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Role of amino acid residues important for nucleic acid binding in human Translin

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ABSTRACT

Translin is a multifunctional DNA/RNA binding protein involved in DNA repair and RNA metabolism. It has two basic regions and involvement of some residues in these regions in nucleic acid binding is established experimentally. Here we report the functional role of four residues of basic region II, Y85, R86, H88, R92 and one residue of C terminal region, K193 in nucleic acid binding using substitution mutant variants. CD analysis of the mutant proteins showed that secondary structure was maintained in all the mutant proteins in comparison to wild type protein. Octameric state was maintained in all the mutants of basic region as evidenced by TEM, DLS, native PAGE and gel filtration analyses. However, K193G mutation completely abolished the octameric state of Translin protein and consequently its ability to bind ssDNA/ssRNA. The mutants of the basic region II exhibited a differential effect on nucleic acid binding, with R86A and R92G as most deleterious. Interestingly, H88A mutant showed higher nucleic acid binding affinity in comparison to the wild type Translin. An *in silico* analysis of the mutant variant sequences predicted all the mutations to be destabilizing, causing increase in flexibility and also leading to disruption of local interactions. The differential effect of mutations on DNA/RNA binding where octameric state is maintained could be attributed to these predicted disturbances.

1. Introduction

Translin is a 228 amino acids multifunctional protein with ability to interact with DNA as well as RNA. It was initially discovered during a search for DNA interacting proteins in human lymphoid malignancies with chromosomal translocations (Kasai et al., 1994). Translin is well conserved across eukarya and homologs in archaea have also been reported (Gupta et al., 2012). Information on the biochemical activities of Translin is generated mainly from Drosophila, Mouse (Testis Brain RNA binding Protein, TB-RBP) and Human orthologues. In addition, Translin protein from *Saccharomyces pombe*, *Neurospora crassa*, chicken, etc. have also been studied. Human and chicken Translin share 86% amino acid homology and shown to form ring like structures (Aoki et al., 1999).

Translin specifically interacts with TRAX (Translin Associated factor X), a 33 kDa protein which has extensive homology with Translin. Translin is a cytosolic protein and it utilizes the nuclear localization signal of TRAX to translocate into the nucleus (Chennathukuzhi et al., 2003; Kasai et al., 1997) under conditions that damage DNA. Localization of Translin into the nucleus in response to DNA damaging agents such as etoposide, mitomycin C etc. has been observed, indicating its involvement in DNA damage and repair process (Kasai et al., 1997). Translin-TRAX constitute the C3PO complex, which is involved in Dicer activation in RNAi mediated gene silencing (Wang et al., 2004). It is also involved in translational control and transport of mRNAs, especially in neurons (Wu et al., 1997; Chiaruttini et al., 2009). Binding studies with G rich oligonucleotides have indicated the involvement of Translin in recombination at microsatellites and telomeric regions (Jacob et al., 2004). Recent reports suggest Translin to play a role in sleep regulation in *Drosophila* (Murakami et al., 2016). Yet, it was reported to be non-essential for survival in fission yeast, *S. pombe* (Laufman et al., 2005; Jaendling et al., 2008). TB-RBP/Translin null mice are viable but have reduced fertility and behavioural abnormalities (Chennathukuzhi et al., 2003). Likewise, in *Drosophila*, Translin is not essential for survival, but is suggested to be involved in neuronal development leading to determination of behaviour (Suseendranathan et al., 2007). Diverse activities of Translin suggest that it is a multifaceted protein, working differently in different cell types. Taken together, although dispensable for viability, Translin is a crucial protein involved in processes linked to nucleic acid metabolism.

Abbreviations: CD, Circular Dichroism; DLS, Dynamic Light Scattering; ss, single strand; TEM, Transmission Electron Microscopy; Tsn, Translin

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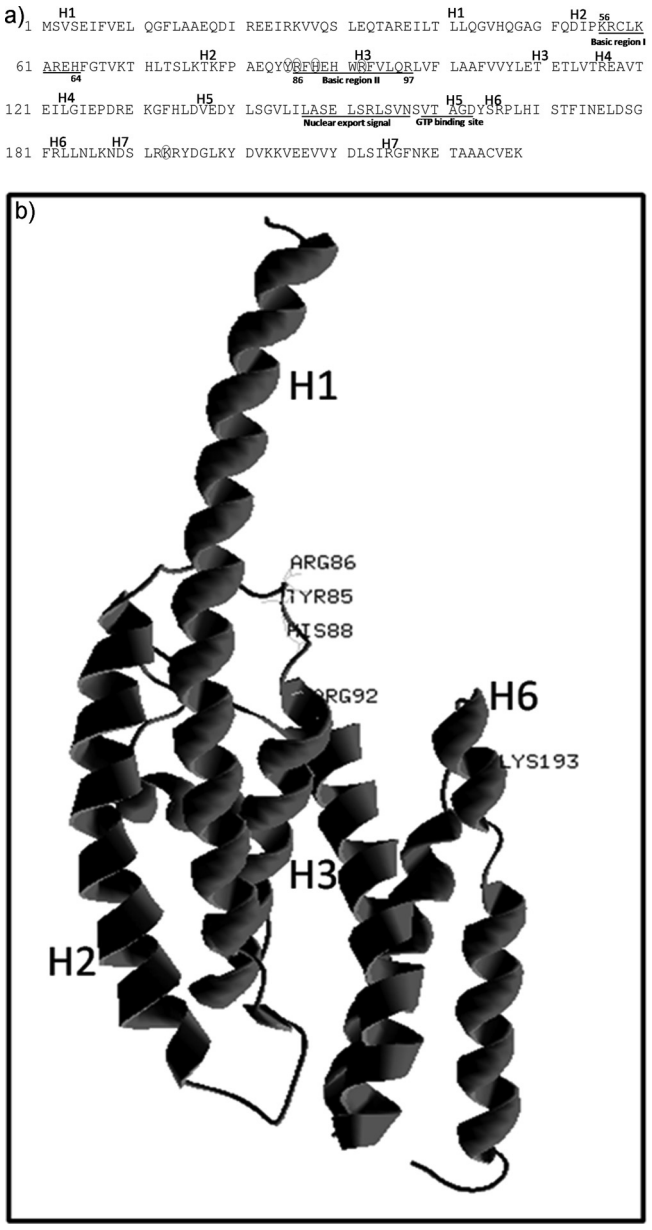

Fig. 1. Primary and secondary structural features of the human Translin protein.
Amino acid sequence of human Translin (HTsn) with the location of point substitution mutations generated in this study are circled. Also, amino acids forming alpha helices are shown as H1-H7. Starting and ending amino acid of each helix is marked with the respective helix number. Known functional regions are underlined. (b) Position of the amino acid residues studied in the present work are indicated on PDB ID 1j1j (VanLoock et al., 2001) structure of human Translin.

Sequence analysis predicted Translin to have two basic regions, basic region–I from 56 to 64 residues and basic region-II from residues 86–97 (Aoki et al., 1999), a putative GTP binding domain 'VTAGD', towards C-terminal half of the protein and hypothetical leucine zipper motif towards the C-terminus. GTP specific conformational changes have been observed in human Translin and attenuation of Translin-nucleic acid interaction upon addition of GTP indicates possible role of GTP as a molecular switch (Sengupta et al., 2006).

Bioinformatic and mutational analysis have revealed certain regions on amino acid sequence of Translin that are critical for its function. Amino acids 86–97 of the basic region-II are found to be important for the formation of DNA binding domain in the Translin multimer (Aoki et al., 1999), although information on dispensability of single amino acids in this region is not available. Deletion of leucine zipper motif abolishes DNA/RNA binding activity of Translin. In addition, bioinformatic and experimental evidence showed the role of some of the residues in RNA binding for *S. pombe* Translin (Eliahoo et al., 2010). Based on this data and conservation between human and *S. pombe* Translin, equivalent residues for human Translin were predicted. In the present study, we selected five residues of human Translin protein (Fig.1), based on the equivalent residues of *S. pombe* Translin, which were experimentally shown to be critical for its RNA binding activity. We therefore generated point substitution mutant variants of human Translin, Y85A, R86A, H88A, R92G and K193G. The effect of these mutations on nucleic acid binding, secondary and quaternary structure structural integrity (by *in-silico* analyses) is reported here. The study provides experimental evidence of the involvement of the above residues in affecting function, either by loss of octameric configuration or by altered ability to bind to nucleic acids. Human Translin is nearly identical with difference in only three amino acids with mouse Translin and they share ~90% similarity at nucleotide level (Wu et al., 1997). Therefore, we presume that the information thus generated in this study will also applicable to TB-RBP.

2. Material and methods

2.1. Materials

Full length human Translin cDNA cloned into pQE9 was obtained as a gift from Dr. Gagan Deep Gupta, BARC, Mumbai and used as template to generate the point mutant variants. The sequence of the oligonucleotides used in this study is mentioned in Table 1. These were synthesized from MWG Biotech Private Limited, Bengaluru, India. Ni-NTA agarose was purchased from Qiagen. All the chemicals and media were purchased from Sigma-Aldrich and RNA oligonucleotide, RNA1 was synthesized from Metabion, Germany. All the bacterial culture work was carried out under aseptic conditions.

2.2. Methods

2.2.1. Cloning, over expression and purification of Translin proteins

Full length human Translin cDNA cloned into pQE9 was used as template for generation of point substitution mutant variants Y85A, R86A, H88A, R92G and K193G by site directed mutagenesis. Hotstart Q5 site directed mutagenesis kit (New England Biolabs) was used and manufacturer's instructions were followed to obtain the mutant plasmids in *E. coli* DH5α. The inclusion of the desired mutation in resulting plasmids was confirmed by their DNA sequencing. Wild type and mutant proteins expression, analyses and purification was carried using sequence confirmed *E. coli* DH5α clones according to Chittela et. al (Chittela et al., 2014). Cell pellets from 100 ml of over-expressing cultures were suspended in 25 ml of lysis buffer (25 mM Tris − HCl, pH 7.8,

Table 1
List of oligonucleotides used in the study.

Oligonucleotide	Sequence (5'–3')	Purpose
BCL-CL1	GCCCTCCTGCCCTCCTTCCGCGGG	Oligonucleotide for DNA binding
RNA 1	UGGUGGUGGAUGCAGAGAUC	Oligonucleotide for RNA binding

1000 mM NaCl, 1.0 mM dithiothreitol, protease inhibitor cocktail) and lysed by freeze thawing followed by sonication. The lysate was centrifuged at 11,000 × *g* for 45 min at 4 °C and the resulting clear supernatant of cell free crude extract was mixed with 1.0 ml Ni-NTA agarose resin pre-equilibrated with equilibration buffer (25 mM Tris − HCl, pH 7.8, 1000 mM NaCl, 5.0 mM imidazole). After incubation of at least 3.0 h under slow mixing condition at 4.0 °C, the mixture was packed in to 10 ml column and flow through (FT) was collected. Column was washed with 10 ml of wash buffer (W) (25 mM Tris − HCl, pH 7.8, 1000 mM NaCl, 20.0 mM imidazole). Elution of the bound protein was carried out by passing 5 ml of elution buffer (E) (25 mM Tris − HCl, pH 7.8, 1000 mM NaCl, 200 mM imidazole) through the column. Imidazole was removed by dialysis against Tris buffer (25 mM Tris − HCl, pH 7.8) containing 100 mM NaCl for 8.0 h at 4 °C. Proteins were analyzed by SDS PAGE at every step and estimated.

2.2.2. SDS/Native PAGE and western blot analysis

SDS-PAGE analysis of purified proteins was carried out using 12% acrylamide gels (Laemmli, 1970). For native PAGE analysis, about 25 µg of the purified proteins were resolved on 8% native PAGE using modified SDS PAGE method. Sample and running buffer without SDS and DDT were used and electrophoresis was carried out at a constant voltage of 100 V at 4 °C. The proteins were visualized by staining the gel with Coomassie brilliant blue R-250. For western blotting, proteins resolved on SDS PAGE as above and were electro-blotted onto PVDF membrane. Blots were probed with the rabbit antiserum raised against human Translin.

2.2.3. Circular dichroism (CD)

Far UV CD spectra of the purified proteins was recorded using Biologic CD spectrometer. The spectra of 0.2 mg ml^{-1} protein samples were recorded at 25 °C from 200 to 260 nm. Average of three spectra was recorded and milli degree versus wavelength was plotted to observe the CD signatures of the Translin proteins. The CD spectra obtained were deconvoluted using K2D3 server (Louis-Jeune et al., 2012; http://www.ogic.ca/projects/k2d3/) (Caroline et al., 2012) for analysis of secondary structure content.

2.2.4. In-silico analyses

The effect of single point substitution mutations on human Translin protein was analyzed using "Dynamut" web server available at http://biosig.unimelb.edu.au/dynamut/ (Carlos et al., 2018). Human Translin crystal structure (PDB 1j1j) was used as a template to analyse the effect of the point substitution mutations. Pymol software was used for the preparation of figures.

2.2.5. Gel filtration

Gel filtration of the purified proteins was carried out using Superdex 200 column (column volume of 24 ml) on an Acta pure system (GE healthcare). The column was equilibrated with two column volumes of degassed buffer containing 25 mM Tris-HCl, pH 7.6 and 100 mM of NaCl. The molecular weight standards from GE health care (Cat No: 17044501) containing Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kda), Lactate dehydrogenase (140 kDa), Bovine serum albumin (66 kDa) were used to calibrate the column. Protein sample was injected in to the column and eluted with 1.5 column volumes of same buffer at a flow rate of 0.5 ml/min. The elution of the protein was monitored by measuring the $OD_{280\,nm}$ online. The column was washed with two column volumes of the same buffer before analyzing the next protein.

2.2.6. Dynamic light scattering (DLS)

DLS analysis of the proteins was carried out using Zeta nanosizer. 0.2 mg ml^{-1} of the protein sample was subjected to the measurement at 25 °C, average of three measurements was represented. Particle size and homogeneity of protein samples was analyzed by the software provided with the instrument.

2.2.7. Transmission Electron Microscopy (TEM)

Electron microscopy of the full length Translin and mutant proteins was carried out according to the procedure published earlier (VanLoock et al., 2001; Gupta et al., 2017) with slight modifications. 1.0 mg ml^{-1} of Translin protein sample was incubated in 10 µl of reaction buffer (25 mM Tris-HCl, pH 7.8, 100 mM NaCl) for 5.0 min. Samples were diluted 10 times with 1.0 X reaction buffer and 5 µl of each sample was spotted on 200 mesh carbon coated copper grids freshly activated under UV lamp for 1.0 h. After 5 min, grids were stained with 2.0% aqueous uranyl acetate solution for 2.0 min. The excess stain was removed using a filter paper wick and allowed to dry. Samples were viewed and digital images were acquired using Carl Zeiss Libra 120 TEM operating at an accelerating voltage of 120 kV.

2.2.8. Nucleic acid binding assay

DNA and RNA binding assays were carried out essentially according to the procedure mentioned in Gupta et al. (2017). For RNA oligonucleotide binding assays, equipment were sterilized and rinsed with autoclaved DEPC-treated water before use. Increasing concentrations of the proteins were incubated in a final volume of 15 µl assay buffer (25 mM Tris-HCl, pH 7.8, 100 mM NaCl) at 37 °C for 5 min. 50 picomoles of DNA or RNA (heat denatured and quick chilled to remove any secondary structures) oligonucleotide were added and incubated for another 15 min. The reaction mixture was mixed with loading buffer and resolved on 2% agarose gel in 0.5 X TBE buffer. Nucleo-protein complexes were visualized by staining the gel with ethidium bromide followed by the quantification using ImageJ software. Bound fraction percentage was calculated by dividing the bound fraction intensity by total intensity in a particular lane.

3. Results

In human Translin, two short basic regions (basic region I and II), and a putative leucine zipper motif in the region 177–212 are present (Fig. 1a). About 82% of the Translin polypeptide chain folds into alpha helix (Fig.1b). In order to pinpoint critical residues in Translin, five single point substitution mutant variants of human Translin, Y85A, R86A, H88A, R92G in the basic region II and K193G in the leucine zipper motif were generated and their effects on the activity of the protein were compared.

3.1. Secondary structure is not disturbed due to the point substitution mutation

The substitution mutant variants of Translin protein were appreciably over-expressed (Fig. 2a) and purified to near homogeneity (Fig. 2b). A band corresponding to dimer appeared on SDS-PAGE in all the variants including wild type protein. Other higher order structures (minor bands) were also observed. On immuno-detection with anti-Translin antisera, the purified proteins showed the expected ~27 kDa protein, in addition to ~54 kDa protein band corresponding to the Translin dimer (Fig. 2c). A CD spectropolarimetric analysis showed no appreciable difference in the secondary structure of the mutant proteins in comparison to the wild type Translin (Fig. 3) with ~86% alpha helicity (Table 2).

3.2. K193G contributes to oligomerization of Translin

To analyze the effect of these mutations on multimerization of the Translin protein, equal amounts of the proteins were analyzed on native PAGE (Fig. 4a). Interestingly, all the mutant proteins, except K193G showed same mobility as the wild type protein, with size corresponding to octamer of ~220 kDa. K193G however showed an appreciable downward shift, indicating disruption of the octamer and formation of

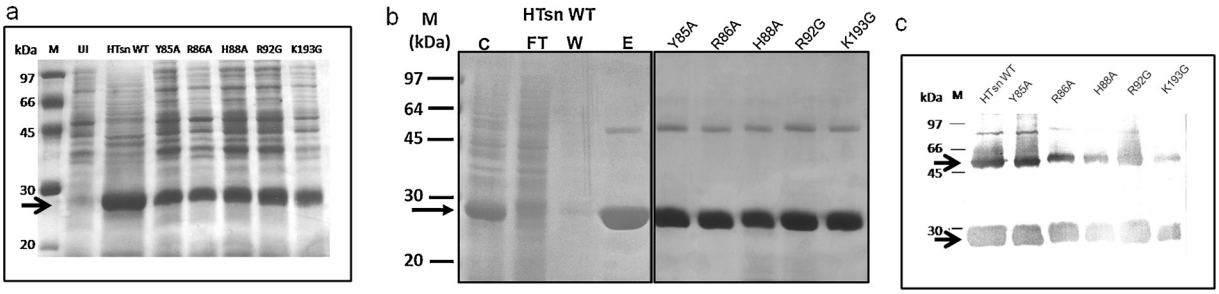

Fig. 2. Expression, purification and western blot analysis of the human Translin wild type (HTsn WT) protein and the mutant variant proteins (Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly and K193G: Lys193Gly).
(a) SDS – PAGE analysis of the over-expressed human translin wild type (HTsn WT) and the mutant Translin proteins. *E. coli* DH5α cells harboring the recombinant pQE9 plasmid with HTsn insert (wild type or mutants) were induced with IPTG and the cell free extracts were analyzed on a 12% SDS PAGE. Band of ~27 kDa corresponding to Translin was observed in all the over expressed lysates which is indicated by an arrow. Uninduced cell extract without adding IPTG was also included. M: Marker, UI: Un-induced.
(b) Purification of human wild type Translin (HTsn WT) and its mutant variant translin proteins. Induced cell cultures were lysed and subjected to Ni-NTA affinity column chromatography as described in methods. C – Crude extract, FT: Flow through, W: Wash, E: Elute of HTsn WT is shown; for mutant variant proteins, only final purified proteins are showed on SDS-PAGE. Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly and K193G: Lys193Gly.
(c) Western blot analyses of the proteins with anti- human Translin anti-sera. Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly, K193G: Lys193Gly and human wild type Translin (HTsn WT).

lower order structures. This was further corroborated by gel filtration analysis where Y85A, R86A, H88A and R92G proteins eluted at the same volume as wild type protein, which corresponds to an octamer. As expected, K193G showed an elution peak at a much higher volume, further suggesting that the protein K193G Translin is not able to octamerize (Fig. 4b) and forms lower order structures, possibly tetramer.

TEM analysis of all the variants and the wild type protein was performed to visualize the octameric ring formation. The wild type protein exhibited the presence of typical ring like structures of about 10–12 nm, with a central hole (Fig. 5). Similar ring structures could be found in all the grid areas scanned under the microscope. The mutant proteins, Y85A, R86A, H88A and R92G also showed typical ring like structure in abundance. However, no rings could be observed in Translin K193G protein. Irregular structures were visible which could be inactive aggregates. Interestingly, R92G Translin rings seemed smaller than that of Y85A, R86A, H88A and wild type proteins in all the areas observed. This was further ascertained by a size distribution analyses of the Translin rings visualized under TEM (Table 3). A DLS analysis of the mutant proteins was also carried out to analyze the size and homogeneity of the octamer state of the proteins. The mutant proteins, Y85A, R86A, H88A and R92G were homogenous solutions containing particle size ranging 10–12 nm, like the TEM image analyses data. However, the K193G protein showed the presence of a large aggregate (Table 3).

Table 2
K2D3 prediction of secondary structure content. The CD spectra were deconvoluted using K2D3 webserver to yield the predicted secondary structure content from the CD signature at ~222 nm and 208 nm.

Sample	Alpha helix Content (%)
HTsnWt	86.1
Y85A	86.15
R86A	86.32
H88A	86.19
R92G	86.11
K193G	86.67

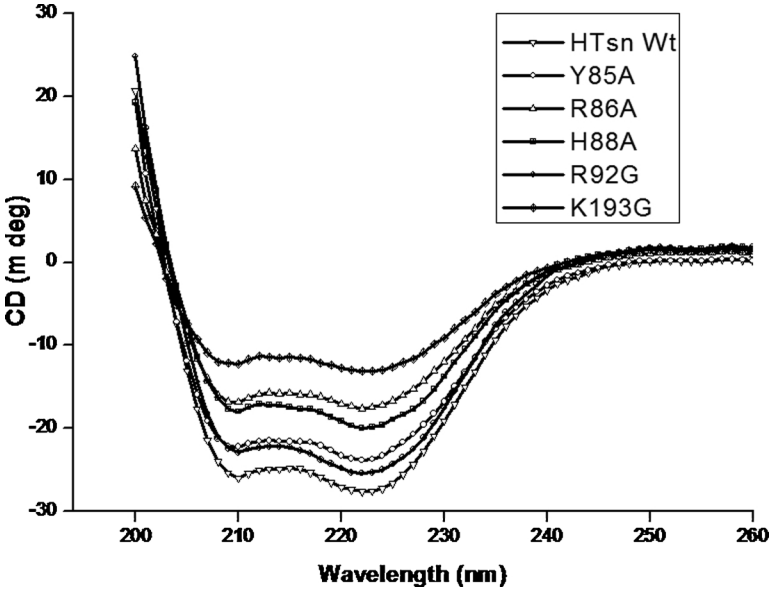

Fig. 3. Circular Dichroism (CD) analyses of wild type and mutant Translin proteins.
0.2 mg/ml of protein samples were subjected to far UV CD analyses in the wavelength range of 200–260 nm at 25 °C. CD spectrum is obtained by plotting CD (in milli degree) versus wave length (nm). All the proteins exhibited signature dips of α – helical proteins at ~222 nm and 209 nm. Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly, K193G: Lys193Gly and human wild type Translin (HTsn WT).

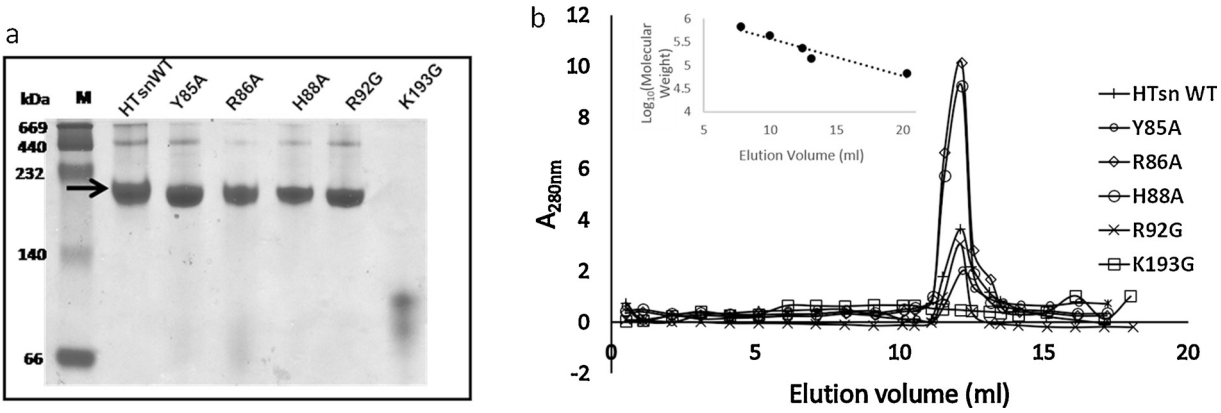

Fig. 4. Size analyses of the humanTranslin proteins in native state.
(a) Native PAGE analyses of the purified proteins was carried out by resolving about 25 μg of proteins on 8.0% native PAGE. HTsn WT and the mutant variants except K193G exhibited a major band of ~220 kDa, corresponding to an octamer. K193G protein showed a protein band at ~110 kDa, corresponding possibly to a tetramer. (b) Gel filtration chromatography of human Translin proteins: Proteins were analyzed on Superdex 200 column as described in methods; OD_{280nm} versus elution volume is plotted. When deduced using standard plot (Log_{10}Molecular Weight versus elution volume), all the proteins except K193G translin showed octameric configuration in native state. K193G translin eluted at a much lower volume, possibly corresponding to a tetramer. Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly, K193G: Lys193Gly and human wild type Translin (HTsn WT).

3.3. The point mutations affect the nucleic acid binding capability of Translin

Octamer formation is necessary for the nucleic acid binding activity of Translin. The above results suggested that like wild type protein, octameric assembly is maintained in all the mutant proteins, except K193G. The nucleic acid binding activity of the Translin variants was analyzed using ssDNA or ssRNA substrates (Figs. 6 and 7). Binding efficiencies of the proteins with both DNA and RNA were in the decreasing order of H88A > WT > Y85A > R92G > R86A > K193G. H88A Translin exhibited higher level of binding to both DNA and RNA substrates in comparison to the wild type Translin protein. As expected, K193G mutation completely abrogated the ssDNA/ssRNA binding capability. Although the mutations Y85A, R92G and R86A did not disrupt the octameric ring formation of Translin, but they abrogated the nucleic acid binding to more than 50% in comparison to the wild type Translin.

Table 3
Size analyses of Translin protein octamer. Protein size in solution was determined by DLS and visualization of the rings was carried out by TEM. Size of about 35 rings were measured and tabulated here.

Protein	Size ± std. dev. (nm)	
	TEM	DLS
WT	11.19 ± 1.20	10.29 ± 3.233
Y85	12.10 ± 1.38	9.588 ± 2.645
R86	11.88 ± 1.17	9.591 ± 2.965
H88	12.10 ± 1.07	9.401 ± 2.785
R92	10.44 ± 1.05	10.58 ± 2.620
K193G	No rings found	196.8 ± 51.52

3.4. In-silico analyses of the effects mutation

As we observed a disruption in protein functioning due to mutation, an assessment of the impact of the point substitution mutations on

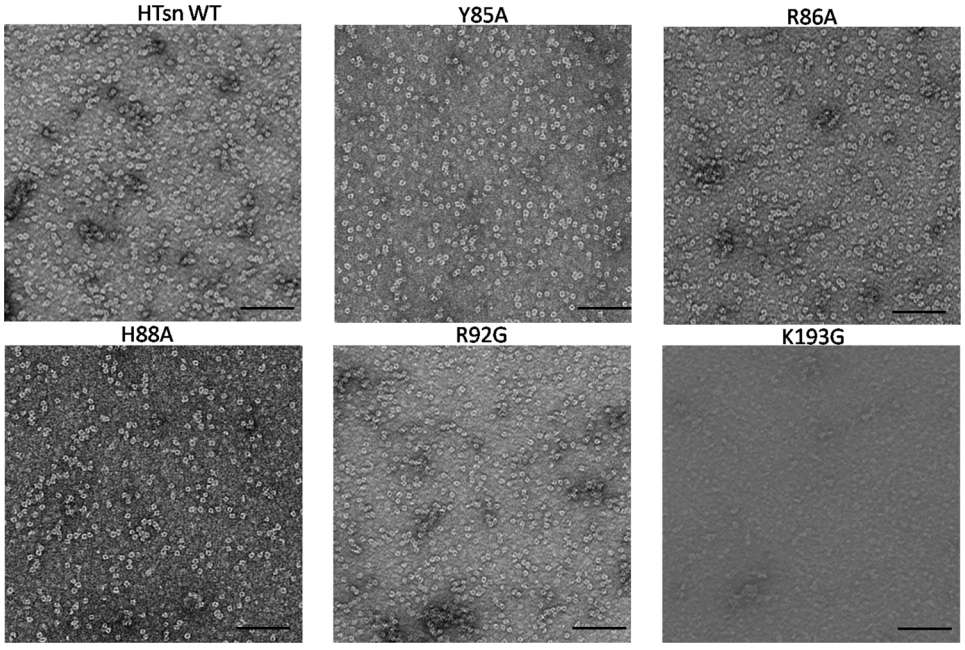

Fig. 5. Transmission Electron Microscopy of human wild type and mutant Translin proteins. Transmission electron microscopy of HTsn WT, Y85A, R86A, H88A, R92G and K193G Translin proteins was performed using negatively stained protein samples as described in methods. Digital Images were recorded using a Libra 120 kEV Transmission Electron Microscope. Octameric rings could be observed in a–e. In K193G, octameric rings were absent. Scale bar represents 100 nm.

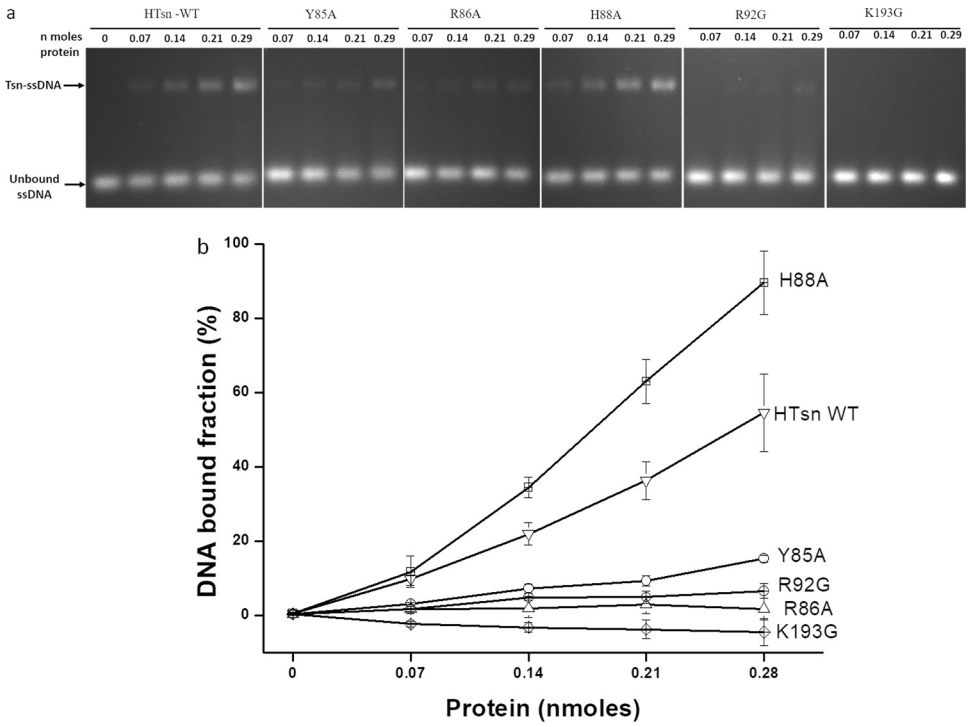

Fig. 6. ssDNA binding activity of wild type human Translin and point mutants.
(a) Indicated amount of proteins (0.07–0.29 nmoles) were incubated with ss DNA oligonucleotides (50 pmoles) at 37 °C for 15 min. The samples were then resolved on 2% agarose gel run at 100 V and protein-DNA complexes were visualized by ethidium bromide staining.
(b) Quantification of gel shifted DNA–protein complexes using image J software. Percentage of ssDNA bound to the proteins was calculated by dividing it's intensity with total intensity of bound and unbound ssDNA.

Translin flexibility was carried out using "Dynamut" web server. Table 4 shows prediction of change in folding free energy change (ΔΔG) and change in vibrational entropy change (ΔΔSVib) due to mutation. Dynamut server predicted all the mutations to increase molecular flexibility at overall protein monomer level. Surprisingly, the H88A mutation caused the maximum flexibility change among the five mutations tested here. The server also provided visual representation of regions of the protein monomer which suffered change in flexibility due to mutation (Fig. S1), in addition to local interactions disrupted due to the mutations. Y85A, R86A and H88A mutations caused a gain in flexibility in the helix 1 to the maximum extent. H88A also suffered a gain in flexibility in the helix 2. The R92G mutation caused highest gain

Table 4
Stability and vibration entropy change analyses due to mutation. Using Dynamut, change in free energy, indicating stability and change in vibrational entropy, indicating flexibility due to point mutation was obtained.

mutation	ΔΔG prediction (kcal/mol)	Δ vibrational entropy (ΔΔSVib) (kcal mol^{-1} K^{-1})
Y85A	−1.371	0.579
H88A	−2.048	0.989
K193G	−0.137	0.233
R86A	−0.047	0.644
R92G	−1.146	0.491

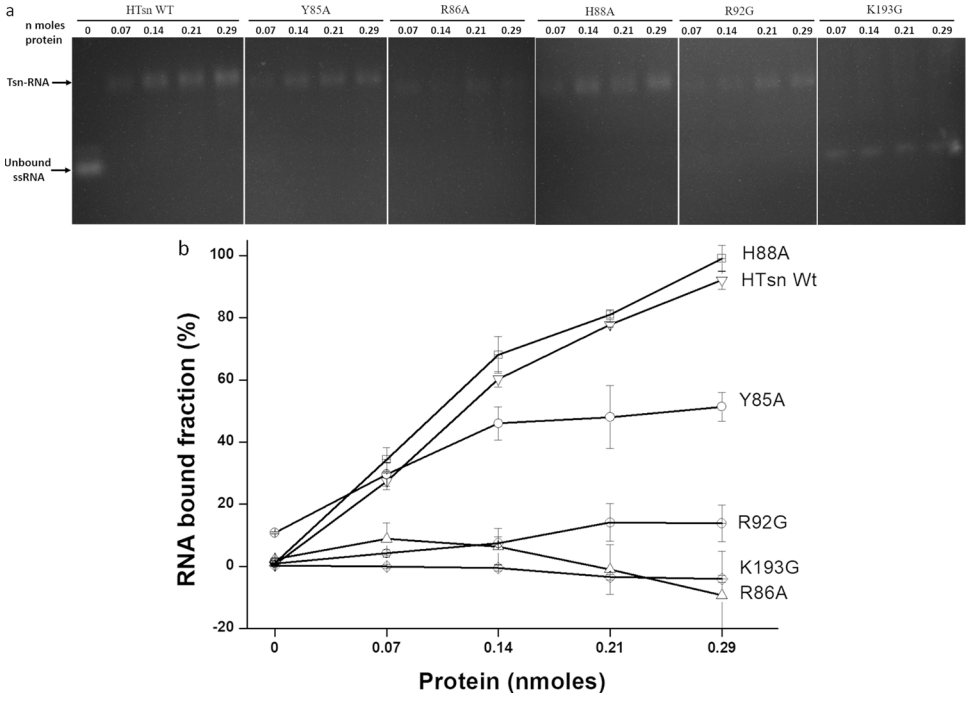

Fig. 7. ssRNA binding activity of wild type human Translin and point mutants.
(a) Indicated amount of proteins (0.07–0.29 nmoles) were incubated with ssRNA oligonucleotides (50 pmoles) at 37 °C for 15 min.The samples were then resloved on 2% agarose gel run at 100 V and protein-RNA complexes were visualized by ethidium bromide staining.
(b) Quantification of gel shifted RNA–protein complexes using image Jsoftware. Percentage of ssRNA bound to the proteins was calculated by dividing it's intensity with total intensity of bound and unbound ssRNA.
Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly, K193G: Lys193Gly and human wild type Translin (HTsn WT).

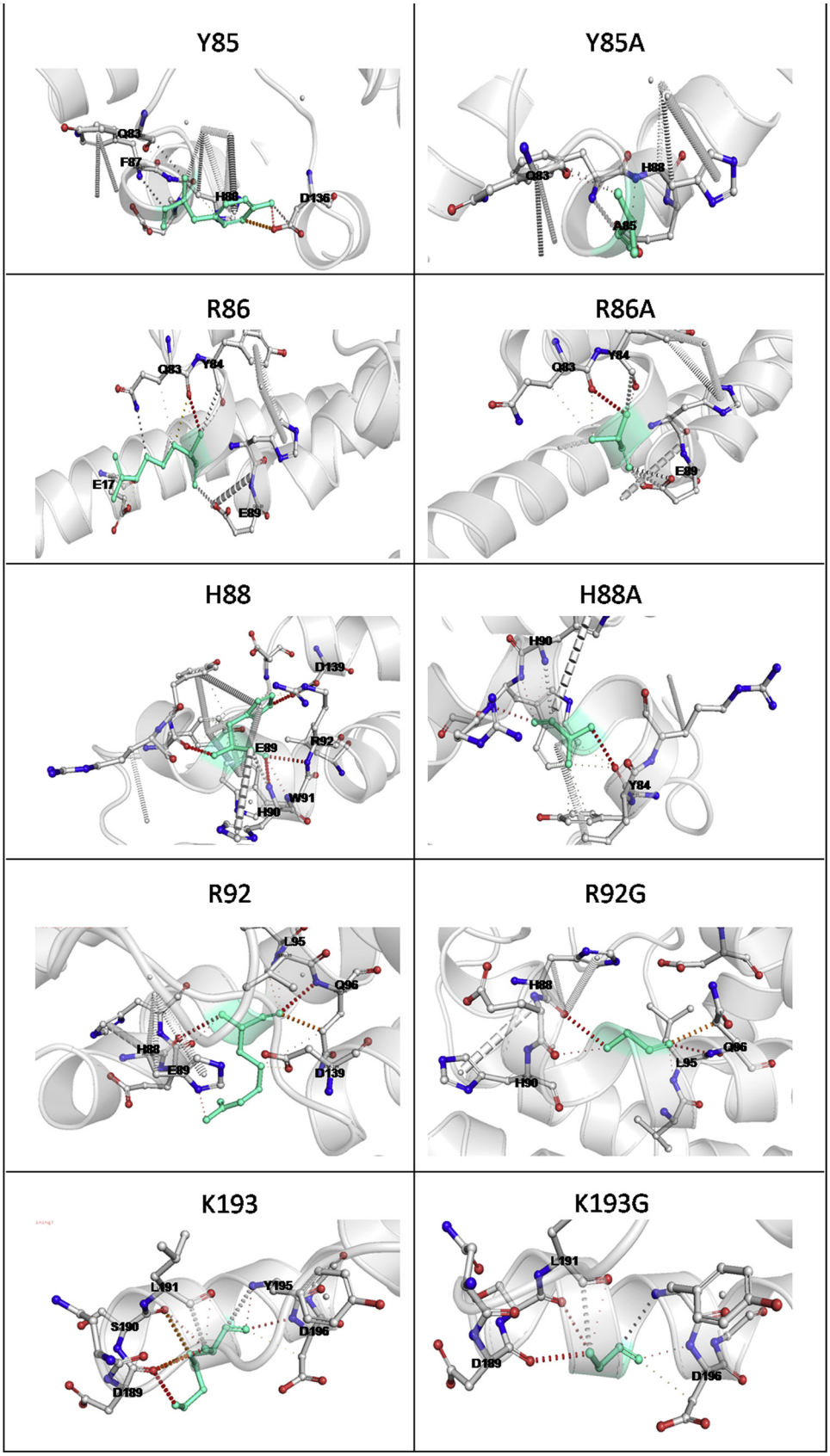

Fig. 8. *In-silico* analyses of the effect of point mutation on Translin protein.
Local interactions of HTsn WT and mutant variant proteins were predicted by analyzing the protein sequences by Dynamut, a bioinformatic package which predicts the local changes in the protein structure upon incorporation of a mutation. Y85A: Tyr85Ala, R86A: Arg86Ala, H88A: His88Ala, R92G: Arg92Gly, K193G: Lys193Gly and human wild type Translin (HTsn WT).

in flexibility throughout the protein. K193G mutation led to gain in flexibility only in the C terminal helix 7. We observed that the major interactions between the residues tested here had weak interactions, like Vander Waal and hydrogen bonding with adjacent as well as far away residues in primary structure (Fig. 8). As such, in the local interacting moieties of all the residues tested, most of the weak interactions and few hydrogen bonding interactions were predicted to be maintained due to the mutation. Due to Y85A mutation, the weak interaction of Y85 with F87 and hydrogen bonding interaction with D136 were disturbed, but the rest of the interactions were maintained. The weak interaction between R86 with E17 is disrupted due to the R86A mutation. H88 has several hydrogen bonding interactions, with R92, H90, Y85 and N139. Of these, only the interaction with N139 is maintained in the mutation. Similarly, the R92G mutation disrupted one of the three hydrogen bonding interactions present in the wild type protein. The K193G mutation led to disruption of hydrogen bonds between K193 and D189, S190 and L191, while maintaining all the other weak interactions. Probably, the loss of most of the hydrogen bonding interactions in K193G mutation led to the failure of the monomers to octamerize.

4. Discussion

Translin is a multifunctional protein with reported involvement in genomic stability, mRNA transport/storage, telomere maintenance etc. (Jacob et al., 2004). Proteins involved in DNA/RNA binding generally act as multimers (VanLoock et al., 2001). Sequence analysis has predicted Translin to have patches of basic residues responsible for nucleic acid binding, and also regions involved in protein-protein interaction (Fig. 1). However, role of individual residues in nucleic acid binding was not known. Based on the predicted critical residues from *S. pombe* Translin, we selected critical residues in the basic region II and also one in the leucine zipper domain, which were not studied earlier in human Translin. Y85, R86, H88 and R92 lie in the loop between helix 2 and helix 3 and K193 is in the leucine zipper motif, located in the C terminal region (Fig. 1b). As expected, Translin appeared as a ~27 kDa band on SDS PAGE in the induced cell lysates and in elute fractions. The dimerization of Translin is suggested to be due to leucine zippers and Cys 225 (Wu et al., 1997). We could detect the ~54 kDa protein band corresponding to Translin dimer in all the mutant variants, suggesting that point substitution mutants, including K193G, which is in the leucine zipper motif did not disrupt the dimer formation and its stability. It is interesting to point out that substitution mutations at leucine 184 and 191 with proline did not affect human Translin dimerization, but disrupted the octamerization (Aoki et al., 1999).

CD signature of the mutant variants was also almost identical to the wild type Translin protein, suggesting that protein folding at monomer level is maintained in the mutants, with about ~86% alpha helix. Earlier we had analysed the point substitution mutations in rice Translin and found that they did not disturb the secondary structure (Gupta et al., 2017). The mutation K193G in human Translin disrupted the interaction between Translin monomers, evidenced by absence of ~220 kDa octamer on native PAGE (Fig. 4a). A lower order multimer of ~120 kDa was observed, as also suggested by the gel filtration analyses (Fig. 4b). However, the DLS analyses showed the presence of a higher order aggregate in K193G mutant variant. This is probably due to aggregation of the inactive protein. Proteins which fail to form active structures generally tend to undergo degradation or form amorphous big multimeric structures. Loss of octamer was confirmed by TEM analysis of the same where ring like structures were absent. All other variants including the wild type protein showed regular, uniform ring like structures (Fig. 5) similar to earlier observation using crystallographic and electron microscopic studies (Kasai et al., 1997). Vanloock et. al (VanLoock et al., 2001) have suggested the presence of an open channel in Translin for DNA/RNA entry. In TEM data, we could find a central channel (dark spot in the middle of the rings), which is known to be involved in nucleic acid binding. As such, K193G variant fails to form octameric complex, it probably may not form the heteromeric complex with its partner TRAX. Taken together, our data suggests that K193 in human Translin is critical for octamer formation. Earlier data on rice Translin also concluded lysine 217th to be critical for octamerization (Gupta et al., 2017). The leucine zipper motif is suggested to be important for protein-protein interaction to form the octamer (Aoki et al., 1999). Probably, in addition to the leucine residues in leucine zipper region, non-Leucine resides are also critical for octamerization.

Multimerization is known to be a prerequisite for nucleic acid binding (Aoki et al., 1999). TB-RBP dimer was suggested to be the minimum essential unit to show DNA/RNA binding activity (Wu et al., 1998). But we did not observe any ssDNA/ssRNA binding activity by K193G Translin, which did not affect the Translin dimer formation (Fig. 2). Possibly, this mutation is causing the structural transitions which are not allowing it to form ring like structures.

The mutant variants deviated from wild type protein in both DNA and RNA binding activity. The corresponding residues of *S. pombe* Translin showed similar effect on both DNA and RNA binding (Eliahoo et al., 2010). H88A Translin showed about 40% higher DNA binding activity in comparison to wild type Translin. However, its RNA binding is comparable to wild type. Probably, Histidine at 88th position hinders the binding of Translin to DNA. When replaced with Alanine, the molecular flexibility increased which could lead to the formation of less efficient octamers. Y85A, R86A and R92G Translin formed octamer with diminished nucleic acid binding activity, indicating that though octamerization is a prerequisite to nucleic acid binding, but may alone not be sufficient, and local amino acid residues too play an important role in interaction with nucleic acids. Nucleic acid binding however, is also dependent on the local ionic interactions which are disrupted in the point mutant variants (Fig. 8). In human Translin, the basic region II is thought to be critical for DNA binding as shown by analysis of the mutant protein, *viz*. R86T, H88N, H90N (Aoki et al., 1999). However, in this mutant protein, all the mutations were introduced together. Hence, it is difficult to decipher the exact role of individual amino acid in Translin function. In the present study, we show that each of the individual amino acid at 85 (Y), 86 (R) and 92 (R) is critical for Translin activity.

Translin is known to form heterogenous configuration when in solution (Pérez-Cano et al., 2013); it may be needed to perform diverse functions, for which conformational flexibility (Sugiura et al., 2004) is also important. Dynamut analyses showed that these mutations resulted in increased flexibility of the protein monomer (Table 4 and Fig. S1). The mutations also disrupted local as well as global interactions (Fig. 8). Since Translin is a dynamic protein, the flexibility changes and disruption of local interactions could be attributed to differential abrogation of nucleic acid binding capability, even though octamers were maintained in the Y85A, R86A, H88A and R92G mutant variants. The data further suggested that the Translin monomers have numerous weak interactions, some of which may be non-specific at many instances as they are not disturbed due to the mutations. Therefore, we observed a gradation in nucleic acid binding activity in the octamer forming mutant variants (Y85A, R86A, H88A and R92G).

5. Conclusion

Translin protein and Translin/TRAX complex has been accounted for diverse functions, mostly related to nucleic acid metabolism. Translin/TRAX which has pre-miRNA degrading activity is considered as a potential target for restoring mi-RNA function in certain cancer and dicer deficient conditions (Asada et al., 2014). It is therefore important to identify the residues of Translin, which has potential functional and clinical relevance. To this end, present study has provided experimental evidence on contribution of the residues Y85, R86, H88 R92 and K193 toward nucleic acid binding activity, for which octamer formation is a

prerequisite. Lysine at 193 position was concluded to be critical for octamerization, in addition to the previously reported Leucine 184 and 191 (Aoki et al., 1999). Since, Translin/TRAX complex plays vital functions pertaining to DNA/RNA, information on amino acid residues on Translin contributing to interaction with other proteins, like TRAX can be explored in future.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

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Author's contribution

AG, RK conceived the idea and designed the experiments. AG, VP and RK performed the experiments and analyzed the data. AG and VP wrote the manuscript and RK reviewed the manuscript.

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Appendix A. Supplementary data

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