3 Materials and Methods

3.1 Cloning

3.1.1 RuvBL1 and RuvBL2

For separation purposes, a 6xHis tag followed by a FLAG tag (MVHHHHHHDYKDDDDKLLV) was added to the N-terminus of RuvBL1 and RuvBL2. The plasmid for expression of these constructs was constructed as follows. First, the NcoI-BamHI region was excised from pET-15b (Novagen) and replaced with the following region coding for 6xHis-FLAG by using the oligonucleotide 5´ GGGCGGCCATGG TTCATCACCATCACCATCACGATTACAAAGACGATGACGATAAACTGCTGGTTCC GCGTGGATCCGGTACCGAGCTCGCTAGCAGATCTGCCGGG-3´ and its complementary sequence. After annealing and digestion with NcoI and BglII (cutting sites are underlined), the double-stranded DNA was introduced into pET-15b. To generate a NheI restriction site in the pET-15b vector a point mutation was introduced after the BamHI site (GCTAAC→GCTAGC). The RuvBL1 coding sequence was PCR-amplified using the forward primer 5´-GGCCGGTTGGATCCAAGATTGAGGAGGTGAAGAGC-3´ and the reverse primer 5´-GCGCGGTTGCTAGCTCACTTCATGTACTTATCCTGC-3´. Accordingly, the RuvBL2 coding sequence was PCR-amplified using the forward primer 5´-GGCCGGTTGGATCCGCAACCGTTACAGCCACAACC-3´ and the reverse primer 5´-GCGCGGTTGCTAGCTCACTTGGAGGTGTCCATGGTC-3´. The products were digested with BamHI and NheI (cutting sites are underlined) and introduced downstream of the 6xHis-FLAG coding region in the modified pET-15b, previously digested with the same enzymes, creating N-terminal 6xHis-FLAG fusion proteins (pET-15b-6xHis-FLAG-RuvBL1 and pET-15b-6xHis-FLAG-RuvBL2). The resulting plasmids were sequenced for verification.

Primer for Walker B mutation	Sequence 5´-3´
R1_D302N_for	CCGGGTGTGCTGTTTGTTAACGAGGTCCACATGCTGGAC
R1_D302N_rev	GTCCAGCATGTGGACCTCGTTAACAAACAGCACACCCGG
R2_D299N_for	CCTGGAGTGCTGTTCATCAACGAGGTCCACATGCTGGAC
R2_D299N_rev	GTCCAGCATGTGGACCTCGTTGATGAACAGCACTCCAGG

Table 2: Primers to introduce Walker B mutation in RuvBL1 (R1) and RuvBL2 (R2).

Mutations were introduced into pET-15b-6xHis-FLAG-RuvBL1 and pET-15b-6xHis-FLAG-RuvBL2 constructs by site directed mutagenesis using the Quick-change® site-directed mutagenesis kit (Stratagene). Aspartate residue 302 of RuvBL1 was mutated to an asparagine

(pET-15b-6xHis-FLAG-RuvBL1D302N) and aspartate residue 299 of RuvBL2 was mutated to an asparagine (pET-15b-6xHis-FLAG-RuvBL2D299N). The primers used for mutagenesis are listed in Table 2.

3.1.2 RuvBL1 and RuvBL2 for co-expression in *E. coli* and insect cells

First the RuvBL1 coding sequence was PCR-amplified using the forward primer 5´-GGCCGGTTCATATGAAGATTGAGGAGGTGAAGAGC-3´ and the reverse primer 5´-GCGCGGTTGGATCCTTACTTCATGTACTTATCCTGC-3´. The PCR product and the vector pET-15b were cut with the restriction enzymes NdeI and BamHI and the RuvBL1 coding region was introduced downstream of the 6xHis site in the pET-15b vector (pET15b-6xHis-RuvBL1). For co-expression of RuvBL1 and RuvBL2 both genes were cloned into the bi-cistronic pETDuet vector (Novagen). 6xHis tagged RuvBL1 was PCR-amplified using pET-15b-6xHis-RuvBL1 as a template (forward primer 5´-GGGGCCATGGTTCATCACC ATCACCATC-3´; reverse primer 5´-GGGGAAGCTTTTATCACTTCATGTACTTATC CTGCT-3´), digested with NcoI and HindIII and inserted into pETDuet, previously cut with the same enzymes. FLAG tagged RuvBL2 was also PCR-amplified using pET-15b-6xHis-FLAG-RuvBL2 as a template (forward primer 5´-GGGGCATATGGATTACAAAGACGAT GACGATAAAGAAAACCTGTATTTTCAGGGCGCAACCGTTACAGCCACAACC-3`; reverse primer 5´-GGGGGGTACCTTATCAGGAGGTGTCCATGGTCTC-3´). Following digestion with NdeI and KpnI, RuvBL2 was inserted into the NdeI and KpnI restriction sites of pETDuet already containing RuvBL1 (resulting plasmid: pETDuet-6xHis-RuvBL1_FLAG-RuvBL2). In order to co-express 6xHis-RuvBL1 and FLAG-RuvBL2 in Hi5 insect cells, both proteins were also cloned into the vector pD-INS3 (resulting plasmids: pD-INS3-6xHis-RuvBL1 and pD-INS3-FLAG-RuvBL2).

In order to obtain the Walker B mutations (RuvBL1_D302N and RuvBL2_D299N) pETDuet-6xHis-RuvBL1_FLAG-RuvBL2 was modified with the same primers mentioned in section 3.1.1 (Table 2) using the Quick-change® site-directed mutagenesis kit (Stratagene).

3.1.3 Domain II of RuvBL1

In order to obtain the 6xHis-tagged Domain II of RuvBL1 amino acids L122 to V238 were cloned into the pET-15b vector. The vector pET-15b-6xHis-RuvBL1 was used as a template for PCR amplification with the forward primer 5´-GGGGCATATGCTGCGAATAAAGGAG ACCAAGGAAG-3´ and the reverse primer 5´-GGGGGGATCCTTACACATCTTGGATGA TTTCTTTCTTTTTG-3´. The PCR product and the pET-15b vector were digested with NdeI and BamHI before ligation.

3.1.4 Deletion mutants of RuvBL1 and RuvBL2

For crystallization purposes and functional studies the domain II of both RuvBL1 and RuvBL2 was truncated variedly using overlap extension PCR. The overlap extension PCR consists of two steps. Since up to 324 oligonucleotides needed to be excised from the coding regions, the flanking regions were PCR-amplified first. The forward and reverse primers that annealed to the regions next to the domain II to be excised, contained 12 additional nucleotides that were complementary to each other. In a second PCR containing both products from the first PCR, the flanking parts were not only amplified, but also ligated because of the complementary sequences (Figure 4). All deletion constructs were first cloned into the pET-15b vector. The primers used for cloning of the deletion constructs are listed in Table 3 and Table 4. A scheme of all cloned deletion constructs is shown in Figure 5.

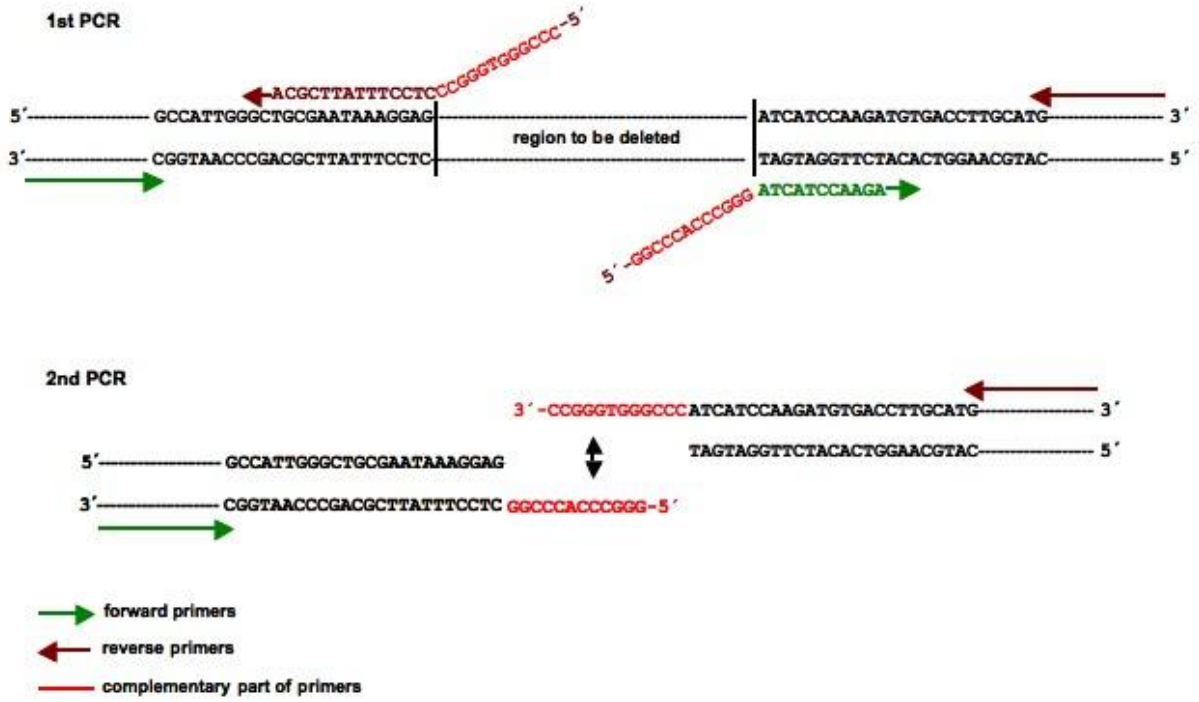


Figure 4: Overlap extension PCR. The overlap extension PCR consists of two steps. First the parts flanking the region to be excised are amplified. In the second step both products of the first PCR are amplified and ligated, because of complementary oligonucleotides at the connecting ends.

Primer RuvBL1-DII	Sequence 5´-3´
R1-DII_NcoI_for	GGGGCCATGGTTCATCACCATCACCATC
R1-DII_rev	CCCGGGTGGGCCCTCCTTTATTCGCAGCCCAATGGC
R1-DII_for	GGCCCACCCGGGATCATCCAAGATGTGACCTTGCATG
R1-DII_XhoI_rev	GGGGCTCGAGTTATCACTTCATGTACTTATCCTGCT

Table 3: Primers for truncation of domain II in RuvBL1.

Primer RuvBL2 mutants	Sequence 5´-3´
R2delNdeI_for	GGGGCATATGGCAACCGTTACAGCCACAACC
R2delBamHI_rev	GGGGGGATCCTTATCAGGAGGTGTCCATGGTCTC
R2-DIIK132-V239_for	GGCCCACCCGGGGTGCACACCGTGTCCCTGCACG
R2-DIIK132-V239_rev	CCCGGGTGGGCCCTTGATGCGAACGCCGATGGACC
R2-DIIE133-V238_for	GGCCCACCCGGGGTTGTGCACACCGTGTCCCTGC
R2-DIIE133-V238_rev	CCCGGGTGGGCCCTCCTTGATGCGAACGCCGATGG
R2-N251-AAA-I276_for	GCCGCTGCCGCGATCAATGCCAAGGTGGCTGAGTGG
R2-N251-AAA-I276_rev	CGCGGCAGCGGCGTTGATGACGTCGATCTCGTGCAG
R2-N251-4xA-I276_for	GCCGCTGCCGCGGCCATCAATGCCAAGGTGGCTGAGTGG
R2-N251-4xA-I276_rev	GGCCGCGGCAGCGGCGTTGATGACGTCGATCTCGTGCAG
R2-N251-AAA-I276A_for	GCCGCTGCCGCTAATGCCAAGGTGGCTGAGTGGCG
R2-N251-AAA-I276A_rev	AGCGGCAGCGGCGTTGATGACGTCGATCTCGTGCAG
R2-N251-4xA-I276A_for	GCCGCTGCCGCAGCTAATGCCAAGGTGGCTGAGTGGCG
R2-N251-4xA-I276A_rev	AGCTGCGGCAGCGGCGTTGATGACGTCGATCTCGTGCAG

Table 4: Primers for cloning diverse truncation mutants of RuvBL2.

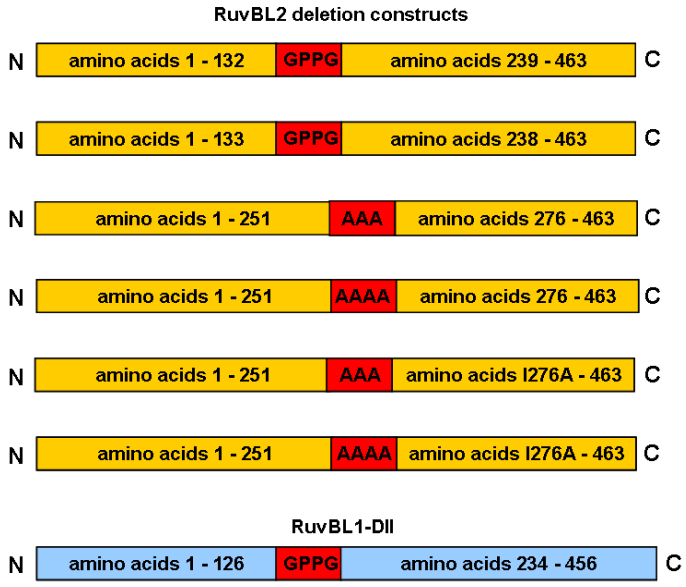


Figure 5: Truncated RuvBL1 and RuvBL2 constructs. The deleted region within domain II was replaced by the red highlighted amino acids, which resulted from the complementary nucleotides that connected both PCR products in the second step of the overlap extension PCR. In addition these amino acids provided a link to connect both protein domains properly.

After expression of the constructs (see below), the solubility was checked and only the genes coding for soluble proteins were used for pETDuet cloning (Figure 6 summarises combinations for co-expression). The primers for cloning the soluble deletion constructs into the pETDuet vector are listed in Table 5.

Mutants pETDuet	Sequence 5´-3´
R1_NcoI_for	GGGGCCATGGTTCATCACCATCACCATC
R1_HindIII_rev	GGGGAAGCTTTTATCACTTCATGTACTTATCCTGCT
R2-DII_NdeI_for	GGGGCATATGGATTACAAAGACGATGACGATAAAGAAAACCTGTAT TTTCAGGGCGCAACCGTTACAGCCACAACCAAA
R2-DII_KpnI_rev	GGGGGGTACCTTATCAGGAGGTGTCCATGGTCTC
FLAG-DIIofR2_NdeI_for	GGGGCATATGGATTACAAAGACGATGACGATAAAGTTCGCATCAA GGAGGAGACGGAG
FLAG-DIIofR2_KpnI_rev	GGGGGGTACCTTATCACACGGTGTGCACCACC

Table 5: Primers to clone the truncation constructs into the pETDuet vector.

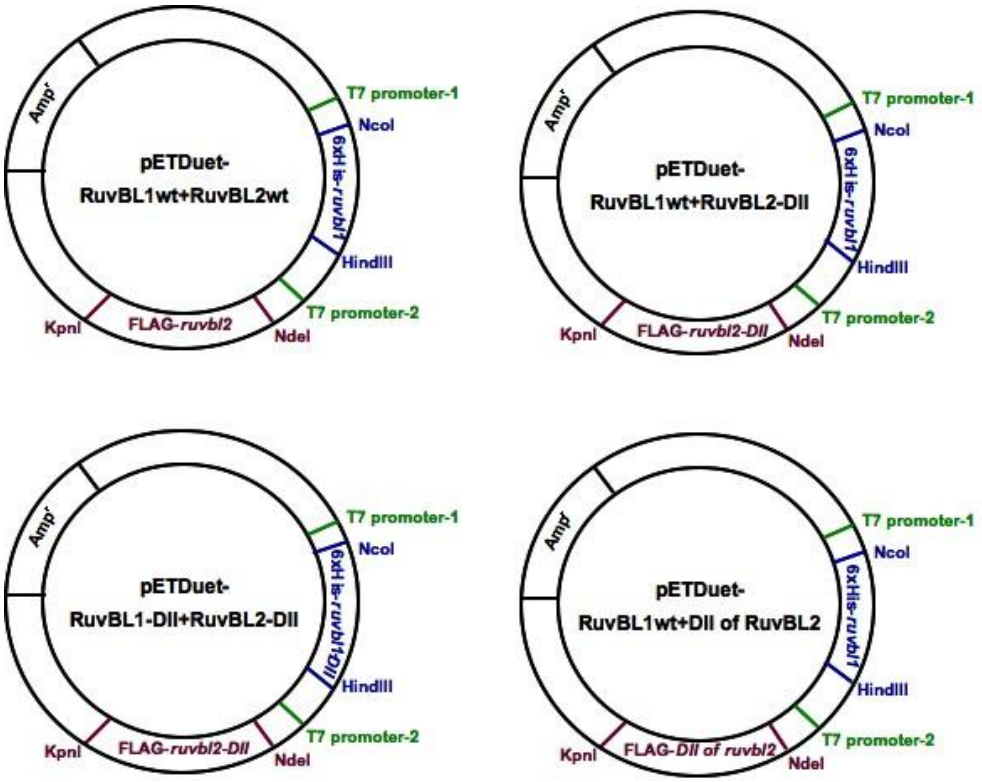


Figure 6: pETDuet vectors for co-expression in *E. coli*. RuvBL1 and RuvBL2 constructs carried a 6xHis and a FLAG tag, respectively.

3.1.5 Surface mutants of RuvBL2

In addition to the truncated constructs, 13 surface mutants of RuvBL2 were created for crystallization studies using the Quick-change® site-directed mutagenesis kit (Stratagene) and the vector pET-15b-6xHis-FLAG-RuvBL2 as a template. All primers are listed in Table 6. The cloned DNA constructs were transformed into TOP 10 cells (Invitrogen) and the purified DNA from single colonies was sent for sequencing (AGOWA).

R2 surface mutants	Sequence 5´-3´
R2-I138Y_for	GGAGGAGACGGAGATCTACGAAGGGGAGGTGGTGGAGATCC
R2-I138Y_rev	GGATCTCCACCACCTCCCCTTCGTAGATCTCCGTCTCCTCC
R2-A198N_for	GGGACGTGATCACCATCGACAAGAACACGGGCAAGATCTCCAAGC
R2-A198N_rev	GCTTGGAGATCTTGCCCGTGTTCTTGTCGATGGTGATCACGTCCC
R2_KEE132AAA_for	GCGGTCCATCGGCGTTCGCATCGCGGCGGCGACGGAGATCATCGAAGG
R2_KEE132AAA_rev	CCCCTTCGATGATCTCCGTCGCCGCCGCGATGCGAACGCCGATGGACCG
R2_EIQ144AIA_for	CATCGAAGGGGAGGTGGTGGCCATCGCGATTGATCGACCAGCAACAGG
R2_EIQ144AIA_rev	CCTGTTGCTGGTCGATCAATCGCGATGGCCACCACCTCCCCTTCGATG
R2_triple144_for	GATCATCGAAGGGGAGGTGGTGGCCATCGCGATTGATGCACCAGCAACA GGGACGGGCTCC
R2_triple144_rev	GGAGCCCGTCCCTGTTGCTGGTGCATCAATCGCGATGGCCACCACCTCC CCTTCGATGATC
R2_KVGK157_for	CAGCAACAGGGACGGGCTCCGCGGTGGGCGCACTGACCCTCAAGACCA CAGAGATGG
R2_KVGK157_rev	CCATCTCTGTGGTCTTGAGGGTCAGTGCGCCCACCGCGGAGCCCGTCCC TGTTGCTG
R2_EME167_for	CTGACCCTCAAGACCACAGCGATGGCGACCATCTACGACCTGGGC
R2_EME167_rev	GCCCAGGTCGTAGATGGTCGCCATCGCTGTGGTCTTGAGGGTCAG
R2_triple180_for	CGACCTGGGCACCAAGATGATTGCGTCCCTGACCGCGGACGCGGTCCA GGCCGGGGACGTG
R2_triple180_rev	CACGTCCCCGGCCTGGACCGCGTCCGCGGTCAGGGACGCAATCATCTT GGTGCCCAGGTCG
R2_KSE269_for	GGTGACACAGGGGAGATCGCGTCAGCAGTCCGTGAGCAGATCAATGCC
R2_KSE269_rev	GGCATTGATCTGCTCACGGACTGCTGACGCGATCTCCCCTGTGTCACC
R2_EK364_for	GTCTCCACCACCCCCTACAGCGCGGCAGACACGAAGCAGATCCTCCGCA
R2_EK364_rev	GATGCGGAGGATCTGCTTCGTGTCTGCCGCGCTGTAGGGGGTGGTGGA GAC
R2_DVE379_for	CCGCATCCGGTGCGAGGAAGAAGCTGTGGCGATGAGTGAGGACGCCTA CACGG
R2_DVE379_rev	CCGTGTAGGCGTCCTCACTCATCGCCACAGCTTCTTCCTCGCACCGGAT GCGG
R2_RK416_for	CCAGCTTGGTGTGCCGGAAAGCCGCGGGTACAGAAGTGCAGGTGG
R2_RK416_rev	CCACCTGCACTTCTGTACCCGCGGCTTTCCGGCACACCAAGCTGG
R2_KE444_for	CCCGCTCCACGCAGTACATGGCGGCGTACCAGGACGCCTTCCTC
R2_KE444_rev	GAGGAAGGCGTCCTGGTACGCCGCCATGTACTGCGTGGAGCGGG

Table 6: Primers for cloning RuvBL2 surface mutants.

3.2 Protein expression

All cloned DNA constructs were transformed into the *Escherichia coli* BL21 (DE3) strain. This strain is deficient in methionine synthesis and could therefore be used for the production of native proteins and the selenomethionine (Se-Met) substituted RuvBL1. *E. coli* cells containing the pET-15b or pETDuet construct were grown overnight at 37 °C in Luria-Bertani broth supplemented with ampicillin (200µg/mL). The cells of this preculture were harvested, washed and grown at 37 °C in Luria-Bertani media containing ampicillin to an A_{600} of 0.8 and induced at 28 °C with 100 µM IPTG for 20 hours.

For the production of the Se-Met substituted RuvBL1, cells from the preculture were harvested and washed twice with medium supplemented with a mixture of amino acids lacking methionine (Overnight Express Autoinduction System 2; Novagen), but containing selenomethionine (125 mg/L) instead. The washed cells were used to inoculate 1 L of this medium lacking methionine and were grown at 37°C for 32 hours (Gorynia et al. 2006).

3.3 Purification of recombinant RuvBL1, RuvBL2 and their surface mutants

Induced cells were harvested by centrifugation (Rotor SLA-3000, Firma: Sorvall; 11000 g; 15 min; 4 °C). The wet cells were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 0.1 % (w/v) Nonidet P40 (Roche), 1 mM β-mercaptoethanol, Complete (protease inhibitor cocktail) without EDTA, Roche) and disrupted twice in a High Pressure Laboratory Homogeniser (RANNIE) at 750 bar. Soluble proteins were collected by centrifugation at 100000 g for 45 min. The supernatant was applied to a Ni-NTA Superflow (QIAGEN) column previously equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 2 mM β-mercaptoethanol, 20 mM imidazole pH 8.0). Unbound proteins were washed out of the column using buffer A. RuvBL1 (or RuvBL2) were eluted in a gradient (elution buffer B: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 2 mM β-mercaptoethanol, 400 mM imidazole pH 8.0) with rising imidazole concentration. The protein pool was loaded onto an anion exchange chromatography (MonoQ) column (Amersham Biosciences), which was equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0, 10 % glycerol, 100 mM NaCl and 2 mM β-mercaptoethanol. A gradient with rising NaCl concentration up to 500 mM was applied for elution of the desired protein. The peak was

pooled and used for structural and biochemical studies. All purification steps were monitored by SDS-PAGE analysis.

3.4 Purification of the RuvBL1/RuvBL2 complexes

Three purification steps were necessary to obtain a clean and uniform complex of RuvBL1 and RuvBL2. Following cell disruption (see 3.3), lysates were cleared by centrifugation at 100 000 g for 45 min in a Beckman 45 Ti rotor. The cleared lysates were loaded onto a Ni-NTA Superflow (QIAGEN) column, equilibrated in buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 20 mM imidazole pH 8.0). The column was washed with buffer A and the bound 6xHis-tagged RuvBL1 was eluted in a 20 mM–400 mM imidazole gradient. Peak fractions of 6xHis-RuvBL1/FLAG-RuvBL2 were collected and loaded onto an Anti-FLAG affinity column (SIGMA), equilibrated in FLAG buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 5 mM $MgCl_2$). The protein was eluted using 2 column volumes of FLAG peptides (SIGMA) dissolved in FLAG buffer (200 µg/ml). To assure that the purified complex was uniform, a size exclusion chromatography was performed as last purification step. The HiLoad^{TM} 16/60 Superdex 200 (Amersham Biosciences) column was equilibrated and run in GF buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 % glycerol, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol). All purification steps were monitored by SDS-PAGE analysis.

3.5 Purification of RuvBL1-DII and RuvBL2-DII

The RuvBL1 and RuvBL2 deletion constructs containing a truncated domain II were purified slightly differently from the wild-type constructs. The first step also consisted of an affinity purification using a Ni-NTA Superflow (QIAGEN) column. Since the deletion constructs with the truncated domain II eluted at higher imidazole concentrations (between 280 and 400 mM), the protein pools were already pure. However, the high amounts of imidazole in the buffer caused protein aggregation. For that reason a buffer exchange (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 7 % glycerol, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol) using a desalting column (HiPrep^{TM} 26/10, Amersham) was necessary. The last purification step was an Anion exchange chromatography (MonoQ) column (Amersham Biosciences), which was equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0, 10 % glycerol, 100 mM NaCl and 2 mM β-mercaptoethanol. In a gradient with rising NaCl concentration up to 500

mM, the deletion mutant was eluted. The peak was pooled and used for structural and biochemical studies. All purification steps were monitored by SDS-PAGE analysis.

3.6 Thermal Shift Assay

The thermal shift assay is a protein stability assay that uses an environmentally sensitive fluorescent dye to monitor protein thermal unfolding and directly measures stability changes due to different buffer conditions or binding of compounds. Since protein stability varies with solution conditions, either due to a binding event (e.g. Mg^{2+} binding) or due to a bulk solvent effect, various buffers were tested to find out the best conditions for protein stability.

The thermal stability of proteins was measured with the 7500 Fast Real-Time PCR System (Applied Biosystems) in a 96-well plate using buffers with varying concentrations of NaCl (100-1000 mM), $MgCl_2$ (2-20 mM) and glycerol (2-10 %), different pH values (6-9) and additives (Additive Screen, Hampton Research). Each well contained 9 µl buffer, 1 µl protein (1-2 µg/µl) and 1µl of 50x SYPRO® Orange dye (Invitrogen). The program consisted of three steps, whereas step 1 was a pre-incubation for 1 min at 20 °C and step 2 and 3 were cycles comprising the temperature increase of 1 °C within 20 sec. The temperature gradient proceeded from 20 to 90 °C and included 70 cycles.

3.7 Dynamic Light Scattering

Dynamic light scattering (DLS) is a method used to determine the size distribution profile of small particles in solution. When light hits small particles the light scatters in all directions. DLS uses the scattered light to measure the rate of diffusion of the protein particles. This motion data is conventionally processed to derive a size distribution for the sample, where the size is given by the "Stokes radius" or "hydrodynamic radius" of the protein particle. This hydrodynamic size depends on both mass and shape. DLS was used for determining whether the native state of a protein is a monomer or a higher oligomer and for sensing the presence of aggregated protein. Prior to crystallization all protein samples were checked with the DynaPro™ DLS (Protein Solutions).

3.8 Nanodrop

To determine protein and DNA concentrations the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) was used. DNA absorbance was measured at 260 nm and protein absorbance at 280 nm. Molecular weights and molar extinction coefficients of the proteins are listed in Table 7.

Protein	Molecular Weight (kDa)	Molar Extinction Coefficient
RuvBL1wt	51,9	16,1
RuvBL1-DII	40,5	9,4
RuvBL2wt	53,0	20,1
RuvBL2-DII	42,4	14,9
RuvBL1wt/RuvBL2wt	52,5	18,1
RuvBL1wt/RuvBL2-DII	47,2	15,5
RuvBL1-DII/RuvBL2-DII	41,5	12,2

Table 7: Molecular weights and molar extinction coefficient of RuvBL1 and RuvBL2 constructs and their complexes.

3.9 ATPase assay

The reaction mixture contained 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM DTT, 2 mM ATP and 0.01 µCi/µl [γ^{33}P] ATP (Amersham Biosciences). Reactions were performed in a total volume of 20 µl, in the presence of 2 µg protein. Double-stranded plasmid DNA and single-stranded oligonucleotides were used as DNA substrates and added to a final concentration of 5 ng/µl. Reactions were incubated for 30 min at 37 °C and stopped by addition of EDTA to 25 mM final concentration. 1 µl aliquots were spotted onto PEI Cellulose TLC plates (Merck), which were developed with 0.5 M LiCl and 1 M formic acid. Plates were dried and exposed on a Phosphorimager screen (KODAK) overnight. Spots were visualised with a Phosphorimager (Personal Molecular Imager FX, Biorad). SV40 large tumour antigen helicase was used as a positive control, while the D302N mutant of RuvBL1 and the D299N mutant of RuvBL2 were used as negative controls. Molecular masses specified in Table 7 and 70 kDa for SV40 were used to calculate the number of moles of hydrolysed ATP per mole of protein.

3.10 DNA helicase assay

Diverse dsDNA substrates and a DNA/RNA hybrid were generated to test helicase activities. To this effect, the ssDNA plasmid M13mp18 (New England Biolabs) was annealed with diverse complementary oligonucleotides in hybridisation buffer (10 mM Tris-HCl pH 7.5, 800 mM NaCl, 5 mM $MgCl_2$). In addition to usual dsDNA, other substrates mimicking intermediates of DNA repair and transcription (a DNA/RNA hybrid and looped DNA) were used.

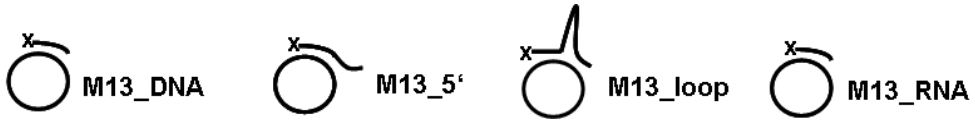
Sequences of oligonucleotides annealed to the single-stranded plasmid M13mp18:

M13: 5´-AGTCACGACGTTGTA-3´

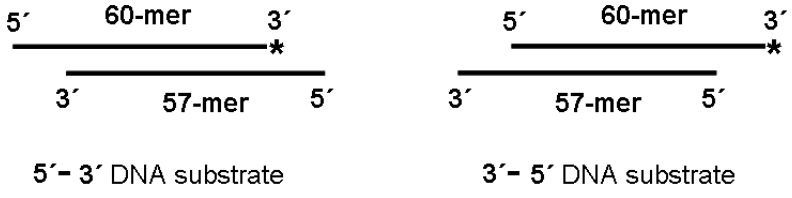
M13_5´: 5´-AAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACT-3´

M13_loop: 5´-GGGTAACGCCAGGGTTTTCCCAGTCACCGCATCGACGTTGT AAAACGACGGCCAGTGCC-3´

M13_RNA: 5´-AGUCACGACGUUGUA-3´



The M13mp18 substrates were labelled with the Klenow Enzyme (Roche) using [α-^{33}P]-ATP in Klenow buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA) at room temperature overnight. In addition a 60-mer oligonucleotide (5´–CAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAC CCTGG–3´) was labelled at the 3´ end and annealed with two different complementary oligonucleotides (57mer: 5´-TTTAAACCAGGGTTTTCCCAGTCACGACGTTGTAAAAC GACGGCCAGTGCCAAGCTT-3´ and 56mer: 5´-TCCCAGTCACGACGTTGTAAAACGA CGGCCAGTGCCAAGCTTGCATGCCTGAAATT-3´). One resulting partial duplex DNA had a 3´ overhang and the other one a 5´ overhang.



All double-stranded nucleic acid substrates were purified from free nucleotides using the NucTrap® Probe Purification Columns (Stratagene).

The DNA helicase assay was performed in a final volume of 20 µl. The reaction mixture, composed of purified protein and 0.1 pmol dsDNA substrate in reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 4 mM $MgCl_2$, 25 µg/ml BSA, 0,5 mM DTT, 1 mM ATP) was incubated at 37 °C for 30 min. The reaction was stopped by adding 5 µl of sample buffer (Novex®, Invitrogen) containing 1 % SDS and 50 mM EDTA. Subsequently, the sample was separated by a 10 % PAGE with 1 x TBE buffer and visualised by autoradiography. SV40 large tumour antigen helicase was used as a positive control.

3.11 Electrophoretic mobility shift assay

Diverse nucleotide oligomers (60mer: 5´-CAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGG-3´; 56mer: 5´-TCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGAAATT-3´; 51mer: 5´-AAAAAGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGAAAAAA–3´; 41mer: 5´-AAAAAACAGUCACGACGUUGUAAAACGACGGCCAGAAAAUU-3´) were labelled with [γ-^{33}P]-ATP using T4 Polynucleotide Kinase (Roche). The labelled nucleic acids were used as ssDNA and ssRNA substrates. To generate a dsDNA substrate, the labelled 60mer was annealed to its complementary oligonucleotide in annealing buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$). Binding reactions were performed in a final volume of 15 µl. Nucleic acids (1 pmol) were incubated with 40 pmol of purified protein in binding buffer (15 mM HEPES pH 6.7; 10 % glycerol; 1 mM EDTA; 500 µM ATP; 1 mM DTT and 2 mM $MgCl_2$) for 30 min at room temperature. Hi-Density TBE Sample Buffer (Invitrogen) was added to the reaction and the sample was separated by a 6 % PAGE with 0.5 x TBE buffer and visualised by autoradiography. Bovine serume albumin (BSA) was used as negative control.

3.12 Protein crystallization

For protein crystallization the peak pools after the last purification steps were concentrated using Amicon Ultra Centrifugal Filters (Millipore) with a 30 kDa cutoff. Different protein concentrations were tested in crystallization trials. Initial crystallization screens were performed on a Phoenix nanoliter-drop dispensing robot in a 96-well plate using the standard Hampton screens (Hampton Research). Refinement screens were done in 24-well plates using hanging or sitting drop vapour-diffusion methods, with a drop composition of 2 µl of protein

solution and 2 µl of reservoir solution, equilibrated against 500 µl of precipitant solution in the well.

3.13 Data collection of the selenomethionine variant of RuvBL1

Prior to data collection, a crystal of Se-Met RuvBL1 was flash-frozen under a stream of nitrogen gas at 100 K using a cryoprotecting buffer composed of 2.0 M sodium malonate pH 6.0 and 5 % PEG 400. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble at beamline ID14-4, using an ADSC Quantum 4 detector at a wavelength corresponding to the maximal value of f" from the Se atoms near the X-ray absorption K-edge, and were processed to 2.2 Å resolution with XDS (Kabsch 1993). The data sets were initially indexed, integrated, scaled and merged using the HKL suite (Otwinowski and Minor 1997) and later with either MOSFLM (Leslie 1992) or XDS (Kabsch 1993) combined with SCALA in the CCP4 suite (Collaborative Computational Project Number 4 1994). The first X-ray measurements showed the RuvBL1 crystals to be sensitive to radiation damage and great care was necessary during data collection in order to minimise such effects while at the same time measuring data to the highest possible resolution. In particular, inverse beam geometry was used in the ID14-4 Se-Met data collection to try to minimise systematic errors in anomalous differences due to radiation damage.

3.14 Structure solution of RuvBL1

Final data scaling, merging and intensity conversion to structure factor amplitudes were carried out with SCALA and TRUNCATE in the CCP4 program package (Collaborative Computational Project Number 4 1994). Initial attempts to solve the structure by the Molecular Replacement method were unsuccessful. The search models tried, chosen on the basis of sequence homology using BLAST (Altschul et al. 1997) and with available coordinates at the Protein Data Bank (PDB) (Berman et al. 2000) were the RuvB molecule from *Thermotoga maritima* (PDB 1IN7) (Putnam et al. 2001) and the FTSH ATPase domain from *Thermus thermophilus* (PDB 1IY1) (Niwa et al. 2002). These search models had a relatively low (*ca.* 30 %) sequence homology with RuvBL1 and their protein chain length differed as well, indicating that not all domains of RuvBL1 were represented in their 3D structures. The three-dimensional structure of RuvBL1 was solved using the Single-Wavelength Anomalous Dispersion method and the ID14-4 Se-Met dataset. Using the

HKL2MAP graphical user interface (Pape and Schneider 2004), the ID14-4 Se-Met dataset was analyzed with SHELXC (G. M. Sheldrick, personal communication), the heavy atom substructure was determined with SHELXD (Schneider and Sheldrick 2002) and the phase problem was solved with SHELXE (Sheldrick 2002). SHELXD found many possible solutions out of 100 trials, containing 29 possible selenium sites in the asymmetric unit of the crystal structure. However, the electron density maps were not of sufficient quality to allow interpretation and model building. The 29 selenium sites located with SHELXD were then input to a maximum-likelihood heavy-atom parameter refinement using SHARP (La Fortelle and Bricogne 1997). The SHARP calculations showed that 8 of the selenium sites were spurious and were therefore discarded. Thus, a total of 21 Se sites were used in the final SHARP calculations, followed by density modification with SOLOMON (Abrahams and Leslie 1996). Attempts at automated model building with ArpWarp (Perrakis et al. 1999) were unsuccessful, but a partial model was obtained, showing parts of the main chain for three RuvBL1 molecules in the asymmetric unit. The initial RuvBL1 model was built with O (Jones et al. 1991) and Turbo-FRODO (Roussel et al. 1990). Clear density was visible in the electron density maps for an ADP molecule per monomer. The structure was refined with REFMAC (Murshudov et al. 1997) using weak non-crystallographic symmetry restraints between the three independent monomers and TLS rigid body refinement (Schomaker and Trueblood 1968) prior to restrained refinement of atomic positions and thermal motion parameters. One rigid body was defined for each of the three domains in each independent monomer. In the final refinement, 158 water molecules, located with Arp/wArp (Lamzin and Wilson 1993) were included in the model, individual restrained B-factors were refined for all non-hydrogen atoms, and hydrogen atoms were included in calculated positions. The final values of R and R-free were 0.206 and 0.257, respectively. The maximum likelihood estimate of overall coordinate error was 0.15 Å. Because their electron density could not be seen, the following residues are absent from the final model: 1-8, 142-155, 248-276 and 450-456 in monomer A, 1-10, 142-154, 245-278 and 449-456 in monomer B, and 1-7, 129-230, 247-276 and 450-456 in monomer C. The main refinement statistics are presented in Table 9. The structure was analysed with PROCHECK (Laskowski et al. 1993) and its stereochemical quality parameters were within their respective confidence intervals. The final coordinates have been deposited with the Protein Data Bank (Berman et al. 2000) under accession code 2c9o.

3.15 Data collection of the RuvBL1/RuvBL2 complex with a truncated domain II

Prior to data collection, a fragment of a thin (*ca.* 20 μm) hexagonal-shaped plate consisting of the RuvBL1/RuvBL2 complex with truncated domains II (RuvBL1-DII/RuvBL2-DII) was flash-frozen under a stream of nitrogen gas at 100 K using a cryoprotecting buffer composed of 0.8 M $LiCl_2$, 10 % PEG 6000, 0.1 M Tris pH 7.5 and 20 % glycerol. In order to allow the crystal to adjust to the glycerol concentration in the cryoprotecting buffer and avoid cracking, it was dipped briefly in drops containing 0.8 M $LiCl_2$, 10 % PEG 6000, 0.1 M Tris pH 7.5 and increasing concentrations (5 %, 10 %, 15 % and 20 %) of glycerol. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble at beamline ID14-2, using an ADSC Quantum 4 detector at a wavelength of 0.933 Å, and were integrated to 4 Å resolution with XDS (Kabsch 1993). Final data scaling, merging and intensity conversion to structure factor amplitudes were carried out with SCALA and TRUNCATE in the CCP4 suite (Collaborative Computational Project Number 4 1994).

3.16 Structure solution of the truncated RuvBL1/RuvBL2 complex

The three-dimensional structure of the RuvBL1-DII/RuvBL2-DII complex was solved by the Molecular Replacement method using the program PHASER (Storoni et al. 2004) and the ID14-2 dataset. The search model was the homolog RuvBL1 hexamer whose structure was already solved at that stage as part of this thesis. RuvBL1 has 65 % sequence similarity with RuvBL2 and their protein chain length is also similar (456 and 463 residues, respectively). The dodecameric structure resulting from the Molecular Replacement calculations was refined for 10 cycles with REFMAC (Murshudov et al. 1997) using tight non-crystallographic symmetry restraints for positional and thermal parameters between all atoms in the twelve independent monomers. The final values of R and R-free were 0.317 and 0.346, respectively. The maximum likelihood estimate of overall coordinate error was 1.23 Å. Because of the low resolution and the relatively high sequence and structural similarity between RuvBL1 and RuvBL2 monomers, it was not possible to distinguish them in the 2|Fo|-|Fc| and |Fo|-|Fc| electron density maps and model re-building was not attempted. Density was visible in the electron density maps for an ADP molecule per monomer.

3.17 Small-Angle X-Ray Scattering

Small-angle x-ray scattering (SAXS) is a tool for structure analysis of native biological macromolecules in solution from individual proteins to large complexes (Svergun and Koch 2002). Since x-ray crystallography requires good crystals and NMR spectroscopy a low molecular mass, a significant fraction of proteins cannot be analysed using these two high-resolution methods. The scattering of x-rays at small angles (close to the primary beam) provides low-resolution structural information on the overall shape and internal structure in the absence of crystals. SAXS experiments require a homogeneous dilute solution of macromolecules in a near physiological buffer without special additives. For dilute protein solutions comprising monodisperse systems of identical particles, the random orientation of particles in solution leads to spherical averaging of the single particle scattering.

All experimental data were recorded at the EMBL beamline X33 on the DORIS storage ring at DESY in Hamburg. The exposure time for all data collections was 120 seconds and the sample container used for this experiment was a vacuum cuvette with two 25 µm thick mica windows. The sample thickness of this cuvette is 1 mm and the sample volume approximately 50 µl. The cuvette was cleaned between each measurement with ethanol, HCl, rinsed with distilled water and dried using nitrogen gas. The corresponding buffer, which consisted of 20 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 % glycerol, 4 mM $MgCl_2$ and 2 mM β-mercaptoethanol, was measured before and after each protein sample. The experimental data were radially integrated to give a 1D x-ray scattering profile of intensity against scattering angle and normalised for changes in intensity caused by the decay of the circulating current in the storage ring. The average of the scattering from the buffer measured before and after each sample was subtracted from the scattering of the protein solution leaving only scattering from the protein under investigation.

Since the samples could be damaged by synchrotron radiation during the collection time, 2 mM DTT were added to all samples before measurement in order to minimise the effects. The degree of dose dependant aggregation caused by radiation damage was checked for each protein complex using multiple exposures of the same sample and was shown to be minimal for the exposure time used in this experiment. For each sample a concentration series was measured (Table 8) in order to verify that the solutions were monodisperse and to check whether concentration-based interactions were observable in the data. The sample concentrations were determined directly at the beamline using the Nanodrop

spectrophotometer. Calibration of the extrapolated scattering at zero angle with molecular weight was checked using a 4 mg/ml solution of bovine serum albumin (BSA).

Sample	Concentration Series in mg/ml
RuvBL1wt	2,7 → 5,1 → 7,8 → 9,7 → 18,2
RuvBL2wt	3.2 → 8,8 → 19,3
RuvBL2-DII	0,9 → 1,8
RuvBL1wt/RuvBL2wt	2,1 → 4,12 → 10,8
RuvBL1wt/RuvBL2-DII	1,8 → 4,2 → 6,5
RuvBL1-DII/RuvBL2-DII	0,9 → 11,2 → 21,9

Table 8: Different concentrations of each construct used for SAXS experiments.