### Structural Biology

# Dimerization of the fungal defense lectin CCL2 is essential for its toxicity against nematodes

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#### Abstract

Lectins are used as defense effector proteins against predators, parasites and pathogens by animal, plant and fungal innate defense systems. These proteins bind to specific glycoepitopes on the cell surfaces and thereby interfere with the proper cellular functions of the various antagonists. The exact cellular toxicity mechanism is in many cases unclear. Lectin CCL2 of the mushroom Coprinopsis cinerea was previously shown to be toxic for Caenorhabditis elegans and Drosophila melanogaster. This toxicity is dependent on a single, high-affinity binding site for the trisaccharide GlcNAc(Fuc $\alpha$ 1,3) $\beta$ 1,4GlcNAc, which is a hallmark of nematode and insect N-glycan cores. The carbohydrate-binding site is located at an unusual position on the protein surface when compared to other β-trefoil lectins. Here, we show that CCL2 forms a compact dimer in solution and in crystals. Substitution of two amino acid residues at the dimer interface, R18A and F133A, interfered with dimerization of CCL2 and reduced toxicity but left carbohydrate-binding unaffected. These results, together with the positioning of the two carbohydrate-binding sites on the surface of the protein dimer, suggest that crosslinking of N-alycoproteins on the surface of intestinal cells of invertebrates is a crucial step in the mechanism of CCL2-mediated toxicity. Comparisons of the number and positioning of carbohydrate-binding sites among different dimerizing fungal β-trefoil lectins revealed a considerable variability in the carbohydrate-binding patterns of these proteins, which are likely to correlate with their respective functions.

Key words: carbohydrate recognition, lectin dimer, fucosylated N-glycan core, nematodes, toxin

#### Introduction

A common feature of animal, plant and fungal innate defense systems is the use of lectins as effector proteins (Gallo and Hooper 2012; Arthur and Cummings et al. 2014; Lannoo and Van Damme 2014; Kunzler 2015). These proteins act by binding to specific glycoepitopes on the surface of the cells and tissues of the antagonist and thereby fulfill a dual defense function: (1) they distinguish between self and non-self based on the organism-specific glycomes, and (2) they affect the viability of the antagonist either directly or in conjunction with additional effector proteins or protein domains. Examples of lectin-mediated toxicity in innate defense are bactericidal galectins recognizing blood group antigens in the intestinal mucosa of mammals (Stowell et al. 2010; Mukherjee et al. 2014), insecticidal vacuolar lectins produced and stored in specific plant tissues recognizing terminal mannose residues on the brush border membrane of midgut epithelial cells of insects (Caccia et al. 2012) and nematicidal and insecticidal cytoplasmic lectins abundant in the fruiting bodies and sclerotia of dikaryotic fungi (Sabotic et al. 2015). The cellular toxicity mechanism of these lectins is often unclear since most of these proteins lack additional domains or interacting proteins carrying established toxicity functions e.g. proteolysis or pore-formation. It appears that, in these cases, mere binding to glycoconjugates on cell surfaces leads to malfunctioning and eventually death of cells.

A key requirement for the toxicity of lectins appears to be the capacity to crosslink glycoconjugates (glycoproteins, glycolipids, polysaccharides) on the cell surface due to multivalency of the lectins i.e. the presence of multiple binding sites for the same or different glycoepitopes on the lectin surface (Brewer et al. 2002; Boscher et al. 2011; Kunzler 2015). Experimental evidence for this mechanism stems from studies with mammalian galectins, which were shown to crosslink specific glycoconjugates on the surface of mammalian cells (Pace et al. 1999; Sacchettini et al. 2001; Mockl et al. 2015). The resulting lattices between lectins and glycoconjugates on the cell surface were shown to interfere with cell function. Two mechanisms for the coupling of lattice formation and cell function are currently discussed. (1) Lectin-mediated multimerization of glycosylated cell surface receptors activates signaling pathways leading to apoptosis (Pace et al. 2000; Stillman et al. 2006; Hamshou et al. 2012); (2) Lectin-mediated clustering of glycoconjugates on cell surfaces interferes with the spatiotemporal dynamics, i.e. the residence time and localization of the glycoconjugates on the cell surface (Partridge et al. 2004; Cha et al. 2008; Lakshminarayan et al. 2014; Torreno-Pina et al. 2014). Indirect evidence for the requirement of lattice formation for lectin-mediated toxicity comes from a report where a fungal  $\beta$ -trefoil lectin, CNL from the mushroom *Clitocybe* nebularis, lost its toxicity due to a loss in multivalency but not carbohydrate-binding (Pohleven et al. 2012).

An exception to these proposed mechanisms seemed to be the recently identified  $\beta$ -trefoil lectin CCL2 that is highly expressed in *Coprinopsis cinerea* fruiting bodies (Plaza et al. 2014). CCL2 has been implicated in the defense of this mushroom against invertebrate predators based on its toxicity towards nematodes and insects (Schubert et al. 2012; Heim et al. 2015). Toxicity of CCL2 towards the model nematode *Caenorhabditis elegans* is dependent on the specific recognition of the  $\alpha$ 1,3fucosylated cores of *N*-glycans on the nematode intestinal epithelium but does not involve endocytosis of the lectin (Stutz et al. 2015). The previously determined three-dimensional NMR structure of monomeric CCL2 explained how the specificity and high affinity of this protein for this unusual

glycan target is achieved (Schubert et al. 2012) but gave, as a monovalent monomer, no hints at the toxicity mechanism. Here, further characterization with notably protein X-ray crystallography and NMR revealed that CCL2 forms in fact a compact dimer and, thus, is bivalent for its ligand. Dimerization occurs in a way that the two carbohydrate binding sites are located on one face of the dimer. Mutations in the dimerization interface did not affect binding of CCL2 to the glycan target in solution but interfered with the dimeric structure and led to a reduction of toxicity. These results suggest that the crosslinking of N-glycoproteins on the surface of intestinal epithelial cells is an essential part of the toxicity mechanism of this fungal  $\beta$ -trefoil lectin towards invertebrates.

#### Results

### Mutation of a conserved residue of CCL2(R18A) significantly reduces nematotoxicity

An alignment of CCL2 with its paralog CCL1 from C. cinerea and homologs encoded in other fungal genomes available via the Mycocosm of the US Department of Energy Joint Genome Institute (DOE-JGI) (Figure 1) revealed a number of highly conserved amino acid residues. Some of these residues, e.g. Y57, W78, L87, Y92 and W94, were previously shown to be involved in binding of the trisaccharide GlcNAc(Fucα1,3)β1,4GlcNAc by the single, high-affinity binding site of CCL2 (Schubert et al. 2012). The conservation of other conserved residues, e.g. R18, K26 and R136, could not be explained by a role in ligand binding, however. We speculated that these positively charged residues may be involved in binding of an additional ligand, e.g. phospholipids, and that this interaction would contribute to the nematotoxicity of the protein. In order to test this hypothesis, we generated individual alanine mutants of these residues and assessed the toxicity of Escherichia coli cells expressing untagged versions of these protein variants compared to the analogous wild type protein towards C. elegans N2. As a control, we used a protein variant with a dysfunctional carbohydrate binding site, CCL2(Y92A). The CCL2(R18A) protein variant showed thereby a significant drop in nematotoxicity while it was expressed in E. coli in soluble form and at similar levels as the wild type and the CCL2(Y92A) protein variant (Figure 2).

#### R18A mutation does not affect carbohydrate binding

To elucidate the molecular basis behind the effect of the R18A mutation on nematotoxicity, we first verified that R18 is not involved in carbohydrate-binding. We performed an NMR titration experiment with recombinant, His-tagged protein in which we monitored binding of the previously used, synthetic ligand GlcNAc (Fucα1,3)β1,4GlcNAcβ1O(CH<sub>2</sub>)<sub>5</sub>COOH (Schubert et al. 2012) on the protein side at residue-specific precision using 2D NMR spectroscopy in combination with <sup>15</sup>N labeled protein (Figure 3). In the presented 2D <sup>1</sup>H, <sup>15</sup>N-HSQC spectrum the NH group of each amino acid is represented by a cross-peak whose spectral position reflects the local chemical environment of that NH group. In the case of CCL2 WT and CCL2(R18A), ligand binding leads to the appearance of new cross-peaks and the disappearance of some initial signals, as ligand concentration is increased. The new cross-peaks originate from amino acids in tight contact to the ligand whereas the initial signals from the same amino acids reporting the absence of ligand disappear. The simultaneous observation of the free and bound form (Figure 3 on the right) indicates slow exchange kinetics

Second. struct.	β1		β2	β3	β4	35	36
			•		<b>•</b>		· ·
	10	20	3	0 40	50 60		70 80
CCL2 A :	MDSPAVTLSACN	IIYNRVLSPRGE	KLAL	YPERQRTPVIVSPLDGSSE	CAMILRSYDSNS	NIWTIS	PVGSPNSOIGWGAG
CCL2 0 :	MDSPA-TLSACH	VI FNRVLSPRGE	KLAL	YPCRORTPVIVSPLDGSSE	CAWILRSYDSNS	NIWSIS	PASSPNSOIGWCAG
CCL1 A :	MDTQAKPP-ACR	VI YNRVLSPNGE	KLAL	YPCYQREALTLTPLHHSAN	OIWILNNWDAT-	-ISSLT	PETARNTOVGWCSG
CCL1 O :	MDTOAKPP-ACR	VIYNRVLSPNGE	KLAL	YPCYOREALHLTPLHHGAN	OIWILNDWDAT-	-KSSLT	PETARNTOVGWCSG
CV L1 :	MSKPTACR	VIYSRVLSSTCO	KISL	YSCNONEGLUITPLANTPK	MOVIKDSSST-	SYIS	PYTSSGDEVGWGAG
CV L2 :	MSKVAACR	VTYSBAL TPTCO	KLAL	FSCTOKEALUISPLANAPK	TRVVKDYNST-	-ISTIG	DTSNRDVGNGGG
PP L1 :	MSRPSACT	TTYNRVLSPKCE	KLAF	FTC-ONKTOUVTPLSNDET	KOTTKDYNS	NUOTIS	ESNANL AAWOD-
PP L2	MSKPSA CT	TTYNEWI SPTCE	KLAF	FTC-ONKTOUVTPLSNNET	KNITKDYNS	NTOTIS	PESNANL AAWOD-
CT T1 .	MCKDCA eT	WT VNDWI DACOD	ZT N T	YMC-ECNYAHATRI SNCDI	WATTRDEDDO-	HOCUT	DATINGCI DACHOGE
CP II .	MCKDCDGD	UTVURTORCOF	DINT		THIT TONYNDT-	TOCTO	ATNOGLOAGWOOL
PO II .	MBNDer	VIIINIQ033CE	TT DI	ENG-EDNTWWWTDI VKUTN	CANCEDNANDO-	Anocuc	A DECONODI
FO_L1 :	CACACAMENDOT	VITUNIDUL ODACO		FNC-ERNIVEVEDICC FUC	AMCIANTIDG-	ALOOVO	PAPKSKLEIGMOPG
PO_LZ :	SASASAMPNPGT	IIVNRVLSPAGQ	NUBL	PNC-ERNIVIVIPLSSFHS	G-WCIRNIIDG-	ALQSVS	PASKSKLEIGMGPG
GC_LI :	MSRPAPEC	ALINKVLSPICE	ALC: I	INC-KGNILIVIPPRN-NSDS	OINQISNIDSN-	- QAVV	PNNDGSLOVGWGNA
C0_L1 :	MPSPSNCD	VIIINRII GHIGD	KLAT.	HNG-NDQIAHVEPLDRSSK	TMILQKSGST-	- FSVS	NKQTNLOAAWGEN
AP_LI :	MSRPNQCS	VIYNRVLSPTGD	KLAT:	FTG-QNNTLTVTPMTKSSN	OVFNLTNNDTN-	-IMFVV	PQSSSGLOVGWGSN
RV_L1 :	MTALPPRPAPEV	AIVNRVLSPNDE	RLAI	FNC-SDQPAHATVRTDSPA	ORWVIADYDTR-	-IQSVS	PQSSQNLOAAWGSG
PO_L3 :	MFNQCT	YIVNRMLSSSGQ	KLAL.	FNG-ENNTVUVT-LINATN	QAMAIRDYSDG-	KIQTVS	PVSSPHLQIGMGSG
ArO_L1 :	MSKPAPCT	KIINRVLSVNNK	RLAI	ANA-EGKASNVTEEAS-SNTA	QQNVIADFDSN-	-IQSMA	PVARPNLOAAWGSG
ArO_L2 :	MSKPAPGT	KIINRVLSVNNK	RLAI	ANA-EGKAAUVTEESS-SNTA	QQNVIADFDSN-	-TQSMA	PIARPSLOAAWGSG
ArO_L3 :	MSKPAPGT	KIINRVLSVNNK	QLAI	ANA-EGKAAUVTEESS-SNTA	QQNVIADFDSN-	-IQSMA	PVARPSLOAAWGSG
PO_L4 :	MANVAPCT	YIISRVLSPECE	KLAL	FNC-LGN-VIVTPFTNNSK	QAMVVKDYNAQ-	-NQMFS	PASQSNYQIGMGEG
RV_L2 :	-MCSSPRPAPGV	VIVNRVLSPNDK	RLAI	FNC-PDQPAHATVRTDGPA	ORNVIADYDTR-	-IQSVS	PTNAQNLOAAWGSG
SS_L1 :	MSKPSAGT	(ILYLRVLSPSGA	KLAL	WKS-SDNTVNLEPLTGDGS	QKWSIKDYNAT-	-TQSIS	PSNDANKOIGFGNG
AP L2 :	MSRPSPCS	VIYNRVLSPNGE	KLAI	FNC-QNNTLIVTPKNDSPG	QIFNLSNYDAN-	-IMFFV	PQSNSGLQVAWGNA
SH L1 :	MTKPPAPST	VIVNKAFSPTNE	RLAI	YNC-LDQPLTVNIKTDSSH	ORMIVQDHDQN-	-IKSLV	PVDATALOAAWGEV
HR L1 :	MSRPTPCT	IITNKARSTECN	ELTI	FNC-KGNALTVDNRTDSPS	ORMKISNYSDG-	RISYVV	PVSAPSLOVAWCRG
DS L1 :	MSSLPDED	FIINRALGYTCA	KLAI	FNG-VNOYAAVEPLASPPIOT	OINTIKVSRDK-	KIFSVT	RGASTDEAGWGSO
AO L1 :	MTKPAPCN	VIVSRVLSCDGQ	KLAL	FCK-KGGAVULTPLTHRQE	CINTISDYSDG-	OTOYII	PKKDDDLOIAIGGK
LB L1 :	AASVHAKVPACT	SIVNKVPSSDCL	SRAI	FNC-EGOTVIVSFDDSTAS	KOWIVEDYDGK-	-AOSIS	PVDGDGLOCAWGOD
LB L2 :	PTITVAYVRPDL	YIVNRVTPRRCA	PLAV	LSC-ENKOAVISTKTMSPT	OWYITEFDGN-	-GOAIV	SASDSSLKAARVGN
-	1.2					-	
	07 00					0.00	
Second. struct.	β7 β8		β9	β10	β11	β12	
Second. struct.	β7 β8		β9 •	β10	β11	β12	
Second. struct.	β7 β8	100	β9 •	β10 110120	β11	β12 140	
Second. struct.	β7 β8 90 NVPVVLPPNNYV	100 TLTLTSGS	β9 Υ-Ν <b>Ι</b>	β10 110 120 DECKRTVSØSLNNPTAGEEV	β11 130 SIGADATFSG	β12 140 RWVIEK	v
Second. struct. CCL2_A : CCL2_0 :	β7 β8 50 NVPVVLPPNNY00 NVPVVLPPNNY00	100 TLTLTSGG	β9 Υ-ΝΙ Υ-ΝΙ	β10 110 120 DCKRTVSSLNNTAGEEV DCKRTVSSLNNATAGEEV	130 SIGADATFSG SIGADATFSG	β12 140 RWVIEK RWVIEK	 v
Second. struct. CCL2_A : CCL2_O : CCL1_A :	β7 β8 90 NVPVVLPPNNVVV NVPVVLPPNNVVV NVPIVLPPGNVVV	100 TLTLTSGG TLTWTSAG TLASSAGG	β9 Υ-ΝΙ Υ-ΝΙ Υ-ΙΙ	β10 110 120 DECKRTVSNSLINNTAGEEU DECKRTVSNSLINNTAGEEU DECKRTVYNNAGEUVANTKV	β11 SIGADATFSG SIGADATFSG SIGGDNGLQA	β12 140 RWVIEK RWVIEK RWIFER	V V V
Second. struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O :	β7 β8 NVPVVVPPNNKVV NVPVVPPNNKVV NVPIVPPNNKVV NVPIVPPNNKVV NVPIVPPNNKVV	100 TLTLTSGC TLTWTSAC TLASSAGC TLASSAGC	β9 Y-NI Y-NI Y-II Y-II	β10 10 120 DCKRTVSNSLNN TAGEEV DCKRTVSNSLNN TAGEEV CGKTVYNAGE VANTKV CGKTVYNAGE VANTKV	β11 SIGADATFSG SIGADATFSG SIGGDNGLQA SIGGDNGLQA	β12 140 RWVIEK RWVIEK RWIFER RWIFER	V V V
CCL2_A : CCL2_O : CCL1_A : CCL1_O : CCL1_O : CV_L1 :	β7         β8           90         NVPV           NVPV         PNNYVO           NVPV         PGNYVO          PVV         PGGYVO	100 TLTLTSGC TLTWTSAC TLASSAGC TLRSGDWC	β9 Y-NI Y-NI Y-II Y-II Y-II Y-TI	β10 110 120 120 120 120 120 120 1	BII 130 SIGADATFSG SIGGDNGLQA SIGGDNGLQA SIGKDNGLLS	β12 140 RWVIEK RWVIEK RWIFER RWIFER RWIFEK	V V V A
CCL2_A : CCL2_O : CCL1_A : CCL1_O : CCL1_O : CV_L1 : CV_L2 :	β7         β8           90         NVPVULPENNYW           NVPVULPENNYW         NVPIULPENYW           NVPIULPENYW         NVPIULPENYW           -PVVLPENYW         -PVVLPENYW	TLTLTSGC TLTWTSAC TLASSAGC TLASSAGC TLRGGDWC TMQVVDSC	β9 Y-NI Y-II Y-II Y-II Y-II Y-II Y-II	βιο           110         120           CKRTVSNSLNNATAGEE         CKRTVSNSLNNATAGEE           DCKRTVSNSLNNATAGE         CKA-TVYNNAGE.VANTKV           CGKTVYNNAGE.VANTKV         CGKTAYNSVGNAVNTKV           CGKTAYNSVGNAVNTKV         CGKSASSYNVUCD TAGTKV	BII SIGADATFSG SIGADATFSG SIGGDNGLQA SIGGDNGLQA SIGKDNGILS SFGODNGILS	β12 140 WVIEK WIFER WIFER WIFER WIFEK	V V Q A
Second.struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : CV_L2 : PP L1 :	β7         β8           NVPVULPPNNYU         NVPVULPNNYU           NVPUVLPPNNYU         PNNYU           NVPIVLPGNYU         - PVULPGGYU           - PVULPGGYU         - QGYTLLPAAYU	TLTTSG TLTWTSA TLASSAG TLASSAG TLQGDW TMQVVDS VIKSTDS	β9 Y-NI Y-NI Y-II Y-II Y-TI Y-TI Y-TI	β10         120           CKR TVSNSLNNTAGEE         CKR TVSNSLNNTAGEE           CKR TVSNSLNNTAGEE         CKR TVSNNAGE           CGK TVYNNAGE         VANTK           CGK TVYNAGVAD         VANTK	β11 130 SIGADATFSG SIGGDNGLQA SIGGDNGLQA SIGGDNGLS SFGQDNGLS TTA-GTGNEKQ	β12 140 VIEK VIEK VIEK VIEK VIEK VIEK VFEK	V V Q Q Q
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Second. struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : CV_L2 : PP_L1 : PP_L2 : SL L1 :	β7         β8           NVPVUL PNNYU         NVPUL PNNYU           NVPVL PNNYU         NVPIL PGNYU           VVPUL PGNYU         PGNYU           VVPUL PGNYU         PGNYU           OUVUL PGNYU         PGNYU           OUVUL PGNYU         PGGYU           OUVUL PGGYU         PGGYU           OUVUL PGGAY         OUVUL PGGAY           OUVUL PGGAY         OUVUL PGGAY           OUVUL PGGAY         OUVUL PGGAY	IQO TLITISG TLATTSA TLASSAG TLASSAG TLRGOW VIKSTDS VIKSTDS VIKSTDS VIKSTDS VIKSTDS	β9 Y-NI Y-II Y-II Y-TI Y-TI Y-TI Y-TI Y-TI Y-TI	βιο           110         120           CKRTVS/05LNNATAGEEV         CKRTVS/05LNNATAGEEV           CGKTVY/05/05LVANTKV         CGKTVY/05UGEVANTKV           CGKTVY/05UGEVANTKV         CGKA-SYNV0GD-TV/05UGEVANTKV           CGKA-SYNV0GD-TDGTKV         CGKA-SYNV0GD-TDGTKV           CGKA-TTY/05UAD-VDNANV         CGQTVY/05UAD-VDNANV           CGQTVY/05UAD-VDNANV         CGQTVY/05UAD-VDNANV	110 SIGADATFSG SIGA-DATFSG SIGG-DNGLQA SIGG-DNGLQA SIGG-DNGLQS SIGC-DNGLS SIGC-DNGLS TITA-GTGNEKQ TVTA-GTGNEKQ	β12 140 RWVIEK RWVIEK RWIFEK RWIFEK RWIFEK RWIFE RWIFEQ RWIFEQ RWIFEQ	V V V A A A
Second.struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : PP_L1 : PP_L2 : SL_L1 : CP_L1 :	β7         β8           NV PV         PNN YO           NV PV         PNN YO           NV PV         PENN YO           NV PV         PENN YO           NV PV         PENN YO           VP VI         PEGAY           QGYTV         PAGAY           GVVT         PAGAY           -QVVT         PAGAY           -QVVT         PAGAY           -QVT         POAY	IN TLTITSG	β9 Y-NI Y-II Y-II Y-TI Y-TI Y-TI Y-TI Y-TI Y-TI Y-TI	β10           10         120           CKR TVSNSLNNTAGEE         CKR TVSNSLNNTAGEE           CKR TVYNNAGE-VANTKV         CKR TYNNAGE-VANTKV           CKK TYNNAGE-VANTKV         CKR TAYNSVGN AVNTKV           CKK TYNNAGE-VANTKV         CKR TAYNSVGN AVNTKV           CKK TYNNAGE-VANTKV         CKR TYNNAGE-VANTKV           CKR TYNNGVAD-VDNANV         CKQ TVYNGVAD-VDNANV           CKQ TVYNGVAD-VDNANV         CKQ TVYNGVAD-VDNANV           CKQ TVYNGVAD-VDNANV         CKQ TVYNGVAD-VDNANV           CKQ TVYNGVAD-VDNANV         CKQ TVYNGVAD-VDNANV	130 SIGA DATFSG SIGA DATFSG SIGG DNGLQA SIGK DNGLQA SIGK DNGLSS SFGQ DNGILS SFGQ DNGILS TTA GTGNEKQ AIGS DTGNEKQ AIGS DTGNEKQ	β12 140 RWVIEK RWIFER RWIFER RWIFEK RWIFEK RWIFFQ RWIFQ SWILKQ	V V A A A V
Second. struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : CV_L2 : PP_L1 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : PO_L1 :	β7         β8           NVPVUL PNNY         PNNY           NVPVL PRNY         PNNY           NVPIL PGNY         PGNY           NVPIL PGNY         PGNY           VVVL PGOY         PGNY           OVVL PGOY         PGNY           AVIV POA         POA           TIRV PGAY         PGAY	TLTLTSG TLTWTSA TLASSAG TLRGGDW TMQVVDS VIKSTDS TIRSSDS TIRSSDS TIRNAS TLRNTPT	β9 Y-NI Y-NI Y-II Y-TI Y-TI Y-TI Y-TI Y-TI Y-TI A-TI	β10           110         120           CKR TVSØSLNN TAGEE         CKR TVSØSLNN TAGEE           CGK TVYØNAGE/VANTK         CGK TVYØNAGE/VANTK           CGK TVYØNAGE/VANTK         CGKSASSYNVGD TDGTK           CGK TVYØVAD VDNAN         CGV TVYØGVAD VDNAN           CGQ TVYØGVAD VDNAN         CGQ TVYØGVAD VDNAN           CGQ TVYØGVAD VNDOE         CQ TVYØGVAD VNDAN	110 SIGADATFSG SIGGDATFSG SIGGDAGLQA SIGGDAGLQA SIGGDAGLQA SIGKDAGLS TTA-GTONEKQ AIGS-DTGNEKQ AIGS-DTGNEKQ	β12 140 RWVIEK RWIFER RWIFER RWIFEK RWIFEK RWIFHQ RWIFQQ RWILER SWILKQ RWILER	V V A A V V V
Second.struct. CCL2_A : CCL2_O : CCL1_A : CV_L2 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L2 : PO_L2 :	β7         β8           90         90           NVPVVL PNNYV         PRNYV           NVPIV PRNYV         PRNYV           NVPIV PRG II         -PVV PRG II           -PVV PRG II         -QVV PRG II           -QVV PRG II         -QVP PRG II           -QVV PRG II         -QVP PRG II           -QVP PRG II         -QVP PRG II	ILTITSG	β9 Y-NI Y-II Y-II Y-TI Y-TI Y-TI Y-TI A-TI A-TI	βιο           110         120           CKRTVS/NSLNM-TAGEE         CKRTVS/NSLNM-TAGEE           CKKTVY/NAGE/VANTK         CKR-TVY/NAGE/VANTK           CKKTAY/NSVGN-AVNTK         CKRTAY/NSVGN-AVNTK           CKKTAY/NSVGN-AVNTK         CKRTAY/NSVGN-AVNTK           CKKTAY/NSVGN-AVNTK         CKRTAY/NSVGN-AVNTK           CKATVY/NAGAD-VD/NAN         CKQTVY/NGVAD-VD/NAN           CKQTVY/NGVAD-VD/NAN         CGVTVY/NGLD-VEG/KI           CGVTVY/NGLD-VEG/KI         CKQTVY/NGLD-VEG/KI           CGVTVY/NGLD-VEG/KI         DIODE           CAVTVFNGLSD-NVDQE         CAVTVFNGLSD-NVDQE	110 SIGA DATFSG SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLS SIGG DNGLS TITA - GTGNEKQ TTA - GTGNEKQ TTA - GTGNEKQ GFGD - EDERGNQ TIKQ - GEGGIFQ GFGD - EDERGNQ	β12 140 RWVIEK RWVIEK RWIFER RWIFEK RWIFEK RWIFQ RWIFQ RWILCR RWILLP RWILLP	V V V A A V V V
Second.struct. CCL2_A : CCL2_O : CCL1_A : CV_L1 : CV_L2 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : PO_L1 : PO_L2 : GC_L1 :	β7         β8           NVPVULPNNY         90           NVPVLPNNY         90           NVPULPNNY         90           NVPILPRNY         90           NVPILPRNY         90           QVVVLPRANY         90           QVVVLPRANY         90           QVVVLPRANY         90           QVVVLPAGAY         90           QVVLPAGAY         90           -AVILPPANY         -11           -TIRUPPANY         -11           -GAVU         90	TLTITSG TLTWTSA TLASSAG TLASSAG TLRGGDW TMQVVDS VIKSTDS TIRSSDS TIRNTPS TLRWTPT TLRWTPS TLRWTPS TLRWTPS TLRWTPS	β9 Y-NI Y-II Y-TI Y-TI Y-TI Y-TI Y-TI A-TI A-TI A-TI	β10           110         120           CKR TVSNSLINN TAGEE         CKR TVSNSLINN TAGEE           CGK TVYNNAGE-VANTK         CGK TVYNNAGE-VANTK           CGK TVYNNAGE VANTK         CGK TVYNNAGE           CGK TVYNNAGE VANTK         CGK TVYNNAGE           CGK TVYNNAGE VANTK         CGK TVYNGVAD-VDNAN           CGC TVYNGVAD-VDNAN         CGC TVYNGVAD-VDNAN           CGQ TVYNGLET VEGOKI         CGQ TVYNGLET VEGOKI           CGAV TVFNGLSD NVDQE         CAV TVFNGLSD NVDQE           CGK TONHLINN TONAL         CGK TONAL	BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLS SIGY DNGLS SIGY DNGLS SIGY DNGLS SIGY DNGLS AIGS DTCNEKQ AIGS DTCNEK	β12 140 RWVIEK RWIFER RWIFER RWIFER RWIFER RWIFER SWILER SWILL RWIF RWIF	V V A A V V V V V V
Second.struct. CCL2_A: CCL2_O: CCL1_A: CV_L2: PP_L1: CV_L2: PP_L2: SL_L1: CP_L1: CP_L1: PO_L2: GC_L1: CU_	β7         β8           NVPVU - PNNY         PNNY           NVPVU - PNNY         NVPI - PONY           NVPI - PGO         -           - VPV - PGO         -           - VVV - PGO         -           - GVE - PGO         -           - TIR - PGA         -           - TIR - PGA         -           - AVK         0.2460	TLTISG TLTWTSA TLASSAG TLRGGDW TMQVVDS VIKSTDS VIKSTDS TIRSDS TIRSDS TLRNTPS TLRNTPS TLRNTPS TIRSGYD TIRSGYD	β9 Y-NI Y-II Y-II Y-II Y-TI Y-TI Y-TI A-TI A-TI A-TI A-TI Y-FI Y	βιο           110         120           CKRTVS/MSLNN-TAGEE         CKRTVS/MSLNN-TAGEE           CGKTVY/NAGE/VANTK         CGKTVY/NAGE/VANTK           CGKTVY/NAGE/VANTK         CGKTVY/NAGE/VANTK           CGKTVY/NAGE/VANTK         CGKASSYNV0G-TDGTK           CGKTVY/NGVAD/VDNAN         CGG/TVY/NGVAD/VDNAN           CGQTVY/NGLGT/VEGCKI         CGQ/TVY/NGLDF/EEKON           CGQTVY/NGLSD-NVQOE         CA/TVY/NGLSD-NIDOE           CGA/TVY/NGLSD-NIDOE         CGG/TVY/NGLSD-NIDOE           CGUTVY/NGLSD-NIDOE         CGA/TVY/NGLSD-NIDOE	130 SIGADATFSG SIGADATFSG SIGGDNGLQA SIGGDNGLQA SIGKDNGLS SIGKDNGLS TTA-GTONEKQ TTA-GTONEKQ AGS-DTONEKQ AFGD-EDERGNQ AFGD-EDERGNQ AFGD-EDERGNQ AFGD-EDERGNQ	β12 140 RWVIEK RWIFER RWIFEK RWIFEK RWIFEQ RWILP RWILLP RWILLP RWILLP RWILLP RWILLP RWILLP	V V A A V V V V V
Second struct. CCL2_A : CCL2_C : CCL1_A : CCL1_C : CV_L1 : PP_L2 : PP_L2 : SL_L1 : PO_L1 : PO_L1 : PO_L2 : GC_L1 : CU_L1 : QP_L1 : PD_L2 : CCL1 : QCL2 : QP_L1 : PO_L2 : QP_L1 : QP_L1 : QP_L1 : QC_L1 : QP_L1 : QC_L1 : QC_	β7         β8           NVPVUEPNNY         90           NVPVEPNY         90           NVPVEPNY         90           -PVVEPGGY         90           -PVVEPGGY         90           QUVTUEPGGY         90           -QVVEPGGY         90           -QVVEPGGY         90           -QVVEPGGY         90           -AVI         90           -TIRVEPGAY         -TIRVEPA           -QAVU         90           -TIRVEPA         90           -QAVU         90	IN TLTITSG	β9 Y-NI Y-II Y-II Y-II Y-TI Y-TI Y-TI A-TI A-TI A-TI A-TI A-TI Y-TI A	β10         120           CKR TVSNSLNN TAGEE         CKR TVSNSLNN TAGEE           CKR TVSNSLNN TAGEE         CKR TVSNSLNN TAGEE           CKR TVSNSLNN TAGEE         CKR TVSNSLNN TAGEE           CKR TVSNSLNN TAGEE         CKR TYNNAGE VANTK           CKR TYNNAGE VANTK         CKR TYNNAGE VANTK           CKR TYNGVAD VDNAN         CKC TVYNGVAD VDNAN           CKC TVYNGUAD VDNAN         CKC TVYNGUAD VDNAN           CKQ TVYNGUAD VDNAN         CGV TVYNGUAD VDNAN           CKQ TVYNGUAD VDNAN         CGU TVYNGUAD VDNAN           CGC TVYNGUAD VDNAN         CGU SONGID VOCON           CGC TQNHLDNNTDNAL         CGU SONGID SPSPGTKI	BII TIA SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLS SIGC DNGLS SIGC DNGLS SIGC DNGLS SIGC DNGLS TA GIGNERQ AIGS DTGNERQ AIGS DTG	β12 140 RWVIEK RWIFER RWIFER RWIFER RWIFER RWIFER RWIFER RWIFER RWIFE RWILLER RWILLER RWILLER	V V A A V V V V V V V V V
Second.struct. CCL2_A : CCL1_O : CCL1_A : CV_L1 : CV_L2 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : PO_L2 : GC_L1 : CU_L1 : AP_L1 : PV_L1 : PV_L1 : PV_L1 : CU_L1 : PV_L1 : CU_L1 : PV_L1 : CU_L1 : PV_L1 : CU_L1 : CU	β7         β8           NVPVUL PNNY         NVPVUL PNNY           NVPVUL PNNY         NVPIL           NVPVUL PGNY         NVPIL           VVVUL PGNY         NVPIL           OVTVL PGGY         OVTVL PGGY           - PVV VE PGGY         OVTVL PGAY           - GVVL PGGY         OVTVL PGAY           - TIRV PGAY         - TIRV PGAY           - GAV LPAHAY         - AVK QSAGG           - AVK QSAGG         - AVK QSAGG	TLTLTSG	β9 Y-NI Y-NI Y-III Y-III Y-TII Y-TII Y-TII A-TII A-TII Y-TII	β10           110         120           CKR TVSØSLNN TAGEE         CKR TVSØSLNN TAGEE           DCKR TVSØSLNN TAGEE         CKR TVYØNAGE-VANTK           CGK TVYØNAGE         VANTK           CGK TVYØNAGE         VANTK           CGK TVYØNAGE         VANTK           CGK TVYØNAGE         VANTK           CGC TVYØSVAD-VDNAN         CGC TVYØGVAD-VDNAN           CGQ TVYØGUAD-VDOAN         CGQ TVYØGLAE           CGQ TVYØGLAE         VEGQKI           CGQ TVFØGLAE         VEGQKI           CGQ TVFØGLAE         VEGQKI           CGQ TVFØGLAE         VEGQKI           CQ TVFØGLAE         VEGQKI           CQ TVFØGLAE	BII SIGADATFSG SIGGDATFSG SIGGDATFSG SIGGDNGLQA SIGGDNGLQA SIGGDNGLS SIGCDNGTLS SIGCDNGTLS SIGCDIGNEKQ AIGS-DTGNEKQ AIGS-DTGNEKQ AIGS-DTGNEKQ AIGS-DTGNEKQ AIGS-DCORENG AIGS-DCORENG AIGS-DQGSETY IGC-DCORENG AIGS-DQGSETY IGC-DCORENG IGC-DCORENG IGC-CORENG	β12 140 RVIEK RVIEK RVIFER RVIFER RVIFER RVIFER RVIFER RVIER	V V A A V V LD V
Second struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : PO_L2 : GC_L1 : CU_L1 : CU_L1 : RV_L1 : RV_L1 : PD_12 : RV_L1 : R	β7         β8           NV PV         PNN PV           NV PV         PNN PV           NV PV         PRN PV           NV PV         PRN PV           NV PV         PRN PV           OV V         PRO           -PV V         PRO           QV V         PRO           -GVE         PRA           -AVI         PRA           -AVK         PRA           -GAV         PAN           -GAV         PAN           -GAV         PAN           -GATPHTPCG         -           -FVT         -	ICTURYSA	β9 Y-NI Y-NI Y- Y- TI Y- Y- TI Y- Y- TI Y- Y- TI Y- Y- TI Y- Y- TI Y- Y- TI Y- Y- Y- TI Y- Y- Y- TI Y- Y- Y- Y- TI Y- Y- Y- Y- Y- Y- Y- Y- Y- Y-	β10         120           CKRTUSNSLNN-TAGEEU         CKRTUSNSLNN-TAGEEU           CKRTUSNSLNN-TAGEU         CKRTAYNSUG           CKKTAYNSUG         VANTKI           CGKTUYNNAGC         VANTKI           CGKTUYNNAGC         VANTKI           CGKTUYNAUGU         VANTKI           CGKA-SSYNVICD         TDGTKI           CGKASSYNVICD         TDGTKI           CGQTUYNGVAD/VDNANI         CGQTUYNGUAD/VDNANI           CGQTUYNGLAD-VEGKI         CGQTUYNGLAD-VEGKI           CGQSQNHLDK-ANNAN         CGMSQNAHLDK-ANNAN           CGMSQNAHLDK-ANNAN         CGQTUYNGLAD-VEGAN           CGMSQNAHLDK-ANNAN         CGNTUYNGLAN-VEGAN	110 SIGADATESG SIGADATESG SIGADATESG SIGGDNGLQA SIGGDNGLQA SIGKDNGLS SIGKDNGLS TITA-GTONEKQ TTA-GTONEKQ GFGD-EDERGNQ ATAP-DQCSERY TIGK-DNGQPSY ATAP-DQCNERY TIGK-DNGQPSY ATAP-DQCNERY	β12 140 VIEK VIEK VIFR VIFR VIFR VIFR VIER V	V V A A V
Second.struct. CCL2_A: CCL2_O: CCL1_A: CCL1_O: CV_L1: CV_L2: PP_L2: PP_L2: PP_L2: PD_L1: PO_L1: PO_L1: CC_L1: CU_L1: AP_L1: RV_L1: PO_L3: DC_L3: CV_L1: CV_L1: CU	β7         β8           NVPVUL PNNY         PNNY           NVPVL PNNY         PNNY           NVPUL PGNY         PONY           -PVVL PGGY         -PVVL PGGY           -VVVL PGGY         -PVVL PGGY           -PVVL PGGY         -PVVL PGGY           -TIRVL PGAY         -           -TIRVL PGAY         -           -AVIVL PGAY         -           -AVKL QSAGY         -           -GAVU PARAY         -           -AVKL QSAGY         -           -FYTN -         -           -FYTN -         -           -FYTN -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -           -         -	TLTITSG	β9 Y-NII Y-II Y-II Y-II Y-II Y-II Y-II Y-II Y-II X-II Y-II X-X X-X	β10           110         120           CKR TVSNSLINN TAGEE         CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE         CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE         CANTK           CKR TVSNSLA         CKR TVSNSLA           CKR TVSNSLA         CKR TVSNSLA           CKR TVSNSLA         CNNTK           CKR TVSNSLA         CNNTK           CKR TVSNSLA         CVNTK           CKQ TVSNSLA         CVNTK           CGU TVSNSLA         CKR TSNSLA           CGU TVSNSLSD         NVDQE           CAV TVFNGLSD         NVDQE	BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLQS SIGC DNGLSS SIGC DNGLSS TITA -GTGNEKQ AIGS DTGNEKQ AIGS DTGNEKQ AIGS DCSSERY TIGK DQGSERY TIGK DQGSERY TIGK DQGSERY TIGC GTGNEKQ GFGK EAANGQU	β12 140 WIEK WIEK WIFER WIFER WIFER WIFER WIE WIE WIEL WILL WILL WILL WILL WILL WILL WILL WILL WIE WILL K WIFER WIE WIE WIE WIE WIE WIE WIE WIE	V V A A V
Second.struct. CCL2_A : CCL1_O : CCL1_A : CV_L1 : CV_L2 : PP_L2 : SL_L1 : CP_L1 : PO_L2 : GC_L1 : CU_L1 : RV_L1 : RV	β7         β8           NVPVUL PNNYU         PNNYU           NVPVUL PNNYU         PNNYU           NVPUL PGNYU         PGNYU           VVVUL PGNYU         PGNYU           -PVUL PGGY         G           -QVVUL PGGY         G           -QVVUL PGGY         G           -QVUL PGGY         G           -QVUL PGGY         G           -QVUL PGGY         -           -QVUL PGGY         -           -AVIU PGAY         -           -GAVU PAHAS         -           -AVK (JSAGG)         -           -FVTU PGGY         -           -FVTU PGGY         -           -FVTU PGGY         -           -FVTU PGGY         -	TLTITSG TLTWTSA TLASSAG TLASSAG THRGGDW TIRGGDW TIRSSDS TIRSSDS TIRSSDS TLRNTPS TIRSGYD TIRSSDA TIRNTDT TIRNTDT TIRNTDT TIRNTDT TIRNTDT TIRNTDT TIRNTDT	β9 Y-NII VY-IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	β10           110         120           CKRTVS/05LINN-TAGEE         CKRTVS/05LINN-TAGEE           DCKRTVS/05LINN-TAGEE         CKRTVY/05LINN-TAGEE           CGKTVY/05LGT-VEQKI         CGKS-SY/05VGN-AVNTKI           CGKTVY/05UGD-TDGTK         CGKS-SY/05VGN-AVNTKI           CGKTVY/05UGD-TDGTK         CGQTVY/05UGD-TDGTKI           CGQTVY/05UGD-TPGKI         CGQTVY/05UGD-TEGKI           CGQTVY/05UGD-TEGKI         CGQTVY/05UGD-TEGKI           CGQTVY/05UGD-	BII IIO SIGA DATFSG SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLSA SIGC DNGLS TTA-GTONEKQ AIGS-DTGNEKQ AIGS-DTGNEKQ AIGC-EDERGNQ AIGC-EDERGNQ AIGC-EDERGNQ AIGC-EDERGNQ TGA-CTGNVQQ IGCK-EAANGNQ TGA-CTGNVQQ	β12 140 WVIEK WIEK WIFER WIFER WIFER WIFER WIFER WIER WILE WILE WILE WULE WILE WULE WILE WULE W	V V
Second struct. CCL2_A : CCL2_C : CCL1_A : CCL1_C : CV_L1 : CV_L2 : PP_L2 : SL_L1 : CP_L1 : PO_L2 : GC_L1 : CU_L1 : CU_L1 : RV_L1 : RV_L1 : PO_L3 : ArO_L1 : ArO_L1 : ArO_L2 : Ar	β7         β8           NVPV/L PNNY         PNNY           NVPV/L PNNY         PNNY           NVPV/L PGG II         -           -PV/L PGG Y         -           QVYVL PGG Y         -           -QVVYL PGG Y         -           -QVVYL PGG Y         -           -QVVYL PGG Y         -           -QVYL PGG Y         -           -TIR V PGAY         -           -QAVU - PGAY         -           -QAVY - PGAY         -           -QAVY - PGAY         -           -PVV - PGAY         -           -TIR V - PGAY         -           -QAVV - PGAY         -           -PVV - PGAY         -           -PV	INTERTAL	β9 VIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	βιο           110         120           CKRTUSISLINIA TAGEE         CKRTUSISLINIA TAGEE           CKRTUSISLINIA TAGEE         CKRTUSISLINIA TAGEE           CKRTUSISLINIA TAGEE         CKRTASIS           CKRTUSISLINIA TAGEE         CKRTASIS           CKRTUSISLINIA TAGEE         CKRTASIS           CKRTUSISSINA         TUSIS           CKQTUSIS         CKR-SSINANG           CKQTUSISLINIA TAGEE         CKQTUSISSINA           CGVTUSISSINA         TUSISSINANDOE           CGVTUSISSINANDOE         CGXTUSISSINANDOE           CGUSONGIDSSPERKINANDOE         CGKSONGIDSSPERKINANDOE           CGUTUSISSINANDANDOE         CGVTUSISSINANDANOE           CGUSONGIDSSPERKINANDOE         CGVTUSISSINANDANOE           CGUTUSISSINANDANO         CGUTUSISSINANDANO           CGUTUSISSINANDANO         CGRTUSISSINANANO           CGRTUSISSINANANO         CGRTUSISSINANANO           CGRTUSISSINANANO         CGRTUSISSINANANO           CGRTUSISSINANANO         CGRTUSISSINANANO           CGRTUSISSINANANO         CGRTUSISSINANANO           CGRTUSISSINANO         CGRTUSISSINANO           CGRTUSISSINANO         CGRTUSISSINANO           CGRATUSISSINANO	BII TIA SIGA DATFSG SIGG DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLS SIGC DNGLS SIGC DNGLS TTA -GTGNEKQ AFGD - EDERGNQ AFGD - EDERGNQ AFGD - EDERGNQ AFGD - EDERGNQ AFGD - EDERGNQ AFGD - GTGNEKQ GFGK - EAANIGNG GFGK - EGTSDVQQ TIGA GTGDVQQ TIGA GTGDVQQ	β12 140 WVIEK WVIEK WVIEK WVFEK WIF2Q WIF2Q WIF2Q WIF2K WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIEK WVI	V V A A V
Second.struct. CCL2_A: CCL2_O: CCL1_A: CCL1_O: CV_L1: CV_L2: PP_L1: PP_L2: PP_L1: PO_L1: PO_L1: CC_L1: CU_L1: AP_L1: CU_L1: PO_L3: ATO_L1: PO_L3: ATO_L2:	β7         β8           90         90           NVPVULPNNY         90           NVPULPENY         90           NVPILPENY         90           NVPILPENY         90           OVVVLPEGY         90           -PVVLPEGY         90           OVVTLPAGAY         -QVVLPEGY           -AVILPEGY         -ANILPEGY           -TIRUPEGAY         -TIRUPEGAY           -GAVLARAY         -QAVLARAY           -GAVLARAY         -AVKOSAGGY           -FYTULACAY         -YIKUSPGGY           -FYTULACAY         -YIKUSPGGY           -FMTULPANNY         -YIKUSPGGY           -FMTULPANSY         -YIKUSPGGY	TLTITSG TLTWTSA TLASSAG TLRGGDW	β9 YNI IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	β10           110         120           CKR TVSOISLINN TAGEE         CKR TVSOISLINN TAGEE           CGK TVYNNAGE VANTK         CGK TVYNNAGE VANTK           CGK TVYNNAGE VANTK         CGK TVYNNAGE VANTK           CGK TVYNNAGE VANTK         CGC TVYNGVAD VDNAN           CGC TVYNGVAD VDNAN         CGC TVYNGUAD VDNAN           CGO TVYNGUAD VDNAN         CGO TVYNGUAD VDNAN           CGO TVYNGLDE EKKNN         CGA TVYNGLDE EKKNN           CGA TVYNGLSD NIDDE         CGA TVYNGLSD NIDDE           CGA TVYNGLSD NIDOE         CGA TVYNGLDE EKKNN           CGA TVYNGLD VGGE         CGA TVYNGLD VGGE           CGA TVYNGLAN SDSCO         CGR TAFNGLAN SGNSCO           CGR TAFNGLAN SGNSCO         CGR TAFNGLAN SGNSCO	BII SIGA DATFSG SIGG DATFSG SIGG DATFSG SIGG DAGLA SIGG DAGLA SIGG DAGLA SIGC DAGLA	β12 140 VIEK WVIEK WIFFER WIFFER WIFFER WIFFER WIFFΩ WIFFΩ WIFFER WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WVIE WIFFΩ WVIE WVIE WIFFΩ WVIE WVIE WIFFΩ WVIE WVI	V V A V
Second struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : PO_L1 : CP_L1 : CD_L1 : CU_L1 : CU_L1 : CU_L1 : RV_L1 : RV_L1 : RV_L1 : RV_L1 : PO_L3 : ArO_L3 : PO_L3 : PO_L4 : PU_C : PU_C : PU_C : PO_L3 : PO_L3 : PO_L3 : PO_L3 : PO_L3 : PU_C : PU_C : PU_C : PO_L3 : PO_L3 : PU_C : PU_C : PO_L3 : PO_L3 : PU_C : PU_C : PU_C : PU_C : PU_C : PU_C : PO_L : PU_C :	β7         β8           NVPV/LPNNY         90           NVPV/LPNNY         90           NVPV/LPNNY         90           NVPV/LPNNY         90           OVV/LPNNY         90           -PV/LPCG1         90           -QVV/LPCG3         90           -QVV/LPCG3         90           -GVE/LPCG3         90           -TIRV         90           -TIRV         90           -GAV/LPCG3         90           -GAV/LPCG3         90           -FIRV         90	TLTISG TLTSG TLASSAG TLASSAG TLRGGDW TIRSSDS TIRSSDS TIRSSDS TIRSSDS TIRNAS TLRNTPT TIRSSDA TIRSSDA TIRSDA TIRTDA TIRETDT TIRETDI TIRETDI TIRETDI TIRETDI TIRETDI TIRETDI TIRETDI TIRETDI		βΙ0           110         120           CKRTVS/05LNNTTAGEE         CKRTVS/05LNNTTAGEE           CKRTVS/05LNNTTAGEE         CKR-TVY/05L02           CKRTVY/05L02         CANTK           CGKTVY/05L02         CANTK           CGKTVY/05L02         CANTK           CGKTVY/05L02         CANTK           CGKA-SSYNV0CD-TVEGCKI         CGKASSYNV0CD-TVEGCKI           CGQTVY/05L02         CEKQASSYNV0CD-TVEGCKI           CGQTVY/05L02         CEKQASSYNV0CDASSYNV0CAN           CGRTAFEGCLANSSYNV0CAN         CEKQASSYNV0CAN	10           SIGA DATFSG           SIGA DATFSG           SIGA DATSG           SIGA DATSG           SIGA DATSG           SIGA DATSG           SIGA DNGLQA           SIGK DNGLGA           SIGK DNGLGA           SIGK DNGLGA           GS-DTGNEKQ           TTVAGEGIFOQ           AIGS-DTGNEKQ           TGO-EDERGNQ           AIAP-DQCSERY           AIAP-DQCSERY           TIGA-GTGNEKQ           TIGA-GTGNEQ           TIGA-GTGDIQQ           PFEE-GNGSDKQ           TIGA-GTGDIQQ           PFEE-GNGSDKQ	β12 140 VIEK VIEK VIFSR VIFSR VIFSR VIFSR VIFSR VIEC VI	V V A V
Second.struct. CCL2_A: CCL2_O: CCL1_A: CCL1_O: CV_L1: CV_L2: PP_L2: PP_L2: PD_L1: PO_L1: PO_L1: PO_L1: QC_L1: CU_L1: AP_L1: AP_L1: AP_L1: ATO_L2: ATO_L2: ATO_L2: ATO_L2: CU_L1: ATO_L2: ATO_L2: ATO_L2: CU_L1: ATO_L2: ATO_L2: CU_L1: C	β7         β8           NVPV/L PNNY         PNNY           NVPV/L PRNY         NVPUL PGCYL           -PV/L PGCYL         -PCGYL           -QVV/L PGCYL         -QCVL           -QV/L PGCYL         -QCVL           -YIR/L PGAYL         -TIR/L PGAYL           -QCAVU-ARAY         -QAVL           -QAVU-ARAY         -QAVL           -QAVU-ARAY         -QAVL           -PYT/L PGAYL         -TIR           -FYT/L PGAYL         <	TLTITSG	β9 YIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	β10           110         120           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CGK TVYNAGE-VANTK           CGK TVYNAGE-VANTK           CGK TVYNAGE-VANTK           CGK TVYNAGE-VANTK           CGK TVYNAGE-VANTK           CGK TVYNAGE-VANTK           CGK TVYNGVAD-VDNAN           CGA TVYNGUAD-VDNAN           CGQ TVYNGLE           CGW TVFNGLESD-NVDQE           CGW TVFNGLE           CGW TVFNGLE           CGW TVFNGLNSDE           CGW TVFNGLANSENSCO           CGR TAFNGLANSENSCO           CGR TAFNGLANSENSCO           CGR TAFNGLANSENSCO           CGR TAFNGLANSENSCO           CGR TAFNGLANSENSCO           CGW TVFNGLE           CGW TVFNGLE	BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLSA SIGC DNGLSA SIGC DNGLSA TTA-GTGNEKQ GFGD-EDERGNQ GFGD-EDERGNQ GFGD-EDERGNQ GFGD-EDERGNQ GFGD-EDERGNQ GFGD-EDERGNQ GFGD-EDERGNQ GFGC-GTGNEKQ GFGC-GTGNE	B12 140 VIEK VI	V V A A V
Second.struct. CCL2_A: CCL2_O: CCL1_A: CCL1_O: CV_L1: CV_L2: PP_L2: PP_L2: SL_L1: CC_L1: PO_L1: PO_L1: PO_L2: CU_L1: AP_L1: CU_L1: PO_L3: ATO_L3: ATO_L2: ATO_L2: ATO_L2: SC_L1: CU_L1: PO_L3: PO_L3: PO_L3: CU_L1: PO_L3: PO_L3: PO_L3: PO_L3: PO_L2: SC_L1: PO_L3: PO_L3: PO_L3: PO_L3: PO_L2: SC_L1: PO_L3: PO_L3: PO_L3: PO_L2: CU_L1: PO_L3: PO_L4: PO_L3: PO_L4: PO_L2: PO_L4: PO_L2: PO_L2: PO_L4: PO_L2: PO_L4: PO_L4: PO_L3: PO_L4:	β7         β8           NVPVUL PNNY         NVPVL PNNY           NVPVL PGNY         NVPIL           NVPIL         PGNY           NVPIL         PGNY           NVPIL         PGNY           QGVTUL         PGAY           -PVUL         PGGY           QVVTUL         PGAY           -QVVTL         PGAY           -TIRUL         PGAY           -GAVUL         PANY           -TIRUL         PGAY           -GAVUL         PANY           -FMTUL         PANY           -FMTUL         PGAY           -FMTUL         PGAY           -FMTUL         PANY           -FMTUL         PANY           -FMTUL         PANY           -FMTUL         PANY           -FMTUL         PANY           -FMTUL         PANY           -FWTUL         PANY           -	TLTUTSG	β9 Y-N111111111111111111111111111111111111	βΙ0           110         120           CKR TVS/05LINN-TAGEE         CKR TVS/05LINN-TAGEE           CKR TVY/05LINN-TAGEE         CKR TVY/05LINN-TAGEE           CKR TVY/05LINN-TAGEE         CKR TVY/05LINN-TAGEE           CKR TVY/05LINN-TAGEE         CKR TVY/05LINN-TAGEE           CKR TVY/05LINN-TAGEE         CKR TVY/05LINN-TOGK           CKQ TVY/05LINN-TVG/05LINN-TVG/05LIN         CKQ TVY/05LINN-TVG/05LIN           CKQ TVY/05LINN-TVG/05LIN         CGR TVY/05LINN-TVG/05LIN           CGU TVY/05LINN-TVG/05LIN         CGU TVY/05LINN-TVG/05LIN           CGU TVY/05LINN-TVG/05LIN         CGR TVY/05LINN-TVG/05LIN           CGU TVY/05LINN-TVG/05LIN         CGU TVY/05LINN-SUDS(0)           CGU TVY/05LINN-SUDS(0)         CGR TVY/05LINN-SUDS(0)           CGR TVY/05LINN-SUDS(0)         CGR TVY/05LINN-SUDS(0)           CGR TVY/05LINN-SUDS(0)         CGR TAF/05LINN-SUDS(0)           CGR TAF/05LINN-SUDS(0)         CGR TAF/05LINN-SUDS(0)           CGR TAF/05LINN-SUN-S	10           SIGA DATFSG           SIGA DATSG           SIGA DATSG           SIGA DATFSG           SIGA DATSG           AIGA DATSG           AIGA DOGERY           GFGK EAANSQ           TIGA GTGDVQQ           TIGA GTGDVQQ           TIGA GTGDVQ           TIGA GTGNEXQ	β12 140 WIEK WIFR WIFR WIFR WFF WFF WIF WIF WIF WIE WIE WIE WIE WIE WIE WIE WIE	V V A V
Second struct. CCL2_A : CCL2_C : CCL1_A : CCL1_C : CV_L1 : CV_L2 : PP_L1 : PP_L2 : SL_L1 : CP_L1 : PO_L1 : CO_L1 : CC_L1 : CU_L1 : CU_L1 : RV_L1 : PO_L2 : AP_L1 : RV_L1 : ATO_L1 : ATO_L3 : PO_L3 : ATO_L3 : PO_L4 : RV_L2 : SS_L1 : AP_L2 : SS_L1 : AP_L2 : CS_L1 : CS_L1 : CD_L3 : CD_L4 : CD_L4 : CD_L3 : CD_L4 : CD_L4 : CD_L3 : CD_L4 : CD_L4 : CD_L3 : CD_L4 : CD_L4 : CD_L3 : CD_L4 : CD_L4 : CD_L3 : CD_L4 : CD_L3 : CD_L4 :	β7         β8           NVPV/LPNNY         PNNY           NVPV/LPRNY         PNNY           NVPV/LPRNY         PRNY           VVPV/LPRS         II           -PV/LPRS         II           QVVY/LPRS         II           -QVV/LPRS         II           -QVV/LPRS         II           -AVX/LPRS         II           -TIR/LPRS         GAV           -TIR/LPRS         GAV           -FIT         ANN           -FIT         ANN           -FIT         ARN           -FIT         AR	INTERDA		βιο           110         120           CKRTUSNSLNN-TAGEE         CKRTUSNSLNN-TAGEE           CKRTVYNSSCVNN-ZVARK         CKR-TYNNAGC VANTK           CGKTVYNNAGC VANTK         CGKTVYNNAGC VANTK           CGKTVYNNAGC VANTK         CGKSASSYNVGO TOTK           CGKTVYNGUATVONAN         CGGVTVYNGUATVONAN           CGQTVYNGUAD-VDNAN         CGQTVYNGUAD-VDNAN           CGQTVYNGUAD-VDNAN         CGQTVYNGUAD-VDNAN           CGQTVYNGUAD-VDNAN         CGQTVYNGUAD-VDNAN           CGQTVYNGUAD-VDNAN         CGQTVYNGUAD-VDNAN           CGQTVYNGUAD-VDQE         CGQTVYNGUAD-VDQE	BII TIA SIGA DATFSG SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLS SIGC DNGLS SIGC DNGLS SIGC DNGLS TITA -GTGNEKQ GIGC DTGNEKQ GIGC DNGLS TIGA GTGNUQQ TIGA GTGDVQQ TIGA GTGDVQ TIGA	ALC	V V A A V
Second.struct. CCL2_A : CCL2_O : CCL1_A : CCL1_O : CV_L1 : CV_L2 : PP_L2 : PP_L2 : PD_L1 : PO_L1 : PO_L1 : PO_L1 : PO_L1 : PO_L1 : AP_L1 : AP_L1 : ATO_L1 : ATO_L1 : ATO_L1 : ATO_L3 : ATO_L3 : ATO_L3 : SS_L1 : SS_L	β7         β8           90         90           NVPVUL PRNY         90           NVPUL PRNY         90           NVPUL PRNY         90           NVPUL PRNY         90           OVTUL PRNY         90           PMTUL PRNY         90           PMTUL PRNY         90           VIA WI PROVI         <	TLTITSG		β10           110         120           CKR TVSNSLINN TAGEE         CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE         CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGE         CANTK           CKR TVSNNAGE         CANTK           CKR TVSNSLINN TAGEE         CANTK           CKR TVSNSLINN TAGE         CANTK           CKR TVSNSLINN TAGE         CANTK           CKR TVSNSLINN TAGE         CANTK           CKR TVSNSLINN TAGE         CANTK           CKQ TVSNSLINN TAGE         CANTK           CKR TSNSLINN TAGE         CANTK           CAV TVFNGLSD         NIDQE           CAV TVFNGLSD         NIDQE           CGAV TVFNGLSD         NIDQE           CGAV TVFNGLSD         NIDQE           CGAV TVFNGLSD         SPGTKI           CGAV TVFNGLANSSES         SPGTKI           CGAV TVFNGLANSSES         SSSS           CGAV TVFNGLANSSES         SSSSS           CGR TAFNGLANSGNSC         SGSSSSS           CGR TAFNGLANS         SGNSS           CGR TAFNGLANS         SGNSSSSS           CGR TAFNGLANS         SGNSSSSSS           CGR TAFNGLANS         SGNSSSSSSS	BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLGA SIGC DNGLIS SIGC DNGLIS SIGC DNGLIS SIGC DNGLIS SIGC DNGLIS TITA GTONEKQ GIGC EDERGNQ AI AP DQGSERY TIGA GTGDVQQ TIGA GTGDVQ TIGA -	B12 140 WIEK WI	V V A V
Second struct. CCL2_A: CCL2_O: CCL1_A: CCL1_O: CV_L1 : PP_L1 : PP_L2 : SL_L1 : PO_L1 : PO_L1 : PO_L1 : CV_L1 : CU_L1 : CU_L1 : RV_L1 : RV_L1 : RV_L1 : APO_L3 : ArO_L3 : ArO_L3 : PO_L4 : RV_L2 : SL_L1 : SL_L1 : APO_L1 : APO_L1 : APO_L1 : APO_L1 : APO_L1 : APO_L1 : SL_L1 : APO_L1 : APO_L1 : APO_L1 : APO_L1 : SL_L1 : APO_L1 : APO_L1 : SL_L1 : APO_L1 : APO_L1 : SL_L1 : APO_L1 : SL_L1 : CU_L1 : APO_L1 : CU_L1 : APO_L1 :	β7         β8           NVPV/LPNNY         90           NVPV/LPNNY         90           NVPV/LPNNY         90           NVPV/LPNNY         90           VVV/LPNY         90           -PV/LPGG10         90           -QVV/LPGG11         90           -QVV/LPGG11         90           -QVV/LPGG11         90           -GVE/LPGG12         90           -TIRV-PGA3         90           -AVK/UPAAN         90           -TIRV-PGA3         90           -GAV/LPAAN         90           -FIRV-PGA3         90           -FIRV-PGA3         90           -FITU-PAAN         90           -FITU-PANN         90           -FITU-PANN         90           -FITU-PANN         90           -FITU-PANN         90           -FITU-PANN         90           -FITU-PARS         90           -FITU-PARS         90           -FITU-PARS         90           -FITU-PARS         90           -FITU-PARS         90           -FITU-PARS         90	TLTISG	δ9 	βΙ0           110         120           CKRTVS/05LINN-TAGEE         CKRTVS/05LINN-TAGEE           CKKTVS/05LINN-TAGEE         CKRTVS/05LINN-TAGEE           CKKTVY/05NGE-VANTK         CGKA-TVY/05NGE-VANTK           CGKTVY/05NGE-VANTK         CGKA-TVY/05NGE-VANTK           CGKATVY/05NGE-VANTK         CGKA-TVY/05NGE-VANTK           CGKA-TVY/05UGT-VEGKEI         CGGA-TVY/05UGT-VEGKEI           CGVTVY/05UGP-EEK(N)         CGAT0NMELDF-EEK(N)           CGATVY/05UGP-SPGTKI         CGGS0NSIDS-SPGTKI           CGGS0NSIDS-SPGTKI         CGG-S0NSIDS-SPGTKI           CGGTVYNGLPA-QUDAN         CGGATAFNGLAN-SENSO           CGGTVFNGLPA-QUDAN         CGGRTAFNGLAN-SGNSO           CGGRTAFNGLAN-SGNSO         CGGRTAFNGLAN-SGNSO           CGGTVFNGLAN-SGNSO         CGRTAFNGVAN-VDQNN           CGGRTAFNGLAN-SGNSO         CGRTAFNGVAN-SONSO           CGGTVFNGLAN-SGNSO         CGRTAFNGVAN-SONSO           CGGTVFNGLAN-SGNSO         CGRTAFNGVAN-SONSO           CGGTAFNGVAN-VDQNAPL         CGASVFNQADN-VDHVN           CGVTAFNGVAN-VDQNAPL         CGATAFNGVAN-VDQNAPL           CGATAFNGVAN-VDQNAPL         CGATAFNGVAN-VDQNAPL           CGVTAFNGVAN-VDQNAPL         CGATAFNGVAN-VDQNAPL	10           SIGA DATFSG           SIGA DATFSG           SIGA DATFSG           SIGA DATSG           AIGA DATSG           TIGA -GTGNEQO           AIA DQCSERY           TIGA -GTGNEQO           AIA DQCSEQO	B12 I40 GVIEK GVIEK GIFER GIFER GIFER GIFER GIFER GUVEK GUVEK GUVEK GUVER GULER GULER GVUER GULER	V V
Second.struct. CCL2_A: CCL2_C: CCL1_A: CCL1_O: CCV_L1: PP_L2: PP_L2: PO_L1: PO_L1: PO_L1: PO_L1: PO_L1: PO_L1: AP_L1: RV_L1: ATO_L1: ATO_L2: ATO_L2: ATO_L2: SS_L1: SS_L1: HRL1: BS_L1: SCL1: SS_L1: SS_L1: SCL1: SS_L1: SCL1: SS_L1: SCL1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SS_L1: SCL1: SS_L1: SCL1: SS_L1: SS_L1: SCL1: SCL1: SS_L1: SS_L1: SCL1: SCL1: SS_L1: SCL1: SS_L1: SS_L1: SCL1: SS_L1:	β7         β8           NVPV/L PNNY         PNNY           NVPV/L PRNY         NVPV/L PRO           -PV/L PRO         -PV/L PRO           -QVV/L PRO         -QV/L PRO           -QVV/L PRO         -QV/L PRO           -TIR/L PRO         -TIR/L PRO           -TIR/L PRO         -TIR/L PRO           -TIR/L PRO         -TIR/L PRO           -FVT/L PRO         -FV/L PRO           -FVT/L PRO         -FV/L PRO           -FVT/L PRO         -FM/L PRO           -FMT/L PRO         -FM/L PRO </td <td>TLTITSG</td> <td></td> <td>β10           110         120           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVYNNAGE -VANTK           CKR TVYNGVAD-VDNAN           CKQ TVYNGUAD-VDNAN           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CR TVYNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET</td> <td>BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLGA SIGG DNGLGA SIGG DNGLGA SIGG DNGLGA SIGG DTGNEKQ GFGD EDERGNQ AIGA DNGLGA SIGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ TIGA GTG</td> <td>β12 140 WIEK WIEK GIFER GIFER GIFER GIFER GIFER GIFER GIFER GIFER GIFER GILR GILR GILR GILR GILR GILR GILR GILR GIR GIR GIR GIR GIR GIR GIR GI</td> <td>V V A V</td>	TLTITSG		β10           110         120           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVSNSLINN TAGEE           CKR TVYNNAGE -VANTK           CKR TVYNGVAD-VDNAN           CKQ TVYNGUAD-VDNAN           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CKQ TVYNGLET           CR TVYNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET           CG TVFNGLET	BII SIGA DATFSG SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLQA SIGG DNGLGA SIGG DNGLGA SIGG DNGLGA SIGG DNGLGA SIGG DTGNEKQ GFGD EDERGNQ AIGA DNGLGA SIGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ GFGA GTGNEKQ TIGA GTG	β12 140 WIEK WIEK GIFER GIFER GIFER GIFER GIFER GIFER GIFER GIFER GIFER GILR GILR GILR GILR GILR GILR GILR GILR GIR GIR GIR GIR GIR GIR GIR GI	V V A V
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Second struct. CCL2_A : CCL2_C : CCL1_A : CCL1_C : CV_L1 : CV_L2 : PP_L2 : SL_L1 : CP_L1 : PO_L2 : GC_L1 : CU_L1 : CU_L1 : PO_L2 : GC_L1 : CU_L1 : PO_L3 : ArO_L1 : ArO_L3 : PO_L4 : RV_L2 : SL1 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L4 : RV_L2 : SL1 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L3 : ArO_L4 : ArO_L3 : ARO_L1	β7         β8           NVPVUEPNNY         PNNY           NVPVEPNY         PCNY           NVPVEPNY         PCNY           -PVUEPCGY         -PVVEPCGY           -VVVUEPCGY         -PVVEPCGY           -VVVUEPCGY         -PVVEPCGY           -VVVUEPCGY         -PVVEPCGY           -VVVEPCGY         -PVVEPCGY           -TIRVEPCGY         -TIRVEPCGY           -TIRVEPCGY         -TIRVEPCGY           -TIRVEPCGY         -FVVEPCGY           -FIRVEPCGY         -FVVEPCGY           -FVTVEPCGY         -FVTVEPCGY           -FVTVEPCGY         -FVTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -FMTVEPCGY           -FMTVEPCGY         -GSSGYEGY           -GLSUSSCONE         -GATPMTAGY           -VATVEPCGA         -VATVEPCGY           -VATVEPCGY         -VATVEPCGY	INTERDATION		βΙ0           110         120           CKR TVS/05LINN TAGEE         CKR TVS/05LINN TAGEE           CKR TVS/05LINN TAGEE         CKR TVY/05LGTVANTK           CGK TVY/05LGTVANTK         CGK TVY/05LGTVEGCKI           CGK TVY/05LGTVEGCKI         CGK TVY/05LGTVEGCKI           CGK TVY/05LGTVEGCKI         CGK TVY/05LGTVEGCKI           CGV TVY/05LGTVEGCKI         CGV TVY/05LGTVEGCKI           CGV TVY/05LGTVEGCKI         CGV TVY/05LSD           CGV TVY/05LSD         VDQE           CGV TVY/05LSD         SPG'KI           CGU SONWILDS         SPG'KI           CGU TVY/05LSD         SVDQE           CGU TVY/05LSD         SVDQE           CGU TVY/05LSP         SPG'KI           CGU TVY/05LSP         SQN           CGU TVF05LSP         SQN           CGW TVF05LSN         SQN           CGW TVF05LNN         SQN           CGW TVF05LNN         SQN           CGW TVF05LNN         SQN           CGW TVF05LNN         SQN	BII SIGA DATFSG SIGA DATFSG SIGA DATFSG SIGA DATFSG SIGA DATFSG SIGA DATFSG SIGA DAGIA SIGA DAGIA SIGA DAGIA AGA DAG	B12 140 WIEKK WIEKK WIEKK WIEKC WIEKC WIE	V V A V

**Fig. 1.** Multiple sequence alignment of CCL2 homologs encoded in different fungal genomes. Sequences were retrieved from the DOE-JGI Mycocosm via a BLASTP search against the filtered model proteins of all available fungal genomes and carry the following abbreviations and JGI gene model numbers: CCL2\_A: CCL2 of *C. cinerea* strain AmutBmut; CCL2\_O: CCL2 of *C. cinerea* strain Okayama7; CCL1\_A: CCL1 of *C. cinerea* strain AmutBmut; CCL2\_O: CCL2 of *C. cinerea* strain Okayama7; CV\_L1: *Crepidotus variabilis* lectin 1 (Crevar1\_905694); CV\_L2: *C. variabilis* lectin 2 (Crevar1\_109630); PP\_L1: *Postia placenta* lectin 1 (Pospl1\_130016); PP\_L2: *P. placenta* lectin 2 (Pospl1\_121916); SL\_L1: *Serpula lacrymans* lectin 1 (SerlaS7\_144703); CP\_L1: *Coniophora puteana* lectin 1 (Conpu1\_119225); PO\_L1: *Pleurotus ostreatus* lectin 1 (PleosPC9\_89828); PO\_L2: *P. ostreatus* lectin 2 (PleosPC15\_1043947); PO\_L3: *P. ostreatus* lectin 3 (PleosPC9\_64199); PO\_L4: *P. ostreatus* lectin 1 (PleosPC15\_1065820); GC\_L1: *Gymnopilus chrysopellus* lectin 1 (Gymch1\_1574453); CU\_L1: *Cerrena unicolor* lectin 1 (Cerun2\_380560); AP\_L1: *Agrocybe pediades* lectin 1 (Agrped1\_766896); AP\_L2: *A. pediades* lectin 1 (Gymch1\_1574453); CU\_L1: *Cerrena unicolor* lectin 1 (Rhivi1\_768162); RV\_L2: *R. vinicolor* lectin 2 (Rhivi1\_700206); ArO\_L1: *Armillaria ostoyae* lectin 1 (Armost1\_894609); ArO\_L2: *A. ostoyae* lectin 1 (Armost1\_93450); SS\_L1: *Sphaerobolus stellatus* lectin 1 (Dicsq1\_137073); AO\_L1: *Arthrobotrys oligospora* lectin 1 (Artol1\_3664); LB\_L1: *Laccaria bicolor* lectin 1 (Lic\_330799); LB\_L2: *L. bicolor* lectin 2 (Lic\_327918). Light gray, dark gray and black shading indicate 60, 80 and 95% sequence identity. On top the secondary structure of CCL2 according to NMR (Schubert et al. 2012) and X-ray crystallography (this work) is indicated. Residues involved in carbohy-drate binding are marked with green triangles, residues involved in the dimerization with yellow filled circles. The figure was generated using GeneDoc (Nicholas et al.1997).

between the free and bound form of the protein in regard to the NMR time scale (slow  $k_{\rm ON}$  and slow  $k_{\rm OFF}$ ). This is in contrast to fast exchanging interactions that are typical for weak protein–carbo-hydrate interactions, in which one averaged signal position is observed for each amino acid with contributions of the free and the

bound form leading to a movement of signal positions with increasing ligand concentration. In the case of the CCL2(Y92A) variant with the dysfunctional carbohydrate binding site, neither the appearance of new signals nor a movement of signals occurred, confirming that the oligosaccharide does not bind. The fact that almost identical



**Fig. 2.** Amino acid substitutions in the N- and C-terminal loops of the β-trefoil fold reduce the nematotoxicity of the fungal lectin CCL2. (**A**) Soluble expression levels of CCL2 wild type, R18A single, F133A single and R18A\_F133A double variant proteins in *E. coli*. Soluble protein extracts of *E. coli* BL21(DE3) transformants expressing wild type CCL2, CCL2(Y92A), CCL2(R18A), CCL2(F133A) and CCL2(R18A\_F133A), respectively, were separated by SDS-PAGE and stained with Coomassie brilliant blue (R-250). Transformants containing pET24 without insert (Vector Control) and expressing CCL2(Y92A), previously shown to be defective in carbohydrate-binding, served as controls. Sizes of marker proteins are indicated. (**B**) Development of *C. elegans* L1 larvae fed with *E. coli* BL21(DE3) transformants expressing wild type CCL2, CCL2(R18A), CCL2(F133A) and CCL2(R18A\_F133A). CCL2-expressing transformants were mixed in different ratios with bacteria containing pET24 vector. The percentage of CCL2-expressing bacteria in these mixtures is indicated on the *X*-axis. Pure cultures of pET24-containing transformants (Vector Control) and transformants expressing CCL2(Y92A) served as controls. Error bars indicate the standard deviation from the average of three biological replicates. This figure is available in black and white in print and in color at *Glycobiology* online.

chemical shift changes and slow exchange kinetics were observed for the R18A variant and the wild type protein shows that the influence of the ligand on the protein resonances at the carbohydrateprotein interface is comparable. Taken together, these results confirmed that the ligand binding in CCL2(R18A) is intact and has structural features that are comparable to the wild type protein.

### X-ray crystallography reveals the formation of CCL2 dimers

Based on previously published NMR and size exclusion chromatography (SEC) data, it was concluded that CCL2 was monomeric (Schubert et al. 2012). In contrast, six other single domain  $\beta$ -trefoil fungal lectins have been structurally characterized so far and been shown to form dimers such as *C. nebularis* lectin CNL (Pohleven et al. 2012), *Boletus edulis* beta-trefoil lectin BEL (Bovi et al. 2013), *Sclerotinia sclerotiorum* agglutinin SSA (Sulzenbacher et al. 2010) and *Rhizoctonia solani* agglutinin RSA (Skamnaki et al. 2013). The MPL lectin from *Macrolepiota procera* was crystallized only under its monomeric form but was shown to form dimers in solution (Zurga et al. 2014). Based on these structures and above results regarding the CCL2(R18A) mutant, we reexamined the possibility of CCL2 oligomerization by determining the crystal structure of the recombinant, His-tagged protein.

The crystallization trials with the Midas screen, which is a screen based on alternative polymeric precipitants (Grimm et al. 2010), resulted in many hits. The CCL2 apoprotein produced clusters of thin needles with a diffraction limited to about 2.2 Å. In the presence of the ligand  $sLe^{X}$  tetrasaccharide (Neu5Aca2,3Gal $\beta$ 1,4 (Fuc $\alpha$ 1,3)GlcNAc) (Schubert et al. 2012), several crystal forms were obtained but the best diffraction was observed for crystals grown

from solutions containing glycerol ethoxylate. All crystals were quite susceptible to radiation damage.

Two and four protein chains were found by molecular replacement in the asymmetric unit of the apoprotein and sLe<sup>X</sup> co-crystals, respectively. The structure of the apoprotein was determined and refined to 2.25 Å resolution to values of R = 17.2% and  $R_{\text{free}} =$ 21.0%. The structure comprised residues 8-142 in both protein chains and has excellent geometry with no outliers in the Ramachandran plot (Table I). The  $sLe^X$  complex structure was determined and refined to 1.95 Å resolution to values of R = 17.5%and  $R_{\rm free} = 20.4\%$ . The protein structure, comprising residues 7-142 in molecule A, 8-142 in molecule B, 10-141 in molecule C and 12-141 in molecule D, present excellent geometry (Table I and validation report available on wwPDB). Protein chains B, C and D presented much higher temperature factors than protein chain A probably as a result of thermal motion as shown from the anisotropic displacement observed with the TLS refinement (Winn et al. 2001) (Table I). The electron density was poor for the side chains of some surface loops so some atoms had to be omitted. As a result, those protein chains present most of the real-space R-value Z-score outliers and very few water molecules were found associated to them contrary to protein chain A for which the thermal motion is more limited (Table I and validation report available on wwPDB). In both structures, the N-terminal His-tag and the first amino acids could not be modeled due to the absence of clear electron density and probably as the result of disorder. In the NMR structures, those residues adopted numerous conformations. Protein chain A is the best defined and small differences to the other protein chains in the asymmetric unit are only observed at the level of surface loops which leads to a rmsd between 0.17 and 0.21 Å. Thus, in the following, we will refer to protein chain A unless otherwise stated.



**Fig. 3.** Amino acid substitutions in the N- and C-terminal loops of CCL2 do not affect carbohydrate-binding. In the presented contour plots of <sup>1</sup>H-<sup>15</sup>N NMR correlations every amino acid residue contributes one signal of its backbone amide (except proline), any change in the environment, e.g. ligand binding, typically leads to changes of the <sup>1</sup>H and <sup>15</sup>N resonance frequencies resulting in a change of position in this 2D plot. Binding of CCL2 wild type, R18A single, R18A\_F133A double variant and Y92A proteins to GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 10(CH<sub>2</sub>)<sub>5</sub>COOH was studied using NMR, as shown in panels A, B, C and D, respectively. <sup>15</sup>N-<sup>1</sup>H HSQC spectra of <sup>15</sup>N-labeled proteins in the absence (blue) and presence of GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 10(CH<sub>2</sub>)<sub>5</sub>COOH (red) are shown on the left. Most prominent chemical shift changes are labeled at the peak positions of the WT. The behavior of the signal of T111 as one representative, is shown on the right in the course of a titration. The simultaneous presence of the free and bound state of T111 indicates slow exchange kinetics compared to the NMR time scale. The slow k<sub>OFF</sub>, which is only seen for tight binders, is unchanged in the R18A and the R18A\_F133A double variant, indicating an intact binding site. No chemical shift changes were observed for Y92A, indicating that the protein is not able to bind the ligand anymore. This figure is available in black and white in print and in color at *Glycobiology* online.

CCL2 apoprotein adopts the same  $\beta$ -trefoil fold in the crystal than the one observed by NMR spectroscopy in solution (Figure 4A). It consists of three  $\beta$ - $\beta$ - $\beta$ - $\beta$  repeats with a pseudo C3 symmetry (Schubert et al. 2012). When compared to the NMR structures, differences are mainly observed at the level of flexible regions such as the surface loops, in particular loops  $\beta$ 1- $\beta$ 2,  $\beta$ 6- $\beta$ 7,  $\beta$ 8- $\beta$ 9 and  $\beta$ 11- $\beta$ 12 as defined in (Schubert et al. 2012) (Supplementary data, Figure S1). Most importantly, the crystal structures of both the apo- and the liganded protein clearly indicate a dimeric arrangement of the protein (Figure 4A).

Analysis of the quaternary crystal structure with PISA (Krissinel and Henrick 2007) confirmed the presence of a dimer interface with a total buried surface of 1344, 1356 and 1371 Å<sup>2</sup> between protein chains A and B in the apoprotein and sLe<sup>X</sup> complex and protein chains C and D in the sLe<sup>X</sup> complex, respectively. This represents around 12% of the solvent accessible surface area of 11774 Å<sup>2</sup> (AB apo), 11631 Å<sup>2</sup> (AB sLe<sup>X</sup> complex) and 11121 Å<sup>2</sup> (CD sLe<sup>X</sup> complex).

Surface loops, in particular loops  $\beta 1-\beta 2$ ,  $\beta 10-\beta 11$ ,  $\beta 11-\beta 12$ , are involved in the formation of the dimer interface. The interface is mainly stabilized by hydrogen bonds formed by the L20 main chain in one protomer and the R18 main and side chain in the other protomer of the dimer (Figure 4B). The side chain of R18 interacts also with the main chain oxygen of N118 and A119 of the other chain. The NH<sub>3</sub> group of K26 is bonded to the main chain oxygen of G24 of the other protomer of the dimer. There are also two Van der Waals stacking interactions implicating the aromatic ring of F133 and the side chain of R18 of one protomer with the side chains of V19 and P22 of the other protomer, respectively. Thus, PISA detailed analysis revealed two main residues involved in dimerization: R18 and F133.

### NMR relaxation and deuterium exchange confirm dimerization of CCL2

Two different NMR experiments confirm the X-ray crystallography data and suggest that CCL2 forms also dimers in solution. On the one hand, we measured <sup>15</sup>N longitudinal and transverse relaxation times using standard experiments (Farrow et al. 1994) and used the ratio between T<sub>1</sub> and T<sub>2</sub> to estimate the rotational correlation time  $\tau_c$  according to Fushman et el. (1994) (Supplementary data, Figure S2). Using a double logarithmic plot of  $\tau_c$  versus the molecular weight (MW) (Serdyuk et al. 2007), the obtained  $\tau_c$  value of 16.4 ns points to a MW in the range of 30–40 kDa (Figure 5A), which agrees with a dimer of CCL2.

Dimerization of CCL2 is further supported by a deuterium exchange experiment. Dissolving a lyophilized CCL2 sample in 100%  $D_2O$  resulted in an NMR spectrum in which only  ${}^{1}\text{H}{}^{-15}\text{N}$  correlations of amides show up that were protected from  ${}^{1}\text{H}{}^{/2}\text{H}$  exchange, typically due the involvement into a hydrogen bond (Figure 5B). Although arginine side chains normally exchange very

#### Table I. Data collection and refinement statistics for CCL2 sLe<sup>X</sup> complex and apoprotein structures

	sLe <sup>X</sup> comple	ex			Аро		
Data							
Beamline (wavelength, Å)	ID23-1/0.97	BM30A/0.9205					
Spacegroup	P6122	P41212					
Unit cell dimensions, a, b, c Å	121.7, 121.7	59.92, 59.92, 202.9					
Resolution (outer shell), Å	29.23-1.95	44.85-2.25 (2.33-2.25)					
Measured/ Unique reflections	292542/460	102330/18296					
Average multiplicity	6.3 (6.6)				5.6 (5.7)		
R <sub>merge</sub>	0.047 (0.424	4)			0.093 (0.543)		
R <sub>pim</sub>	0.029 (0.271	0.061 (0.359)					
Completeness (%)	99.2 (100)	99.5 (99.8)					
Mean I / σI	18 (4.4)	15.7 (3.7)					
CC1/2	0.999 (0.892	0.998 (0.900)					
Wilson B (truncate)	34.1				29.5		
Refinement							
R <sub>cryst</sub> / R <sub>free</sub>	17.5/ 20.4	17.2/21.00					
nb reflections/free reflections	43754/2327	17392/892					
R <sub>msd</sub> bonds, Å	0.015				0.015		
$R_{\rm msd}$ angles, <sup>°</sup>	1.61				1.69		
Rmsd chiral, Å <sup>3</sup>	0.094				0.096		
Atoms (chain)	Α	В	С	D	А	В	
Protein	1052	1022	962	967	1033	1033	
Bfac Å <sup>2</sup>	27.4	49.2	58.9	66.6	26.1	34.4	
Water molecules	152	39	28	10	123	72	
Bfac, Å <sup>2</sup>	39.8	46.1	45.5	52.5	34.8	36.0	
Ligand	56						
Bfac, Å <sup>2</sup>	43.1						
Ramachandran (Molprobity) Allowed	100%	100%					
Favored	97%					96.6%	
Outliers :	0%					0%	
PDB code	4USO				4USP		

rapidly, one arginine side chain remained protected for >50 min. That signal originates from R18 He of which a protection cannot be explained by the determined monomeric NMR structure. However, in the crystal structure R18 is located in the center of the dimer interface and Ne forms a hydrogen bond to the backbone carbonyl of L20 of the partner molecule (Figure 4B). The exchange protected side chain NeHe of R18 indicates thus the presence of an intermolecular hydrogen bond at the dimer interface and a very tight dimer interaction because any dissociation event would lead to immediate exchange. A fast equilibrium between dimer and monomer can be excluded because no significant dissociation of the dimer occurs within 50 min. In conclusion, the exchange protected intermolecular hydrogen bond at the dimer interface NMR undoubtedly confirms the presence of a very tight dimer in solution.

#### CCL2 dimerizes in solution in a R18and F133-dependent fashion

Based on the above results, we produced untagged and His-tagged versions of CCL2(F133A) single and CCL2(R18A\_F133A) double protein variants for further analysis. To assess the oligomerization state of CCL2 wild type protein and R18A, F133A and R18A\_F133A variants in solution, several experimental approaches were used. On the one hand, we analyzed the proteins using a previously used, partially denaturing gel electrophoresis procedure (Schutz et al. 2010; Singh et al. 2015). For this procedure, identical samples of purified His-tagged proteins were mixed with Laemmli sample buffer. One of these samples was heated to 95°C for 5 min

whereas the other one was left at room temperature. Both samples were analyzed on a regular denaturing sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie brilliant blue. In case of the CCL2 wild type protein, the gel showed a clear protein band at the expected size of the monomeric protein in the heated sample (Figure 6A). In the non-heated sample, however, most of the CCL2 protein ran at an apparent MW of 50 kDa indicating oligomerization interpreted as dimerization. In contrast, the non-heated sample of the R18A, F133A and R18A\_F133A variants did not show any formation of dimers suggesting that both the single and double substitutions of these residues sufficiently weaken dimerization under these conditions.

As a second approach to assess the oligmeric state of the WT and protein variants, we used Diffusion Ordered nuclear magnetic resonance SpectroscopY (DOSY) (Johnson 1999) to measure the translational diffusion coefficients (D) of CCL2 variants at 298 K (Supplementary data, Figure S3) and correlated the obtained values with the MW using protein standards ranging from 6.5 to 66 kDa according to Groves et al. (2004). The measured logD value of CCL2 wild type falls in the expected range for a dimer (Figure 6B). The R18A and F133A variants yielded diffusion constants not significantly different from wild type, indicating that those point mutants diffused still as dimers. In contrast, the CCL2(R18A\_F133A) double variant displayed a significantly smaller logD value indicative of a monomer.

As a third approach to demonstrate the dimerization of CCL2 in solution, SEC using an ENrich SEC 70 column (Bio-Rad Ltd) was performed in a buffer composed of 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH



Fig. 4. CCL2 forms dimers in crystals. (A) Representation of the crystallographic CCL2 dimer complexed with sLe<sup>X</sup> (4USO). The molecules are colored by chain and sLe<sup>X</sup> is depicted as balls and sticks. (B) Wall-eyed stereo representation of the CCL2 dimeric interface as revealed by X-ray crystallography. Residues R18 and F133 are part of the CCL2 dimerization interface. Carbon atoms and labels are colored according to their chain (A in forest and B in marine). Hydrogen bonds are represented as dotted lines. This figure is available in black and white in print and in color at *Glycobiology* online.

5.7 and 150 mM NaCl (Figure 6C). According to the calibration curve (Supplementary data, Figure S4A), the calculated MWs were 30.8 kDa for CCL2 WT, 29.8 kDa for CCL2(R18A), 31.5 kDa for CCL2(F133A) and 17.1 kDa for CCL2(R18A F133A). The expected MW being of 33.1 kDa for a dimer and 16.6 kDa for a monomer, the elution volumes are in accordance with CCL2 WT, R18A and F133A forming dimers and CCL2 (R18A\_F133A) forming monomers. Similar results were obtained when the buffer was changed to MES pH 6.0 or Hepes 7.5 (data not shown). For comparison, the same samples were subjected to SEC on a Superdex 75 column (GE Healthcare) in KH2PO4/K2HPO4 pH 5.7 and 150 mM NaCl (Supplementary data, Figure S4B). According to the calibration curve (Supplementary data, Figure S4C), the calculated MW was 17.8 kDa for CCL2 WT, 16.2 kDa for CCL2(R18A), 18.3 kDa for CCL2(F133A) and 11.7 kDa for CCL2(R18A\_F133A). On this resin, the elution volumes of the WT and the single variant proteins are rather in accordance with a monomeric protein as described previously (Schubert et al. 2012). The difference between the two resins is likely due to a weak interaction between the lectin CCL2 and the carbohydrate-based Superdex resin, which leads to a delayed elution of the proteins and gives rise to erroneous conclusions on their MW and hence on their oligomeric state. The ENrich resin is not carbohydrate-based and, hence, no interactions with the CCL2 protein appear to occur. Despite this difference between the resins, both resins reveal a clear difference in the elution profile of the WT and single variants compared to the double variant (Figure 6C and Supplementary data, Figure S4B). Finally, size measurements by dynamic light scattering (DLS) were performed before and after SEC. SEC fractions were analyzed directly if the concentration allowed it  $(>0.2 \text{ mg mL}^{-1})$  or after their concentration. The hydrodynamic radius (Rh) measured for the various samples of CCL2 WT, CCL2(F133A) and CCL2(R18A) was between 2.81 and 3.08 nm which is in agreement with the radius of 3.45 nm measured for the elongated CCL2 dimer from the X-ray structure (Figure 6D). These results were also obtained for the CCL2 WT and single variant proteins eluted from the Superdex column (data not shown),



**Fig. 5.** CCL2 forms dimers in solution. (**A**) NMR relaxation data support a protein dimer in solution. Shown is a correlation of the rotational correlation time  $\tau_c$  and the MW of 34 proteins. Values of  $\tau_c$  scaled to 310 K were taken from Dayie et al. (1996), MWs were calculated with the protein sequence. A linear regression resulted in  $\tau_c = 0.887$ \*MW–0.182 (R = 0.89). The rotational correlation time of CCL2 estimated from the average  $T_1/T_2$  ratio (Supplementary data, Figure S2) and the corresponding approximate MW are shown in red. (**B**) The side chain of R18 is protected from proton–deuterium exchange. Left panel: <sup>15</sup>N-<sup>1</sup>H HSQC spectrum of WT CCL2 in a solution containing 95% H<sub>2</sub>O recorded at 900 MHz. The N $\epsilon/H\epsilon$  signals of the arginines are aliased and appear with a different sign (maroon). Right panel: Comparable <sup>15</sup>N-<sup>1</sup>H HSQC spectrum of wild type CCL2 after 50 min in 100% D<sub>2</sub>O. Only amides that do not exchange their proton to deuterium within that time show up—they are protected from exchange typically due to their involvement in a hydrogen bond. The H $\epsilon$  of R18 is protected and labeled with R18sc. Folded signals a colored in magenta. This figure is available in black and white in print and in color at *Glycobiology* online.

supporting above hypothesis about the abnormal mobility of the CCL2 protein on this resin. The  $R_{\rm h}$  measured for CCL2 (R18A\_F133A) at a concentration below 2 mg mL^{-1} was between 2.03 and 2.12 nm which is in agreement with the monomer radius of 2.05 measured from the X-ray structure. At concentrations higher than 2 mg mL^{-1}, however, the  $R_{\rm h}$  was in the range of the dimer and the solution was polydisperse indicating a mix of monomers and dimers.

To further analyze the monomer-dimer equilibrium we used concentration dependent NMR measurements of the monomeric CCL2 (R18A\_F133A) double variant (Supplementary data, Figure S5). At a protein concentration of 50  $\mu$ M narrow line widths are observed, typical for a monomer and at 450  $\mu$ M concentration some signals move, broaden or disappear. These effected signals originate from the dimer interface indicating a fast to intermediate exchange equilibrium between monomers and dimers.

Taken together, these results demonstrate that CCL2 forms dimers in solution and that this dimerization is weakened in the R18A and F133A single variants and practically abolished in the CCL2(R18A\_F133A) double variant.

### Interference with CCL2 dimerization goes along with reduction of nematotoxicity

Based on above results, we concluded that the reduced toxicity of the CCL2(R18A) variant was due to a reduced stability of the protein dimer rather than reduced binding to a second ligand.

To confirm this hypothesis, we analyzed the nematotoxicity of the dimerization-impaired CCL2(F133A) single and CCL2 (R18A\_F133A) double variants. In agreement with our conclusion, the nematotoxicity of the F133A single variant was also reduced in comparison to the WT protein, albeit not as much as the R18A variant, and the double variant protein was further reduced in comparison to the R18A single variant since a 50%/50% mixture of "empty" vector-containing and CCL2(R18A\_F133A)-expressing bacteria showed hardly any toxicity any more (Figure 2). In order to exclude any effects by general folding defects, we confirmed that purified, His-tagged versions of the R18A and R18A\_F133A variant proteins folded properly according to <sup>15</sup>N-HSQC finger print spectra (Supplementary data, Figures S6 and S7) and bound to their carbohydrate target comparably to WT as monitored by NMR titration experiments (Figure 3). The concentration dependence of the NMR spectra of CCL2(R18A\_F133A) described above, indicated a monomer at concentrations of 50 µm or lower. In summary, it appears that the degree of dimerization of CCL2 correlates with its nematotoxicity.

### The carbohydrate-binding sites of CCL2 are oriented towards one face of the dimer

For the simultaneous binding of multiple binding sites of the lectin to multiple ligands displayed on a cell surface, e.g. in a nematode intestinal epithelium, the location of the binding sites on the lectin surface may be crucial. For this reason, we studied the characteristics and the location of the two carbohydrate binding sites on the CCL2 dimer and compared their location with other structurally characterized fungal  $\beta$ -trefoil lectins.

Inspection of the electron density map after rebuilding with Arp-Warp (Langer et al. 2008) revealed without ambiguity the presence of the Gal and GlcNAc moieties of sLe<sup>X</sup> bound to the molecule A of CCL2. The sialic acid moiety appeared in the next refinement step and showed some disorder especially for the glycerol substituent for whose only clear density was observed at 0.7  $\sigma$  and was therefore modeled with a reduced occupancy (Figure 7).

The sLe<sup>X</sup> is found in the binding site previously described for GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 1O(CH<sub>2</sub>)<sub>5</sub>COOH (Schubert et al. 2012) and all interactions are summarized in Table II, Figure 7 and Supplementary data, Figure S8. Upon binding of sLe<sup>X</sup> to CCL2, only minor differences are observed with the apoprotein structure especially at the level of the binding site. There is a small rigid body movement of the  $\beta$ 9- $\beta$ 10 loop which moves away by 0.8 Å in order to avoid close contact and form correct H-bonding between G108



**Fig. 6.** Amino acid substitutions in the CCL2 dimer interface impair dimerization. (**A**) Affinity-purified wild type CCL2, CCL2(R18A), CCL2(F133A) and CCL2 (R18A\_F133A) proteins were dissolved in 2× Laemmli sample buffer and run out on a denaturing SDS-PAGE without (RT) and with preheating (95°C) of the samples. Sizes of marker proteins (M) are indicated in kDa. (**B**) Diffusion Ordered nuclear magnetic resonance SpectroscopY measured at 298 K. Shown is a correlation between experimental translational diffusion coefficients D of CCL2 variants and the MW. A linear fit of five protein standards is indicated. Solutions of 0.31 mM aprotinin (6.5 kDa), 0.3 mM lysozyme (14.3 kDa), 0.3 mM carbonic anhydrase (29 kDa), 0.23 mM ovalbumin (44 kDa) and 0.34 mM bovine serum albumin (BSA, 68 kDa) were used as standards. The measured logD of the CCL2 variants are indicated in red together with their apparent logMW. The MWs of monomer and dimer of CCL2 are indicated as dotted lines. (**C**) Size exclusion chromatograms of wild type CCL2, CCL2(R18A), CCL2(F133A) and CCL2(R18A\_F133A) proteins on an ENrich SEC 70 10 × 300 mM column equilibrated with 20 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 5.7 and 150 mM NaCl. Elution volumes and calculated MW based or wild type CCL2, CCL2(R18A), CCL2(F13A), CCL2(F13A) and CCL2(F13A) and CCL2(F13A) proteins in 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 5.7 and 150 mM NaCl. Elution volumes and calculated MW based for wild type CCL2, CCL2(R18A), CCL2(F13A) and CCL2(F13A) and CCL2(R18A\_F133A) proteins in 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 5.7 and 150 mM NaCl. Support and the set of the set

and the fucose O4 hydroxyl. There are also slight changes at the level of loop  $\beta 6$ - $\beta 7$  that lead to optimal Van der Waals interaction between W78 side chain and the sialic acid moiety. The Fuc, Gal and GlcNAc moieties present orientations equivalent to the one observed in GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 1O(CH<sub>2</sub>)<sub>5</sub>COOH (Supplementary data, Figure S8). The rotamer of the hydroxymethyl of the GlcNAc is however different in both complexes. There are 8 direct hydrogen bonds and 10 water-mediated hydrogen bonds. The Van der Waals interactions involve Y57, W78, Y92, and W94 like in the case of GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 1O(CH<sub>2</sub>)<sub>5</sub>COOH. The binding site accommodates the galactose moiety very well and in the absence of the acetamido group, the side chain of Y92 reorients a little since there is no more stacking interaction with it. Comparison of the two complex structures shows clearly that there are some rearrangements of the loop  $\beta 6$ - $\beta 7$  as a result of movements of G79

and G81 (Supplementary data, Figure S8). The orientation observed in the fucosylated chitobiose complex cannot permit the accommodation of the Sialyl moiety and would result in steric conflict. The new orientation allows the side chain of W78 to optimize stacking with the sialyl moiety.

The sLe<sup>X</sup> is bound in a similar conformation observed in many other lectin complexes with this oligosaccharide such as the staphylococcal superantigen-like protein 11 (Chung et al. 2007). The torsion angles for the different glycosidic linkages all fall in low energy regions, in particular the glycosidic linkage torsional angles  $\Phi$  (O6-C2-O-C'x) and  $\Psi$  (C2-O-C'x-C'x+1) for the  $\alpha$ Neu5Ac(2–3)Gal linkage with values of 48.03° and 100°, respectively (Supplementary data, Table S1) (Imberty and Perez 2000). The Le<sup>x</sup> trisaccharide is present in a closed conformation, in which the galactose moiety is stacked onto the fucose moiety as described earlier (Zierke et al. 2013).



**Fig. 7.** Coordination of sLeX by CCL2. Representation of the maximumlikelihood weighed 2mFo-DFc electron density around the sLe<sup>X</sup> complexed to CCL2 contoured at 0.9 Å (0.205 eA<sup>3</sup>). Hydrogen bonds are represented as dotted lines and waters as magenta spheres. This figure is available in black and white in print and in color at *Glycobiology* online.

Table II. Contacts between CCL2 and sLe<sup>X</sup>

Sugar atom	Protein atom	Distances (Å)	
Fucose site			
O2	Wat2149-GalO6	3.11-2.59	
	Wat2149-GlcNAcO3	3.11-2.87	
O3	Gly108 N	3.05	
	Wat2117-Asp107 OD2	2.60-2.74	
O4	Gly108 N	2.76	
	Val93 O	2.60	
O5	Val93 N	3.08	
Van der Waals	Trp94		
GlcNAc site			
O1	Wat2099-Asn91 ND2	2.62-3.02	
	Wat2099-Asn90 OD1	2.62-2.84	
N2	Asn91 O	2.67	
O3	Wat2149-FucO2	2.67-3.11	
Van der Waals	Tyr57, Tyr92		
Gal site			
O2	Wat2091-Trp78 O	2.72-2.87	
	Wat2091- Trp78 N	2.72-3.12	
O4	Wat2148-Wat2120-Thr111 OG1	3.16-3.07-2.89	
O6	Wat2148-Wat2117-Asp107 OD2	3.16-3.14-2.74	
Van der Waals	Trp78		
Sia site			
O4	Trp78 O	3.18	
	Gly79 O	3.20	
	Ala80 N	3.12	
Van der Waals	Trp78		

This rigid low energy conformation is the one found bound to most lectins apart of two special cases: the lectins RSL from *Ralstonia solanacearum* and BambL from *Burkholderia ambifaria* (Topin et al. 2016).

The residues involved in the glycan-binding interface residues of CCL2 are spatially well separated from those implicated in the dimerization. By using the analogy between the  $\beta$ -trefoil structure

with a tree (Renko et al. 2010), the ligand binding occurs mainly in the upper stem, the lower and upper crown at the interface between subdomains  $\beta$  and  $\gamma$  and therefore on the side of CCL2 (Figure 8A). This is very different when compared to the other dimerizing fungal  $\beta$ -trefoil lectins like CNL fom *C. nebularis* (Pohleven et al. 2012), BEL from *B. edulis* (Bovi et al. 2013), SSA from *S. sclerotiorum* (Sulzenbacher et al. 2010) and RSA from *R. solani* (Skamnaki et al. 2013) where binding occurs at the top and in the upper crown of one or several subdomains (Figure 8A).

## Dimeric $\beta$ -trefoil lectins from fungi differ considerably in their three-dimensional arrangements

Since a considerable number of dimerizing fungal β-trefoil lectins have been structurally characterized, we compared the threedimensional arrangements of the carbohydrate-binding sites and the dimerization interfaces in these lectins with the one of CCL2. This comparison revealed striking differences and a large variety among those lectins (Figure 8). They all present different dimerization interfaces, which often involve some surface loops. Those are dissimilar in length and orientation from one lectin to the other whilst the beta-strands are structurally well conserved. In CCL2, the  $\beta 1-\beta 2$ loop for example plays a critical role in the dimer formation. It includes an insertion of four to five residues compared to the above lectins, which allows this loop to have the proper orientation and to bring some necessary residues for CCL2 dimerization like L20, P22 and G24. The stem and the lower crown of subdomains  $\alpha$  and  $\gamma$  are associated with CCL2 dimer formation whereas other subdomains are used by the other  $\beta$ -trefoil dimers. Thus, the CCL2 dimer is not only unique in the localization of its dimer interface but also in the orientation of the two molecules: one molecule is in a side-by-side contact with another up-side down oriented one (Figure 8). The pseudo-symmetry axes of both molecules are perpendicular to the axis connecting both molecules and are thus not tilted. However, both monomers are twisted by a torsion angle of 49°. The two glycan-binding sites are located at the extremities of the dimer in a front view (Figure 8B, left panel), but are facing the same side in a side view (Figure 8B, right panel), which will enable binding to two ligands exposed on a surface. The orientations of the molecules reflected by tilt and torsion angles vary extremely. Like SSA, CCL2 presents only one binding surface whilst the others present two, but the distances between the binding sites are distinct with 55 Å in CCL2 compared to 38 Å for SSA (Figure 8B).

#### Discussion

In retrospect, we are surprised that we obtained such high quality NMR data with a 30 kDa dimer complex (Schubert et al. 2012). Normally deuteration and TROSY techniques are required to study proteins of a size >25 kDa. The compactness of the dimer and the elevated temperature of 37°C are likely the reasons for the fairly sharp NMR signals. The previously obtained, misleading size exclusion results seem to be due to very weak interactions of the lectin with the polysaccharide column material resulting in slightly longer elution time and thus smaller apparent size. Although we did not notice the presence of a dimer in the previous NMR structure, the main conclusions of that study, namely the  $\beta$ -trefoil fold, the unusual binding location and the structural details of the carbohydrate recognition interface responsible for specificity and affinity are not questioned with the present paper. However, the results of this



**Fig. 8.** Orientation of the binding sites in the dimer and three-dimensional arrangements of single-domain  $\beta$ -trefoil lectin dimers of fungi. (**A**) Locations of the dimerization interface and the carbohydrate-binding sites in CCL2 as compared to other  $\beta$ -trefoil fold lectins from fungi. CLN, *Clitocybe nebularis* lectin; BEL, *Boletus edulis* lectin; SSA, *Sclerotinia sclerotiorum* agglutinin; RSA, *Rhizoctonia solani* agglutinin. See text for references. As an inset the monomers with the pseudo C<sub>3</sub> symmetry are shown schematically indicating which subdomains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) are mainly involved at the dimerization interface. This scheme is very simplified, any tilt and twist between the two molecules is ignored. The bottom of the  $\beta$ -trefoil fold (root in tree model) is indicated in gray, ligands in green and the dimerinterface in yellow. (**B**) Schematic representation with the lectin name and the corresponding PDB accession code in brackets. Tilt and torsion angles are derived from visual inspection and *x*-, *y*- and *z*-rotations in MOLMOL (Koradi et al. 1996). Distances between different ligand binding sites were measured between the O1 atoms of the monosaccharide that is in closest contact with the protein. This figure is available in black and white in print and in color at *Glycobiology* online.

study clearly demonstrate that the formation of dimers is essential for the function of the lectin as a toxin.

Previous hypotheses with regard to the toxicity mechanism of CCL2 towards invertebrates were based on the assumption of a monomeric state of CCL2 and the absence of additional carbohydrate binding sites on the protomer. Here, we show that mutations in the dimerization interface, revealed by X-ray crystallography and confirmed by NMR, interfere with dimer formation as demonstrated by SEC combined with DLS, partially denaturing gel electrophoresis and NMR analysis. Although these mutations do not affect the binding of the protein to the carbohydrate ligand, they clearly reduce the nematotoxicity of the protein. These results suggest that the bivalency of CCL2 is a requirement for its nematotoxicity and that the toxicity mechanism involves crosslinking of N-glycoproteins on the surface of intestinal epithelial cells of the nematode. The orientation of the carbohydrate binding sites pointing towards the same face of the protein dimer may thereby facilitate the simultaneous binding to two N-glycoproteins that are fixed to the same cell

surface. As mentioned in the introduction, the crosslinking of N-glycoproteins of the plasma membrane or the glycocalyx may either lead to lectin-induced activation of intracellular signaling pathways or to defects in plasma membrane or glycocalyx function possibly by changed vertical (in/out) and lateral (rafts) trafficking of the target glycoproteins. According to a recent study, this mechanism does appear to involve surface binding but not internalization of CCL2 (Stutz et al. 2015).

The major characteristics i.e. the structure ( $\beta$ -trefoil, homodimer), the orientation and valency (one binding site per monomer, both binding sites located on one face of the dimer) and the toxicity mechanism (dependency on carbohydrate-binding, binding to microvilli but no apparent internalization) of CCL2 are very similar to another fungal lectin SSA from *S. sclerotiorum*, which shows strong toxicity towards insects (Hamshou et al. 2010; Sulzenbacher et al. 2010). The only difference is in the architecture of the dimer interface (different geometry and residues involved), the location of the carbohydrate binding site (canonical site in case of SSA) and the

carbohydrate specificity since SSA is specific for B-1,3 or B-1,4linked terminal Gal or GalNAc residues. In contrast, the β-trefoil lectin BEL from B. edulis with a different dimer geometry and six carbohydrate binding sites was shown to be internalized into mammalian epithelial Caco-2 cells (Bovi et al. 2013) although it has the same carbohydrate specificity as SSA. Thus, it is possible that toxic β-trefoil lectins with a low number of carbohydrate binding sites may exert toxicity by mere crosslinking of glycoconjugates at the cell surface whereas the toxicity mechanism of lectins with a higher valency may involve internalization. On the other hand, the observed differences in toxicity mechanisms might also be due to differences in the target glycoconjugate between the different  $\beta$ -trefoil lectins. Nematotoxicity of CCL2 was previously shown to be dependent on the binding of N-glycoproteins on the surface of intestinal epithelial cells of C. elegans (Schubert et al. 2012; Stutz et al. 2015). In contrast, the chimeric β-trefoil lectin MOA from Marasmius oreades was previously shown to exert nematotoxicity by binding to glycosphingolipids of these cells (Wohlschlager et al. 2011). Accordingly, bacterial toxins and entire bacteria were recently shown to trigger their own endocytosis by binding and clustering of glycolipids (lipid zipper mechanism) (Eierhoff et al. 2014; Aigal et al. 2015). Similarly, binding of galectin-3 to glycoproteins was recently shown to trigger endocytosis of these proteins by additional interaction of galectin-3 with glycolipids in a mechanism independent of clathrin (CLIC mechanism) (Lakshminarayan et al. 2014). Based on the differences in the number and the arrangement of the carbohydratebinding sites between the characterized dimerizing β-trefoil lectins from fungi, we hypothesize that these lectins act by simultaneous binding of multiple glycans and forcing these glycans into a spatial arrangement that is determined by discrete distances of the carbohydrate binding sites on the lectin surface. If these glycans are part of membrane-bound glycoconjugates, this lectin-enforced spatial arrangement may have a profound effect on the function of the respective biological membrane and the underlying cell. The effect likely depends on the nature of the glycoconjugate (glycoproteins, glycolipids) and the specific spatial arrangement of the glycoconjugates enforced by the lectin.

In summary, after CNL (Pohleven et al. 2009; Pohleven et al. 2012), CCL2 is only the second example of a lectin for which multivalency was shown to be essential for biological function (toxicity). The significance of this finding might go beyond basic research as CCL2 was recently shown to bind specifically to cancerous epithelia from pancreatic tumors, which are characterized by the display of sialyl-Lewis<sup>X</sup> (Singh et al. 2015). CCL2 has not been tested for toxicity towards these cells yet but an application of this lectin in cancer diagnostics or therapy may be feasible.

#### Materials and methods

#### Cultivation conditions

*Escherichia coli* was cultivated on LB or NGM medium as described (Sambrook and Russell 2001; Stiernagle 2006). Cultivation of *C. elegans* was performed according to Stiernagle (2006).

#### Site-directed mutagenesis

Mutant versions of the CCL2-expressing plasmids pET24-CCL2 and pET24-His<sub>8</sub>-CCL2 were generated using the QuikChange<sup>TM</sup> site-directed mutagenesis kit according to the manufacturer's recommendations (Stratagene).

#### Protein expression and purification

Untagged and His<sub>8</sub>-tagged versions of CCL2 were expressed in the cytoplasm of *E. coli* BL21(DE3) and His<sub>8</sub>-tagged versions were purified by metal-affinity chromatography as described previously (Schubert et al. 2012).

#### Crystallization and structure determination

Crystals of CCL2 were obtained by the hanging drop vapor diffusion method using 2 µL of drops containing a 50:50 (v/v) mix of protein and reservoir solution at 20°C. For the complex with sLe<sup>X</sup>. the protein at 5 mg mL<sup>-1</sup> in 10 mM Hepes/NaOH buffer pH 7.5 and 150 mM NaCl was incubated with sLeX 1.2 mM during 1h at room temperature prior to co-crystallization. Crystals were obtained in 2 days from solution 2-28 from the Midas screen (Molecular Dimensions Ltd): 30% glycerol ethoxylate and 100 mM Tris/HCl pH 8.5 (Grimm et al. 2010). Crystals of the apoprotein were obtained from a protein solution at  $5.1 \text{ mg mL}^{-1}$  in 100 mM sodium citrate pH 5.8 150 mM NaCl. Clusters of long and thin needles were obtained in 24 h from solution 1-14 of the Midas screen: 15% pentaerythritol propoxylate 5/4 PO/OH, 0.2 M sodium thiocyanate and 0.1 M Hepes pH 7.5 (Grimm et al. 2010). A single crystal or broken needle was directly mounted in a cryoloop and flash-freezed in liquid nitrogen. Diffraction data for the apoprotein and sLe<sup>X</sup> complex were collected at 100 K at the European Synchrotron Radiation Facility (Grenoble, France) at beamlines BM30A-FIP and ID23-1 using a ADSC Q315r CCD and a Pilatus detector, respectively. The data were processed using XDS (Kabsch 2010). All further computing was performed using the CCP4 suite (Winn et al. 2011). Data quality statistics are summarized in Table I. Both structures were solved by molecular replacement using PHASER (McCoy et al. 2007). For the sLe<sup>X</sup> complex, the 2LIE coordinates of model 1 comprising residues 20-153 were used as search model to find four molecules in the asymmetric unit (Schubert et al. 2012). The dimer coordinates of the sLe<sup>X</sup> complex were then used as search model to solve the apoprotein structure. Five percent of the observations were set aside for cross-validation analysis, and hydrogen atoms were added in their riding positions and used for geometry and structure-factor calculations. After initial rebuilding using ARP/WARP (Langer et al. 2008), the structures were refined with TLS and restrained maximum likelihood refinement using REFMAC 5.8 (Murshudov et al. 2011) iterated with manual rebuilding in Coot (Emsley et al. 2010). Incorporation of the ligand was performed after inspection of the 2mFo-DFc weighted maps. Water molecules, introduced automatically using Coot, were inspected manually. The stereochemical quality of the models was assessed with the program Molprobity (Chen et al. 2010), and coordinates were deposited in the Protein Data Bank under codes 4USO and 4USP for the sLe<sup>X</sup> complex and apo CCL2 structure, respectively.

#### SDS-PAGE-based oligomerization assay

The oligomerization assay employing partially denatured protein samples for SDS-PAGE was performed as described previously (Singh et al. 2015). In short, protein samples were mixed with  $2 \times$  SDS-PAGE (Laemmli) loading dye and left at RT or heated to  $95^{\circ}$ C for 5 min, run on regular SDS-PAGE and stained with Coomassie Brilliant blue R-250.

#### SEC and DLS

SEC was performed on an ENrich SEC 70 10 × 300 column (Bio-Rad) at a flow rate of 1 mL min<sup>-1</sup> in a buffer composed of 50 mM sodium KH2PO4/K2HPO4 pH 5.7 and 150 mM NaCl. 100 µL of protein solutions at concentrations of 6 mg mL<sup>-1</sup> for R18A\_F133A,  $4.2\,mg\,mL^{-1}$  for F133A,  $9\,mg\,mL^{-1}$  for R18A and  $3\,mg\,mL^{-1}$  for WT were injected and the eluate was monitored at 280 nm. Another SEC was performed on an Superdex 75 10 × 300 column (GE Healthcare) at a flow rate of 0.5 mL min<sup>-1</sup> in the same buffer. 100  $\mu$ L of protein solutions at concentrations of 5 mg mL<sup>-1</sup> for R18A\_F133A, 4 mg mL<sup>-1</sup> for F133A, 4 mg mL<sup>-1</sup> for R18A and 3 mg mL<sup>-1</sup> for WT were injected and the eluate was monitored at 280 nm. A calibration curve was done on each column with the following standards: Bovine serum albumine (BSA) 66.5 kDa, ovalbumin 43 kDa and RNAse A 13.7 kDa. The CCL2 elution peaks were concentrated on a Vivaspin 500 (5 kDa cut off, Sartorius) to a volume around 90 µL and size measurement were done using DLS on a zetasizer Nano ZS (Malvern instrument Ltd) using small size quartz cuvettes. Proteins were also subjected to DLS analysis prior to SEC.

#### Carbohydrates

Chemical synthesis of GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc $\beta$ 1O(CH<sub>2</sub>)<sub>5</sub>COOH was described previously (Schubert et al. 2012). sLe<sup>X</sup> was purchased from Elicityl (Crolles, France).

#### NMR spectroscopy

All NMR spectra were recorded on Bruker Avance HD spectrometer operating at 500, 600, 700 or 900 MHz equipped with tripleresonance cryogenetic or room temperature probes at 313K in a buffer of 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 5.8 and 100 mM NaCl. Standard <sup>15</sup>N-HSQC spectra of WT and mutant proteins were recorded at protein concentrations between 240 and 485 µM using  $1024 \times 256$  points. The detection of exchange protected amide protons was achieved with a 15N-HSQC recorded on a lyophilized CCL2 sample after dissolving it in D<sub>2</sub>O at a concentration of 280 µM (buffer conditions identical to samples in H2O/D2O). Four number of scans and  $1024 \times 128$  points were recorded. Binding to  $\alpha$ 1,3-fucosylated chitobiose was tested by titrating concentrated chitobiose solution (28 mM in identical buffer) into protein solutions of WT, R18A and R18A F133A at concentrations of 400, 240 and 330 µM, respectively. 2D DOSY experiments were measured with aqueous samples of WT, R18A, F133A and R18A/F133A protein variants (unlabeled) at 300 µM concentration. The standard Bruker pulse sequence stebpgp1s19, with stimulated echo, bipolar gradient pulses, one spoil gradient and 3-9-19 water suppression, was used with a linear gradient (53.5 G  $cm^{-1}$ ) stepped between 2 and 95%. Protein standards were measured at concentrations ranging from 200 to 400 µM. They included aprotinin, lysozyme, carbonic anhydrase, ovalbumin and BSA. Typical parameters were: td = 32, a gradient duration of  $\delta = 1.0-1.4$  ms and an echo delay  $\Delta$  between 200 ms and 400 ms.  $T_{\rm 1}$  and  $T_{\rm 2}$  relaxation times were measured with <sup>13</sup>C/<sup>15</sup>N-labeled CCL2 WT at a concentration of 1 mM using the Bruker pulse sequences hsqct1etf3gpsi.2 and hsqct2etf3gpsi, respectively. Seven T<sub>1</sub> relaxation delays ranging from 1 ms to 2 sec and six T<sub>2</sub> relaxation delays ranging from 4 to 130 ms were applied using ns = 32 and td = 200.

Spectra were processed using Topspin 3.2 (Bruker Biospin) and analyzed by Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, USA). <sup>1</sup>H chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid. <sup>15</sup>N chemical shifts are indirectly referenced using a scaling factor of 0.101329118 (Markley et al. 1998).

#### Nematotoxicity assays

Toxicity assays using CCL2-expressing *E. coli* BL21(DE3) and *C. elegans* N2 were performed as described previously (Kunzler et al. 2010). In brief, ~30 L1 larvae were added to 100  $\mu$ L of bacterial suspension (final  $A_{600} = 2$ ) in sterile PBS in 96-well plates. In order to detect subtle differences in nematotoxicity between the CCL2 variants, CCL2-expressing *E. coli* were mixed at different percentages with vector control containing *E. coli* as described previously (Yan et al. 2012). The percentage of individuals reaching L4 stage was quantified after 48 h of incubation at 20°C. Each assay was performed in quintuplicate. Welch's *t*-test was performed to validate statistical significance of the differences observed.

#### Comparing dimer structures of β-trefoil lectins

The arrangements of the dimers were analyzed using Molmol (Koradi et al. 1996). Molecules were oriented till a symmetry axis was visible, followed by *x*-, *y*- and *z*-rotations till the pseudo-symmetry axis of an individual  $\beta$ -trefoil molecule was visible. The derived angles have an estimated error of 1°.

#### Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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#### **Conflict of interest statement**

None declared.

#### Abbreviations

2D-[<sup>1</sup>H-<sup>15</sup>N]-HSQC, two-dimensional [<sup>1</sup>H-<sup>15</sup>N] Heteronuclear Single Quantum Coherence; sLe<sup>X</sup>, Sialyl-Lewis<sup>X</sup>.

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