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Supplemental Information

The BID Domain of Type IV Secretion Substrates Forms a Conserved Four-Helix Bundle Topped with a Hook

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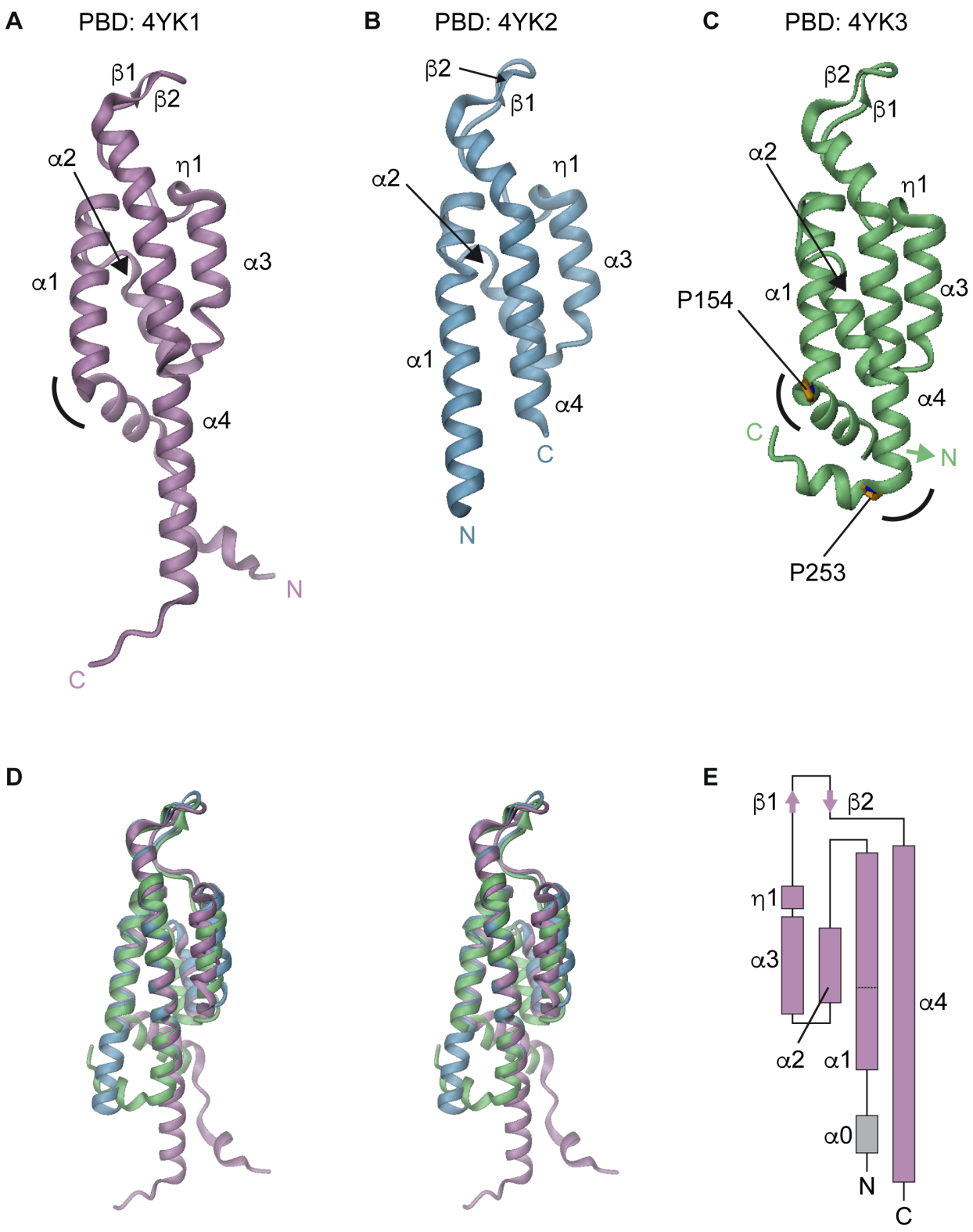

Figure S1, related to Figure 1. Secondary structure element annotations for the three BID structures. The BID domain structures are colored according to their BID domain subclass (see Figure 1A and 3B) and the numbering of the secondary structure

elements is shown. (A) tBID1 domain from *Bro*Bep6, (B) tBID1 domain from *Bcl*Bep9 and (C) BID1 domain from *Bhe*BepE. Prolines P154 and P253 located respectively at the kink of helices α1 and α4 are shown as sticks with their carbon atoms colored in orange. A black arch indicate the position of the kinks in helix α1 and α4 in panels A and C. (D) Stereoview of the three superposed BID domains shown in (A-C). (E) Topology diagram of the structure of *Bro*Bep6_tBID1 (PDB: 4YK1) shown in panel A.

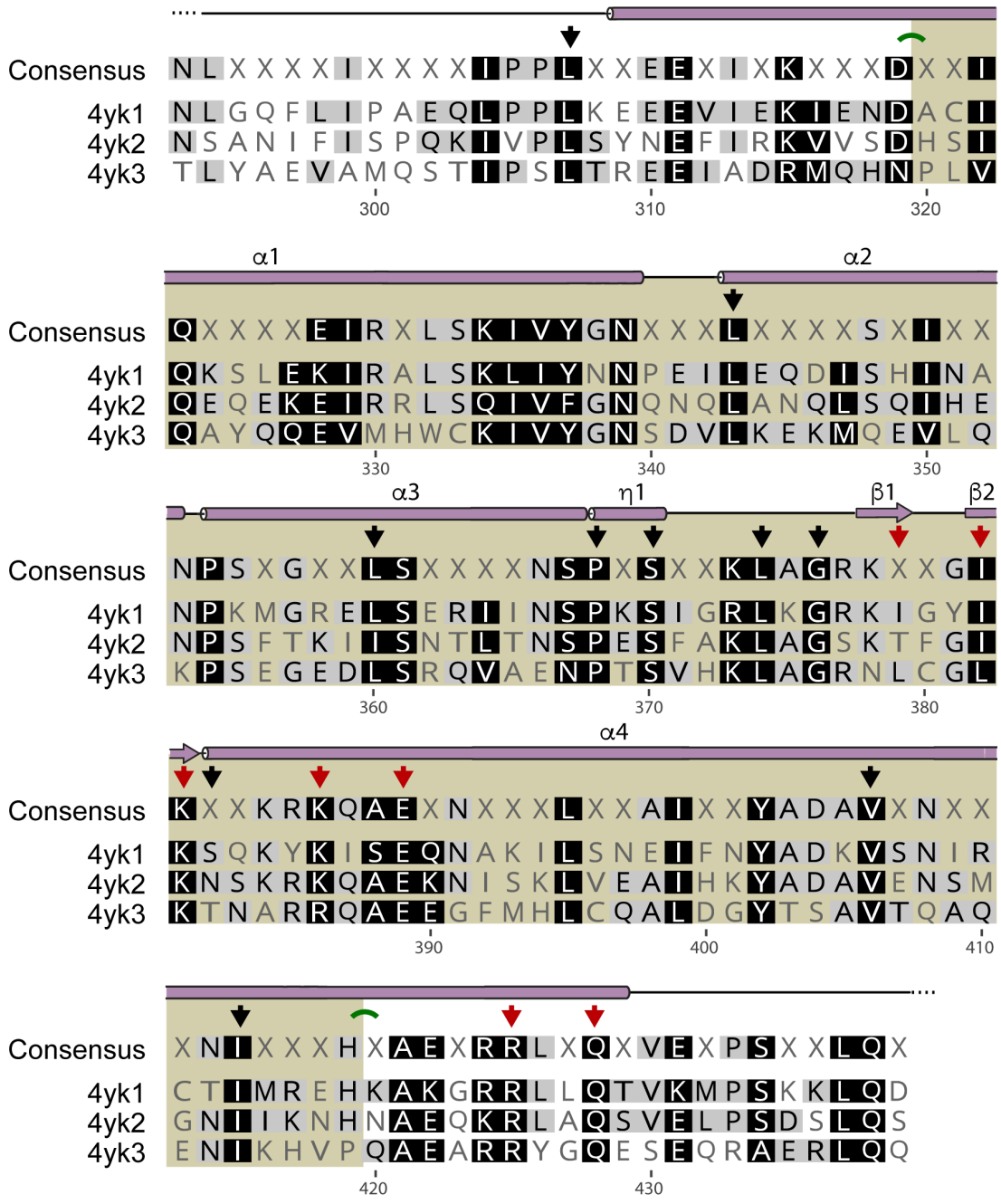

Figure S2, related to Figure 1. Sequence alignment of the three structures determined in this study. The consensus and secondary structure elements of the reference structure (PDB: 4YK1) are indicated on top of the alignment. Residues of structural importance are marked with black triangles and residues of potential functional relevance are marked with red triangles. Additionally, green arches indicate kinks in helices α1 and α4. The core of the BID domain is highlighted in beige.

Figure S3, related to Figure 2. High-resolution image of the neighbor-joining distance based tree shown in Figure 2. This tree includes the individual species names and sequence references**.**

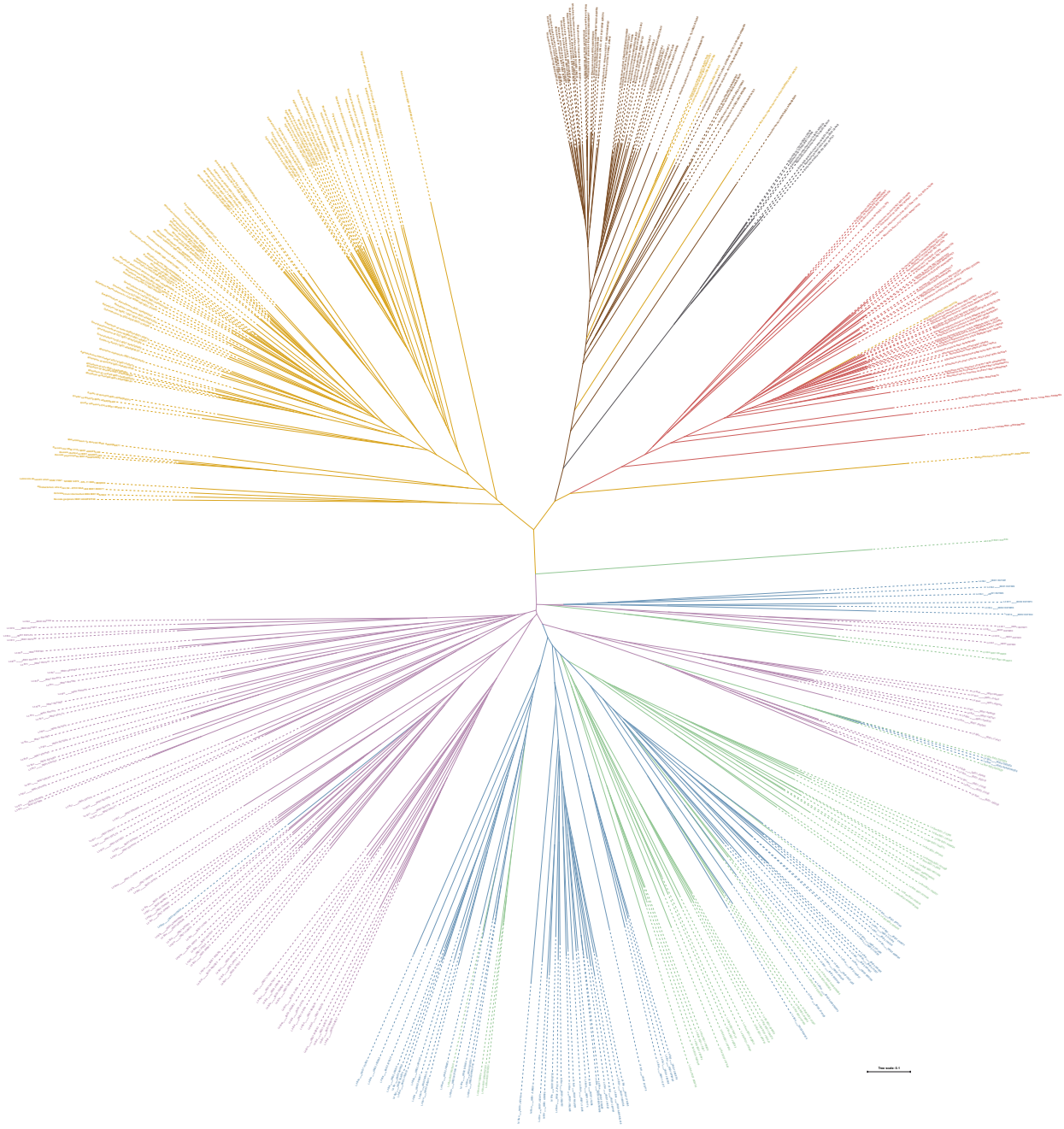

Figure S4, related to Figure 2. High-resolution image of the neighbor-joining distance based tree of the core BID domain. This tree includes the individual species names and sequence references**.**

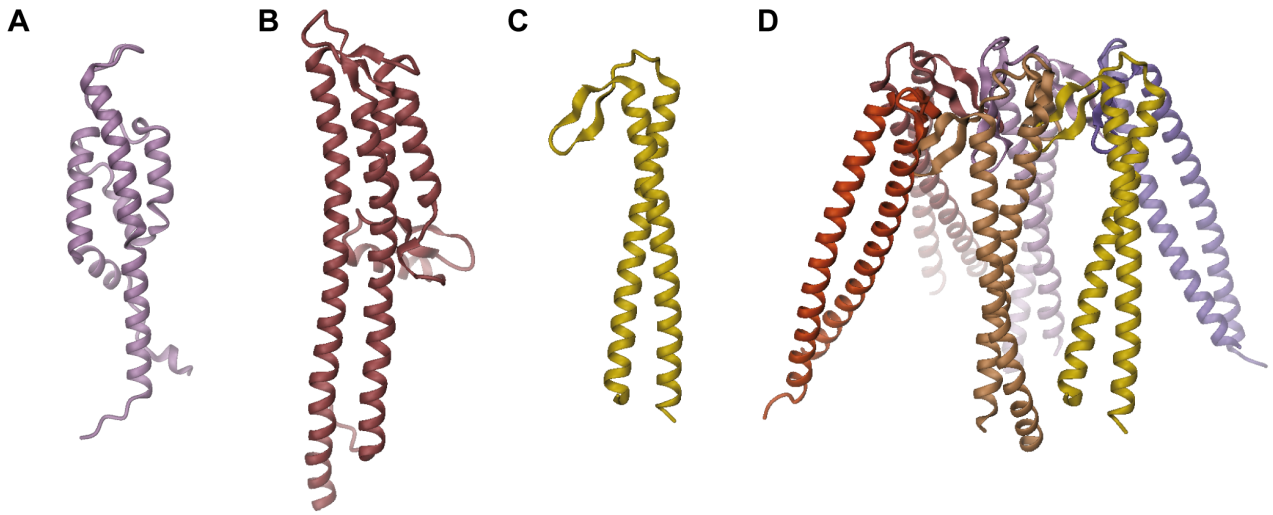

Figure S5, related to Figure 1 and Discussion. Structural comparison of the BID domain with resembling folds. (A) Structure of the BID domain of *Bro*Bep6 as shown in Figure 1A as reference. (B) Monomer of IpaD as observed in the tetrameric structure of IpaD from *Shigella flexneri* (PDB:4D3E) in a similar orientation as the BID domain shown in A. For clarity, only the monomer is shown. (C) Structure of one monomer of archaeal prefoldin (*Methanothermobacter thermautotrophicus*) as observed in the hexametric structure (PDB: 1FXK) displayed in (D).

Supplemental experimental procedures

Protein Expression and Purification

The BID domains from three different *Bartonella* effector proteins, *Bartonella rochalim*ae Bep6 (UniProt: E6YLF3 residues 298-434), *Bartonella clarridgeiae* Bep9 (UniProt: E6YIM5 residues 64-201), and *Bartonella henselae* BepE (UniProt: Q5QT01 residues 131-268) were introduced by ligation independent cloning (Aslanidis and de Jong, 1990) into the *E. coli* expression vector BG1861, which results in the fusion of a non-cleavable His_6-tag to the N-terminus of the BID domains (Myler et al., 2009) yielding *Bro*Bep6_tBID1, *Bcl*Bep9_tBID1 and *Bhe*BepE_BID1, respectively. *Bro*Bep6_tBID1 was overexpressed using BL21(DE3)-pLysS *E. coli* cells in M9 media supplemented with SeMet and induced with 1 mM IPTG overnight at 16˚C, shaking at 220 rpm. Cells were harvested by centrifugation and frozen at -80˚C. The ~10 g bacterial pellet was resuspended in 50 ml of buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, 50 mM arginine, 10 mM imidazole, 0.25% glycerol, 1 mM TCEP (VWR), 1% CHAPS (JT Baker), 1/2 tablet of EDTA-free protease inhibitor (Roche), 75 U benzonase (Novagen), 75 mg lysozyme (Sigma) and sonicated at 4˚C for 45 minutes. The resulting slurry was clarified by centrifugation at 4˚C for 30 minutes and the supernatant was the loaded onto a HiTrap Ni Chelating Column (GE Healthcare) attached to an AEKTA FPLC and washed with buffer A (25 mM Tris pH 8.0, 200 mM NaCl, 50 mM arginine, 0.25% glycerol, 1 mM TCEP) at 4˚C. The protein was eluted with a gradient of eluting buffer (25 mM Tris pH 8.0, 200 mM NaCl, 500 mM imidazole, 1 mM TCEP). The eluted protein was pooled, concentrated to 22.36 mg/ml via centrifugation using a 3 kDa molecular weight cutoff membrane (Amicon). The protein was then loaded onto a Sephacryl S-100 (GE Healthcare) size exclusion chromatography column pre-equilibrated with a buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, 1%

glycerol, 1 mM TCEP. The protein was concentrated to a final concentration of 19.68 mg/mL.

After transformation into chemically competent *E. coli* BL21(DE3) Rosetta cells, starter cultures for each *Bcl*Bep9_tBID1 or *Bhe*BepE_BID1 construct were grown for 18 hours at 37°C. The protein was expressed in a LEX bioreactor in the presence of ampicillin (50 $\mu g.ml^{-1}$) (Studier, 2005). The cells were grown for 24 hours at 25°C and the temperature was reduced to 15°C for another 60 hours. The pellet was flash frozen in liquid nitrogen and stored at -80°C. Cells were resuspended in lysis buffer (20 mM HEPES pH 7.4, 300 mM NaCl, 5% glycerol, 30 mM imidazole, 0.5% CHAPS, 10 mM $MgCl_2$, 3 mM β-mercaptoethanol, 1.3 µg/ml protease-inhibitor cocktail, 0.05 mg/ml lysozyme) at 4°C. The cells were sonicated and incubated with Benzonase (20 µL of 25 unit/µL) at 37°C for 40 minutes. The soluble fraction was loaded onto a 5 mL Ni-NTA His-Trap FF column (GE Biosciences, Piscataway, New Jersey, USA). The column was washed with binding buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol, 30 mM imidazole, 1 mM TCEP) and eluted with 500 mM imidazole in the same buffer. The collected protein was concentrated and further resolved by size-exclusion chromatography (SEC) using a Hiload 26/60 Superdex 75 prep grade column (GE Biosciences) pre-equilibrated with a buffer containing 25 mM HEPES pH 7.0, 500 mM NaCl, 5% glycerol, 0.25% azide and 2 mM DTT for *Bcl*Bep9_tBID1 and 20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol and 1 mM TCEP for *Bhe*BepE_BID1. Peak fractions were collected and pooled based on purity-profile assessment by SDS-PAGE. *Bcl*Bep9_tBID1 was concentrated to 24.7 mg/mL and *Bhe*BepE_BID1 was concentrated to 28.7 mg/mL. All concentrated pure proteins were flash-frozen in liquid nitrogen and stored at -80°C. The three purified BID domains eluted as monomers from gel filtration columns.

Protein Crystallization

All proteins were thawed and crystallized using the sitting drop vapor diffusion method at 289 K with 0.4 µl protein and 0.4 µl precipitant equilibrated against 80 µl of reservoir solution. *Bro*Bep6_tBID1 crystals grew within days (in 0.1 M sodium cacodylate-HCl pH 6.5 and 1 M sodium citrate tribasic (MCSG3 (Anatrace) A1)) and were then soaked in a reservoir solution supplemented with 20% (v/v) ethylene glycol and subsequently flash frozen in liquid nitrogen. *Bcl*Bep9_tBID1 crystals grew within days in 200 mM ammonium sulfate, 100 mM sodium citrate-HCl pH 5.6, 25% (w/v) PEG 4000 (MCSG1 (Anatrace) C8) and were immediately harvested into liquid nitrogen for flash freezing. *Bhe*BepE_BID1crystals grew in 200 mM $MgCl_2$, 100 mM HEPES-NaOH, pH 7.5, 25% (w/v) PEG 3350 (MCSG1 (Anatrace) A9). Crystals grew within days and were harvested and soaked in a solution containing the prior crystallization solution supplemented with 15% (v/v) ethylene glycol before flash freezing

X-ray data collection and structure determination

Data for *Bro*Bep6_tBID1 was collected at the Canadian Macromolecular Crystallization Facility beamline 08ID-1 with a Marmosaic 300 CCD detector. Data for *Bcl*Bep9_tBID1 and *Bhe*Bep6_BID1 were collected at the Advanced Photon Source on beamline 21-ID-G on a Marmosaic 300 CCD detector. All data were reduced using XDS/XSCALE (Kabsch, 2010). For *Bro*Bep6_tBID1 Friedel pairs were not merged, and the unmerged data provided an anomalous signal that was used to phase the data of *Bro*Bep6_tBID1 with Phaser (McCoy et al., 2007) from the CCP4 program suite (Winn et al., 2011). Density modification was performed with Parrot (Zhang et al., 1997) on the resulting electron density and the initial model was built into this modified map with ArpWarp (Morris et al., 2003). This structure was then used as a model for molecular replacement to determine the structure of *Bcl*Bep9_tBID1 using MR-Rosetta (Terwilliger et al., 2012). *Bcl*Bep9_tBID1 was then used as a molecular replacement model to determine the structure of

*Bhe*BepE_BID1 using MR-Rosetta. All structures were completed using iterative rounds of refinement in Phenix (Adams et al., 2010) followed by manual structure rebuilding with COOT (Emsley et al., 2010). All models were quality checked by Molprobity (Chen et al., 2010). All data reduction and refinement statistics are reported in Table 1.

Sequence and structure analysis

To generate our BID sequence working dataset, the sequences of the three newly determined BID domain structures were searched in Uniprot (UniProt Consortium, 2015) against the UniprotKB database using BLAST (Altschul et al., 1990) with a maximal e-value threshold of $1e^{-3}$. This resulted in 203, 196 and 197 homologous sequences for *Bro*Bep6_tBID1, *Bcl*Bep9_tBID1 and *Bhe*BepE_BID1, respectively. To remove redundant sequences (90% level of redundancy), we merged the three datasets and obtained 211 unique sequences. The FIC, OB and BID domains were then annotated using Geneious v7.1.7; Biomatters. Similarly, we BLASTed the BID domain of the relaxase of At-pRi1724 and the first and second BID domains of pATC58 (Schulein et al., 2005). After merging the three relaxase datasets, it resulted in 140 unique sequences. Combining the *Bartonella* and the relaxases a total of 351 sequences were retrieved.

Relaxase sequences were annotated with their species name, followed by the domain classification and ending with the UniProt sequence reference. For the *Bartonella* species, we annotated them with the lineage (L3/L4) followed by a three letter abbreviation for the species (see accession numbers section) followed by the domain classification and then the UniProt sequence reference.

All the sequences of our working dataset were then aligned using ClustalX 2.0 (Larkin et al., 2007) with a gap opening penalty of 10 and a gap extension penalty of 0.2 using BLOSUM matrices (S. Henikoff and J. G. Henikoff, 1992). Neighbor-joining distance based trees were constructed and visualized with iTOL (Letunic and Bork, 2011). Sequence

logos were generated with Weblogos (Crooks et al., 2004) and alignments visualized with Aline (Bond and Schüttelkopf, 2009). Conservation scores were generated using ConSurf (Ashkenazy et al., 2010). Electrostatics were calculated with the APBS-Tools and PDB2PQR (Dolinsky et al., 2007; 2004) plugins for PyMOL using the default settings.

The following servers were used to compare the structure of the newly determined BID domain structures and revealed no structural homology to any known structure currently available: ProFunc (Laskowski et al., 2005), InterProScan (Jones et al., 2014), PDBeFold (Krissinel and Henrick, 2004), MarkUs (Fischer et al., 2011) and ProBIS (Konc and Janezic, 2010).

Abbreviations

The abbreviations for the *Bartonella* sequences are: *Bartonella clarridgeiae* – L3_*Bcl*, *Bartonella rochalimae* - L3_*Bro*, *Bartonella sp.* AR 15-3 - L3_B15, *Bartonella sp.* 1-1C - L3_B11, *Bartonella alsatica* - L4_*Bal*, *Bartonella birtlesii* - L4_*Bbi*, *Bartonella doshiae* - L4_*Bdo*, *Bartonella elizabethae* - L4_*Bel*, *Bartonella henselae* - L4_*Bhe*, *Bartonella grahamii* - L4_*Bgr*, *Bartonella koehlerae* - L4_*Bko*, *Bartonella quintana* - L4_*Bqu*, *Bartonella rattimassiliensis* - L4_*Bra*, *Bartonella taylorii* - L4_*Bta*, *Bartonella tribocorum* - L4_*Btr*, *Bartonella washoensis* 085-0475 - L4_*Bwa*085, *Bartonella washoensis* Sb944nv - L4_*Bwa*Sb, *Bartonella washoensis* - L4_*Bwa*, *Bartonella sp. DB5-6* - L4_*Bdb*, *Bartonella vinsonii* subsp. Arupensis - L4_*Bva*, *Bartonella vinsonii* subsp. Berkhoffii - L4_*Bvb*.

Accession numbers

A0A024J204, I3QKD8, P55418, J0Q2C9, J0QJW8, J0R9F3, K0PZL2, E6YHI3, E6YHI2, H0HGV1, J1J4K1, W3TX69, J1JMC5, J1JQX9, A0A087LYR2, A0A060I368, E6YW78, M5JSQ2, F8C170, E6YS53, E6YS54, A0A031LX45, J0K3V5, A0A0Q7Y262, J0ZU19, W8IF22, K0Q5J8, J1K5A2, B9JPA6, J0QS37, H0GBP1, J1J5R5, J0R175, J0WFK0,

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