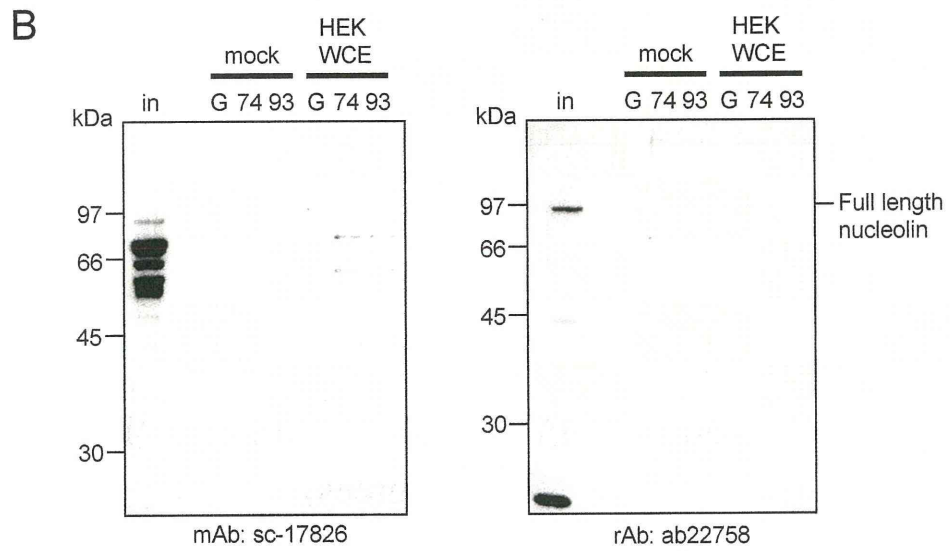
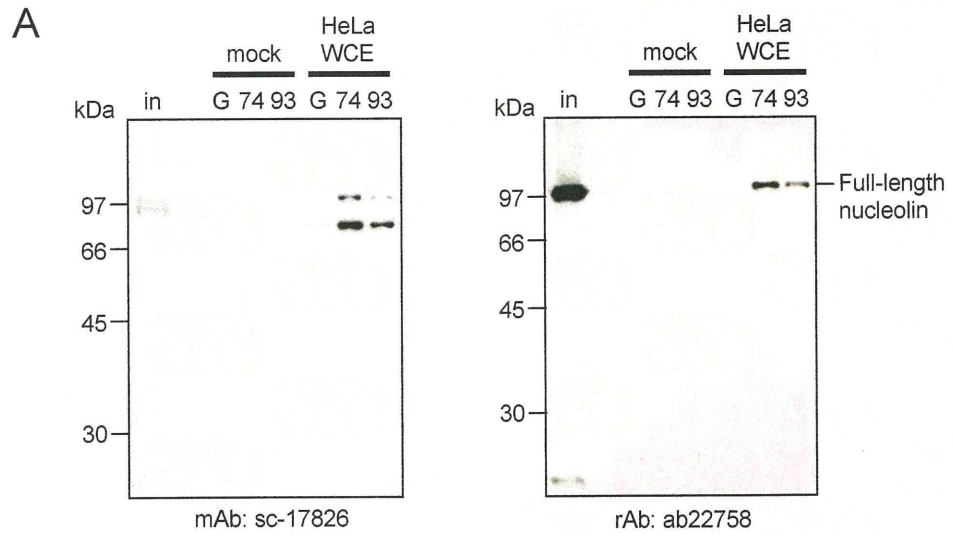
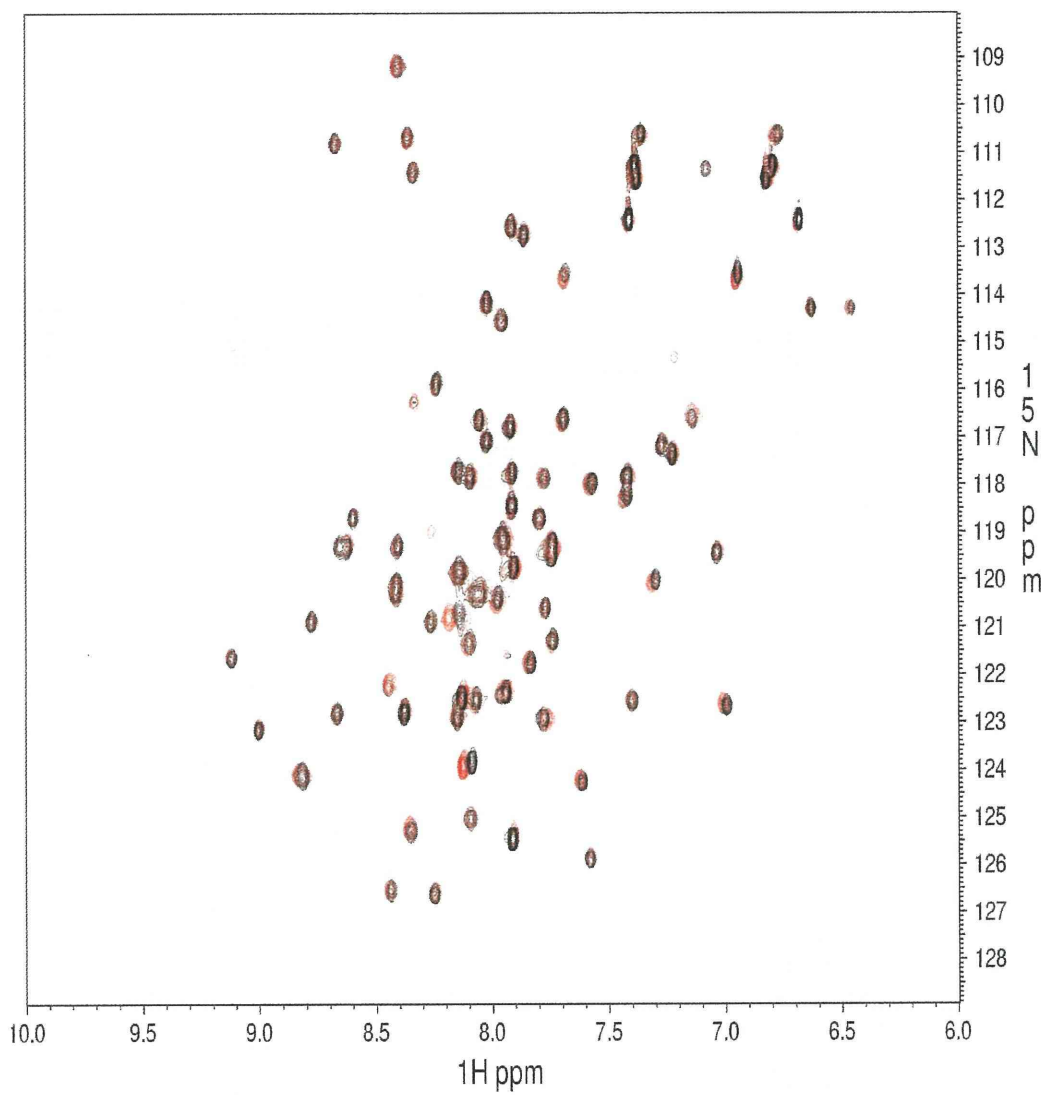


Supplementary Figure S1



Supplementary Figure S2

		RNP2		RNP1																																															
RRM1	308	N	L	F	V	G	N	L	N	F	N	K	S	A	P	E	L	K	T	G	I	S	D	V	F	A	K	N	D	L	A	V	V	D	V	R	I	G	M	T	R	K	F	G	Y	V	D	F	E	S	356
RRM2	394	T	L	L	A	K	N	L	P	Y	K	V	T	Q	D	E	I	K	E	V	F	E	D	--	A	A	E	I	R	L	V	S	K	--	D	G	K	S	K	G	I	A	Y	I	E	F	K	T	438		
RRM3	487	T	L	V	L	S	N	L	S	Y	S	A	T	E	E	T	L	Q	E	V	F	E	K	--	A	T	F	I	K	V	P	Q	N	-	Q	N	G	K	S	K	G	Y	A	F	I	E	F	A	S	532	
RRM4	573	T	L	F	V	K	G	L	S	E	D	T	T	E	E	T	L	K	E	S	F	D	G	--	S	V	R	A	R	I	V	T	D	R	E	T	G	S	S	K	G	F	G	F	V	D	F	N	S	619	
RRM1	357	A	E	D	L	E	K	A	L	E	L	T	G	-	L	K	V	F	G	N	E	I	K	L	E	-	379																								
RRM2	439	E	A	D	A	E	K	T	F	E	E	K	Q	G	T	E	I	D	G	R	S	I	S	L	Y	Y	463																								
RRM3	533	F	E	D	A	K	E	A	L	N	S	C	N	K	R	E	I	E	G	R	A	I	R	L	E	L	557																								
RRM4	620	E	E	D	A	K	A	A	K	E	A	M	E	D	G	E	I	D	G	N	K	V	T	L	D	W	644																								



Supplementary Figure S4

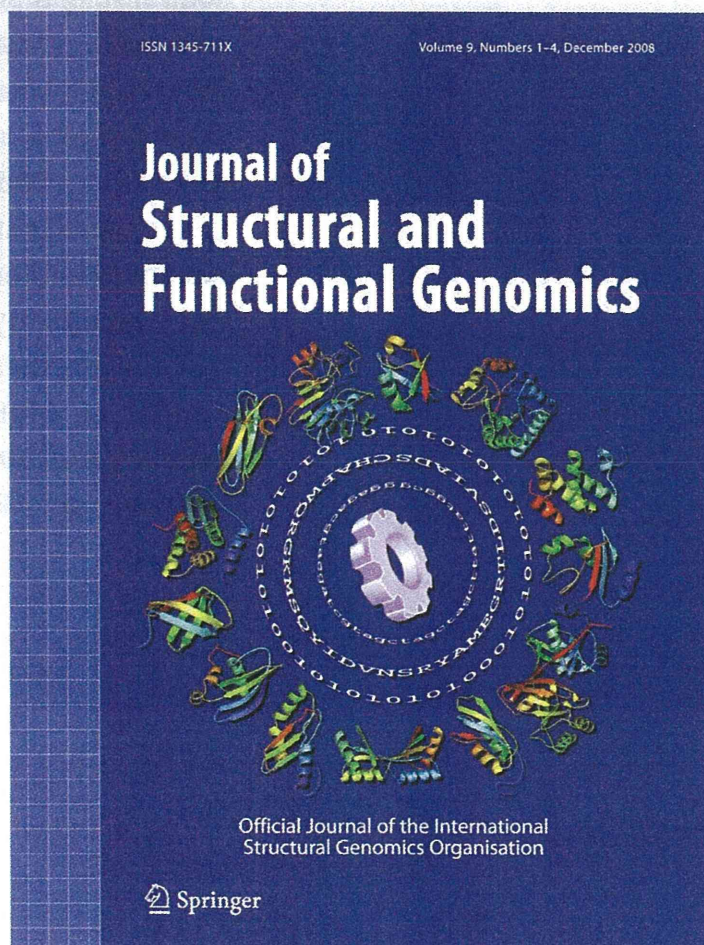
*A simplified recipe for assigning amide  
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# A simplified recipe for assigning amide NMR signals using combinatorial $^{14}\text{N}$ amino acid inverse-labeling

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**Abstract** Assignment of backbone amide proton resonances is one of the most time-consuming stages of any protein NMR study when the protein samples behave non-ideally. A robust and convenient NMR procedure for analyzing spectra of marginal-to-low quality is helpful for high-throughput structure determination. The  $^{14}\text{N}$  selective- and inverse-labeling method is a candidate solution. Here, we present a simplified protocol for assigning protein backbone amide NMR signals. When  $^{14}\text{N}$  inversely labeled residues are present in a protein, their backbone NH cross peaks vanish from the protein's  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum, and thus, their chemical shifts can be readily identified by a process of

elimination. Some metabolically related amino acids, for example, Ile, Leu, and Val, cannot be individually incorporated but can be inversely labeled together. We optimized and simplified the protocol and M9-based medium formula for the  $^{14}\text{N}$  selective- and inverse-labeling method without any additives. Our approach should be cost-effective, because the method could be additively applied stepwise, even when the proteins of interest were found to be non-ideal.

**Keywords** Combinatorial inverse-labeling · A $\beta$ (1–40) peptide · NMR sample preparation · Isotope labeling

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## Abbreviations

HSQC	Heteronuclear single quantum coherence spectroscopy
IPTG	Isopropyl- $\beta$ -D-galactoside
IL1 $\beta$	Interleukin-1 $\beta$
SOFAST-HMQC	Band-selective optimized flip-angle short-transient heteronuclear multiple quantum coherence spectroscopy
BEST	Band-selective excitation short-transient
TROSY	Transverse relaxation optimized correlation spectroscopy

## Introduction

The development of a robust and cost-effective NMR method that is applicable to assignment of backbone amide proton resonances of proteins with non-ideal properties is a challenge in structural genomics research. Usually, assignment of the backbone resonances is one of the most time-consuming but indispensable stages of any NMR study. A  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum (and its variants, for

example, SOFAST-HMQC [1] and TROSY [2]) provides a fingerprint of the protein of interest and is thereby important as a reference spectrum for other triple resonance experiments. The information obtained from a  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum is not only necessary for 3D structure determination but also indispensable for examining protein dynamics, H/D exchange, and protein–ligand interactions. A process for assignment of the backbone resonances of proteins with molecular weights as large as 20 kDa routinely starts with preparation of a uniformly  $^{13}\text{C}/^{15}\text{N}$ -labeled non-deuterated protein. Using this sample, several pairs of 3D NMR experiments are recorded, for example, HNCA/HN (CO)CA, HNCACB/CBCA (CO)NH, and HNCO/HN (CA)CO [3–5]. Among these pairs, one provides information on the intraresidual spin systems while another provides details of the interresidual connectivities. Thus, analysis of these data sets leads to sequence-specific assignment of all main chain signals along the peptide sequence.

Nevertheless, non-ideal properties of a protein sample often hamper the analysis of a protein's NMR data set because some key resonances are often missing. Such undesired behaviors include limited solubility, nonspecific self-association, chemical exchange, and internal motion of the protein. These properties often result in low signal-to-noise ratios of certain cross-peaks in 3D NMR experiments, for example, those arising from  $^{13}\text{C}\beta\text{s}$  in HNCACB and CBCA (CO)NH. As a result, assignment of the backbone resonances becomes difficult or even impossible because connectivities between residues are not uniquely identified. In addition, some non-ideal experimental conditions provide similar results. Examples of non-ideal NMR conditions of physiological or biophysical interest include highly viscous conditions, NMR analysis of a protein inside a living cell, presence of lipid micelles, and a high level of denaturants or salts.

At present, there are several options for solving the problem of missing resonances: (a) use a SOFAST-/BEST-NMR pulse scheme to gain signal intensity per experimental time [1, 6]; (b) use a nonlinear, sparse sampling method for the indirect dimension of a 3D/4D NMR experiment to minimize the measurement time and to maximize S/N [7–9]; and (c) use protein samples that contain selectively  $^{15}\text{N}$ -labeled residues [10–12]. However, there exist many drawbacks associated with these solutions in terms of the costs. For example, using the third strategy, the cost and effort associated with sample preparation is a major concern when projects like structural genomics that require massive protein preparation and NMR data acquisition are involved. The cost and effort required to introduce selectively labeled amino acids by using cell-free translation systems are now relatively minimized, but are still relevant [12].

In this study, we describe a simple method to obtain the information necessary for residue-type assignments that uses “combinatorial inverse-labeling” of sets of specific amino acids, an idea that was initially proposed by Shortle [13]. In the original method, MOPS minimal media [14], modified such that glycerol was the sole carbon source, was employed with the combinatorial inverse-labeling method. A matrix of four combinations of amino acids redundantly covering 17 amino acids was designed and inversely labeled. The resulting five HSQC data sets (four inversely labeled and one uniformly labeled) were processed with the “labeling pattern matrix” to identify each amino acid type independent from the other 3D experiments. One major drawback of this method is the requirement of a special processing application.

Here, we further optimized the method for high-throughput structural determination in the current manner. By this method, we prepared  $^{15}\text{N}$  uniformly labeled and  $^{14}\text{N}$  selectively and inversely labeled protein samples as recombinant proteins expressed in *Escherichia coli* BL21(DE3) that were grown in M9 minimal media containing  $^{15}\text{NH}_4\text{Cl}$  as the sole nitrogen source with or without supplementation of unlabeled ( $^{14}\text{N}$ ) amino acids. In addition, we proposed some new sets of combinations of metabolically related amino acids, which were found to be useful.

## Materials and methods

### Protein techniques

The *E. coli* expression plasmid for IL1 $\beta$ , which has an added N-terminal methionine and whose synthesis is under the control of the *ptac* promoter, was generously provided by Dr. Y. Kikumoto [15].  $^{15}\text{N}$ -labeled recombinant IL1 $\beta$  and  $^{14}\text{N}$  selectively and inversely labeled IL1 $\beta$ s were expressed in *E. coli* BL21(DE3) that had been transformed with the plasmid. Cells were grown at 30°C in 0.1 L M9 minimal media containing  $^{15}\text{NH}_4\text{Cl}$  as the sole nitrogen source with or without unlabeled amino acid(s). Protein production was induced by adding IPTG to a final concentration of 1 mM when the OD600 of a culture reached 0.4. Cells were harvested after 4 h. The details of this expression protocol are outlined in Table 1. Each cell-free extract was applied to a S-Sepharose fast flow column (GE-Healthcare Biosciences), and the IL1 $\beta$  was eluted using a NaCl gradient containing 20 mM sodium phosphate (pH 6.5).

The *E. coli* expression plasmid for human A $\beta$ (1–40) peptide, which has a N-terminal maltose-binding protein tag and a linker containing tobacco etch virus protease cleavage site followed by A $\beta$ (1–40), was generously



**Table 1** The procedure for  $^{14}\text{N}$  selective- and inverse-labeling of a protein

1. Prepare  $^{15}\text{N}$  M9 medium containing glucose (4 g/L),  $^{15}\text{N}$ -ammonium chloride (0.7 g/L),  $\text{NaH}_2\text{PO}_4\cdot 12\text{H}_2\text{O}$  (15 g/L),  $\text{K}_2\text{HPO}_4$  (3 g/L), NaCl (0.5 g/L),  $\text{MgSO}_4$  (0.24 g/L),  $\text{CaCl}_2\cdot\text{H}_2\text{O}$  (15 mg/L), vitamins, nucleobases (each at 100 mg/L), and ampicillin (50 mg/L)
2. Pick three to five colonies of *E. coli* BL21(DE3) to add into 1 mL LB medium and grow the culture overnight at 30–37°C
3. Add 1 mL of the bacterial culture to 50 mL M9 medium containing  $^{15}\text{NH}_4\text{Cl}$  and incubate at 30–37°C until the OD600 of the culture is 0.8–1.0 (pre-culture medium)
4. Add the pre-culture medium to 450 mL of M9 medium containing  $^{15}\text{NH}_4\text{Cl}$  and incubate at 30–37°C until the OD600 of the culture is 0.2–0.4 (main culture medium)
5. Add 100 mg/L (final concentration) of each  $^{14}\text{N}$  selected-labeled amino acid\* and incubate the culture at 30–37°C for an additional 30–60 min
6. Induce protein expression with the addition of 0.5–1.0 mM IPTG (final concentration)
7. Incubate the culture at 30–37°C for an additional 2–4 h
8. Harvest the cells

\* Use of a stock solution of 20 mg/mL ( $\sim\text{pH}$  10) is recommended for Tyr and Phe. Other amino acids can be added in powder form

provided by Dr. D. Hamada.  $^{15}\text{N}$ -labeled recombinant  $\text{A}\beta(1\text{--}40)$  and  $^{14}\text{N}$  selectively and inversely labeled  $\text{A}\beta(1\text{--}40)\text{s}$  were expressed in *E. coli* BL21(DE3) that had been transformed with the plasmid. Cells were grown at 37°C in 0.1 L M9 minimal media containing  $^{15}\text{NH}_4\text{Cl}$  as the sole nitrogen source with or without unlabeled amino acid(s). Protein production was induced by adding IPTG to the final concentration of 1 mM when the OD600 of a culture reached 0.45.  $^{14}\text{N}$ -amino acids were added 45 min prior to IPTG induction. Cells were harvested after 18 h at 20°C in order to prevent aggregation of  $\text{A}\beta(1\text{--}40)$ . Each cell-free extract was applied to an amylose resin column (New England Biolabs), and the fusion protein was eluted by maltose. The fusion protein was treated with tobacco etch virus protease (New England Biolabs), and the resultant peptide was finally purified by reversed-phase HPLC using a linear acetonitrile gradient containing dilute ammonium hydroxide.

#### NMR spectroscopy

Samples used for NMR spectroscopy were approximately 0.5 mM and 0.1 mM in fully  $^{13}\text{C}/^{15}\text{N}$ -labeled and inversely labeled protein, respectively, 5%  $\text{D}_2\text{O}$ –95%  $\text{H}_2\text{O}$ , and 20 mM sodium phosphate (pH 5.5). For assignment of the backbone resonances, initially, HNCA, HN (CO)CA, HNCACB, CBCA (CO)NH, HNCO, HN (CA)CO, and 3D  $^{15}\text{N}$ -edited NOESY-HSQC spectra of fully  $^{13}\text{C}/^{15}\text{N}$ -labeled proteins were recorded at 25°C using a 600 MHz Bruker DMX600 spectrometer or a 600 MHz Bruker AVANCE-III spectrometer [1]. Then,  $^1\text{H}$ – $^{15}\text{N}$  HSQC spectra or  $^1\text{H}$ – $^{15}\text{N}$  SOFAST-HMQC spectra of several inversely labeled proteins were recorded for residue-type assignment according to the backbone resonance assignment circumstances. Data were processed using NMRPipe [16]. IL1 $\beta$  backbone signal assignments were taken from the literature [17].

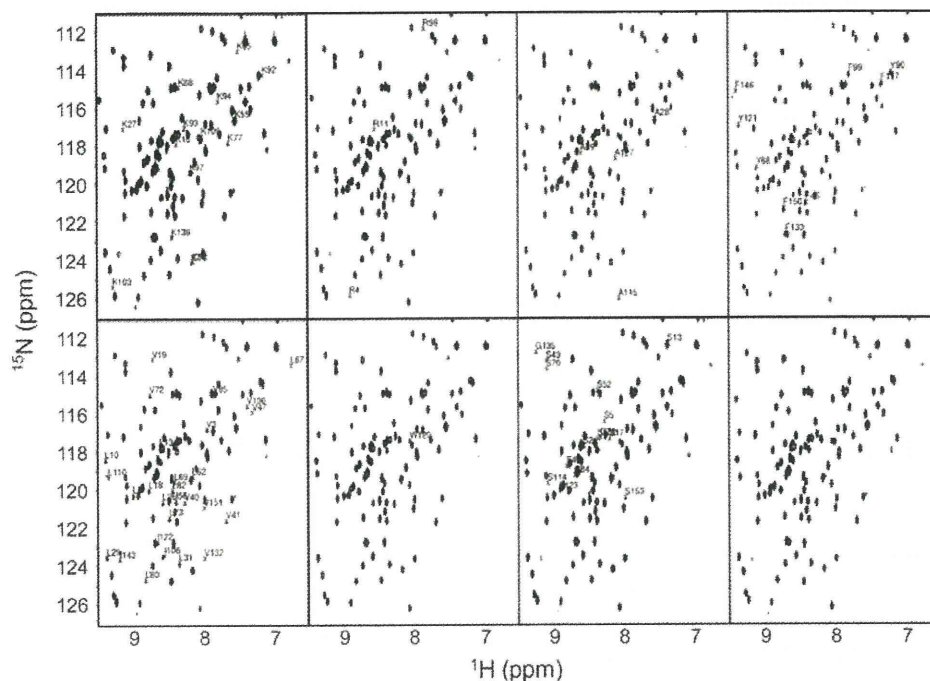
$\text{A}\beta(1\text{--}40)$  backbone signal assignments were taken from the literature [18] and confirmed by this method.

#### Results and discussion

To obtain the information necessary for backbone signal assignments when using data sets derived from low-quality 3D NMR spectra, we developed an assignment strategy using a “combinatorial inverse-labeling” method. According to this method, we prepared  $^{15}\text{N}$  uniformly labeled and  $^{14}\text{N}$  selectively and inversely labeled IL1 $\beta\text{s}$ . We expressed them in *E. coli* BL21(DE3) grown in M9 minimal media that contained  $^{15}\text{NH}_4\text{Cl}$  as the sole nitrogen source with or without supplementation of specific  $^{14}\text{N}$ -labeled amino acids at concentrations of 100 mg/L each. The  $^{14}\text{N}$  amino acids were added to the cultures approximately 30–60 min prior to IPTG-induced protein expression. Because *E. coli* BL21(DE3) was a competent host strain, no special auxotrophic host strains were needed. The simplified fermentation protocol is summarized in Table 1.

We carefully selected combinations of amino acids to be inversely labeled. In detail, these amino acids are the final products of isolated metabolic pathways or are synthesized in metabolically related pathways. The expression of  $^{15}\text{N}$  uniformly labeled IL1 $\beta\text{s}$  was possible in *E. coli* BL21(DE3), which produces large amounts of recombinant proteins in the minimal media [19]. We expressed the labeled IL1 $\beta$  in BL21(DE3) cells from plasmid DNA under the control of the *ptac* promoter. Next, expression for other  $^{14}\text{N}$  inversely labeled proteins was examined. We chose three metabolically isolated amino acids (Arg, Lys, and Ala) and four combinations of amino acids (Phe/Tyr, Ile/Val/Leu, His/Trp, and Gly/Ser/Cys) to label IL1 $\beta$ . Note that the biosynthetic pathways for His and Trp are metabolically distinct from each other, and hence a protein need

**Fig. 1** Expanded regions of the  $^{14}\text{N}$  selectively and inversely labeled IL1 $\beta$ 's  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra. The inversely labeled proteins, named according to the  $^{14}\text{N}$ -labeled residues that they contain, are **a** Lys $^-$ , **b** Arg $^-$ , **c** Ala $^-$ , **d** (Phe/Tyr) $^-$ , **e** (Ile/Val/Leu) $^-$ , **f** (His/Trp) $^-$ , and **g** (Gly/Ser/Cys) $^-$ . The control spectrum, which is that of fully labeled IL1 $\beta$ , is shown in **h**. The full range expansion spectrum of **(h)** with assignments is shown in Supplementary Fig. 1. The eliminated *cross peaks* are marked by *crosses* and residue names



**Table 2** Cross peak identification in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC of IL1 $\beta$  inversely labeled with the selected  $^{14}\text{N}$  amino acid(s)

Amino acid(s)	Number of expected cross peaks	Number of eliminated cross peaks	Number of overlapping cross peaks <sup>a</sup>
K	15	14	1
R	3	3	0
A	4	3	1
I/V/L	5/11/15	31	0
H/W	1/1	2	0
G/S/C	8/14/2	23	1
F/Y	9/4	9 <sup>b</sup>	0

<sup>a</sup> The positions of these peak(s) overlap with other peaks. It was not possible to identify them as eliminated peaks by manual inspection

<sup>b</sup> Four of these peaks were barely visible but could be identified as phenylalanine or tyrosine cross peaks

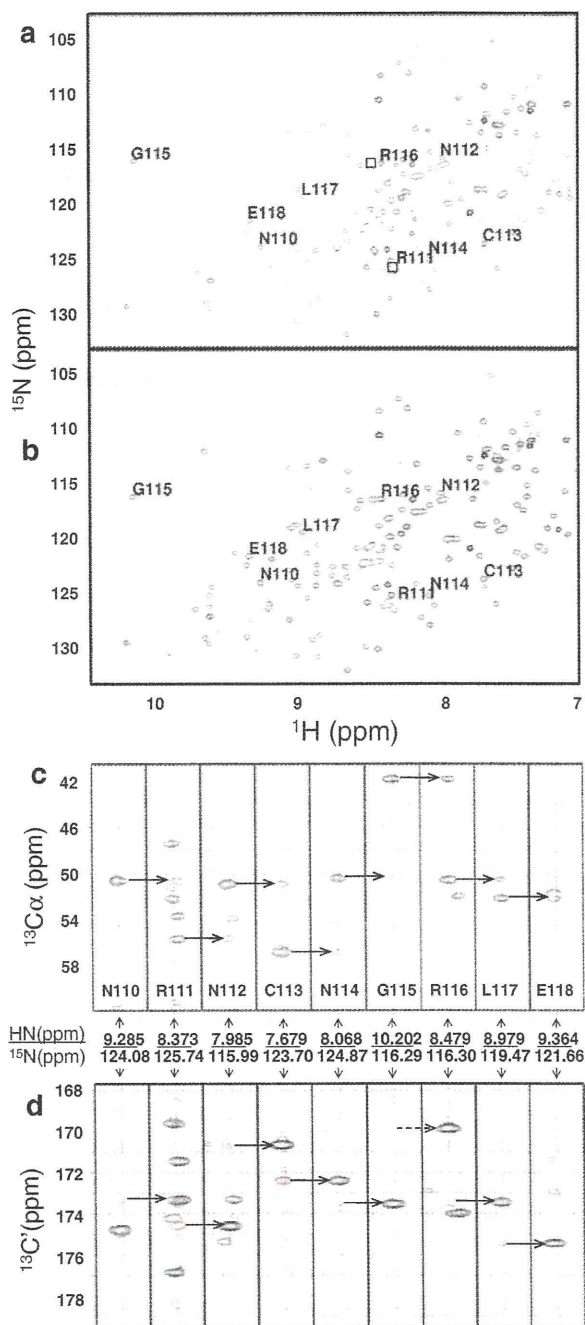
not be labeled with both amino acids at once. Nevertheless, in this study, we used these two amino acids simultaneously because His and Trp are usually present in low concentrations in the protein. We could identify each residue type by its absent or weakened cross peak in the reference  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum (Fig. 1). The targeted cross peaks' intensities decreased by greater than 95% for all spectra, while the method did not affect the other cross peak intensities (Table 2). The incorporation ratio of  $^{14}\text{N}$  inversely labeled amino acids by each method was quantified (Supplementary Table 1). Thus, our method can provide a large chunk of the missing information,

specifically, the residue-type information associated with amide cross peaks, even if many  $^{13}\text{C}\beta$  chemical shifts in the HNCACB and CBCA (CO)NH spectra are absent.

We also employed our method for de novo assignment of several protein NMR spectra, for example, those of the PX domain from human p47<sup>phox</sup> NADPH oxidase (PX-p47<sup>phox</sup>, residues 1–128, 130 total residues) [20], the DNA binding domain of the *Drosophila melanogaster* gcm transcriptional factor (gcm-DBD, 158 residues) [21, 22], the TIR domain from MyD88 (residues 148–296, 149 residues) [23, 24], and the second LOV domain (LOV2) from *Arabidopsis thaliana* phototropin 2 (LOV2<sup>phot2</sup>, residues 363–500, 140 total residues; manuscript in preparation). In all cases, 3D-NMR data sets were analyzed using only spectra recorded with fully  $^{13}\text{C}/^{15}\text{N}$ -labeled protein samples prepared at concentrations of 0.3–0.6 mM—per-deuterated samples were not needed. For PX-p47<sup>phox</sup>, all observable amide signals were assigned when the data sets for PX-p47<sup>phox</sup> that had been selectively and inversely labeled with arginines and lysines (designated Arg $^-$  and Lys $^-$ , respectively) were used to eliminate ambiguities. For gcm-DBD, more than 95% of the backbone signals were unambiguously assigned by incorporating the information contained in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of Arg $^-$  and Lys $^-$  samples during an iterative computer assignment followed by manual examination. The assignment results were further confirmed using the data obtained from the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of (Phe/Tyr) $^-$  and (His/Trp) $^-$  samples. Assignment of the gcm-DBD resonances proved to be our most difficult test case because of poor signal-to-noise

ratios found for many of the 3D NMR spectra. Only 60% of the intra-residue CO, 30% of the inter-residue C $\beta$ , and 10% of the intra-residue C $\beta$  cross peaks were observed in the HN (CA)CO, CBCA(CO)NH, and HNCACB spectra, respectively (Fig. 2). For LOV2<sup>phot2</sup>, more than 95% of its backbone signals were unambiguously assigned when the data sets obtained from the <sup>1</sup>H-<sup>15</sup>N HSQC spectra of (Ile/Val/Leu)<sup>-</sup> and (Phe/Tyr)<sup>-</sup> were included in the assignment procedure. Note that these examples included not only a T7 promoter-driven pET-based expression system, which was originally described by Shortle [13], but also a *tac*-promoter-based pGEX GST-fusion protein expression system. We further confirmed the expression of the inversely labeled sample with a pMal MBP-fusion system as well. In essence, there is no difference between these expression systems.

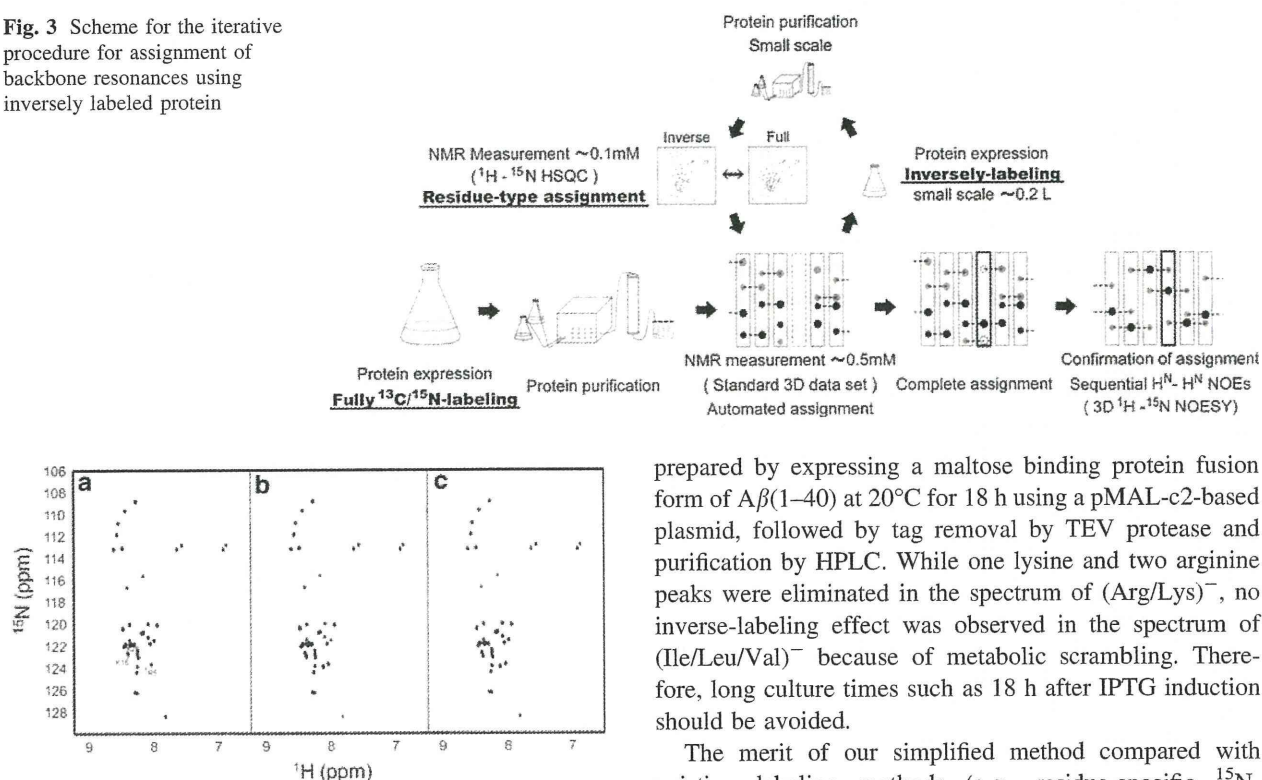
One possible problem associated with this combinatorial labeling method concerns potential ambiguity of assignment(s) when an eliminated cross peak overlaps another cross peak of any residue type. Usually, it will be easy to classify resonance(s) according to residue type without ambiguity when the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of the inversely labeled sample and the reference spectrum are compared. If there are not many unassigned backbone resonances to begin with, a visual examination of the two spectra should suffice. However, it often becomes difficult to begin to assign backbone resonances because many of the necessary inter- and intra-residue cross peaks, especially those involving C $\beta$ s and COs, are missing within a data set. These NMR cross peaks are needed to make connectivities between two successive residues; however, the cross peaks between backbone amides and the intra-residual C $\beta$ s and COs tend to vanish. This is because the two triple-resonance experiments HNCACB and HN(CA)CO are remarkably insensitive compared to CBCA(CO)NH and HNCO, respectively, in cases of non-ideal samples. When the aforementioned difficulty occurs, it may be resolved if an automated assignment program (e.g., AUTOASSIGN [25], MAPPER [26], MARS [27], KUIRA [28], or Olivia [29]) is used in conjunction with the additional data acquired from inverse-labeling experiments. For the experimental work reported herein, we used a program developed in-house that relies on a simple exhaustive search algorithm for residue assignment. The data set inputted into these assignment programs usually consists of peak lists obtained from six 3D NMR experiments: HNCA, HNCO, HN(CO)CA, HN(CA)CO, CBCA(CO)NH, and HNCACB. The residue-type information obtained from <sup>1</sup>H-<sup>15</sup>N HSQC spectra of inversely labeled proteins would be added to these input data. Using our simplified method, selected combinations of amide signal elimination data were added in a stepwise manner according to the completion of the assignment. During the



**Fig. 2** An example of the backbone assignment of GCM-DBD using inversely labeled protein. Expanded regions of the of the <sup>14</sup>Arg-labeled and fully labeled references and GCM-DBD's <sup>1</sup>H-<sup>15</sup>N HSQC spectra are shown in **a** and **b**, respectively. The eliminated signals for R111 and R116 are indicated as *open squares*. Selected *strip plots* of **c** HNCA and **d** HNCO (*black*)/HNCACO (*red*) spectra overlays corresponding to the residues N110 to E118 are shown. Sequential connectivities between strips are indicated by *arrows*

assignment procedure, the cross peak data generated from a 3D <sup>15</sup>N-edited NOESY spectrum should not be used as additional input because this data can be used later to

**Fig. 3** Scheme for the iterative procedure for assignment of backbone resonances using inversely labeled protein



**Fig. 4** Expanded regions of the  $^{14}\text{N}$  selectively and inversely labeled  $\text{A}\beta(1-40)\text{s}'$   $^1\text{H}-^{15}\text{N}$  SOFAST-HMQC spectra. The inversely labeled proteins, named according to the  $^{14}\text{N}$ -labeled residues that they contain, are **a**  $(\text{Arg}/\text{Lys})^-$  and **b**  $(\text{Ile}/\text{Val}/\text{Leu})^-$ . The control spectrum, which is that of fully labeled  $\text{A}\beta(1-40)$ , is shown in **c**. The inversely labeled *cross peaks* are marked by crosses and residue names

eliminate any assignment ambiguities. Many (e.g., 10–30) sequential assignment possibilities may be generated by a semi-automatic program, but often the correct assignment can be confirmed using the  $^{15}\text{N}$ -edited NOESY connectivity data. Finally, backbone resonance assignments can be further confirmed by manually tracing the connectivity of the sequential NOEs. Interpretation of the spectra of non-ideal protein samples should benefit from the use of the strategy outlined above because NOESY experiments usually have greater signal-to-noise ratios compared with HNCACB and HN(CA)CO experiments. Our NMR assignment procedure using inversely labeled protein is summarized in Fig. 3.

Another limitation of this method is the harvesting time of the bacteria after the addition of the  $^{14}\text{N}$  amino acids. For example, some aggregation-prone proteins are available in their soluble form only when the proteins are expressed at a low temperature. For assessing this problem, we prepared  $(\text{Arg}/\text{Lys})^-$  and  $(\text{Ile}/\text{Leu}/\text{Val})^-$   $\text{A}\beta(1-40)$  peptide (40 residues, Fig. 4) as a good example of an aggregation-prone protein sample. The sample was

prepared by expressing a maltose binding protein fusion form of  $\text{A}\beta(1-40)$  at  $20^\circ\text{C}$  for 18 h using a pMAL-c2-based plasmid, followed by tag removal by TEV protease and purification by HPLC. While one lysine and two arginine peaks were eliminated in the spectrum of  $(\text{Arg}/\text{Lys})^-$ , no inverse-labeling effect was observed in the spectrum of  $(\text{Ile}/\text{Leu}/\text{Val})^-$  because of metabolic scrambling. Therefore, long culture times such as 18 h after IPTG induction should be avoided.

The merit of our simplified method compared with existing labeling methods (e.g., residue-specific  $^{15}\text{N}$ -labeling methods [30, 31] and a side chain/residue-specific  $^{12}\text{C}$ -inverse-labeling method, where other carbons are  $^{13}\text{C}$ -labeled [32]) is in the preparation of proteins with specific, metabolically related amino acids that are  $^{14}\text{N}$  inversely labeled in combination. Although our method requires two to seven additional protein samples with different  $^{14}\text{N}$  inversely labeled amino acids, the required concentration of each sample is less than 0.1 mM, which suffices for a single experiment of  $^1\text{H}-^{15}\text{N}$  HSQC. Such samples are routinely prepared economically with a small scale fermentation (i.e., 0.2 L). In addition, inverse-labeling is routinely achieved by expressing the target protein in *E. coli* strain BL21(DE3) with the normal glucose-based M9 minimal medium—auxotrophic host strains, cell-free protein expression systems, or specialized glycerol-based MOPS medium are not required. This implies that one can skip the step for optimizing the conditions of individual experiments to prepare the additional inversely labeled samples. Thus, we found our method experimentally advantageous because it can be used additively after the standard NMR assignment experiments using the fully labeled sample, even when the quality of some of the 3D NMR spectra were found to be insufficient to complete the full assignment. Finally, this approach is not necessarily limited by sample viscosity (suitable for highly viscous samples) or protein locality (compatible with an in-cell NMR approach with *E. coli*) [33].

## Conclusion

Our simplified combinatorial  $^{14}\text{N}$  selective- and inverse-labeling method has enormous potential for NMR-based structural biology studies of proteins, because the strategy should be widely applicable for proteins with non-ideal properties at any stage of the research project. The information from the spectra of  $^{14}\text{N}$  selectively and inversely labeled proteins could be applied to compensate for an otherwise incomplete 3D NMR data set in a stepwise manner. The method reduces the ambiguity often present during the initial sequential assignment trials and can confirm the tentative assignments derived from 3D-NMR spectra of marginal quality because the specific residue type of a cross peak can be readily identified as a result of the elimination of its cross peak in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum.

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# IDEAL: Intrinsically Disordered proteins with Extensive Annotations and Literature

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## ABSTRACT

**IDEAL, Intrinsically Disordered proteins with Extensive Annotations and Literature (<http://www.ideal.force.cs.is.nagoya-u.ac.jp/IDEAL/>), is a collection of knowledge on experimentally verified intrinsically disordered proteins. IDEAL contains manual annotations by curators on intrinsically disordered regions, interaction regions to other molecules, post-translational modification sites, references and structural domain assignments. In particular, IDEAL explicitly describes protean segments that can be transformed from a disordered state to an ordered state. Since in most cases they can act as molecular recognition elements upon binding of partner proteins, IDEAL provides a data resource for functional regions of intrinsically disordered proteins. The information in IDEAL is provided on a user-friendly graphical view and in a computer-friendly XML format.**

## INTRODUCTION

The discovery of intrinsically disordered proteins (IDPs) has brought about a paradigm change in structural biology (1,2). Although proteins were believed to adopt unique 3D structures to function, IDPs do not, by themselves, assume any stable 3D structure under physiological conditions, and yet they participate in crucial biological processes such as signal transduction and transcription control (3–5). Some proteins contain long intrinsically disordered regions (IDRs) while others are fully disordered. In contrast to the long studied 3D structures of proteins, investigations on IDPs started only about 10 years ago and, as yet, knowledge of IDPs is not well collected and integrated. Although the first database of IDPs, Disprot

(6), has more than 600 well-annotated entries, this number is much smaller than the over 70 thousands entries in the Protein Data Bank (PDB) (7). Considering that the protein 3D structural databases such as PDB, SCOP (Structural Classification of Proteins) (8) and CATH (9), have played important roles in deepening our understanding of the nature of protein structures and functions, the development of IDP databases are essential to the progress of IDP research.

We have developed a database, IDEAL (IDPs with Extensive Annotations and Literature) in which experimentally verified IDRs are collected. In the database construction process, we paid special attention to the functional regions in IDRs, for example, regions that interact with other molecules and post-translational modification sites. In particular, we have extensively curated IDRs that adopt unique 3D structures when they bind to other molecules by the ‘coupled folding and binding’ process (10–16). We have called these IDRs the protean segments (ProS). The information in IDEAL is provided on a user-friendly web-interface and in computer-friendly XML files.

## CONTENTS OF IDEAL

### Summary of the annotation process

We used the UniProt amino acid sequence (17) as the reference, and marked structural and functional features along the sequences. A unique serial identifier, IID (IDEAL Identification), was assigned to each protein in IDEAL, starting with IID0001 for human proteins, IID5001 for other eukaryotic proteins and IID9001 for all other proteins including virus proteins. Ordered and disordered regions were annotated as follows: First, ordered regions were obtained from the structural regions atomically detailed in the PDB. Then, disordered

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regions were located by careful assessment of PDB coordinates and by reading the literature. After identifying the ordered and disordered regions, the ProSs were manually determined. Finally, miscellaneous information, such as binding sites and post-translational modifications, was derived mainly from UniProt annotations, and structural domains were assigned by homology searches.

### Proteins stored in IDEAL

As a starting point for the annotation, we chose UniProt human nuclear proteins with PDB structures (712 proteins), because eukaryotic nuclear proteins are known to contain long IDRs (18,19). We have annotated more than 120 human nuclear proteins. Out of them, the overlap with DisProt is only one-third at most, indicating IDEAL and DisProt complement each other. Most of the PDB structures for these proteins are hetero-oligomers in which the protein was associated with its binding partners. Annotations for these partner proteins are also in IDEAL, regardless of the source organism or the presence or absence of IDRs.

### Ordered/disordered regions

The most important part of the IDEAL annotation is to identify the ordered and disordered amino acid segments. Ordered regions can be assigned by referring to the PDB. It is not straightforward to identify the disordered regions. In IDEAL, disordered regions are judged using several criteria; (i) missing residues in the X-ray structures, (ii) regions that interfere with protein crystallization in X-ray experiments, (iii) regions that fluctuate greatly in ensemble number of NMR model structures and (iv) regions that have been shown to be flexible in experiments using NMR, CD and other methods, and that have no corresponding structures in PDB. Of the four categories, (i) can be automatically obtained from PDB. Regions that were identified using the other three categories could only be judged manually. Although fluctuating regions in category (iii) could be found automatically by comparing the PDB coordinates of a group of models, the regions were only accepted as IDRs after curators confirmed the fluctuations by examining the corresponding literature. Category (iv) requires the most laborious procedure to be obtained, but provides variable information. Curators conduct manual literature searches to obtain such information as much as possible.

### Protean segment

One of the reasons why IDPs have drawn so much attention is the discovery of the phenomenon known as coupled folding and binding in which a short flexible segment binds to its binding partner by forming a specific structure which acts as the molecular recognition element (10–16). In IDEAL, we explicitly annotated this short flexible region as ProS when both unstructured and structured information is available for the region. We defined two categories for ProS, verified ProS and possible ProS. A verified ProS is a sequence for which there is evidence of both a disordered isolated state and an ordered binding state. A possible ProS is a sequence for which there is only evidence of an ordered

binding state, but circumstantial evidence suggests that the sequence is disordered in the isolated state. A possible ProS is, for example, a sequence from a protein whose homolog contains a verified ProS in the corresponding position. Another example would be the one in which the binding partner of a possible ProS binds a verified ProS using the same interface.

Sequences involved in coupled folding and binding have been addressed in several ways, for example, molecular recognition features (MoRFs) (20) and eukaryotic linear motifs (ELMs) (21) have been studied. Although ProS, MoRF and ELM are similar concepts, MoRF has a length limitation of 70 residues and an ELM should have a motif that can be described in a regular expression. On the other hand, the definition of ProS depends only on evidence of a disorder-order transition. Although most ProSs bind to a partner protein, by its definition, ProS can include IDRs whose structures are induced upon binding to small ligands. ProSs do not necessarily assume secondary structures in the binding state, and long IDRs or IDRs without a motif can also be ProSs. Some relatively long IDRs, such as p27Kip1 (PDB:1jsu) and Tcf3 (PDB:1g3j), can transform into ordered states (22). ProSs can also cover these IDRs.

### MISCELLANEOUS INFORMATION

We integrate the miscellaneous information from UniProt, namely, regions interacting with other molecules, motifs and post-translational modifications. During the annotation process, the curators find interaction sites, sequence motifs or other information that has not been described in UniProt, the new information is included in IDEAL. IDEAL also provides SCOP (version 1.75) and Pfam (23) (version 24.0) domain assignments using reverse PSI-Blast (24) and HMMer (25). Note that ordered regions assigned in the order/disorder annotation process are experimentally verified ordered regions, while the structural domain assignments were done using homology searches.

### USING IDEAL

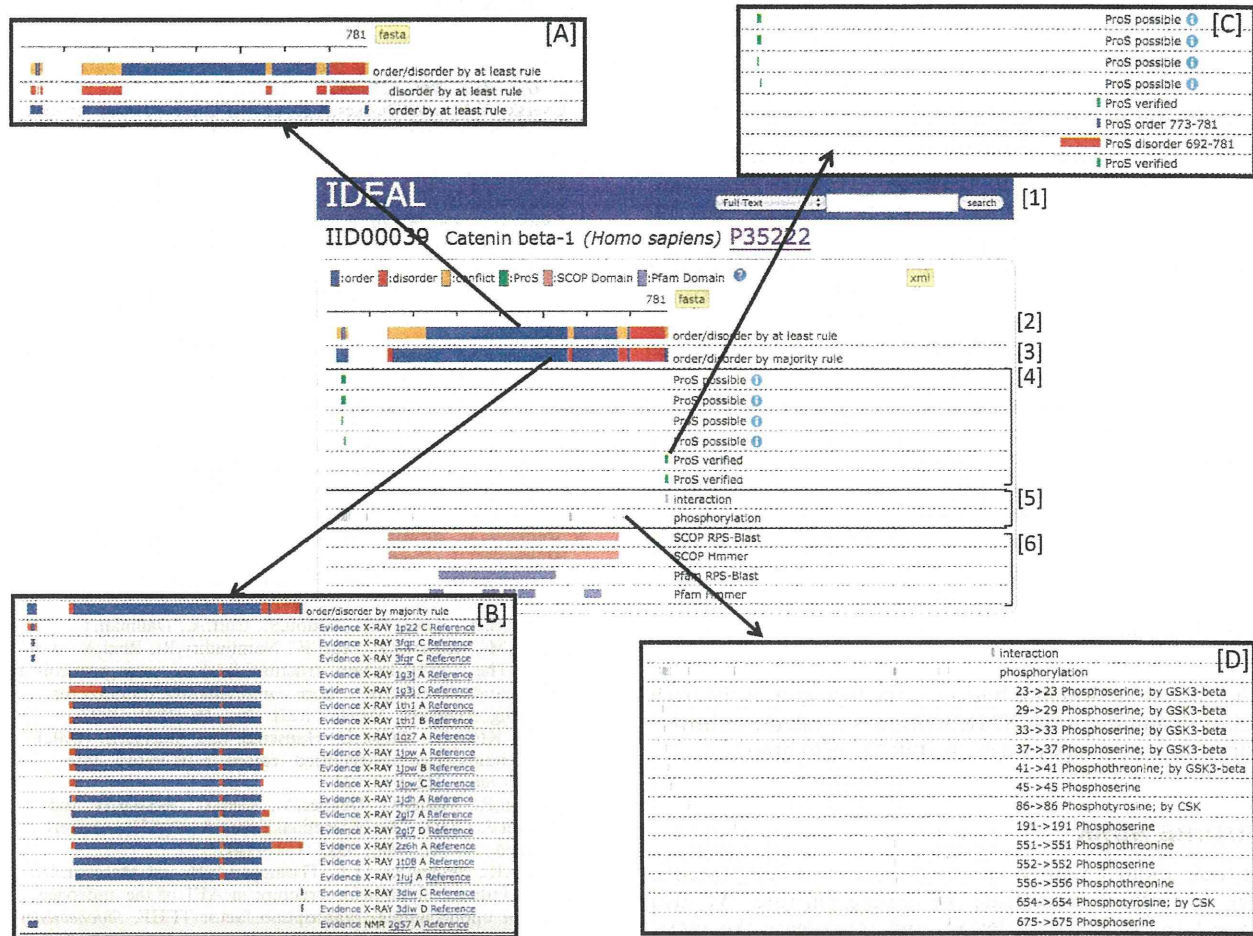
#### Browse and search entries

'The list' on the top page of IDEAL provides an easy way to access any of the entries in IDEAL. The list enumerates all entries in IDEAL, where IID, protein name, organism, total sequence length and the presence/absence of ProS are tabulated. IDEAL also provides a search tool, which always appears in the blue bar at the top of each page ([1] in Figure 1). Users can choose from 'Full text', 'UniProt accession', 'Protein Name' and 'PDB id' categories, and enter some words or an ID into the input field. The BLAST search is available through the 'BLAST search' link button, and the user can input an amino acid sequence to find homologs in the IDEAL entries.

#### Representation of each entry

IDEAL provides a user-friendly web interface for each entry. An example, a page for catenin  $\beta$ -1, is shown





**Figure 1.** IDEAL annotation for catenin  $\beta$ -1. The identifier, IID, protein name, source organism and the link to UniProt are shown below the blue bar which contains the search tool ([1]). Bars [2] and [3] show a summary of the annotated regions using the ‘at least rule’ and ‘majority rule’ criteria. Ordered, disordered and conflict regions are colored in blue, red and orange, respectively. The inner boxes (A) and (B) show a detailed breakdown of the regions in [2] and [3], respectively. These diagrams appear by clicking on the bars. The inner box (B) provides the link to the experimental evidence and the technique supporting the order/disorder regions. ProSs are represented in section [4] as the green bars, which can be expanded by clicking to reveal the inner box (C). Interaction sites and post-translational modification sites are shown in section [5]. These bars expand to show the detailed information in the inner box (D). The bars in section [6] show the domain assignments.

in Figure 1. The annotated regions are presented in a bar diagram to help make annotations intuitively understandable. Two color bars at the top ([2] and [3] in Figure 1) summarize ordered/disordered information in the distinctive ways shown. A protein may have multiple PDB entries and other information without accompanying PDB entries from different experimental techniques such as CD, H/D exchange, etc. Because IDEAL contains all PDB entries together with the other structural information associated with a query protein, all the associated information are not necessarily consistent due to different experimental conditions and other reasons. To summarize these diverse situations, IDEAL uses two representations:

(1) The bar [2] in Figure 1 shows the summary of ordered/disordered regions by the ‘at least rule’. Here an ordered (blue bar) or disordered (red bar)

site is shown if the site has at least one ordered or one disordered annotation. When a single site has both an ordered and a disordered annotation, the site is in ‘conflict’ (orange bar). The inner box [A] in Figure 1, opened by clicking the bar, shows the detailed breakdown of the annotations. The first and the second bars correspond to the at least ordered regions, and the at least disordered regions, respectively. All of the data sources supporting each of order/disorder regions can be presented by clicking the ‘majority rule’ bar explained below.

(2) The bar at [3] shows the summary of ordered/disordered regions by the ‘majority rule’, in which majority decision is employed to show the annotation. The inner-box (B), opened by clicking the bar, shows all the evidence of annotations used to in the majority vote. They include ordered and disordered

regions derived from the literature and PDB structures. The experimental methods supporting the order/disorder regions ('X-ray', 'NMR', etc) are also shown together with the links to the PubMed Abstracts ('Reference').

A unique feature of IDEAL is the explicit description of IDRs with the ability to undergo structural transformation, the ProSs, which are shown by the green bars ([4] in Figure 1). Each of the bars expands by a click to show the ordered and disordered regions that account for the 'verified ProS' status [inner box (C)]. For 'possible ProS', only ordered regions are presented. Note that a verified ProS should match one of the conflict regions in the bar at [2].

Below the ProS annotation, the miscellaneous information from UniProt, is summarized. These bars can be clicked on to open up the detailed information shown in box [D]. The results of the domain assignment ([6] in Figure 1) show the SCOP and Pfam domains identified by the reverse PSI-Blast and HMMer. The bars show a summary of the results and expand to show the details.

#### The XML files

The XML files are provided and can be downloaded by clicking on the xml link button at the top right of the page [2]. A definition of the XML schema is available at <http://idp1.force.cs.is.nagoya-u.ac.jp/IDEAL/help.html>.

#### FUTURE WORK

It took about 1 year to annotate more than 120 proteins. We now plan to accelerate the annotation rate. We also expect to collect more ProSs, and investigate the interaction mechanism of the ProS. To do this, we aim to develop an interface showing the binding partner proteins associated with ProSs and to illustrate their interaction networks. As in any databases, updating the contents is a key issue. We will address this by developing an update system to keep information in IDEAL as current as possible.

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# Effect of Ca<sup>2+</sup> on the microtubule-severing enzyme p60-katanin

## Insight into the substrate-dependent activation mechanism

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### Keywords

AAA ATPase; heteronuclear NMR; microtubule-severing enzyme; molecular modeling; three-dimensional domain rearrangement

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Katanin p60 (p60-katanin) is a microtubule (MT)-severing enzyme and its activity is regulated by the p80 subunit (adaptor-p80). p60-katanin consists of an N-terminal domain, followed by a single ATPase associated with various cellular activities (AAA) domain. We have previously shown that the N-terminal domain serves as the binding site for MT, the substrate of p60-katanin. In this study, we show that the same domain shares another interface with the C-terminal domain of adaptor-p80. We further show that Ca<sup>2+</sup> ions inhibit the MT-severing activity of p60-katanin, whereas the MT-binding activity is preserved in the presence of Ca<sup>2+</sup>. In detail, the basal ATPase activity of p60-katanin is stimulated twofold by both MTs and the C-terminal domain of adaptor-p80, whereas Ca<sup>2+</sup> reduces elevated ATPase activity to the basal level. We identify the Ca<sup>2+</sup>-binding site at the end of helix 2 of the N-terminal domain, which is different from the MT-binding interface. On the basis of these observations, we propose a speculative model in which spatial rearrangement of the N-terminal domain relative to the C-terminal AAA domain may be important for productive ATP hydrolysis towards MT-severing. Our model can explain how Ca<sup>2+</sup> regulates both severing and ATP hydrolysis activity, because the Ca<sup>2+</sup>-binding site on the N-terminal domain moves close to the AAA domain during MT severing.

### Structured digital abstract

- [p80-CTD binds to p60-vMIT](#) by [pull down](#) ([View interaction](#))
- [p80-CTD and p60-vMIT bind](#) by [nuclear magnetic resonance](#) ([View interaction](#))

## Introduction

Katanin, spastin and fidgetin are three major microtubule (MT)-severing enzymes [1–5]. Katanin is a hetero-

dimeric ATPase composed of two subunits, p60 (p60-katanin) and p80 (adaptor-p80). p60-katanin is the

### Abbreviations

AAA, ATPase associated with various cellular activities; GST, glutathione S-transferase; MBP, maltose binding protein; MIT, microtubule interacting and trafficking; MT, microtubule; p60-vMIT, variant MIT domain of p60-katanin; p80-CTD, C-terminal domain of adaptor-p80.