Chapter 4

Functional analysis of the sirtuin 2A of D. discoideum
4.1: INTRODUCTION

We have successfully identified and classified the 9 HDACs present in *D. discoideum* and studied their mRNA expression profile and effect of their modulators (see chapter 2). Out of the 9 HDACs we have chosen the class III HDACs (sirtuins) namely *sir2A, sir2B, sir2C, sir2D* and *sir2E* and observed their role during growth and development. In the present study, we have carried out detailed analyses of both *sir2A* and *sir2D*. Our phylogenetic analysis suggests that *sir2A* has closer homology with human *SIRT2* and *sir2D* shows homology to human *SIRT1*. In this chapter we shall emphasize on *sir2A* and its functional role during growth, development and differentiation of *D. discoideum*. A detail of functional analysis of *sir2D* is given in chapter 5 of this thesis.

Sirtuins are highly conserved from bacteria to humans. Since their diverse functions in the cell, especially in extending the life span, they have been paid high level of attention. There are seven sirtuins (*SIRT1–7*) found in humans which display diversity in their cellular localization as well as their functions (Fyre, 1999). They have emerged as therapeutic targets for age-related diseases.

The SIRT2 deacetylases are known to play important roles during cell cycle (Dryden et al., 2003) and have many substrates including α-tubulin and histone H4K16Ac (Finnin et al., 2001; North et al., 2003). It has also been suggested that SIRT2 acts as a tumor suppressor gene in human gliomas (Hiratsuka et al., 2003). They regulate adipocyte differentiation through FOXO deacetylation (Jing et al., 2007). Yeast Hst2, an orthologue of human SIRT2 is responsible for inducing Sir2p independent lifespan extension and rDNA silencing in yeast (Lamming et al., 2005). Human SIRT2 is cytoplasmic but binds to the nucleus during G2/M phase (Vaquero et al., 2006) of the cell cycle (Figure 4.1).
Figure 4.1: **Targets of human SIRT2.** It is found to activate α-tubulin (responsible for oligodendroglia proliferation), FOXO1 (responsible for white adipose tissue differentiation) and glioma cells. It also plays an important role in Parkinson’s disease, oxidative stress etc. It inactivates H4K16 residue, which further regulates the mitotic exit during cell cycle (Nakagawa and Guarente, 2011).

SIRT2 is located on chromosome 19q13.2 (Dali-Youcef et al., 2007). Human SIRT2 contains acatalytic core of 304 amino acid residues and a 19-residue N-terminal helical extension. Its catalytic core has two elongated shaped domain: a larger domain and a smaller domain. Larger domain is a variant of Rossmann fold which is present in many NAD/NADP binding enzymes (Finnin et al., 2001).

Inhibitors of SIRT2 are used for the treatment of the neurodegenerative disorders like Parkinson's disease (Horio et al., 2011). It was shown that SIRT2 inhibition was moderately protective in alpha-synuclein toxicity. However, modulations in the expression of other sirtuins were also observed (Outeiro et al., 2007). Inhibitors of SIRT2 ameliorate dopaminergic cell death both in *in vitro* and *in vivo* Drosophila model of Parkinson's disease. Action mechanisms of these inhibitors are unknown but it can be speculated that they act by promoting the formation of enlarged inclusion bodies, which are supposed to provide a cell-survival advantage (Adam et al., 2007).
SIRT2 is a tubulin deacetylase acting on the non-histone substrate, tubulin. Hst2 acts as a chromatin silencer whereas human SIRT2 co-localize with microtubule network where it deacetylates Lys40 of α-tubulin (North et al., 2003). NAD$^+$ independent HDAC6 and NAD$^+$ dependent histone deacetylase SIRT2 are the isotypes of tubulin deacetylases (Hubbert et al., 2002; Schemies et al., 2009).

It is known that necrosis takes place when tumor necrosis factor (TNF)-α activates the formation of a complex containing receptor-interacting protein 1 (RIP1) and receptor-interacting protein 3 (RIP3). Recently in 2012, Narayan and colleague have shown that SIRT2 constitutively binds to RIP3 and knockdown of SIRT2 inhibits the formation of RIP1-RIP3 complex. These findings suggest that SIRT2 is an important regulator of programmed necrosis. The inhibitors of SIRT2 deacetylase are being used as therapeutic drugs for necrotic injuries like ischaemic stroke and myocardial infarction (Narayan et al., 2012).

SIRT2 plays an important role in the control of mitotic exit (Dryden et al., 2003) where it acts as a mitotic checkpoint protein preventing the chromosomal instability during division and inhibits the formation of hyperploid cells during early metaphase. Overexpression of SIRT2 leads to the delayed cell cycle progression during mitosis (Inoue et al., 2007).

SIRT2 plays an important role in myelinogenesis where the oligodendrogial cytoplasmic protein that is localized in the outer and juxtanodal loops of the myelin sheath help decrease the cell differentiation via α- tubulin deacetylation (Li et al., 2007). Donath (2013) has showed that SIRT2 is linked to the activation of NLRP3 inflammasome apart from its role in metabolic process and cytoskeleton remodeling (Figure 4.2).
Figure 4.2: **Action mechanism of human SIRT2 during inflammation.** The accumulation of glucose, fatty acids, cholesterol and uric acid results in higher level of metabolic stress by the accumulation of reactive oxygen species (ROS). This process impairs the deacetylase activity of SIRT2 and results into the accumulation of acetylated α-tubulin which further facilitate the activation of the inflammasome by opposing ASC on mitochondria to NLRP3 on the endoplasmic reticulum (ER) (Donath, 2013).

In case of *E. coli*, CobB, an orthologue of human SIRT2 is involved in the regulation of chemotaxis via deacetylating the response regulator CheY (Li et al., 2010). Similarly in Drosophila, sirtuins were showed to play an important role during oxidative stress and lifespan. In Drosophila there are 5 sirtuins which are orthologous to human SIRT1, SIRT2, SIRT4, SIRT5 and SIRT6. The SIRT2 orthologue of Drosophila is found to suppress the pathogenesis in Huntington's disease. It protects against the neurodegeneration (Pallos et al., 2008) and its inhibition leads to neuroprotection by decreasing sterol biosynthesis (Luthi-Carter et al., 2010).

### 4.2: OBJECTIVES

1. To carry out *in silico* analyses of sir2A gene from *D. discoideum*.

2. To analyze the temporal and spatial expression patterns of Ddsir2A mRNA and protein during development.
Chapter 4

3. To find the role of \(Ddsir2A\) during growth, development and differentiation of \(D. discoideum\).

4.3: EXPERIMENTAL DESIGN

Objective 1:

- Homology and phylogenetic analysis.
- Architectural analysis of core domain and N- and C-terminals

Objective 2:

- Temporal and spatial expression patterns of \(Ddsir2A\) mRNA.
  - Spatial and temporal expression of reporter gene (both stable and labile \(lacZ\)) under the putative \(sir2A\) promoter.
  - \(\beta\)-galactosidase activity measurement during development in the strains developed.
- Temporal and spatial expression pattern of \(Ddsir2A\) protein.
  - Expression of \(Ddsir2A\) gene fused with reporters genes (both stable and labile \(lacZ\)) under the \(sir2A\) putative promoter to check the spatial and temporal expression pattern.
  - \(\beta\)-galactosidase activity measurement during development.

Objective 3:

- Creating the following strains for the analysis of the function of \(sir2A\) during growth, development and differentiation of \(D. discoideum\): \([sir2A^-/Ax2]\) (knockout), constitutively expressing \([\text{act15/sir2A-eyfp}/Ax2]\) (overexpressor), \([\text{act15/sir2A-eyfp/sir2A}^-]\) (rescued) and random blastcidin integrant (RI) strains.
- Sirtuin activity measurement in the above created strains.
- Study the effect of \(Ddsir2A\) on cell type specific gene expression.
Performing mixing experiments to test whether sir2A is involved in regulating cell-to-cell signaling during development.

4.4: MATERIALS AND METHODS

4.4.1: In silico analyses for the identification and characterization of Ddsir2A

Protocols and tools are similar to that mentioned in chapter 2 and 3.

4.4.2: Strategy for the promoter driven reporter expression analysis

The 587 bp upstream (intergenic sequence) of the sir2A ORF harboring the putative promoter was PCR amplified using the primers shown in Figure 4.3C. Ddsir2A promoter was cloned into XbaI/BglII site of PsA-iDQgal vector by replacing its original promoter to drive the expression of lacZ (stable gal) (Figure 4.3A). Similarly Ddsir2A promoter was cloned into XbaI/BglII site of 63ubi-IlacZ vector by replacing its original promoter to drive the expression of ubi-IlacZ (ubiquitinated ile gal) (Figure 4.3B). In case of labile (ubi-IlacZ), the chimeric gene encodes the ubiquitin β-galactosidase fusion protein, which is when expressed, the ubiquitin gets cleaved off the nascent fusion protein and leaves the β-galactosidase with an exposed isoleucine, and decreases the protein stability with a half-life of approximately 30min (Detterbeck et al., 1994). Whereas in stable lacZ, β-galactosidase activity stays for more than 24 hours. Vector maps are given in the appendix. Constructs were confirmed by restriction digestion and further by sequencing. Constructs prepared were transformed into Ax2 cells by electroporation and the transformants were selected on the antibiotic G418.

The transformants were developed on treated dialysis membranes and processed for β-galactosidase staining (as per the protocol of Escalante and Sastre, 2006). The stages were photographed under stereomicroscope (Olympus SZ61).
4.4.3: Strategy for the promoter driven self and reporter expression analysis

The promoter with ORF region (2.36 Kb) of the *Ddsir2A* was amplified by PCR using the primers given in Figure 4.4C. The PCR product was cloned into XbaI/BglIII site of Psa-iDQgal vector by replacing its own promoter to drive the expression of *lacZ* (stable *gal*) (Figure 4.4A). Similarly *Ddsir2A* promoter was cloned into XbaI/BglIII site of 63*ubi-IlacZ* vector by replacing its own promoter to drive the expression of *ubi-IlacZ* (ubiquitinilated ile *gal*) (Figure 4.4B). Constructs were confirmed by restriction digestion and further by sequencing. Final constructs were transformed into Ax2 cells and the transformants were selected on G418. The transformants were developed and processed for β-galactosidase staining.
Chapter 4

Figure 4.4: Strategy for the construct preparation for Ddsir2A putative promoter driving the expression of sir2A open reading frame fused to reporter gene. (A) sir2A expressed under its own promoter as a fusion with stable lacZ reporter gene. (B) sir2A expressed under its own promoter as a fusion with labile lacZ reporter gene. (C) Primers used for the above cloning along with their restriction sites and the expected size of the amplicon.

4.4.4: Creation of sir2AΔ/Ax2 strain (knockout)

The sir2A gene was disrupted using the blasticidin cassette by homologous recombination in the genome and the transformants were selected on the basis of their resistance to blasticidin-S antibiotic. Cultures were maintained at a concentration of 10µg/mL of blasticidin-S. In brief, two regions from the 5’ and 3’ ends of the gene were PCR amplified from the genomic DNA as fragments of NotI/XbaI and HindIII/KpnI with amplicon sizes of 742 bp and 766 bp, respectively. The fragments were cloned in pBS-Bsr vector (see Appendix) with the blasticidin (Bsr) cassette inserted between XbaI and HindIII. The 5’ and 3’ fragments of the sir2A gene were PCR amplified using the primer combinations as shown in the Figure 4.5D.
Figure 4.5: Cloning strategy and the list of primers used for creating a knockout construct. (A) The gene was disrupted by the blasticidin cassette (1.44 Kb) and the two fragments 1 (0.74 Kb) and 2 (0.76 Kb) corresponding to 5′ and 3′ end of the gene was used. (B) Shows the process of homologous recombination. (C) Diagrammatic representation of sir2A knocked out construct obtained (D) Primer sequences with restriction sites (underlined) used for the amplification of KO first fragment (5′) and second fragment (3′) needed for creating the knockout construct. The genomic positions and expected amplicons sizes are also given.

4.4.5: Confirmation of knockout strains

The knockout strains obtained were first checked by appropriate PCR amplifications and further confirmed by Southern hybridization. The semi-quantitative RT-PCR and sirtuin activity measurement was also carried out to check their expression levels.

Genomic DNA amplification

Genomic DNA was isolated from the knockout and random integrants. Wild type Ax2 and the linearized vector genomic DNA was taken as control. List of primers used for confirmation are mentioned in Figure 4.6C. Their respective
positions in the construct are shown in Figure 4.6A. Their expected results in different strains and their product sizes are given in Figure 4.6B.

![Figure 4.6](image)

Figure 4.6: List of primers used for the validation of \([Ddsir2A/Ax2]\) knockout strain. (A) strategy used for the validation of knockout strain (B) primer combinations used for the validation of various strains that include wild type Ax2, random integrants, vector as a positive control and \(sir2A/Ax2\) with their respective product size (C) Primers used for the validation which are numbered according to their positions assigned in section (A) of this figure.

**Semi-quantitative RT-PCR**

Log phase cells were collected from the overexpressor, knockout, random integrant and rescued strains of \(sir2A\) and wild type Ax2 strains. RNA was isolated followed by cDNA synthesis. PCR was carried by the combination of primers mentioned in chapter 2.

**Southern hybridization**

For the confirmation of knockout strain Southern hybridization was carried using the DIG High prime DNA labeling and Detection starter Kit from ROCHE (cat# 11 745 832 910). Probe was synthesized as per the protocol mentioned in the manual.
Chapter 4

Blasticidin cassette was used as the template for the probe synthesis which was excised from the vector after the digestion with XbaI and HindIII. Product size of blasticidin cassette was approximately 1.44 Kb. Genomic DNA was isolated from the wild type Ax2 strain, knockout strains and the random integrants. Isolated genomic DNA with empty vector as a positive control was digested with KpnI and NotI. Digested samples were run in 0.8% agarose gel. Samples were transferred to the nylon membrane (MDI # SNNPZ) by capillary action. Hybridization was carried as per the instructions from the manufacturer. Color was allowed to develop and then photographed.

4.4.6: Growth and development of *D. discoideum*

A comparison of growth and development was carried for the strains developed.

4.4.7: Sirtuin activity assay

To measure the sirtuin activity in [(act15/sir2A-eyfp)/Ax2], (sir2A/Ax2), [(act15-sir2A-eyfp)/sir2A] and random integrant strains, human SIRT2 deacetylase substrate provided by the kit was used (See protocol chapter 3).

4.4.8: Role of Dd*sir2A* in cell type patterning

The role of cell type patterning in the sir2A/Ax2 cells was studied by transforming them with various plasmids having cell type specific gene promoters (ecmA, ecmB, ecmAO and D19) driving the expression of the reporter gene, lacZ. The transformed sir2A/Ax2 and Ax2 strains were developed and stained for β-galactosidase. The stages were photographed under stereomicroscope (Olympus SZ61). The expression levels were verified by quantitating the level of β-galactosidase activity in the migrating slug stage and compared with the wild type level of expression. The RNA expression of the specific genes was measured by RT-PCR. The list of primers used for this study is shown in Table 4.1. RNA from different developmental stages of (act15/sir2A-eyfp)/Ax2, sir2A/Ax2 and Ax2 wild type strain were isolated.
Table 4.1: List of primers used for the expression analysis of cell type specific marker genes.

4.4.9: Mixing experiments for creating chimeras for lineage tracing

Contributions of autonomy/non-autonomy by lineage tracing of Eyfp marked cells during slug and terminal differentiation of chimeras with varying mixtures of labeled wild type (act15/eyfp/Ax2) and unlabeled sir2A/Ax2 and vice versa was carried. Varying ratios of these cells as mentioned below was carried.

For chimeric studies we have used four different strains and divided them into two groups. The strains are as mentioned below:

1) sir2A/Ax2 (sir2A\(^{-}\) knockout unmarked)
2) (act15-eyfp)/Ax2 (Ax2 marked)
3) (act15-eyfp)/sir2A\(^{-}\) (sir2A\(^{-}\) knockout marked)
4) Ax2 (Ax2 unmarked)

Group 1 contains sir2A/Ax2 knockout (unmarked) and [(act15-eyfp)/Ax2] (marked) with Eyfp were mixed in the following ratio i.e. 1:1, 1:4, 1:9, 4:1 and 9:1. Likewise group 2 contains [(act15-eyfp)/sir2A\(^{-}\)] (marked) and Ax2 (unmarked) and mixed in the same ratio as mentioned above. Cells well mixed and plated for...
development after synchronization. Photographs of different stages of development were taken at regular intervals under fluorescent microscope. Spores from single fruiting body were picked and fluorescent vs. non-fluorescent spores were scored. Minimum 10-15 spore heads per experiment per ratio was calculated. A minimum of 3-5 such individual experiments were carried.

4.5: RESULTS AND DISCUSSION

4.5.1: *Putative sir2A in D. discoideum show homology to known SIRTs*

We have already discussed in chapter 3 that Ddsir2A show close homology to human SIRT2, SIRT3 and yeast Hst2. The protein sequences were retrieved from Uniprot Database and their domains were deduced using SMART online tool. The results obtained are shown in Figure 4.7.

<table>
<thead>
<tr>
<th>Siruins</th>
<th>Uniprot ID</th>
<th>N-terminal</th>
<th>C-terminal</th>
<th>SIR2 domain</th>
<th>Other domains</th>
<th>Protein structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsSIRT2</td>
<td>Q8IX6</td>
<td>1-83</td>
<td>269-389</td>
<td>84-268</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HsSIRT3</td>
<td>Q9NTG7</td>
<td>1-145</td>
<td>327-399</td>
<td>145-326</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ddsir2A</td>
<td>Q54QE6</td>
<td>1-257</td>
<td>444-512</td>
<td>258-443</td>
<td>34-96 (Zf-UBP)</td>
<td></td>
</tr>
<tr>
<td>ScHst2</td>
<td>P53686</td>
<td>181-301</td>
<td>1-82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.7: Schematic representation of conserved domain architecture of the Ddsir2A, HsSIRT2, HsSIRT3 and ScHst2. [Zf-UBP-Zinc finger ubiquitin hydrolases; red block- signal peptide sequence; pink block-low complexity regions; Sc- Saccharomyces cerevisiae; Hs-Homo sapiens; Dd- Dictyostelium discoideum]

We could observe from Figure 4.7 that Ddsir2A has an additional UBP-type Zn finger (zinc-finger ubiquitin-specific protease) domain at its N-terminal apart from the SIR2 domain. The function of Zn finger domain is not well characterized but from what is known in other organisms it regulates various cellular activities. They were first identified as a DNA-binding motif in transcription factor TFIIIA from *Xenopus laevis*, but now they are recognized to bind DNA, RNA, protein and lipid substrates.
In addition to this, Ddsir2A possess the longest N-terminal whereas it is absent in ScHst2.

Both the full protein and the core domain sequence of Ddsir2A were used as a query for BLASTp at NCBI (Table 4.2). Percent homology is higher with core domain sequences than with complete protein and shows maximum homology to the human SIRT2.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Complete protein</th>
<th>Core domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsSIRT2</td>
<td>54%</td>
<td>65%</td>
</tr>
<tr>
<td>HsSIRT3</td>
<td>50%</td>
<td>57%</td>
</tr>
<tr>
<td>ScHst2</td>
<td>44%</td>
<td>63%</td>
</tr>
</tbody>
</table>

Table 4.2: List of proteins showing high homology to core domain sequence of putative Ddsir2A as a query for BLASTp at NCBI. Ddsir2A show high homology to the core domain sequence of the human SIRT2. [Sc- Saccharomyces cerevisiae; Hs- Homo sapiens]

Sequence similarity suggests that the putative D. discoideum protein could be functionally similar to the human SIRT2 and therefore we carried out a multiple sequence alignment of the core domain of Ddsir2A and other known homologs from different organisms using ClustalW2 at EBI server to check for the conserved residues which possibly could be responsible for the activity.
Chapter 4

Figure 4.8: Multiple sequence alignment of core domain sequences of sir2A of D. discoideum and other known homologs using ClustalW2. Only the highly conserved region around the core domain is shown in red colour.

ClustalW2 results show the conserved sequences of human SIRT2, SIRT3, yeast Hst2 and Ddsir2A. Humans SIRT2, SIRT3 and yeast Hst2 are members of class Ib of sirtuin and according to the above observation Ddsir2A also falls in class Ib of sirtuins which validate our findings mentioned in chapter 3. In Ddsir2A, signature sequences GAGISXXXGIPXXR reads as GAGISVAAGIPDFR and PXXXH read as PTTVH. These variations place the sir2A of D. discoideum in class I. The HG motif required for ADP-ribosylation is present and responsible for its further classification into subclass as the sequence present before this motif determines that. Presence of A before this HG motif belongs to class Ib i.e. AHG, in case of sir2A of D. discoideum.

4.5.2: Expression analysis of Ddsir2A during development

4.5.2.1: RNA expression analysis

Spatio-temporal expression of Ddsir2A both at the RNA and the protein levels were monitored during development. Temporal expression pattern of the Ddsir2A
transcript during development as carried out by a semi-quantitative RT-PCR showed the levels to be high during the vegetative cells with a steep decrease at the aggregate stage and again at the culmination. The levels were very high at the mound and migrating slug stage (chapter 2). The spatial expression as analyzed by in situ hybridization results showed largely prespore specificity (chapter 3) during late development it is present in both cell types which is more obvious at slug stage.

The spatial expression of sir2A was further confirmed by sir2A promoter (putative) driven expression of reporter genes (lacZ and ubi-IlacZ). The putative promoter region ~0.58 Kb was PCR amplified (Figure 4.9A) and cloned into XbaI/BglII site of the PsA-iDQgal and 63ubi-IlacZ vectors, replacing their original promoters with sir2A promoter to drive the expression of reporter genes lacZ and ubi-IlacZ respectively. Since the promoter region of the genes present in D. discoideum are of approximately in between 300 to 7/800 bp in length we had cloned approximately 600 bp intergenic sequence upstream of the sir2A gene presuming that it may host the putative promoter region of this gene. The constructs sir2A/lacZ and sir2A/63ubi-IlacZ were confirmed by appropriate restriction digestions (Figure 4.9B and C respectively) and further by sequencing.

![Figure 4.9: Construct preparation for Ddsir2A promoter driven expression of lacZ and ubi-IlacZ reporter genes.](image)

(A) PCR amplification of ~ 0.58 Kb putative Ddsir2A promoter (B) Restriction analysis of [sir2A/lacZ] construct; digestion with XbaI/BglII yield fragments 8.7 and 0.58 Kb in size, BglII/XhoI yield 5.87 and 3.4 Kb, XbaI/XhoI yield 5.3 and 3.98 Kb. (C) Restriction analysis of [sir2A/ubi-IlacZ] constructs. Digestion with
XbaI/BglII yield fragments 8.0 and 0.58 Kb in size, BglII/XhoI yield 5.78 and 3.3 Kb, XbaI/XhoI yield 5.2 and 3.87 Kb. M- 1 Kb plus ladder (Fermentas)

Figure 4.10: Promoter activity in multicellular structures. Wild type cells were transformed with the construct (A) [sir2A/lacZ] which contains fusion of sir2A promoter with lacZ gene, encoding stable β-galactosidase enzyme and (B) [sir2A/ubi-IacZ] encoding labile β-galactosidase enzyme. Different developmental stages like (a) aggregate, (b) first finger, (c) fruiting body (d) early culminants (e) slug (Images are not according to scale).

The [sir2A/lacZ] expression could be seen at all stages of development. It was found dispersed throughout during the aggregate stage but slowly got concentrated in the prespore region of the first finger stage. The expression pattern was localized to the prespore region of later developmental stages. Figure 4.10A (e), migratory slug stage shows the well define prespore and prestalk regions. Figure 4.10A (d) shows the stalk tube to be devoid of any expression. The upper and lower cups in the fruiting body and the stalk are also devoid of any expression. The labile β-galactosidase activity also shows similar effects but in lesser concentration. The labile β-galactosidase activity is quite less in early culminant as compare to migratory slug and fruiting body stage.
The expression pattern observed was found to be very similar to that observed with in situ hybridization studies. The intensity of expression of promoter under \textit{ubi-IIacZ} is lower than the stable \textit{lacZ}. The main finding here is that the \textit{sir2A} RNA expression is largely in the prespore cells. The promoter analysis (Figure 4.9-4.10) gave results similar to that obtained by \textit{in situ} therefore, we were quite confident that the 700 bp region upstream of the \textit{Ddsir2A} ORF contained the putative promoter. This still needs further validation.

4.5.2.2: Protein expression analysis

The spatial expression of the \textit{Ddsir2A} protein was analyzed by driving the expression of \textit{sir2A} ORF fused with \textit{lacZ} \textit{[sir2A/sir2A-lacZ]} or \textit{ubi-IIacZ} \textit{[sir2A/sir2A-ubi-IIacZ]} gene under by \textit{sir2A} promoter. The 2.36 Kb amplicon of \textit{sir2A} promoter plus ORF was amplified (Figure 4.11A) and the construct was confirmed by restriction digestion (Figure 4.11B and C) and sequencing.

![Figure 4.11: Construct preparation for the expression of \textit{Ddsir2A} as a fusion protein with \textit{lacZ} and \textit{ubi-IIacZ} reporter genes under \textit{sir2A} promoter. (A) PCR amplification of 2.36 Kb \textit{Ddsir2A} promoter plus ORF (B) Restriction analysis of \textit{[sir2A/sir2A-lacZ]} construct; Digestion with XbaI/BglII yield fragments 8.4 and 2.3 Kb in size, XhoI/BglII yield 7.3 and 3.2 Kb, XbaI/XhoI yield two fragments of 5.0 Kb each. (C) Restriction analysis of \textit{[sir2A/sir2A-ubi-IIacZ]} constructs; digestion with XbaI/BglII yield fragments 8.4 and 2.3 Kb in size, XhoI/BglII yield 7.3 and 3.2 Kb, XbaI/XhoI yield two fragments of 5.0 Kb each. [M- 1 Kb plus ladder; Fermentas]}
Figure 4.12: Ddsir2A was expressed as a fusion protein with lacZ under sir2A promoter. (A) (sir2A/sir2A-lacZ)/Ax2 cells expressing stable β-galactosidase. (B) (sir2A/sir2A-ubi-lacZ)/Ax2 expressing labile β-galactosidase enzyme. Different developmental stages like (a) aggregate, (b) first finger/mound, (c) fruiting body (d) early culminants (e) slug (Images are not according to scale).

Above observations show that in wild type *D. discoideum* Ax2 cells, the Ddsir2A promoter is almost exclusively active in the prespore cells. Both the RNA and the protein were found to have similar patterns of expression. In order to confirm their expression patterns we have measured the β-galactosidase activity every two hours of development in both (sir2A/sir2A-ubi-lacZ)/Ax2 and (sir2A-ubi-lacZ)/Ax2 strains (Figure 4.13).
Figure 4.13: β-galactosidase activity measurement during development. β-galactosidase activity was estimated every two hour time intervals from the strains namely (sir2A/ubi-lacZ)/Ax2 and (sir2A/sir2A-ubi-lacZ)/Ax2 which measures indirectly the transcript and protein levels respectively.

Figure 4.13 shows an indirect estimation of the transcript and the protein levels of Ddsir2A during development. The expression pattern is more or less similar except that the protein levels are much lower than the transcript levels. The results show that both the transcript and the protein are present throughout development. The transcript shows a gradual increase from the time of starvation (t₀) to the loose aggregate stage (t₄) and then drops down. It again shows an increase to peak at the slug stage (t₁₆) and drops by the time it reaches the early culminant (t₂₀) to again increase at the fruiting body stage (t₂₄). The protein level shows a decrease till the loose aggregate stage but thereafter the pattern of expression is similar to the transcript profile. Both the slug and the early culminant to fruiting body stage requires high sir2A levels suggesting that they may be required for the differentiation programme. Since they are present in the prespore/spore stage, we could speculate that they may be required for the prespore differentiation which is the surviving cell population.
4.5.3: Creating a Ddsir2A knockout strain

4.5.3.1: Construction of sir2A'/Ax2 strain

To obtain a knockout mutant of Ddsir2A, we prepared a construct bearing 5' and 3' arms of sir2A made separately by PCR amplification from genomic DNA. By using the forward and reverse primers for knockout fragment 1 (KOs') and fragment 2 (KO3') the genomic sequence was PCR amplified to give an amplicon of sizes 0.74 Kb and 0.76 Kb respectively (Figure 4.14A). The fragment 1 was digested with the restriction enzyme combination NotI/XbaI and ligated with the similarly digested vector. After the fragment 1 was successfully inserted into the vector bearing the blasticidin cassette, this construct was digested with the restriction enzyme combination HindIII/KpnI and ligated to the fragment 2 (KO3') amplicon subjected with the same restriction digestions. The amplicons were inserted in the pBS-Bsr cloning vector with a blasticidin resistance cassette. The ligated construct was then transformed in E coli DH5a. The positive clone was confirmed by a combination of restriction digestions (Figure 4.14B) and further by sequencing.

Figure 4.14: PCR amplification and restriction digestion of [sir2A'] knockout construct. (A) Fragments 1 and 2 were amplified from the genomic DNA as 0.74 Kb and 0.76 Kb sizes, respectively. (B) The construct was confirmed by restriction digestions. The enzymes used with the respective sizes of the releaseare NotI/XbaI (5.16 and 0.74 Kb), HindIII/KpnI (5.14 Kb and 0.76 Kb), XbaI/HindIII (4.5 Kb and 1.44 Kb), NotI/KpnI (3.0 Kb and 2.9 Kb). [M denotes the 1 Kb plus DNA ladder; Fermentas].

153
The above plasmid was linearized by using appropriate restriction enzymes and purified by using DNA extraction kit (Fermentas). Since the construct linearized by NotI/KpnI digestion yielded the desired fragment of 2.9 Kb and the vector backbone of 3.0 Kb which was difficult to separate, we had to amplify the construct by using the following primers bearing the NotI and KpnI site. ~10µg of the PCR amplified construct was transformed into Ax2 cells by electroporation. After 24 hours, 5µg/mL of blasticidin was added and subsequently increased to 10µg/mL. Gene disruption by the blasticidin cassette confers resistance upon the cells which have successfully taken up the (sir2A⁻) knockout construct to be resistant against the drug when present in the growth medium. As D. discoideum feeds on bacteria, selection of the clones was carried out on a lawn of dead (heat killed) Klebsiella aerogenes. Transformed D. discoideum cells were serially diluted and plated on NNA agar plates with blasticidin at a concentration of 5µg/mL with 200µL of a dead Klebsiella aerogenes. After a few days of incubation at 22°C clear plaques were seen on the bacterial lawn. The clear plaque represents a single clone which is subsequently picked up and inoculated in HL5 supplemented with 10µg/mL of blasticidin. The cells were allowed to grow at 22°C and genomic DNA was isolated to check for positional and random integrants by PCR amplifications.

The validation of the (sir2A⁻/Ax2) strain was carried by PCR amplification using different primer combinations to distinguish between the wild type Ax2, the positional integrants and the random integrant strains.
Figure 4.15: **Confirmation of the [sir2A/Ax2] knockout strain by PCR amplifications.**

Different primer combinations (pc) and their respective sizes for each lane of all 4 A-D are: Lane 1= pc 2 and 3; size=0.74 Kb; Lane 2= pc 2 and 4; size=1.2 Kb; Lane 3= pc 1 and 4; size=1.77 Kb; Lane 4= pc 1 and 3; size=1.31 Kb; Lane 5= pc 7 and 6; size=0.76 Kb; Lane 6= pc 7 and 5; size=1.77 Kb; Lane 7= pc 8 and 5; size=2.05 Kb; Lane 8= pc 8 and 6; size=1.03 Kb. (A) Ax2 strain lane 1, 4, 5 and 8 are positive (B) sir2A/Ax2; lane 1-8 show positive (C) random integrant strain lane 1, 2, 4, 5, 6 and 8 are positive (D) vector strain lane 1, 2, 5 and 6 are positive [M denotes the 1 Kb plus DNA ladder; Fermentas].

Figure 4.15 shows the PCR amplification of Ax2, vector, knockout, and random integrant strains by using different primer combinations. In case of Ax2, primer combination 2 and 3, amplifies the 0.74 Kb fragment of 5’ end of the gene (Lane 1 of Figure 4.15A). Similarly, primer combination 6 and 7 yields the 0.76 Kb of 3’ end of the gene (Lane 5 of Figure 4.15A). These two combinations are present in all the strains namely, Ax2, random integrant, knockout and vector.

Primer combination 1 and 4, (product size of 1.77 Kb) corresponding to 0.56 Kb fragment of the 5’ upstream sequence and 0.74 Kb 5’ end of the gene and 0.47 Kb of the Bsr fragment (Lane 3 of Figure 4.15B) and primer combinations 5 and 8 (product size of 2.05 Kb) corresponding to 0.27 Kb fragment of the 3’ downstream
sequence and 0.76 Kb 3’ end of the gene and 1.01 Kb of the Bsr fragment (Lane 7 of Figure 4.15B). The above two combinations are present in the knockout strain only.

Primer combination 1 and 3; (product size of 1.31 Kb) corresponds to 0.56 Kb fragment of the 5’ upstream sequence and 0.74 Kb 5’ region of the gene (Lane 4 of Figure 4.15A). Similarly, primer combinations 8 and 6 (product size of 1.03 Kb) corresponding to 0.27 Kb fragment of the 3’ downstream sequence and 0.76 Kb 3’ end of the gene (Lane 8 of Figure 4.15A). The above two combinations are present in wild type, knockout strain and random integrant.

Primer combination 2 and 4; (product size of 1.2 Kb) corresponds to 0.74 Kb 5’ region of the gene and 0.47 Kb fragment of the Bsr fragment (Lane 2 of Figure 4.15D). Similarly, primer combinations 5 and 7 (product size of 1.77 Kb) corresponding to 0.76 Kb fragment of the 3’ downstream sequence and 1.01 Kb fragment of the Bsr (Lane 6 of Figure 4.15C). The above two combinations are present in knockout, random integrant and vector and absent from the wild type.

We have thus successfully made a knockout strain of this gene which could be further used for the functional analysis.

**Figure 4.16:** Confirmation of the [sir2A*/Ax2] knockout strain by RT-PCR and Southern hybridization. (A) Semi-quantitative RT-PCR of all the strains. (B) Graph showing the relative level of the transcript as shown in (A). (C) Southern hybridization results. [Ax2- wild type; OE- (act15/sir2A-eyfp/Ax2); KO- (sir2A/Ax2); RI- Random integrant; Res- [(act15/sir2A-eyfp)/sir2A*]
We confirmed further the knockout strain by semi-quantitative RT-PCR and Southern hybridization (Figure 4.16). The transcript level in the various strains was measured using the primers as given below. Their product sizes in gDNA and cDNA are 1134 bp and 910 bp respectively.

F: 5' TGTACCATCAGCCTCAATTACTACA 3'
R: 5' TGATTTAATTCATTTCCCAACCTA 3'

The graph shows the RNA expression in all the strains tested except the knockout strain. For the Southern hybridization, the probe synthesis was done by using blasticidin cassette as a template. Blasticidin cassette was excised from the vector by XbaI/HindIII digestion and purified by Fermentas purification kit. Product size was approximately 1.44 Kb. gDNA of wild type Ax2 strain, sir2A/Ax2 and random integrant was isolated and digested with NotI/KpnI site. Vector was also digested with the same combination of enzyme. Selection of digestive enzymes was done on the basis of the presence of our construct in between NotI and KpnI sites (see Figure 4.5A). We could observe the 3 Kb band present in both vector as well as our knockout (Figure 4.16C). In general, Bsr probe should be present in random integrants, but in our result we could not see any band. This needs to be repeated but since we did other experiments to confirm the various strains we did not do so. Further the sirtuin activity in each strain developed was measured and is shown in Figure 4.19.

4.5.3.2: Effect of sir2A on growth and developmental profiles

In order to understand the functionality of sir2A we have compared the growth and development of the sir2A overexpressors, knockout, random integrant and rescue strains with wild type Ax2 cells. We have grown all strains under similar conditions and counted their cell numbers after the time interval of 12 hours till 96 hours (Figure 4.17).
As can be seen, both the wild type and the random integrant used in this study show similar profiles. The overexpressing strain showed slightly faster growth especially after 24 hours of inoculation. They also show early senescence by 60 hours as compared to 72 hours for the control. Knockout shows slow growth profile and reaches the stationary phase at about slightly over $6 \times 10^6$ cells/mL.

Developmental profiles obtained are shown in Figure 4.18 for all the above mentioned strains. Developmental timings and morphologies were observed and the number of mounds, migrating slugs and fruiting bodies per unit area was calculated. Minimum of 5-7 such areas were observed and the results obtained are shown in Table 4.3 and Table 4.4 respectively. Minimum of three independent experiments were done.
Figure 4.18: Role of sir2A on developmental profile of \textit{D. discoideum}. [Ax2- wild type; OE- (act15/sir2A-eyfp/Ax2); KO- sir2A/Ax2; RI- Random integrant; Res- [(act15/sir2A-eyfp)/sir2A'; Agg-loose aggregate; M- mound; MS- migratory slug; EC- early culminant; FB- fruiting body].

<table>
<thead>
<tr>
<th>Dev. stages</th>
<th>Ax2</th>
<th>OE</th>
<th>KO</th>
<th>RI</th>
<th>Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mound</td>
<td>125 ± 5.8</td>
<td>120 ± 8.3</td>
<td>117 ± 4.7</td>
<td>122 ± 10.2</td>
<td>115 ± 8.4</td>
</tr>
<tr>
<td>Migratory slug</td>
<td>85 ± 7.3</td>
<td>79 ± 6.7</td>
<td>80 ± 9.06</td>
<td>59 ± 8.8</td>
<td>87 ± 7.1</td>
</tr>
<tr>
<td>Fruiting Body</td>
<td>71 ± 5.9</td>
<td>65 ± 9.1</td>
<td>69 ± 6.7</td>
<td>47 ± 5.4</td>
<td>70 ± 6.2</td>
</tr>
</tbody>
</table>

Table 4.3: Number of multicellular structures developed per unit area. The representative stages of development formed were counted in a given unit area and the average is shown. [n=3]
Table 4.4: Comparison of development. Time point of each respective stage was noted and morphology was observed. [Ax2- wild type; OE- \((act15/sir2A-eyfp)/Ax2\); KO- \(sir2A/Ax2\); RI- Random integrant; Res- \([(act15/sir2A-eyfp)/sir2A]/\) [n=3].

Table 4.3 and 4.4 represents the various developmental aspects of the strains developed. There were no significant changes in the number of structures developed except in case of random integrants where they were comparatively lower. This could be due to the integration in the regions which may be responsible for development. The rescue phenotypes were slightly larger as compared to the control. The rescue was done by transforming the plasmid \((act15/sir2A-eyfp)\) which is expressing constitutively the \(sir2A\) gene. This suggests that the level of sir2A protein in high amount but not as high as that present in the constitutively expressing sir2A was responsible for this. As can be observed from Figure 4.18 not many cells took part in the development of fruiting body in the overexpressing cells suggesting that a threshold level was required for development.

4.5.3.3: Measurement of sirtuin activity

We then measured the actual levels of sirtuin in these strains. Sirtuin activity was measured in two ways, first total sirtuin activity was measured by using HDAC
fluorimetric kit (Figure 4.19A). And second SIRT2 specific activity was measured by using SIRT2 specific deacetylase substrate (Figure 4.19B). For these two experiments, we have taken the various sir2A strains developed and compared them with the wild type Ax2 strain. We have followed the protocol as per instructions given in manual.

**Figure 4.19:** *Sirtuin activity measurement in the various strains developed.* (A) Total sirtuin activity measurement in sir2A overexpressing, knockout, random integrant and rescue strains with respect to wild type Ax2 cells (B) Specific SIRT2 activity measurement in sir2A overexpressing, knockout, random integrant and rescue strains with respect to wild type Ax2 cells [Ax2- wild type; OE- (act15/sir2A-eyfp/Ax2); KO- sir2A/Ax2; RI- Random integrant; Res- [(act15/sir2A-eyfp)/sir2A] [*p-value <0.001 is statistically significant difference with respect to Ax2 cells].

In Figure 4.19A, we could observe the total sirtuin activity as measured using nicotinamide as an inhibitor in the overexpressing strain as ~2.5 fold higher as compared to the wild type vegetative cells. The knockout strain is comparable to the wild type as the other endogenous sirtuin activity may be contributing towards it. We then utilized SIRT2 specific deacetylase substrate to measure the activity of SIRT2 in the various strains (Figure 4.19B). We could observe ~4 fold increase in the overexpressing strain. The sir2A knockout strain show activity similar to the wild type Ax2 cells. During homology search, we have already noticed that human SIRT2 share closer homology to sir2A and sir2C of *D. discoideum* (see Chapter 3). Therefore, high activity of SIRT2 in sir2A overexpressing strains could be also contributed by sir2C. Similarly, the expression in sir2A knockout strains is similar to Ax2 wild type cells which could be because of the presence of endogenous sir2C.
activity which also show homology to human SIRT2. The random integrant also show significantly higher activity which could be due to the integration in a place which ultimately increase the sirtuin activity. This was also evident from the developmental profile where it resembled the overexpressing strain more than the wild type. The sir2A rescue strain also showed a statistically significant enhanced level of activity, which could be because of the presence of sir2A constitutively overexpressing gene in sir2A knockout strains. So, the resulting expression could be the combined effect of both endogenous sir2C and constitutively expressing sir2A gene.

4.5.3.4: Role of sir2A in cell type patterning

During development of *D. discoideum* the cells differentiate into two cell types due to changes in gene expression which begins early in development. The cells that would become the stalk cells are called as prestalk cells and that which become spores are called as prespore cells. They can be distinguished by particular prestalk and prespore marker genes. We have used these marker genes to determine if the pattern and localization of prestalk and prespore cells are disrupted in the sir2A cells. Wild type cells and sir2A cells were transformed with plasmids that contained the promoter sequence for *ecmA*, *ecmB*, and *ecmA*O (prestalk specific) and *D19* (prespore specific) marker genes upstream of the *lacZ* gene. The transformed cells were developed on filters, fixed and then stained with X-Gal to reveal either prespore or prestalk patterning at various stages of development (Figure 4.20).
Figure 4.20: Changes in the expression pattern of the cell type specific marker genes in the sir2A/Ax2 cells. Expression pattern of the prespore marker (D19-lacZ); prestalk markers (ecmA-lacZ), (ecmB-lacZ) and (ecmAO-lacZ) were observed in the wild type cells and the sir2A knockout cells. The β-galactosidase staining pattern and the activity were checked in both the strains. In each strain, figures a-e show the staining pattern in the knockout strain and f-h shows the staining pattern in the wild type cells. The panel on
the right shows the β-galactosidase activity for each strain. [a-aggregate; b-first finger; c-slug; d-early culmination; e-fruiting body; f-first finger; g-migrating slug h-fruiting body. Second column for every figure: β galactosidase activity in the slugs of control and sir2A/Ax2 cells transformed with cell type specific marker]. [*p-value < 0.001]

The two cell types can be distinguished by particular cell type specific marker genes. The slug contains several different prestalk populations that are defined by their patterns of expression of reporters driven by ecmA and ecmB promoter fragments. pstA cells comprise the front 1/3 of the prestalk region, pstO cells occupy the remainder of the prestalk region and there are scattered gene expression pattern in the anterior-like cells (ALC). The ecmB gene is expressed at low levels but its expression increases when culmination begins. The prespore marker D19 expresses in the posterior 4/5th region of the slug which comprises largely of the prespore cells.

When the prespore specific promoter D19 is used to drive the lacZ expression, staining is seen in the entire prespore/spore zone in the wild type slugs and fruiting bodies (Figure 4.20; panel 1) formed. In the sir2A- slugs and the terminal structures developed, the expression was comparatively less than that observed in the wild type. The prestalk area was comparatively larger than that observed in wild type. The sir2A mutants showed reduced pspA promoter activity, especially in the slugs where no well-defined prespore region was observed. In the culminating structures, the mutant sori were thinner than the wild type sori. The β-galactosidase activity during the slug stage was significantly low in that formed from the knockout cells.

In the wild type, ecmA (as visualized by lacZ reporter) is expressed in the anterior-most regions with minimal expression in the posterior of the slug (Figure 4.20; panel 2) and in the terminal structure, the stalk and the upper cup region show the expression. In sir2A- slugs, the staining appears to be comparatively lower throughout development. The β-galactosidase activity present during the slug was found to be significantly lower in the knockout cells.

The stalk specific ecmB gene is expressed in the pstB cells as a centralized cone of cells and to a lesser degree in the ALCs of the multicellular structures developed from wild type cells. In the fruiting body staining appears in the upper and
lower cups, the entire stalk and the basal disc (Figure 4.20; panel 3). However, in the sir2A– slugs staining was seen in both the pstB and pstO regions of the first finger and slug stage. The ALCs also showed higher staining in the knockout cells. At the slug stage the β-galactosidase activity was not significantly higher but at other stages the levels appear to be higher than the wild type. The pstAB cells move to the rear to be shed from the slug during long periods of migration or also in the presence of large number of cells expressing prestalk specific genes. This was not evident in the fruiting body developed from sir2A– cells.

The ecmAO, the full promoter of the ecmA gene drives expression in the pstA and AB cells and to a lesser extent in the pstO cells and ALCs in the wild type strain. In the terminal structures formed, expression is seen in the upper and lower cups, stalk and the basal disc regions (Figure 4.20, panel 4). In the sir2A– slugs, the ecmAO expression was found to be significantly lower than that observed with the wild type. In the terminal structures formed, expression was also seen to a lesser extent than that observed in the slugs expressing ecmA only.

To confirm our findings we have analyzed the role of sir2A on the various transcript levels of the genes mentioned above in development and differentiation in the different strains developed in the present study. We carried out RT-PCR to measure the transcript levels of the genes like ecmA, ecmB and D19 gene markers (Figure 4.21). The primers used for the amplification and their details are given in Table 4.1).

Figure 4.21: Expression levels of cell type specific genes during development of various strains. Different developmental stages were collected from the wild type Ax2, sir2A overexpressing and the sir2A knockout strains and the transcript levels of (A) ecmA-prestalk specific marker; (B) ecmB-a prestalk specific marker and (C) D19- prespore specific marker were measured by RT-PCR.
The developmental phenotype of the mutant was further characterized by studying the expression of cell type specific marker genes during development. In this experiment the endogenous levels of \( ecmA \) and \( ecmB \) prestalk genes and the \( pspA \) (D19) prespore gene activity was measured during various developmental stages in the overexpressing and knockout structures developed and compared to that developed from the wild type cells. We found the appearance of \( ecmA \) transcript at the mound stage onwards which is generally not seen in case of wild type. The levels were higher in the structures developed from overexpressing cells as compared to those developed from the knockout cells. The expression was negligible after the early culminant stage in case of the knockout strain. The \( ecmB \) transcript also appeared earlier in the mound stage but only in the knockout cells and became negligible in the early and late culminant stages. The transcript level was higher than that observed in the wild type in case of overexpressing strain. The \( D19 \) transcript appeared only in the mound stage in case of the knockout strain and was absent at the aggregate stage.

Taken together the \( \beta \)-galactosidase staining patterns and the transcript level measurements during development we find that the expression of both the \( ecmA \) and \( D19 \) are disturbed in the \( sir2A/-Ax2 \) cells. As seen the level of \( ecmA \) appeared earlier but decreased with development. Similarly the \( D19 \) was absent during the aggregate stages. The results indicate that the \( sir2A \) is involved in the determination and differentiation of the prespore cells and a group of prestalk cells in \( D. discoideum \).

These defects are cell-non-autonomous because the presence of wild type cells is able to induce differentiation of the \( sir2A^- \) mutant cells. The differences observed could be due to the participation of \( sir2A \) in the process of prespore differentiation but could also be due to defective intercellular signaling in the mutant structures. If the \( sir2A^- \) mutant cells were defective in generating the intercellular signals required for cell differentiation, their mixture with wild type cells would provide the defective signal and induce correct differentiation of the mutant cells.

Since the \( sir2A \) is prespore specific during normal development and in its absence the prespore cell differentiation is altered we went ahead to study it in more
Chapter 4
details. The \textit{sir2A} transcript (chapter 2) is maximally present during aggregation and the slug and early culminant stages. The \textit{D19} is also expressed earlier in development and therefore in the null background we find the expression of \textit{D19} reduced suggesting that absence of \textit{sir2A} may be disrupting the expression of \textit{D19} which is required for prespore differentiation. Therefore, \textit{sir2A} is required at a very early stage of development to induce the expression of prespore genes. Later again at the slug and early culmination this gene is expressed. Hopper et al., (1993) have suggested the presence of minimum two classes of prespore specific genes that can be distinguished by the requirement of PKA for their expression. \textit{D19} expression is PKA independent and is induced earlier than the other prespore coat proteins like \textit{SP60/SP70} etc which require PKA for its expression. Expression of \textit{D19} in the wild type is seen during the aggregation stage but here they show negligible expression in the \textit{sir2A}− aggregates. We, therefore suggest that \textit{sir2A} activity may be required very early in the prespore differentiation pathway.

To get better insight into the effect of \textit{sir2A} mutants on induction of prespore genes we went ahead to study the changes in the transcript profile of the two prespore genes namely \textit{cotA} and \textit{cotB}. They both code for the major spore coat proteins, SP60, SP70 respectively. These genes are transcribed throughout the post-aggregation stages, exclusively in prespore cells. The induction of most prespore genes, such as \textit{cotB}, requires both extracellular cAMP acting on cARs and intracellular cAMP acting on PKA (Schaap and Van Driel, 1985; Hopper et al., 1995). However, as mentioned earlier, the prespore gene \textit{pspA} is less sensitive to ablation of PKA function (Hopper et al., 1993).

![Figure 4.22: Expression levels of prespore specific genes during development of various strains.](image)

Different developmental stages were collected from the wild type \textit{Ax2}, \textit{sir2A} overexpressing and the \textit{sir2A} knockout strains and the transcript levels of (A) \textit{cotA}; (B) \textit{cotB} was measured by RT-PCR.

168
cAMP signaling is also responsible for inducing the expression of genes essential for aggregation (Mann and Firtel, 1987) as well as for subsequent prespore and prestalk cell specific gene expression. In addition, intracellular cAMP is thought to be important in activating cAMP-dependent protein kinase (PKA) by binding to the regulatory subunit (R) and releasing an active catalytic subunit. PKA plays essential roles in gene expression throughout development, and an increase in its activity is thought to trigger culmination (Harwood et al., 1992; Williams et al., 1993). Another important signaling molecule is a low molecular mass DIF. In addition to these two well-known signaling molecules, Anjard and co-workers (1997) have identified two small peptide factors, SDF1 and SDF-2 that appear to act as intercellular signals at culmination.

Prestalk cell differentiation requires DIF-1 along with cAMP. DIF-1 induces pstA and pstB specific gene expressions, while cAMP inhibits pstB specific expression. Similarly, cAMP relay response and prespore-differentiation is inhibited by DIF. The mutant strain has no cotA and cotB expression at the aggregate stage. Prespore cells are defined by the expression of pspA and the coordinately regulated spore coat genes cotA, B and C (Early et al., 1988; Fosnaugh and Loomis, 1991; Haberstroh et al., 1991). Expression of the spore coat genes is induced several hours before the expression of ecmA, suggesting that prespore cell differentiation precedes prestalk cell differentiation.

Our results suggest that the prespore differentiation (both early and late) which is dependent on the cAMP signaling is reduced suggesting that there must be some aberration in cAMP signal transduction. Since cAMP is also responsible for prestalk differentiation which occurs later than the prespore differentiation ecmA gene expression is also reduced. cAMP inhibits ecmB expression. If cAMP signaling is aberrated the ecmB expression will not be inhibited which is seen in our case but ecmA expression which is dependent on DIF is reduced. cAMP relay response and prespore-differentiation is inhibited by DIF.
Chapter 4

Taken together our results show that sir2A is required for the prespore cell differentiation and proper proportioning of the prestalk cells. Lack of sir2A, result in reduced expression of both prespore and part of prestalk differentiation.

4.5.3.5: In chimeras with wild type cells

We next studied the contributions of autonomy/non-autonomy by lineage tracing of YFP-marked cells during slug and terminal differentiation of chimeras with varying mixtures of labeled wild type (act15/eyfp/Ax2) and unlabeled sir2A− cells. During normal development of wild type cells, approximately 80-85% of the total population forms spores.

To distinguish whether the developmental defects observed was cell autonomous or non-cell autonomous we allowed the development to be followed in the chimeras. We mixed labeled wild type cells (act15/eyfp/Ax2) and unlabeled sir2A− cells (sir2A−/Ax2) at various ratios and plated the cell mixtures on non-nutrient agar plates (Figure 4.23).

Figure 4.23: sir2A regulate prespore/spore differentiation non-cell-autonomously. The labeled (act15-eyfp) wild type cells were mixed with unlabeled sir2A− cells in
various ratios and allowed to develop. The percentage of fluorescent spores from each ratio was calculated 4 times and averaged. Error bars indicate standard error. Marked \((act15\text{-eyfp})/Ax2\) cells mixed with unmarked \(sir2A^-/Ax2\) in (A) 9:1 ratio (10% population is marked); (B) 1:4 ratio (20% population is marked); (C) 1:1 ratio (50% population is marked); (D) 1:4 ratio (80% population is marked); (E) 1:9 ratio (90% population is marked) (F) 100% marked cells; (G) Histogram showing the percentage of fluorescent spores/fruiting body from each ratio. [a-aggregate; b-mound; c-migratory slug; d-early culminant; e-fruiting body] (n=3)

When 1:1 ratio of cells was taken we found that the multicellular structures developed were near normal and comparable to the wild type. The size and number of the aggregates formed was similar to that observed by the wild type cells only. We found that the cells in the aggregates formed were contributed by both the strains, and the mutant as well as the wild type could form the center of the aggregate (Figure 4.23). This suggests that in the developing co-aggregates of 1:1 ratio, the wild type was able to correct the observed failure in development. The defects seen during the development of \(sir2A^-\) cells are non-cell autonomous as it can be corrected by the wild type cells during chimera formation.

When 10% of the mutant cells were mixed with 90% labeled wild type cells the prespore/spores were formed largely by the wild type cells (66±7.9%) and the mutant cells occupied the prestalk/stalk region in both slugs/fruiting bodies, respectively. When the ratio of \(sir2A^-\) to the wild type cells were as 1:4, we found that both the cells contributed towards the formation of the spores (63±1.8% fluorescent spores) but the \(sir2A^-\) cells contributed largely to the prestalk/stalk cell formation. When equal ratio of both the strains was taken for development we found fluorescence throughout the structure with slightly more in the posterior prespore region suggesting that both strains contributed towards the formation of both the cell types but wild type was comparatively more to the prespore/spore cells (46±5.2% fluorescent spores). Our results suggest that the \(sir2A\) in wild type cells regulates a non-autonomous pathway that stimulates spore differentiation of cells that lack \(sir2A\).

We did reverse experiments, in which we mixed small percentage of wild type cells (labeled with \(act15\text{-eyfp}\)), with higher percentage of \(sir2A^-\) cells (unlabeled) (Figure 4.24). When we mixed 10% of the wild type cells with 90% of the mutant
Chapter 4

cells we found that the wild type cells preferentially located themselves in the anterior prestalk region (7.2±1.44% fluorescent spores). When we increased the ratio of wild type cells to that of sir2A− cells we found an increase in the proportion of prespore/spore cell contribution by the wild type cells (19±2.5% fluorescent spores). The results of these mixing experiments indicate that the sir2A− cells preferentially differentiate into prestalk/stalk cells in the slugs/fruiting bodies formed but do have a potential to form prespore/spore cells. When the percentage of the mutant cells is high the wild type preferentially moves to the prestalk region during slug formation. Since the mutant strain has low ecmA activity, the wild type probably takes over the function as pstA cells move in following the pstB to form the stalk tube.

Figure 4.24: sir2A regulate prespore/spore differentiation non-cell-autonomously. The unlabeled Ax2 wild type cells were mixed with labeled act15-eyfp/sir2A− cells in various ratios and allowed to develop. The percentage of fluorescent spores from each ratio was calculated 4 times and averaged. Error bars indicate standard error. Marked sir2A−/Ax2 cells mixed with unmarked Ax2 in (A) 9:1 ratio (10% population is marked); (B) 1:4 ratio (20% population is marked); (C) 1:1 ratio (50% population is marked); (D) 1:4 ratio (80% population is marked); (E) 1:9 ratio (90% population is marked)(F) 100% marked cells; (G) Histogram showing the percentage of fluorescent spores/fruiting body from each ratio. [a-aggregate; b-mound; c-migratory slug; d-early culminant; e-fruiting body] (n=3)
100% labeled knockout cells makes nearly 80±2.7% of the fluorescent spores. When 90% of the labeled mutant cells are mixed with 10% unlabeled cells we find 78±1.6% fluorescent spores. The results of these mixing experiments indicate that the \textit{sir2A} cells preferentially make spore cells. When the percentage of the mutant cells is high the wild type preferentially moves to the prestalk region during slug formation. Since the mutant strain has low \textit{ecmA} activity, the wild type probably takes over the function, as pstA cells move in following the pstB to form the stalk tube. Our results are in perfect agreement to that observed earlier.

Further experiments are still required to find out the kind of functions that is supported by sir2A. We have earlier observed that a knockout of sir2A is probably supporting prespore differentiation and a part of the prestalk mainly \textit{ecmA} differentiation. Even though they have the tendency to form the fruiting body on their own, in the presence of the wild type (specially observed in higher ratios) the prestalk/stalk is generally composed of the wild type. It could be possible that the sir2A codes for a secreted protein which can take over the functions of sir2A when small proportion of them are mixed with large amount of wild type cells. But when the proportion of mutant cells are increased in comparison to the wild type, then the wild type takes over the function which is required by the prestalk cells.

4.6: CONCLUSIONS

In this chapter we have expanded our knowledge on \textit{sir2A} of \textit{D. discoideum}, which is a homolog of human SIRT2. We have carried out a detailed bioinformatics analysis of sir2A of \textit{D. discoideum} and found that it shows maximum homology to human SIRT2. By protein architecture analysis, we could observe that Ddsir2A contains UBP-Zn finger as an additional domain at its longest N-terminal region. Signature sequence analysis clearly shows that Ddsir2A belongs to class Ib. sir2A along with sir2C was found to be closer to human SIRT2 and SIRT3, yeast Hst2 and Sir2 (1) of \textit{S. pombe}, which are the representatives of subclass Ib.
Chapter 4

*sir2A* expression is present throughout growth and development and is found to be prespore specific. Biochemical measurement of *sir2A* RNA and protein by β-galactosidase activity measurement, show almost similar expression patterns throughout the development. RNA pattern is slightly higher at the aggregate stage and follows the similar pattern as that of protein expression. This result validates our findings obtained by RT-PCR analyses in chapter 2 which also show that *sir2A* is highly conserved throughout the development and its expression rises during some important phase of the development like early mound stage, where initiation of cell type specific gene expression takes place and then migratory slug stage where differentiation initiates and fruiting body stage where the differentiation terminates.

We have created the knockout of *sir2A* to study the detailed functional analysis of the gene. We have compared *sir2A* overexpressor, knockout, rescued and random integrant strains to the wild type Ax2 strain during growth and development. We have observed that overexpression of *sir2A* caused early senescence and the growth of the knockout was retarded as compared to wild type Ax2 strain. During development there was not any significant changes observed except in case of random integrants where they were comparatively lower in number. This could be because of the random integration taking place in the regions influencing development. Phenotypic expression was also same but in case of overexpressors and knockout, numbers of cells taking part in the development of fruiting body was less.

Total sirtuin activity was high in *sir2A* overexpressing strain. Similarly, SIRT2 specific activity was also high in *sir2A* overexpressing strain. In contrast, knockout strain showed the SIRT2 activity, which could be due to the presence of another homolog of HsSIRT2 in *Dictyostelium* i.e. *sir2C*. Rescued strain also showed higher expression. This could be because of the cumulative effect of endogenous *sir2C* activity and the constitutively expressing *sir2A*.

Our cell type specific expression analyses data shows that *D19* (prespore marker) expression is low in *sir2A* knockout strain. This signifies the role of *sir2A* in prespore cells. We have taken two extra spore specific marker genes i.e. *cotA* and *cotB*. Their expression was found to be absent in *sir2A* knockout. This could be
because of the fact that the prespore differentiation is dependent on the cAMP signaling is reduced which suggests that cAMP signal transduction must be altered. In prestalk marker genes, *ecmA* and *ecmAO* are low in *sir2A* knockout strain, whereas *ecmB* expression was similar to the control. This suggests that cAMP signaling is responsible for prestalk differentiation so the expression of *ecmA* gene was also reduced. cAMP also inhibits *ecmB* expression. So, if cAMP signaling is altered the *ecmB* expression should not be reduced, which is reflecting in our results.

In chimeras study various ratios of cells were taken in different combinations and we have found that the multicellular structures developed were normal and comparable to the wild type. This suggests that the defects seen during the development of *sir2A* - cells were non-cell autonomous as it could be corrected by the wild type cells during chimera formation.

### 4.7: LITERATURE CITED


