Chapter 5:

ADPRT1A knockout and its possible role on *D. discoideum* growth and multicellularity

***ADPRT1A* knock-out and its possible role in *D. discoideum* growth and multicellularity**

5.1 Introduction

Dictyostelium discoideum is a social amoeba used as a model system for development, and differentiation studies owing to the presence of unicellular as well as multicellular life stages in its life cycle (Schaap, 2011). On the basis of our previous studies, we analyzed the role of an abundant nuclear protein, ADPRT1A, homologous to human Poly ADP-ribose polymerase-1 (PARP-1) in survival and multicellular development of *D. discoideum* (specifically during unicellular growth and aggregation during developmental morphogenesis). PARP-1 is stimulated significantly in the presence of a range of activators like damaged DNA, nucleosomes, non B-DNA structures, and various protein-binding partners (D'Amours *et al*., 1999; Oei and Shi, 2001; Kun *et al*., 2002; Kim *et al*., 2005). Developmental and environmental stimuli have also been reported to induce activation of PARP-1 (Pinnola *et al.,* 2007). Evidences establish the role of PARP-1 in plants, fungi as well as in mice (De Block *et al*., 2005; Semighini *et al*., 2006; Kothe *et al*., 2010; Jubin et al., 2016b). de Murcia *et al.,* also reported that PARP-1 and PARP-2 double mutant mice died at the onset of gastrulation (de Murcia *et al*., 2003). PARP-1 overexpression hampered the cytoskeletal organization [F-actin] and disrupted tissue polarity suggesting the regulation of PARP and its plausible role in the developmental process in *Drosophila* (Uchida *et al*., 2002). It has been suggested that PARP is necessary for viability and organization of chromatin, heterochromatin, and other sequences during development of *Drosophila* (Tulin *et al*., 2002). However, role of PARP-1 in differentiation still remains obscure.

Dictyostelium possesses eight potential *PARP* genes (Kawal *et al*., 2011). We have demonstrated PARP's role in development by downregulation wherein development was blocked at the initial aggregation stage signifying the involvement of PARP during development of *D. discoideum* (Rajawat *et al*., 2011). ADPRT1A (PARP-1 ortholog) overexpression led to delayed growth and development in *Dictyostelium.* Moreover, nutrient starvation also resulted in PARP activation (Jubin *et al*., 2016a). ADPRT is

known to participate in cellular proliferation (Colon-Otero *et al*., 1987; Kaiser *et al*., 1992, Jubin *et al*., 2016a), cell differentiation (Exley *et al*., 1987) and DNA repair (Satoh and Lindahl, 1992; Couto *et al*., 2011). However, the role of ADPRT in development and differentiation is not yet fully understood. ADPRT1A overexpression indicated a certain link between PARP-1 and growth and multicellularity in *Dictyostelium*. Hence, the present study aims to unravel the role of ADPRT1A in cellular processes as well the developmental morphogenesis of *Dictyostelium* via a loss-of-function mutant namely *ADPRT1A* knockout. The study would help us to identify how ADPRT1A regulates cellular proliferation, survival and its requirement in developmental morphogenesis.

5.2 Results

In order to unravel the role of ADPRT1A in growth and multicellularity, an *ADPRT1A* KO *D. discoideum* cells was generated by homologous recombination followed by the study of the effect of *ADPRT1A* KO in *D. discoideum* growth and development.

5.2.1 Sequential cloning of *PARP* (*ADPRT1A*) 5' and 3' HR into pDrive vector

The *ADPRT1A* knockout (*ADPRT1A* KO) construction was done by homologous recombination as shown in Fig. 5.1. The 5' homologous region (HR) and 3' HR of *ADPRT1A* were cloned directionally on both sides of blasticidin cassette (*Bsr*) of the pDrive cloning vector.

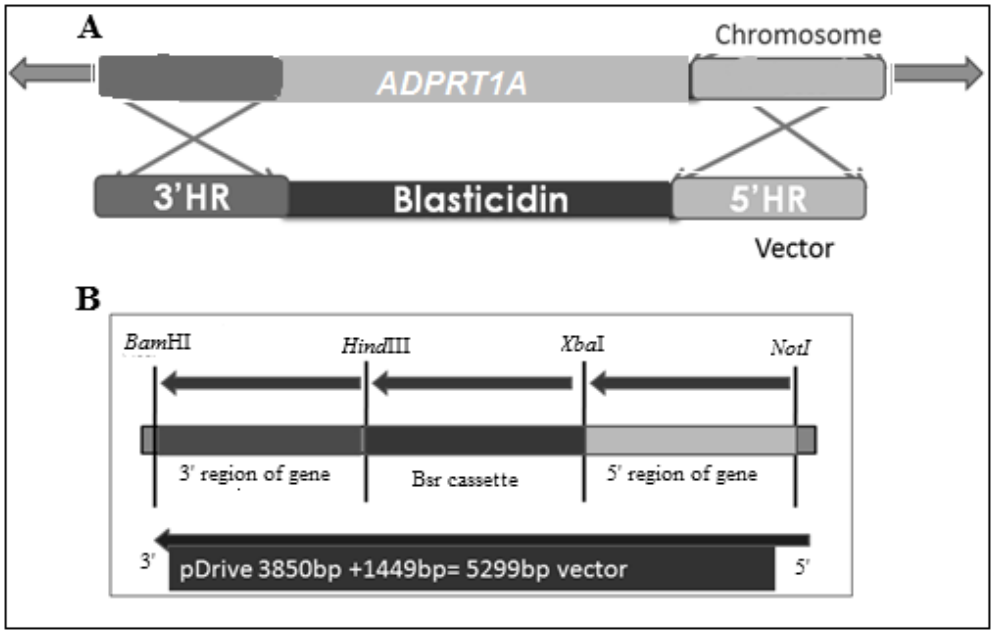


Fig. 5.1: Strategy for Sequential cloning of *PARP* (*ADPRT1A*) 5' and 3' HR into pDrive vector. (A) Homologous recombination strategy for *ADPRT1A* KO construct (B) Organization of *ADPRT1A* KO construct in pDrive cloning vector.

D. discoideum genomic DNA was used for the PCR amplification as a template for 5' homologous region- 5'HR (889 bp) and 3' homologous region- 3'HR (875 bp) using respective specific primers (Fig. 5.2). The confirmed 5' HR amplicon and pDrive vector were digested with *Not*I and *Xba*I and subjected to ligation. This ligation mixture was transformed into *E. coli DH5α* and the transformants were subjected to selection on Luria Bertani Agar containing ampicillin (100µg/ml). Randomly selected colonies were screened for the presence of recombinant plasmid 5' HR-*Bsr* pDrive which were confirmed by restriction digestion with *Not*I and *Xba*I enzymes as shown in Fig. 5.3. This positive clone was used for further sequential cloning of 3' HR. 3' HR amplicon and the above mentioned positive clone (5' HR-*Bsr* pDrive) were digested with *BamH*I and *Hind*III followed by ligation and transformation into *E.coli DH5α* cells. Ampicillin resistant colonies obtained were randomly selected for the presence of recombinant plasmid (5'HR-*Bsr*-3'HR pDrive) and screened by restriction digestion pattern (Fig.5.4).

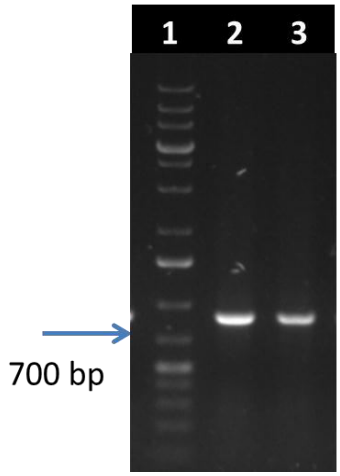


Fig. 5.2: PCR amplification of 5' and 3'HR regions of *ADPRT1A*. Lane 1:1kb DNA ladder; Lane 2: 5' HR *ADPRT1A* PCR (889 bp); Lane 3: 3' HR *ADPRT1A* PCR (875 bp).

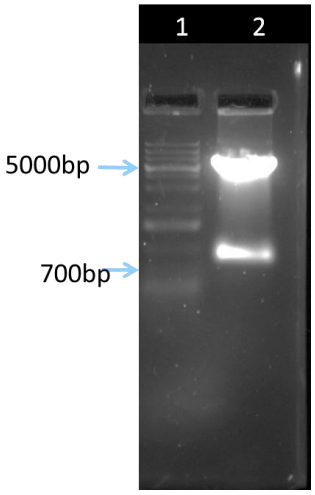


Fig. 5.3: Screening of 5'HR-Bsr pDrive by RE digestion. Lane 1: 1kb DNA ladder; Lane 2: Positive clone digested with *Not*I and *Xba*I.

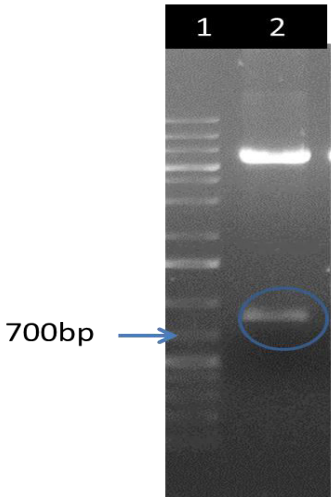
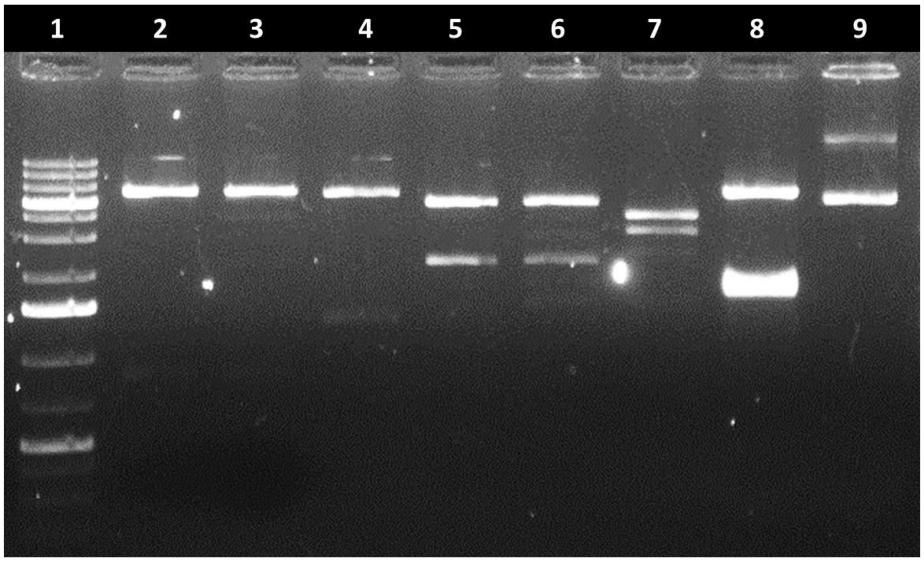


Fig. 5.4: Screening of 5'HR-Bsr-3'HR pDrive by RE digestion. Lane 1: 1kb DNA ladder; Lane 2: Clone digested with *Hind*III and *BamH*I.

5.2.2 Confirmation of *ADPRT1A* knockout (*ADPRT1A* KO) constructs

The resultant pDrive knockout construct that was obtained is comprised of the 3' homologous region (3'HR) and 5' homologous region (5'HR) of *ADPRT1A* along with Blasticidin Selection Cassette (*Bsr*). To confirm *ADPRT1A* knockout construct, various combinations of restriction enzymes were used and the positive *ADPRT1A* knockout clone restriction digestion pattern is shown in Fig. 5.5.



Expected	**5'HR**	**3'HR**	**Bsr**	**5'HR Bsr**	**3'HR Bsr**	**5'HR - Bsr- 3'HR**	**pDrive**	**uncut**
Enzymes used	**N& X**	**H& B**	**X&B**	**N& H**	**X& B**	**N&B**	**N& X**	
Release after digestion in bp	**889**	**875**	**1449**	**2338**	**2324**	**3203**	**5299**	

Fig. 5.5: RE digestion pattern for confirmation of *PARP* (*ADPRT1A*) knockout construct. Using various combinations of restriction enzymes for digestion of resultant positive clone, *ADPRT1A* knockout was confirmed (Enzymes used: *Not*I-N, *Xba*I-X, *Hind*III-H and *BamH*I-B).

5.2.3. Transformation of *ADPRT1A* knockout construct in *D. discoideum*

D. discoideum cells were transformed with *ADPRT1A* knockout construct using electroporation (Gaudet *et al.,* 2007). Restriction enzyme (RE) double digestion was done using *Not*I and *BamH*I and knockout fragment of 3.2 Kb was successfully released which was then used for transformation (Fig.5.6). Blasticidin antibiotic at a concentration of 10µg/mL was used as the selection pressure.

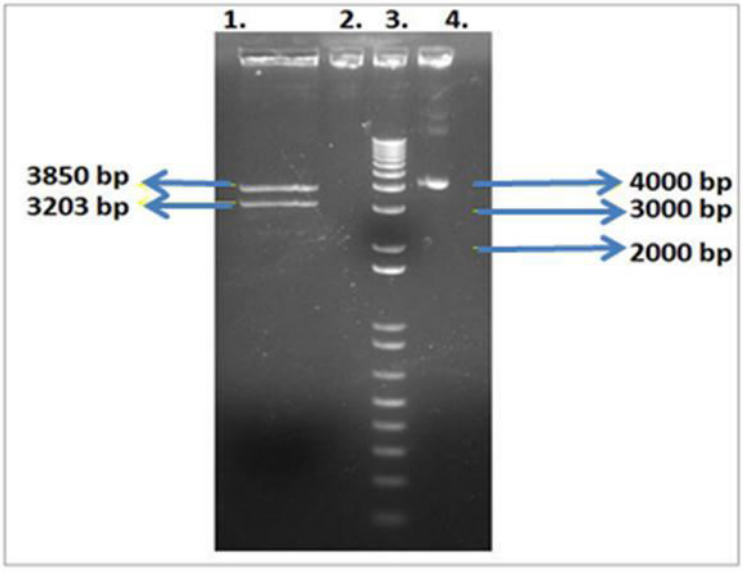
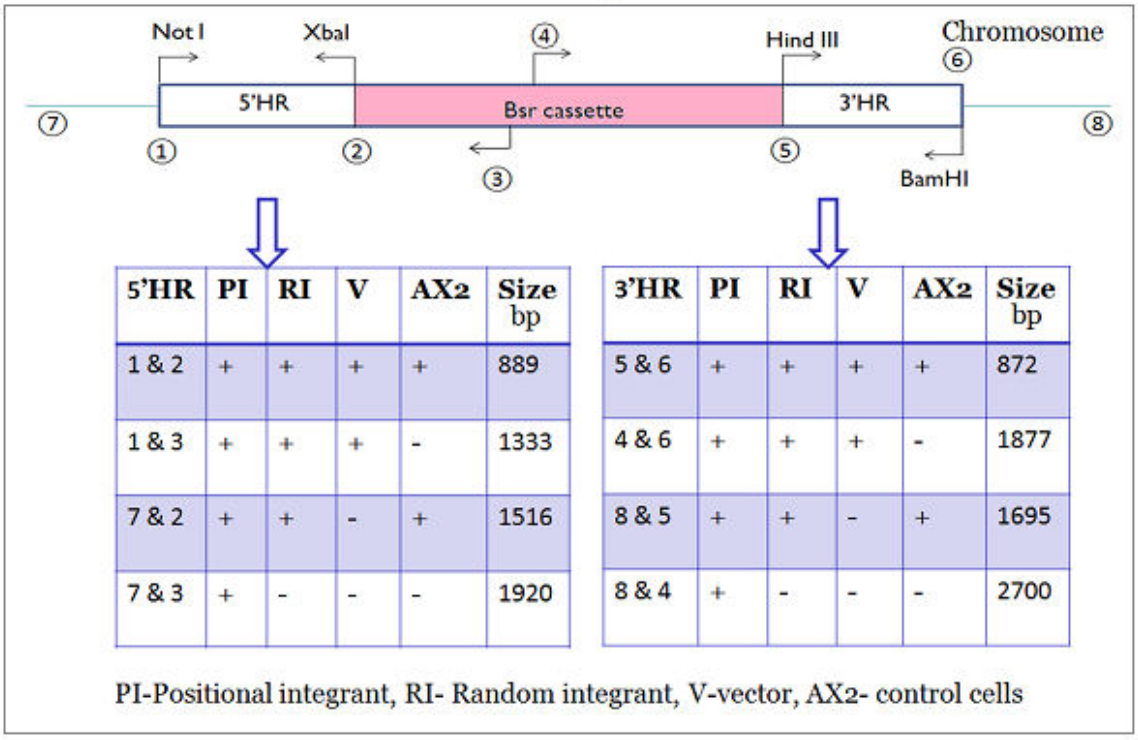


Fig. 5.6: RE digestion pattern of *ADPRT1A* knockout construct. Lane 1: RE digestion with *Not*I and *BamH*I; Lane 3: 1 kb Ladder; Lane 4: Uncut pDrive vector.

5.2.4. Standardization of PCR for screening of positional and random integrant knockout clone

As *D. discoideum* feeds on bacteria, selection of the clones was carried out on a lawn of heat killed *Klebsiella aerogenes* cells. Transformed *D. discoideum* cells were serially diluted and plated on Non-nutient agar (NNA) plates with blasticidin at a concentration of 5µg/mL with 200 µL of a dead *Klebsiella aerogenes*. After a few days of incubation at 22^{o}C clear plaques were seen on the bacterial lawn. The clear plaque representing a single clone was subsequently picked and inoculated in HL5 supplemented with 10µg/mL of blasticidin. The cells were allowed to grow at 22^{o}C and genomic DNA was isolated to check for positional and random integrants by PCR amplifications. The validation of [*ADPRT1A* KO/Ax2] strain was carried by PCR amplification using eight different primer combinations to distinguish between the Control Ax-2, the positional integrants and the random integrant strains as shown in Fig. 5.7.

In case of Ax2, primer combination 1 and 2, amplifies 889 bp fragment of 5′end of the gene. Similarly, primer combination 5 and 6 yields 872 bp of 3′end of the gene. PCR amplification using these two combinations would be obtained in all the strains namely, Ax2, random integrant, knockout and vector. However, PCR from primer combinations 7 and 3, (product size of 1.92 Kb) and 8 and 4 (product size of 2.7 Kb) would be in the knockout strain only. PCR with all 8 combinations were standardized by gradient PCR using genomic DNA of cells from single spore obtained on plates containing blasticidin.



5'HR	PI	RI	V	AX2	Size bp
1 & 2	+	+	+	+	889
1 & 3	+	+	+	-	1333
7 & 2	+	+	-	+	1516
7 & 3	+	-	-	-	1920

3'HR	PI	RI	V	AX2	Size bp
5 & 6	+	+	+	+	872
4 & 6	+	+	+	-	1877
8 & 5	+	+	-	+	1695
8 & 4	+	-	-	-	2700

PI-Positional integrant, RI- Random integrant, V-vector, AX2- control cells

Fig. 5.7: Screening of random integrant knockout clone by PCR

5.2.5. Screening of *ADPRT1A* knockout clones for positional and random integrants and confirmation

Using this strategy (Fig. 5.7), *ADPRT1A* knockout strain was successfully screened out which was further used for the functional analysis. Confirmation of *ADPRT1A* KO was done by PCR at genomic as well as at transcript level. Eight combinations of primers were used to confirm PCR using genomic DNA. Two clones (named *ADPRT1A* KO1

AND *ADPRT1A* KO2) had all eight positive PCR confirming knockout of *ADPRT1A* as shown in Fig. 5.8 which was further used for all knockout downstream experiments.

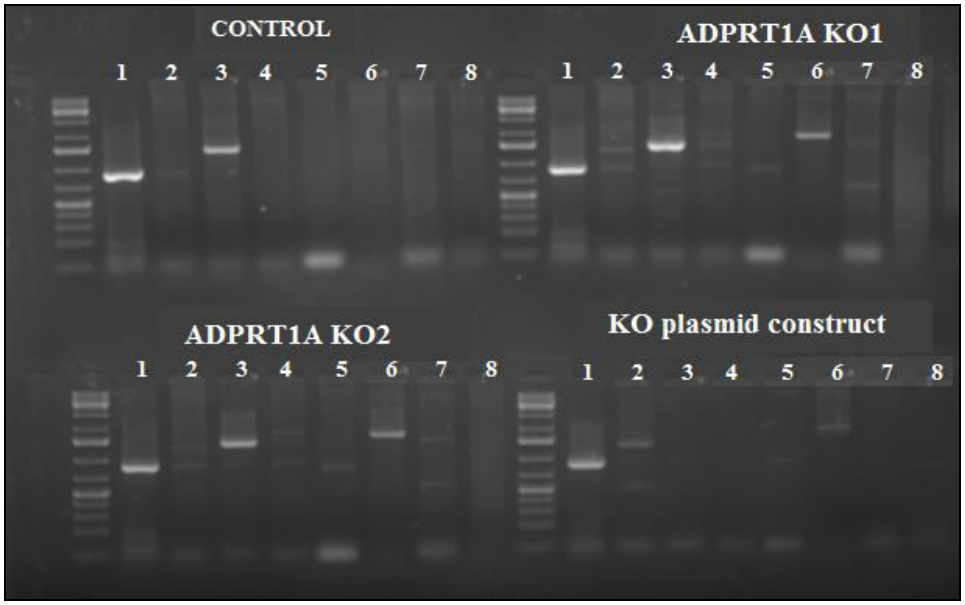


Fig. 5.8: Confirmation of knockout of *PARP* (*ADPRT1A)* by PCR at genomic level. Lane1: Primers 1&2, Lane2: Primers 1&3, Lane3: Primers 7&2, Lane4: Primers 7&3, Lane5: Primers 5&6, Lane6: Primers 4&6, Lane7: Primers 8&5 and Lane8: Primers 8&4 (Primers combinations as explained in Fig. 5.7).

5.2.6 Functional Characterization of *ADPRT1A* KO

ADPRT1A KO was confirmed by real time PCR using *ADPRT1A* specific primers wherein transformed cells exhibited loss of ~93% expression of *ADPRT1A* as compared to control cells as seen in Fig 5.9. In addition, NAD^+ levels in the cell were also estimated. PARP-1 being an avid consumer of NAD^+ (Houtkooper *et al.*, 2010), *ADPRT1A* knockout may lead to less consumption of NAD^+. This was established by significantly high NAD^+ levels in *ADPRT1A* KO cells ($p<0.005$) as compared to wild type *D. discoideum* cells (Fig. 5.10).

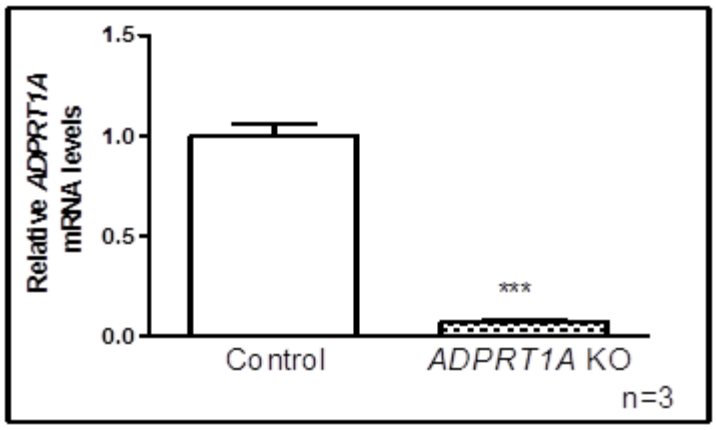


Fig. 5.9: Functional characterization of *ADPRT1A* KO. Real time analysis in *ADPRT1A KO* cells showed significantly decreased (>90%) *ADPRT1A* transcripts as compared to control. Data is a representation of SEM values of three independent experiments.

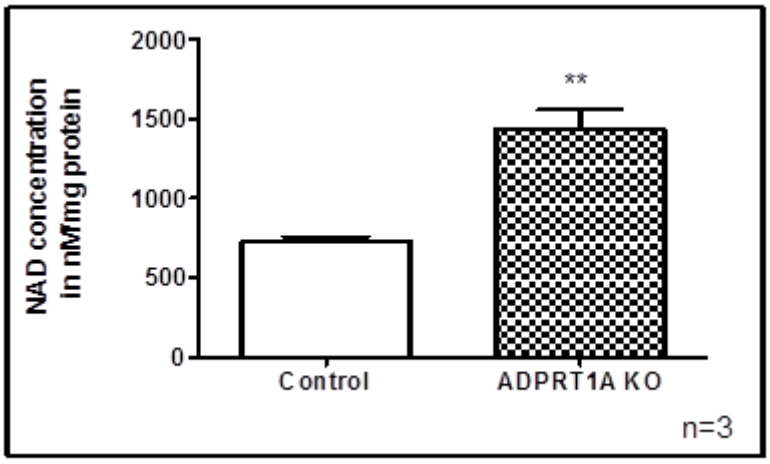


Fig. 5.10: NAD^+ concentration in control and *ADPRT1A KO* cell using Bernofsky's Enzymatic recycling method. Data is a representative of three independent experiments. ** *p* value for *ADPRT1A KO*<0.01 as compared to control as compared to control.

5.2.7: Growth profiling and cell cycle analysis in *ADPRT1A* KO

Next, the effect of *ADPRT1A* KO was checked on cellular proliferation of *D. discoideum.* Our results showed that *ADPRT1A KO* exhibited an extended lag phase as seen in Fig. 5.11A compared to control cells. *ADPRT1A KO* cells showed doubling time of 24.95 hrs ± 2.167 as opposed to 12.26 hrs ± 0.6703 for control *D. discoideum* cells. Microscopic images also showed bigger cell size and stressed phenotype of *ADPRT1A* KO as compared to control cells as shown in Fig. 5.11B. This stressed phenotype was also confirmed by TEM wherein *ADPRT1A KO* cells showed vacuolar phenotype as seen in Fig. 5.11C compared to control thereby illustrating requirement of ADPRT1A in cellular growth and cell survival.

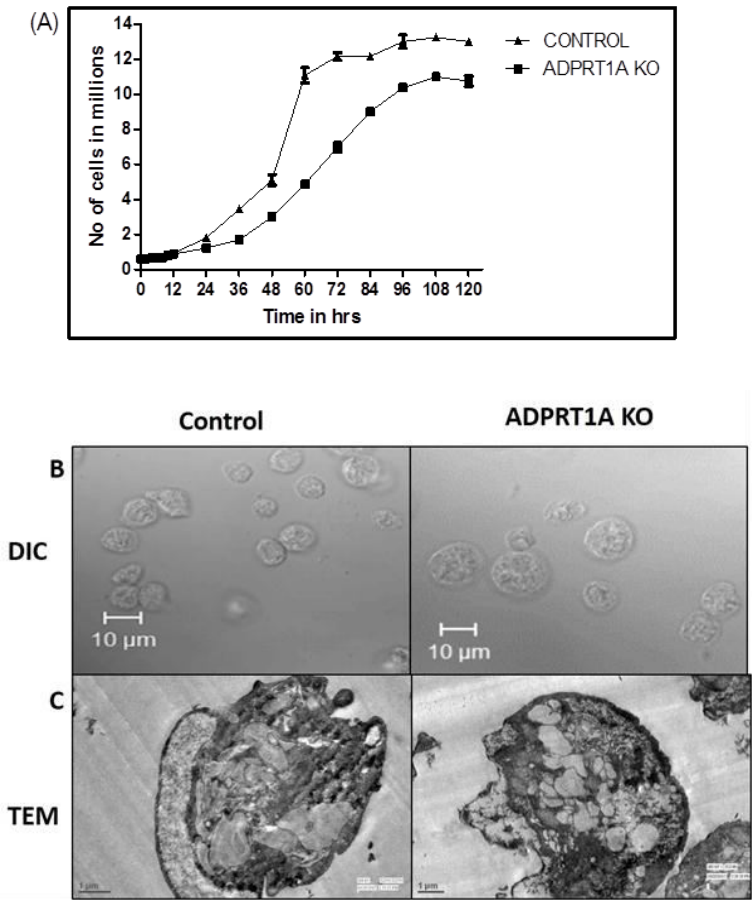
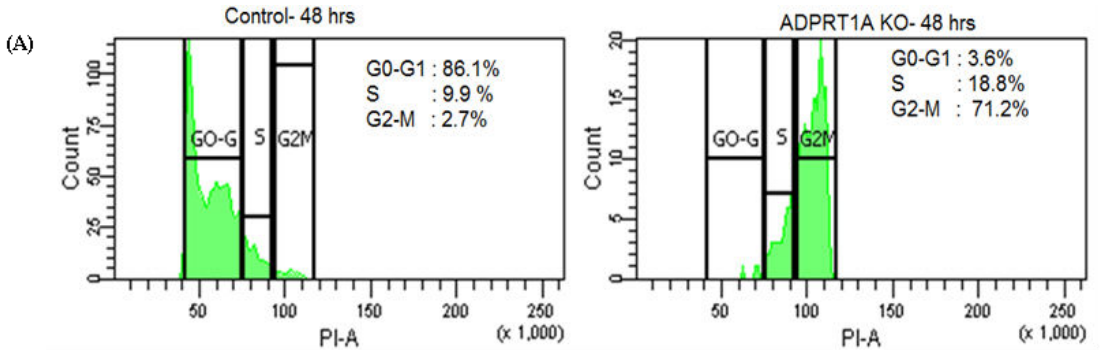


Fig. 5.11: Growth analysis of Control and *ADPRT1A KO* cells. (A) *ADPRT1A KO* cells showed doubling time of 24.95 hrs ± 2.167 as opposed to 12.26 hrs ± 0.6703 for

control *D. discoideum* cells. (B) Differential interface images DIC and (C) Electron Microscopic images of control and *ADPRT1A KO* cells wherein the *ADPRT1A KO* cells showed stressed and vacuolar phenotype. Data is a representative of three independent experiments.

Further to explain the slow proliferation of *ADPRT1A KO*, cell cycle analysis was carried out in a time dependent manner as there are reports of role of PARP-1 in induction of G1 arrest and regulation of G2 arrest (Masutani *et al*., 1995). *ADPRT1A KO* cells showed significant number of cells in G2/M phase at 48 hrs of growth (Fig. 5.12A). However, this G2/M arrest continued till 72 hrs wherein significantly high number of cells was stalled in G2/M phase as compared to control *D. discoideum* cells (Fig. 5.12B). Additionally, PARP-1 has been reported to regulate cell cycle regulating proteins for e.g. *Cyclin A, Cyclin B* etc. (Simbulan-Rosenthal *et al*., 2000). Therefore, to understand the G2/M arrest exhibited by *ADPRT1A KO* cells, *Cyclin B* transcript levels were checked in these cells and interestingly, *Cyclin B* transcript levels were found to be significantly lower as compared to its levels in control cells (Fig. 5.12C). PARP-1 may regulate *Cyclin B* expression thus controlling cell cycle which eventually affected cellular proliferation.



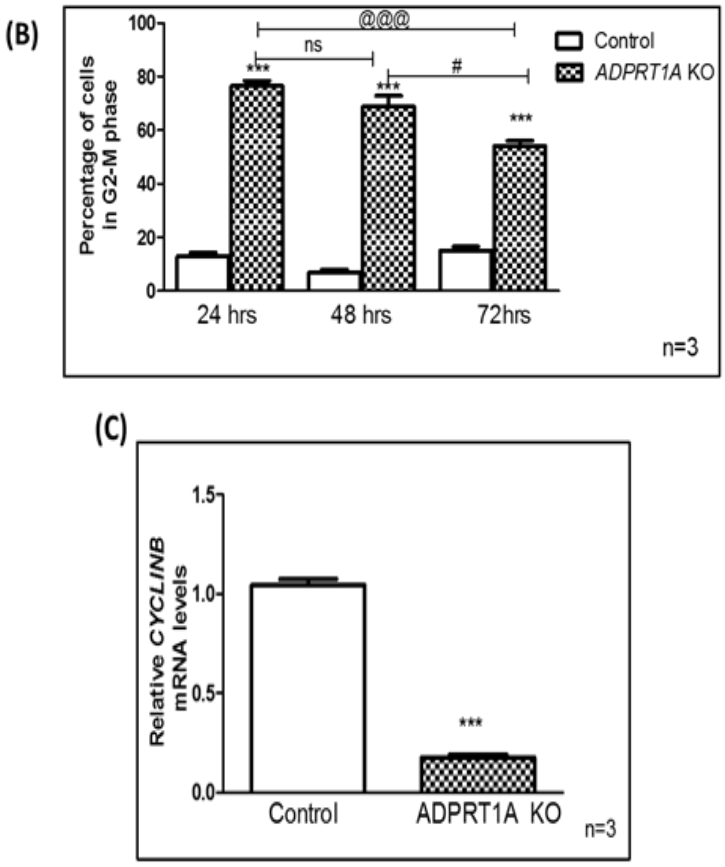


Fig. 5.12: Cell cycle analysis and *CYCLINB* transcript levels in *ADPRT1A KO* cells. (A) Analysis of cells cycle by FACS with Propidium Iodide. *ADPRT1A KO* cells showed a marked population of cells in G2/M phase as compared to control, at 48 hrs of growth. Data is a representative of three independent experiments. (B) Time dependent cell cycle analysis in *ADPRT1A KO* cells show cells arrested in G2-M phase till 72 hrs of growth. *** *p* value for *ADPRT1A KO*<0.001 as compared to control as compared to control. (C) Real time analysis of *CYCLIN B* transcripts in *ADPRT1A KO* cells showed significantly decreased levels as compared to control. Data is a representation of SEM values of three independent experiments.

5.2.8: Effect of oxidative stress on *ADPRT1A* KO

Evidences demonstrate *PARP-1* KO to confer resistance to endotoxic shock (Oliver *et al*., 1999) while reports also suggest susceptibility to environmental stress (Wang *et al*., 1997). Thus, to understand the effect of ADPRT1A deficiency in *D. discoideum* in response to oxidative stress, growth profile was checked in presence of apoptotic dose of cumene H_2O_2 for 120 hrs. Interestingly, it was seen that H_2O_2 treated *ADPRT1A KO* cells showed significantly higher growth at 24 hrs (p<0.001) and almost comparable growth as untreated control cells at 48 hrs even in the presence of H_2O_2 (Fig. 5.13).

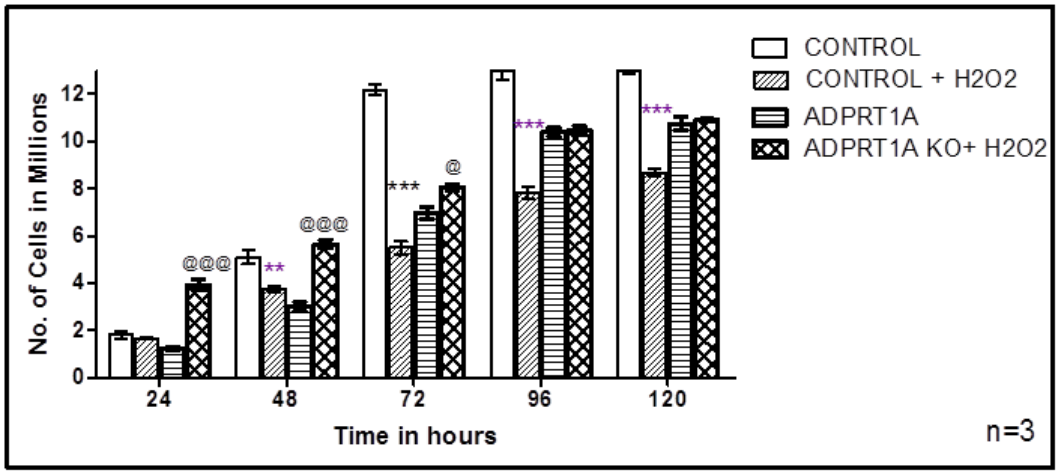


Fig. 5.13: Effect of oxidative stress on *ADPRT1A KO* cells. (A) Growth analysis in presence of 0.03 mM H_2O_2. *ADPRT1A KO* cells show significant growth in presence of oxidative stress as compared to untreated *ADPRT1A KO* cells till 72 hrs exhibiting a resistant phenotype. ** p value for treated control cells<0.01, *** p value for treated control cells <0.001 as compared to untreated control cells. $^{@}p$ value for treated *ADPRT1A KO*<0.05, $^{@@@}p$ value for treated *ADPRT1A KO*<0.001 as compared to untreated *ADPRT1A KO* cells.

Interestingly, it was observed that after 48hrs, growth rate of the treated *ADPRT1A KO* cells reduced as compared to control which may be due to the accumulating DNA damage. This was evident as significantly higher pH2AX (marker of DNA damage) is

seen in *ADPRT1A KO* untreated and treated *D. discoideum* cells (Fig. 5.14) as compared to control cells. Therefore, it is clear that *ADPRT1A* knockout *D. discoideum* cell line showed resistance to oxidative stress.

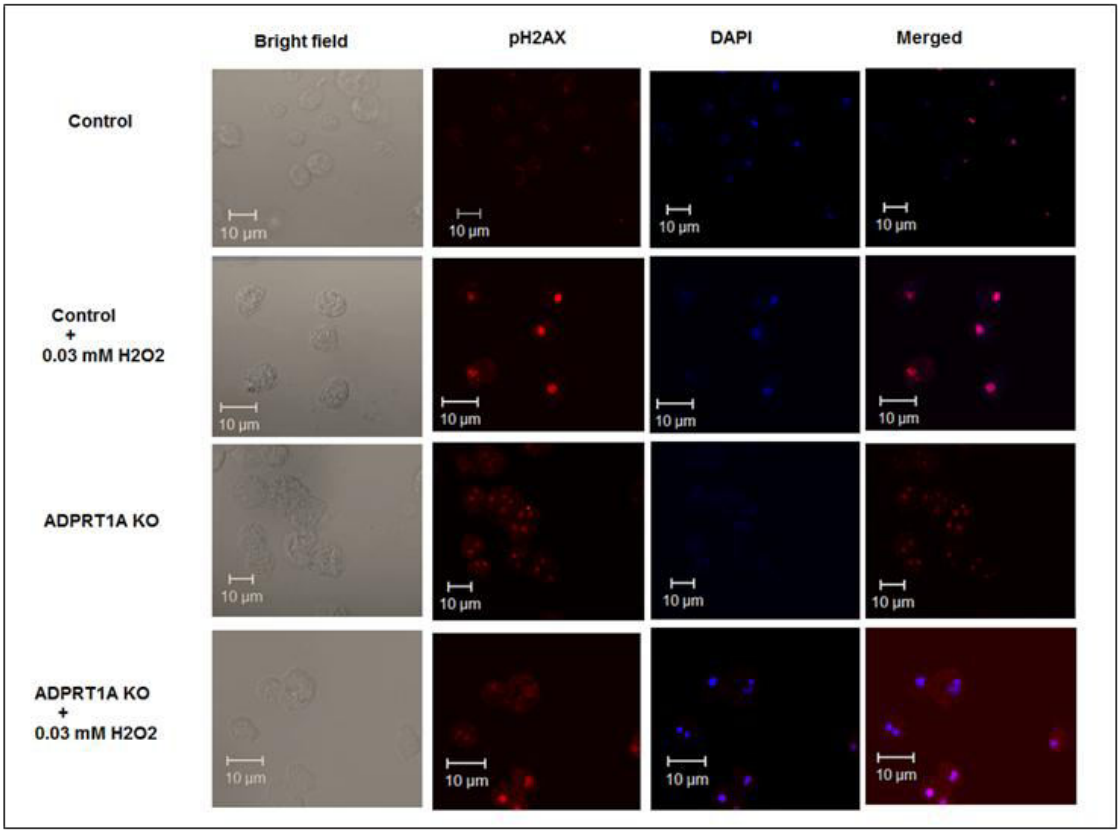


Fig. 5.14: DNA damage in *ADPRT1A* KO. DNA damage analysis was carried out in control and *ADPRT1A* KO post 5 min of 0.03mM cumene H_2O_2 stress by immunofluorescence using antibody against pH2AX. Data is a representative of three independent experiments. Scale: 10 µm.

5.2.9 Role of ADPRT1A in development and multicellularity

Till now, *ADPRT1A* KO has been demonstrated to have affected the unicellular stage significantly. Also, earlier reports illustrated PARP inhibition, downregulation and specifically *ADPRT1A* overexpression to affect developmental morphogenesis of *D.*

discoideum. In this study we aimed to pinpoint the role of ADPRT1A in development of *D. discoideum* in *ADPRT1A* KO as loss of function mutants help us to understand the underlying mechanism. *ADPRT1A* KO cells were subjected to starvation and subjected to development and it was found that the aggregation process of development was significantly affected as seen in Fig. 5.15. Loose aggregates were seen only at 12 hrs in *ADPRT1A KO* cells as opposed to 6 hrs seen in control cells (Fig. 5.15A) leading to further delay in developmental morphogenesis which finally culminated into smaller fruiting bodies at 45 hrs (Fig. 5.15C). It is also surprising that *ADPRT1A* KO cells formed discontinuous streaming structures during aggregation leading to smaller and more number of aggregates as compared to control cells (Fig. 5.15B).

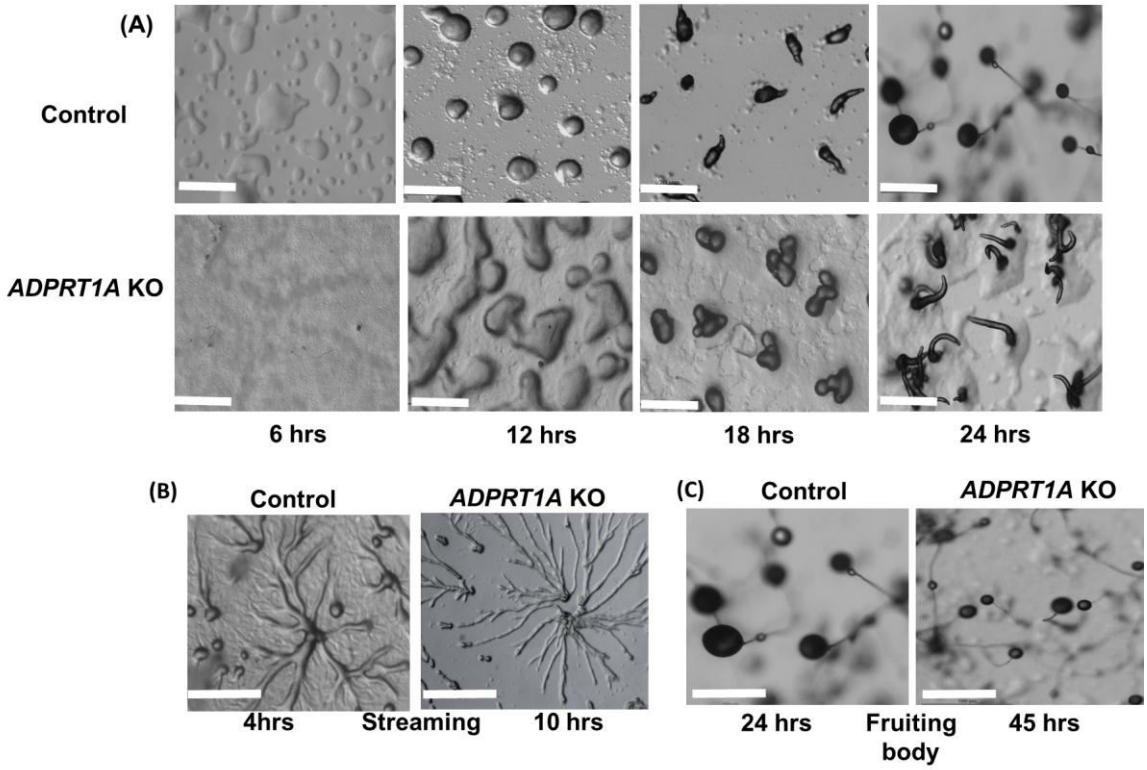


Fig. 5.15: Role of ADPRT1A in *D. discoideum* development. (A) Cells were developed on the NNA plates at a density of 1×10^8 cells/ml. *ADPRT1A* KO cells (bottom panels) showed aggregation delay compared to control, Ax-2 cells (top panels). Bars indicate scale: 100µm. Data is representative of three independent experiments. (B) Streaming

structures of Control Ax-2 cells and *ADPRT1A* KO cells developed on the NNA agar plates at a density of 1×10^8 cells/ml. Bars indicate scale: 100µm. (C) Fruiting body of Control Ax-2 cells and *ADPRT1A* KO cells developed on the NNA agar plates at a density of 1×10^8 cells/ml. Bars indicate scale: 100µm.

As the result of forming small aggregates, fruiting bodies were smaller in size (smaller spore heads) than in control cells and were also more in number. Hence, our results in the *ADPRT1A* knockout background establish ADPRT1A's role during early development of *D. discoideum*

5.2.10 cAMP estimation and chemotaxis analysis in *ADPRT1A* KO

Cyclic-AMP (cAMP) acts as the signaling molecule during *Dictyostelium* development particularly during aggregation and culmination (Kessin, 2001). Thus, in order to explore whether delayed and defective aggregation in *ADPRT1A* KO may be due to defective cAMP signaling, intracellular and extracellular levels of cAMP were estimated at 6 hrs after initiation of development. Comparative analysis of our previously studied *ADPRT1A* OE*, PARP* down regulation (*PARP* dR) and PARP inhibition cells were also carried out along with *ADPRT1A* KO as observed in Fig. 5.16 A and B. It was seen that *ADPRT1A* KO and OE show decreased cAMP levels as compared to control cells, at 6 hrs of starvation (time point at which control cells initiate aggregate formation). *PARP* dR and PARP inhibition also showed decreased intracellular (Fig. 5.16A) as well as extracellular cAMP levels (Fig. 5.16B). This further validates the need of regulated levels of PARP and more specifically ADPRT1A in cAMP production. Thus, it can be comprehended that alteration in cAMP levels could be the reason for delayed initiation of aggregation in *ADPRT1A* mutant phenotypes.

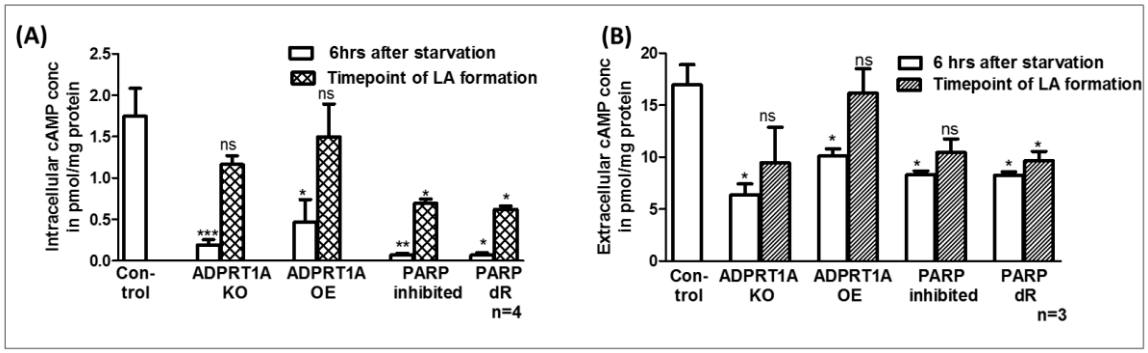
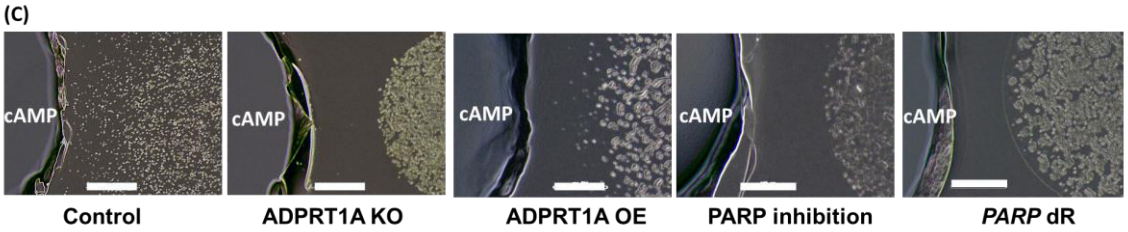


Fig. 5.16: cAMP estimation and chemotaxis assay. (A) Intracellular cAMP concentration after 6 hrs of nutrient starvation and at the time point of loose aggregate (LA) formation in Control, *ADPRT1A* KO, *ADPRT1A* OE, PARP inhibited cells and *PARP* dR cells. **p* value <0.05, ***p* value <0.01 and *** *p* value <0.001 as compared to control. Data is a representation of SEM values of four independent experiments. (B) Extracellular cAMP concentration after 6 hrs of nutrient starvation and at the time point of loose aggregate (LA) formation in Control, *ADPRT1A* KO, *ADPRT1A* OE, PARP inhibited cells and *PARP* dR cells. **p* value <0.05, ***p* value <0.01 and *** *p* value <0.001 as compared to control. Data is a representation of SEM values of three independent experiments.



(C) *ADPRT1A* KO, *ADPRT1A* OE, PARP inhibited cells and *PARP* dR cells failed to move towards 1 µM cAMP. Wells were formed on NNA plates using a cup borer and were filled with 70 µl of 1µM cAMP; cells were placed at a distance of 3 mm from wells. Photographs were captured 5 hrs after plating the cells at 40 X magnification. Results are representative of three independent experiments.

This hypothesis was further supported by significant rescue in cAMP levels at the time point at which aggregation initiated in *ADPRT1A* OE and KO (12 hrs). However, PARP inhibition and downregulated cells did not show comparable rescue as OE and KO cells. This could be due to inhibition of all PARP homologs by down regulation (antisense against conserved catalytic domain) and chemical inhibition using Benzamide (substrate analog for PARP) whereas in *ADPRT1A* KO, other close isoforms viz., ADPRT2 and ADPRT1B could compensate for the loss of function.

5.2.11: Effect of exogenous cAMP on chemotaxis and development

Subsequent to cAMP estimation, we explored whether the delayed aggregation could be due to faulty chemotactic response to cAMP, using the two droplet chemotaxis assay (Mir *et. al.,* 2015) *ADPRT1A* KO and *PARP* dR cells showed significant chemotaxis defect towards 1 µM cAMP as compared to control cells which showed chemotaxis to cAMP as early as 5 hrs after plating on non-nutrient agar plates (Fig 6C). While *ADPRT1A* OE and PARP inhibited cells showed few cells that chemotax towards cAMP. However, it was not comparable to control cells indicating alteration in ADPRT1A and specifically ADPRT1A knockout to hamper chemotactic response towards cAMP. Thus, it can be concluded that both decreased cAMP levels and chemotaxis defect clearly indicate defective cAMP signaling in absence of PARP activity alone as well as physical presence of ADPRT1A.

The role of ADPRT1A was further reinstated by pulsing cells with 50 nM cAMP pulses every 6 min for 6 hrs. The pulsed cells were washed followed by the chemotaxis assay and developed on non-nutrient agar plates. It was seen that cAMP pulsed *ADPRT1A* KO cells showed rescue in chemotaxis and also showed fruiting bodies at ~22 hrs as opposed to 20 hrs in control cells (Fig. 5.17 A and B). *ADPRT1A* OE, PARP inhibited cells and PARP dR cells also showed rescue in a similar manner but it was not as significant as *ADPRT1A* KO. The above results thus strengthen *ADPRT1A*'s involvement in cAMP signal relay along with regulation of the synthesis and release of cAMP in the early stage of development.

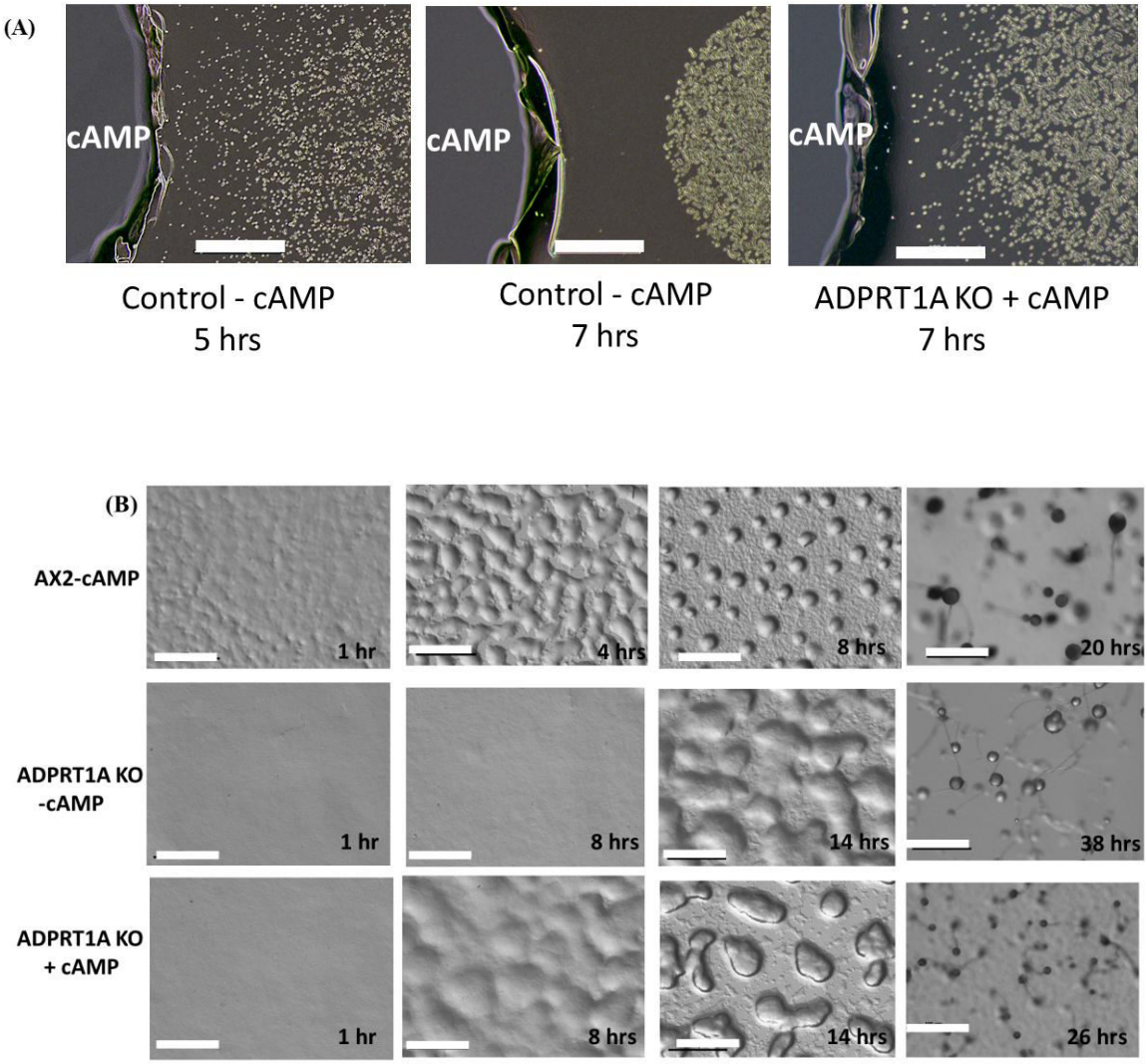


Fig. 5.17: Effect of exogenous cAMP pulsing on chemotaxis and development in *ADPRT1A* KO cells. (A) Cells were starved at 2 ×10^7 cells/ml in PB with or without 50 nM cAMP pulses every 6 min for 6 hrs. Droplets of pulsed cells were spotted next to 1µM cAMP droplets on NNA agar plates. Photographs were taken after 6 hrs. Bars indicate scale: 100 µm. (B) cAMP pulsed *ADPRT1A* KO cells show normal aggregation in the absence of *ADPRT1A*. Cells were stimulated by 50 nM cAMP pulses every 6 min for 6 hrs and then were developed on NNA plates at 1×10^8 cells/ml. Time points on panels indicate time elapsed since cells were washed following the cAMP pulsing

completion. cAMP pulsed *ADPRT1A* KO cells showed normal development without significant developmental delay. Bars indicate scale: 100 µm.

5.3 Discussion

PARP-1 is a major ADP-ribosylating enzyme involved in various biological pathways viz., DNA repair, cell death, mitotic apparatus function, transcriptional regulation etc. (Virag and Szabo, 2002, Soldani and Scovassi, 2002; Krishnakumar *et al*., 2008; Kashima *et al*., 2012; Jubin *et al*., 2016b). In view of the earlier lab studies that indicated PARP to play a role in *Dictyostelium* growth and development, ADPRT1A was identified as the PARP-1 ortholog in *D. discoideum* and in the course of analyzing the overexpression phenotype, it was seen that *ADPRT1A* overexpression affected both growth and development of *D. discoideum* (Rajawat *et al*., 2011; 2014; Jubin *et al.,* 2016a). Thus, to understand how ADPRT1A is involved in these processes, a loss of function-*ADPRT1A* knockout was generated in *D. discoideum* by homologous recombination. *ADPRT1A* KO cells showed absence of full length ADPRT1A at genomic level via PCR (Fig. 5.8). However, only ~93% decrease in *ADPRT1A* transcript was observed as compared to control cells as shown in Fig. 5.9. This ~7% residual *ADPRT1A* levels could be due to the mixture of a very small proportion of wild type Ax-2 cells in formation of the fruiting body of *ADPRT1A* KO during selection process. The difference in the doubling time of *ADPRT1A* KO cells further substantiates the effect of *ADPRT1A* KO and also nullifies any interference by the wild type Ax-2 cells. In addition, significantly high NAD^+ levels in the cell also indicated the absence of *ADPRT1A*. PARP-1 being an avid consumer of NAD^+ (Houtkooper *et al*., 2010), *ADPRT1A* knockout may lead to less consumption of NAD^+ (Fig. 5.10). Deletion or inhibition of PARP-1 has been reported to cause an increase in the NAD^+ levels in animal and cellular models (Bai *et al*., 2011). Thus, it is clearly illustrated that *ADPRT1A* has been successfully knocked out.

PARP-1 has been reported to be involved in a myriad of cellular processes essential for cell growth and survival (Jubin *et al.,* 2016b). In mouse PARP-1 and PARP-2 double knockouts exhibit embryonic lethality (Henrie *et al*., 2003), suggesting that PARP has a crucial role to play in development. Our results showed that *ADPRT1A* KO exhibited slower cellular proliferation rate with an extended lag phase as seen in Fig. 5.11A compared to control cells. Also, these cells displayed a bigger cell size and stressed vacuolar phenotype as compared to control cells as seen in Fig. 5.11B thereby illustrating a definite role of ADPRT1A in cellular growth and cell survival. Further to understand the slow proliferation of *ADPRT1A* KO, cell cycle analysis was carried out owing to reports on role of PARP-1 in induction of G1 arrest and regulation of G2 arrest (Masutani *et al*., 1995). *ADPRT1A* KO cells showed significantly high number of cells to be stalled at the G2/M phase as compared to control *D. discoideum* cells (Fig. 5.12 A, B). Additionally, PARP-1 has been reported to regulate cell cycle regulating proteins for e.g. *Cyclin A, Cyclin B* etc. (Simbulan-Rosenthal *et al*., 2000). Also, it has been demonstrated that *Cyclin B* fluctuates throughout growth of *Dictyostelium* with the highest accumulation at G2/M boundary (Luo *et al*., 1994; Evans *et al*., 1983). G2M- transition is also prevented by inhibition of cyclin B1 transcription (Innocente *et al*., 1999). Interestingly, *CYCLIN B* transcript levels were found to be significantly lower as compared to their levels in control cells (Fig. 5.12C). PARP-1 may regulate *CYCLIN B* expression via binding to its enhancers like p300 or by aiding in the maintainence of open conformation of promoter region during transcription.

Additionally, the absence of ADPRT1A leading to stressed and vacuolar phenotype was intriguing which led to the study of the effect of external stress namely oxidative stress on *ADPRT1A* KO cells. Reports demonstrate *PARP-1* KO to confer resistance to endotoxic shock, (Oliver *et al*., 1999) while conversely it has been reported to confer sensitivity to environmental stress (Wang *et al*., 1997). Treated *ADPRT1A KO* cells showed comparable growth as untreated control cells till 48 hrs even in the presence of H_2O_2 (Fig. 5.13). This may be due to the absence of ADPRT1A which is required for DNA damage sensing (Couto *et al*., 2013) which may result in normal growth in *ADPRT1A* KO cells initially. Also, ADPRT1A isoforms may compensate for ADPRT1A

function. Accumulating evidences however prove that the functions of PARP-2 cannot compensate functions of PARP-1 completely. Although significant PARP activity was observed due to PARP-2 in PARP-1 mouse knockout models following genotoxic stimulation (Shieh *et al*., 1998; Ame *et al*., 1999), these knockouts present several phenotypes associated with genomic instability (Trucco *et al*., 1999), demonstrating that PARP-2 cannot completely compensate for the loss of PARP-1. Interestingly, it was observed that after 48hrs, growth rate of treated *ADPRT1A* KO cells reduced as compared to control which may be due to the mounting up of DNA damage. This was evidently seen in significantly pH2AX stained (marker of DNA damage) *ADPRT1A* KO untreated as well as treated *D. discoideum* cells (Fig. 5.14). Therefore, it is clearly seen that *ADPRT1A* knockout led to resistance to oxidative stress in *D. discoideum* due to loss of ADPRT1A- the DNA damage sensor.

The life cycle of *D. discoideum* is divided into the unicellular as well as the multicellular stages (Raper, 1935). *ADPRT1A* KO has been demonstrated to affect the unicellular stage significantly. Also, earlier report illustrated PARP inhibition, down regulation and specifically *ADPRT1A* overexpression to affect developmental morphogenesis of *D. discoideum. ADPRT1A* KO cells showed delayed and aberrant structures during development (Fig. 5.15) with the aggregation process being most significantly affected. Loose aggregates were seen only at 12 hrs in *ADPRT1A* KO cells as opposed to 6 hrs in the control cells (Fig. 5.15A) while fruiting bodies formed at 45 hrs in *ADPRT1A* KO cells as opposed to 12 hrs in the control cells (Fig. 5C). *ADPRT1A* KO cells formed discontinuous and transient streams during aggregation which led to smaller and more number of aggregates as compared to control cells (Fig. 5.15B). This goes in line with reports of PARP-1 gene alterations in fungus resulting in defective development and decreased life span (Semighini *et al*., 2006; Kothe *et al*., 2010; Muller-Ohldach *et al*., 2011). Also, it was seen that AtPARP-1 and/or AtPARP2 knockdown reported to alter *Arabidopsis* development (Block *et al*., 2005). Furthermore, PARP-1and PARP-2 double knockout in mice proved to be lethal at the onset of early embryogenesis (de Murcia *et al*., 2003). It was recently reported that PARP-1 may be required in demethylation of

IL17d promoter which in turn controlled the genes required for early embryogenesis (Hamazaki *et al*., 2015). Also, *ADPRT1A* overexpression as well as PARP inhibition and downregulation were reported to affect aggregation during *Dictyostelium* development (Rajawat *et al*., 2007; 2011; Jubin *et al*., 2016a). Our present results in the knockout background establish ADPRT1A's role during early development.

Cyclic AMP acts as the signaling molecule during *Dictyostelium* development particularly during aggregation and culmination (Kessin, 2001). Defects in cAMP synthesis and chemotaxis could lead to small aggregates and delayed aggregation (Tang and Gomer, 2008; Garciandia *et al*., 2013). Thus, in order to explore whether delayed and defective aggregation in *ADPRT1A* KO is due to defective cAMP signaling, intracellular and extracellular levels of cAMP were estimated at 6 hrs after initiation of development. *ADPRT1A* KO and OE showed decreased cAMP levels at 6 hrs of starvation (time point at which control cells initiate aggregate formation). *PARP* dR and PARP inhibition also show decreased intracellular (Fig 5.16A) as well as extracellular cAMP levels (Fig. 5.16B). This further validates the need of regulated levels of PARP and more essentially ADPRT1A in cAMP production. cAMP triggers initiation of development (Schulkes and Schaap, 1995). Thus, it can be comprehended that alteration in cAMP levels could be one of the reasons for the delayed initiation of aggregation in ADPRT1A mutant phenotype. This was further substantiated by rescue in cAMP levels at a time point when aggregation was initiated leading to continued development in *ADPRT1A* OE and KO (12 hrs). However, PARP inhibition and *PARP* downregulated cells did not show comparable rescue as OE and KO cells. This could be due to inhibition of all PARP homologs by both downregulation (as antisense was against conserved catalytic domain) and chemical inhibition using Benzamide (the substrate analog for PARP) whereas in *ADPRT1A* KO, other close isoforms viz., ADPRT2 and ADPRT1B could compensate for the loss of function. It is also indicative of the possibility of both PARP activity and the physical presence of ADPRT1A protein being essential in *Dictyostelium* development. Not only was cAMP production affected in *ADPRT1A* KO, but also these cells displayed faulty chemotactic response to cAMP (Fig. 5.16C). Therefore, both decreased cAMP levels and

chemotaxis defect clearly indicate defective cAMP signaling in absence of both ADPRT1A and its activity. This was further confirmed by exogenous addition of cAMP pulses to ADPRT1A starved cells. The cAMP pulsed *ADPRT1A* KO cells showed rescue in chemotaxis and also showed fruiting bodies at ~22 hrs as opposed to 20 hrs in control cells (Fig. 5.17 A and B). Thus, our results strengthen *ADPRT1A's* involvement in cAMP signal relay along with regulation of the synthesis and release of cAMP in the early stage of development.

Our present study thus provides the primary evidence wherein ADPRT1A, PARP-1 ortholog is specifically shown to be involved in controlling cell growth, chemotaxis, development as well as differentiation in *D. discoideum*. This adds a new viewpoint to the multitasking functions of PARP-1.

5.4 References

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