Annals of Plant and Soil Research 25(4): 549-563 (2023)
https://doi.org/10.47815/apsr.2023.10305

Evidence for parvulin *pinA*-mediated control of cellular events in *D. discoideum* by mutational analysis

VIPUL YADAV[1] AND ARUNA NAOREM[*1]

[1]Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India

Received: August, 2023; Revised accepted: October, 2023

ABSTRACT

D. discoideum, a single-celled haploid soil amoeba, feeds on bacteria and transits into a multicellular developmental program upon starvation, starting with the aggregation of individual cells into mounds which undergoes morphogenesis to give fruiting body consisting of live spores supported on top of a stalk. In this organism, the role of parvulins is yet to be identified. Parvulins are unique ubiquitous enzymes belonging to the family of peptidyl-prolyl cis/trans isomerases (PPIases) in catalyzing the cis/trans isomerization of Ser/Thr-Pro or phosphorylated Ser/Thr-Pro bonds in peptides and proteins thus control various cellular processes. In our earlier study, we found that PinA is a D. discoideum homolog of Ess1/Pin1 parvulin-type PPIase. Here, we report the findings of a mutational analysis that pinpoints PinA-mediated developmental processes in D. discoideum. Different random pinA mutants were generated using error-prone PCR resulting in single to multiple amino acid changes in the protein sequence. In silico structure modeling predicted that these pinA mutants have altered tertiary structures compared to wild-type PinA. Complementation analysis confirmed the structure prediction by their inability to substitute for wild-type counterparts in either yeast ESS1 mutant or D. discoideum cells lacking pinA. D. discoideum cells expressing these pinA mutants exhibited developmental defects forming aggregation centers with small territories, numerous stream breakup and small fruiting bodies. Confocal analysis found that these pinA mutants are mostly in the cytoplasm as opposed to its normal nuclear localization. Together, this study indicates that PinA can regulate multiple cellular events by modulating key target proteins and developmental gene expression in D. discoideum.

Keywords: cyclic AMP, *Dictyostelium discoideum*, PPIases, Parvulins, PinA

INTRODUCTION

Cellular processes are dynamic events involving specific proteins in a series of regulated interactions and enzymatic reactions. The outcome of these processes is determined by the modulation of protein function. The regulatory mechanisms such as post-translational modifications, allosteric regulation, and controlled protein degradation contribute to the intricacies of these cellular controls. Conformational changes mediated by prolyl *cis*/*trans* isomerization in proteins are also an essential contributor to the regulation of protein functions. As proline residues within proteins can adopt distinct *cis* and *trans* peptide bond conformations, switching of the backbone is regulated by prolyl *cis*/*trans* isomerization. This conversion is a rate-limiting step but is accelerated by enzymes called peptidyl prolyl *cis*/*trans* isomerases (PPIases) or rotamases (Fischer and Aumüller, 2003; Lu and Zhou, 2007; Yaffe *et al.,* 1987). They facilitate protein folding by catalyzing proline imidic peptide bond *cis*/*trans* isomerization in oligopeptides. PPIases are classified into four structurally unrelated families: cyclophilins (Cyps), FK506-binding proteins (FKBPs), parvulins and PTPA (protein Ser/Thr phosphatase 2A activator). Each PPIase family possesses unique catalytic domains and exhibits variations in substrate specificity (Innes *et al.,* 2013; Pemberton and Kay, 2005; Schmidpeter *et al.,* 2011; Zoldák *et al.,* 2009). PPIases are distributed across various cellular compartments in eukaryotic cells, encompassing the cytoplasm, endoplasmic reticulum, mitochondrion, nucleus, and nucleolus (Pemberton & Kay, 2005). Their widespread presence in different cellular locations underscores their crucial involvement in numerous cellular processes.

Among PPIase families, cyclophilins and FKBPs have been extensively investigated after being recognized as targets of immunosuppressive drugs, namely cyclosporin A (CsA) and FK506, respectively (Fischer *et al.,* 1989; Harding *et al.,* 1989). They are also referred to as immunophilins due to their ability

*[1]*Corresponding author Email: aruna.naorem@south.du.ac.in*
Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India

to bind to specific immunosuppressant drugs and also serve as multifunctional enzymes in vertebrates. Previous research has indicated that these enzymes function by sequestering calcineurin, rather than having a solely immune system-specific role (Shaw, 2002). Furthermore, CyPs and FKBPs have been associated with diverse activities, including neuroprotection or neuro-regeneration, Ca^{2+}-mediated intracellular signaling, chaperone functions, and involvement in HIV infection (Wang and Etzkorn, 2006). In contrast to other PPIases, parvulins have a lower molecular mass and possess an extended PPIase catalytic domain (Rahfeld *et al.,* 1994a). They exhibit the unique ability to recognize both phosphorylated Ser/Thr and unphosphorylated substrates and play crucial roles in various cellular signalling events. The members of this family have neither shown sequence similarity to cyclophilins or FKBPs nor bind to the immunosuppressant drugs; cyclosporin A, FK506 and rapamycin (Hanes, 2015). Within the PPIase domain, the signature sequence shared by parvulins consists of conserved amino acids such as histidine, isoleucine, leucine, and cysteine (Born *et al.,* 2020). Par10 parvulin was initially identified as a novel PPIase family in *Escherichia coli* (Rahfeld *et al.,* 1994b). Ess1 was recognized as the first eukaryotic member of the parvulin family in *S. cerevisiae* (Hani *et al.,* 1995). Pin1 was subsequently identified as an Ess1 homolog in humans (Lu *et al.,* 1996). The discovery of Pin1 reveals the diverse functions with its unique substrate recognition and its significant roles in various cellular processes (Lu *et al.,* 2007). For instance, Pin1 regulates gene transcription by directly interacting with the C-terminal domain (CTD) of the largest subunit, Rpb1, of RNA polymerase II (RNAP II) (Xu *et al.,* 2003). Previous studies have established the critical role of Ess1/Pin1 in regulating the G2/M transition of the cell cycle through interactions with cell-cycle regulatory proteins in eukaryotic cells (Crenshaw *et al.,* 1998; Lu *et al.,* 1996). Furthermore, the absence of Ess1 led to an upregulation of a cyclophilin A gene indicating a functional crosstalk between PPIases in yeast cells (Fujimori *et al.,* 2001). Among the four PPIases, protein-Ser/Thr-phosphatase 2A activator (PTPA) plays a pivotal role in the regulation of PP2A and the PP2A-like enzymes in the presence of ATP and Mg^{2+}. Its involvement is crucial for the regulation of these enzymatic processes (Jordens *et al.,* 2006).

PPIases families possess distinct structural features. Cyclophilins (CyPs) possess a tight hydrophobic pocket formed by eight antiparallel β-strands encircling a barrel surface, enabling the binding of CsA (Ke *et al.,* 1991). FKBPs are characterized by an amphipathic, five-stranded β-sheet enveloping a single short α-helix(Van Duyne *et al.,* 1991). On the other hand, parvulins fold their PPIase domain into a half β-barrel structure, comprising four antiparallel β-strands surrounded by four α-helices (Ranganathan *et al.,* 1997). Several studies have introduced mutations in Ess1 and Pin1 to examine their isomerase activity, ligand binding, and interdomain allostery (Born *et al.,* 2020; Hanes *et al.,* 1989). For instance, the change of isoleucine at 28 position to alanine in Pin1 (I28A) induces changes in substrate binding affinity and isomerase activity (Wilson *et al.,* 2013). A change of glycine at 6 position to arginine (G6R) in cyclophilin B results in autosomal recessive hyperelastosis cutis, commonly known as hereditary equine regional dermal asthenia in the American Quarter Horse (Ishikawa *et al.,* 2012). Additionally, a substitution of phenylalanine at 36 position to methionine (F36M) in FK506-binding protein (FKBP) leads to antiparallel FKBP dimers that can dissociate to monomers when bound to ligands (Barrero *et al.,* 2016). These investigations shed light on the implications of specific mutations in determining the function of these proteins.

Our research focuses on elucidating the regulatory function of PPIases in *D. discoideum*. Although *D. discoideum* has a huge repertoire of PPIases encoded in the genome, only a few studies are available on the function of *D. discoideum* PPIases. *D. discoideum* cells exist as single-celled haploid amoebae, feeding on soil bacteria. When the bacteria get exhausted, cells enter a multicellular developmental program by secreting and responding to cyclic AMP (cAMP) as a chemoattractant and aggregate into an aggregation center. This aggregate further develops into a fruiting body consisting of live spores supported on top of a stalk (Loomis, 2014). The progression and morphogenesis throughout development are accompanied by tightly regulated changes in gene expression. In our earlier study, we identified PinA, encoded by *pinA* gene, as a

novel member of the Ess1/Pin1-type parvulin and was the first report on parvulin PPIase from *D. discoideum*. Also, PinA is unique in possessing an FHA domain at its N-terminus for mediating protein-protein interactions instead of the WW domain in Ess1/Pin1 and a PPIase catalytic domain at its C-terminus (Haokip and Naorem, 2017). Thus, apart from the potential role of PinA in cell differentiation and development, the study of PinA may reveal its novel substrate due to the presence of the FHA domain. It is noteworthy that the crystal structure of PinA and its activity are yet to be determined.

Cells lacking *pinA* or (*pinA*$^{-}$) show developmental defects by forming large number of small mounds that further develop into smaller fruiting bodies at the same amount of time as wild-type cells. In order to examine the events/processes requiring PinA function in *D. discoideum*, we carried out a study using *pinA* mutants. This study involves the generation of *pinA* mutants, complementation in yeast and *D. discoideum*, effect of their expression on growth and development of *D. discoideum*. Firstly, multiple *pinA* mutants were generated having single to multiple mutations in the open reading frame of *pinA* by error-prone PCR. Computational structure modeling predicted that the changes in the primary amino acid sequence of these mutants affect the native protein structure of PinA. Complementation analysis confirmed that these *pinA* mutants differ from PinA by their inability to replace the function of Ess1 or PinA in yeast and *D. discoideum* respectively. Furthermore, expression studies in *D. discoideum* wild-type Ax2 cells indicated that these mutants seem to disrupt the normal function of PinA by exhibiting growth and developmental defects. Confocal analysis also showed that these mutants are localized in the cytoplasm in contrast to PinA thus they may not function similar to PinA. For further studies on understanding its structure, substrate and activity, these mutants are expressed in bacteria as recombinant fusion proteins. Together, these results confirm PinA is crucial for *D. discoideum* growth and developmental processes and also studying these *pinA* mutants can give insight into the mechanism of how it regulates these processes.

MATERIALS AND METHODS

Amplification and construction of plasmids

All the plasmid manipulations were done in *Escherichia coli* DH5α strain, following the standard protocols (Ausubel *et al.,* 1995).

Yeast culture, transformation and complementation analysis

Haploid yeast wild type (W303A) or *ESS1* mutant (*ess1*^{H164R}) (Wu *et al.,* 2000) strains were used in all experiments. Yeast culture, transformation and complementation analysis were performed following the protocol as described previously (Haokip and Naorem, 2017). For complementation analysis, five independent transformants of each construct in W303A or *ess1*^{H64R} were patched on fresh YEPD plates and incubated at 25°C overnight. A loop of cells was resuspended in water, serially diluted and spotted on YEPD plates in triplicates. The plates were then incubated at permissive (25°C) and restrictive (34°C or 37°C) temperatures. The plates were photographed after 2-3 days.

D. discoideum cell culture, maintenance and development

All gene deletion (*pinA*$^{-}$) or expression of all constructs was done in *Dictyostelium discoideum* Ax2 as a wild-type parent. Cells lacking pinA (*pinA*$^{-}$) were generated by homologous recombination using *bsr* cassette (Haokip and Naorem, 2017). *pinA*$^{-}$ cells were maintained in HL5 containing 10 µg/ml Blasticidin S final concentration. Hereafter, vector as GFP; different constructs GFP-wild-type *pinA* as PinA; GFP-*pinA*^{K111N} as PinA (K111N); GFP-*pinA*^{L227I} as PinA (L227I) will be used interchangeably throughout the text. For development, actively growing cells were harvested and washed twice with KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.5) and plated on 1.5% non-nutrient agar plates ($5x10^5$ cells/cm^2). Incubation was at 22°C in a dark, moist chamber (Fey *et al.,* 2007). For development in submerged condition, cells were seeded in KK2 buffer ($1x10^6$ cells/cm^2) and incubated at 22°C.

Error-prone PCR

Mutations in *pinA* were introduced using

error-prone PCR using gene-specific primers: forward with BamHI site 5′-GGGGATCCATGGGCGATAATAATAATAATAA TAATAATG-3′ and reverse with XhoI site 5′-GGCTCGAGTTATGCTAATCTTTCAATAATATG AACACCTG-3′ following protocol as described previously with few modifications (Stark, 1998). The *pinA* open reading frame was PCR amplified with varying $MnCl_2$ concentrations (0.1 to 0.5 mM) using a 5:1 ratio of (dTTP+dCTP): (dATP+dGTP). Purified PCR products were cloned into pGEM-T Easy. Recombinants were confirmed by BamHI and XhoI digestion, followed by sequencing. Mutations were identified by aligning with wild-type *pinA* DNA sequence. Finally, the DNA sequence was translated using TRANSLATE program revealed amino acid changes, confirmed by protein sequence alignment using CLUSTAL Omega (Sievers *et al.,* 2011). PinA mutants were cloned into *S. cerevisiae* and *D. discoideum* vectors for complementation and expression studies. The list of plasmids generated and used in this study is available on request.

in silico structure modeling and secondary structure analysis

The three-dimensional (3D) structure of PinA was predicted using homology modeling due to the unavailability of a crystal structure. Phyre2, utilizing Hidden Markov Method, aligned the sequence with known structures to generate 3D models (Kelley *et al.,* 2015). Phyre2 was used to create PDB format models for PinA in 'intensive' mode.

Transformation of *D. discoideum* Cells

The transformation of *D. discoideum* cells was carried out following the protocol outlined in (Gaudet *et al.,* 2008) with minor modifications. All transformants were selected and maintained in HL5 with either G418 (20 µg/ml final concentration) and/or Blasticidin S (10 µg/ml final concentration).

Growth assay

For growth assay on bacteria, 100 cells of each *D. discoideum* cell line to be analyzed were plated on SM/5 agar with *Klebsiella aerogenes* and incubated the plates at 22°C. After 5 days, the numbers of plaques formed were counted and also measured the size of plaques for comparison. The size of the plaques reflects the growth of cells incubated for a given amount of time.

SDS-PAGE and Western Blots

Total cell lysate of bacteria was separated on 10% SDS-PAGE gel using standard protocol described previously (Haokip and Naorem, 2017). For western blotting, proteins equivalent of 1×10^6 cells were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were developed as per previous protocol (Haokip and Naorem, 2017) and imaged with ChemiDoc Imaging System (Bio-Rad). Protein band quantification utilized Fiji/ImageJ software.

Confocal microscopy

For *in vivo* protein localization studies by confocal microscopy, following a previous protocol (Haokip and Naorem, 2017), actively growing Ax2 cells expressing GFP, GFP-PinA, or *pinA* mutants were harvested, washed and resuspended in 1x PBS. Cells were counterstained with 1 µg/ml DAPI for 5 minutes and images were captured by Leica TCS SP8 confocal microscope. LasX software aided image analysis, observing 10 cells per sample.

Statistical analysis

Data analysis was performed using Prism software (GraphPad, San Diego, CA) and Microsoft Excel, employing t-tests. The mean ± SD was used to represent the data.

RESULTS AND DISCUSSION

pinA mutants are generated and these mutants are predicted to have alteration in their structure by Phyre2 structure model prediction tool

D. discoideum PinA has high amino acid sequence conservation with both human Pin1 and ScEss1 in the C-terminal PPIase domain with FHA as protein-protein interacting domain at its N-terminus instead of WW domain in Pin1 or Ess1 (Haokip and Naorem, 2017). The domain architecture of PinA is shown along with Pin1 and Ess1 in Fig. 1. However, the details of PinA function in various cellular pathways, activities or interacting proteins during growth and developmental phases are yet to be elucidated. To examine further into the function of PinA

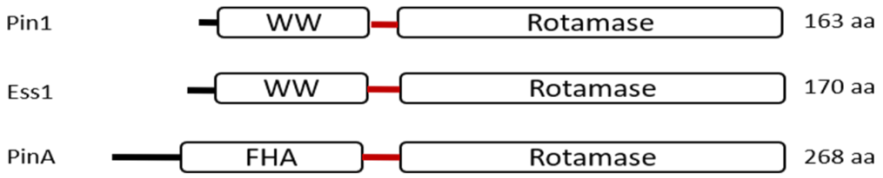

Figure 1: Domain architecture. Schematic representation of domains and number of amino acids in human Pin1, *S. cerevisiae* Ess1 and *D. discoideum* PinA. PinA has PPIase domain at C-terminus FHA domain at N-terminus instead of WW domain in both Pin1 and Ess1 proteins. Besides the two domains, PinA has an N-terminal extension (black bold line) as compared to human and yeast counterparts. The red bold line indicates the linker connecting WW/FHA and PPIase domains in these parvulin proteins

during growth and development, we carried out mutational analysis by generating *pinA* mutants and studying the effect of expression of these mutants on growth and development in *D. discoideum*. For this, nucleotide substitutions were introduced randomly in *pinA* ORF by error-prone PCR and any changes, single or multiple base substitutions or any base deletion were confirmed by DNA sequencing. DNA and protein alignments of these mutants with wild-type nucleotide and amino acid sequences of *pinA* revealed that single or multiple base substitutions as well as single base deletion were introduced in *pinA* coding region leading to synonymous and non-synonymous changes in the amino acid sequence of the protein. The list of *pinA* mutants thus generated with the changes in amino acid sequences are indicated as compared to wild-type PinA protein sequence (Table 1).

Table 1: PinA and its mutants Shown is the information on the number of mutations in each mutant, changes in amino acid residues, location and also their ability to substitute Ess1 in *ess1*^{H164R} *ts* mutant yeast

Protein Name	Amino acid change	No. of mutations	Location	Phenotype in yeast
PinA	wild type PinA	-	-	WT
VY30	L38S, I47L, K54R, P74S, T103S, T117A, S155X, K158R, C162R, S176L, L211F, K214R, Q236L, P238L, F239L, E250V, V261I	17	FHA, PPIase	ts[b]
VY29	K54R, A82V, G137S, H169Y, R237P, C242R	06	FHA, PPIase	ts[b]
VY28	D48N, K54R, F122I, V167I, Q170R	05	FHA, PPIase	ts[b]
PinA (K54R, A206T, G260V)	K54R, A206T, G260V	03	FHA, PPIase	ts[a]
PinA (P19L, I52F, K54R, G260V)	P19L, I52F, K54R, G260V	04	FHA, PPIase	ts[a]
PinA (K54R, G260V)	K54R, G260V	02	FHA, PPIase	ts[b]
PinA(G260V)	G260V	01	PPIase	ts[a]
PinA(A192V)	A192V	01	PPIase	ts[b]
PinA(L227I)	L227I	01	PPIase	wt
PinA(K111N)	K111N	01	FHA	ts[a]
PinA(K54R)	K54R	01	FHA	wt

Note: wt, wild-type; ts, temperature sensitive; a, temperature sensitive at 37°C only; b, temperature sensitive at 34°C, and 37°C

To check the effect of these amino acid changes on the tertiary structure of PinA, we performed *in silico* structure modeling using the available Phyre2 tool. The tool predicted the native structure of PinA with N-terminal FHA with PPIase/rotamase domain at C-terminus linked by a flexible disordered stretch. Interestingly, structure prediction programs indicated that most of these mutants were found to have distorted structures compared to wild-type PinA structure indicating that these mutants may not function like PinA (Fig. 2).

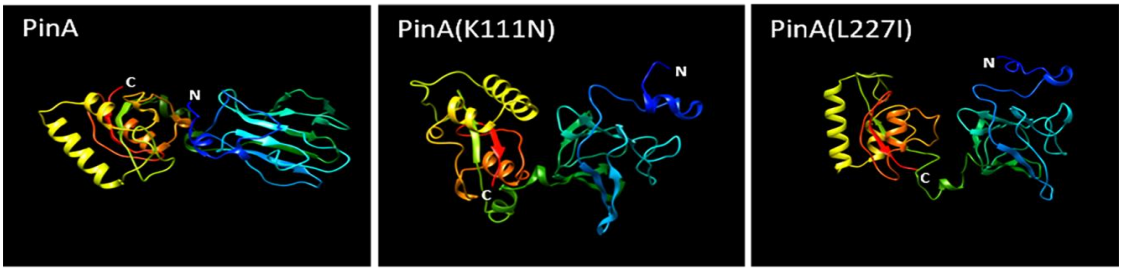

Figure 2: Predicted structures of PinA and its mutants. The secondary structures of PinA, PinA(K111N) and PinA(L227I) were predicted using Phyre2. Amino- and Carboxy- termini are marked as N and C respectively in the protein structures. A single amino acid change in PinA(K111N) or PinA(L227I) can induce a significant change in the overall alignment of N- terminal FHA and C-terminal PPIase domains in PinA

pinA mutants show varying abilities to complement the lethality phenotype associated with _S. cerevisiae ESS1_ temperature-sensitive mutant (_ess1_^{H164R}) compared with complete complementation by PinA

Further, these *pinA* mutants were subcloned in *S. cerevisiae* vector to carry out complementation analysis in *ESS1* temperature-sensitive mutant yeast (*ess1*^{H164R}). For complementation analysis, *ess1*^{H164R} cells were transformed with vector, vector having *ESS1* or *pinA* or *pinA* mutants and selected on synthetic complete medium lacking leucine. It was observed that few of the *pinA* mutants could support the growth of *ess1*^{H164R} cells similar to Ess1 or PinA at all temperature tested while other could partially support the growth of *ess1*^{H164R} till 34°C. However, similar to the vector, PinA(K111N) mutant could not support the growth of *ess1*^{H164R} at high temperatures while PinA(L227I) mutant can completely rescue the growth of *ess1*^{H164R} cells at high temperatures (Fig. 3). The results of the complementation analysis of all *pinA* mutants are given in Table 1. Taken together, different *pinA* mutants were generated and these mutations affect the function of PinA. *pinA* mutants were categorized according to no rescue or partial or complete rescue of the lethality phenotype associated with *ess1*^{H54R} yeast mutants at high temperatures respectively. Thus, these results indicate that a single amino acid change at a critical position in PinA can affect the protein function. Thus, the two *pinA* mutants, PinA(K111N) in which the amino acid change from lysine to arginine is in FHA domain and PinA(L227I), the amino acid mutation is at conserved leucine residue to isoleucine in the PPIase domain, were analyzed for their ability to function in *D. discoideum* as compared to PinA.

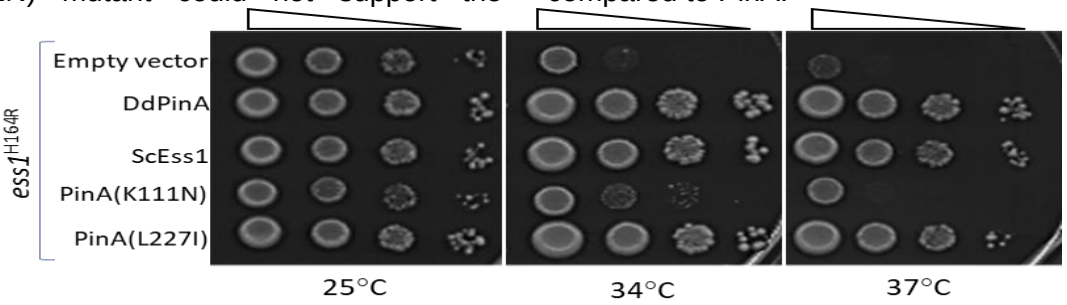

Figure 3: Complementation analysis in yeast *ESS1* temperature sensitive mutant (*ess1*^{H164R}) strain. Wild-type W303A or *ess1*^{H164R} transformed with indicated constructs were serially diluted and replica plated on SC-Leu plates and incubated at indicated temperatures. The growth was observed and photographed after 3 days of incubation. All transformants in wild-type W303A could grow at all temperatures (data not shown). As expected, *ess1*^{H164R} expressing empty vector could grow only at 25°C but showed temperature sensitivity (*ts*) and could not grow at 34°C and 37°C whereas *ess1*^{H164R} expressing Ess1 or PinA could grow at all temperatures thus rescuing the *ts* phenotype associated with *ess1*^{H164R} at high temperatures. Similar to Ess1 and PinA, PinA (L227I) could support the growth of *ess1*^{H164R} at all temperatures. Unlike Ess1 and PinA, PinA (K111N) could support the growth of *ess1*^{H164R} till 34°C. Triangles show the decrease in cell number by serial dilution. The picture shown here is a representative of three independent experiments using five independent transformants

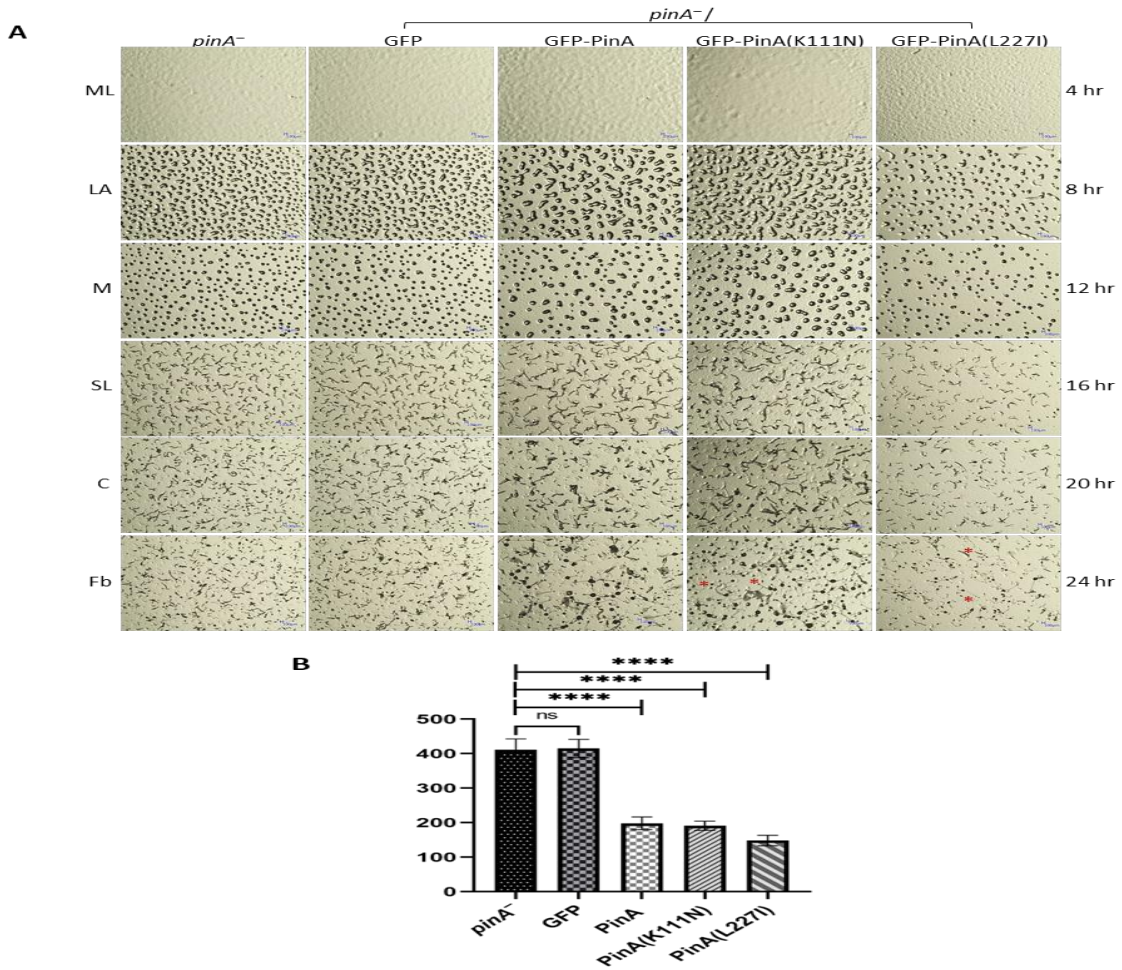

Figure 4: *pinA* mutants could not rescue the phenotypes of *pinA*$^{-}$ cells. (A) Developmental profile. *pinA*$^{-}$ cells expressing GFP or PinA, PinA(K111N) or PinA(L227I) were starved on 1.5% non-nutrient KK2 agar plates at a density of 1x10^{6} cells/ square centimeter and developed in a dark, moist chamber at 22°C. Photographs of developmental structures were taken at 10x magnification at every 4 hr intervals for 24 hr indicated at *right*. Normal developmental structures of Ax2 are shown in Fig. 6A for reference. Developmental stages of *pinA*$^{-}$ cells shown at *left*, ML-Monolayer of cells, LA-loose aggregates, M-mounds, SL-slug, C- culminants, Fb-fruiting body with corresponding time on *right* after starvation for comparison. Scale bar=100 µm. As reported, PinA can rescue most of the phenotypes of *pinA*$^{-}$ cells whereas *pinA* mutants could not rescue and showed small mound and small fruiting body phenotypes at 12 hr and 24 hr respectively. The severity of the phenotypes is greater in *pinA*^{L227I} than in *pinA*^{K111N}. (B) Number of aggregates. The number of aggregates observed in the view at 12 hr of development were counted. The graph is a representative of three independent experiments. Error bars represent ±SD of three biological replicates. Asterisks indicate the statistical significance of the difference from the WT using Student's *t*-test: ****, *P*-value< 0.0001

pinA mutants fail to rescue the growth and developmental phenotypes of *pinA*$^{-}$ cells

To test whether the selected *pinA* mutants could complement the phenotypes of *pinA*$^{-}$ cells, *pinA* and *pinA* mutants were overexpressed as GFP-fusion proteins under the control of constitutively expressed actin 15 promoter from an extrachromosomal plasmid in *pinA*$^{-}$ cells. Three independent transformants of each strain were assessed for development with *pinA*$^{-}$ or *pinA*$^{-}$ expressing GFP cells as controls. As reported earlier, upon starvation, cells lacking *pinA* (*pinA*$^{-}$) or *pinA*$^{-}$/GFP showed developmental phenotypes by forming smaller aggregates which further develop into small fruiting bodies compared with wild-type Ax2 cells (Fig. 4A and Fig. 6A). As reported earlier, the phenotypes of *pinA*$^{-}$ cells could be rescued by the reexpression of PinA although not completely. *pinA*$^{-}$/PinA cells exhibited aggregation by 8 hr, mounds by 12 hr, followed by the formation of thin slugs at 16 hours, and normal fruiting bodies at 24 hours.

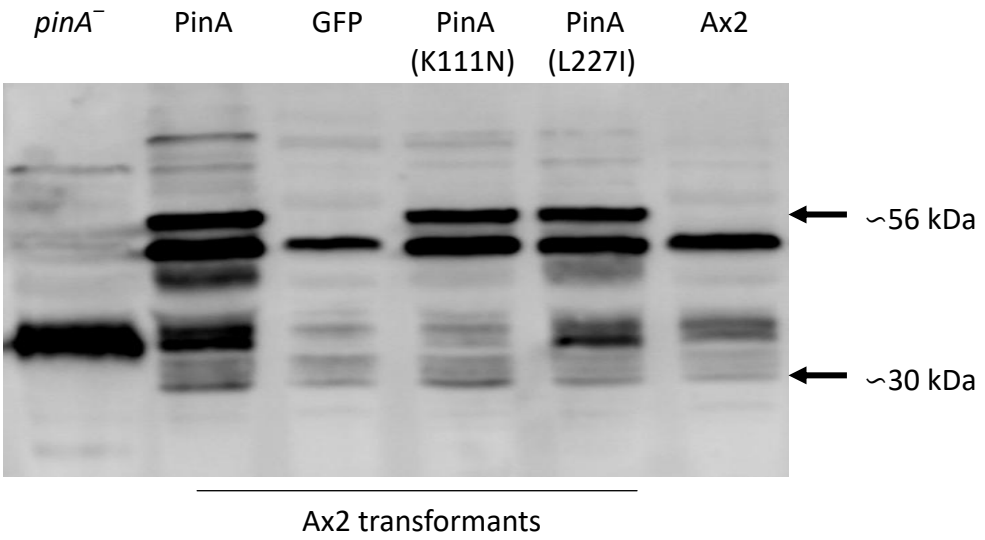

Figure 5: Confirmation of expression of GFP fusion proteins in Ax2 transformants by using polyclonal anti-PinA antibody. Proteins equivalent of 1×10^6 cells of all cell lines were separated on 12% SDS-PAGE gel, transferred on PVDF membrane and probed with polyclonal anti-PinA antibody. Total lysates of Ax2 and *pinA*⁻ cells were loaded as controls. Protein bands corresponding to endogenous PinA (∽30 kDa) in all lanes except *pinA*⁻ lane and GFP-PinA or PinA mutant fusions (∽56 kDa) were observed in the respective lane and are indicated at *right*. No band corresponding to a ∽30 kDa in *pinA*⁻ lane reconfirms the absence of PinA in *pinA*⁻ cells as indicated by an *asterisk*

pinA⁻/PinA(K111N) cells exhibited a similar developmental profile with *pinA*⁻/PinA whereas *pinA*⁻/PinA(L227I) cells exhibited developmental phenotypes similar to *pinA*⁻ cells by forming tiny mounds, thin slugs and small fruiting bodies. However, the number of aggregates formed by each cell lines at 12 hr after starvation is shown in Fig 4B and the number is still more than that of Ax2 cells (compare 12 hr of Fig. 4B with Fig 6B). It was also observed that slugs formed by *pinA*⁻ cells expressing either *pinA* or *pinA* mutants showed delayed culmination by remaining slugs till 20 hr when Ax2 cells formed culminants. Interestingly, *pinA*⁻/PinA (L227I) cells formed more fruiting bodies than by *pinA*⁻ cells but most of them seemed to fallen on agar surface (Fig. 4A). Taken together, many of the phenotypes associated with *pinA*⁻ cells could not be rescued by overexpression of *pinA* mutants suggesting the function of these *pinA* mutants are compromised and cannot substitute for PinA in *D. discoideum*. Incomplete rescue of *pinA*⁻ phenotypes by reexpression of PinA under the control of constitutively active actin 15 promoter can be explained by the fact that any regulation of PinA, temporal and spatial, is absent resulting in the incomplete rescue observed here. The mutations leading to change of amino acids either lysine at 111 position to asparagine in FHA domain or conserved leucine at 227 position to isoleucine in PPIase domain of PinA indicate that both domains are important for the normal function of PinA. The next study will be to understand the critical residues in PinA for binding to its substrate and also for its activities.

Cells overexpressing *pinA* mutants exhibit abnormal growth as well as aberrant development showing delayed aggregation, stream breakup, delayed or arrested culmination

Further, we investigated the effect of expression of these *pinA* mutants as GFP fusion proteins described before on wild-type cells. We found that these mutants affected the growth of wild-type Ax2 cells by exhibiting slow growth phenotype on *K. aerogenes* lawn or in liquid medium as compared to PinA thus indicating they cannot function like PinA (data not shown). We then investigated the effect of their *pinA* mutants on development with respect to PinA. For this, Ax2 cells expressing *pinA* or *pinA* mutants were developed on non-nutrient agar plates and observed the developmental structures as compared to Ax2 cells expressing vector. All plasmid constructs having *pinA* or

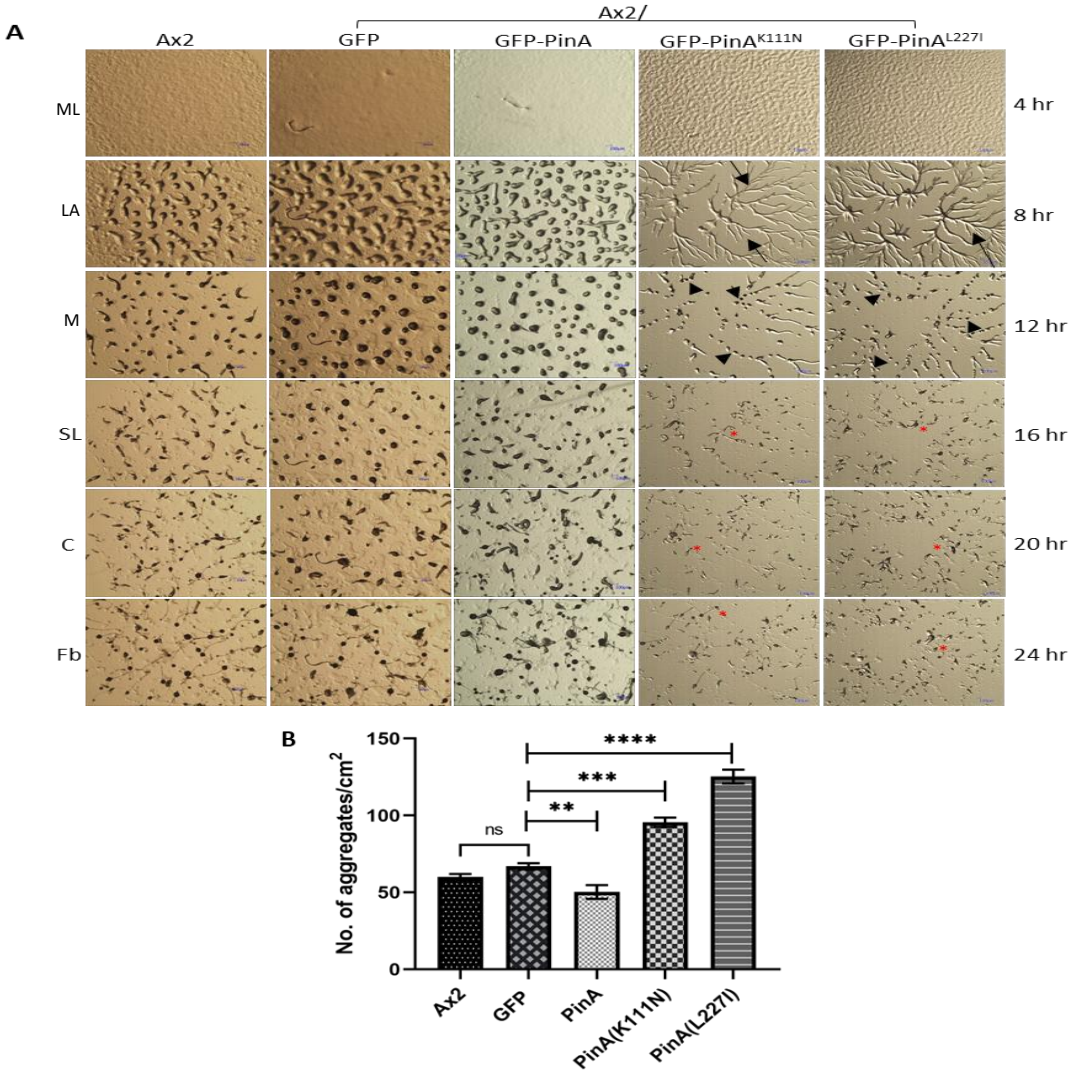

Figure 6: Ax2 cells expressing *pinA* mutants exhibited delayed aggregation, stream breakup and developmental phenotypes compared to control cells. (A) Developmental profile of different cell lines. Ax2 and Ax2 transformed with GFP, PinA, PinA(K111N) and PinA(L227I) were starved on 1.5% non-nutrient agar plates at a density of 5x10^5 cells/ square centimeter and developed in a dark, moist chamber at 22°C. Photographs of developmental structures were taken at 10x magnification at every 4 hr intervals for 24 hr indicated at right. Developmental stages of Ax2 cells shown at left, ML-Monolayer of cells, LA-loose aggregates, TM-tip-mounds, SL-slug, C-culminants, Fb-fruiting body with corresponding time at right after starvation for comparison. At 8 hr, PinA(K111N) and PinA(L227I) formed aggregation streams (arrows) but these streams broke up at numerous places (see 12 hr) indicated by black arrowheads. Scale bar=500 µm for AX2, GFP and PinA and 100 µm PinA (K111N) & PinA (L227I) (n = 3). Asterisks indicate decreased size structures of pinA mutants, PinA(K111N) and PinA(L227I), corresponding to control structures of Ax2 or Ax2 expressing GFP or PinA. (B) Number of aggregates in different cell lines. Aggregates were counted in the observed view at 12 hr of development for all cell lines. The graph is a representative of three independent experiments. Error bars represent ±SD of three biological replicates. Asterisks indicate the statistical significance of the difference from Ax2 cells expressing GFP using Student's t test:**, 0.001 < P-value < 0.01; ***, P-value < 0.001; ****, P-value< 0.0001

pinA mutants with vector were transformed into Ax2 cells and their expressions were confirmed by western blotting with Polyclonal α-PinA antibodies (Fig. 5). On agar, Ax2 and Ax2 cells expressing vector/GFP or PinA aggregated with proper streams with regular territories by 8 hr after starvation and formed fruiting bodies by 24 hr after starvation. It was also observed that GFP alone cells showed a slight delay in development. Interestingly, PinA(K111N) or PinA(L227I) cells showed not only delayed aggregation but also formed a smaller number of aggregation centres with larger aggregation territories (Fig. 6A, 8 hr). However, aggregation streams in PinA(K111N) or PinA(L227I) cells broke up into numerous smaller aggregates at

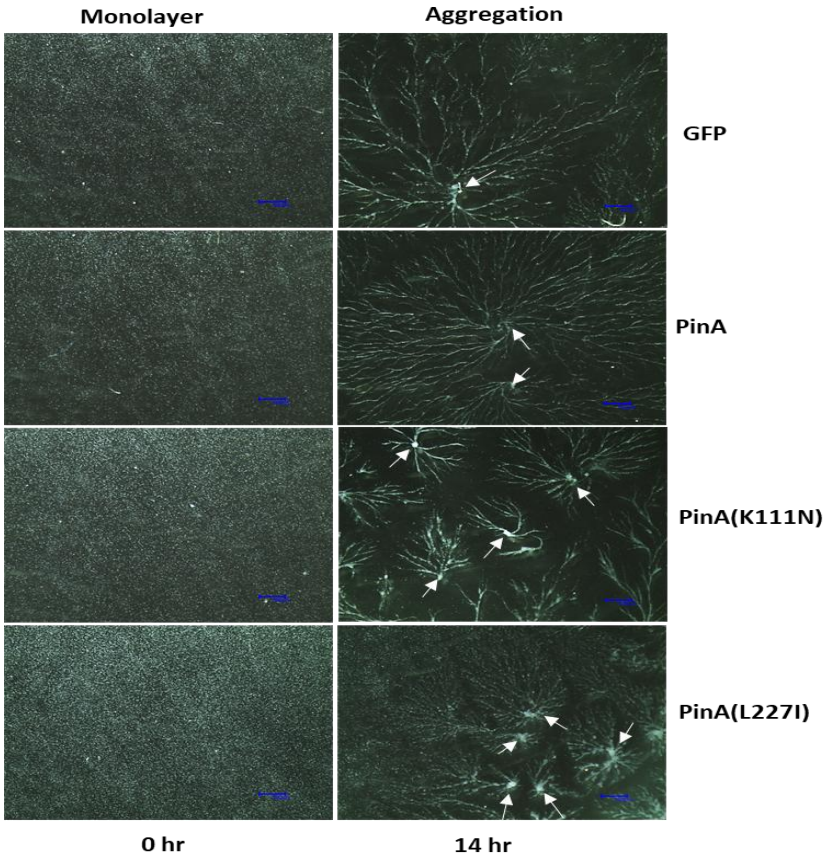

Figure 7: Ax2 cells expressing *pinA* mutants exhibited abnormal aggregation with increased number of aggregation centers with small territories in submerged condition. Ax2 cells expressing GFP or GFP- fusions; PinA or PinA(K111N) or PinA(L227I) were harvested, washed and resuspended in KK2 buffer. Cells were inoculated in different wells in a 6-well tissue culture plate at a density of 1 x 10^6 cells/square centimeter in KK2 buffer. The plate was then incubated and kept stationary for 16 hr at 22°C. Images were taken for developing cells at the indicated times. Cells adhere on plastic as monolayer at the start of the experiment, 0 hr. At 14 hr after incubation, cells form dendritic streams that flow toward aggregation centers, marked by *white arrows*. The number of aggregation centers are more in number than that in GFP or PinA cells. The space between aggregating cells is referred to as the aggregation territory. Scale bar = 500 µm. Images are the representative of three independent experiments

12 hr, followed by formation of small slugs at 16 hr and eventually tiny fruiting body with short stalks and small sori at 24 hr in contrast to normal fruiting bodies formed by wild-type or GFP or PinA cells (Fig. 6A). The number of aggregates was counted for all cell lines at 12 hr after starvation and is given in Fig. 6B. It was also observed that final structures formed by PinA(K111N) or PinA(L227I) cells at 24 hr after starvation were mostly arrested in slugs and culminants compared with Ax2/GFP or Ax2/PinA (Fig. 6A, 24 hr).

A similar starvation-induced development experiment was carried out in submerged condition to observe aggregation process. In submerged condition, as expected, we observed that wild-type cells initiate aggregation at around 12 hr after inoculation later than aggregation observation on agar. Unlike on agar, the delayed aggregation phenotype of *pinA* mutants was not visible. However, aggregation was observed to proceed with numerous aggregation centres having small territories compared to that of vector and PinA cells which formed few aggregation centres with large territories (Fig. 7). It is likely that there is an involvement of factor/s which is getting diluted in submerged condition but not on agar due to which the phenotypes of small aggregation territories could be observed. Together, these findings support the fact that the function of PinA in these mutants is compromised and also confirms the requirement of normal function of PinA at aggregation and culmination stage of development. Further experiments will be carried out to examine the events during growth to development transition,

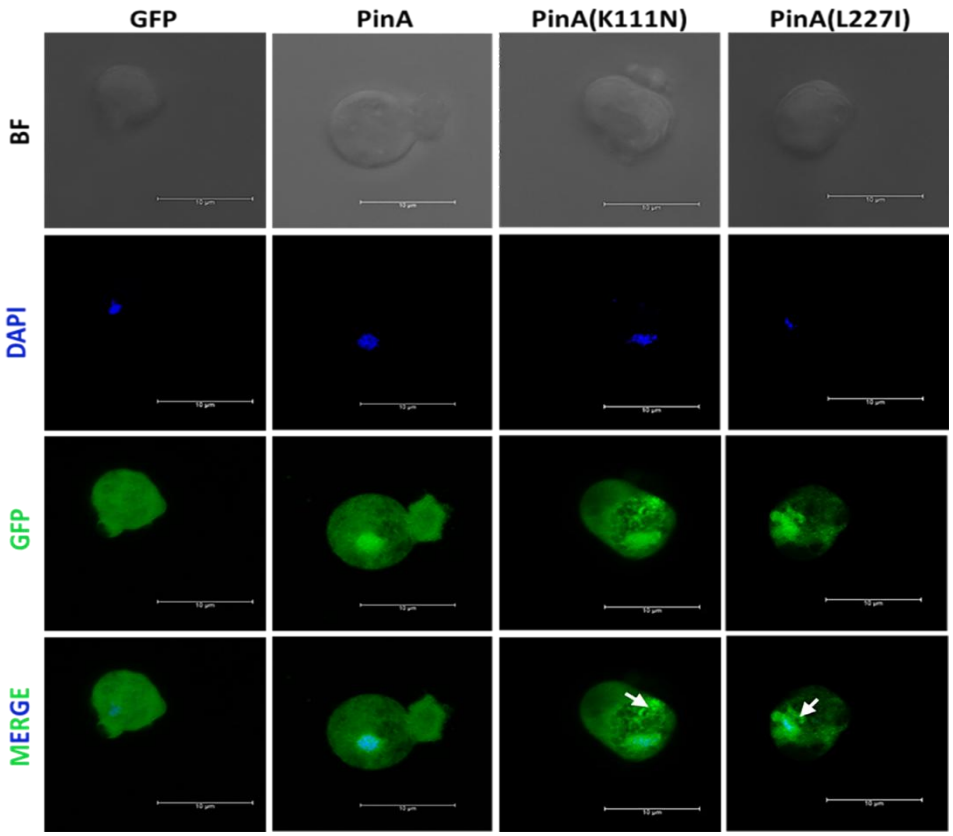

Figure 8: PinA mutants are mainly localized in the cytoplasm in contrast to the nuclear localization of PinA. Ax2 cells expressing GFP or PinA or PinA(K111N) or PinA (L227I) were harvested, washed and resuspended in 1X PBS and counterstained with 1µg/ml DAPI concentration. The cellular localization of GFP was visualized under a Confocal microscope. Brightfield images are also shown along with DAPI (blue) and merge images for reference. Arrows showing the aggregation of proteins in the cytoplasm of cells expressing *pinA* mutants. Blebs are visible in cells of GFP, PinA and PinA(K111N), which may be due to processing before taking these images, cells are otherwise normal. Scale bar = 10 µm

cyclic AMP signalling and also late developmental stages to understand the exact role of PinA.

Confocal analysis shows that some of the phenotypes observed in *pinA* mutants may be due to alteration in PinA localization

PinA and Pin1 are also reported to be predominantly localized in the cell nucleus (Haokip and Naorem, 2017; Lu *et al.,* 2002). To determine whether the developmental phenotypes displayed by the expression of *pinA* mutants in Ax2 cells are due to any alteration in the localization of PinA, confocal microscopy was used to check the protein localization in growing cells. As seen earlier, the localization of PinA was mainly nuclear and also detectable in cytoplasm in Ax2 cells. On the other hand, PinA(K111N) and PinA(L227I) mutants were found mostly in the cytoplasm as aggregates while a very little signal was observed in the nucleus (Fig. 8). These results suggest that the growth and developmental phenotypes observed in Ax2 cells expressing PinA(K111N) or PinA(L227I) could be due to alteration in the protein localization. Further, this alteration can affect the expression of genes for early developmental events resulting in the observed defect in either *pinA*$^{-}$ or *pinA* mutant cells.

***pinA* mutants are expressed in bacteria for their structures and activity analysis**

Although the crystal structure of Pin1 is available, understanding the mechanism of PinA requires determining its crystal structure. Notably, PinA features a unique FHA domain for protein-protein interactions, unlike the WW domain found in Pin1/Ess1. After identifying structural differences between wild-type PinA and PinA mutants, PinA(K111N) and

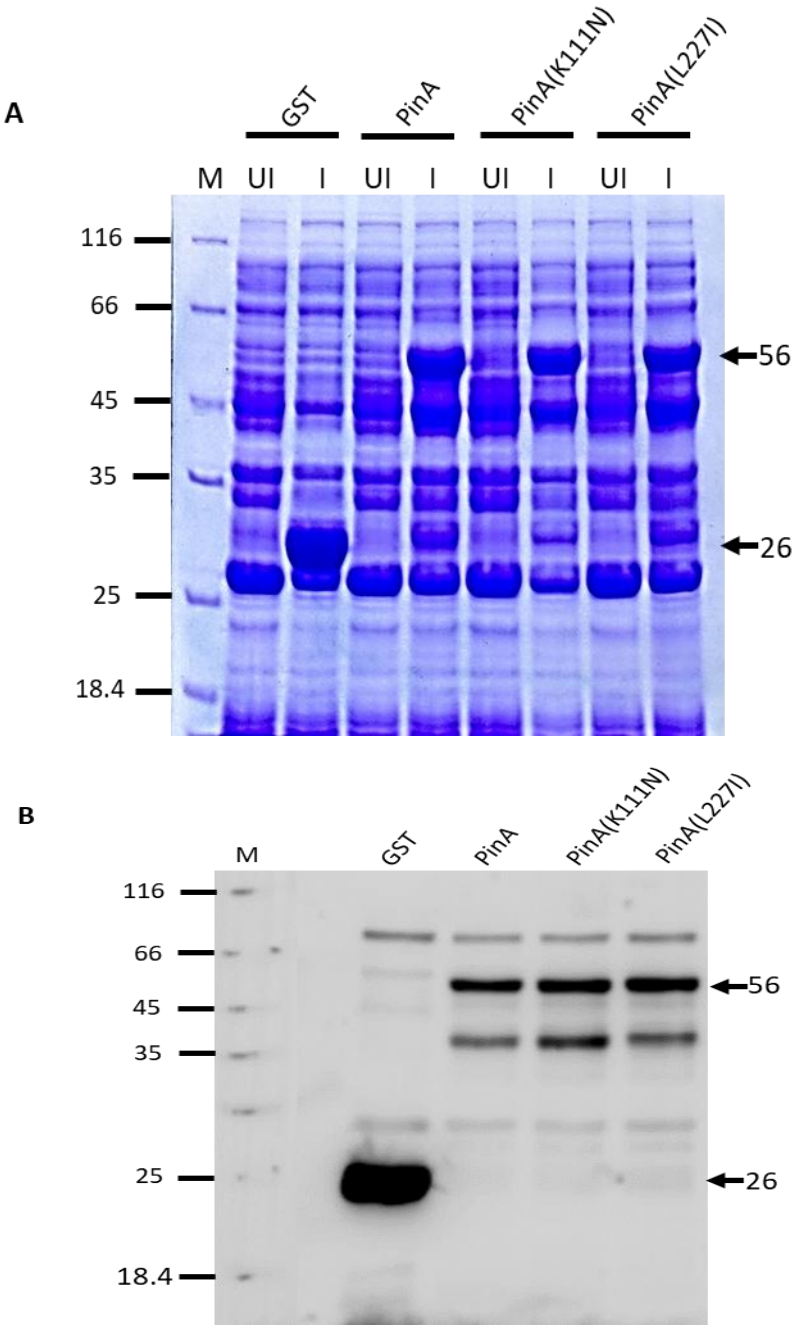

Figure 9: Expression of *D. discoideum* PinA and PinA mutants as GST fusion proteins in *E. coli*. BL21 codon plus cells transformed with pGEX4T1 (GST) and pGEX4T1 having the open reading frame (ORF) of *pinA* or *pinA*^{K111N} or *pinA*^{L227I} in frame with GST at N-terminus were induced with 0.2 mM IPTG and separated on 10% SDS-PAGE. (A) Coomassie stain gel showing the expression of GST (~26 kDa) and recombinant GST tagged PinA mutants, PinA(K111N) and PinA(L227I) as ~ 56 kDa proteins in induced samples (I) as indicated with expected size indicated by arrows at *right*, compared to uninduced samples (UI). (B) Western blot confirming the expression of expected size GST (~26 kDa) and GST fusion proteins; PinA, PinA(K111N) and PinA(L227I) of ~ 56 kDa proteins as detected by Polyclonal anti-GST antibody. M in (A) and (B) indicates Unstained Protein Markers with size in kDa at *left*

PinA(L227I) using Phyre2 (Fig. 2), we attempted to express these mutants, along with others (listed in Table 1). For this, PinA and PinA mutants were expressed as GST-fusion proteins in bacteria by cloning in frame with GST in pGEX-4T1 vector. GST-fusions, PinA (K111N) or PinA(L227I) mutant protein and wild-type PinA, were expressed in *E. coli* BL21 codon plus and observed after separating on 10% SDS PAGE gel stained with Coomassie

dye (Fig. 9A). The expressed proteins were also confirmed by western blots with anti-GST antibody and detected the corresponding protein bands ~ 56 kDa (Fig. 9B). Further, these *pinA* mutants were also expressed as SUMO-fusions which will be used for studying their structure and also for their activity assays (data not shown). Expression of these mutants and purification along with appropriate controls will then be used to examine PinA structure, interacting proteins, activity and critical amino acid residues for its binding and also activity etc.

CONCLUSION

In conclusion, this study emphasizes on the power of mutation in elucidating the significance of PinA in regulating diverse cellular processes governing growth and development in *D. discoideum*. Generation of the *pinA* mutants and complementation analysis in yeast and *D. discoideum* cells lacking PinA indicated the compromised function of *pinA* mutants. Supporting the above observation, *D. discoideum* cells overexpressing *pinA* mutants exhibited compromised function of PinA, leading to aberrant growth and developmental phenotypes. It can be envisaged that alteration in the subcellular localization of these *pinA* mutants to the cytoplasm rather than its normal nuclear localization may lead to alteration in developmental gene expression which is crucial for normal development. Thus, analyzing the *pinA* mutants indicated that PinA regulates specific cellular processes operating in growth, growth to developmental transition, aggregation, multicellular establishment and the final culmination to fruiting body. Further investigations are needed to examine the activity and interacting proteins of PinA in specific cellular pathways operating during growth and development processes. It will also be worthwhile to investigate its structure-function using these mutants in *D. discoideum*. Further, these mutants were successfully expressed in bacteria as recombinant proteins to elucidate the structure of PinA, its critical amino acid residues for protein-protein interactions and activity. Overall, this study on PinA in *D. discoideum* highlights the novel function of parvulin PPIase in regulating various cellular processes governing growth and developmental events.

ACKNOWLEDGEMENTS

We thank the members of the Naorem laboratory for helpful discussion and CIF, UDSC, for the Confocal facility. This work was supported by grants (SB/EMEQ-203/2013) and (EEQ/2022/000330) from Science and Engineering Research Board, Department of Science and Technology, Govt. of India. V.Y thank University of Delhi and ICMR for the fellowship.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1992) *Short Protocols in Molecular Biology*. ***275***, 28764-28773.

Barrero, J. J., Papanikou, E., Casler, J. C., Day, K. J., & Glick, B. S. (2016) An improved reversibly dimerizing mutant of the FK506-binding protein FKBP. *Cellular Logistics*, ***6***(3), e1204848.

Born, A., Henen, M. A., & Vögeli, B. (2019) Activity and affinity of Pin1 variants. *Molecules*, ***25***(1), 36.

Crenshaw, D. G., Yang, J., Means, A. R., & Kornbluth, S. (1998) The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. *The EMBO Journal*, ***17***(5), 1315-1327.

Fey, P., Kowal, A. S., Gaudet, P., Pilcher, K. E., & Chisholm, R. L. (2007) Protocols for growth and development of *Dictyostelium discoideum*. *Nature Protocols*, ***2***(6), 1307-1316.

Fischer, G., & Aumüller, T. (2003) Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes. *Reviews of Physiology, Biochemistry and Pharmacology*, ***148***, 105-150.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., & Schmid, F. X. (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature*, ***337***(6206), 476-478.

Fujimori, F., Gunji, W., Kikuchi, J., Mogi, T., Ohashi, Y., Makino, T., Oyama, A., Okuhara, K., Uchida, T. and Murakami,

Y. (2001) Crosstalk of prolyl isomerases, Pin1/Ess1, and cyclophilin A. *Biochemical and Biophysical Research Communications*, ***289***(1), 181-190.

Gaudet, P., Williams, J. G., Fey, P., & Chisholm, R. L. (2008) An anatomy ontology to represent biological knowledge in *Dictyostelium discoideum*. *BMC Genomics*, ***9***, 1-12.

Hanes, S. D. (2015) Prolyl isomerases in gene transcription. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1850*(10), 2017-2034.

Hanes, S. D., Shank, P. R., & Bostian, K. A. (1989) Sequence and mutational analysis of ESS1, a gene essential for growth in *Saccharomyces cerevisiae*. *Yeast*, ***5***(1), 55-72.

Hani, J., Stumpf, G., & Domdey, H. (1995) PTF1 encodes an essential protein in Saccharomyces cerevisiae, which shows strong homology with a new putative family of PPIases. *FEBS Letters*, ***365***(2-3), 198-202.

Haokip, N., & Naorem, A. (2017) Functional characterisation of parvulin-type peptidyl prolyl cis-trans isomerase, PinA in *Dictyostelium discoideum*. *Biochemical and Biophysical Research Communications*, ***482***(2), 208-214.

Harding, M. W., Galat, A., Uehling, D. E., & Schreiber, S. L. (1989) A receptor for the immuno-suppressant FK506 is a cis–trans peptidyl-prolyl isomerase. *Nature*, ***341***(6244), 758-760.

Innes, B. T., Bailey, M. L., Brandl, C. J., Shilton, B. H., & Litchfield, D. W. (2013) Non-catalytic participation of the Pin1 peptidyl-prolyl isomerase domain in target binding. *Frontiers in Physiology*, ***4*****,** 18-27.

Ishikawa, Y., Vranka, J. A., Boudko, S. P., Pokidysheva, E., Mizuno, K., Zientek, K., Keene, D. R., Rashmir-Raven, A. M., Nagata, K., Winand, N. J., & Bächinger, H. P. (2012) Mutation in cyclophilin B that causes hyperelastosis cutis in American Quarter Horse does not affect peptidylprolyl cis-trans isomerase activity but shows altered cyclophilin B-protein interactions and affects collagen folding. *Journal of Biological Chemistry*, ***287***(26), 22253-22265.

Jordens, J., Janssens, V., Longin, S., Stevens, I., Martens, E., Bultynck, G., Engelborghs, Y., Lescrinier, E., Waelkens, E., Goris, J., & Van Hoof, C. (2006) The protein phosphatase 2A phosphatase activator is a novel peptidyl-prolyl cis/trans-isomerase. *Journal of Biological Chemistry*, ***281***(10), 6349-6357.

Ke, H. M., Zydowsky, L. D., Liu, J., & Walsh, C. T. (1991) Crystal structure of recombinant human T-cell cyclophilin A at 2.5 A resolution. *Proceedings of the National Academy of Sciences*, ***88***(21), 9483-9487.

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, ***10***(6), 845-858.

Loomis, W.F. (2014) Cell signaling during development of *Dictyostelium. Developmental Biology*, ***391***(1), 1-16.

Lu, K.P., Finn, G., Lee, T. H., & Nicholson, L. K. (2007) Prolyl cis-trans isomerization as a molecular timer. *Nature Chemical Biology*, ***3***(10), 619-629.

Lu, K. P., Hanes, S. D., & Hunter, T. (1996) A human peptidyl–prolyl isomerase essential for regulation of mitosis. *Nature*, ***380***(6574), 544-547.

Lu, K.P., & Zhou, X. Z. (2007) The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nature Reviews Molecular Cell Biology*, ***8***(11), 904-916.

Lu, P. J., Zhou, X. Z., Liou, Y. C., Noel, J. P., & Lu, K. P. (2002) Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. *Journal of Biological Chemistry*, ***277***(4), 2381-2384.

Pemberton, T. J., & Kay, J. E. (2005) Identification and comparative analysis of the peptidyl-prolyl cis/trans isomerase repertoires of *H. sapiens*, *D. melanogaster*, *C. elegans*, *S. cerevisiae* and *S. pombe. Comparative and Functional Genomics*, ***6***(5-6), 277-300.

Rahfeld, J. U., Rücknagel, K. P., Schelbert, B., Ludwig, B., Hacker, J., Mann, K., & Fischer, G. (1994a) Confirmation of the existence of a third family among peptidyl-prolyl cis/trans isomerases

Amino acid sequence and recombinant production of parvulin. *FEBS Letters*, ***352***(2), 180-184.

Rahfeld, J. U., Schierhorn, A., Mann, K., & Fischer, G. (1994b) A novel peptidyl-prolyl *cis*/*trans* isomerase from *Escherichia coli. FEBS Letters*, ***343***(1), 65-69.

Ranganathan, R., Lu, K. P., Hunter, T., & Noel, J. P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. *Cell*, ***89***(6), 875-886.

Schmidpeter, P. A., Jahreis, G., Geitner, A. J., & Schmid, F. X. (2011) Prolyl isomerases show low sequence specificity toward the residue following the proline. *Biochemistry*, ***50***(21), 4796-4803.

Shaw, P. E. (2002) Peptidyl-prolyl isomerases: a new twist to transcription. *EMBO Reports*, ***3***(6), 521-526.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, ***7***(1), 539.

Stark, M. J. (1998) 6 Studying Essential Genes: Generating and Using Promoter Fusions and Conditional Alleles. *Methods in Microbiology*, ***26***, 83-99.

Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., & Clardy, J. (1991) Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. *Science*, ***252***(5007), 839-842.

Wang, X. J., & Etzkorn, F. A. (2006) Peptidyl-prolyl isomerase inhibitors. *Peptide Science: Original Research on Biomolecules*, ***84***(2), 125-146.

Wilson, K. A., Bouchard, J. J., & Peng, J. W. (2013) Interdomain interactions support interdomain communication in human Pin1. *Biochemistry*, ***52***(40), 6968-6981.

Wu, X., Wilcox, C. B., Devasahayam, G., Hackett, R. L., Arévalo-Rodríguez, M., Cardenas, M. E., Heitman, J., & Hanes, S. D. (2000) The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery. *The EMBO Journal*, ***19***(14), 3727-3738.

Xu, Y. X., Hirose, Y., Zhou, X. Z., Lu, K. P., & Manley, J. L. (2003) Pin1 modulates the structure and function of human RNA polymerase II. *Genes & development*, ***17***(22), 2765-2776.

Yaffe, M. B., Schutkowski, M., Shen, M., Zhen Zhou, X., Todd Stukenberg, P., Rahfeld, J.-U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., & Ping Lu, K. (1987) Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science*, ***278***(5345), 1957-1960.

Zoldák, G., Aumüller, T., Lücke, C., Hritz, J., Oostenbrink, C., Fischer, G., & Schmid, F. X. (2009) A library of fluorescent peptides for exploring the substrate specificities of prolyl isomerases. *Biochemistry*, ***48***(43), 10423-10436.