml. At higher concentrations RuvBL1 assembled into a hexamer. Some $AAA^+$ proteins like RuvB require adenine nucleotides for oligomerisation (Mitchell and West 1994). The addition of ADP or ATP to the monomeric solution of RuvBL1 had no influence on its oligomerisation. In contrast RuvBL2 was only a monomer at concentrations below 2 mg/ml and rapidly assembled into a hexamer after addition of ATP. These findings suggest that the RuvBL2 monomer was able to bind to ATP and assembled into a hexamer in order to hydrolyse it. The nucleotide-binding pocket of RuvBL2 has probably a higher accessibility than its homolog RuvBL1, which had a very low solvent-accessible area within its nucleotide-binding pocket indicating a tightly bound ADP molecule. RuvBL1 can therefore not easily exchange with ATP, and this may be the cause for its low *in vitro* ATPase activity. Since ATP hydrolysis was also marginal in RuvBL2, it can be concluded that the hexamerisation upon ATP addition blocked the nucleotide-binding pocket, as it happens in RuvBL1, allowing only a weak ATPase activity. At concentrations above 2 mg/ml, RuvBL2 only consisted of hexamers and dodecamers in solution, also without the addition of ATP. In contrast RuvBL1 was not able to form dodecameric structures. These differences between the closely related proteins RuvBL1 and RuvBL2 could be essential for their functional specialisation.

Given that RuvBL1 and RuvBL2 can also form hexameric and dodecameric structures separately, the positive effect on ATPase activity when two hexamers assemble into the RuvBL1/RuvBL2 complex might be due to a mutual stabilisation of the hexameric rings. The need for both proteins to be catalytically active and the synergistic effect on enzymatic activity clearly suggest intimate communications between the two proteins coupled to conformational effects. It would have been very important to crystallize the RuvBL1/RuvBL2 complex in the presence of ATP in order to visualise the conformational changes that occur during ATP hydrolysis. Unfortunately, co-crystallization with ATP or the non-hydrolysable

ATP analog AMP-PNP was not successful, suggesting that the conformation of the complex is clearly different in the presence of ATP. Since the RuvBL1/RuvBL2 complex only crystallized in the presence of ADP, it can be concluded that the structure is more stabilised in its ADP-bound state. There is also evidence in the literature that significant conformational changes occur within the RuvBL1/RuvBL2 complex, when it is bound to ATP. For example RuvBL1 and RuvBL2 bound to ATP are in the correct conformation to associate with the INO80 chromatin remodelling complex and initiate recruitment of Arp5 (Jonsson et al. 2004), which is essential for the activity of the INO80 complex. These findings suggest that RuvBL1 and RuvBL2 also may play a structural role for the assembly and activity of other proteins.

Surprisingly, the RuvBL1/RuvBL2 complexes with truncated domains II exhibited higher ATPase activities than the wild-type complex, whereas the activity was stimulated even further when domain II was not only truncated in RuvBL2 (R1wt/R2-DII), but in both RuvBL1 and RuvBL2 (R1-DII/R2-DII). Also, the individual proteins RuvBL1-DII and RuvBL2-DII with a truncated domain II showed an almost 2-fold increase compared to the activity of the wild-type proteins. These findings indicate that *in vivo* domain II may play a regulatory role to control ATP consumption. Cofactors present in the cell could bind to domain II and change its position, thereby clearing the way to the nucleotide-binding pocket for a more efficient ADP/ATP exchange. As a result, the ATPase activity of RuvBL1 and RuvBL2 could be stimulated. Diverse examples support the notion that ATPase consumption of RuvBL1 and RuvBL2 is activated when they are part of multiprotein complexes. The p400 complex participates in chromatin-remodelling events and displays ATPase and helicase activities (Fuchs et al. 2001), which are at least in part contributed by RuvBL1 and RuvBL2. In addition it was shown that the ATPase activity of RuvBL1 is needed for the chromatin-remodelling function of the TIP60 complex in order to allow transcription of TCF-dependent cellular genes, like *ITF-2*. The Walker B mutant RuvBL1_D302N inhibits *ITF-2* gene expression (Feng et al. 2003), which is linked to decreased acetylation of histones in the vicinity of the TCF-binding sites in the *ITF-2* promoter region. These findings not only suggest that the ATPase activity of RuvBL1 is important for β-catenin/TCF gene regulation, but are also in line with a direct role of RuvBL1 in chromatin remodelling.

Given that the ATPase activity of RuvBL1 and RuvBL2 is needed for several *in vivo* functions, it can be speculated that cofactors regulate the ADP/ATP exchange by changing the position of domain II. It is possible that I have mimicked an *in vivo* situation by truncating domain II since the ATPase activity of RuvBL1 and RuvBL2 was higher in these constructs. This could be a sophisticated regulatory mechanism taking place in the cell to allow ATP

hydrolysis of RuvBL1 and RuvBL2 only when it is needed and prevent an useless waste of the precious energy source.

## 5.5 The evolutionarily conserved AAA$^+$ proteins RuvBL1 and RuvBL2 unwind DNA

The sequence homology to RuvB which is clearly reflected in the hexameric ring structures of RuvBL1 and the RuvBL1/RuvBL2 complex suggests a functional homology with the DNA-dependent ATPase and helicase RuvB which plays a role in homologous recombination and recombinational repair of damaged DNA (Tsaneva et al. 1993). Also, the channel formed at the centre of RuvBL1 and the RuvBL1/RuvBL2 complex is wide enough to accommodate ssDNA. The interaction with single-stranded nucleic acid molecules in the inner channel may possibly be similar to that described for T7 gp4D (Singleton et al. 2000). T7 gp4D has a preference for forked DNA substrates with two single-stranded tails of sufficient length that allow the hexamer to assemble on the DNA and begin unwinding of the duplex (Ahnert and Patel 1997; Kaplan and Steitz 1999). The 5' tail of the forked DNA passes through the centre of the ring (Yu et al. 1996), whereas the 3' tail is thought to contact the outside of the ring (Ahnert and Patel 1997). In T7 gp4D there is no obvious DNA-binding surface on the outside of the ring for the 3' tail of the forked dsDNA. In RuvBL1 however, the novel domain II is likely to represent such an interaction region. RuvBL1 and RuvBL2 could function as a helicase during transcription and DNA repair when access to the genetic information is necessary. In addition helicase activity might be needed for snoRNP assembly to dissolve stable tertiary RNA structures.

Since there is a debate in the literature about the helicase activities of RuvBL1 and RuvBL2, I tested such activity with highly purified RuvBL1 and RuvBL2. In agreement with the results of Ikura *et al.* and Qiu *et al.* (Ikura et al. 2000; Qiu et al. 1998), I could not detect helicase activity of recombinant wild-type RuvBL1, RuvBL2 or their complex. Interestingly, my biochemical experiments showed that RuvBL1 and RuvBL2 individually or in complex can process DNA *in vitro* when their domain II is truncated, however at a low rate. RuvBL1 containing a truncated domain II exhibited a higher helicase activity than its truncated RuvBL2 homolog. This may be due to their different behaviour in DNA binding. While RuvBL1 interacted with diverse DNA substrates, RuvBL2 did not bind to DNA under my assay conditions. However, it is possible that RuvBL2 can bind to other DNA substrates like plasmid DNA used in the helicase assay. Unfortunately, binding of RuvBL2 to plasmid DNA

could not be tested in the EMSA experiments because only smaller oligonucleotides were shifted in the gel. In the cellular context RuvBL2 may be recruited and loaded onto DNA as part of a large multi-protein complex.

Based on the ATPase assay results, I also expected an increase in helicase activity when both RuvBL1 and RuvBL2 were truncated in domain II in comparison to the RuvBL1wt/RuvBL2-DII complex. In contrast to my expectations, the complex of RuvBL1-DII/RuvBL2-DII exhibited a slightly lower helicase activity than the complex of RuvBL1wt and RuvBL2-DII, although the RuvBL1-DII/RuvBL2-DII complex had a higher ATPase activity than the complex of RuvBL1wt and RuvBL2-DII. The results of the nucleic acid-binding studies might explain these findings. RuvBL1, but not RuvBL2 bound to the tested nucleic acids suggesting that RuvBL2 either did not bind, or had a very low affinity to DNA. For that reason I assume that RuvBL1 was mainly responsible for DNA binding of the complex, guiding it to the helicase substrate. Wild-type RuvBL1 had a higher affinity to DNA than its truncated version. This may result in a weaker binding of the RuvBL1-DII/RuvBL2-DII complex to the helicase substrate compared to the complex containing wild-type RuvBL1 (RuvBL1wt/RuvBL2-DII). Therefore, the helicase activity of RuvBL1-DII/RuvBL2-DII could be weaker due to less binding of the complex to the substrate. Since the wild-type proteins exhibited no helicase activity *in vitro*, it can be concluded that *in vivo* cofactors bind to RuvBL1 and RuvBL2 within chromatin remodelling complexes such as those involved in the control of gene transcription, thereby altering the conformation of both proteins and allow them to exert their helicase activity.

This situation would be similar to that of bacterial RuvB which has weak ATPase and helicase activities *in vitro* and requires RuvA as a partner for its *in vitro* and *in vivo* activities. RuvB has a low intrinsic affinity for DNA that might be similar to that of RuvBL2. In contrast RuvA is a structure-specific DNA-binding protein and has a high affinity for Holliday junctions. It interacts with RuvB to form specific complexes with DNA and facilitates RuvB-mediated ATP hydrolysis and branch migration. Importantly, the bacterial helicase RuvB does not contain the insertion of 174 residues, which forms domain II of RuvBL1 and RuvBL2. Since helicase activity was only detectable for the RuvBL1 and RuvBL2 constructs with a truncated domain II, it is conceivable that DII is not needed for this activity. Also, I cannot exclude, that I reconstituted the RuvB activity by truncating domain II in RuvBL1 and RuvBL2. In case wild-type RuvBL1 and RuvBL2 cannot exert helicase activity *in vivo* because of the insertion of domain II which is not present in the helicase RuvB, both proteins may equally well act as molecular chaperones in processes where no helicase activity is needed. For example helicase

activity of RuvBL1 and RuvBL2 is not needed for their function in assembling the multi-protein INO80 complex. It was shown that RuvBL1 and RuvBL2 bound to ATP are in the correct conformation to assemble that complex (Jonsson et al. 2004) without the need of any activities. In addition RuvBL1 and RuvBL2 acting as molecular chaperones may be required for the association of the snoRNPs that implies several restructuring events (Watkins et al. 2004).

The three-dimensional structures of RuvBL1 and of the RuvBL1/RuvBL2 complex combined with the results of all biochemical studies presented here demonstrate that the structural organisation of these highly conserved proteins is typical for helicase members of the $AAA^+$ family and that these two proteins possess all features characteristic of molecular machines. However, there are still some open questions about RuvBL1 and RuvBL2: how do they influence the activity of the different complexes listed above?; do they always act as hexamers or dodecamers, or are some functions performed by monomers?; why does RuvBL2 function as a repressor in some situations where RuvBL1 activates gene expression?

Due to the promiscuous nature of these proteins, it will be most important to identify their specific partners for each particular biological process in which they are involved. Given that RuvBL1 and RuvBL2 are part of several important complexes that require molecular machines to perform their function, it is very likely that the cellular activities of these proteins are regulated by cofactors. It will be an exciting challenge for the future to identify these cofactors and determine the structure of the complex they form with RuvBL1 or RuvBL2. The understanding of how the function of RuvBL1 and RuvBL2 is regulated by the different steps of ATP hydrolysis and by interacting proteins will deepen our understanding of the essential role RuvBL1 and RuvBL2 play in biological processes.