Purification and Characterization of Hrp1, a Homolog of Mouse CHD1 from the Fission Yeast *Schizosaccharomyces pombe*

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Hrp1, of *Schizosaccharomyces pombe*, is a new member of the SWI2/SNF2 protein family that contains a chromodomain and a DNA binding domain as well as ATPase/7 helicase domains. This configuration suggests that Hrp1 could be a homolog of mouse CHD1, which is thought to function in altering the chromatin structure to facilitate gene expression. To understand the enzymatic nature of Hrp1, we purified the 6-Histidine-tagged Hrp1 protein (6×His-Hrp1) to homogeneity from a *S. pombe* Hrp1-overexpressing strain and then examined its biochemical properties. We demonstrate that the purified 6×His-Hrp1 protein exhibited a DNA-binding activity with a moderate preference to the (A+T)-rich tract in double-stranded DNA via a minor groove interaction. However, we failed to detect any intrinsic DNA helicase activity from the purified Hrp1 like other SWI2/SNF2 proteins. These observations suggest that the DNA binding activities of Hrp1 may be involved in the remodeling of the chromatin structure with DNA-dependent ATPase. We propose that Hrp1 may function in heterochromatins as other proteins with a chromo- or ATPase/helicase domain and play an important role in the determination of chromatin architecture.

More than thirty proteins with comprehensive amino acid sequence similarity to SWI2/SNF2 have been grouped into a protein family. This family contains diverse proteins that have various intracellular functions (Bork and Koonin, 1993; Eisen et al., 1995), such as transcriptional regulation (SNF2, BRM and MOT1), maintenance of chromosome stability during mitosis (lodestar), and various aspects of processing DNA damages (RAD5, RAD16, RAD26, ERCC6 and RAD54). Every protein in the SWI2/SNF2 family has a conserved region of about 60 kDa that includes consensus motifs required for the binding and hydrolysis of ATP (Henikoff, 1993). Furthermore, it has been proposed that these proteins, with the exception of RAD5, may function as ATP-driven motors that translocate along DNA and destabilize protein-DNA interactions (Eisen et al., 1995; Pazin and Kadonaga, 1997).

In the SNF2/SWI2 family, CHD1 was identified as a mammalian DNA-binding protein that contains three signature sequence motifs. The motifs include a chromodomain found in the heterochromatin protein HP1 and the homeotic repressor Polycomb, the ATPase/helicase domain conserved in SWI2/SNF2 proteins with diverse functions, and DNA binding motifs characteristic of minor-groove binding proteins histone H1, HMgI/Y, D1 and datin (Delmas et al., 1993; Stokes and Perry, 1995). CHD1 shows a sequence-selective preference for binding to (A+T)-rich tracts *in vitro* (Richmond and Peterson, 1996). Homologs of the CHD1 protein have been reported in *Drosophila*, bird, mouse, and human (Starr and Hawley, 1991; Stokes et al., 1996; Griffths and Korn, 1997; Woodage et al., 1997). However, biochemical and genetic analyses of the CHD1 protein have not yet been reported.

In this work, we show that Hrp1, a homolog of CHD1 from the fission yeast, has a DNA-binding activity with preference of (A+T) rich tract as shown in a mouse CHD1. This result may provide a primary *in vitro* assay system to investigate a requirement of DNA-binding activity and DNA-dependent ATPase for the alteration of nucleosome or chromatin structure in a way that facilitates gene expression.

Materials and Methods

Strains and media

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Haploid *Schizosaccharomyces pombe* strain JY746 was provided by Dr. Yamamoto (University of Tokyo, Japan). The Hrp1 overproducing strain, JYK672 (*h*$^+$ *ade6-M216 leu1-32 ura4-D18 hrp1::nmt1-6(His)-hrp1*$^+$), was constructed as described in Jin et al. (1998). Transformation of *S. pombe* was performed by the lithium method (Ito et al., 1983). *S. pombe* cells were grown in minimal medium (MM) supplemented with appropriate amino acids (Alfa et al., 1993).

Preparation of 6His-tagged Hrp1

The Hrp1 overexpressing cells, JYK672, grown in one liter of TMM (MM with 1 mM of thiamine) to an OD_{595} of 2.0 were harvested and washed three times with fresh MM. After dilution to an OD_{595} of 0.1 into 15 liters of fresh MM, cells were further cultured at 30℃ for 12 h, collected and washed with extraction buffer (20 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 10% glycerol, pH 8.0). The cells were lysed in 150 ml of extraction buffer using glass beads with 6×30 sec pulses on a BioSpec Products beadbeater in the presence of some inhibitors (0.5 μg/μl leupeptin, 0.7 μg/μl pepstatin A, 35 μg/μl PMSF and 1 μg/μl aprotinin). The cell extracts were then clarified by centrifugation for 2 h at 43 K in a Beckman Ti70 rotor. The supernatant (whole cell extract, WCE) was bound batchwise with 20 ml of Ni^{2+}-NTA agarose (Qiagen) for 2.5 h at 4℃. The resin was poured into a column and washed sequentially with 300 ml of extraction buffer, and 100 ml of extraction buffer with 10 mM imidazole. The proteins were then eluted with 100 ml of extraction buffer with 500 mM imidazole. Peak fractions (fractions 6 - 10, 20 ml) were pooled and dialyzed against equilibration buffer (20 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, pH 8.0) and loaded onto an DEAE Sepharose column (2×15 cm). After washing the column, bound proteins were eluted with 50 ml of linear salt gradient (100 mM to 400 mM NaCl) in equilibration buffer. A single peak of ATPase activity appearing at about 250 mM NaCl was pooled (30 ml) and then concentrated to 2 ml using an Amicon concentrator (Amicon). The concentrated sample was applied onto a Sephacryl S-200 column (4×60 cm) at 0.2 ml/min. Peak fractions containing Hrp1 were pooled (fractions 14 - 17, 16 ml) and stored for further use.

ATPase activity assay

Standard reaction mixtures (20 μl) contained 40 ng of Hrp1 in 20 mM Tris-HCl (pH 7.0), 5% glycerol, 0.05% Tween-20, 30 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.5 mg/ml BSA, 1 mM ATP, 1 μCi of [γ-^{32}P] ATP (3000 Ci/mmole, Amersham), and 100 ng of pBluescript II KS(+) double-stranded DNA. After incubation at 30℃ for 15 min, an aliquot (1 μl) was spotted onto a polyethyleneimine-cellulose plate (MERCK) and developed in 0.5 M LiCl, 1 M formic acid. The products were analyzed using a Bio-Imaging Analyzer BAS-1500 (Fujifilm).

Preparation of dsDNA substrates for the helicase assay

The standard, ds DNA substrate, used to assay the DNA helicase activity, was prepared by annealing two oligomers as follows: the 98-mer (upper strand), 5′-GAATACAAGCTTGGGCTGCAGGTCGACTCTAGAGGA TCCCCGGGCGAGCTCGAATTCGGGTCTCCCTATAGT GAGTCGTATTAATTTCGATAAGCCAG-3′, and the 38-mer (lower strand), 5′-GAATACACGGAATTCGAGCTC GCCCGGGGATCCTCTAG-3′. The flush-ended duplex DNA was also prepared by annealing two oligomers as follows: the 30-mer (upper strand), 5′-AGAGTCGA CCTGCAGCCCAAGCTTGTATTC-3′, and the 30-mer (lower strand), 5′-GAATACAAGCTTGGGCTGCAGGTC GACTCT-3′. With two substrates, the lower DNA strands were end-labeled with [γ-^{32}P] ATP. In most cases, the specific activity of the labeled substrates was 1.4-1.5× 10^3 cpm/fmol of dsDNA substrate. For annealing, two oligomers were mixed together in 0.2 ml of hybridization buffer (20 mM Hepes-KOH, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS, pH 7.4). The mixture was heated at 100℃ for 5 min and then incubated at 65℃ for 30 min followed by cooling slowly for 12 h to room temperature (RT). The dsDNA was then separated by electrophoresis on a native 8% polyacrylamide (30:1) gel and eluted from the gel. The gel slice containing dsDNA was ground with a micropestle in 0.4 ml of elution buffer (0.5 M ammonium acetate, pH 7.0, 0.1% SDS, 10 mM EDTA) and incubated for 2 h at RT. After a brief centrifugation, the supernatants were extracted with phenol/chloroform (1:1, v/v), chloroform and then treated with 2.5 volumes of cold absolute ethanol. After 1 h at -70℃, the substrate was collected by centrifugation at 4℃ for 15 min. The pellet was dried under vacuum. The purified dsDNA substrates were adjusted to a final concentration of 50 fmol/μl in a solution containing 20 mM Hepes-KOH (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 0.05% Nonidet P-40 and stored in 50 μl aliquots at -20℃.

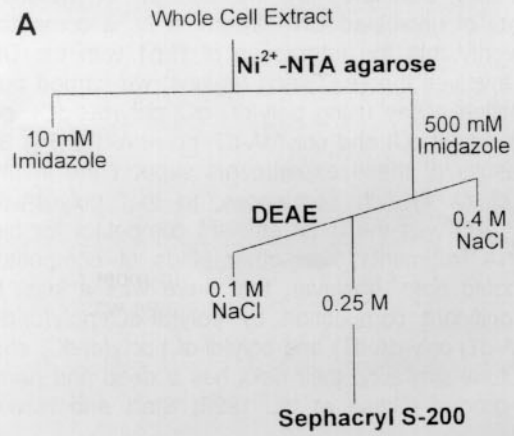
DNA helicase assay

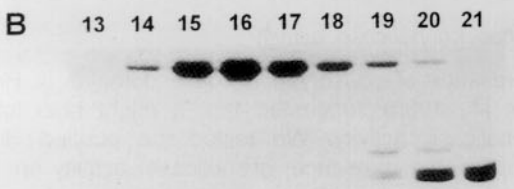
DNA helicase activity was assayed according to Lee et al. (1997). The measurement of helicase activity was carried out in a reaction mixture (20 μl) containing 20 mM Hepes-KOH (pH 7.4), 2 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mg/ml BSA, 50 fmol of dsDNA substrate and various amounts of the Hrp1 protein. Mixtures were incubated for 30 min at 30℃, and then stopped by the addition of 5 μl of a mixture (5×) containing 0.1 M Tris-Cl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol. Aliquots (10 μl) of each reaction were loaded onto a native 8% polyacrylamide (30:1) gel and electrophoresed at 15 mA for 1.5 h. The gel was then vacuum dried on Whatman DE81 paper, and the dsDNA substrate and the displaced single-

stranded DNA(ssDNA) product were visualized by autoradiography.

Gel electrophoresis mobility shift assay (EMSA)

DNA binding assay was performed in a reaction mixture (20 μl) containing 20 mM Hepes-KOH (pH 7.4), 2 mM DTT, 0.2 mg/ml BSA, 100 mM NaCl, 5% glycerol, 50 fmol of ^{32}P-labelled-DNA probe (5′-GGATCCATAT ATATATATATATATATATATGCAAGCT-3′) and the indicated amount of Hrp1. After 30 min of incubation at 30℃, the complexes formed were analyzed by 4% polyacrylamide gel in 0.5 TBE.





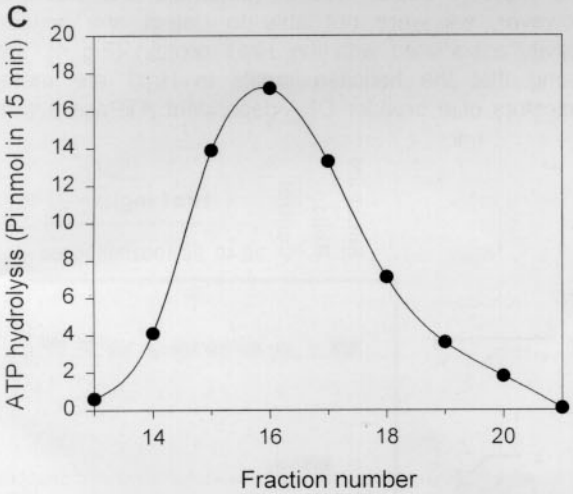


Fig. 1. Purification of Hrp1 protein. A, Purification flow chart for over-expressed Hrp1 protein. The peak fractions from DEAE-Sepharose eluate were pooled, concentrated and loaded onto a Sephacryl S-200 gel filtration column. B, Eluents were collected (4 ml × 40) and each fraction (50 μl) was subjected to SDS-PAGE (8%) and the gel was stained with Coomassie brilliant blue. The fraction numbers (13 - 21) are indicated at the top of the figure. The sizes of marker proteins are 158, 116, 97.2, 66.4, 55.6, 42.7, 36.5, and 26.6 kDa. C, The ATPase activity coeluted with the protein (1 μl, the same fractions as in figure B) under standard conditions as described in Materials and Methods.

Table 1. Purification of Hrp1 ATPase from *S. pombe* extracts

Fraction	Volume (ml)	Concentration (mg/ml)	Total protein (mg)	Total units[a]	Specific units (units/mg)
WCE	200	18.84	3768.0	ND[b]	ND
Ni^{2+} eluate	20	38.87	777.4	1217.8	1.6
DEAE	2	68.25	182.5	965.0	5.3
Sephacryl	16	0.27	4.4	436.2	99.5

[a]One unit of ATPase activity is defined as the amount of enzyme that hydrolyzes 1 μmol of ATP after 30 min incubation period at 30℃.
[b]Not determined

Results

Purification of Hrp1 protein

To examine the biochemical and biophysical properties of the Hrp1, we purified the protein from a yeast strain (JYK672) overexpressing the 6 × His-Hrp1.

The scheme toward purification of Hrp1 protein is summarized in Fig. 1A. The peak fractions from Sephacryl S-200 gel filtration chromatography were subjected to SDS-PAGE (Fig. 1B) and then Western blot analysis using affinity purified anti-Hrp1 and anti-6 × Histidine monoclonal antibody (data not shown). Analysis of ATPase activity on each fraction was proportional to the intensity of the 159 kDa Hrp1 protein band (Fig. 1C). Thus, the corresponding fractions (14 -

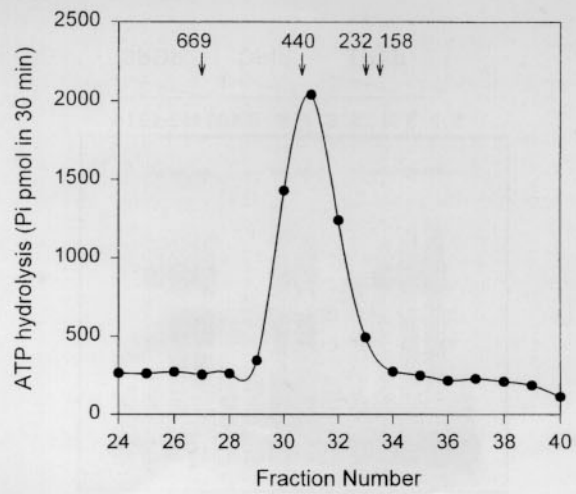


Fig. 2. Gel filtration of the Hrp1 on Superose-6 column. To determine the size of Hrp1 protein under native conditions, the Hrp1 (200 μg) obtained from the Sephacryl S-200 column of Fig. 1 were chromatographed on a Superose-6 column (1 × 30 cm) equilibrated with buffer A (20 mM Tris-Cl, pH 7.8, 5 mM $MgCl_2$, 100 mM NaCl). Fractions of 0.5 ml were collected at a flow rate of 20 ml/h, and an aliquot (3 μl) was assayed for the Hrp1 ATPase activity under standard conditions described in Materials and Methods. The size markers used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) as indicated with arrows.

17) shown in Fig. 1B were pooled and used in further experiments. The three-step purification yielded a nearly homogeneous Hrp1 protein and increased 62-fold in ATPase specific activity over that present in the Ni^{2+}-NTA agarose pooled fractions (Table 1).

To determine the size of purified Hrp1 under non-denaturing conditions, the pooled Hrp1 (200 g) was subject to gel filtration on a Superose-6 column. Fig. 2 shows that the peak ATPase activity of Hrp1 was eluted in the fractions corresponding to about 330 kDa. This result suggests that Hrp1 protein may form a homodimer under native conditions, considering that the size of Hrp1 under denatured conditions is 159 kDa.

Hrp1 is a DNA binding protein with a moderate preference to (A+T) rich tract

The mouse CHD1, a homolog of Hrp1, is a DNA binding protein, which binds to the minor groove of (A+T)-rich DNA (Stokes and Perry, 1995). Thus, we examined whether Hrp1 has the activity to interact with ds oligonucleotides. As shown in Fig. 3A, the Hrp1 protein does indeed bind to the DNA fragment, as evidenced by the formation of a single retarded band. The amount of binding activity increased with increasing amounts of protein and the binding activity was sequentially eliminated by the addition of increasing amounts of unlabeled DNA fragment as a competitor.

To verify that the interaction of Hrp1 with the DNA probe involves the (A+T)-rich regions, we carried out a competitive assay using poly(dG-dC)·poly(dG-dC), poly (dI-dC)·poly(dI-dC) and poly(dA-dT)·poly(dA-dT) (Fig. 3B). The results of these experiments support the involvement of (A+T) rich sequences, in that poly(dA-dT)·poly(dA-dT) was the most efficient competitor for binding DNA fragments than other kinds of competitors. We should note, however, that there was a less, but still significant competition by poly(dI-dC)·poly(dI-dC). Poly(dA-dT)·poly(dA-dT) and poly(dI-dC)·poly(dI-dC) share a structural similarity: their helix has a deep and narrow minor groove (Giese et al., 1992; Starr and Hawley, 1991).

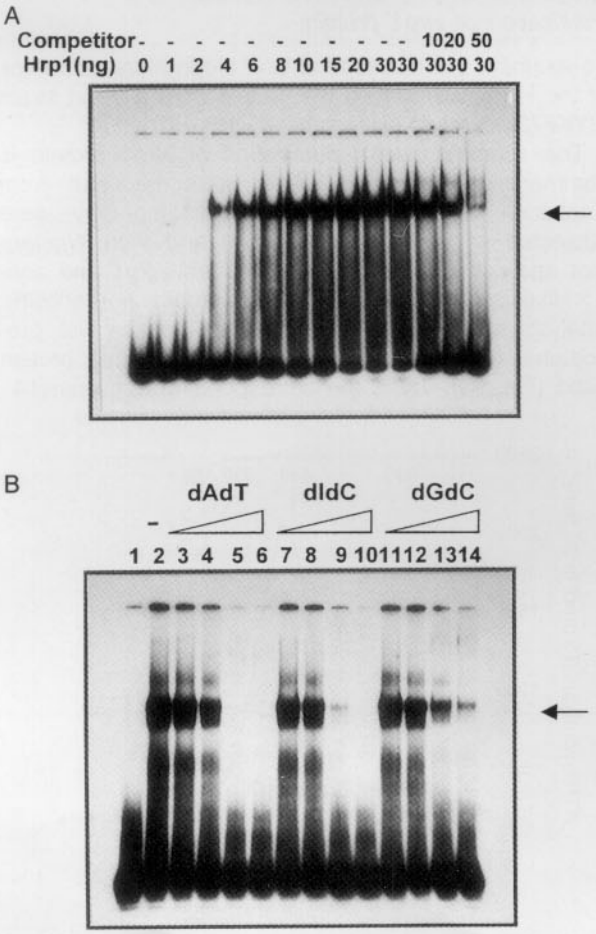


Fig. 3. DNA binding of Hrp1. As described in Materials and Methods, an EMSA was carried out with a ^{32}P-labeled dsDNA probe (50 fmol per lane). A, The numbers at the top of the figure indicate the fold molar excess of proteins to the DNA probe. The competitor is the cold flush-end duplex DNA. B, Binding competition EMSA with different polynucleotides. Lane 1 contained probe alone and lane 2 contained no competitor. Lanes 3 through 6, 7 through 10 and 11 through 14 contained increasing amounts (1, 2, 10 and 20 ng) of indicated cold polynucleotides. The bound DNA-protein complexes are indicated with arrows.

Hrp1 has no helicase activity

The presence of conserved helicase domains in Hrp1 (Jin et al., 1998) suggested that it might also have DNA helicase activity. We tested the purified Hrp1 protein for the presence of helicase activity in an oligonucleotide release assay using two types of substrates: an annealed oligonucleotide with flushed-ends and standard duplex dsDNA (Materials and Methods). However, we were not able to detect any helicase activity associated with the Hrp1 protein (Fig. 4), indicating that the helicase motifs in Hrp1 are merely indicators of a broader DNA-dependent ATPase activity.

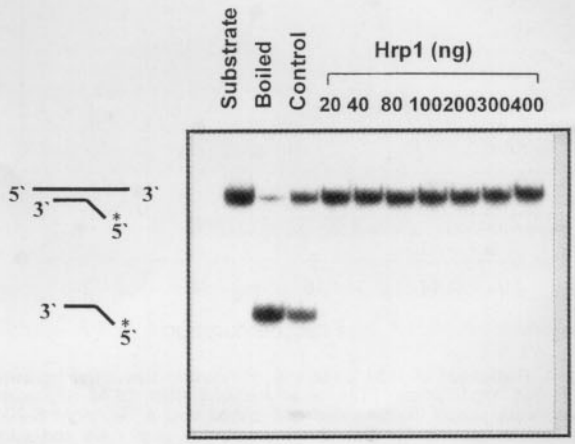


Fig. 4. DNA helicase activity assay. The standard helicase assay was performed as described in Materials and Methods with amount of Hrp1 protein indicated. However, no helicase activity was detected. The RHA (20 ng) helicase protein was used in a control lane.

Discussion

In this report, we present the purification and the enzymatic activities of the Hrp1 protein and also discuss their implication for *in vivo* function. Until now, there are no results reporting on the purification of the CHD1 protein family, which are essential for the elucidation of *in vivo* functions of the protein. We purified the Hrp1 protein and provided the first analysis of its enzymatic activities. The high conservation of amino acid sequence in the SNF2 domain has led to much speculation about whether any particular biochemical activity is shared by all members of the SWI2/SNF2 family.

First, we purified 6His-tagged Hrp1 from a Hrp1-overexpressing strain to near homogeneity. We suggested the dimerization of Hrp1 protein in native conditions. Although we did not show the complex formation of Hrp1 protein with other proteins, preliminary unpublished evidence suggested that CHD1 is a component of a ~650 kDa complex (Eisen et al., 1995). Thus, it may be safe to say that Hrp1 may also exist as a multiprotein complex in the nucleus and we are currently investigating the component polypeptides of the complex.

We also found that Hrp1 is capable of preferentially binding to ds DNA via minor groove interactions. Together with other results indicating that Hrp1 is a nuclear protein which has DNA-dependent ATPase activities (unpublished data), this observation provides a possibility that Hrp1 may be one of the proteins that determine the architecture of chromatin.

The presence of the helicase motifs in the SNF2 domain has been used to suggest that the helicase activity is conserved. But helicase activity *in vitro* has never been detected in any of the proteins in the SWI2/SNF2 family (Auble et al., 1994; Côte et al., 1994), even though it was proposed that this activity is needed for processes (i.e. transcription, recombination and DNA repair) in which these proteins are known to be involved. We also failed to detect any helicase activity in the Hrp1 protein. So we suggest a possible explanation for that as follows. Henikoff proposed that the SWI2/SNF2 family proteins are not helicases and that the helicase motifs indicate broader, DNA-dependent ATPase activity, of which helicase activity is a subset. Consistent with this proposal, the Hrp1 protein, as well as SWI2/SNF2, STH1, ISWI, MOT1 and RAD5, have no detectable helicase activities. Despite his proposal, we cannot exclude a possibility that other cofactors are required for activation of dormant helicase activity in Hrp1.

Although the proteins in CHD1 subfamily are postulated to function in the remodeling of chromatin architecture, the direct evidence on this function has not been reported yet. The null and overexpressing strains indicated that Hrp1 is involved in chromosome segregation (unpublished data). Using these strains and Hrp1 protein purified from *S. pombe*, we are currently trying to understand the *in vivo* roles of the protein.

Acknowledgements

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