

***Dictyostelium discoideum*: A Model System to Study Autophagy Mediated Life Extension**

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Abstract Autophagy is a major catabolic process in eukaryotes that help degrade and recycle macromolecules and organelles. Recent evidences suggest autophagy to mediate cytoprotection and also help increase longevity. It acts as a central regulatory mechanism for aging/longevity in diverse eukaryotic species. In the present study we have exploited the lower eukaryotic model organism, *Dictyostelium discoideum* to study longevity, which could use autophagy as a major mechanism for increasing lifespan. This organism is unique as it follows a developmental programmed cell death by autophagy that is independent of caspases. It could be a beneficial organism to delineate the mechanism of increased longevity that follows autophagy.

1 Introduction

Aging is not a disease but an accumulation of damaged molecules over a lifetime, which leads to a decline in normal functions of the organism as well as lays a platform for many diseases. Thus, the central question that arises is “can we increase our lifespan without trading with its quality”. Recent researches have emphasized on this aspect and today we have to some extent knowledge on the conserved genetic as well as biochemical pathways that are involved in longevity/aging. Today we know that tinkering with some signaling pathway or nutrition has led to an increase in longevity over a range of organisms.

Much of what we know about aging/longevity comes from the study of model organisms like yeast, *Drosophila*, *C. elegans* and mouse; even when we are sure that

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they are not representatives of human aging. It has been proposed that similar mechanisms operate across species and possibly there could be an underlying conserved common mechanism of aging. In recent times there has been an explosion of research in the area of aging/longevity. The key pathways and molecules that have emerged out to play a role in life extensions, in most cases follow autophagy to increase lifespan. Some known conserved pathways that regulate lifespan in most organisms studied so far are target of rapamycin (TOR), AMPK, insulin/IGF signaling and dietary controls. Few small molecules like rapamycin, spermidine and resveratrol may directly or indirectly affect the above pathways to increase lifespan.

The terms “aging” and “longevity” is usually used interchangeably but there exists a clear demarcation (McDonald and Ruhe 2011). Aging deals with the functional decline of biological processes while longevity is a parameter to measure the length of time an individual remains alive in the absence of death due to extrinsic factors. It is for sure that aging is not a disease state. Recently Lepez-Otin et al. (2013) has laid emphasis on “nine tentative hallmarks” representing aging, which include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. An understanding of all these hallmarks and their inter-relationship leading to aging still remains a challenge. Therefore, the need of the hour is to identify many more model systems for longevity studies, which could help identify and investigate the longevity genes as well as involvement of other signaling pathways. Model organisms having orthologs of many human genes could be best suited for such studies. Organisms having unique mode of life cycle could provide added advantage. Keeping this in view new model systems are coming up for researches in aging.

2 Model Systems to Study Longevity

The aging research community has been trying to measure the effects of potential treatments to carry out lifespan studies, which is next to impossible in humans due to varied known reasons. Studies in various model organisms suggest a surprising degree of similarity in the fundamental biology of cells and mechanism of aging. Thus, research in lower organisms remains relevant to mechanisms of human aging. It is still true that a mouse remains a mouse and yeast remains yeast and still maintains a difference from humans. Literature suggests that the translation of the outcomes from unicellular to multicellular, lower to higher organisms and ultimately to humans is quite different but the mechanisms remain similar. Since there are no available alternatives, it is useful to utilize as many different model systems as possible for understanding the mechanism(s) of aging. An added advantage in studying these organisms is their associated medical conditions, which can be created by genetically altering their lineages. Below, we discuss few of the model organisms that have provided immense knowledge regarding the mechanisms of aging/longevity.

2.1 *Saccharomyces Cerevisiae*

The advantage of using yeast as an aging model includes its fast growth and easy maintenance under laboratory conditions. Despite being unicellular organisms, it has been useful in identifying the conserved aging pathways that is shared amongst various organisms. In addition, easy genetic manipulations make yeast a powerful model system to study longevity. Two different types of aging are studied in yeast: replicative and chronological which allows the analysis of aging process in both dividing and non-dividing cells (Wasko and Kaerberlein 2014).

2.2 *Caenorabditis Elegans*

The analysis of the dauer larvae provided the first genetic evidence for longevity/aging. The involvement of insulin like growth factor 1 signaling in aging laid the foundation of research in modulating longevity. Also, the use of disease model and drug screens has helped in improving the quality of life and understanding of the molecular mechanisms of aging. This nematode is evolutionarily distant from human making it limiting as a model system (Bansal et al. 2015).

2.3 *Drosophila Melanogaster*

Fruit flies have many advantages as a model system for aging studies. Apart from having a short lifecycle it has more distinct tissues and organs. Infact, they also have proliferating stem cell populations in their gut, which allows analysis in proliferating populations. Flies share about 60 % of disease-related genes with humans, which makes them a desirable model. The only drawback is the maintenance of transgenic flies (Matthews et al. 2005).

2.4 *Danio Rerio*

Zebra fish is being successfully used as a model system for understanding human aging as it draws lot of parallels at the internal organization in terms of organs as compared to the whole organism level. It lives for about 2–3 years, which is not particularly beneficial, because its lifespan is similar to rodents, but it is more evolutionary distant from humans. The advantage of this model organism is its ability to regenerate its tissues, which help elucidate the mechanism of tissue regeneration and longevity (Lepilina et al. 2006).

2.5 Rodents

Mice are invaluable in aging research as they show approximately 99 % of human orthologs, which is significantly advantageous than the invertebrate model. Usually inbred mice are used for aging research, which shows high genetic relatedness, a fact that is significantly different from the human population. Thus, the results obtained may or may not hold true for humans.

Naked mole rats can provide clues to mechanisms of longevity and potential therapies in humans and hence are an extremely valuable model animal. These rats live for nearly 30 years and show age related pathologies similar to humans. There are several disadvantages of using them as laboratory animals including specific housing conditions like low light levels, high temperature and humidity. Very long lifespan also poses an obvious limitation on the variety of experiments that could be performed (Phelan 1992).

2.6 Primates

Rhesus macaque is a good model system to study longevity but unfortunately we do not have many age related studies with them. One of the main reasons is its housing under laboratory conditions and its long lifespan. The biggest advantage of using non-human primates for studying age related pathological conditions that cannot be recapitulated in mouse models (Mitchell et al. 2015).

Usage of various model organisms for aging studies has generated enormous useful information regarding the mechanism of aging/longevity. Observations from the animal models were helpful in the identification of the conserved pathways that regulate human aging/longevity. Model organisms are fundamental for aging research as there are limitations in using humans as subjects. Therefore, every model system does answer some questions in human aging/longevity. Thus, results obtained from many model systems would help understand few aspects in this area of research.

3 Autophagy Promotes Longevity

Aging is a multifactorial process and many mechanisms contribute to its decline, which lead to accumulation of damaged proteins and other cellular constituents, which is removed by autophagy. Autophagy, (a cellular self eating) lysosome mediated catabolic process to digest their own cellular content to the smallest components to be reutilized for survival. Many reports show that autophagy is required for life extension in various model organisms (Rubinsztein et al. 2011) especially those which are exposed to prolonged starvation. Literature suggests that

lifespan extensions can be achieved by genetic manipulations of cellular processes like insulin/Insulin like growth factor 1 pathway, histone acetylations, p. 53 pathways etc. Other factors responsible are calorie restriction and target of rapamycin (TOR) pathway inhibition by drugs like rapamycin, resveratrol and spermidine. All these pathways lead to autophagy either directly or indirectly to increase the lifespan.

3.1 Genetics Manipulations Leading to Life Extension via Autophagy

Sirtuin 1, a class III histone deacetylase that require NAD^+ for its activity has been identified as a major gene responsible for longevity. Overexpression of *sirtuin 1* or its orthologs increases lifespan in flies, yeast and nematodes (Burnett et al. 2011). Under in vitro mammalian cells induce autophagy when *sirtuins* are overexpressed. Sirtuins mediate deacetylation of histones and several other cytosolic proteins like AMP-dependent kinase, AMPK and autophagy related genes like Atg8, Atg5 etc. Inhibition of *sirtuin 1* prevents autophagy. Resveratrol probably acts via the Sirtuin 1 and is its positive regulator.

A knockdown of p.53 in *C. elegans* showed induction of lifespan extensions and autophagy (Tavernarakis et al. 2008). TOR inhibition induces autophagy to increase lifespan and the same has been observed in many organisms (Madeo et al. 2010). TOR is an intracellular signal transduction pathway, which is conserved from lower to higher organisms that integrates various signals namely starvation, nutrients, growth factors, stress and hormones (Inoki et al. 2005). It increases protein synthesis, inhibits autophagy thus promoting growth.

3.2 Pharmacological Manipulations Affecting Longevity via Autophagy

Many anti-aging drugs are known today which increase lifespan by promoting autophagy. One such molecule, rapamycin, an inhibitor of TOR signaling, has already been shown to increase lifespan in a variety of organisms, including most recently, mice (Lamming et al. 2013). Rapamycin is known to have profound effects on health and longevity in various model organisms (Swier et al. 2014) and are being studied in humans as therapies for treating age-related diseases. Rapamycin (sirolimus) is a natural product initially identified as a fungicide (Sehgal 2003) and later found to be a potent immunosuppressant (Muthukkumar et al. 1995). It has a wide usage but its effects on other pathways or molecules are not understood. In recent years, signaling through the target of rapamycin (TOR) kinase has emerged as a key pathway involved in lifespan extension. Reduction in TOR

signaling is sufficient to increase lifespan in yeast (Powers et al. 2006), *C. elegans* (Vellai et al. 2003), *Drosophila* (Bjedov et al. 2010) and mice (Stanfel et al. 2009) probably by modulating the mRNA translation and enhanced degradation of damaged macromolecules. Mice with reduced TOR signaling in adipose tissue showed resistance to diet induced obesity and metabolic diseases. Rapamycin is known to induce autophagy, which is independent of Sirtuin 1, in other words they promote autophagy by non-overlapping mechanisms (Madeo et al. 2010). Rapamycin is now known to confer protection in model animals and cell based models of neurodegenerative diseases, cardiovascular disease and against a variety of cancers (Johnson et al. 2013a, b).

Resveratrol, a polyphenol present in red wine, prolongs lifespan via autophagy by inducing the *sirtuin 1* expression only if animals are fed with a high-calorie diet leading to obesity (Baur and Sinclair 2006). Studies show the potential of resveratrol in inducing both canonical and non-canonical pathways (Scarlatti et al. 2008). In *C. elegans* or in animals that express a functional *sirtuin 1* gene show lifespan extension mediated by resveratrol, whereas resveratrol abrogated the favourable effects on longevity when *Beclin 1/Atg 6* are knocked down (Morselli et al. 2011). Assembling the two different studies confirms that resveratrol elicits lifespan extension in *C. elegans* in *sirtuin 1*-dependent manner.

The polyamine, spermidine has been recently shown to increase lifespan in various model organisms by inducing autophagy (Eisenberg et al. 2009). Exogenous addition of spermidine also shows the same effect in various organisms tested. It significantly reduces the age-related oxidative protein accumulation in mice (Minois et al. 2011) and thus has been proposed as an anti-aging drug. Inhibition of autophagy by knockout of autophagy related genes abolish the lifespan promoting effect of spermidine in yeast (Eisenberg et al. 2009). Medical application of spermidine as a natural autophagy inducer is of most interest for future research and applications.

Many questions still need to be answered. It is true that autophagy related proteins decline with age and so does the efficiency of autophagy process. It suggests the possibilities of genetic, pharmacological and nutritional strategies that work via this pathway to serve as good targets for longevity. One that still remains to be answered is whether all longevity-promoting manipulations require autophagy? This may be true in lower organisms but does it still hold true for humans? Results obtained with neurodegenerative diseases also raise the question whether autophagy induction works better than interventions in the central nervous system (Kesidou et al. 2013). Much more rigorous researches in different model systems are required to answer these questions.

4 *Dictyostelium discoideum*: A Good Model System to Study Autophagy

Dictyostelium discoideum is a simple eukaryote that lives in the soil. It feeds on bacteria by phagocytosis and divides mitotically till there is sufficient food (Raper 1935). Upon depletion of food, the amoebae chemotax to common collecting points in response to the chemoattractant cAMP and initiate multicellular development (Konijn and Raper 1961). The unicellular amoebae, under nutrient starvation conditions enter multicellularity, which is not by repeated cell division but by coming together of spatially segregated cell types. Terminal differentiation in this organism leads to the formation of two cell types namely, the stalk and the spore cells. The spores are viable and each of them can germinate into an amoeba under favorable conditions whereas the stalk is made up of dead vacuolated cells, which fail to regrow under similar conditions. Multicellularity in *D. discoideum* is different from the other metazoans and genes involved during both the phases of life cycle are different and mutations in the developmentally regulated genes do not affect the viability of the organism (Gross 1994; Weeks 2000; Kessin 2001). Thus, it displays distinct characteristics of true cellular specialization, coherent cell movement, cell death and altruism.

The terminally differentiated stalk and spore cells are present in its precursor kinds as prestalk and prespore cells respectively during earlier developmental stages. These cells are specified and not terminally differentiated and also has the potential to show transdifferentiation (Browne and Williams 1993). At any given time during development, 15–20 % of the total cell population is destined to die by becoming stalk cells. A clear anterior-posterior pattern formation is visualized in the slugs where the anterior 1/5 is composed of prestalk cells while the rest is composed of prespore cells. Interspersed in the prespore region are the anterior-like cells (ALC) which biochemically is similar to the prestalk cells. The fruiting body formed includes a stalk that is made of vacuolized cellulose walled dead cells (Williams et al. 1987; Cornillon et al. 1994; Giusti et al. 2009) (Fig. 1).

D. discoideum occupies a strategic phylogenetic position which emerged after the divergence of the plants but before the animals and fungi. *Dictyostelium* represents one of the distinct successful transitions in evolution from unicellular eukaryotes to multicellularity. Since the fruiting body formed shows death with respect to stalk cells is thus considered as one of the most ancient developmental programmed cell death in eukaryotes. This death is neither by apoptosis nor necrotic but is vacuolar. Induction of this developmental cell death requires two signals: one being starvation that induces the complicated cAMP signal transduction pathway and second being the polyketide Differentiation inducing factor (DIF), a prestalk pathway promoter (Luciani et al. 2011). This also requires inositol 1, 4, 5-triphosphate receptor (IP3R), which governs the calcium fluxes from the endoplasmic reticulum into the cytosol. The prestalk cells show higher levels of intracellular free calcium (Saran et al. 1994) as compared to prespore cells; also high

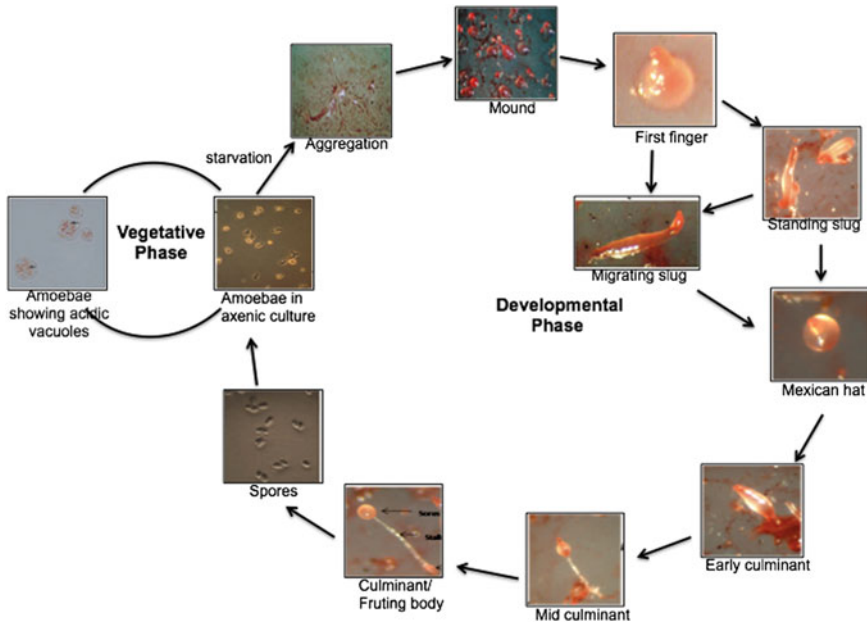


Fig. 1 Life cycle of *Dictyostelium discoideum*. Amoebae remain in vegetative cycle till the food is abundant. Starvation triggers the developmental cycle which undergoes various morphogenetic movements to ultimately form a fruiting body comprising two terminally differentiated cell types: stalk and spore. Here the vegetative cells were stained with the vital dye, neutral red that stains acidic vacuoles and appear red in colour under visible light. The acidic vacuoles are present in the prestalk/stalk cells during development

calcium levels can bias the cells towards the prestalk pathway (Abe and Maeda 1989).

The *Dictyostelium* genome has been fully sequenced and annotated (<http://dictybase.org/>). It is a 34 Mb genome having many genes that are homologous to the higher eukaryotes but are missing in yeast. Many genes are homologous to human genes making them a very useful model organism. Also approximately 33 genes are homologous to genes involved in human disease (Eichinger et al. 2005). Because of its similarities, it is now used to understand complex human diseases (Williams et al. 2006) and test anticancer drugs, immune cell diseases and bacterial intracellular pathogenesis. Behaviour of individual cells accounts for many phases of health and diseases, for example, cytokinesis for tissue maintenance and cell proliferation for cancer. Chemotaxis deals with inflammation, lymphocyte trafficking and axon guidance (Bozzaro 2013). Phagocytosis can be analyzed to understand antigen presentation. General basic features of embryogenesis like pattern formation, cell fate determination and differentiation can be always analyzed.

The ability to synchronize morphogenesis and a well-developed molecular genetics allows it to become a good model system for the analysis of various cell

signaling as well as for the understanding of cellular and biological processes involved in multicellular development. Myosin mutations that cause cardiac myopathies (Fujita et al. 1997) and mechanism of action of lithium have recently been analyzed (Williams et al. 2002).

Multicellular development in case of *Dictyostelium* occurs in the absence of any external food source. The energy required for aggregation and morphogenesis comes from glycogenolysis and autophagy (Rabinowitz and White 2010). In case of *D. discoideum*, the stalk cell formation is due to autophagic cell death (ACD). With respect to programmed cell death, *Dictyostelium* offers advantages over other systems as it shows ACD in the absence of apoptosis, which is caspase dependent. There are no caspase or metacaspase genes identified in the *Dictyostelium* genome. Inactivation of the single paracaspase gene (Roisin-Bouffay et al. 2004) did not show any alteration in the cell death program but showed some degree of multicellular developmental defects. No ortholog of any known caspases or its family members were found in *Dictyostelium*, thus it offers a good model system to study non-apoptotic cell death in the absence of apoptosis. ACD is described as cell death with an autophagic component. The autophagic components are quite well known in *D. discoideum* but the mechanism of cell death (ACD) is still poorly defined. Out of the two signals, the first signal of starvation/cAMP initiates autophagy while the second signal, DIF-1 is required for the transition from autophagy to ACD. Autophagy is insufficient to trigger ACD as it requires an intact *Atg1*. Inactivation of *Atg1* blocks vacuolization as well as autophagy but not cell death (Giusti et al. 2009). All characteristics of necrotic cell death like generation of reactive oxygen species, depletion of ATP, rupture of plasma membrane are observed.

Autophagy induction and regulation is regulated by the energy and nutritional status of the cell. The nutritional sensor is the target of rapamycin (TOR), which is a serine threonine kinase and receives signals like nutrients, energy, calcium, amino acids, growth factors etc. There is one *Tor* gene in *D. discoideum*, which forms both the C1 and C2 complexes of which TORC1 is primarily involved in autophagy (Soulard et al. 2009). The autophagosomes originate from multiple sites in the cytoplasm and appear as punctate pattern in the cytoplasm, which can be quantitated. Several autophagy genes have been isolated in *Dictyostelium*, which can be grouped according to their functions. Some of the genes required for the origin, elongation, completion and degradation of the autophagosomes are known but their regulation is still not understood (Levine et al. 2011). *Atg1* and the lipid kinase *Vps34* (Maiuri et al. 2007) are required for the initiation of nucleation of the autophagosomes. *Atg8* and *Atg12* are required for the vesicle expansion and completion while *Atg2*, *Atg9* and *Atg18* play a role in the biogenesis of autophagy (Yorimitsu and Klionsky 2005). TORC1 functions upstream of *Atg1* complex and is involved in cell growth and metabolism (Wullschleger et al. 2006).

5 *Dictyostelium discoideum*: As a Model to Study Longevity

Nearly all metazoans undergo the process of aging. Many model organisms have been used to elucidate the mechanism of aging especially to understand human aging. It is true that observations from all model organisms have added on to the understanding of the process of aging but as yet it has to be seen if they hold true for humans. One thing, which has emerged is that considerable number of pathways involved in longevity are conserved throughout evolution. Invertebrate model systems have given maximum answers in the field of aging research but the degree to which it is conserved between organisms is yet to be answered. Comparative functional genomics approach between yeast and *C. elegans* show conserved pathways involved in longevity suggesting that they may play similar roles in human aging. Also, there exists orthologs of aging related genes in mammals.

Organisms from various habitats across eukaryotic species are influenced by different extrinsic factors that may be responsible for age-associated mortality. However, many studies have shown the existence of few similar patterns of aging across species. Thus, the question remains is why evolution selected these processes in organisms, which are very divergent. Additionally, to what extent is the mechanism of longevity/aging conserved over evolution.

As discussed earlier many more model system for analyzing aging/longevity would prove beneficial. In fact the mechanism(s) involved in autophagy by the anti-aging drugs and their interactions still need to be elucidated. In the present study, we intend to present *D. discoideum* as a new model system to analyze longevity. There are many reasons contributing towards the process of aging. Some of the significant observations, point to dietary restrictions, as a measure for longevity but the mechanistic understanding of how it works is not yet understood. Using this model system we could understand the correlation between autophagy, TOR signaling, sirtuins, anti-aging drugs and their role in longevity. *Dictyostelium* provides a very good model system to delineate the mechanistic of these processes, as its genome is known to be close to the human genome. It follows a caspase independent cell death mechanism, which is ACD. Nutrient plays a major role in the transition from uni-to multicellularity.

By all means, *Saccharomyces cerevisiae* has identified the maximum number of genes that are involved in the control of aging/longevity. Aging in yeast is assayed primarily by measurement of replicative or chronological life span (Longo and Fabrizio 2012; Fabrizio et al. 2005). These are well-established methods to measure longevity. Yeast replicative lifespan is defined as number of times a single cell divides before its senescence. This method is useful to study aging process of mitotically active cells in multicellular organisms (Kaeberlein. 2010). Chronological life span is a suitable model for post-mitotic cells in multicellular organisms as it determines the length of time a cell can survive under non-proliferative conditions. i.e. number of days cells remain viable in stationary phase culture (Kaeberlein et al. 2007). Chronological aging helps investigate the

different activities required for somatic maintenance, which is well conserved across species. The most important of them all is stress protection by anti-oxidants. In most cases mitochondrial dysfunction is associated with aging (Morino et al. 2006), which contributes to reactive oxygen species (ROS) production leading to damage of proteins, lipids and DNA (Bhat et al. 2015). This study is now being taken to other organisms. Even in yeast, the results obtained is limiting as we still do not have a clear picture of how the genetic factors controlling aging/longevity works.

Similar to yeast chronological aging/longevity model, chronological life span determinations was performed in *Dictyostelium discoideum*, wild type ($A \times 2$) cells grown in axenic media containing standard amount of glucose. Chronological aging experiment was conducted by inoculating fresh $A \times 2$ (-80°C) spores in axenic medium at 22°C and allowed to grow till stationary phase was reached. The midpoint of stationary culture was considered as the beginning of the chronological ageing or t_0 h. Subsequently viability of non-proliferative cells from stationary culture was determined at different time points 12, 24, 36, 48 h to measure viability of the cells. This was carried out by three different methods (Fig. 2): MTT reduction assay (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (Swier et al. 2014), propidium iodide (PI) staining followed by flow cytometry analysis (FCM) (Ocampo and Barrientos 2011) and regrowth assay (Giusti et al. 2009). The methodologies followed are described in brief below.

- MTT assay: Cell viability was monitored at various time points by a slightly modified method of Mosmann (1983). Absorbance of the reduced dye was measured at 570 nm and the background reading at 630 nm was taken.
- Regrowth assay: A serial dilution of the stationary culture cells was taken for plating with bacteria on non-nutrient agar and subsequently allowed to incubate at 22°C for 72 h. The clearing zone or plaques formed were counted for the analysis.
- Propidium iodide staining and flow cytometry analysis: Cell viability was analyzed using propidium iodide (PI), a fluorescent dye extensively used from bacteria to mammalian cells to discriminate between live (PI^-) and dead (PI^+) cells based on membrane permeability. Ocampo and Barrientos (2011) used it to determine the viability of stationary phase cells thus making it suitable for studying chronological aging. Approximately, a density of 1×10^6 cells/ml was incubated for 30 min at 22°C in the presence of $1 \mu\text{M}$ PI. Cells were then subjected to flow cytometry. Analysis of cells at different time points ranging from 12–60 h was carried. The data obtained was utilized for the construction of a survival curve by plotting the percentage of PI^- cells versus time.

Our data shows that *Dictyostelium* could be used for the measurement of longevity using chronological lifespan extension model similar to yeast. The major genes involved in chronological aging pathways in almost all organisms studied so far are the TOR/S6 K pathway (Fabrizio et al. 2001) and the Ras/adenylate cyclase/PKA pathway (Longo et al. 1999; Fabrizio and Longo 2003). The TOR/S6 K pathway is activated by nutrition like amino acids etc. while the

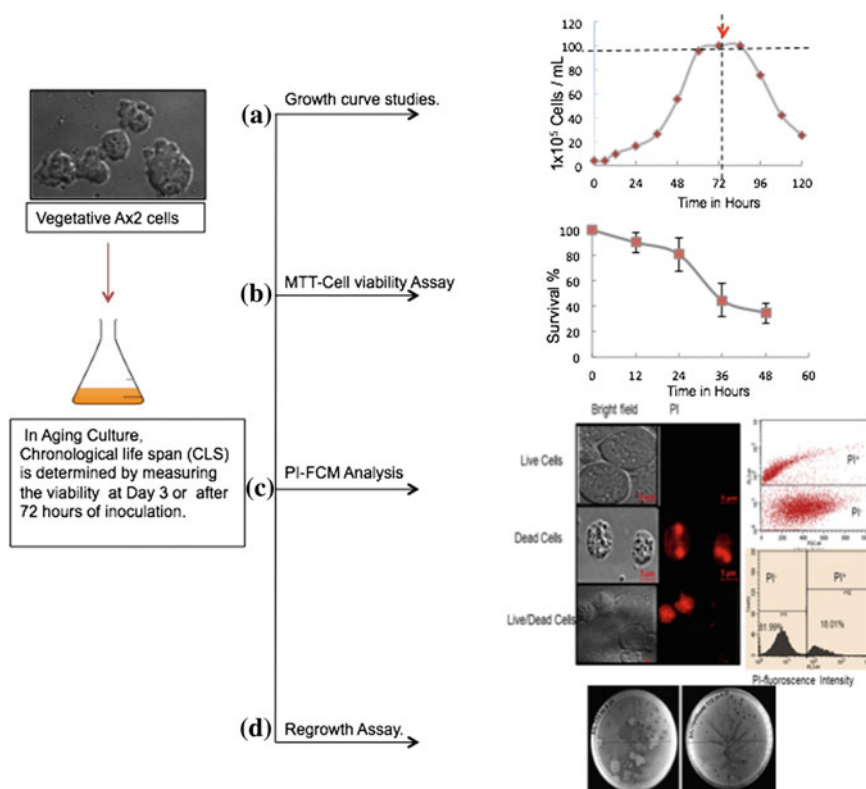


Fig. 2 Chronological longevity assay in *Dictyostelium discoideum*. The assay was based on that used for yeast longevity test. **a** Growth kinetics: $A \times 2$ cells were grown in HL-5 medium till it reached the stationary phase ($\sim 1 \times 10^7$ cells/mL) after, which the rate of cell multiplication decreases and approaches zero due to lysing of cells. Here, the dashed lines represent extrapolations of stationary phase. Intersection of this plot represents the onset of stationary phase. This time point is considered as t_0 h to determine cell viability at different time points by MTT, PI-FCM and regrowth methods. **b** Cell viability assay using MTT: Cell viability of stationary phase cells is considered as initial survival (100 %) and was monitored by MTT-reduction assay, at different time points 12, 24, 36, 48 h. Here, data represents percentage of survival relative to t_0 as function of time in stationary phase. **c** Propidium iodide staining and Flow cytometer analysis: Both live (PI⁻) dead (PI⁺) cells were visualized in a given population. The stationary culture, which had a mixture of live and dead cells were visualized and further taken for FACS analysis. The percentage of cells in each population was accounted for. **d** Regrowth assay: Chronological survival of aged cells on *solid* medium was determined by counting the number of plaques or clearing of food seen

AC/PKA pathway is modulated largely by glucose etc. These signaling pathways interact with each other and regulate metabolism and stress. In case of *D. discoideum*, starvation activates the nutrient signal transduction pathway by inhibiting the TOR to induce autophagy (Swier et al. 2015). Also the energy sensor, AMPK is activated upon starvation, which decreases the level of ATP to ADP. The

mechanistic of this is still not understood. NAD^+ , an energy donor is utilized by Sirtuin for its activity, gets affected by nutrient depletion but how it regulates the Sirtuin 1 and AMPK is not yet known.

6 Sir2D of *D. discoideum* Promotes Lifespan Extension by Inducing Autophagy

As discussed earlier Sirtuin 1 promotes longevity in many organisms. There are five Sirtuins present in this organism, which may have a role to play in longevity. The Sirtuin family of proteins is both functionally and structurally conserved and is found in organisms ranging like eubacteria, archaea, eukaryotes and viruses (Raffaelli et al. 1999; Martin et al. 2001; Miller et al. 2003). Sirtuins are involved in both metabolic and chromatin regulations throughout evolution. The Sir2 was first discovered in *Saccharomyces cerevisiae* and was named after its ability to relieve gene silencing (Dali-Youcef et al. 2007). Phylogenetic analysis of sirtuins has divided the family into five different classes. Four of the five sirtuins from fall into class I and one in class III. We found that the sir2A and sir2C fall in one subclass while sir2B and sir2D fall in another subclass. sir2E could be seen as a separate clade from the rest of the sirtuins of *D. discoideum*. The *sirtuin* genes encode an important and complex family of proteins that participate in a wide array of physiological processes (North and Verdin 2004). In several species, caloric restriction has been shown to increase lifespan and decrease spontaneous rates of illness, such as insulin resistance, neurodegenerative disease, cancer and oxidative stress (Merksamer et al. 2013). Caloric restriction generally activates specific cellular signaling networks and therefore is thought that all chemicals that can induce sirtuin activity could possibly prove beneficial for therapeutics (Imai and Guarenete 2010; Johnson et al. 2013a, b). Sir2 is known to prolong the lifespan in budding yeast (Kaeberlein et al. 1999; Imai et al. 2000), flies and worms (Tissenbaum and Guarenete 2001; Rogina and Helfand 2004). Humans have seven sirtuins namely SIRT1-7. SIRT1 of human plays a very important role in longevity. It is also reported that during fasting SIRT1 modulates gluconeogenesis in liver by deacetylating few important factors like PGC-1 α , FOXO1, etc. (Brunet et al. 2004; Rodgers et al. 2005; Liu et al. 2008). SIRT1 is also found to function during inflammation, neurodegenerative diseases, cardiovascular disease and cancer (Luo et al. 2001; Yeung et al. 2004). SIRT2 (also called α -tubulin deacetylase) knockout delay entry into S-phase of the cell cycle (Li et al. 2007). It has been reported that SIRT2 specific inhibitor can ameliorate α -synuclein—mediated toxicity in Parkinson's disease (Outeiro et al. 2007). SIRT3, 4, and 5 are mitochondrial sirtuins as they have mitochondrial targeting sequence in their N-termini (Haigis et al. 2006). SIRT3 increases the acetyl-CoA by interacting with acetyl-CoA synthetase (Hallows et al. 2006). SIRT4 is known to control insulin secretion in pancreatic β -cells (Haigis et al. 2006) while SIRT5 is known to regulate urea cycle (Nakagawa

et al. 2009). SIRT6 is nuclear localized and is known to be involved in DNA double strand break repair by regulating C-terminal binding protein (CtBP) and DNA protein kinase (McCord et al. 2009). SIRT7 is the least known sirtuin and possibly involved in regulating RNA polymerase I transcription (Ford et al. 2006). Thus, it seems reasonable to propose that the sirtuin protein family members could prove to represent novel molecular targets for the treatment of a variety of human diseases having a strong association with increasing age.

Sir2D, an ortholog of human SIRT1 was used to test this model of longevity in *D. discoideum* (Lohia 2015). We created an overexpressor and a knockout of *sir2D* by gene disruption followed by homologous recombination (Sutoh 1993) and tested for longevity (Fig. 3). Chronological ageing mechanism was studied in overexpressing cell line of *Ddsir2D*. As done with wild type cell population cell viability studies were conducted at different time points and as expected, at consecutive time intervals the percentage of PI⁺ cells increased in comparison to wild type cells. This was further confirmed by the regrowth assay, which showed that the *sir2D* overexpressing cells survived 24 h more than the wild type cells confirming that in *D. discoideum* also Sirtuin 1 homolog increases longevity.

Longevity extension by overexpression of *sir2D* in *D. discoideum* is by autophagy induction (Fig. 3). Autophagic flux in cells can be monitored by microscopic visualization of the RFP-GFP-Atg8 tandem probe (Calvo-Garrido et al. 2011). Atg8 marks the autophagosomes and is taken as a marker for autophagy studies. RFP fluorescence is resistant to acidic pH of the lysosome whereas, GFP fluorescence gets quenched rapidly is a well-known fact. Thus, the red-green puncta

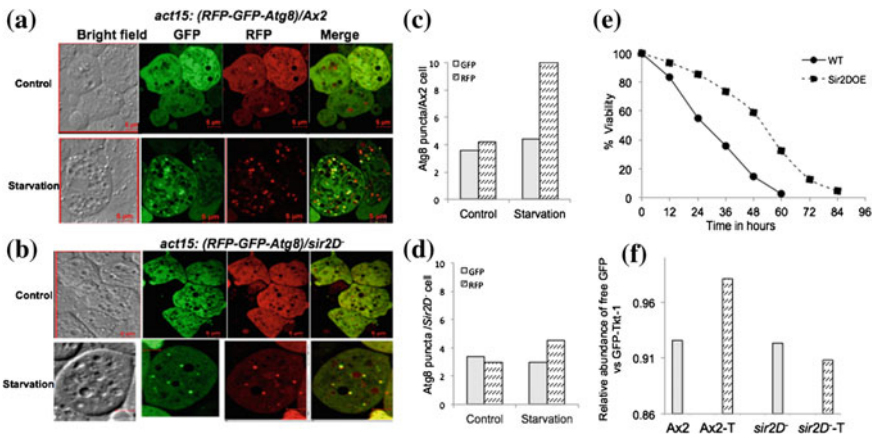


Fig. 3 Sir2D of *Dictyostelium discoideum* increases lifespan by inducing autophagy. Both wild type (a) and *sir2D*⁻ (b) cells were transformed with tandemly tagged RFP-GFP-Atg8 (a marker for studying autophagic flux). Atg8 puncta/cell was counted using NIS elements software. The wild type c showed higher autophagy flux in comparison to the knockout cells (d). Scale bar: 5 μ m. e Sir2D overexpressing cells show increased chronological lifespan as compared to wild type. f Autophagic flux by Western hybridization show lower ratio between free GFP and GFP-Tkt-1 in *sir2D* knockout cells

marks the early autophagosomes and the red puncta that lack green fluorescence marks the fusion of autophagosomes with lysosomes. The *sir2D⁻* cells showed lower red puncta after starvation (Fig. 3) as compared to the control wild type. The percentage decrease in the red puncta count suggests a role for Sir2D in the process of autophagy. We found an increase in the red puncta in the wild type upon starvation as compared to the *sir2D⁻* cells. The *sir2D⁻* cells failed to activate autophagy in response to nutrient starvation as compared to wild type. Further, autophagic flux was also performed based on proteolytic cleavage (Calvo-Garrido et al. 2011). A significant decrease in free GFP levels in the *sir2D⁻* cells as compared to wild-type confirmed the decrease in autophagic flux (Fig. 3).

We also checked lifespan extensions by treating these cells with anti-aging drugs like spermidine, resveratrol and rapamycin. All these treatments induce autophagy and extend the chronological lifespan. Rapamycin treatment in *Dictyostelium* induces autophagy by the accumulation of ROS and intracellular free calcium (Swier et al. 2014) and vice versa. Figure 4 show the longevity extension in this organism in a dose dependent manner after spermidine treatment. We find that low

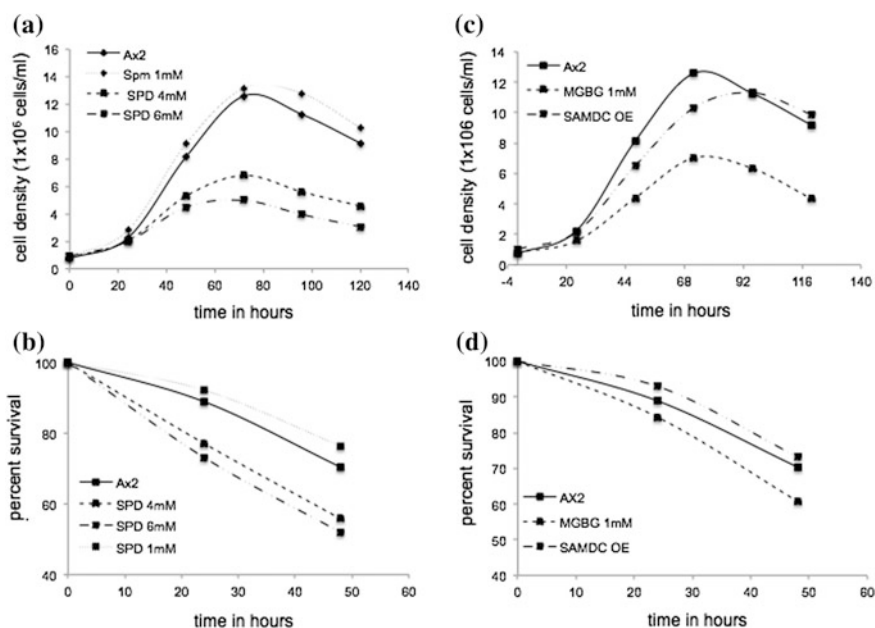


Fig. 4 Spermidine increases lifespan extension in *Dictyostelium discoideum*. **a** Shows the cell proliferation after addition of exogenous spermidine. Lower concentrations improve the proliferation over the control wild type cells. Higher concentrations inhibited cell proliferation. **b** Chronological life extensions were better in lower concentration of spermidine treatment. **c** Cell proliferation of overexpressing SAMDC that increases the spermidine levels in cells and treatment with MGBG that decreases the spermidine levels in comparison to wild type cells. **d** Chronological life extensions were better in cells having slightly higher net spermidine levels that are those which were treated with MGBG

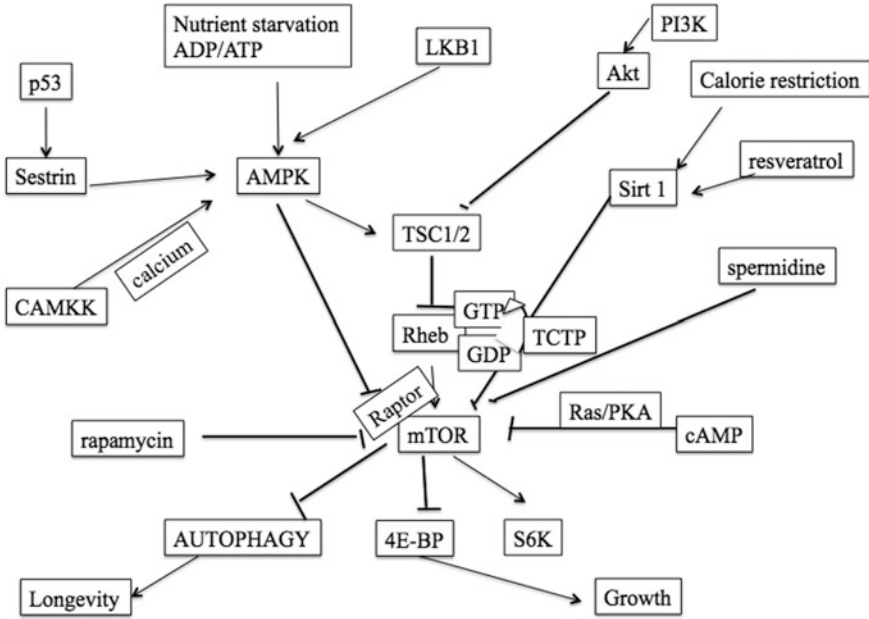


Fig. 5 Molecules and pathways that may control longevity in case of *Dictyostelium discoideum*. All possible interactions between the molecules are shown in the figure

concentrations of spermidine increases lifespan while higher doses induce cell death. The mechanism of these drugs is yet to be delineated.

We have earlier reported that both the transcript and the protein of the rate limiting enzyme ornithine decarboxylase were high in the prestalk/stalk cells in *D. discoideum* (Kumar et al. 2014). In the present study we show that exogenous addition of spermidine to $A \times 2$ cells shows dose dependency where lower concentrations increase lifespan while higher doses inhibited proliferation and decreased lifespan (Fig. 4a, b). The results were similar to that observed with yeast, flies, worms and human cells. Our unpublished results show that at lower concentration spermidine treatment induces autophagy. A rate-limiting step in polyamine biosynthesis is the decarboxylation of *S*-adenosylmethionine (SAM) by SAMDC that yields decarboxylated SAM, which, in turn, donates its propyl amine moiety to form spermidine and spermine by two specific aminopropyl transferases, spermidine synthase and spermine synthase (Gilmour, 2007). Methylglyoxalbis-guanyldrazon (MGBG) is a potent inhibitor of SAMDC (Regenass et al. 1992) and an antitumor drug with extremely toxic side effects. Apart from addition of spermidine exogenously we created SAMDC overexpressing cells, which results in higher intracellular spermidine levels ultimately higher longevity (Fig. 4c, d). MGBG, which decreases net spermidine levels results in decreased lifespan.

Some of the signaling pathways and other molecules that may control longevity in *D. discoideum* by bringing about autophagy is shown in Fig. 5. Our preliminary unpublished data on the genes and other small molecules have confirmed their involvement in autophagy.

7 Conclusions

A major challenge is to find the interaction of these signaling pathways in inducing autophagy to increase the lifespan of an organism. Many intracellular signals recognized in *Dictyostelium* development are shown to be involved during vertebrate embryogenesis, synaptic transmission, or cellular interactions in plants or bacteria. We thus expect that they may show many parallel between the mechanisms of longevity with humans. We consider *D. discoideum* as one efficient model system to delineate these pathways and find the mechanism of action of these drugs. The beauty of this organism is to study autophagy in the absence of apoptosis as there are no known caspases identified in this organism. Since this organism shares high homology with humans, the result obtained here may hold true to certain extent in humans. Nutrient signaling plays an important role in the uni-to multicellularity in this organism the TOR pathway could be delineated well. The efforts made with this model organism would help understand the field of age-related research. Studies that could utilize genetic, nutritional and pharmacological manipulations to address the longevity issues could use *D. discoideum* as a model system to expand our knowledge.

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