

The N-terminal intrinsically disordered region of Ncb5or docks with the cytochrome b5 core to form a helical motif that is of ancient origin

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Abstract

Ncb5or (NADH cytochrome b5 oxidoreductase) is a cytosolic ferric reductase implicated in diabetes and neurological conditions. Ncb5or comprises cytochrome b5 (b5) and cytochrome b5 reductase (b5R) domains separated by a CHORD-Sgt1 (CS) linker domain. Ncb5or redox activity depends on proper interdomain interactions to mediate electron transfer from NADH or NADPH via FAD to heme. While full-length human Ncb5or has proven resistant to crystallization, we have succeeded in obtaining high-resolution atomic structures of the b5 domain and a construct containing the CS and b5R domains (CS/b5R). Ncb5or also contains an N-terminal intrinsically disordered region of 50 residues with a distinctive, conserved L³⁴MDWIRL⁴⁰ motif that has no homologs in animals but is present in root lateral formation protein (RLF) in rice and Increased Recombination Center 21 (IRC21) in baker's yeast, and in these proteins, it is likewise attached to a b5 domain. After unsuccessful attempts at crystallizing a human Ncb5or construct comprising the N-terminal region naturally fused to the b5 domain, we were able to obtain a high-resolution atomic structure of a recombinant rice RLF construct corresponding to residues 25-129 of human Ncb5or (52% sequence identity; 74% similarity). The structure reveals Trp120 (corresponding to invariant Trp37 in Ncb5or) to be part of an 11-residue α -helix (S¹¹⁶QMDWLKLRTR¹²⁶) packing against two of the four helices in the b5 domain that surround heme ($\alpha 2$ and $\alpha 5$). The Trp120 side chain forms a network of interactions with the side chains of four highly conserved residues corresponding to Tyr85 and Tyr88 ($\alpha 2$), Cys124 ($\alpha 5$), and Leu47 in Ncb5or. Circular dichroism (CD) measurements of human Ncb5or fragments further support a key role of Trp37 in nucleating the formation of the N-terminal helix, whose location in the N/b5 module suggests a role in regulating the function of this multidomain redox enzyme. This study revealed for the first time an ancient origin of a helical motif in the N/b5 module as reflected by its existence in a class of cytochrome b5 proteins from three kingdoms among eukaryotes.

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ABBREVIATIONS

CD, circular dichroism; Cyb5A, type A cytochrome b5; Cyb5R3, NADH cytochrome b5 reductase isoform 3; IRC21, Increased Recombination Center 21; NADH, nicotinamide adenine dinucleotide (reduced); Ncb5or, NADH cytochrome b5 oxidoreductase; RLF, reduced lateral root formation.

ABSTRACT

Ncb5or (NADH cytochrome b5 oxidoreductase) is a cytosolic ferric reductase implicated in diabetes and neurological conditions. Ncb5or comprises cytochrome b5 (b5) and cytochrome b5 reductase (b5R) domains separated by a CHORD-Sgt1 (CS) linker domain. Ncb5or redox activity depends on proper interdomain interactions to mediate electron transfer from NADH or NADPH via FAD to heme. While full-length human Ncb5or has proven resistant to crystallization, we have succeeded in obtaining high-resolution atomic structures of the b5 domain and a construct containing the CS and b5R domains (CS/b5R). Ncb5or also contains an N-terminal intrinsically disordered region of 50 residues with a distinctive, conserved L³⁴MDWIRL⁴⁰ motif that has no homologs in animals but is present in root lateral formation protein (RLF) in rice and Increased Recombination Center 21 (IRC21) in baker's yeast, and in these proteins, it is likewise attached to a b5 domain. After unsuccessful attempts at crystallizing a human Ncb5or construct comprising the N-terminal region naturally fused to the b5 domain, we were able to obtain a high-resolution atomic structure of a recombinant rice RLF construct corresponding to residues 25-129 of human Ncb5or (52% sequence identity; 74% similarity). The structure reveals Trp120 (corresponding to invariant Trp37 in Ncb5or) to be part of an 11-residue α -helix (S¹¹⁶QMDWLKLTRT¹²⁶) packing against two of the four helices in the b5 domain that surround heme ($\alpha 2$ and $\alpha 5$). The Trp120 side chain forms a network of interactions with the side chains of four highly conserved residues corresponding to Tyr85 and Tyr88 ($\alpha 2$), Cys124 ($\alpha 5$), and Leu47 in Ncb5or. Circular dichroism (CD) measurements of human Ncb5or fragments further support a key role of Trp37 in nucleating the formation of the N-terminal helix, whose location in the N/b5 module suggests a role in regulating the function of this multidomain redox enzyme. This study revealed for the first time an ancient origin of a helical motif in the N/b5 module as reflected by its existence in a class of cytochrome b5 proteins from three kingdoms among eukaryotes.

Keywords

cytochrome b5, circular dichroism, crystallography, helix motif, intrinsically disordered region.

Supporting Information Available .

This includes sequence alignments of Ncb5or in animals (N-terminal region and cytochrome b5 domain) and of the homologs in plants, fungi/yeasts, protists and parasites, along with prediction plots of intrinsically disordered regions in human Ncb5or.

INTRODUCTION

Ncb5or (NADH cytochrome b5 oxidoreductase, also called Cyb5R4, b5/b5R and b5+b5R) is a cytosolic ferric reductase implicated in diabetes, neurologic diseases and iron homeostasis.¹⁻⁵ Ncb5or is widely expressed in animal cells and localized to endoplasmic reticulum.^{2, 6} Ncb5or contains two redox domains and one linkage domain that are homologous to cytochrome b5 (Cyb5), cytochrome b5 reductase (Cyb5R), and members of *C* HORD and *S* GT1 (CS) family, respectively.^{6, 7} Ncb5or mediates electron transfer from NADH or NADPH to a redox substrate via FAD and heme cofactors in the b5R and b5 domains, respectively, and proper inter-domain interactions are essential to this process.⁶ Although full-length Ncb5or from human has proven resistant to crystallization to date, we have succeeded in obtaining high-resolution structures of the b5 domain (PDB 3LF5) and a fragment containing the CS and b5R domains (CS/b5R) in complexes with NAD+ and NADP+ (PDB 6MV1 and 6MV2, respectively).^{8, 9} Despite having the same general fold, the b5 domain exhibits substantially different heme ligation from that of microsomal cytochrome b5 (Cyb5A).⁸ Similarly, the b5R domain differs from microsomal cytochrome b5 reductase (Cyb5R3) in having several multi-residue deletions and insertions that support extensive interactions with the CS domain and reflect a closer relationship to Cyb5R proteins from plants, fungi and protists than to Cyb5R3 from animals.⁹

The b5 domain of Ncb5or is preceded by a 50-residue N-terminal region that is unique among animal proteins but has homologs in Reduced Lateral Root Formation (RLF) proteins in plants and Increased Recombination Center 21 (IRC21) proteins in fungi. As in Ncb5or, the N-terminal region in the largely unexplored RLF and IRC21 proteins precedes a b5 domain. Herein we report the results of studies aimed at elucidating the structural properties of the N-terminal region of human Ncb5or both in isolation (hereafter N-term), and when natively fused to the b5 domain (hereafter N/b5). Circular dichroism (CD) spectroscopic data revealed that N-term is intrinsically disordered but exhibits significant helical content in N/b5, with an invariant tryptophan residue (Trp37) playing a key role in inducing the helical structure. CD studies further suggested that Trp37, located in a highly conserved L³⁴MDWIRL⁴⁰ motif in mammalian orthologs, resides in a highly organized environment. While N/b5 constructs representing human Ncb5or failed to form crystals, a recombinant construct of rice RLF comprising residues K101-E218 crystallized readily. The resultant high-resolution X-ray crystallographic structure showed Trp¹²⁰ (corresponding to Trp³⁷ in Ncb5or) to be part of an 11-residue α -helix (S¹¹⁶QMDWLKLTRT¹²⁶) packing against two of the four helices that surround heme (α 2 and α 5 in Ncb5or). The Trp¹²⁰ side chain forms a network of interactions with the side chains of four highly conserved residues that are equivalent to Ncb5or residues Tyr⁸⁵ and Tyr⁸⁸ in α 2, Cys¹²⁴ in α 5, and Leu⁴⁷. In addition to advancing our understanding of Ncb5or structure and drawing our attention to several surface-exposed side chains that may be functionally important, the studies also establish that the N/b5 unit of this multi-domain protein contains a helix motif that is of ancient origin and part of a well-conserved protein structural module.

MATERIALS AND METHODS

Molecular cloning and site-directed mutagenesis. Human Ncb5or contains 521 amino acid residues that comprise the N-terminal region and the b5, b5R and CS domains as previously defined.⁸ These segments of the protein contain 50, 113, 111, and 247 residues, respectively, that also include linker sequences in the middle two fragments (**Figure 1A**). Nomenclature of the constructs used herein is as follows: N/b5 (Met¹ through Lys¹³⁷); N/b5- Δ 21 (Gly²² through Lys¹³⁷); N/b5- Δ 34 (Met³⁵ through Lys¹³⁷); b5 (Lys⁵¹ through Lys¹³⁷); and N-term (Met¹ through Leu⁵⁰). The cDNAs of all Ncb5or constructs (except N-term) and rice RLF (residues 101-218, XP_015647767) were expressed with no epitope tag (pET19b or pET22b). The cDNA of N-term was cloned into the pE-SUMOpro Kan vector (Life Sensors, Malvern, CA), which contains the 6XHis-SUMO gene immediately in front of the multiple cloning site previously described.¹⁰ Missense point

mutants, N/b5-LMAA (Leu³⁴Ala/Met³⁵Ala) and N/b5-W37A (Trp³⁷Ala) (**Figure 1B**), were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). All mutagenesis primers were synthesized by Integrated DNA Technology (Coralville, IA). All constructs were confirmed by DNA sequencing (ACGT, Inc., Wheeling, IL).

Protein preparation. Except for N-term, all constructs of human Ncb5or and rice RLF were expressed in *E. coli* BL21(DE3) or BL21(DE3)pLysS or BL21(DE3)pRARE cells and purified as previously described.⁸ In combination with size exclusion chromatography on a Superdex 75 10/300 GL column, ion-exchange chromatography was performed for b5 and N/b5- Δ 34 on a Q HP column, N/b5 and variants (N/b5-W37A, Nb5-LMAA, and N/b5- Δ 21) on a SP HP column, and rice RLF on both Q and SP HP columns. Full-length Ncb5or and its variant Ncb5or- Δ 50 were prepared as previously described², except that 0.5 mM IPTG was used for induction in TB media at 15°C. All purification steps were performed at 4°C. SDS-PAGE was used to estimate the purity of each protein used in spectroscopic analyses, kinetics studies and crystallization screening (all greater than 95%). All purified proteins were flash frozen in liquid nitrogen and stored as aliquots at -80°C until use, except for samples prepared for crystallization screening which were stored at 4°C. The size of each polypeptide product was confirmed by mass spectrometry. Expected sizes are: N/b5, N/b5-W37A, N/b5-LMAA, ~15.6 kD; N/b5- Δ 21 and RLF, ~13.6 kD; N/b5- Δ 34, 11.9 kD; and Ncb5or-b5, 10.2 kD. The ratios of A₄₁₃/A₂₈₀ for purified heme-containing constructs were as follows: b5 [?] 4, N/b5 and variants, RLF [?] 3.6, Ncb5or ~1.0, and Ncb5or- Δ 50 ~1.1. The FAD content of Ncb5or-b5R was determined by A₄₆₁ and used to represent enzyme concentrations as previously described.⁸ Final protein yields (mg/L): 8 (b5), 5 (N/b5 and variants), 2 (b5R), [?]1 (Ncb5or, Ncb5or- Δ 50, RLF). Solubility in low-salt buffer: N/b5, RLF > b5 > Ncb5or > Ncb5or- Δ 50 > Ncb5or-b5R. Protease inhibitor (Sigma, St. Louis, MO) was used during protein preparation to prevent proteolytic cleavage at the b5-CS border of Ncb5or and Ncb5or- Δ 50. A SUMOpro Expression System was used to prepare N-term.¹⁰ Briefly, a 6XHis-SUMO-N-term fusion protein was expressed in BL21(DE3) cells and purified by Ni-NTA affinity resin (Qiagen, Valencia, CA). It was then cleaved by a 6XHis-SUMO protease¹¹ to release 6XHis-SUMO tag, both of which were removed by nickel resin. The purity and size of the N-term peptide were confirmed by SDS-PAGE and mass spectrometry (data not shown).

Spectroscopy. UV/visible spectra were obtained on a Cary 100 Bio spectrophotometer (Varian). The concentrations of oxidized heme in all constructs were determined with $\epsilon_{413} = 130 \text{ mM}^{-1}\text{cm}^{-1}$ as previously described.² Circular dichroism (CD) analyses were performed on a JASCO-815 spectropolarimeter using protein concentrations of 4-10 μM . Far-UV (190-250 nm) spectra were recorded using a 1 mm cuvette, whereas a 1 cm cuvette was used for near-UV (250-350 nm) and visible (350-550 nm) CD spectra. All CD spectra are reported in units of molar ellipticity ($[\theta]$, $\delta\epsilon\gamma\text{-cm}^2\text{-dmol}^{-1}$).

RLF crystallization. A purified RLF construct (Lys¹⁰¹-Glu²¹⁸) was concentrated to 44.6 mg/mL in 20 mM Tris pH 8.0 for crystallization screening. All crystallization experiments were set up using an NT8 drop setting robot (Formulatrix Inc.) and UVXPO MRC (Molecular Dimensions) sitting drop vapor diffusion plates at 18°C. 100 nL of protein and 100 nL of crystallization solution were dispensed and equilibrated against 50 μL of the latter. Crystals approximately 300-500 μm long that displayed a needle morphology were observed within one day from the JCSG+ screen (Molecular Dimensions) condition A6 (20% (w/v) PEG 1000, 100 mM phosphate-citrate pH 4.2, 200 mM lithium sulfate). A cryoprotectant solution composed of 80% crystallization solution and 20% (w/v) glycerol was dispensed (2 μL) onto the drop, crystals were harvested with a cryoloop immediately and stored in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source beamline 17-ID using a Dectris Pilatus 6M pixel array detector.

Data collection and structure refinement. Intensities were integrated using XDS^{12, 13} via Autoproc¹⁴ and the Laue class analysis and data scaling were performed with Aimless¹⁵ which indicated that the crystals belong to the $2/m$ Laue class with possible space groups $P 2$ or $P 2_1$. Structure solution was conducted by molecular replacement with Phaser¹⁶ using the Ncb5or b5-domain structure (PDB 3LF5⁸) as the search model. The top solution was obtained in the space group $P 2$ and contained a dimer in the asymmetric unit. Initial refinement with Refmac¹⁷ yielded $R/R_{\text{free}} = 42.1\%/42.5\%$ and the model was improved by automated

building with Arp/wARP.¹⁸ Further refinement and manual model building were conducted with Phenix^{19, 20} and Coot²¹ respectively. TLS parameters^{22, 23} were refined in the later stages to model anisotropic atomic displacement parameters. Disordered side chains were truncated to the point for which electron density could be observed. Structure validation was conducted with Molprobity²⁴ and figures were prepared using the CCP4MG package²⁵. Polder omit electron density maps were calculated using Phenix.²⁶ Structure superposition was carried out with GESAMT.²⁷ Crystallographic data are described in **Table S1**, and the best data set was at 1.55 Å resolution.

RESULTS AND DISCUSSION

The N-terminal region of Ncb5or is predicted to be intrinsically disordered.

The amino acid sequence of the N-terminal region of human Ncb5or is shown in **Figure 1B**, and an alignment of this 50-residue polypeptide with all known Ncb5or examples in animals is included in **Figure S1**. The alignment revealed an overall homology, which is weaker in the first 21 residues than the subsequent 29-residue stretch which is rich in Arg and Lys residues and contains five invariant residues (Lys25, Leu28, Gly31, Trp37 and Gly49). Trp37 is part of a highly conserved motif that has the sequence L³⁴MDWIRL⁴⁰ in mammalian orthologs.

The PONDR VL-XT program²⁸⁻³⁰ predicts that the N-terminal region of Ncb5or is intrinsically disordered, in sharp contrast to the b5, b5R and CS domains (**Figure S2A**). Secondary structure prediction algorithms generally gave inconsistent predictions for the N-terminal region, but several of them indicated a preference for α -helical structure for the L³⁴MDWIRL⁴⁰ sequence. This included PROF³¹ and PSIPRED^{32, 33} (**Figure S2B**), as well as SPINE-X³⁴ (not shown). The latter two *ab initio* algorithms (*i.e.*, those not utilizing homology modeling) have been shown to yield the most accurate predictions.³⁵ AGADIR³⁶, an algorithm that predicts helical content of peptides on the basis of helix/coil transition theory, also predicts some helical tendency for the L³⁴MDWIRL⁴⁰ sequence, albeit with a population of less than 10% at pH 7 and 25°C (**Figure S2C**).

Interactions with the b5 domain induce helical structure in the N-terminal region.

To compare intrinsic structural properties of the N-terminal region of human Ncb5or with those it exhibits in the native protein, we performed CD spectroscopy studies of the 50-residue N-terminal polypeptide (hereafter N-term) and protein constructs representing the b5 domain with and without the N-terminal region attached (hereafter N/b5 and b5, respectively). Far-UV CD spectra of b5 and N/b5 are shown in **Figure 2A** (summarized in **Table 1**) and presented in molar ellipticity units to better reflect differences in total secondary structure content. The N/b5 spectrum differs from that of b5 in exhibiting enhanced negative ellipticity in the vicinity of 200 nm. The dominant feature in far-UV CD spectra of unstructured (random coil) polypeptides is a broad band of negative ellipticity centered near 200 nm.³⁷ The data in **Figure 2A** therefore suggests that N/b5 differs from b5 in having a larger number of disordered residues. This was confirmed by subtracting the b5 spectrum from the N/b5 spectrum to give the difference spectrum shown in **Figure 2C**. However, this difference spectrum also exhibits positive ellipticity near 190 nm and negative ellipticity in the vicinity of 220 nm, both suggestive of the presence of helical structure.³⁸ In contrast, the corresponding N-term spectrum (**Figure 2C**) is dominated by a negative band centered at 200 nm, revealing it to be largely unstructured. These results strongly suggest that the N-terminal region of Ncb5or is intrinsically disordered, consistent with the predictions described above, but that it adopts some helical structure when connected to the b5 domain.

The Trp37 side chain resides in a well-structured environment.

When located in highly asymmetric (*i.e.*, well-structured) environments, side chains of aromatic residues exhibit CD signals in overlapping regions of the near-UV (Phe, 250-270 nm; Tyr, 270-290 nm; Trp, 280-300 nm).³⁸ As shown in **Figure 2B**, b5 displays signals in the near-UV region (250- 350 nm) attributable to the fact that it contains 2 Phe, 4 Tyr, and 2 Trp residues. Comparatively stronger signals, with significant fine structure, appear in the near-UV CD spectrum of N/b5 (**Figure 2B**). The N-terminal region contains

only two aromatic residues, Phe9 and Trp37. Given that the strongest feature in the near-UV CD signature of N/b5 appears at 286 nm, we conclude that the major contributor is Trp37, which is part of the invariant mammalian Ncb5or L³⁴MDWIRL⁴⁰ sequence noted above. In contrast, the corresponding spectrum of N-term exhibits no such signal at 286 nm (**Figure 2D**).

Aromatic residues in well-ordered regions of proteins often contribute to far-UV CD spectra as well. In the case of Trp, positive bands in the 225-250 nm region are a particularly common feature.^{39, 40} Trp37 is therefore a likely source of the positive feature in the difference CD spectrum of N-term centered at 230 nm (**Figure 2C**), another conjecture which is supported by additional CD data described below. Notably, N-term exhibited no signals in the near-UV region (**Figure 2D**) and displays negative ellipticity in the vicinity of 230 nm (**Figure 2C**). The results described in this section allow us to conclude that interactions between the N-terminal region and the b5 domain induce secondary structure in the former, and that this structural transition is accompanied by adoption of tertiary structure involving Trp37. This conclusion was further tested with truncation and point mutants of N/b5.

Trp37 plays a key role in the interactions between the N-terminal region and the b5 domain.

We generated two truncation mutants of N/b5 (**Figure 1**) and examined the effects of the truncations on CD spectra. The first mutant involved the removal of residues 1-21, the portion carrying the lowest conservation among animals, to yield N/b5- Δ 21. Residues 1-34 were also removed to produce a domain fragment (N/b5- Δ 34) analogous to the truncated Ncb5or that was initially cloned.⁶

Deleting the first 21 residues of N/b5 had no significant effect on the near-UV CD spectrum, or on the intensity of the positive far-UV CD band near 230 nm (**Figures 2E-F, Table 1**). The most notable effect on CD spectra caused by removing residues 1-21 was a decrease in negative ellipticity of the lowest wavelength far-UV band, along with a slight red shift of that band. These observations suggest that residues 1-21 are almost exclusively disordered and show that they play no role in the secondary and tertiary structure formation resulting from the interactions between the N-terminal region and the b5 core. They also support our conclusion that the near-UV CD signals and the positive far-UV feature near 230 nm arise from Trp37, with no contribution from Phe9.

Comparison of the far-UV CD difference spectra for N/b5- Δ 21 and N/b5- Δ 34 (**Figure 2E, Table 1**) shows that deleting the additional residues 22-34 decreased, but did not abolish, secondary structure content. In addition, **Figure 2F** (also **Table 1**) reveals diminished intensity for the near-UV CD signals attributable to Trp37 in N/b5- Δ 34, but the signals in this region are much more like those in the spectrum of N/b5 than of b5. This suggests that Trp37 is involved in tertiary structure in both truncation mutants, but that removal of residues 22-34 causes subtle disruption of that tertiary structure.

The evidence obtained with the truncation mutants strongly indicates a key role for Trp37 in interactions between the N-terminal region and the b5 domain of Ncb5or. We therefore examined the effect of mutating Trp37 to Ala on CD spectra of N/b5. Subtracting the far-UV CD spectrum of b5 from that of N/b5-W37A yielded a difference spectrum similar to the spectrum of N-term (**Figure 2G**). Moreover, the W37A mutation in N/b5 resulted in loss of the near-UV CD features present in the N/b5 spectrum (**Figure 2H**). It can therefore be concluded that the W37A mutation in N/b5 abolishes both secondary and tertiary structure. In comparison, the difference spectrum obtained by subtracting the far-UV CD spectrum of N/b5-LMAA from that of N/b5 exhibits modestly increased intensity of the negative band near 220 nm (**Figure 2G, Table 1**) and of the positive band near 190, suggesting a small increase in helix content. This may be attributable to the fact that Ala has a much higher helix propensity than do Leu and Met.⁴¹ More notably, the LM-AA mutation does not significantly alter the shape or intensity of the near-UV and far-UV CD features attributable to Trp37 (**Figure 2G-H**). However, the CD Soret band signal for the LMAA mutant is virtually identical to that of Ncb5or-b5 (**Figure 2H**). These observations suggest that the LM-AA mutation does not alter the ability of N-term to dock with the b5 core, with associated adoption of tertiary structure involving Trp37, but that it alters (or perhaps weakens) interactions between the N-terminal region and the b5 core.

The results described in this section indicate that residues spanning Met35 through Leu50 are sufficient to induce secondary structure (likely helical) and accompanying tertiary structure in the N-terminal region when it docks with the b5 core. The results also show that Trp37 plays an essential role in inducing this structural transition, suggesting a specific recognition site on the b5 domain surface. These results further indicate that the induced secondary structure is propagated toward the N-terminus but does not extend beyond Gly22. This additional secondary structure appears to stabilize the tertiary structure.

Plant and fungal proteins identified as potential models for N/b5

Efforts to confirm the results of the CD studies described above via X-ray crystallographic studies of the N/b5 constructs were unfortunately not successful, prompting us to seek potential homologs that might crystallize more readily. BLAST searches using human Ncb5or residues 1-50 as the query revealed only a few other proteins that contain a homologous polypeptide, all from plants and fungi. In all cases the homologous polypeptide precedes a b5 domain having significant homology to that of Ncb5or and contains a Trp residue analogous to Trp37 of Ncb5or but no additional domains or regions (**Figures S1, S3, S4**). No structural characterization has been performed for these homologues, and only two have been characterized functionally: RLF (*R* educed *L* ateral root *F* ormation protein) from plants (e.g., rice, *Arabidopsis* and glycine) and IRC21 (*I* ncreased *R* ecombination *C* enter 21) protein from yeast (e.g., *Saccharomyces cerevisiae* and *S. pombe*). RLF appears to regulate lateral root formation independently of ARF7/19-mediated auxin signaling⁴², whereas IRC21 functions in chromatin remodeling⁴³ and DNA repair⁴³⁻⁴⁵ likely through its binding to lipid and protein phosphatase.^{46, 47}

The N-terminal regions of plant RLF proteins are approximately 130-residues in length (**Figure 3A-B**) and contain a sequence spanning residues 115-121 (Q¹¹⁵MDWLK¹²¹) that is strikingly similar to residues 34-40 of Ncb5or which encompass Trp37 (L³⁴MDWIRL⁴⁰). The N-terminal regions of IRC21 proteins are approximately 77 residues in length, and in that respect are more like Ncb5or than are the plant RLF proteins. However, there is weaker homology for residues 61-67 (A⁶¹LDWHS⁶⁷) that correspond to residues 34-40 of Ncb5or (**Figure 3A-B**). In addition, the sequence separating this signature from the b5 core is approximately 40 residues longer in IRC21 proteins than in Ncb5or and RLF, an insertion that may have arisen subsequent to divergence of the plant, fungi and animal kingdoms.⁴⁸ Yeast IRC21 and rice RLF were used for these comparisons (**Figures 3B**). For these reasons we selected rice RLF protein for our structural studies, specifically residues 101-218, corresponding to residues 22-137 of human Ncb5or.

The b5 cores of Ncb5or and the rice RLF protein have identical folds.

The rice RLF construct crystallized readily as a non-crystallographic dimer and could be modeled from Phe¹¹¹-Glu²¹⁸ in subunit A and Lys¹¹³-Phe²¹³ in subunit B. The fold comprises six α -helices and four β -sheets (**Figure 4A-B**) that are arranged as follows: α 1 (Ser¹¹⁶-Thr¹²⁶), β 1 (Arg¹⁴⁰-Ile¹⁴²), α 2 (Leu¹⁴⁴-Lys¹⁴⁸), β 2 (Trp¹⁵⁷-Leu¹⁶⁰), β 3 (Arg¹⁶³-Asn¹⁶⁶), α 3 (Ala¹⁶⁸-Phe¹⁷³), α 4 (Val¹⁷⁸-Met¹⁸²), α 5 (Thr¹⁹⁰-His¹⁹⁷), α 6 (Phe²⁰²-Leu²⁰⁵), β 4 (Leu²¹⁰-Leu²¹⁴). Note that the numbering is based on the NCBI reference sequence XP_015647767.1. The b5 core of RLF is identical to that of the Ncb5or b5 domain, with superposition yielding an RMSD deviation of 1.01 Å (80 residues) between C α atoms (**Figure 5**).

The heme molecules are positioned within a cleft formed by helices α 3- α 6. It is important to note that the corresponding helices in the published structure of the Ncb5or b5 domain, and in structures of single domain b5 family members, are numbered α 2- α 5. In the comparison of the b5 and N/b5 structures we will refer to the relevant helices exclusively as α 3- α 6. Like all cytochrome b5 family members, the heme iron is coordinated by the side chains of two histidine residues (His174 and His197). His174 (His89 in Ncb5or) is located in the loop separating α 3 and α 4, while His197 (His112 in Ncb5or) constitutes the C-terminal residue in helix α 5. As observed for His89 and His112 in the crystal structure of the Ncb5or b5 domain, the imidazolyl moieties of His174 and His197 in the rice RLF structure are nearly orthogonal to one another (**Figure 6B**). The angle between the mean planes defined by the indole rings is 77.6° (subunit A) and 77.2° (subunit B) as calculated using Mercury.⁴⁹ The corresponding angles for His89 and His112 in the two subunits of the Ncb5or b5 domain structure (3LF5) are 83.2° and 81.3°. ⁸ This structural feature distinguishes these proteins

(and likely IRC21 as well), from the better known microsomal and mitochondrial isoforms of cytochrome b5, in which angles between the His ligand side chains are closer to 20°. ⁸

We reported that the b5 core of Ncb5or differs from the better known microsomal and mitochondrial isoforms of cytochrome b5 in having a strictly conserved tryptophan residue (Trp114) at the mouth of the heme binding pocket, two residues removed from heme ligand His112. The intervening residue, Arg113, is invariant among mammalian Ncb5or isoforms. In the crystal structure of the b5 domain of Ncb5or the side chain of Trp114 is located on the protein exterior and is substantially solvent-exposed (**Figure 6A**), with its only inter-protein interactions involving the side chain of adjacent residue Arg113. The rice RLF residues corresponding to the Ncb5or His¹¹²ArgTrp¹¹⁴ sequence (His197, Ala198, and Trp199) are strictly conserved among known members of this protein family. In the RLF crystal structure the Trp199 side chain projects into solvent, but as shown in **Figure 6A** its side chain conformation differs from that of Trp114 in the Ncb5or b5 domain structure, enabling hydrogen bonding between its Ne-H and one of the heme propionate groups. Manually changing the side chain torsional angles of Trp114 in the Ncb5or b5 domain crystal structure ($\chi^0 = -53.9$; $\chi^2 = 115.7^\circ$) to match those of Trp199 in the new rice RLF structure ($\chi^0 = 58.9$; $\chi^2 = 85.4^\circ$), using PyMol, showed that Trp114 could form an analogous hydrogen bond with heme without introducing unfavorable steric interactions. Given that this solvent exposed Trp residue is strictly conserved among all known Ncb5or and RLF proteins (and in IRC21 proteins as well), its ability to form a hydrogen bond with heme suggests that it serves an essential functional role.

Yet another distinguishing feature shared by the b5 cores of Ncb5or and rice RLF is an irregular helix ($\alpha 6$) in the heme-binding pocket, featuring a central kink that leaves Leu205 (Met120 in Ncb5or) without an intra-helical hydrogen bond. As will be noted in the following section, this kink plays an important role in interactions with the N-terminal region.

While carrying out the studies reported herein, we became aware of reports of b5 family members in *Giardia* and some other parasitic organisms with strong homology to the b5 domains of Ncb5or, RLF and IRC21 proteins. As shown by some representative examples in **Figure S5**, these proteins feature an N-terminal region, albeit generally shorter than those in Ncb5or, RLF and IRC21 and without a Trp residue analogous to Trp37 in Ncb5or. ⁵⁰ Many of them have also maintained a surface tryptophan residue analogous to Trp114 of Ncb5or. The *Giardia* proteins were shown to exhibit redox potentials ⁵¹ similar to those determined for Ncb5or, ² which are considerably more negative than for microsomal b5s. The *Giardia* proteins and Ncb5or also share EPR spectroscopic signatures that are characteristic of orthogonal His ligands. ⁵¹ It is reasonable to conclude that these proteins have evolved from the common N/b5 ancestor of Ncb5or, RLF, and IRC21, perhaps with divergent functions. ^{50, 51} This notion is supported by the observation of cytochrome b5 proteins in protists (*Trypanosoma* and *Dictyostelium*) that share the same b5 core ⁵⁰ and the N-terminal motif as that in Ncb5or, including the Trp114 and Trp37 residues, respectively (**Figure S5**).

The rice RLF structure confirms N-terminal helix formation involving the Trp120 side chain.

Trp120 in RLF (corresponding to Trp37 in Ncb5or) is part of an 11-residue α -helix, designated $\alpha 1$ (S¹¹⁶QMDWLKLRTR¹²⁶), that packs against the side of the heme-binding pocket defined by helices $\alpha 3$ and $\alpha 6$ (**Figure 7**). In this and subsequent sections, all corresponding residues in Ncb5or are listed in parentheses. A 17-residue stretch connects $\alpha 1$ to $\alpha 2$, the first element of defined secondary structure in the “classic” b5 core ($\alpha 2$ is the only helix in the b5 core that does not comprise part of the heme binding pocket). The corresponding stretch in Ncb5or is two residues shorter, with the gap appearing close to the junction with $\alpha 2$. There are multiple conserved residues in the RLF and Ncb5or loops, and in the RLF structure several of them exhibit specific backbone and side chain interactions both within the loop and with the b5 core. These include two three-residue turns featuring a single backbone hydrogen bond, the first between Asp129 and Gly132 (Asp46 and Gly49), and the second between Leu133 and Gln136 (Leu 50 and Arg53). The Asp129 (Asp46) side chain in the first turn extends this motif via hydrogen bonds to the backbone amide N-H groups of Leu133 and Lys134 (Leu50 and Lys51), residues which can be considered to represent the transition between N-term and the b5 core. The side chain of Leu133 (Leu50) is buried and engages in hydrophobic interactions with several nearby residues, namely C β of Ala131 (Thr48), C β of Gln136 (Arg53),

and C α of Asn138 (Ile55). It also packs against the side chain of Val211 (Val126) which is in strand β 4 of the b5 core. All the residues in this hydrogen bonded array other than solvent exposed Gln136 (Arg53) are conserved in RLF and Ncb5or proteins. Likely because of these interactions, this polypeptide segment is well-ordered. Packing between the N-terminal helix and the b5 core involves primarily hydrophobic interactions and does not noticeably impact the b5 core fold. Consistent with the results of far-UV CD studies described above, the Trp120 side chain is situated in an environment with well-defined tertiary structure that includes hydrophobic packing interactions with the side chains of four residues in α 3 and α 6, each corresponding to a residue that is highly conserved in Ncb5or: Tyr170 and Phe173 in α 3 (Tyr85 and Tyr88); and Leu206 and Cys209 in α 6 (Leu121 and Cys124). The Trp120 side chain engages in additional hydrophobic interactions with the nearby side chains of Leu121 and Thr124 in α 1 (Ile38 and Thr41) and with the side chain of Leu130 (Leu47), located in the first 3-residue turn in the 17-residue loop connecting α 1 to α 2.

It is worth noting that the interaction between the Trp120 and Cys209 side chains (Trp37 and Cys124) involves the cysteine thiol (SH group) packing against the tryptophan pi system. Such interactions are relatively common and are thought to play a stabilizing role.^{52, 53} Both residues are invariant in Ncb5or proteins.

The Trp120 (Trp37) side chain has one additional contact, a hydrogen bond between its N-H group and the backbone carbonyl of Leu205 in α 6 (Met120). As noted in the previous section, the backbone carbonyl of that residue does not form an intra-helix H-bond due to a kink in α 6. The present results suggest that the α 6 kink may have evolved to serve a distinct functional role in these proteins.

The only other polar contacts between α 1 and the b5 core comprise a network of hydrogen bonds involving the side chains of the first two α 1 residues Ser116 and Gln117 (Ser31 and Leu32), the phenolic side chain of Tyr170 in α 3 (Tyr85), and the backbone carbonyl of Phe173 in α 3 (Tyr88). While this network of hydrogen bonds may be important in stabilizing α 1, it is noteworthy that the polar OH group of the Tyr170 side chain also makes van der Waals contact with one of the heme *meso* positions. This counterintuitive positioning of a polar group suggests a potential functional role. An intriguing possibility is that oxidation of ferrous heme to ferric heme during reduction of the downstream substrate is accompanied by deprotonation of the phenolic group. This could conceivably facilitate a coupled electron/proton transfer process during substrate reduction. Alternatively, it could allow for electrostatic stabilization of the b5 domain when heme is in the ferric oxidation state and bears a formal positive charge.

Several conserved residues in the N-terminal helix have solvent-exposed side chains.

The side chains of several other highly conserved residues in the N-terminal helix project into solvent (**Figure 8**), including three in the vicinity of Trp120: Met118, Asp119, and Lys122 (corresponding to Met35, Asp36, and Arg39 in Ncb5or). Particularly notable in this group is Met118 (Met35). The thioether side chain of methionine is considered nonpolar, and indeed methionine is often subject to evolutionarily conservative replacement by residues with aliphatic side chains via single codon mutations (leucine, isoleucine, and valine). Only a few examples of such a mutation, specifically Met to Leu, exist among known Ncb5or proteins, and only in non-mammalian organisms (**Figure S1**). Methionine differs from the aliphatic amino acids it often replaces in that its thioether functional group can serve functional roles (for example, as a ligand to heme iron in cytochrome *c*). The highly conserved nature of Met35 in Ncb5or, considered in the context of its solvent-exposed location, is suggestive of an essential functional role.

CONCLUSION

This study represents the first *in vitro* characterization of the N-terminal region of Ncb5or, a polypeptide segment not found in any other animal proteins, but which shares a common ancestor with RLF proteins in plants and IRC21 proteins in fungi. The N-terminal regions in all three of these protein families precede highly homologous cytochrome b5 domains, and share sequence similarities that suggest that the results we have obtained using N/b5 constructs derived from human Ncb5or and rice RLF protein will pertain to the IRC21 proteins as well. Specifically, we have shown that the N-terminal region is intrinsically disordered but that a 11-residue motif adopts a helical conformation when natively attached to the b5 core, a structural

transition that is nucleated by a strictly conserved tryptophane residue (Trp37 in human Ncb5or). Such docking-induced folding is a common theme for intrinsically disordered regions of mammalian proteins that are often involved in DNA or RNA binding, substrate binding, and protein-protein interactions⁵⁴⁻⁵⁷, of which the specificity is generally mediated by motif structures.^{58, 59} In this context, it is worth investigating whether IRC21 utilizes the helical motif in the Nb5 module to activate protein phosphatase 2A for DNA damage response in yeasts.⁴⁷ Intrinsically disordered regions appear to be enriched in disease-related proteins, such as the N-terminal transactivation domain of tumor suppressor p53,⁶⁰ alpha-synuclein in Parkinson’s disease⁶¹, tau protein in Alzheimer’s disease⁶², and in a number of proteins associated with cardiovascular disease.⁶³ Mutation of the intrinsically disordered region of carboxyl ester lipase in pancreatic acinar cells can cause lean diabetes, likely as a result of protein mis-folding.⁶⁴ Functional contribution of the N-terminal intrinsically disordered region and the helical motif to Ncb5or’s cellular function and related disease pathways is being investigated.

The presence of the helical motif within the N/b5 module in three kingdoms of eukaryotes that diverged about 1.6 billion years ago^{48, 65} suggests an ancient heritage of Ncb5or. This is consistent with our previous observation of closer similarities of its b5R domain to monomeric b5R proteins from plants, fungi and protists than to mammalian Cyb5R3 proteins.⁹ The nucleated N-terminal helix lies between two of the four helices that comprise the heme binding pocket and is connected to what we have previously referred to as the b5 core by a well-structured polypeptide. The N-terminal region in Ncb5or and the plant and fungal proteins could conceivably comprise an integral part of their b5 cores, which would thus be expanded relative to other modern cytochrome b5 superfamily members. Alternatively, the N-terminal regions of these proteins could function to regulate their enzymatic functions, through reversible docking. Suggestive of this latter scenario is the observation that the 17 residues separating the N-terminal helix $\alpha 1$ from $\alpha 2$ in rice RLF protein exhibit few interactions with the “classic” portion of the b5 core, in contrast to the 17-residue helix that is nucleated by the Trp37-dependent docking interactions. These possibilities will be the subject of future studies. A low homology in the N-terminal region of cytochrome b5 proteins of parasites, including the absence of the helical motif, is likely a result of diminishing selection pressure for the above-mentioned motif-mediated function due to the unique parasite-host interaction and pathophysiology.

Future studies will also be aimed at probing potential functional roles of other strictly conserved residues in Ncb5or emphasized above. These include Met35 in the N-terminal helix, and Trp114 which is located two residues from heme ligand His112. The side chains of both these residues project conspicuously into solvent, locations that would subject them to random mutation were they not essential for protein function, especially since they are encoded by a single codon.⁶⁶ Functional roles could include modulating heme reduction by the b5R domain or reduction by heme of the still unknown substrate.

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STRUCTURE DEPOSITION

Coordinates and structure factors for the root lateral formation protein (RLF) b5-domain structure were deposited to the Worldwide Protein Databank (wwPDB) with the accession code 8TGB.

Table 1 . Signature signals of CD spectra for N/b5, its variants, and b5 of human Ncb5or.

Molar ellipticity (deg*cm ² /dmol)	Far-UV (minus b5)	Far-UV (minus b5)	Helix	Near-UV	Tertiary structure	Soret	Heme contact
	190 nm	220 nm		286 nm		413 nm	
N/b5	544543	-310424	Yes	-35041	Yes	-82501	Yes
N/b5- Δ 21	778363	-312167	Yes	-36453	Yes	-84650	Yes
N/b5-LMAA	764345	-396791	Yes	-34688	Yes	-69546	No

Molar ellipticity (deg*cm ² /dmol)(minus b5)	Far-UV (minus b5)	Far-UV (minus b5)	Helix	Near-UV	Tertiary structure	Soret	Heme contact
N/b5-Δ34	636442	-136573	Yes	-27727	Partial	-70516	No
N/b5-W37A	-132660	-245259	No	-346	No	-72577	No
b5	N/A	N/A	N/A	2262	No	-71620	No

Molar ellipticity values of far-UV signals (190 and 220 nm) are obtained from difference CD spectra (**Figures 2C,E,G**) and indicate secondary structure in the N-terminal domain. Its tertiary structure and impact on the heme center are suggested by molar ellipticity values of near-UV (286 nm) and Soret (413 nm) signals, respectively, from CD spectra (**Figures 2B,F,H**). All Molar ellipticity values are presented in deg*cm²/dmol. N/A: not applicable. **Table S1**. Crystallographic data for RLF.

RLF	
Data Collection	
Unit-cell parameters (Å, °)	$a=58.95, b=26.14, c=82.87, \beta=95.5$
Space group	$P2$
Resolution (Å) ¹	45.79-1.55 (1.58-1.55)
Wavelength (Å)	1.0000
Temperature (K)	100
Observed reflections	150,129
Unique reflections	36,463
$\langle I/\sigma(I) \rangle^1$	11.0 (2.0)
Completeness (%) ¹	97.6 (98.1)
Multiplicity ¹	4.1 (4.0)
R_{merge} (%) ^{1, 2}	7.1 (66.6)
R_{meas} (%) ^{1, 4}	8.1 (76.6)
R_{pim} (%) ^{1, 4}	3.9 (37.1)
$CC_{1/2}$ ^{1, 5}	0.998 (0.763)
Refinement	
Resolution (Å) ¹	41.25-1.55
Reflections (working/test) ¹	34,634/1,819
$R_{\text{factor}} / R_{\text{free}}$ (%) ^{1,3}	17.5/20.0
No. of atoms (Protein/Heme/Water)	1,686 / 86 / 164
Model Quality	
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	0.961
Mean B -factor (Å ²)	
All Atoms	23.5
Protein	22.9
Heme	20.2
Water	31.3
Coordinate error (maximum likelihood) (Å)	0.12
Ramachandran Plot	
Most favored (%)	97.6
Additionally allowed (%)	2.4

Values in parenthesis are for the highest resolution shell.

$R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measured for the i th reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflections with indices hkl .

$R_{\text{factor}} = \sum_{hkl} (|F_{\text{obs}}(hkl)| - |F_{\text{calc}}(hkl)|) / \sum_{hkl} |F_{\text{obs}}(hkl)|$; R_{free} is calculated in an identical manner using 5% of randomly selected reflections that were not included in the refinement.

$R_{\text{meas}} = \text{redundancy-independent (multiplicity-weighted)} R_{\text{merge}}^{15, 67}$ $R_{\text{pim}} = \text{precision-indicating (multiplicity-weighted)} R_{\text{merge}}^{68, 69}$

$CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data.^{70, 71}

FIGURE LEGENDS

Figure 1. (A) Schematic diagram of Ncb5or constructs. Except Ncb5or- $\Delta 34$, all are used in the current study. (B) Amino acid sequences of the N-terminal region in human Ncb5or and its point-mutation variant.

Figure 2. Far-UV (A) and near-UV/visible (B) CD spectra of Ncb5or-N/b5 and Ncb5or-b5. Far UV (C) and near-UV/visible (D) spectra of Ncb5or-N as a stand-alone peptide (alone) or when fused to Ncb5or-b5 (difference, N/b5 – b5). Difference far UV (E, G) and near-UV/visible (F, H) CD spectra of Ncb5or-N/b5 variants. Difference spectra were presented by smoothing raw data, which are shown in **Figure S3**, with the use of negative exponential program in SigmaPlot 10. Molar ellipticity is used in all CD spectra. Values of signature bands are summarized in **Table 1**.

Figure 3. (A) Comparison of the domain arrangements in Ncb5or and RLF. (B) Amino acid sequence alignment of human Ncb5or (residues 1 – 137), rice RLF (*Oryza sativa*, residues 72 – 218), and baker’s yeast IRC21 (*Saccharomyces cerevisiae*, residues 17-201). Identical and similar residues are coded in yellow and green, respectively.

Figure 4. (A) Structure of RLF (subunit A) with the secondary structure elements annotated. The heme molecule is rendered as gold cylinders. (B) RLF sequence with the secondary structure elements annotated. The red arrows indicate the His residues that coordinate the heme.

Figure 5. Superposition of rice RLF (magenta) with the Ncb5or b5-domain (PDB 3LF5, green). RMSD deviation between Ca for b5 core residues (see Figure 4) is 1.01 Å.

Figure 6. (A) Comparison of conserved, surface exposed tryptophan residue in Ncb5or (green) and RLF (magenta). Trp¹⁹⁹ in the RLF structure forms a hydrogen bond with a heme propionate group, while Trp¹¹⁴ in Ncb5or extends into solvent. This flexibility suggests a possible role in modulating electron flow. (B) Comparison of the heme orientation and the coordinating His residues. His⁸⁹/His¹¹² are colored green for 3LF5 and His¹⁷⁴/His¹⁹⁷ are colored magenta for RLF.

Figure 7. View of the RLF structure highlighting helix $\alpha 1$ and locations/interactions of residues. Corresponding Ncb5or residues are shown in parentheses. Black dotted lines indicate hydrogen bonds.

Figure 8. Residue Trp¹²⁰ in RLF, corresponding to Trp³⁷ in Ncb5or, forms a network of hydrophobic packing interactions with other conserved residues (shown with carbon atoms rendered in yellow). The hydroxyl group of Tyr¹⁷⁰ (Tyr⁸⁵ in Ncb5or) makes van der Waals contact with the heme, and the thiol group of Cys²⁰⁹ (Cys¹²⁴ in Ncb5or) makes van der Waals contact with Trp¹²⁰.

Figure S1. Sequence alignment of N-terminal region and cytochrome b5 domain of known Ncb5or proteins of a diverse background. Asterisk and “_” indicate conserved and chemically similar residues, among mammals, respectively. Non-conserved residues at key positions are indicated in red. Species (GenBank accession number) are as follows: (mammals in yellow) human (NM_016230), monkey (XP_518614.3), monkey Old World (XP_011920356.1), guinea pig (XP_005006001), mouse (NM_024195), rat (NM_133427), cow (NM_001038159), horse (XM_001499913), sheep (XP_027818354), pig (XP_013848110), dog (XM_532219),

minke whale (XM_007165020.1), ferret (XP_004773650), bat/Molossus (XP_036104100), bat/Pteropus (XP_011363184), brown bat (XM_008145048), opossum (XM1375853), platypus (XP_028903113); (birds, reptiles and amphibians in green) Zebra Finch (XP_030123394), golden eagle (XP_029856007), peregrine falcon (XP_013152060), chicken (XM_001233870), Dalmatian pelican (KFQ624870), crested ibis (KFR09350), seriema (KFP68580), swan goose (XM_013180133), turkey (XP_010705963), turtle/Terrapene (XP_026510156) turtle/Pelodiscus (XP_006133410), garter snake (XP_032072321), Xenopus (XP_031757575), Coelacanth (XP_014343350); (fishes in blue) Cyprinidae (XM_016550377), Zebrafish (NP_001018496), Pimephales (XP_039507272), Salmon (XP_020361560), bony fish (XM_008279520), yellow croaker (XM_019265374), Fugu (TNN02071), Pufferfish/Japan (XM_003965751), tire track eel (XR_003294861), swamp eel (XM_020621659), Gasterosteus (XP_040020873), rice fish (AM140533), rice fish India (XP_024117733), Lamprey (XP_032829495), Skate (XP_032877801), seahorse (XM_019880106), (invertebrates in grey) Lancelet (XM_002603916), Trichoplax (XM_002112883), tunicate (XR_053035), Hydra (XM_002165807), sea anemone (XP_048577331), coral (EZ038676, Acropora), coral/Exaiptasia (KXJ05517), sea urchin/Lytechinus (XP_041463305), sea urchin (XP1198870), Teladorsagia (PIO73582), hookworm/Necator (RCN51358), C.elegans (NM_001026613), pig worm (KFD50571c, Trichuris suis), Trichuris (KHJ45328); (insects in purple) Drosophila (NM_137575), Mosquito/Anopheles (XP_041781397), Mosquito/Aedes (KXJ83737), Honeybee (XM_394412), Louse (XM_002428283), Jewel wasp (XM_001601866), Beetle (XM_963135), Pea aphid (XM_001948299), Deer tick (XM_002401084),

Figure S2. Prediction of disordered domains in Ncb5or by PONDR VL-XT (A). Prediction of secondary structure in the 50 N-terminal residues of Ncb5or by PSIPRED and PROF (B) and by AGADIR (C). AA (amino acid): Target sequence, Pred: Predicted secondary structure (H=helix, E=strand, C=coil), Conf: Confidence (0=low, 9=high).

Figure S3. Sequence alignment of the N-terminal region and b5 core (partial) of human Ncb5or against RLF proteins in plants. Asterisk (*) indicates conserved residues, whereas “_” represents chemically similar residues.

Figure S4 . Sequence alignment of the N-terminal region of human Ncb5or against IRC21 proteins in fungi. Asterisk (*) indicates conserved residues, whereas “_” represents chemically similar residues.

Figure S5 . Sequence alignment of the N-terminal region and cytochrome b5 domain of human Ncb5or against cytochrome b5 proteins in protists (*Dictyostelium* and *Trypanosoma*) and parasites (all others). Asterisk (*) indicates conserved residues, whereas “;” represents chemically similar residues. The conserved “HPGG” heme binding site is highlighted, and the N-terminal motif in human and protists is indicated by “_____”.

REFERENCES

- [1] Xie, J., Zhu, H., Larade, K., Ladoux, A., Seguritan, A., Chu, M., Ito, S., Bronson, R. T., Leiter, E. H., Zhang, C. Y., Rosen, E. D., and Bunn, H. F. (2004) Absence of a reductase, NCB5OR, causes insulin-deficient diabetes, *Proc Natl Acad Sci U S A* 101 , 10750-10755.
- [2] Zhu, H., Larade, K., Jackson, T. A., Xie, J., Ladoux, A., Acker, H., Berchner-Pfannschmidt, U., Fandrey, J., Cross, A. R., Lukat-Rodgers, G. S., Rodgers, K. R., and Bunn, H. F. (2004) NCB5OR is a novel soluble NAD(P)H reductase localized in the endoplasmic reticulum, *J Biol Chem* 279 , 30316-30325.
- [3] Wang, W. F., Guo, Y., Xu, M., Huang, H.-H., Novikova, L., Larade, K., Jiang, Z.-G., Thayer, T. C., Frontera, J. R., Aires, D., Ding, H., Turk, J., Mathews, C. E., Bunn, H. F., Stehno-Bittel, L., and Zhu, H. (2011) Development of diabetes in Ncb5or-null mice is associated with manifestations of endoplasmic reticulum and oxidative stress in beta cells, *Biochimica et Biophysica Acta* 1812 , 1532-1541.
- [4] Stroh, M. A., Winter, M. K., Swerdlow, R. H., McCarson, K. E., and Zhu, H. (2016) Loss of NCB5OR in the cerebellum disturbs iron pathways, potentiates behavioral abnormalities, and exacerbates harmaline-induced tremor in mice, *Metabolic brain disease* 31 , 951-964.

- [5] Stroh, M. A., Winter, M. K., McCarson, K. E., Thyfault, J. P., and Zhu, H. (2018) NCB5OR Deficiency in the Cerebellum and Midbrain Leads to Dehydration and Alterations in Thirst Response, Fasted Feeding Behavior, and Voluntary Exercise in Mice, *Cerebellum* 17 , 152-164.
- [6] Zhu, H., Qiu, H., Yoon, H. W., Huang, S., and Bunn, H. F. (1999) Identification of a cytochrome b-type NAD(P)H oxidoreductase ubiquitously expressed in human cells, *Proc Natl Acad Sci U S A* 96 , 14742-14747.
- [7] Garcia-Ranea, J. A., Mirey, G., Camonis, J., and Valencia, A. (2002) p23 and HSP20/alpha-crystallin proteins define a conserved sequence domain present in other eukaryotic protein families, *FEBS Lett* 529 , 162-167.
- [8] Deng, B., Parthasarathy, S., Wang, W., Gibney, B. R., Battaile, K. P., Lovell, S. W., Benson, D. R., and Zhu, H. (2010) Study of the individual cytochrome b5 and cytochrome b5 reductase domains of Ncb5or reveals a unique heme pocket and a possible role of the CS domain, *J Biol Chem* 285 , 30181-30191.
- [9] Benson, D. R., Lovell, S., Mehzabeen, N., Galeva, N., Cooper, A., Gao, P., Battaile, K. P., and Zhu, H. (2019) Crystal structures of the naturally fused CS and cytochrome b5 reductase (b5R) domains of Ncb5or reveal an expanded CS fold, extensive CS-b5R interactions and productive binding of the NAD(P)(+) nicotinamide ring, *Acta Crystallogr D Struct Biol* 75 , 628-638.
- [10] Prasannan, C. B., Tang, Q., and Fenton, A. W. (2012) Allosteric regulation of human liver pyruvate kinase by peptides that mimic the phosphorylated/dephosphorylated N-terminus, *Methods Mol Biol* 796 , 335-349.
- [11] Reverter, D., and Lima, C. D. (2009) Preparation of SUMO proteases and kinetic analysis using endogenous substrates, *Methods Mol Biol* 497 , 225-239.
- [12] Kabsch, W. (1988) Automatic indexing of rotation diffraction patterns, *J Applied Crystallography* 21 , 67-72.
- [13] Kabsch, W. (2010) Xds, *Acta Crystallogr D Biol Crystallogr* 66 , 125-132.
- [14] Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Paciorek, W., Womack, T., and Bricogne, G. (2011) Data processing and analysis with the autoPROC toolbox, *Acta Crystallogr D Biol Crystallogr* 67 , 293-302.
- [15] Evans, P. R. (2011) An introduction to data reduction: space-group determination, scaling and intensity statistics, *Acta Crystallogr D Biol Crystallogr* 67 , 282-292.
- [16] McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software, *J. Appl. Cryst.* 40 , 658-674.
- [17] Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method, *Acta Crystallogr D Biol Crystallogr* 53 , 240-255.
- [18] Langer, G., Cohen, S. X., Lamzin, V. S., and Perrakis, A. (2008) Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7, *Nat Protoc* 3 , 1171-1179.
- [19] Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H., and Adams, P. D. (2012) Towards automated crystallographic structure refinement with phenix.refine, *Acta Crystallogr D Biol Crystallogr* 68 , 352-367.
- [20] Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: building new software for automated crystallographic structure determination, *Acta Crystallogr D Biol Crystallogr* 58 , 1948-1954.
- [21] Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot, *Acta Crystallogr D Biol Crystallogr* 66 , 486-501.

- [22] Painter, J., and Merritt, E. A. (2006) Optimal description of a protein structure in terms of multiple groups undergoing TLS motion, *Acta Crystallogr D Biol Crystallogr* *62* , 439-450.
- [23] M.D. Winn, M.N. Isupov, and Murshudov, G. N. (2001) Use of TLS Parameters to Model Anisotropic Displacements in Macromolecular Refinement, *Acta Crystallogr D Biol Crystallography* *57* , 122-133.
- [24] Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography, *Acta Crystallogr D Biol Crystallogr* *66* , 12-21.
- [25] Potterton, L., McNicholas, S., Krissinel, E., Gruber, J., Cowtan, K., Emsley, P., Murshudov, G. N., Cohen, S., Perrakis, A., and Noble, M. (2004) Developments in the CCP4 molecular-graphics project, *Acta Crystallogr D Biol Crystallogr* *60* , 2288-2294.
- [26] Liebschner, D., Afonine, P. V., Moriarty, N. W., Poon, B. K., Sobolev, O. V., Terwilliger, T. C., and Adams, P. D. (2017) Polder maps: improving OMIT maps by excluding bulk solvent, *Acta Crystallogr D Struct Biol* *73* , 148-157.
- [27] Krissinel, E. (2012) Enhanced fold recognition using efficient short fragment clustering, *J Mol Biochem* *1* , 76-85.
- [28] Li, X., Romero, P., Rani, M., Dunker, A. K., and Obradovic, Z. (1999) Predicting Protein Disorder for N-, C-, and Internal Regions, *Genome Inform Ser Workshop Genome Inform* *10* , 30-40.
- [29] Romero, Obradovic, and Dunker, K. (1997) Sequence Data Analysis for Long Disordered Regions Prediction in the Calcineurin Family, *Genome Inform Ser Workshop Genome Inform* *8* , 110-124.
- [30] Romero, P., Obradovic, Z., Li, X., Garner, E. C., Brown, C. J., and Dunker, A. K. (2001) Sequence complexity of disordered protein, *Proteins* *42* , 38-48.
- [31] Rost, B., and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy, *J Mol Biol* *232* , 584-599.
- [32] Jones, D. T. (1999) Protein secondary structure prediction based on position-specific scoring matrices, *J Mol Biol* *292* , 195-202.
- [33] Buchan, D. W., Ward, S. M., Lobley, A. E., Nugent, T. C., Bryson, K., and Jones, D. T. (2010) Protein annotation and modelling servers at University College London, *Nucleic Acids Res* *38* , W563-568.
- [34] Faraggi, E., Yang, Y., Zhang, S., and Zhou, Y. (2009) Predicting continuous local structure and the effect of its substitution for secondary structure in fragment-free protein structure prediction, *Structure* *17* , 1515-1527.
- [35] Zhang, H., Zhang, T., Chen, K., Kedariseti, K. D., Mizianty, M. J., Bao, Q., Stach, W., and Kurgan, L. (2011) Critical assessment of high-throughput standalone methods for secondary structure prediction, *Brief Bioinform* *12* , 672-688.
- [36] Munoz, V., and Serrano, L. (1994) Elucidating the folding problem of helical peptides using empirical parameters, *Nature structural biology* *1* , 399-409.
- [37] Greenfield, N., and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation, *Biochemistry* *8* , 4108-4116.
- [38] Kelly, S. M., Jess, T. J., and Price, N. C. (2005) How to study proteins by circular dichroism, *Biochim Biophys Acta* *1751* , 119-139.
- [39] Woody, R. W. (1978) Aromatic side-chain contributions to the far ultraviolet circular dichroism of peptides and proteins, *Biopolymers* *17* , 1451-1467.

- [40] Freskgard, P. O., Martensson, L. G., Jonasson, P., Jonsson, B. H., and Carlsson, U. (1994) Assignment of the contribution of the tryptophan residues to the circular dichroism spectrum of human carbonic anhydrase II, *Biochemistry* *33* , 14281-14288.
- [41] Pace, C. N., and Scholtz, J. M. (1998) A helix propensity scale based on experimental studies of peptides and proteins, *Biophys J* *75* , 422-427.
- [42] Ikeyama, Y., Tasaka, M., and Fukaki, H. (2010) RLF, a cytochrome b(5)-like heme/steroid binding domain protein, controls lateral root formation independently of ARF7/19-mediated auxin signaling in *Arabidopsis thaliana*, *Plant J* *62* , 865-875.
- [43] Alvaro, D., Lisby, M., and Rothstein, R. (2007) Genome-wide analysis of Rad52 foci reveals diverse mechanisms impacting recombination, *PLoS genetics* *3* , e228.
- [44] Lee, W., St Onge, R. P., Proctor, M., Flaherty, P., Jordan, M. I., Arkin, A. P., Davis, R. W., Nislow, C., and Giaever, G. (2005) Genome-wide requirements for resistance to functionally distinct DNA-damaging agents, *PLoS genetics* *1* , e24.
- [45] Guenole, A., Srivas, R., Vreeken, K., Wang, Z. Z., Wang, S., Krogan, N. J., Ideker, T., and van Attikum, H. (2013) Dissection of DNA damage responses using multiconditional genetic interaction maps, *Mol Cell* *49* , 346-358.
- [46] Gallego, O., Betts, M. J., Gvozdenovic-Jeremic, J., Maeda, K., Matetzki, C., Aguilar-Gurrieri, C., Beltran-Alvarez, P., Bonn, S., Fernandez-Tornero, C., Jensen, L. J., Kuhn, M., Trott, J., Rybin, V., Muller, C. W., Bork, P., Kaksonen, M., Russell, R. B., and Gavin, A. C. (2010) A systematic screen for protein-lipid interactions in *Saccharomyces cerevisiae*, *Mol Syst Biol* *6* , 430.
- [47] Ferrari, E., Bruhn, C., Peretti, M., Cassani, C., Carotenuto, W. V., Elgendy, M., Shubassi, G., Lucca, C., Bermejo, R., Varasi, M., Minucci, S., Longhese, M. P., and Foiani, M. (2017) PP2A Controls Genome Integrity by Integrating Nutrient-Sensing and Metabolic Pathways with the DNA Damage Response, *Mol Cell* *67* , 266-281 e264.
- [48] Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L., and Hedges, S. B. (2001) Molecular evidence for the early colonization of land by fungi and plants, *Science* *293* , 1129-1133.
- [49] C.F. Macrae, I. J. Bruno, J. A. Chisholm, P. R. Edgington, P. McCabe, E. Pidcock, L. Rodriguez-Monge, R. Taylor, J. van de Streek, and Wood, P. A. (2008) Mercury CSD 2.0 - new features for the visualization and investigation of crystal structures, *Journal of Applied Crystallography* *41* , 466-470.
- [50] Pyrih, J., Harant, K., Martincova, E., Sutak, R., Lesuisse, E., Hrdy, I., and Tachezy, J. (2014) *Giardia intestinalis* Incorporates Heme into Cytosolic Cytochrome b(5), *Eukaryotic Cell* *13* , 231-239.
- [51] Pazdzior, R., Yang, Z. A., Mesbahuddin, M. S., Yee, J., van der Est, A., and Rafferty, S. (2015) Low reduction potential cytochrome b5 isotypes of *Giardia intestinalis*, *Exp Parasitol* *157* , 197-201.
- [52] Samanta, U., Pal, D., and Chakrabarti, P. (2000) Environment of tryptophan side chains in proteins, *Proteins* *38* , 288-300.
- [53] Pal, D., and Chakrabarti, P. (1998) Different types of interactions involving cysteine sulfhydryl group in proteins, *J Biomol Struct Dyn* *15* , 1059-1072.
- [54] Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) Intrinsic disorder and protein function, *Biochemistry* *41* , 6573-6582.
- [55] Dyson, H. J., and Wright, P. E. (2005) Intrinsically unstructured proteins and their functions, *Nat Rev Mol Cell Biol* *6* , 197-208.
- [56] Sugase, K., Dyson, H. J., and Wright, P. E. (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein, *Nature* *447* , 1021-1025.

- [57] Liu, Y., Matthews, K. S., and Bondos, S. E. (2008) Multiple intrinsically disordered sequences alter DNA binding by the homeodomain of the *Drosophila* hox protein ultrabithorax, *J Biol Chem* *283* , 20874-20887.
- [58] Wang, X., Bajaj, R., Bollen, M., Peti, W., and Page, R. (2016) Expanding the PP2A Interactome by Defining a B56-Specific SLiM, *Structure* *24* , 2174-2181.
- [59] Singh, J. P., Li, Y., Chen, Y. Y., Hsu, S. D., Page, R., Peti, W., and Meng, T. C. (2022) The catalytic activity of TCPTP is auto-regulated by its intrinsically disordered tail and activated by Integrin alpha-1, *Nature communications* *13* , 94.
- [60] Joerger, A. C., and Fersht, A. R. (2008) Structural biology of the tumor suppressor p53, *Annu Rev Biochem* *77* , 557-582.
- [61] Breydo, L., Wu, J. W., and Uversky, V. N. (2012) alpha-Synuclein misfolding and Parkinson's disease, *Biochim Biophys Acta* *1822* , 261-285.
- [62] Uversky, V. N., Oldfield, C. J., and Dunker, A. K. (2008) Intrinsically disordered proteins in human diseases: introducing the D2 concept, *Annu Rev Biophys* *37* , 215-246.
- [63] Cheng, Y., LeGall, T., Oldfield, C. J., Dunker, A. K., and Uversky, V. N. (2006) Abundance of intrinsic disorder in protein associated with cardiovascular disease, *Biochemistry* *45* , 10448-10460.
- [64] Johansson, B. B., Torsvik, J., Bjorkhaug, L., Vesterhus, M., Ragvin, A., Tjora, E., Fjeld, K., Hoem, D., Johansson, S., Raeder, H., Lindquist, S., Hernell, O., Cnop, M., Saraste, J., Flatmark, T., Molven, A., and Njolstad, P. R. (2011) Diabetes and pancreatic exocrine dysfunction due to mutations in the carboxyl ester lipase gene-maturity onset diabetes of the young (CEL-MODY): a protein misfolding disease, *J Biol Chem* *286* , 34593-34605.
- [65] Woese, C. R. (2000) Interpreting the universal phylogenetic tree, *Proc Natl Acad Sci U S A* *97* , 8392-8396.
- [66] Barik, S. (2020) The Uniqueness of Tryptophan in Biology: Properties, Metabolism, Interactions and Localization in Proteins, *Int J Mol Sci* *21* .
- [67] Evans, P. (2006) Scaling and assessment of data quality, *Acta Crystallogr D Biol Crystallogr* *62* , 72-82.
- [68] Diederichs, K., and Karplus, P. A. (1997) Improved R-factors for diffraction data analysis in macromolecular crystallography, *Nature structural biology* *4* , 269-275.
- [69] Weiss, M. S. (2001) Global indicators of X-ray data quality, *Journal of Applied Crystallography* *34* .
- [70] Karplus, P. A., and Diederichs, K. (2012) Linking crystallographic model and data quality, *Science* *336* , 1030-1033.
- [71] Evans, P. (2012) Biochemistry. Resolving some old problems in protein crystallography, *Science* *336* , 986-987.

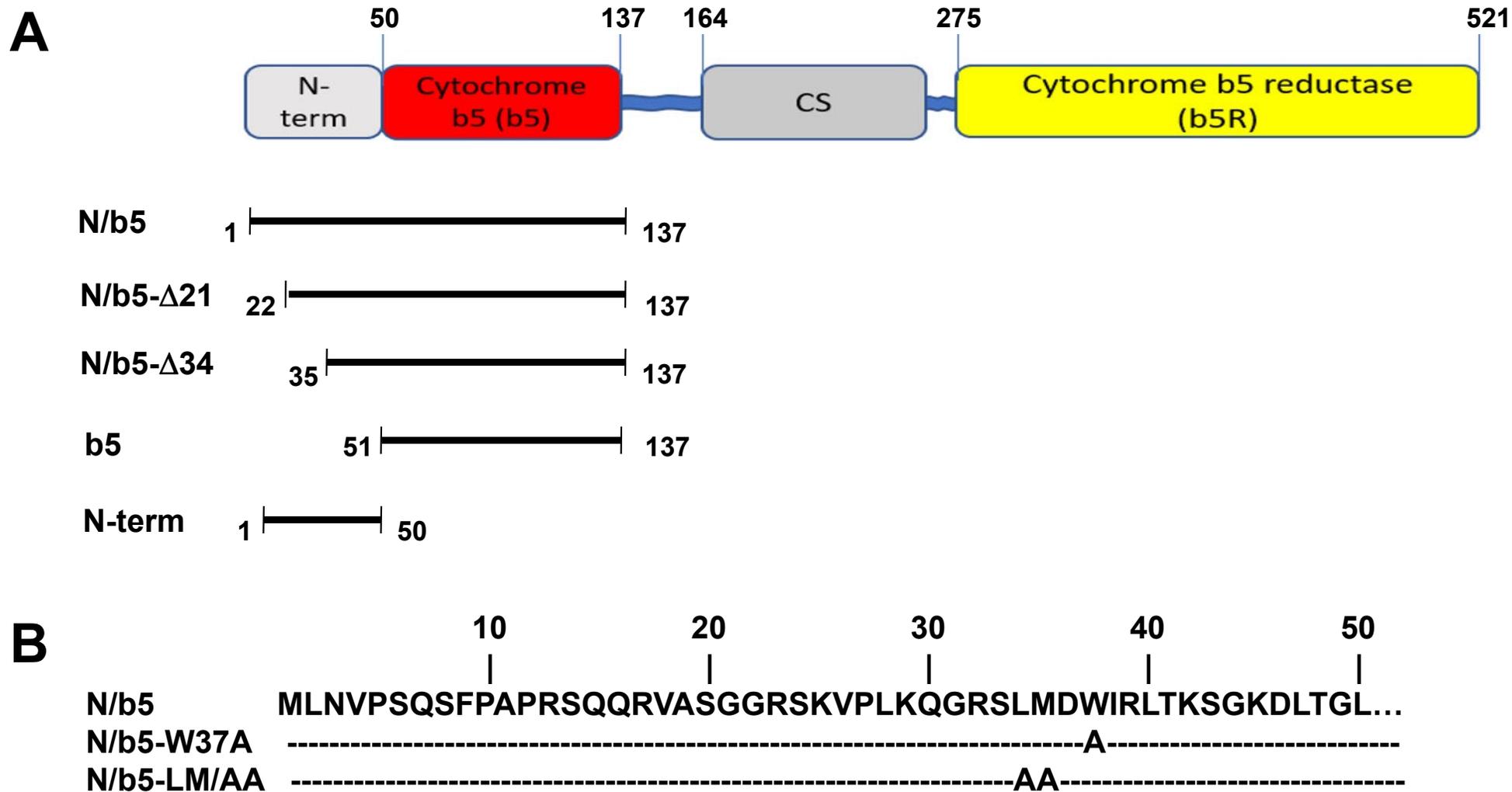


Figure 1

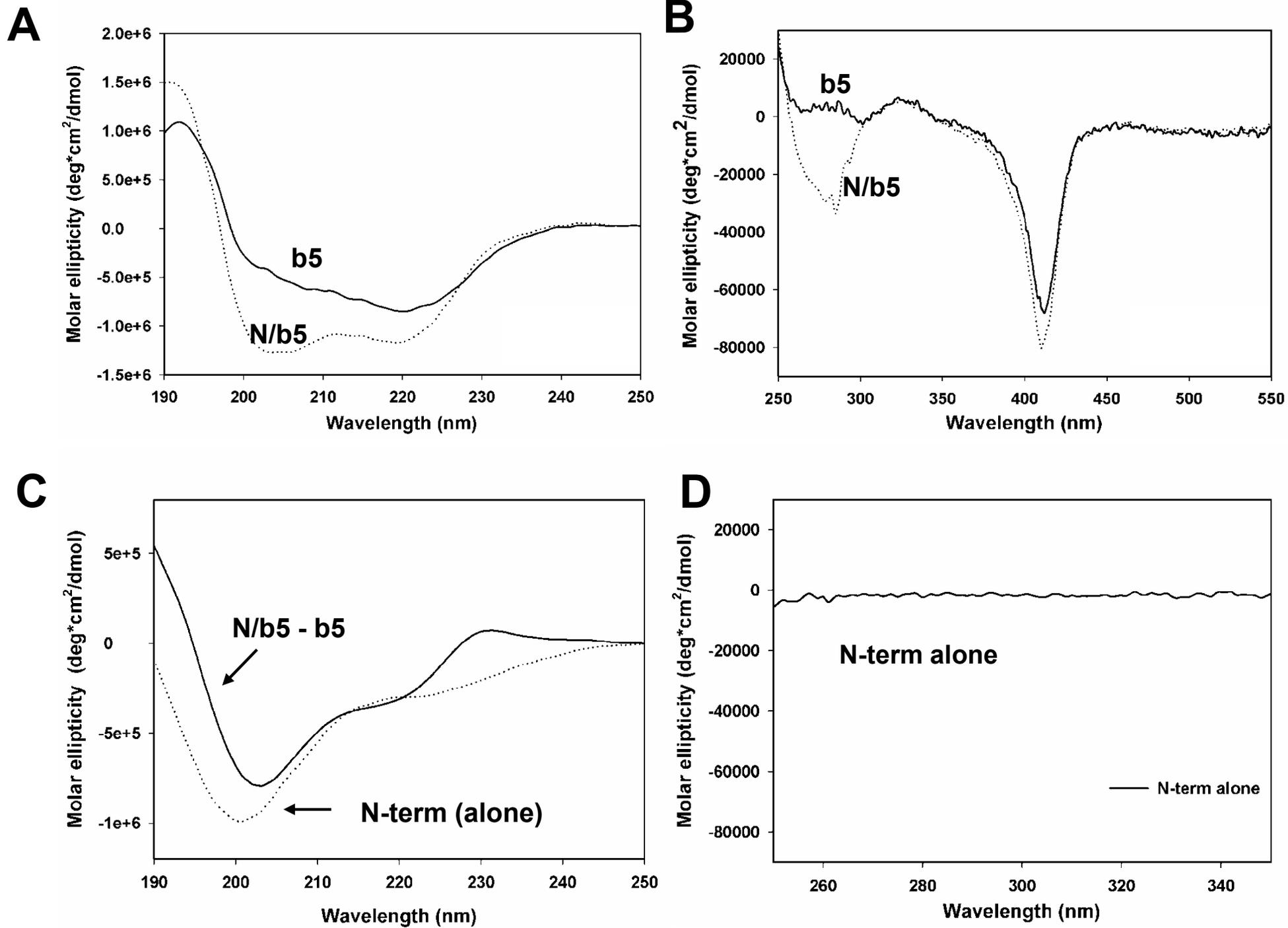


Figure 2

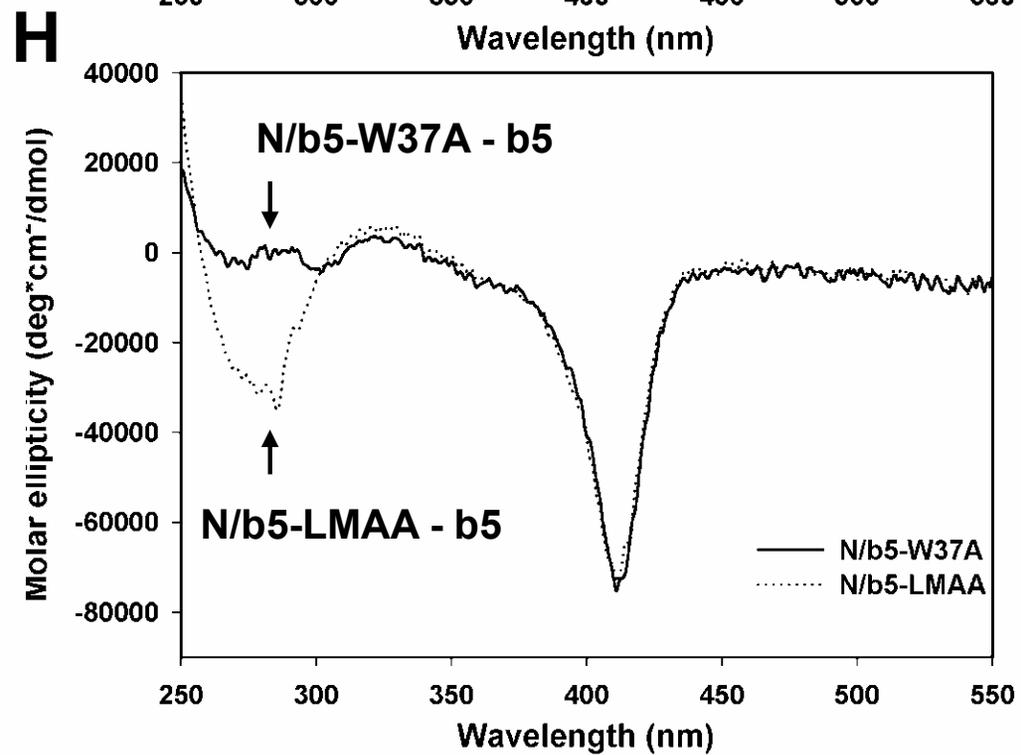
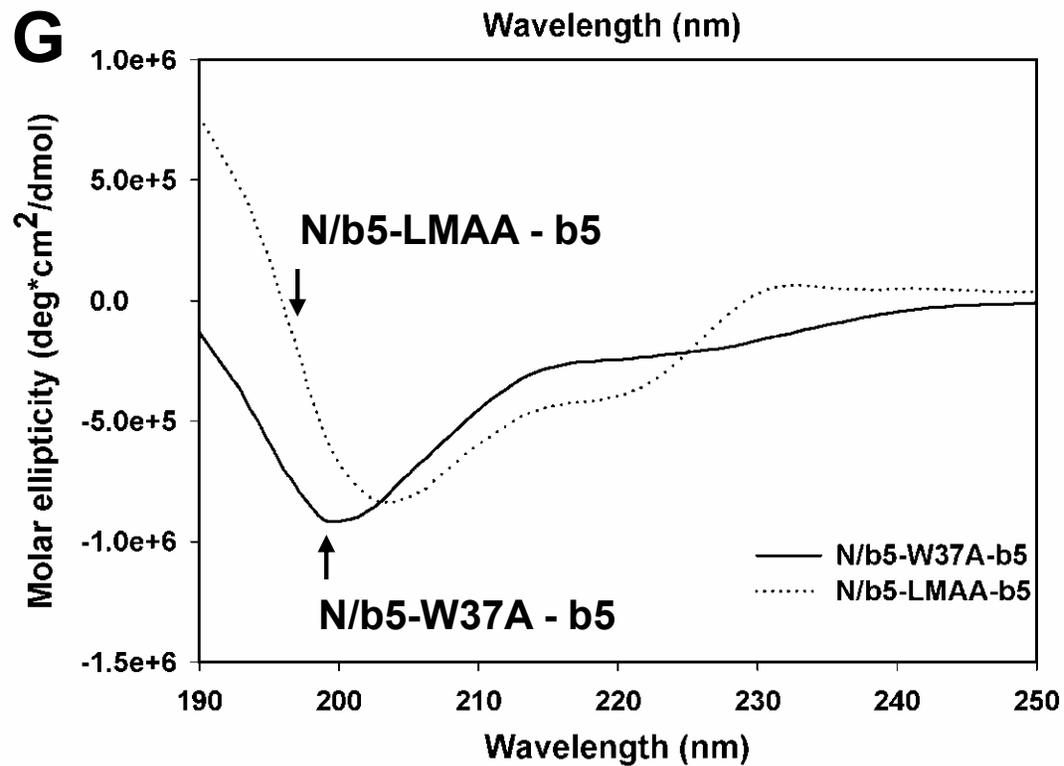
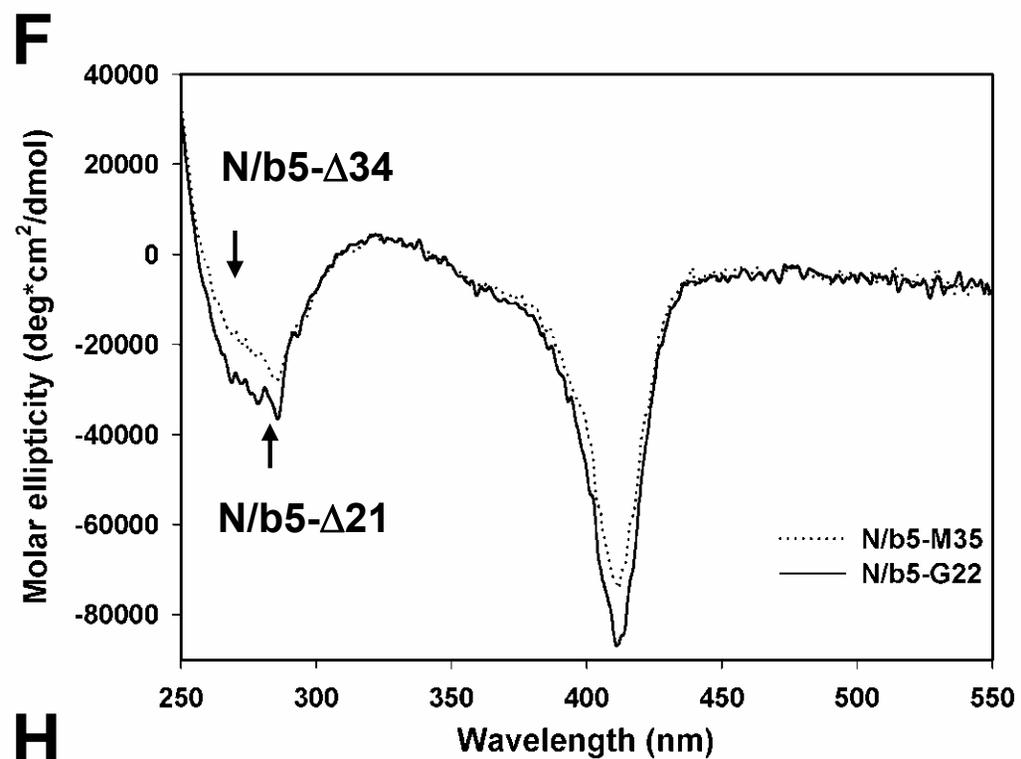
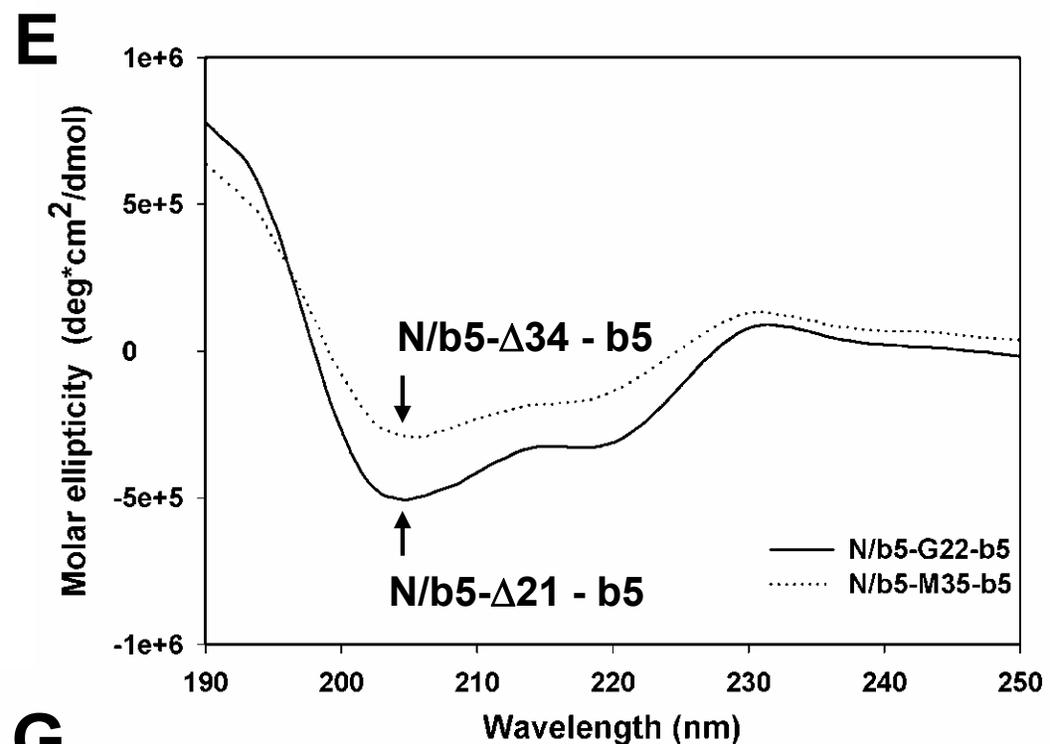


Figure 2 (cont'd)

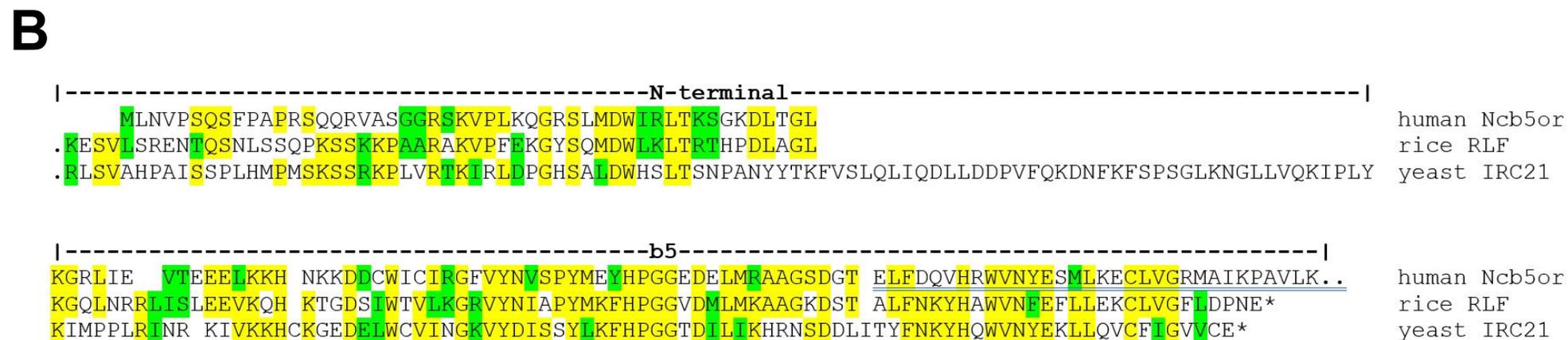
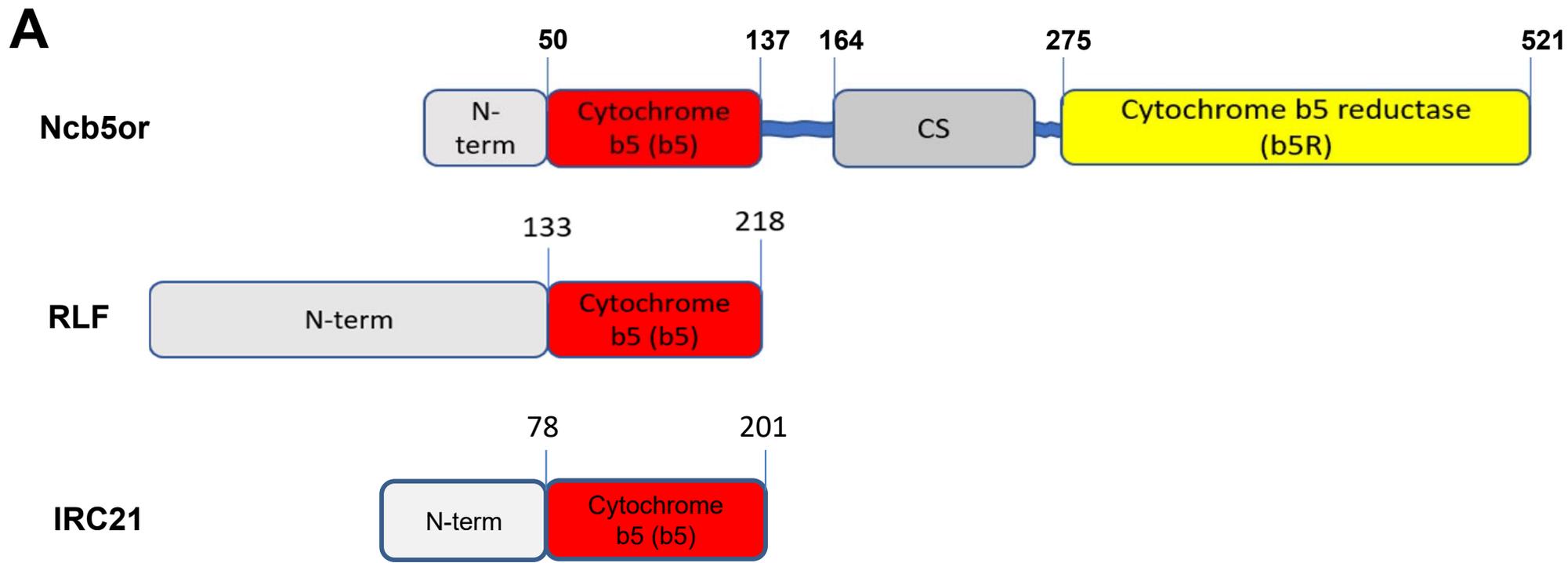


Figure 3

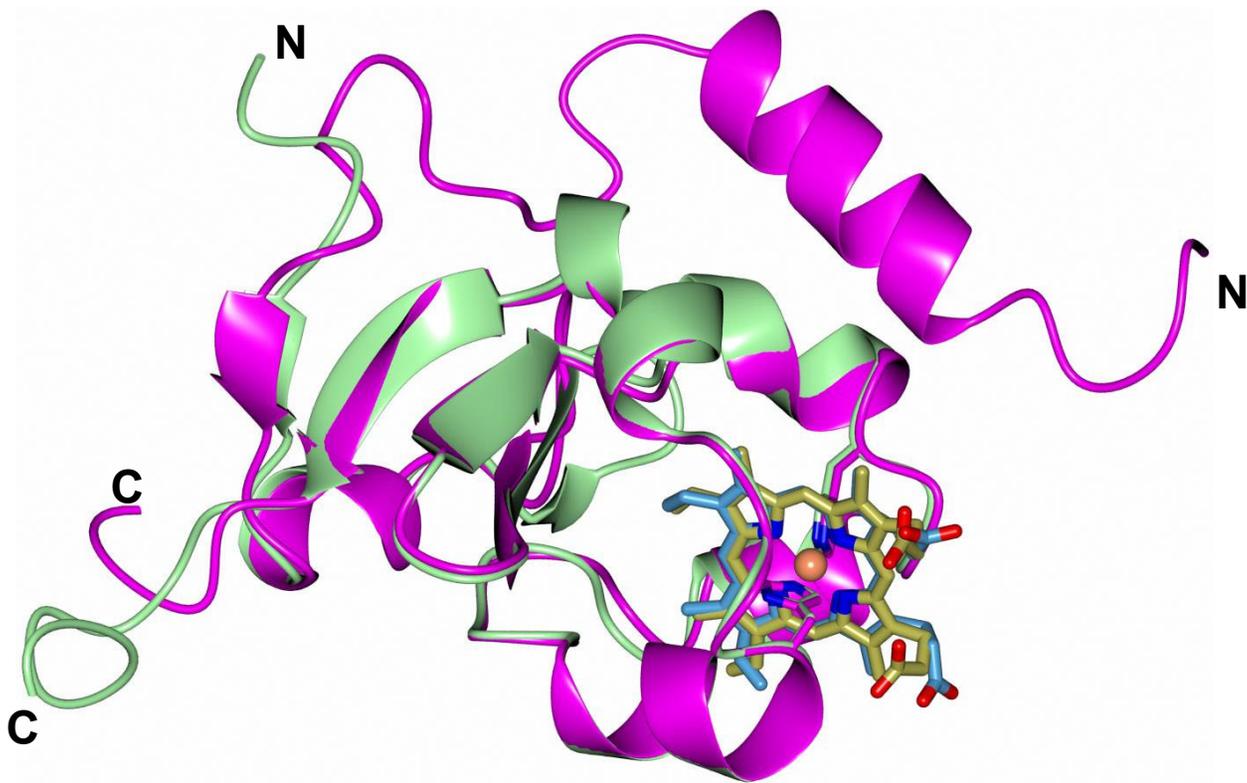


Figure 5

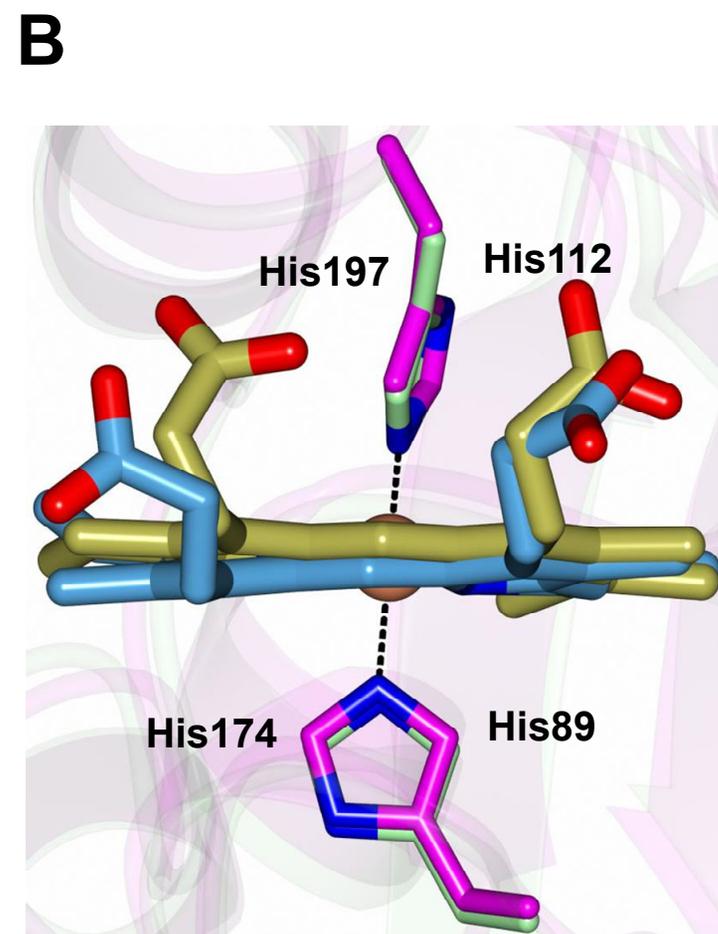
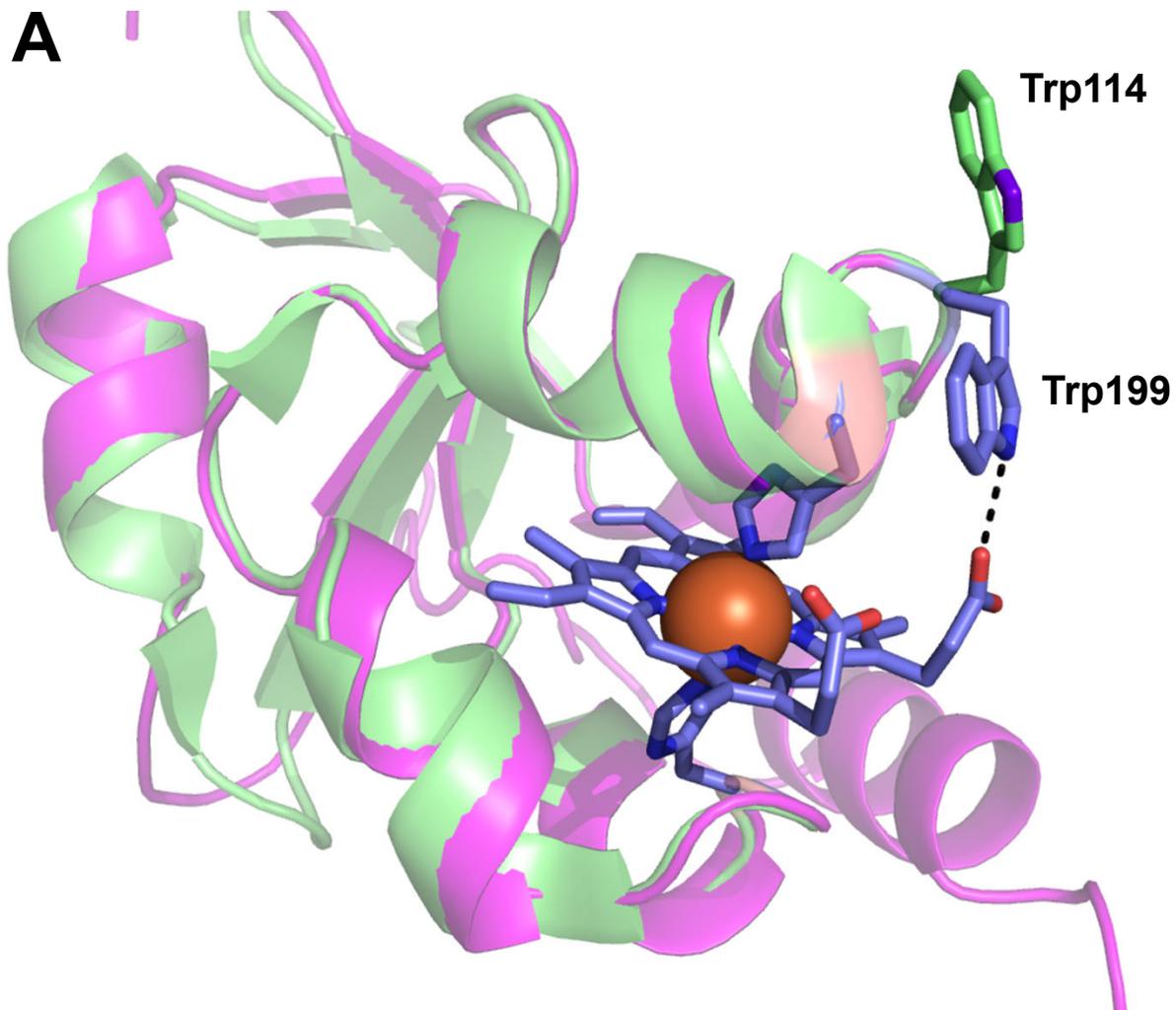


Figure 6

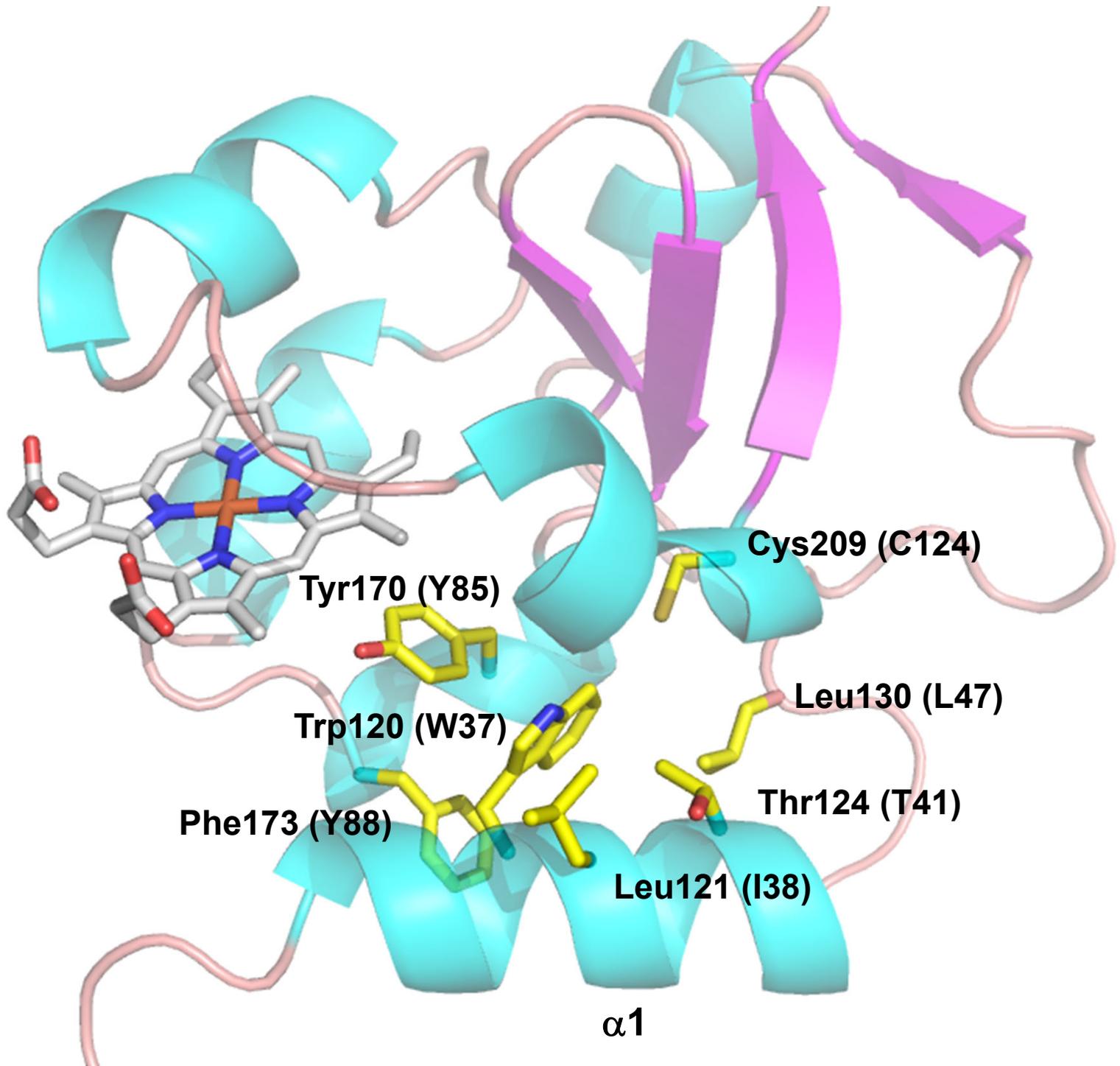


Figure 7

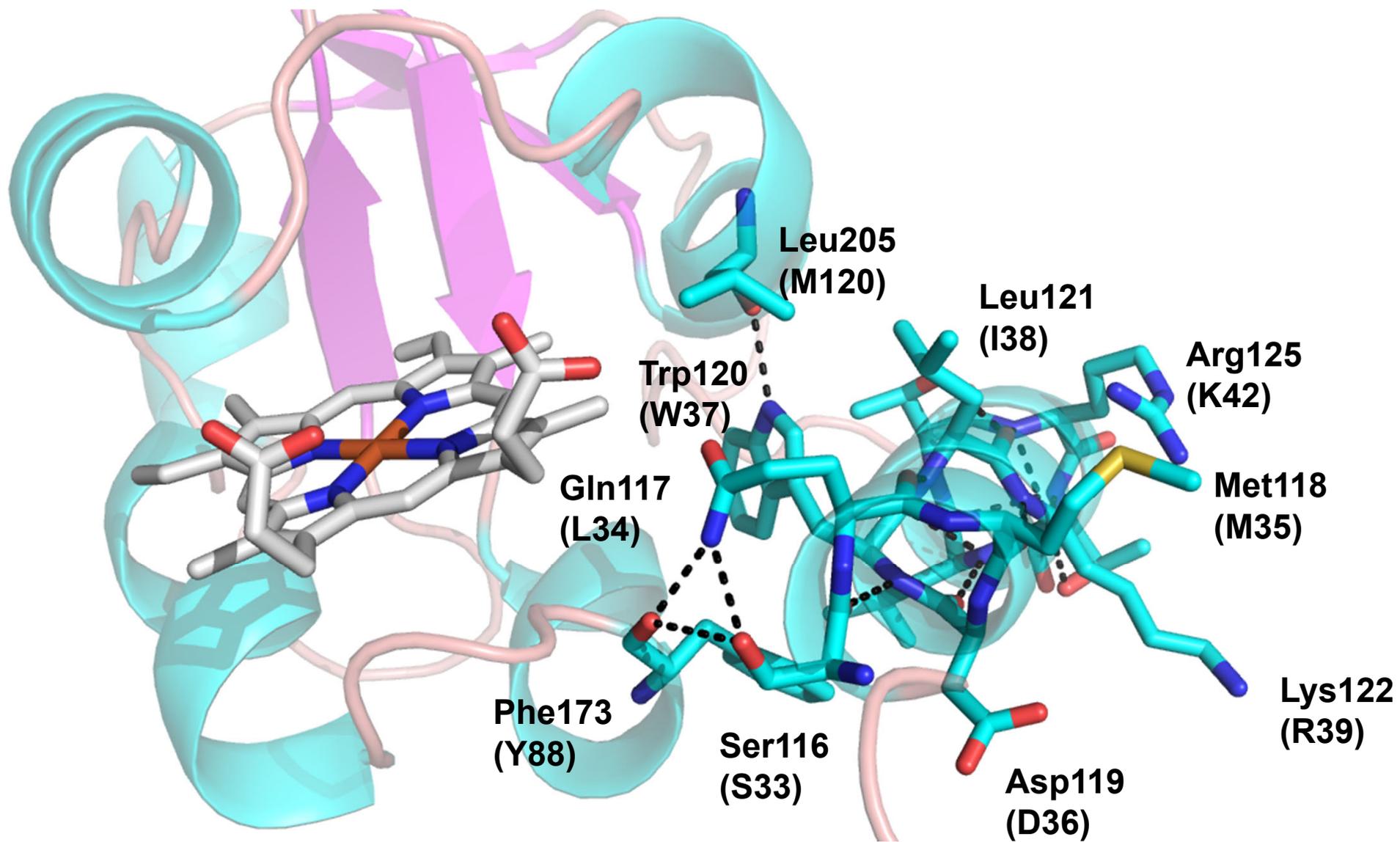
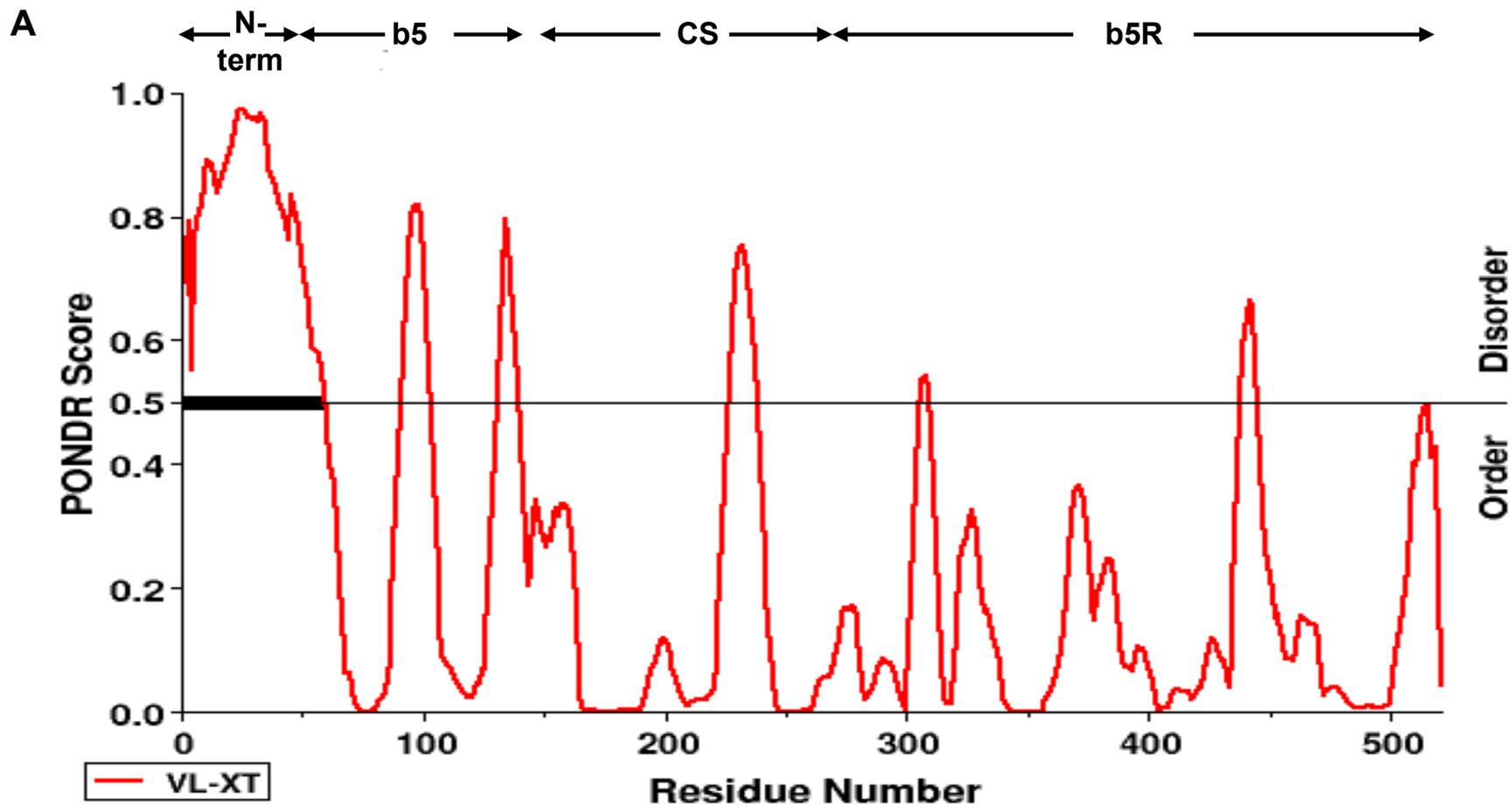


Figure 8

Sequence alignment of N-terminal region and cytochrome b5 domain of known Ncb5or proteins.

----- N-terminal-----	W37	----- ---b5---		
- * * *- * *- * *-	-** * *- * *- * *-	* *- * *- * *-		
MLNVPSQSFPAPRSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	human
MLNVPSQSFPAPRSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	monkey
MLNVPSQSFPAPRSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	monkey Old World
MLNVPSQSFPAPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	guinea pig
MLNVPSQAFPAPGSQQRVSSQ	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGGLIEVT	mouse
MLNVPSQAFPAPGSQQRVASQ	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGGLIEVT	rat
MLNVPSQSFPGPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	cow
MLNVPSQSFPAPSSQQRVAVAG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	horse
MLNVPSQSFPGPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	sheep
MLNVPSQSFPGPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	pig
MLNVPSQSFPAPSSQQRVAASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	dog
MLNVPSQSFPGPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	minke whale
MLNVPSQSFPAPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	ferret
MLNVPSQSFPAPSSQQRVASG	GRSKVPLKQGRSLMDWIRLSK	SGKDLTGL	KGR LIDVT	squirrel
MLNVPSQSFPAPSSQQRVSSSE	GRSKVPLKQGRSLMDWIRMTK	SGKDLTGL	KGR LIEVT	bat (Molossus)
MLNVPSQSFPAPSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	bat (Pteropus)
MLNIPQSFPASSSQQRVASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	RGR LIEVT	brown bat
MLNVPSQAFPAPGSQQRVAASG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	opossum
MLNVPAQAFPAAGSQQRVAAPP	ARTKVPLKQGRSLMDWIRLTK	SGKDLTGL	RGR LIEVT	platypus
MLNVPAQAFPAAGSQQRVAAPP	ARSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	Zebra Finch
MLNVPAQAFPAAGSQQRVAAG	GRSKVPLKQGRSLMDWIRLTK	SGRDLTGL	KGR LIEVT	golden eagle
MLNVPAQAFPAAGSQQRVAAG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	peregrine falcon
MLNVPAQAFPAAGSQQRVAAG	GRTKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	chicken
	..VPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	Dalmatian pelican
	..VPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	crested ibis
	..VPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	seriema
	..MDWIRLTK	SGKDLTGL	KGR LIEVT	swan goose
	..MDWIRLTK	SGKDLTGL	KGR LIEVT	turkey
MLNVPAQAFPAAGSQQRVAAG	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIDVT	turtle (Terrapene)
	MGEQTGVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	turtle (Pelodiscus)
MLNVPSQSFPAPDSQQRVALA	GRSKVPLKQGRSLMDWIRLTK	SGQDLTGL	KGR LIEVT	garter snake
MLNVPSQSFPAPSSQQRVAAI	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIDVT	Xenopus
MLN FPAPKSQQRVSSSS	GRSKVPLKQGRSLMDWIRLTK	SGKDLTGL	KGR LIEVT	Coelacanth
MLNVPSQSFPAPAGSQQRVAPT	GQ RNKVALKPGHSLLDWIRLTK	SGRDLTGL	RGR LIEVT	Cyprinidae
MLNVPSQAFPAAGSQQRVAPA	GQSRNKVVLKPGHSLLDWIRLTK	SGQDLTGL	RGR LIEVT	Zebrafish
MLNVPSQSFPAPAGSQQRVSPA	GQSRNKVVLKPGHSLLDWIRLTK	SGRDLTGL	RGR LIEVT	Pimephales
MLNVPSQSFPAPASSQQRVAPA	GQSGRNKVALKPGHSLMDWIRVAK	SGRDLTGL	RGR LIDVT	salmon
MLNIPTQSFPAPSSQQRVAPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	bony fish
MLNVPTQSFPAPGSQQRVAPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	yellow croaker
MLNIPTQSFPAPSSQQRVSPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	Fugu
MLNIPTQSFPAPSSQQRVSPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	pufferfish.Japan
MLNIPTQSFPAPSSQQRVAPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	tire track eel
MLNIPTQSFPAPSSQQRVAPS	GQSGRNKVALKPGHSLMDWIRFSK	SGKDLTGL	RGR LIEVT	swamp eel
MLNIPSPSFPAPARSQQRVAAS	GQSGRNKVALKPGHSLMDWIRFSK	SATDLTGL	RGR LIEVT	Gasterosteus
MLNVPAQSFPAPAGSQQRVASS	GAGRNKVVLPKPGHSLMDWIRFAK	SGKDLTGL	RGR LIDVT	Rice Fish
MLNVPAQSFPAPAGSQQRVASS	GAGRNKVVLPKPGHSLMDWIRFAK	SGKDLTGL	RGR LIEVT	Rice Fish India
MLNVPAQSFPAPAGSQQRVASS	GAGRNKVVLPKPGHSLMDWIRFAK	SGKDLTGL	RGR LIEVT	Rice Fish India
..FPAPASQQRVSESGLA	GTPRNKVSLKPGHSLMDWIRLAK	SGRDLTGL	RGR LIEVT	Lamprey
MDGPSQ FPAVGSQQRVAAPRTR	GGWTRKMLLKPGRSLMDWIRLIK	SGKDLTGL	RGR LIEVT	Skate
MLNIPTQSFPAPSSQQRV	GQSGRTKVALKPGHSLMDWIRYSK	SGKDLTGL	RGR LIEVT	seahorse
MSQNLGVPQFPALASPQRAGGGK	PRNKVPLKQGRSLMDWIRLSK	SKNLSGT	GGKLLNVT	lancelet
MSNPKGQQLFPANSPQRSSAGS	TANGGRKVVILKGYGLLDWITLGT	KHPDLGT	GGIIRKIT	Trichoplax (metazoa)
MSELFPAPANSQRLGVPAPV	LNTTKVPLKQGRSLMDWIRLSK	SNVDLRGT	GPRPLKIS	tunicate
MGEALFPSPSSSQRLL	VRNKVPLKQGRSLMDWIRFVN	ENKVAGV	EGRNGSIT	Hydra
MLSPKINFPANSPQRQGG	SSNSVRAKVALKPGHSLMDWIRLGA	SGKDLTGV	NGQHRPVT	sea anemone
MEAPKLSFPANSPQRQGG	SSNSVRAKVALKPGHSLMDWIRLGA	SGKDLTGV	GGIRRPVT	coral (Exaiptasia)
	..VPLKQGRSLMDWIRLTK	SARDLAGN	GGIVRPIT..	coral
MMNPKMSLSPQQQFPAMNSPQRAGSSS	LSPVGGSSSRIKVALKPGHSLMDWIRLTK	GGQRRNGV	QQQRMEVT	sea urchin (Lytechinus)
	MNSPQRAGSSSLSVPSGSSSRIKVALKPGHSLMDWIRLTK	GGQRRNGV	QQQRMEVT	sea urchin
MPLLNLPPLTAYFYFTSLNWFQKPP	IGRSEYGRVKVALLPGKGLMDWIRLTK	SGKDLTGL	KRMSVD	Teladorsagia
	MGSNDSKPAVGRSEYGRVKVALLPGKGLMDWIRLTK	SGKDLTGL	KRMSVD	hookworm (Necator)
MNLSVNGSSNGLFAKPTIGRSEYGRVKVALLPGKGLMDWIRLTK	TN KHLAK	RVSGGVD		C.elegans
	..MDCWKEFVSNLEDPST	GGKLLSVT		pig worm (Trichuris suis)
	..SLTDPST	GGKLLSVT		Trichuris
..APAGSGGLQLPVTAPQKLNSSASGSATGNPRNKCALKPGYSLMNWIRLTK	SGADLSGT	GGRLVPS		Drosophila
..NKEVKHVTTPMRSPVNSLPPKQSSGSATGNPRNKALKPGHSLMDWIRLTK	SGTDLTGT	GGRIVPS		mosquito (Anopheles)
	MVRTLDYHAEDLGSNPTPKLAKCNPRNKALKPGHSLMDWIRYGN	SGVDLAGT	GGVRPVQ	mosquito (Aedes)
MPRCCGASLVSMVDGDSQPQSLGLQIQDGNPRNKALAPGHSLMDWIRLTK	SGVDLTGV	GGVPRIVT		honeybee
	MLSSGNPRNKVALNPGHSLMDWIRLTK	SGKDLTGV	GGVLDVP	Louse
..KQLRDAALNEASPSGSSASGSATGNPRNKALAPGHSLMDWIRLTK	SGVDLTGV	GGKLLSVT		Jewel Wasp
..KTPISEQGGDADQEDLETGYKIGKELERNLRNKYALAPGHSLMDWIRLTK	SGKDLTGV	GGKLLSVT		beetle
	MATGTRNKVALAPGHSLMDWIRLTK	SGSDLTGV	GGKLLSVT	Pea aphid
	..MDWIRLTK	ANPNLSGV	GGKLLSVT	Deer Tick



B

10 20 30 40 50 60 70

AA: MLNVPSQSFPAPRSQQRVASGGRSKVPLKQGRSLMDWIRLTKSGKDLTGLKGRLEIEVTEEELKKHNKKDD

PSIPRED results:

Pred: CCCCCCCCCCCCCCCCCCCCCCECCCCCHHHHHHHHCCCCCCCCCCCCCCCCCHHHHHHHCCCC

Conf: 9888998999998755558999643105899985359987428999889888421039999976518889

PROF results:

Pred: _____ HHHHHHH _____ EEEE_HHHHHH _____

Figure S2

C

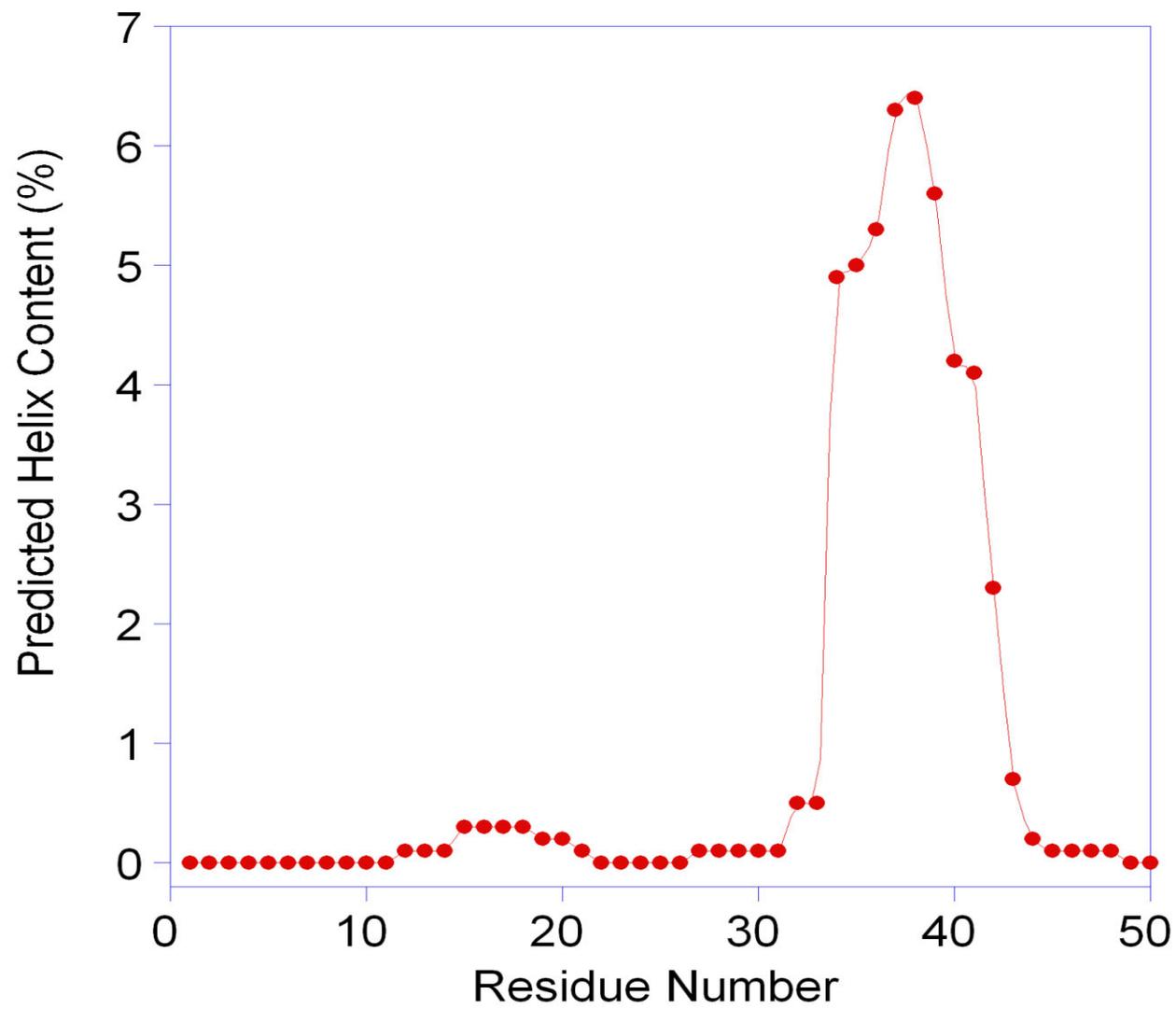


Figure S2 (cont'd)

Sequence alignment of N-terminal region of human Ncb5or against RLF in plants.

N-terminal | b5

*** * * - * - * - * * * * - * - *

MLNVPSQSFAPRSQQ RVASGGRSKVPPLKQGRSLMDWIRLTKSGKDLTGLKGRLEIVTEEELKKNK.
SVLSRENTQSNLSSQPKS SKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
SVIGGENAQSNLSSQPKP SKKPSVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
TDSSSSKKQSNESSETFKT PAKKPITRTKVPFEKGYSQMDWLKLRTRHPDLAGLKGESNRRLIPMDEVKHHRT.
PKSSRESVQLNVPPEPKP TKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQTNRRLLISLEEVKQHKHT.
PSSSGESVQPNVSGQPKP SKKSARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
PTSSGESVQSNVAQPKP SAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
PTSSGESVQSNVAQPKP VKKSARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
PTSSGESVQSNVLAQPKP LKKSARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
ETSKG AESNIQSQPKP SLKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLITLEEVKQHKHT.
QTSKG AESNVSSQAKP SAKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
QTSKG AESNVSSQTKS SSKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
QTSSE EESNVPSQGES SSKKPAARAKVPFEKGYSQMNWLKLRTRHPDLAGLKGHSNRRLISLEEVKQHKHT.
QTSKG AESNVPSQGEF SSKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGHSNRRLISLEEVKQHKHT.
QTSKG AESNVSSQAKP SSKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
QTSKG AESNVPSQAKR SSKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
HTGSG AESNVPLQAKR SSKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
AKDS PQKLSEQKS SVKKPTVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
APGKANLSSKLPEEKN SAKKPTVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ITSKDNLSSKLPEEKN SAKKPTVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
PSKAKELLSKLPEEKN STKKPTVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISIDEVVRKHKHT.
STEMQETTAGSTFKQPV SIAKPVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
LMEKTGHAQEGVQEKPP TVKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLGRSNRRLLISLEEVKQHKHT.
IGSRVGNLKIKEPV VIAKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
RDFGDSFLDAQDEKT VTKKPIARQKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
KPDGEADLDAQKQA KVNPNVGRTKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
DLVTGSQEQKPV IKKSTVRSKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
VGKSLSVNEPTQKPP LAKKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASGDAGTSV NIEQKI SARKTVRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASKEVGTSVKKSQETKI SARKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISINEVVRKHKHT.
AV DVGSSVDTSQRPKI VAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
QKQAGKPLKDPAEQKP VAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
PKQAGNSVKDPADQKP VAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
QEGAGSSSKASVELKA PSRKPATRTKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
SGNIKTPVKASQGPIN SARKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGHSNRRLISINEVVRKHKHT.
SLDVGTAVTNSKQKPP SARKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
STNVGASAGVPQEQKT VNRKPVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
TSVKV ASRKPASRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
VTGDSG KLKPAARAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
GGSNVGSSKETKS AVKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
AHEGATSSAKAPSEQKS PVKSTPRKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
GGVAGSSNKASPETKT PARKSAPRAKVPFEKGYSQMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
TNEVPGGSIKASQAQKT PSKKPVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
GDVGAAPAKNAEKPKI STRKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASGDADASGKAEKPKI STRKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
SVDAGAPAMKSEKARS STRKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASKDGVFSVQTSQKQIK SARKPVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASKDGVFSVQTSQKQIK SARKPVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
SRDGGSSVTSKEQIK PARKSAPRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISINEVVRKHKHT.
SRDGGSSVTSKEQIK PARKSAPRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISINEVVRKHKHT.
SRVSGSSVTSKEQIK PGRKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISINEVVRKHKHT.
SANAAGLVNNSGKQKS AVKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
NEYSQFPKVPSPGDDG KSVKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
NRKSELKQPSGGGAG KSVKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISINEVVRKHKHT.
KKKSEVPKQLKSGDAG KSVKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
ASGDTGSDVNIQERT VARKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASGDAGGSVNEFQEQT VAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ATKDAGSSVVPQKS ATRKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASLDVGTSTVNSQGGKS SARKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASGNAGTSKAFDEKKV SARKPVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
ASKDGSKSVNSQQQKA PAKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
VSVDAGSSVKISQEQKG PARKKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISLEEVKQHKHT.
AKNSSQKSSEQKN SVKKPTVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMAEVVRKHKHT.
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PTQANISLKLSPAQKN SVKKPTVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
PSEANNLPLKLPEQRN SAKKPTVRAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.
PGDARTSATNPQELKA GGKPAARAKVPFEKGFSPMDWLKLRTRHPDLAGLKGQSNRRLLISMEVVRKHKHT.

NP_057314, Human (Ncb5or)
NP_001059238.1, Oryza (RLF)
NP_006657577, Oryza b.
NP_196530.1, Arabidopsis
XP_003580032, Brachypodium
BAJ85812, Hordeum
VAI62358, Triticum
VAI50862, Triticum
XP_020148719, Aegilops
TVU02026, Eragrostis
RLN35912, Panicum
PUZ69075, Panicum h.
XP_025793114, Panicum h.
XP_004981424, TKV90817, Setaria
XP_004955848, TKW31251, Setaria
OQU88741, Sorghum
XP_008667419, Zea
XP_013442808, Medicago
XP_027924497, Vigna
XP_014490185_017438620, Vigna
XP_007151014, Phaseolus
ADE77709, Picea
XP_020265306, Asparagus
KMZ64569, Zostera
RWR77736, Cinnamomum
XP_009418486_5, Musa
XP_008791871, Phoenix
XP_020102204/OAY74390, Ananas
GAV59466, Cephalotus
XP_012065594, Jatropha
XP_024983163, Cynara
XP_020102204, Chenopodium
XP_021838595, Spinacia
TEY81349, Salvia
XP_031127095, Ipomoea
XP_021638891, Hevea
XP_010257153, Nelumbo
XP_006439075/KDO83474, Citrus
XP_024022048, Morus
XP_021996810, Helianthus
XP_011091663, Sesamum
XP_011087944, Sesamum
XP_022869457/Olea
XP_022770099, Durio
XP_021277165, Herrania
KJB49187, Gossypium
XP_016538486/PHT94674, Capsium
PHT60531, Capsium
XP_009776784, Nicotiana
XP_019238920, Nicotiana
XP_09630912_016508308, Nicotiana
XP_030547385, Rhodamnia
XP_024169624, Rosa
VVA25167, Prunus
KAB2627536, Pyrus
XP_023910016_030965286, Quercus
XP_018817051_8, Juglans
XP_010659437, Vitis
XP_021619057, Manihot
TKR58968, Populus
PSS09978, Actinidia
KAA8520222, Nyssa
XP_004513673, Cicer
XP_019464874, Lupinus
XP_027355423, Abrus
XP_020234706, Cajanus
XP_028248018_03534395, Glycine
XP_3539832_006591987, Glycine
TKY67970, Spatholobus
PKI62938, Punica

Sequence alignment of N-terminal region of human Ncb5or against IRC21 in fungi.

- - * - * * - * * - - -

MLNVPSQSFPAPRSQQRVA SGGRSKVPLKQGRSLMDWIRL TKSQKDLTGL.
 ...KPNVRFAP QRLSVAHPAIISSPLHMPMSKSSRKPLVVRTKIRLDPGHSALDWHSL TSNPNANYTKF.
 ...SNRGLFPAVNSAQRAA GSAVNQRKKVVLQPGHSPLDWAHLNRTQPRHKLRGV.
 ...SNRGLFPAINSAQRAANATTT SSAAANQRKKVILQPGHSPLDWAQLNRTQPRHKLRGV.
 ...SNRGLFPLLNSAQRA SSASETRKKVHLKPGHSPLDWARINRTEPQYKLRGV.
 ...MFPPSPNSQQRVN SSNSSLKIPQRSSGSRKVVLEPGFSPLDWASLTSKTPKHQLRGV.
 ...SANSFLPPPNSAQQRVS GNSSSGGTQRQKVALKPGHSPLDWAHLNSTPFHILRGV.
 ...KPPTFPSPFSAQRAAPPITA SSSSTPQAEARARQKVVLPQPGHSPLDWANLNRNAPKHKLRGV.
 ...TLGTLFPPSAQSAQRVQ SLPDGTKKARRKVELAPGHSPLDWAHLNSTASRGQMRGV.
 ...TLGTLFPPSAQSAQRVQ SLPDGTKKARRKVELAPGHSPLDWAHLNLTALRGQMRGV.
 ...IFPAPNSAQRVS RLGIIPGNSRERQKVVLSPGHSPLDWANLNSKTPKHVLRGV.
 ...APGRFPATSSLRVPPPGTGTGVGGV LTQPSGASKAREKVVLPQPGYSPLDWAKL RNSGKNLRGV.
 ...SSLRAPPSCTPAGSKLSSSTLSPLG LPGKPQRSSRQ VMLEPGHSPLDWAAL TADPRNNLRGK.
 ...SSLRAPPSTRTPAGSKLSSSTLSPLG LAGKPQKSSRQ VTLEPGHSPLDWAAL TSDPRNNLRGK.
 ...STLRVPPGMRPGTTNSNSNSLSPNS VTLKPKVKSQR AVLEPGFSPLDWAAL TSNPNHKLGRGA.
 ...ASLRVPPG SRPASNNLAPIS LTLKPSKAACK AVLEPGFSPLDWAAL TANPNNNLRGA.
 ...ASLRVPP SRAASNNLAPLPTQ VTLKPSSTTARK AILEPGYSPLDWAAL TAKPKNNLRGA.
 ...ASLRVPP SSRPTSNSLAPVK LIAQPSNSSKR AVLEPGYSPLDWAAL TSNPKNNLRGA.
 ...LSSSTLAPTK VTAKPSSSSKR AVLEPGYSPLDWAAL TSNPNNNLRGA.
 ...SSLCVPP SRGLTSSTLGPAQ IHGKTQ PSRQ VKLEPGHSPLDWAAL SSNPNNHKLGRGE.
 ...ASLRVPL SRGLTSSTLAPVQ IHGKTQ PSRQ VKLEPGHSPLDWAAL SSNPNNHKLGRGE.
 ...STLRAPR PRGLTSSTLAPVH VQGKQ PSRQ VVLEPGHSPLDWAAL TSNPNHKLGRGD.
 ...STLRGPL PRGLTSSTLAPIQ VQGKQ PSRQ VALEPGRSPLDWAAL TSQPNHKLGRGE.
 ...STLRGPL LRGLTSSTLAPIQ VQGKQ PSRQ VALEPGRSPLDWAAL TSQPNHKLGRGE.
 ...STLRGPL PRGLTSSTLAPIQ VQGKQ PSRQ VALEPGRSPLDWAAL TSQPNHKLGRGE.
 ...ASLRVPPG SRAASNNLAPLPTQ VTLKPSSTTAKK AILEPGYSPLDWAAL TAKPNNNLRGA.
 ...ASLRVPPG SR LTTSTL PVLV LPGKPQRSSRQ VILEPGHSPLDWAAL TSNPNNNLRGA.
 ...QRGLGPPS SGRSLNSSTLAPLPTQ VSLKSS SRK VILEPGFSPLDWAAL AANPKNNLRGK.
 ...ATLRNP VTALTTSTLAPLPTQ RPSRK VILGPGHSPLDWATL TKDPRNNLRGA.
 ...SALRAPPA TTNGLTTSTLAPLPTQ RASRK VILGPGHSPLDWAAL TKDPRNNLRGA.
 ...LPNRGPPS SSSGGGSSSLFPPP THSTKPAKPSRA VVLTTPGHSPLDWARL SGHPTADLRGL.
 ...IPNRSPNRTPTSSLTAPSLAPPP THNSIPTKPRK VLLTPNHSPLDWARL TTSPNSNLRLGL.
 ...LSPAPSRSLPAPARGPGCSTLAPPP THSTKPSKPSKA VVLTTPGHSPLDWARL SGNPSADLRGL.
 ...PLPNRGPP SNGSLGLPPS AGPTQKARGGK VLLSPGHSPLDWAHL QKSGKNLSGV.
 ...PVPNRGPPS SSLGGGLAPPP HSAKPNKPSRK VLLTPGHSPLDWARI SGPNADLRGV.
 ...IPSRGPP GASSLAPPP HTNGPAPSRSL VALAPGHSPLDWARI SGPNADLRNL.
 ...MKPPHSTVKKPNFGIQPGGEDKLGEKEKSEVSTKKKRAK VALTPGHSPLDWARL TSSGQNLRGV.
 ...MKPPHSTVKKPNFGIQPGGEDKVGEKEKSEVSKKKKRAK VALTPGHSPLDWARL TSSGQNLRGV.
 ...PSSALAPPPQSSSSSLALPPSTT KPPPKPSKKREK VALAPGHSPLDWANL KNSGADLRGV.
 ...SSRRPGISSTPGSLAVPLTTS RIPPKPS GRGK VALAPGHSPLDWANL KKSQDLRGV.
 ...NSLRVPPMRGPPNSSSRKPLETSLAPGAGPRRQK IGLKPGHSAMDWAQL KQNKGNALKGN.
 MGYLGRKLAVSNRLRSQREP VALAAGHSPLDWARL VASKQNLGSV.

Human, Ncb5or
NP_013789, yeast (IRC21)
 XP_002416862, Candida
 EEQ42953, Candida
 XP_002550312, Candida
 XP_002616018, Clavispora
 CAG86857, Debaryomyces
 XP_001526828, Lodderomyces
 EDK41487, Pichia
 XP_001482565, Pichia
 XP_001384271, Scheffersomyces
 XP_501731, Yarrowia
 XP_001240913, Coccidioides
 XP_002542840, Uncinocarpus
 XP_661402, Aspergillus
 XP_001272797, Aspergillus
 XP_749520, Aspergillus
 XP_002373662, Aspergillus
 XP_001389090, Aspergillus
 EEH41743, Paracoccidioides
 EEH18593, Paracoccidioides
 EEQ92276, Ajellomyces
 XP_001536862, Ajellomyces
 EER40089, Ajellomyces
 EEH04658, Ajellomyces
 XP_001260021, Neosartorya
 EEQ29684, Microsporium
 XP_002562331, Penicillium
 XP_002149052, Penicillium
 XP_002485125, Talaromyces
 XP_959814, Neurospora
 XP_001596435, Sclerotinia
 XP_001905357, Podospora
 XP_001937768, Pyrenophora
 XP_389554, Gibberella
 XP_364392, Magnaporthe
 XP_568744, Cryptococcus
 XP_771928, Cryptococcus
 XP_001838747, Coprinopsis
 XP_002474966, Postia
 XP_002493579, Pichia
 XP_002173985, Schizosac

Sequence alignment of N-terminal region and cytochrome b5 domain of human Ncb5or against cytochrome b5s in parasites and protists.

Babesia/GFE55910.1	MDASSHGNRR-ERAP	14
Plasmodium/XP_002808938.1	MKENDKNKLNVFSAQ-----NEKSEKSSRT-ERRT	29
Hepatocystis/VWU48363.1	MEPDKNKLPVTPQ-----RDD-DLINKS-NRKP	27
Dictyostelium/XP_643535.1	MMMTDKEQLNNNVQFQQLSTEMKEAIKSIMVSPTEQE-----IEDKKNKPKR-RKVP	51
Trypanosoma/XP_845676.1	MSLPDGGNPESEPNELSTDSEKEAFSQNPSTAGRSDDEP	39
Human Ncb5or/NP_057314.2	MLNVPSQSFPAPR-----SQQRVASGGR-SKVP	27

Giardia/XP_001708782.1	MSEHHGRPSG--VAFLMQHRRIKGVP---AHVAANTNYTANQVYEH	41
Giardia/XP_001704041.1	MASHGRVHRATQEFLDLYSSAKIPT---KQELAAANPITPEEVLRH	42
Giardia/XP_001708389.1	MKGGTIAQRRDAMMRAHILSKVRQRT---DAEINSTFYTPPEEIASH	43
Trichomoniasis/XP_001310855.1	MDVYERREMQRQWRENA--KQCEWIGDITIEEVMKH	35
Babesia/GFE55910.1	-----SNNPVLGSAQFRFAQLMDSNKDSV---VQRATGPIDITEVAKH	54
Plasmodium/XP_002808938.1	-----PNEP---NQLGYINASEDFKNSI-----IKSDKPITKEEVAKH	64
Hepatocystis/VWU48363.1	-----LNSA---NQMVYVNLSESLKKNNT-----SDKKKIFTRREEVKKH	62
Dictyostelium/XP_643535.1	-----LPHGHSQLDWMKK-QSAAQPCF---DTSNGGKITIQLKKH	89
Trypanosoma/XP_845676.1	FRMEVTTRETLDVMLRAKSSCRAGFSMVDWGGILQSIWRKMKRETNEKKLPYLSISEIRRH	99
Human Ncb5or/NP_057314.2	-----LKQGRSLMDWIRLTKSGKDLT---GLKGRLEIVTEEELKKH	65
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Giardia/XP_001708782.1	RSEDDCWVTYRGRVYDITQYLDWHPAGKDILRPFPGYDITEACNVAHSWVGIIHKMIEPLH	101
Giardia/XP_001704041.1	RDVNDWCWVSHKGIYVNLTPYLRYPHAGIAPIEDYYGYDITAVTAAVHGFVQVEQIIAPLA	102
Giardia/XP_001708389.1	ASMDDAWMSYRKGVDITHYVRYHPGGLQCMQBYMGKDMTHAADSVMKWNVATMLRPLA	103
Trichomoniasis/XP_001310855.1	RTPKDCWVILDGIVYNWTQYVYNHPGGSSHFLGK-NPDITVPPHNFHRGMDI-SFVEKLK	93
Babesia/GFE55910.1	TSENDWCWTIYKGVYDITRYLDMHPGGRQHLLDYAGMDITEEFSDIHPWVNAEFLKLSLY	114
Plasmodium/XP_002808938.1	NKKDDAWVIYENKVVYEVTHYLKYHPGGKRILLGKSGKDIKTVKMMHPWVNIIEILKHSF	124
Hepatocystis/VWU48363.1	NTYEDAWVIYENKVVYNITHYFKYHPGGEDVLLLEYAGQDITMKVAEQHSYVNVKLILENSY	122
Dictyostelium/XP_643535.1	NNENDAWTVYKRVYVINITDYFQFHPPGGKIELLRAAGNDCTQMFEEFTHSWVNFAMMLKYL	149
Trypanosoma/XP_845676.1	NTPDDLWIVIGSVVYDCTKFQHFHPGGERMLQLCGGRDATELFFNYHRWVSCESMLQPPFA	159
Human Ncb5or/NP_057314.2	NKKDDCWICIRGFVYNVSPYMEYHPGGDELMRAAGSDGTELFDQVHRWVNYESMLKECL	125
	. * * . * * : : * * * : . * * * : :	

Giardia/XP_001708782.1	IGMLQGPPRLQGYDYDALRTRDLRRGSPA*	131
Giardia/XP_001704041.1	VGVLNGDPCVPPEKKLKE-TGVLRRRY*	129
Giardia/XP_001708389.1	IGTVKTHVDNSNAFSCLPRTIAEVAEEENNKDAS*	136
Trichomoniasis/XP_001310855.1	IGRLVSSKYLYS*	105
Babesia/GFE55910.1	VGELKSEDVATDLPPTKGTAPDN*	137
Plasmodium/XP_002808938.1	IGYVEV*	130
Hepatocystis/VWU48363.1	LGDVED*	128
Dictyostelium/XP_643535.1	VGYSIEDN*	158
Trypanosoma/XP_845676.1	VGLVKPEDEERGEREKEKEKPG*	182
Human Ncb5or/NP_057314.2	VGRMAIKPAVLKDYR..	140
	: * :	