

**POTENTIAL ROLE AND APPLICATION OF TREHALOSE IN THE
MICRORNA DEPENDENT EMBRYONIC DEVELOPMENT OF
*CAENORHABDITIS ELEGANS***

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*CAENORHABDITIS ELEGANS***

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This is to certify that I have examined this copy of master's thesis by

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ABSTRACT

MicroRNAs (miRNAs) are an essential class of non-coding RNAs that post-transcriptionally repress mRNAs. miRNAs were first identified in *Caenorhabditis elegans* as important regulators of developmental timing. Since then, miRNAs have been shown roles in nearly all biological processes. Most important process in which miRNAs take place is developmental processes.

Caenorhabditis elegans is a terrestrial nematode and a widely used model organism for studying biological processes development and aging related to miRNA gene function. A conditional allele of miRNA biogenesis gene, *pash-1* allows to regulate miRNA production by simply changing the growth temperature in worms. Grown at 15°C, *pash-1* mutant animals have normal miRNA levels and shifting the temperature to 25°C ceases miRNA production and leads to rapid ageing, motility defects and embryonic lethality. In our efforts to understand the roles of miRNAs in ageing, we observed a highly interesting phenomenon with one of the chemicals under investigation. Growing the *pash-1* mutant animals in the presence of trehalose rescued the embryonic lethality phenotype at 25°C.

Trehalose is a non-reducing disaccharide [α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside] of glucose. Trehalose is present in a variety of organisms such as bacteria, yeasts, fungi, insects, invertebrates and plants. *C. elegans* accumulate detectable amount of trehalose in embryonic and dauer stages of *C. elegans*. However, the importance of trehalose for developmental processes or its physiology remains to be explored in this model organism. In this study, we focused on the effects of trehalose on the embryogenesis of *C. elegans* that lacks miRNA biogenesis. While trehalose rescued the embryonic

ABSTRACT

lethality of *pash-1(mj100)* and *mir-35* loss of function mutants, it failed to rescue embryonic lethality of miRNA independent temperature-sensitive embryonic lethal mutants, indicating the specificity of the trehalose effect.

Trehalose can function as a chemical chaperone, autophagy inducer, a metabolite and a signaling molecule. In order to understand how trehalose alleviates embryonic lethality phenotype, we tested various chemicals in parallel to trehalose. We showed that autophagy inducer rapamycin does not have the same effect on the embryonic lethality. However, two chemical chaperones proline and serine have similar effects on embryonic lethality as trehalose does. Further efforts will be required to understand how trehalose affects miRNA function in embryogenesis, however, as this is the first time a chemical has been shown to rescue miRNA-related defect, this will have great impact on efforts in developing therapies in various diseases resulting from the loss of miRNA gene function.

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Key words: Trehalose, *C. elegans*, miRNAs, embryonic lethality, *pash-1(mj100)*, *mir-35(ndf50)*, *mir-52(ndf58)*

ÖZET

miRNA'lar neredeyse tüm biyolojik süreçlerde rol oynayan önemli bir protein kodlanmayan RNA sınıfıdır.

Caenorhabditis elegans bir karasal nematoddur ve miRNA'lar bu organizmada bu organizmada bulunmuş ve neredeyse bütün gelişim süreçlerinde önemi gösterilmiştir. Çalışmada *pash-1(mj100)* suşu kullanılmıştır. *pash-1* geni miRNA biyosentezinde görevli olan PASH-1 (PASHA) proteinini kodlamaktadır. *pash-1ts* suşunda sıcaklığın yükseltilmesiyle, miRNA biyosentezinin ilk aşamasının engellenmesinden dolayı miRNA üretilmemektedir. *pash-1ts* suşu, 25°C'de büyütüldüğünde erken yaşlanmaktadır. Ayrıca, *pash-1ts* suşunun 25°C'de üretilen embriyoların hiçbirisi L1 evresine geçemez. miRNA'ların yaşlanmadaki rollerini anlamaya çalışırken kullandığımız kimyasalların biriyle son derece ilginç bir fenotip gözlemledik. Trehalozun varlığında *pash-1* mutant hayvanlarının embriyonik ölüm fenotipi kurtarılabildi.

Trehaloz, indirgeyici olmayan disakkarid [α -D-glukopiranosil- (1 $\rightarrow$ 1) - α -D-glukopiranozid] iki glüköz molekülü taşımaktadır. Trehaloz bakteriler, mayalar, mantarlar, böcekler, omurgasız ve bitkiler gibi çeşitli organizmalarda mevcuttur. Bu çalışmada, biz miRNA biyogenezinin yoksun *C. elegans* embriyogenezin üzerindeki trehalozun etkileri üzerinde duruldu. Trehalozun embriyo ölüm fenotipini neden hafiflettiğini anlamak amacıyla, trehaloza paralel olarak, çeşitli diğer kimyasallar da test edilmiştir. Ancak, diğer kimyasallar trehaloz gibi kurtarıcı etki göstermemiştir. Sadece, prolin ve serin trehalozla benzer etkilere sahiptir. Bu çalışmada ilk kez bir kimyasalın miRNA ilişkili bir fenotip üzerinde iyileştirici etkisi gösterilmiştir.

Anahtar kelimeler: Trehaloz, *C. elegans*, miRNA'lar, embriyonik ölüm fenotipi, *pash-1(mj100)*

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ABBREVIATIONS

ABBREVIATIONS

A	Alanine
AGO	Argonaute
bp	base pair
C	Cystine
cDNA	Complementary DNA
<i>C. elegans</i>	<i>Caenorhabditis Elegans</i>
CGC	<i>Caenorhabditis</i> Genetics Center
CL	Chitin Layer
D	Aspartic Acid
DNA	Deoxyribonucleic Acid
DTC	Distal Tip Cell
E	Glutamic Acid
<i>E.coli</i>	<i>Escherichia Coli</i>
EEM	Extraembryonic Matrix
F	Phenylalanine
g	Gram
G	Glycine
GFP	Green Fluorescent Protein
H	Histidine

ABBREVIATIONS

I	Isoleucine
K	Lysine
L	Leucine
LB	Luria Broth
lincRNAs	Long Intergenic Non-coding RNAs
LrL	Lipid-rich Layer
M	Methionine
M	Molar
Mg	Milligram
Min	Minutes
ml	Milliliter
mM	Millimolar
μ M	Micromolar
mRNA	Messenger RNA
mTOR	Mammalian Target Of Rapamycin
N	Asparagine
ncRNA	Non-coding RNA
NGM	Nematode Growth Medium
nt	nucleotide
P	Proline

ABBREVIATIONS

PCR	Polymerase Chain Reaction
Pri-miRNA	primary miRNA
Pre-miRNA	precursor miRNA
PrP ^{sc}	intracellular pathological prion protein
Q	Glutamine
QPCR	Quantitative PCR
R	Arginine
RDE-4	RNAi-Defective 4
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rRNA	ribosomal RNA
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse transcription polymerase chain reaction
S	Serine
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis
shRNA	Small Hairpin RNA

ABBREVIATIONS

siRNA	Small Interfering RNA
T	Threonine
TRBP	Human immunodeficiency virus Trans activating Response RNA- Binding Protein
Trna	transfer RNA
UTRs	Untranslated Regions
UV	Ultraviolet light
TRE	Trehalase
W	Tryptophan
WT	Wild Type
Y	Tyrosine
V	Valine
VL	Vitelline Layer

INTRODUCTION

MicroRNAs (miRNAs) are an essential class of non-coding RNAs that post-transcriptionally repress messenger RNAs (mRNAs). Lately, miRNAs have been shown roles in nearly all biological processes. Most important process in which miRNAs take place is developmental processes.

Using the temperature sensitive *mj100* allele of *pash-1* gene that encodes an essential protein for miRNA biogenesis, it is now possible to inhibit miRNA production and investigate developmental and post-developmental roles of miRNAs. Phenotypic characterization of *pash-1(mj100)* mutant animals revealed that miRNA biosynthesis is required for normal lifespan. In an attempt to identify the roles of miRNAs in ageing, a small scale chemical screen was performed prior to this thesis work. The chemicals known to extend lifespan were tested for their ability to rescue the shortened lifespan of *pash-1(mj100)* mutant animals. Trehalose has been shown to extend lifespan of *C. elegans* (Honda, Tanaka, & Honda, 2010). When *pash-1(mj100)* mutant animals were grown in the presence of trehalose, we made an interesting observation. *pash-1(mj100)* embryos die in late stages of embryogenesis at restrictive temperature, 25°C; we observed that trehalose rescues embryonic lethality of *pash-1(mj100)* mutant animals by increasing their hatching from 0% to 40%. In this project, we aimed to investigate the effect of trehalose on the embryonic lethality of *pash-1(mj100)* mutants and other miRNA loss of function mutants. We showed that trehalose rescues embryonic lethality of all *pash-1 (mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)*. Osmolytes, proline and serine have similar effect on embryonic lethality as trehalose does. The most interesting results we found is that trehalose probably acts in specifically miRNA related pathway of embryonic development, because it didn't

rescue embryonic lethality of miRNA independent temperature-sensitive embryonic lethal mutants.

Chapter 1 gives literature review about model organism, *Caenorhabditis elegans*, its physiology and genetics; miRNA biogenesis and importance in development of *C. elegans*; lastly about trehalose and its particular features. **Chapter 2** summarizes chemicals used and methods performed in this study. Results of this study are given in **Chapter 3** and discussed in **Chapter 4**.

CHAPTER 1

LITERATURE REVIEW

1.1 Non-coding RNAs, their importance and regulation

Non-coding RNAs (ncRNAs) are RNA molecules which are not translated into proteins, consisting of abundant and important RNAs such as transfer RNA (tRNA), ribosomal RNA (rRNA), and other RNAs such as small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) and the long non-coding RNAs (lncRNAs). Non-coding RNAs have many important roles in various biological processes such as gene silencing, heterochromatin formation and histone modification. For instance; *let-7* is one of the firstly identified miRNA in *C. elegans* that regulates lineage specific development of many species by gene silencing and it has several roles in stem cell maintenance.

Human Genome Project (Bergsma et al., 2001) revealed that the protein coding genes reflects only a small part of the genome. In addition, Encyclopedia of DNA Elements (ENCODE) project (Carninci et al., 2005) supported this phenomenon by showing that 68% of actively transcribed genes of the Human and Mouse genomes does not encode proteins. These recent findings showed that there are more non-coding RNA loci than protein coding loci. Each day, new classes of non-coding RNAs are discovered with next generation sequencing. Although functions of many non-coding RNAs are not known, there is high degree of similarity between small RNAs' genes in different species showing a high degree of conservation. Therefore, it will be of a great interest to study functions of small non-coding RNAs using model organisms.

1.2 *Caenorhabditis elegans* as a model organism to study miRNA gene functions.

Caenorhabditis elegans (*C. elegans*) is a terrestrial nematode, which is mostly found in rotten fruits and roots. It was firstly used as a model organism by Sydney Brenner for studying development of the nervous system, since then it has become a widely used model organism to study a wide range of biological processes (Brenner, 1974). For example, its short lifespan lasting about two weeks has allowed studying ageing. Developmental biologists use *C. elegans* for its invariant development and transparency. In addition to that, *C. elegans*' cell lineage is invariant with 953 somatic cells and 302 neurons in hermaphrodites and 1031 somatic cells and 391 neurons in males. They are very easy to grow and are cheap to maintain. Therefore, it enables the researches to study genomic and phenotypic research.

C. elegans' genome was sequenced in 1998. The genome of *C. elegans* is smaller than that of human genome (*C. elegans* genome is approximately 100 Mb; *H. sapiens* genome is approximately 3000 Mb), but the gene number of *C. elegans* and human is very similar (*C. elegans* have approximately 20000 genes; humans have approximately 23 000) (Hulme & Whitesides, 2011). Moreover, about 80% of human genes have clear, conserved *C. elegans* homolog (Chang Lin et al., 2000).

C. elegans have two possible sexes, hermaphrodite and male which make *C. elegans* an optimal organism for studying genetics with both self-fertility and cross-fertility. These two possible sexes enable to perform crosses for generating different genotypes.

C. elegans is a diploid organism with 5 autosomal (chromosomes I-V) and one X chromosome (chromosome X). XX animals develop as self- fertile hermaphrodites. Hermaphrodites dominate the overall population. Spontaneous non-disjunction of X chromosomes at meiosis generates XO animals that become males. They compromise the 0.1% of the population (Braendle & Felix, 2006).

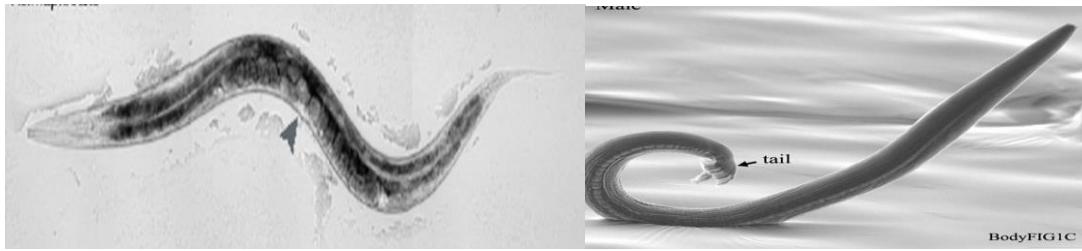


Figure 1.1: Anatomy of hermaphrodite and male *C. elegans*. (wormatlas.org)

The life cycle of *C. elegans* hermaphrodites starts from fertilized egg, goes through embryonic and four larval stages (L1 to L4) before reaching to fertile adulthood seen in the **Figure 1.2** below.

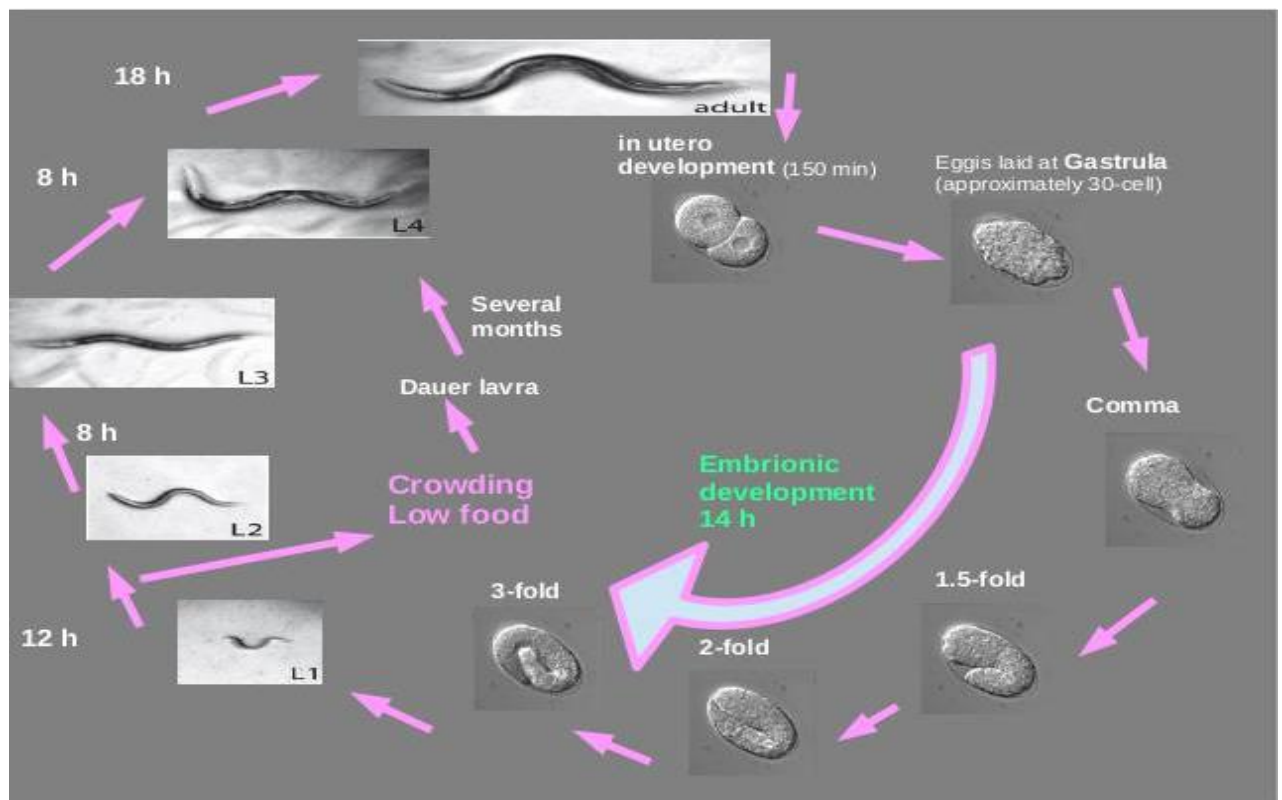


Figure 1.2: The life cycle of *C. elegans* at 22°C. (Adapted from www.wormatlas.org).

The duration of the *C. elegans* life cycle depends on environmental conditions such as temperature, food access and pH. At 20°C, *C. elegans* develops from an embryo to adulthood in almost three days, and lays eggs for 3 or 4 days after reaching adulthood, and reaches “a post-reproductive period” (Herndon et al., 2002).

C. elegans can arrest during development and enter another larval stage called dauer (in German means “endures”) larva at either L1 or L3 stages. The decision to go into dauer depends on the population density, food supply, and temperature. Dauer larvae develop a thick cuticle that protects them from harsh chemicals such as sodium dodecyl sulfate (SDS). They can resist high temperatures and various stresses. Interestingly, when the conditions are reversed, the animals exit dauer and continue their development. Dauer larva has extended lifespan, living approximately 4 months, in which it minimalizes almost all metabolic synthesis and only depends on its own energy source such as fats (Riddle, Swanson, & Albert, 1981).

1. 3 Reproductive System of *Caenorhabditis elegans*

In *C. elegans*, the gonad is a U-shaped tube that is called syncytium. Germline of *C. elegans* is consisted of self- renewing cells at the distal end and differentiating gametes at the proximal end of the gonad. The distal tip self- renewal cells called germline stem cells. Each hermaphrodite has two distal tip cells (DTCs), one located at the distal end of each gonadal arm.

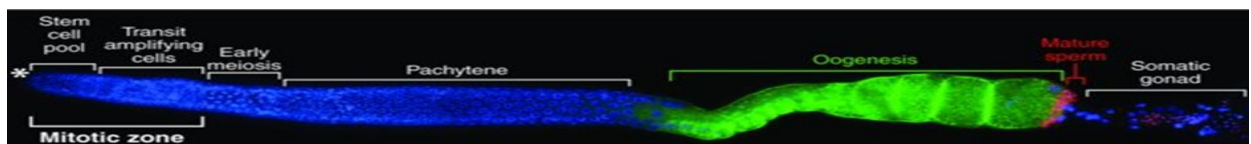


Figure 1.3: Adult germline organization. Gonadal arms (blue), oocyte marker (green) and sperm marker (red). (Judith Kimble, Hannah Seidel).

Oogenesis takes place within the syncytium as apical stem cells, which undergo mitosis to create nuclei that progress through meiosis and they mature to form oocytes. Fertilization takes place as the oocyte pass through spermatheca, a torroid shaped organ that contains the sperms. (Ward & Carrel, 1979). After fertilization, fertilized egg develops in the uterus till gastrulation and embryos are released through the vulva.

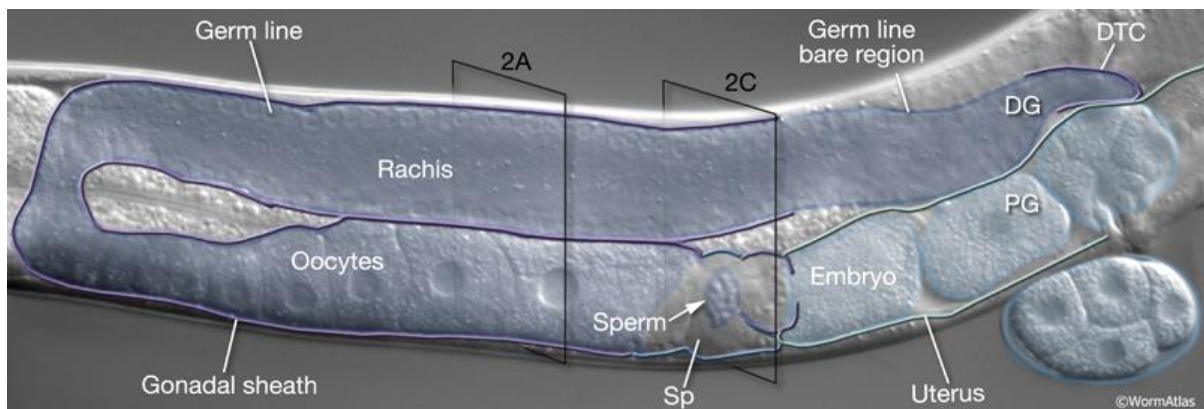


Figure 1.4: DIC image of an adult hermaphrodite anterior gonad arm. (From wormatlas.org)

1. 4 Embryonic Development of *Caenorhabditis elegans*

Embryogenesis is the first stage of development. The first division occurs asymmetrically that separates anterior cell from posterior cell. The first division of the embryo takes place 30 minutes after deposition. It forms a founder cell (AB) and a stem cell (P1). In the second division, the anterior founder cell (AB) and the P1 cell divide to produce another founder cell (EMS) and a posterior stem cell (P2).

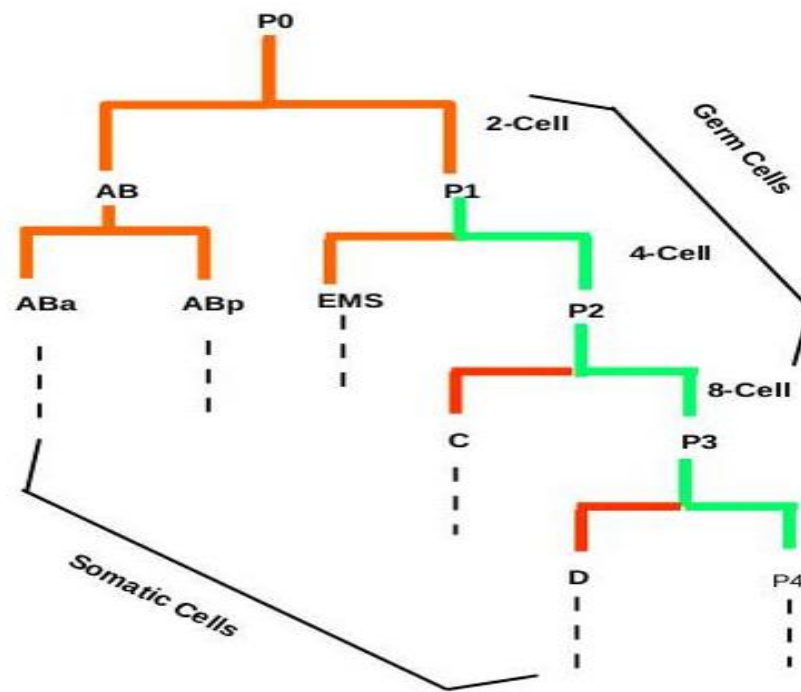


Figure 1.5: Embryogenesis Cell Fate.(Adapted from Skiba & Schierenberg, 1992)

The dorsal-ventral axis of the nematode is appeared in AB cell division. With divisions, eggshell becomes larger causing cells to slide with one anterior AB cell and one posterior AB cell (ABa and ABp, respectively). ABa makes neurons, hypodermis, and anterior pharynx cells, while ABp gives rise to neurons and hypodermal cells. Gastrulation takes place after P4 cell formation in the 24-cell embryo (Skiba & Schierenberg, 1992) when Ea and Ep move to the center, embryonic mass to form the gut tube. AB-derived cells make the pharynx. Afterwards, the cells are formed into organs, and the embryo starts to have worm-shaped structure after they followed comma, 1.5 fold, 2 fold, 2.5 fold, 3 fold stages.

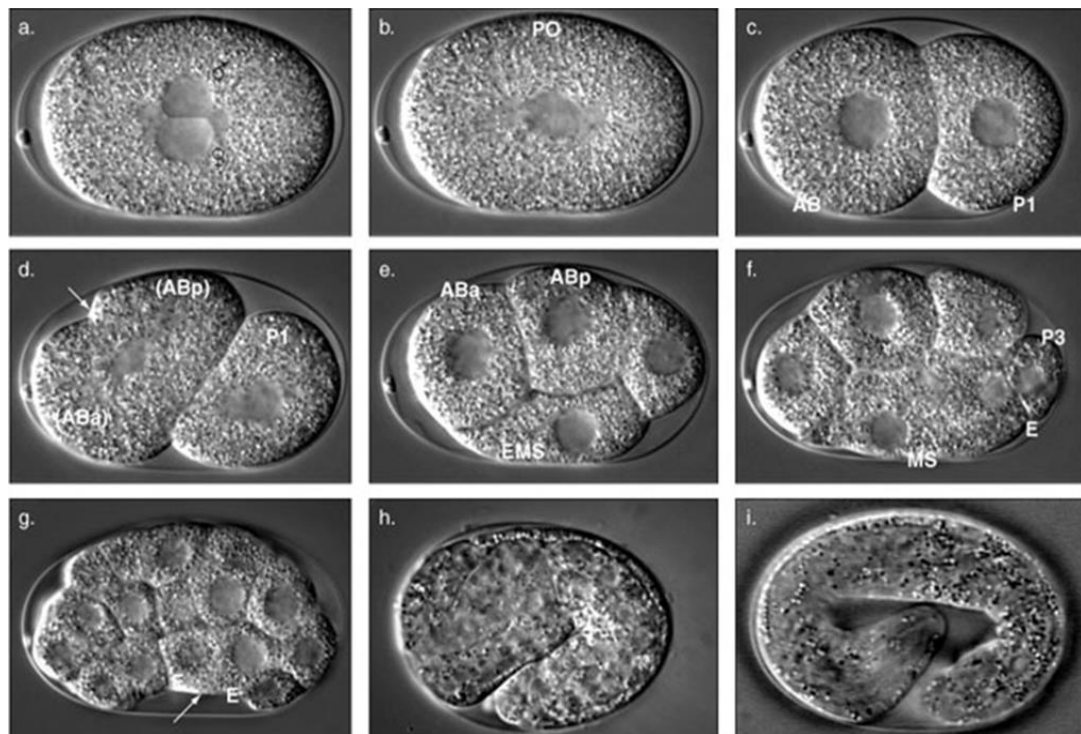


Figure 1.6: Embryonic Stages of Development in *C. elegans*. (Elena Rugerli, Development, 2002)

Using *C.elegans* for morphology analysis of living embryos has several advantages because their simple and transparent cell growth.

1.4.1 Eggshell of *C. elegans*

Eggshell of *C. elegans* is protective structure which consists of outer layer that provides physical support, and semipermeable layers that maintain the osmotic pressure. The outside layer called the vitelline layer (VL) is protein-rich. The middle layer called chitin layer (CL), contains chitin. The inner layer called lipid-rich layer (LrL), contains lipids and proteolipids. Inside the eggshell, a space which separates the LrL and the embryo

plasma is called extraembryonic matrix (EEM). The extraembryonic matrix (EEM) consists of outer clear zone and fibrous embryonic layer (Benenati et al., 2009).

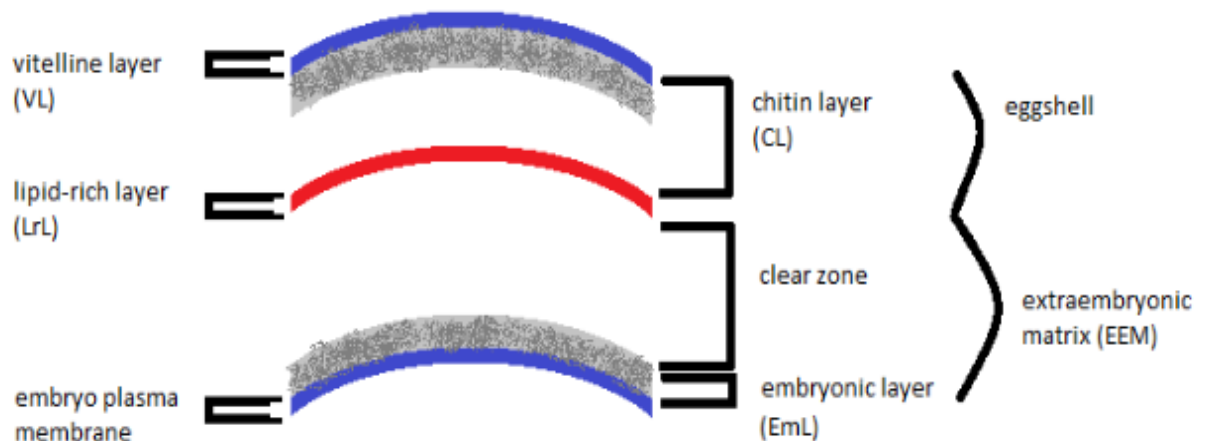


Figure 1.7: *C. elegans* eggshell and EEM (adapted from Benenati et al., 2009)

1.4.2 Embryonic Elongation of *C. elegans*

The *C. elegans* embryo is formed of an outer epidermal layer. Digestive tube goes from head to rectum in the tail inside the epidermal layer. Muscles are located between the epidermis and the digestive tract. Morphology of the embryo is carried out by actin microfilaments in the epidermis and sarcomeres in body wall muscles. Together, they form attached structure called fibrous organelles (FOs) between muscles and cuticle in the epidermis (Kim et al., 2011). Epidermal cells are connected via adherence junctions located apically. *C. elegans*' adherens junctions consist of E-cadherin, β -catenin and α -catenin. Microfilaments are attached to adherens junctions. They are very short at the beginning and organized in parallel bundles during elongation. They are essential for asymmetrical cell cycles in the zygote and they form thick bundles to reduce elongation.

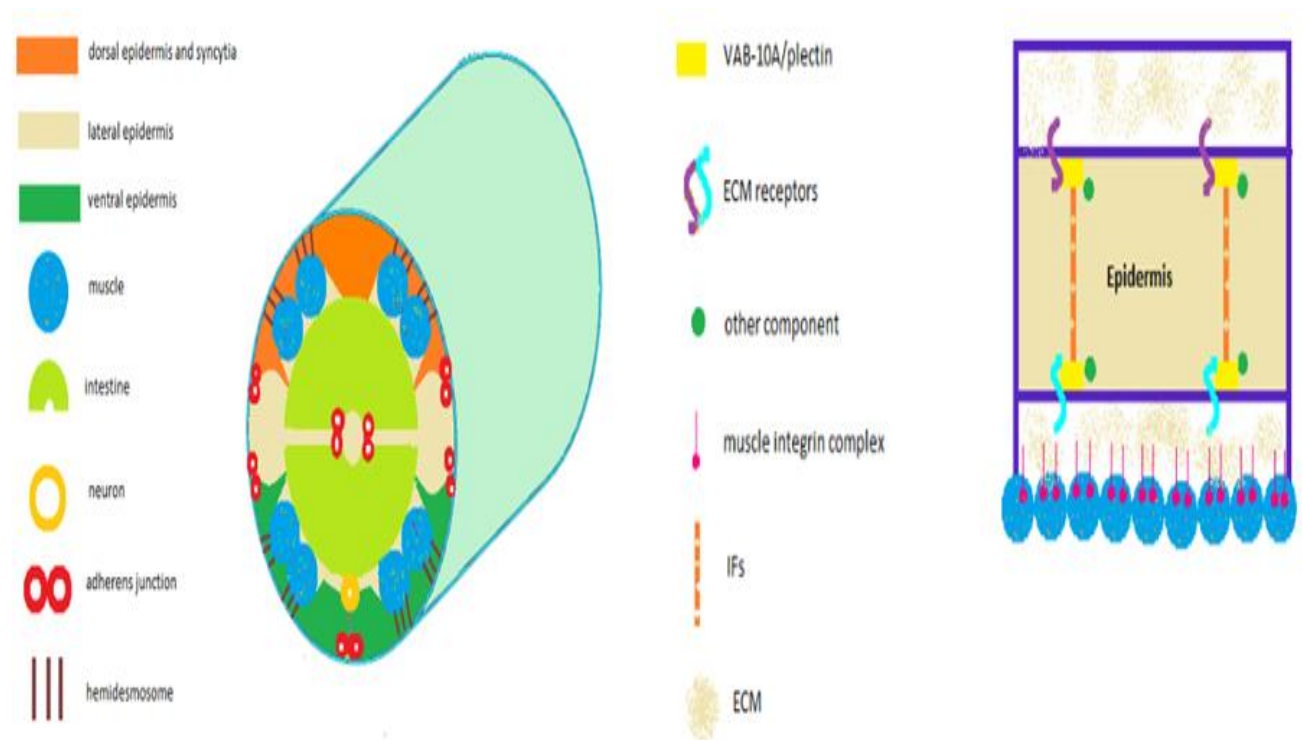


Figure 1.8: Cross section through the body showing the internal parts. (Adapted from M. Labousse, Phil. Trans. R. Soc. A, 2009)

FOs are attached to cuticle and muscle cells. Muscle cells are held on the extracellular matrix and separated from the basal plasma membrane of the dorsal and ventral epidermis. There is an important FO gene called *vab-10* which encodes spectraplakin cytoskeleton crosslinking protein. It encodes a large protein consisting of VAB10A and VAB10B isoforms. VAB10A is plectin and required for IF attachment. VAB10B is plakin and found between muscles and epidermis and it is required for thickening of the epidermis. M. Labousse and his group identified a mutation in *vab-10* gene. While the loss of VAB10A and VAB10B proteins cause embryonic defects in *C.*

elegans. Loss of VAB10A results in epidermal detachment from the cuticle, loss of VAB10B increases epidermal thickness during embryonic development (Bosher JM, Hahn B-S, Legouis R, et al., 2003).

1.5 miRNA biogenesis and their regulation in *Caenorhabditis elegans*

First miRNA, *lin-4* was identified in *C. elegans* (Wightman, Ha, & Ruvkun, 1993). Wightman and his coworkers showed that *lin-4* regulates *lin-41* by binding to its 3'UTR. *lin-4* controls crucial hypodermal cell-fate decisions from L1 stage to L2 stage transition in *C. elegans*. In loss-of function mutants of *lin-4*, cell divisions in L1 stage are reiterated.

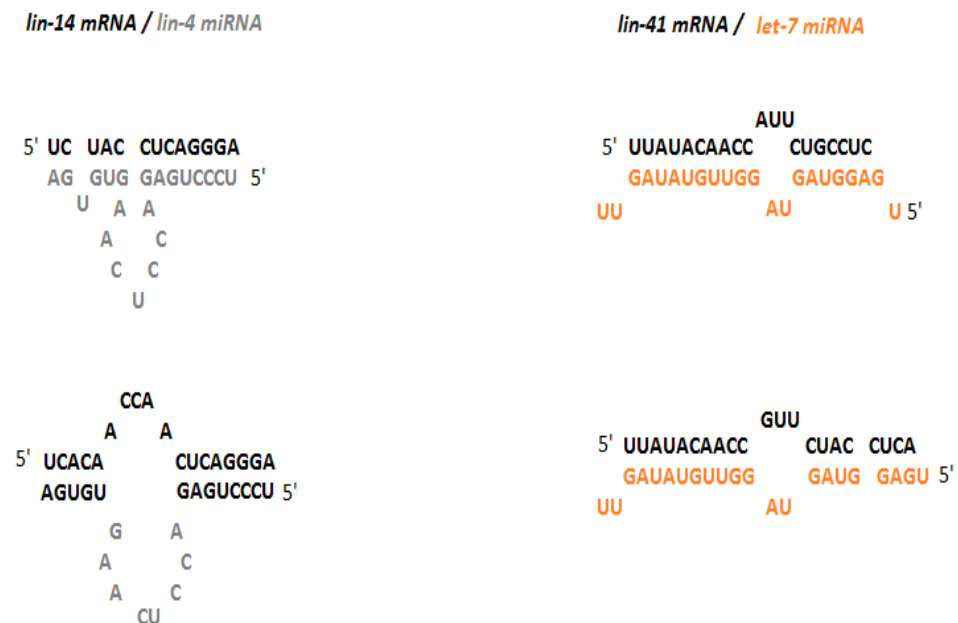


Figure 1.9: Target mRNA recognition of *lin-4* and *let-7*. (Adapted from Wightman et al., 1993)

As a second miRNA, Reinhart et al. identified *let-7* in 2000 again in *C. elegans*. *let-7* post- transcriptionally regulates the transition from the L4 to the adult stage. Unlike *lin-4*, *let-7* is one of the most conserved miRNA, found in a variety of species from nematodes to humans. In *Drosophila*, *let-7* is important in cell-cycle timing. In zebrafish (*Danio rerio*), *let-7* is expressed in neuronal development. In human, *let-7* regulates differentiation in stem cells and tumor growth. Deletion of *let-7* decreases the number of differentiated cells and results in tumor formation (Zhao et al., 2011).

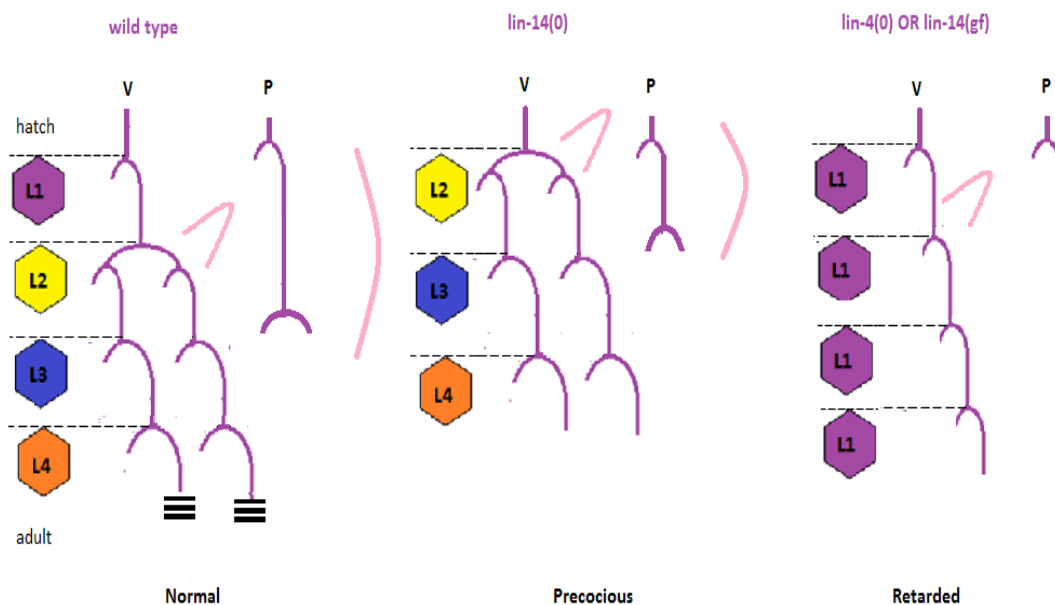


Figure 1.10: Hypodermal cell lineage with *lin-4* and *lin-14* mutants. (Moss E. 2007. Current Biology)

1.5.1 Micro RNA Biogenesis

miRNAs are 21-22 nt long small non-coding RNAs, with a 5' phosphate and 3' hydroxyl groups. They are linked to nearly every biological pathway that has been investigated so far. Gene regulation by miRNAs consists of several steps from production of miRNAs to silencing of the target mRNA.

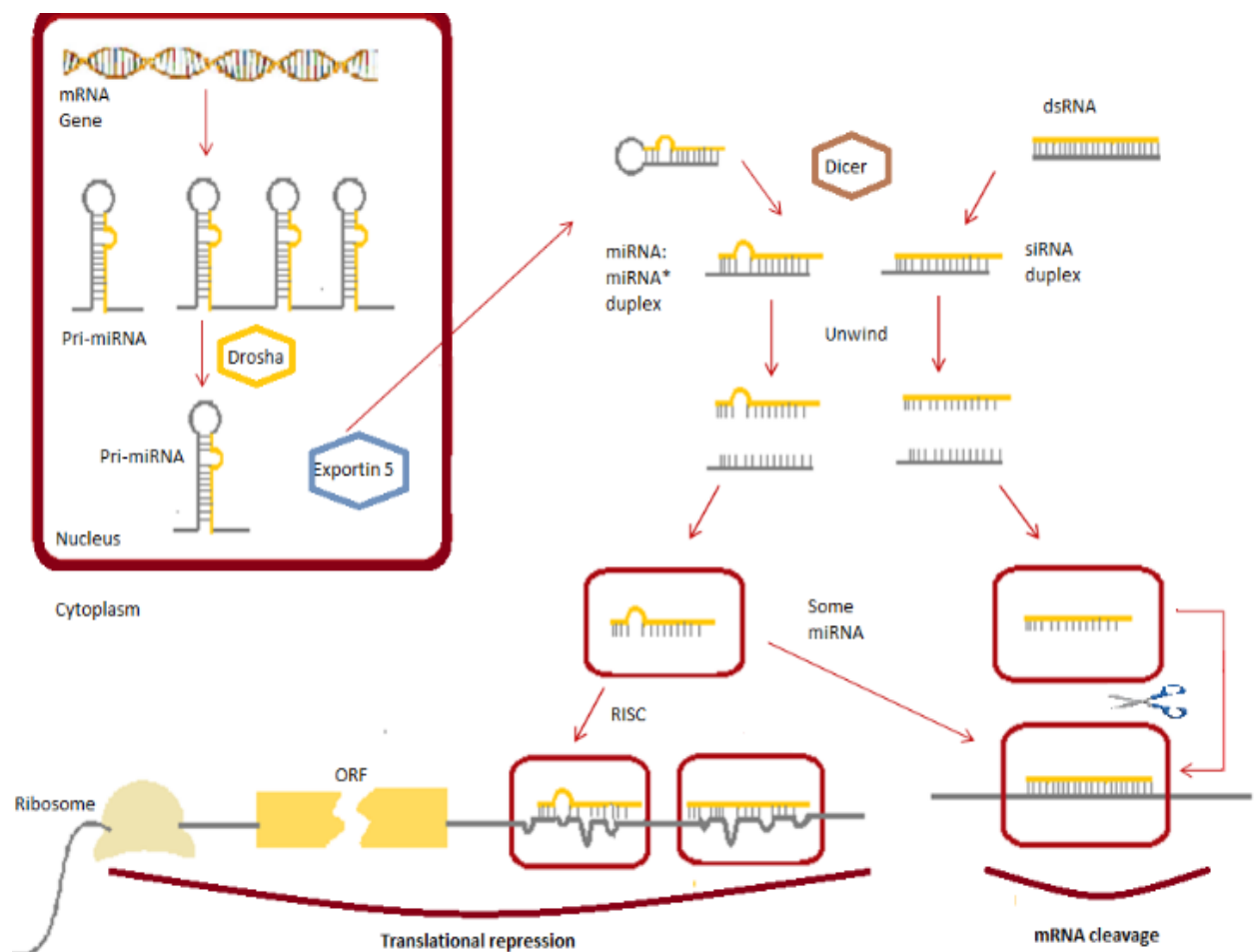


Figure 1.11: Biogenesis of miRNAs (Adapted from He and Hannon, 2004).

The biogenesis takes place as shown in **Figure 1.11**; primary miRNAs (pri-miRNAs) are transcribed by RNA Polymerase II, they are 3' poly-adenylated and 5' capped (Cai, Hagedorn, & Cullen, 2004). Pri-miRNAs form stem-loop secondary structure and they are cleaved by microprocessor complex; Drosha, an RNase III enzyme and (Lee, Jeon, Lee, Kim, & Kim, 2002) PASH-1 (DGCR8 in human), a RNA binding protein (Gregory et al., 2004). PASH-1 binds to a single-stranded portion of the pri-miRNA located at the base of the double-stranded stem. With the help of PASH-1, DROSHA cleaves the pri-miRNA ~11 nt into the stem, releasing a ~70 nucleotide long precursor miRNA (pre-miRNA) with a hairpin structure. Dysregulation of Pasha protein function in *C. elegans* or *Drosophila* inhibits pri-miRNA processing and causes accumulation of pri-miRNAs and inhibits production of miRNAs (Carmell & Hannon, 2004).

Pre-miRNAs are exported from the nucleus by Exportin-5 (Bohnsack, Czapinski, & Gorlich, 2004). In the cytoplasm, pre-miRNAs are cleaved by Dicer, RNase III endonuclease that forms a complex with RDE-4 (RNAi-Defective 4). The result of this cleavage is a ~ 22 nucleotide long double stranded mature miRNA with 2 nucleotide overhangs. Dicer helps mature miRNA duplex to bind into an Argonaute (AGO) protein, usually AGO-1 in plants and AGO-2 in animals, forming the RNA-induced Silencing Complex (RISC). There are four AGO protein while only AGO-2 has “slicer” activity in which it can act as a site specific RNA endonuclease. One of the two miRNA strands is exactly loaded into RISC as the antisense (guide) strand. The other strand which is the sense strand is unwound by an unidentified RNA helicase.

In animals, 6-7 nt complementarity between a miRNA and target mRNA is sufficient for the silencing activity of RISC which causes degradation of the transcript or inhibition of translation (Sontheimer & Carthew, 2005). Conserved region of complementarity between miRNA and mRNA termed as the “seed region”. This seed

region starts in 5' region from the second nucleotide of the mature miRNA. Size of seed sequence varies as 7mer-m8 (7 nt matches with one of the two 7 nt matches), 7mer-1A (7 nt matches with one match at position 1) and 8mer (8 nt matches). Site potency goes up as following: 8mer, 7mer-m8, 7mer-A1, 6mer, no site (Bartel, 2009). According to previous studies mRNA downregulation by miRNAs can be due to translational repression and degradation. miRNAs can induce deadenylation of target mRNAs, causing translational repression and reduction in mRNA levels (Wu, L., J. Fan, and J.G. Belasco, 2006). The process depends on imperfect complementary binding between target mRNA and miRNA-RISC complex and removal of the poly-A tails of mRNAs and finally degradation of target mRNAs.

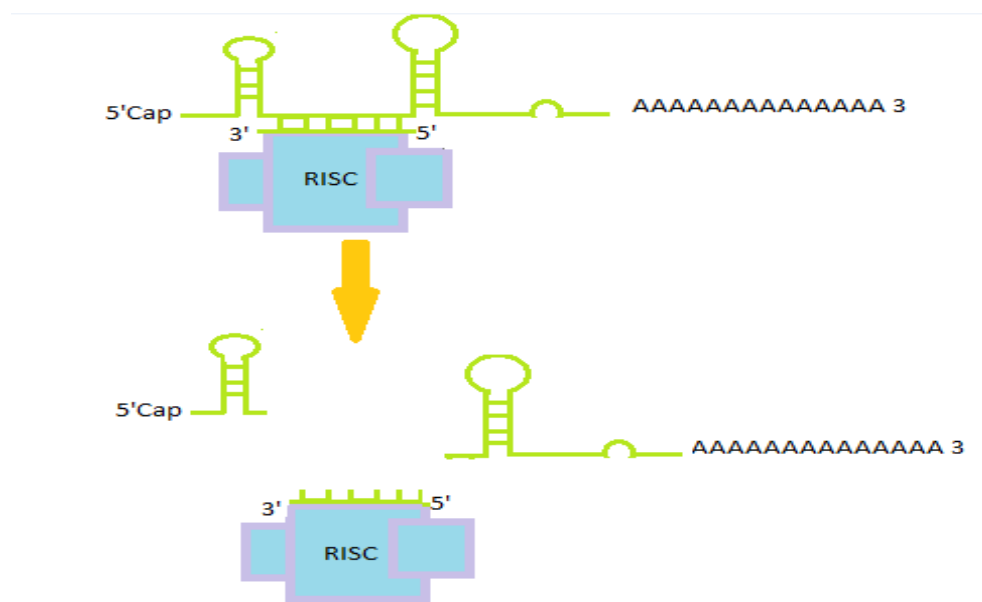


Figure 1.12: Target mRNA cleavage by miRNAs. (Adapted from www.ambion.com)

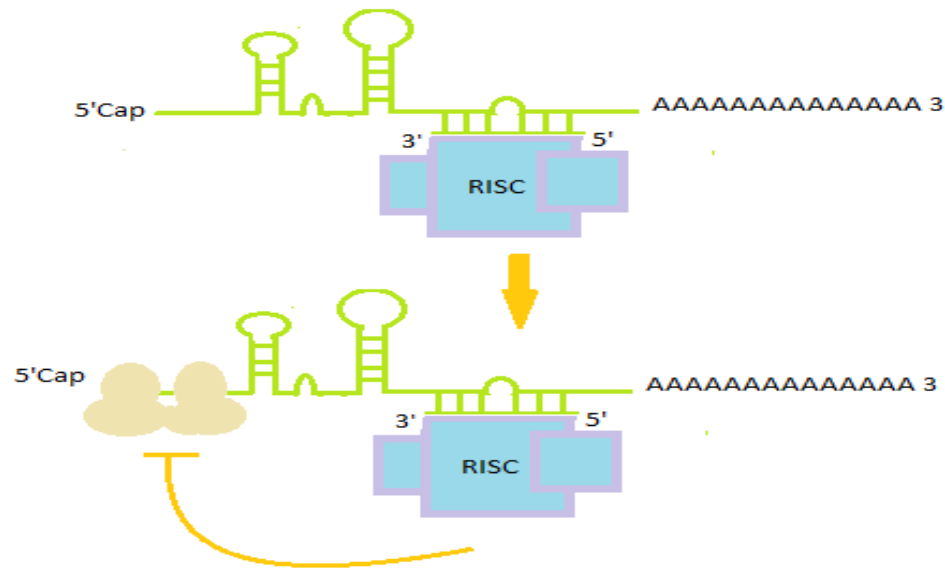


Figure 1.13: Repression of target mRNA by miRNAs. (Adapted from www.ambion.com)

Figure 1.14 below lists the different types of seed sequences.

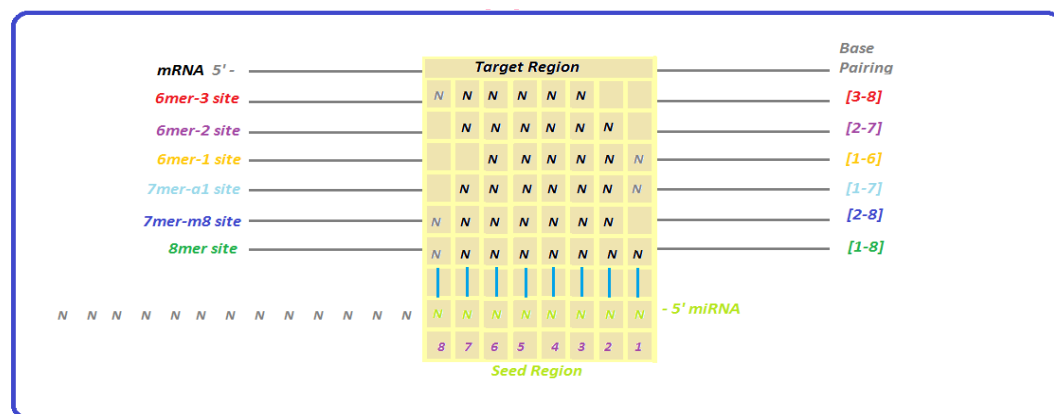


Figure 1.14: Different types of seed sequences. (Adapted from mirvestigator.systemsbiology.net)

Many miRNAs and their targets are conserved across different species. This conservation among species together with their targets might mean an evolutionary conserved way of gene regulation.

1.5.2 miRNAs and embryonic development of *C. elegans*

Studies with loss-of function mutants of miRNA biogenesis genes have provided the first line of evidence that showed miRNAs are essential for embryonic development in various organisms. In mice, knockout of both Dicer and Drosha results embryonic lethality at approximately 7th day of embryonic development (Bernstein et al., 2003). In *C. elegans*, similar observation has been made using *alg-1*, *alg-2* double mutant animals. ALG-1 and ALG-2 are two Argonaute family proteins that are required miRNA gene function in *C. elegans* (Jannot, Boisvert, Banville, & Simard, 2008). Although single *alg-1* and *alg-2* mutant animals do not display an obvious abnormal phenotype, *alg-1/2* double mutation causes embryonic lethality as they arrest at the 2- fold stage of embryonic development. Embryonic lethality is not detected in single mutation of Drosha and Dicer like in the *alg-1/2* double mutation. This observation has been explained by the fact that maternal contribution of *alg-1/2* is not sufficient to complete whole embryogenesis while maternal contribution of Drosha and Dicer is enough to complete their development before they become sterile adults (Vasquez-Rifo et al., 2012).

Loss of function of *mir-35* (*ndf50*) family (*mir-35* to *mir-41* cluster) causes embryonic lethality in the worms by affecting cell cycle in intestinal cells (Alvarez-Saavedra, E., and H. R. Horvitz, 2010). The *mir-51* family is also required for embryonic development. Loss of function of *mir-51* family (*mir-51* to *mir-56* cluster) causes penetrant pharynx unattached (Pun) phenotype which the pharyngeal muscle dissociates from the mouth (Shaw, W. Robert et al., 2010). Embryos lacking *mir-35* and *mir-51* family

members arrest at 2-fold to 3-fold stage, similar to the *alg-1/2* double mutants. Detailed information about *mir-35* and *mir-51* family will be given in following part of the chapter.

Various miRNAs are involved in development as well as in embryogenesis of *C. elegans*. **Table 1.1** shows the miRNAs up- or down- regulated in embryogenesis. As shown in **Table 1.1**, *mir-35* and *mir-51* family miRNAs and *mir-48* are up-regulated and miRNAs *mir-239a*, *mir-241* and *mir-392* are down-regulated.

Table 1.1: Several miRNAs up- or down-regulated in embryogenesis of *C. elegans*. (Karp, X. et al, 2011)

Fold change of 107 miRNAs between embryo and L1					
miRNA	E/L1	+/-	miRNA	E/L1	+/-
<i>let-7</i>	UD	UD	<i>miR-48</i>	3,59	10,9
<i>lin-4</i>	UD	UD	<i>miR-49</i>	1,58	5,2
<i>lsy-6</i>	UD	UD	<i>miR-50</i>	0,61	4,53
<i>miR-1</i>	1,09	4,28	<i>miR-51</i>	2,29	11,9
<i>miR-124</i>	0,46	0,23	<i>miR-52</i>	1,44	2,53
<i>miR-2</i>	0,78	6,17	<i>miR-53</i>	1,45	3,61
<i>miR-228</i>	0,58	2,07	<i>miR-54</i>	0,93	7,6
<i>miR-229</i>	1,2	4,2	<i>miR-55</i>	1,05	6,42
<i>miR-230</i>	0,11	0,3	<i>miR-56</i>	1,22	4,16
<i>miR-231</i>	1,27	4,8	<i>miR-57</i>	0,78	2,77
<i>miR-232</i>	0,9	4	<i>miR-58</i>	1,06	7,16
<i>miR-233</i>	0,91	12,6	<i>miR-59</i>	UD	UD

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<i>miR-234</i>	0,28	0,84	<i>miR-60</i>	0,46	1,43
<i>miR-235</i>	0,34	1,71	<i>miR-61</i>	1,49	2,06
<i>miR-236</i>	1,82	2,16	<i>miR-62</i>	1,08	4,06
<i>miR-237</i>	UD	UD	<i>miR-63</i>	0,25	0,31
<i>miR-238</i>	0,11	0,28	<i>miR-64</i>	2	4,68
<i>miR-239a</i>	0,01	0	<i>miR-65</i>	2,37	5,8
<i>miR-239b</i>	UD	UD	<i>miR-66</i>	1,57	4,61
<i>miR-240</i>	UD	UD	<i>miR-67</i>	2,63	1,91
<i>miR-241</i>	0	0,33	<i>miR-70</i>	0,46	0,48
<i>miR-242</i>	UD	UD	<i>miR-71</i>	0,06	0,15
<i>miR-243</i>	0,32	0,54	<i>miR-72</i>	0,99	14,3
<i>miR-244</i>	1,21	2,34	<i>miR-73</i>	1,64	5,98
<i>miR-245</i>	0,45	0,62	<i>miR-74</i>	1,98	2,48
<i>miR-246</i>	UD	0	<i>miR-75</i>	UD	UD
<i>miR-247</i>	UD	UD	<i>miR-76</i>	0,53	1,96
<i>miR-248</i>	0,23	0,23	<i>miR-77</i>	UD	UD
<i>miR-249</i>	UD	UD	<i>miR-78</i>	UD	UD
<i>miR-250</i>	2,09	3,25	<i>miR-784</i>	UD	UD
<i>miR-252</i>	1,11	4,11	<i>miR-788</i>	0,82	2,03
<i>miR-253</i>	1,59	1,68	<i>miR-789</i>	0,1	0,07
<i>miR-254</i>	0,41	2,08	<i>miR-79</i>	0,83	1,46

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<i>miR-259</i>	0,64	3,32	<i>miR-791</i>	UD	UD
<i>miR-34</i>	0,04	0,08	<i>miR-792</i>	UD	UD
<i>miR-35</i>	22,6	28,9	<i>miR-793</i>	0,16	0,15
<i>miR-358</i>	0,55	0,24	<i>miR-796</i>	UD	UD
<i>miR-36</i>	41,8	50,6	<i>miR-797</i>	0,84	1,04
<i>miR-37</i>	49,2	60,4	<i>miR-798</i>	2,78	3,11
<i>miR-38</i>	46,1	29,9	<i>miR-799</i>	UD	UD
<i>miR-39</i>	25,6	31	<i>miR-80</i>	0,76	56,7
<i>miR-392</i>	0	0,06	<i>miR-800</i>	UD	UD
<i>miR-40</i>	20,5	30,2	<i>miR-81</i>	1,4	25,2
<i>miR-41</i>	14,3	23,3	<i>miR-82</i>	1,41	18,8
<i>miR-42</i>	11,5	39,1	<i>miR-83</i>	0,32	0,67
<i>miR-43</i>	3	9,69	<i>miR-84</i>	0,11	0,31
<i>miR-44</i>	1,13	10,3	<i>miR-85</i>	0,82	0,49
<i>miR-45</i>	1,31	7,45	<i>miR-86</i>	0,75	5,29
<i>miR-46</i>	1,56	1,66	<i>miR-87</i>	0,93	3,89
<i>miR-47</i>	0,05	0,02	<i>miR-90</i>	1	6,69
E= Embryo, L1 = L1.					
UD = Undetected					

1.6 Temperature Sensitive miRNA related mutations in *C. elegans* which lead to embryonic lethality

1.6.1 *pash-1* temperature sensitive mutation in *C. elegans*

PASH-1 is 719 amino acids long, 84 kDa protein that is essential for miRNA biogenesis. There is a temperature sensitive allele of *pash-1(mj100)* in *C. elegans* which is efficient for studying the roles of miRNAs with conditional inactivation of miRNA production, because miRNA levels are gradually decreasing with temperature rise and almost zero at restrictive temperature, 25°C in *pash-1(mj100)* while miRNA biogenesis goes on normally at permissive temperature, 15°C (Lehrbach et al., 2012).

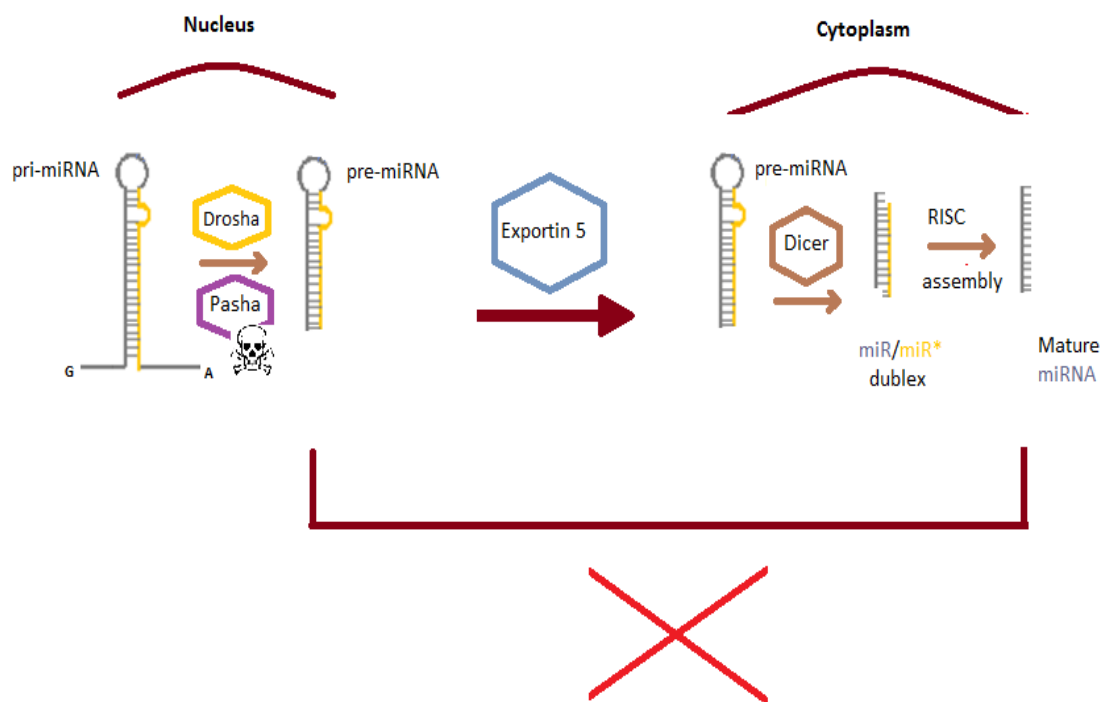


Figure 1.15: *pash-1* mutation in miRNA biogenesis.

pash-1(mj100) allele contains a mutation that causes one amino acid change from cysteine to tyrosine at the amino acid 515 (C515Y). C515Y mutation is in the loop region between the two RNA binding domains. How this mutation affects the function of PASH-1 protein has not been characterized yet.

<i>C. elegans</i>	Q	Q	G	F	E	E	L	F	K	K	V	K	I	D	S	P	N	L	V	Q	M	C	T	Q	C	A	I	P	K	P
<i>C. briggsae</i>	E	E	E	A	A	E	L	F	K	Q	V	K	I	D	S	S	S	V	V	S	M	C	T	R	F	A	I	P	K	P
<i>D. melanogaster</i>	A	Q	S	D	L	S	V	F	D	D	I	R	I	E	D	P	R	V	T	E	F	C	N	K	T	T	E	P	S	P
<i>D. rerio</i>	E	G	D	E	L	E	Y	F	N	H	I	S	I	E	D	S	R	V	Y	E	L	T	N	K	A	G	L	L	S	P
<i>H. sapiens</i>	D	S	E	E	L	E	Y	F	N	H	I	S	I	E	D	S	R	V	Y	E	L	T	S	K	A	G	L	L	S	P
<i>B. taurus</i>	D	S	E	E	L	E	Y	F	N	H	I	S	I	E	D	S	R	V	Y	E	L	T	S	K	A	G	L	L	S	P
<i>M. musculus</i>	D	S	E	E	L	E	Y	F	N	H	I	S	I	E	D	S	R	V	Y	E	L	T	S	K	A	G	L	L	S	P

C515Y

Figure 1.16: Substitution mutation in the conserved residue of PASH-1 in *pash-1(mj100)*. (Adapted from Lehrbach et al., 2012)

While at 15°C embryos laid by *pash-1 (mj100)* mutant animals develop normally, at 25°C they develop till late stages of embryogenesis and die shortly after without hatching. They also show Pun (pharynx unattached) phenotype. They have also short lifespan at 25°C which is approximately eight days after they reach adulthood and they become paralyzed after fourth day of their adult stage. *pash-1(mj100)* males cannot mate at

restrictive temperature. This proved that post-developmental expression of miRNAs is required for lifespan, reproduction and locomotion.

pash-1(mj100) phenotypes can be rescued by a transgene expressing the wild-type protein. This confirmed that these phenotypes are due to the loss of PASH-1.

Table 1.2: Phenotypes of *pash-1(mj100)* *ts* mutation and its rescue at different temperatures. (Lehrbach et al., 2012)

	<i>pash-1</i>				<i>pash-1 rescue</i>
	15°C	20°C	22.5°C	25°C	25°C
Embryonic lethality (%)	2	7	30	100	0
Pharynx unattached (%)	0	0	5	55	4
Larval lethality (%)	0	0	50	100	0
Burst (%)	0	24	67	67	3
Gapped alae (%)	6	43	100	100	2

1.6.2 *mir-35* family and its function in *C. elegans*

mir-35 family contains 7 miRNA genes, *mir-35* to *mir-42*. *mir-35-41* is found in a cluster on chromosome II (**Figure 1.18**), whereas *mir-42* is not present in the chromosome II. The *mir-35* family is expressed in all larval stages, but highest expression has been observed in embryos. Loss of this family results in embryonic lethality in the worms. Mutant embryos develop slower than wild-type embryos till 2-fold stage and they die shortly after (Alvarez-Saavedra, E., and H. R. Horvitz, 2010).

mir-35-41 cluster deleted in nDf50	mir-35	UCACCGGGUGGAAACUAGCAGU
	mir-36	UCACCGGGUGAAAAUUCGCAUG
	mir-37	UCACCGGGUGAACACUUGCAGU
	mir-38	UCACCGGGAGAAAAACUGGAGU
	mir-39	UCACCGGGUGUAAAUCAGCUUG
	mir-40	UCACCGGGUGUACAUCAGCUAA
	mir-41	UCACCGGGUGAAAAAUCACCUA
	mir-42	UCACCGGGUUAACAUCUACAGA

Figure 1.17: *mir-35* family members (McJunkin & Ambros, 2014).

Using temperature sensitive alleles of *mir-35* family, researchers examined the effects of *mir-35* family members on post-embryonic development. McJunkin and his colleagues used a temperature sensitive allele of *mir-35* family, which lacks *mir-35-41* cluster, but have intact *mir-42*. *mir-35-41* mutants have diminished intestine and germline growth. The brood size of the mutant animals is also reduced. They detected that *mir-35* is needed for G1/S transition of intestinal cells and germ cell differentiation.

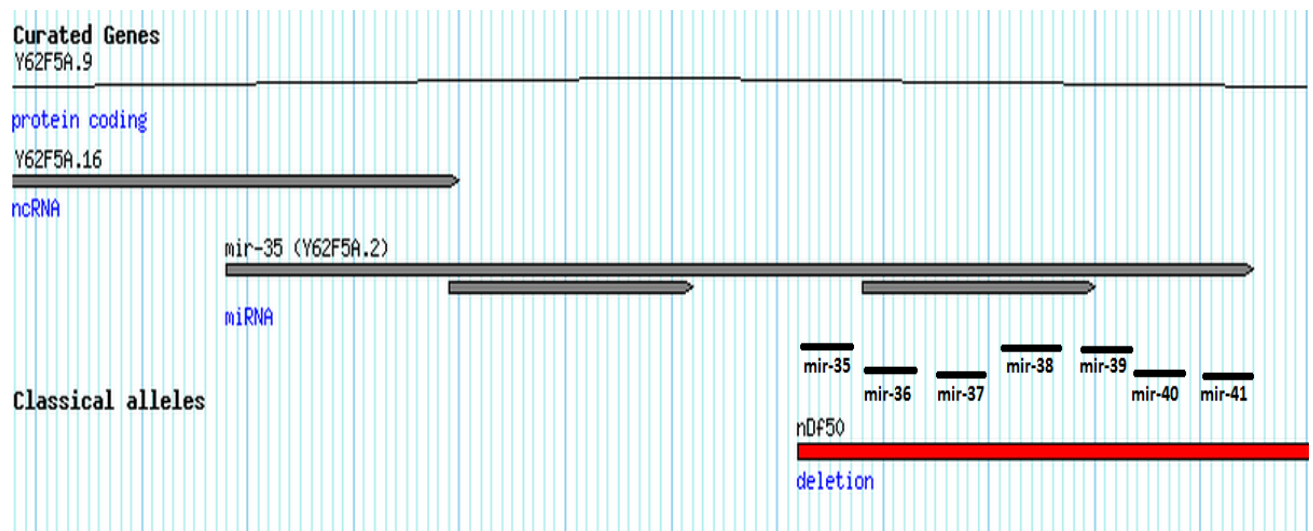


Figure 1.18: *mir-35* family deletion placed on the genome. (From www.wormbase.org)

Lack of *mir-35* causes abnormalities in intestinal nuclei number and distal mitotic gonad (Liu et al., 2011). In another study, *Suppressor-26* (*sup-26*) was identified as a *mir-35* family target gene. Deletion of *sup-26* rescues the “fecundity” of *mir-35-41* hermaphrodites, however, it does not rescue the embryonic lethality (McJunkin & Ambros, 2014).

1.7 Trehalose Metabolism in *Caenorhabditis elegans*

Trehalose is a non-reducing disaccharide of glucose. It has three stereoisomers: α , α -trehalose, α , β -trehalose and β , β -trehalose. So far, only α , α -trehalose [α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside] has been shown in biological organisms such as bacteria, yeasts, fungi, insects, invertebrates and plants. Presence of other forms has not been analyzed in detail.

α , α -1,1-glycosidic linkage

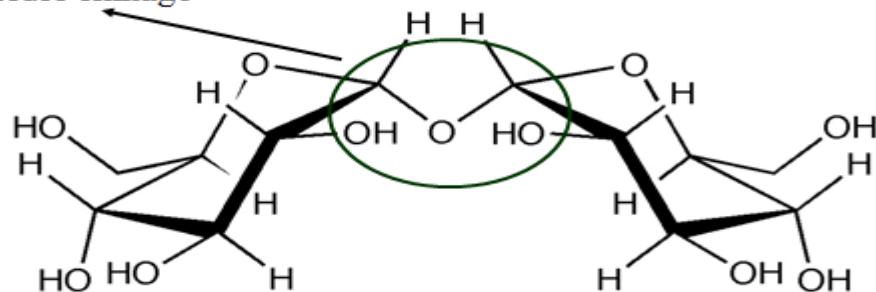


Figure 1.19: Structure of trehalose. (Behm, 1997)

1.7.1 Trehalose biosynthesis

In bacteria, yeasts, fungi, insects and nematodes, trehalose is synthesized by a pathway consisting of two steps: in the first step, from UDP-glucose and glucose-6-phosphate trehalose phosphate synthase generates trehalose-6-phosphate. In the second step, trehalose-6-phosphate is dephosphorylated by trehalose-6-phosphate phosphatase to produce trehalose (Kormish & McGhee, 2005).

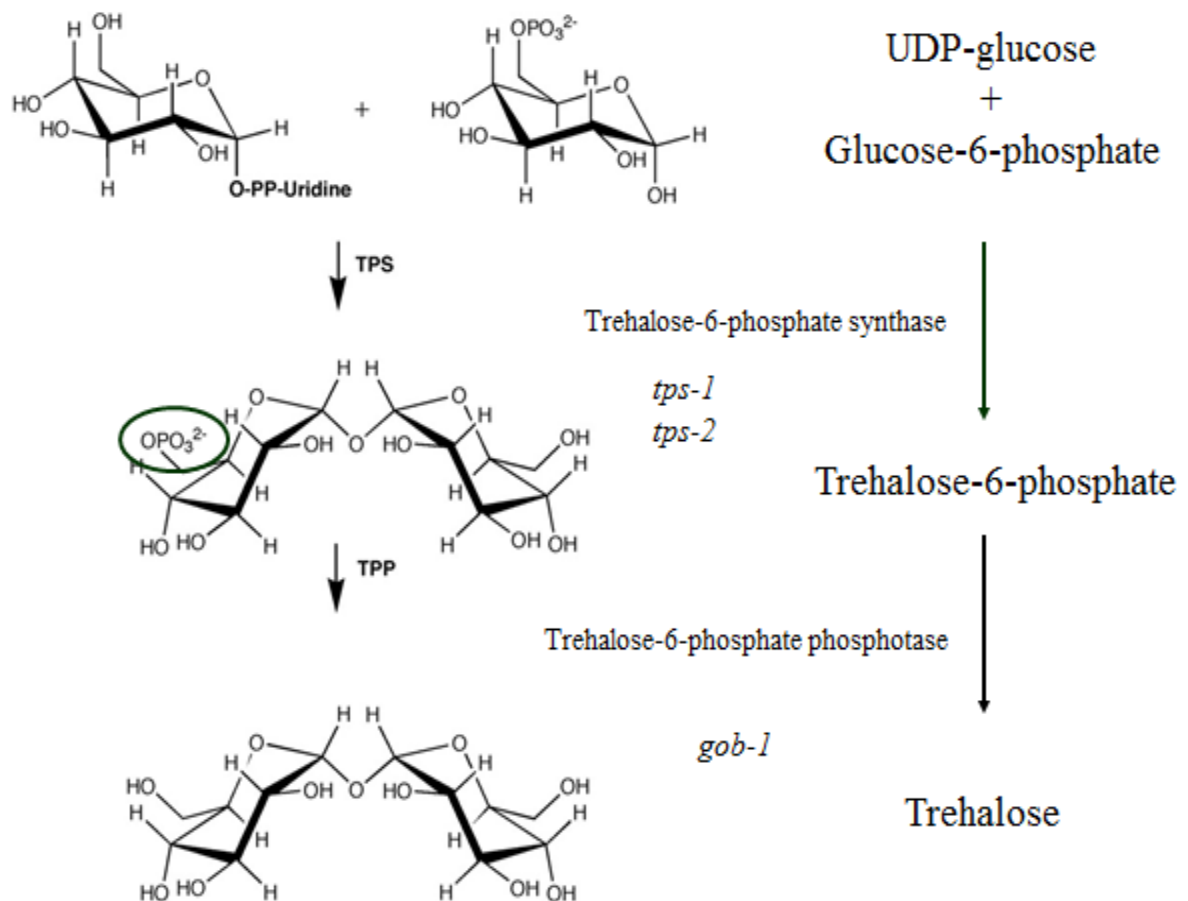


Figure 1.20: Formation of trehalose in nematodes. (Adapted from Behm, 1997)

LITERATURE REVIEW

In *E. coli*, the two enzymatic activities involved in trehalose biosynthesis are encoded by OtsA (TPS) and OtsB genes (TPP). In addition to a TPS (TPS1) and a TPP (TPS2) protein, it also contains a regulatory subunit encoded by TSL1 named as TPS3 (Goddijn and Dun, 1999). Some bacterium species, *Pimelobacter Sp* and *Thermus Sp* have an alternative pathway for trehalose synthesis where trehalose is produced from maltose by trehalose phosphorylase and maltose phosphorylase enzymes (Eleutherio E et al., 2014). Vertebrates do not have the enzymes to synthesize trehalose, but they can break trehalose to glucose by trehalase enzyme. In humans, trehalase is found in small intestine and kidney. It is thought (Alvarez-Peral, Zaragoza, Pedreno, & Arguelles, 2002) that trehalose has a role in sugar transport across the membrane and breakdown of ingested trehalose is provided by the trehalase enzyme. Trehalose has not been detected in the blood so far. Why vertebrates have trehalase in the absence of trehalose synthesizing genes remains unanswered.

Table 1.3: Genes important for trehalose biosynthesis in *C. elegans* (Adapted from Pellerone et al., 2003).

Enzyme	Gene name	Chromosome	Mass of predicted protein
TPS	<i>tps-1</i>	X	150.8
	<i>tps-2</i>	II	139.9
TRE	<i>tre-1</i>	I	66.9
	<i>tre-2</i>	IV	66.5
	<i>tre-3</i>	V	67.8
	<i>tre-4</i>	X	73.5
	<i>tre-5</i>	II	77.9

Two genes encoding TPS and five genes encoding for TRE have been identified in *C. elegans*. RNAi loss of function experiments of *tps* genes in *C. elegans* did not cause obvious abnormal phenotype in the worms and experimentally trehalose amount could not be completely eliminated (Pellerone et al., 2003). This could be explained by redundant pathways for trehalose synthesis in *C. elegans*.

1.7.2 Trehalose functions

Trehalose can act as an energy source, chemical chaperone, autophagy inducer or an osmolyte. Therefore, these properties open up a wide range of industrial and biological applications to this special sugar.

Following table summarizes some crucial applications of trehalose:

Table 1.5: Applications of trehalose. Adapted from (Gabriel Iturriaga, 2009)

Trehalose Applications	Description	Reference
Protecting enzyme activity	storing enzymes such as DNA polymerase, restriction enzymes and DNA ligase	(Colaco, Sen, Thangavelu, Pinder, & Roser, 1992)
Stabilizing multiple molecules	maintaining the activity of molecules like antibodies after long time in storage	(Colaco, Kampinga, & Roser, 1995)
Preserving foods	preserving aromas and other properties of foods such as fruits	(Behling, Perez, Kidd, Staton, & Hunter, 1993)
Preserving cells, tissues and organs:	preserving tissues and organs for several months	(Guo, Puhlev, Brown, Mansbridge, & Levine, 2000)

Improving of plants shelve-life	Trehalose improves shelve-life of tulips after cutting	(Otsubo, M.; Iwaya-Inoue, 2000)
Usage in Cosmetics industry	reducing bad odors emitting from human skin by up to 70%, candidate for deodorants	(Higashiyama, T. Novel. Pure Appl. Chem. 2002)
Possible medical uses	possible therapeutic usage in Huntington's chorea and in osteoporosis	(Higashiyama, T. Novel. Pure Appl. Chem. 2002)
Selection marker in the production of transgenic plants	using AtTPS1 as a selective agent	(Leyman et al., 2006)

1.7.2.1 Trehalose as an osmolyte and chemical chaperone

Trehalose can act as a stress protectant against heat, cold, desiccation, anoxia and oxidation. It stabilizes proteins, lipid membranes in anhydrobiotic organisms with the help of its particular structure. The reducing ends of trehalose form glycosidic bond. The glycosidic bond that joins the two hexose rings has low energy (1 kcal/mol), causing stable organization compared to sucrose with a high-energy bond (27 kcal/mol) (Crowe, Crowe, & Chapman, 1984). Therefore, it is more resistant to acidic hydrolysis and high temperatures. It has high optical rotation and melting point as 203°C. Trehalose can replace water molecule by establishing hydrogen bonds with macromolecules during dehydration and freezing by forming a glass state (Fernandez, Bethencourt, Quero, Sangwan, & Clement, 2010). For instance; when yeast trehalose 6-phosphate synthase gene (TPS1) was introduced to tobacco, it improved drought tolerance. The leaves of these transgenic plants

were larger and showed better growth under drought stress. (Romero, Belles, Vaya, Serrano, & Culianez-Macia, 1997)

In addition to that, the absence of one of the *Tps* genes in plants, AtTPS1 leads to embryonic lethal phenotype in plants. They suggested that TPS genes can have a role in plant development (Avonce, Mendoza-Vargas, Morett, & Iturriaga, 2006). Embryonic lethal phenotype of *tps-1* could be explained by the fact that *tps-1* embryos have thicker cell walls than wild-type embryos because regulation of trehalose synthesis is needed for cell wall formation in the embryos.

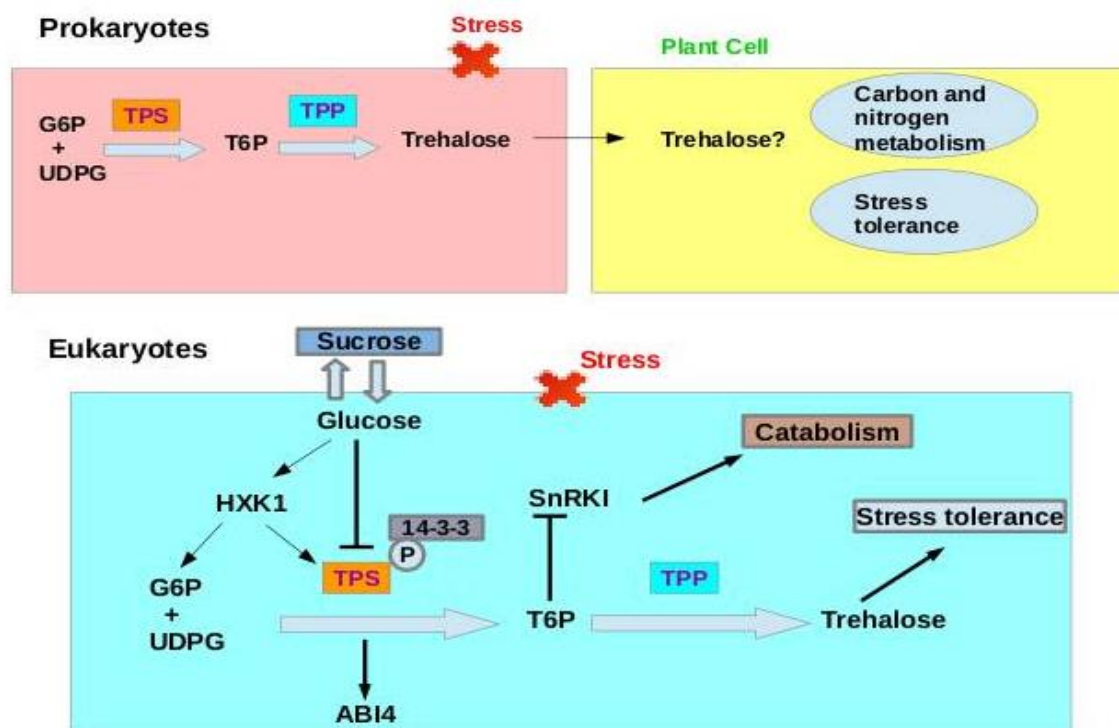


Figure 1.21: Trehalose pathways in prokaryotes and eukaryotes. (Adapted from Iturriaga, Suarez, & Nova-Franco, 2009)

Trehalose is used to generate vaccines and trehalose can be potentially used to treat diseases like Huntington because it has been shown that it decreases Huntington aggregates in mice after oral uptake of trehalose (Iordachescu & Imai, 2008).

1.7.2.2 Trehalose as an autophagy inducer

Trehalose has been shown that it activates autophagy in mTOR-independent manner by reducing intracellular pathological prion protein (PrP^{sc}) levels. PrP^{sc} particles are aggregated and misfolded isoform of the normal prion protein PrP^c in prion diseases. They demonstrated that its effect is reduced by known autophagy inhibitor 3-methyladenine and enhanced with rapamycin, a known autophagy inducer which is mTOR-dependent. It decreases the cytochrome c in cells without causing enhanced cell death and triggers autophagosome formation. They suggested that trehalose binds to the enlarged polyglutamines and stabilizes the unfolded proteins (Aguib et al., 2009). Castillo and his coworkers have demonstrated that trehalose recovers motor neurons function in mouse models of ALS and prolongs their lifespan (Castillo et al., 2013). Trehalose has been also demonstrated in *Drosophila* and mammals to depress the anoxic damages (Q. Chen & Haddad, 2004).

1.7.2.3 Trehalose as an carbon source

Trehalose is a simple disaccharide of glucose and also used as energy source in many organisms such as insects, plants and nematodes. Trehalose maintains energy balance in some organisms in similar way like sucrose, other non-reducing disaccharides.

In plants, trehalose synthesis is important for chitin formation. Although the most important energy source in plants is sucrose, trehalose is also used as energy source in plants. For instance; in maize, trehalose pathway genes are regulated in the respect of day-night cycle. At night, trehalose is accumulated more than sucrose and other hexose sugars (Henry et al., 2014). Trehalose acts as rapid energy resource of glucose in insects found 80-90% in their hemolymph (Wyatt & Kale, 1957). In the starvation conditions, glucose and trehalose levels rapidly decreases in the hemolymph of *Manduca sexta* (Thompson & Lee, 1994). In fungi, trehalose also used as energy source in germination of spores (Sussman & Lingappa, 1959). In budding yeast and in plants, the intermediate in trehalose synthesis, trehalose-6-phosphate, has also an important role in controlling glycolysis. It has been also demonstrated that trehalose-6-phosphatase inhibits hexokinase in yeasts.

1.7.3 Trehalose Metabolism in *Caenorhabditis elegans*

There are few studies that investigated the roles of trehalose in *C. elegans*. Trehalose is present in all stages of the life cycle up to 2.5 % of its dry weight of *C. elegans*. In embryos and dauer larvae, trehalose has the highest concentration (Arguelles, Rodriguez, & Alvarez-Peral, 1999). Trehalose is thought to contribute the stress resistance of dauer larvae. This has been supported by the findings of Erkut et al. which showed that *C. elegans* dauer larva can survive after losing 98% of its body water. This ability is established by several fold increase in the trehalose concentration.

In *C. elegans*, trehalose is synthesized by trehalose-6 phosphate phosphatase (T6PP) and trehalose-6-phosphate synthase (*gob-1* and *tps-1/2*, respectively in *C. elegans*). Trehalose-6-phosphate synthase (*tps-1* and *tps-2* in *C. elegans*) produces trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate. Afterwards, trehalose-6-phosphate phosphate is dephosphorylated to generate trehalose by trehalose-6-phosphate phosphatase

(*gob-1*). Knockdown of *gob-1* (gut- obstructed 1) causes larval lethality as the intestine fails developing completely. In mutant animals, ingested bacteria is clogged in the abnormally develop intestine (Kormish & McGhee, 2005). Loss of function mutants in *C. elegans* *tps-1* and *tps-2* genes, encode trehalose phosphate synthase suppress the lethality of *gob-1* mutant (Erkut et al., 2011).

Worms can generate glycogen from glucose. Glycogen makes up approximately 3.3% of the dry mass of adult worms that is stored in the gut of worms. In mammals, glucose-6-phosphatase converts glycogen into glucose. However, *C. elegans* does not have this enzyme, and therefore, it is thought that *C. elegans* synthesizes glucose from glycogen using trehalose as an intermediate from glucose-6-phosphate (Hulme & Whitesides, 2011).

Trehalose has been reported that it increases lifespan of *C. elegans* 30% through *daf-2* suppression. It inhibits polyglutamine (polyQ) and poly alanine (polyA) mediated protein aggregation by enhancing thermotolerance. (Honda, Tanaka, & Honda, 2010)

There is little report about the exact mechanism of trehalose in the embryonic development of *C. elegans*. On the other hand, trehalose seems to be important in embryonic development and in the hatching process of the embryos of other nematode species. For instance; *Ascaris* ' eggs contain high concentration of trehalose (0, 3- 0, 5 M). Trehalose is present in the perivitelline space of the egg in high concentration which might help to protect egg-shell and perivitelline space under harsh environmental conditions (Carolyn A. Behm, 1997). Because trehalase is present in the all tissues of *C. elegans* except non-luminal parts of intestinal cells, trehalose can be used by all tissues and circulates through the whole body of *C. elegans* (Behm, 1997b) .

Trehalose has been also shown to be important for embryonic development of other species. In *Arabidopsis*, trehalose-6-phosphate has been shown to regulate metabolism and

embryonic development. They found three *tps-1* alleles which are embryonic lethal with decreased root growth and flowering. (Eastmond et al., 2002) It has been reported that when *mir-156* that is shown to be important in flowering and *tps-1* loss of functions are transgenically coupled together, their reducing effect in flowering process is also doubled (Wahl et al., 2013). In *Drosophila*, it has been also shown that trehalose level increases during the later stages of embryonic development, although its level is not significant in the early stage embryos (Matsuda, Yamada, Yoshida, & Nishimura, 2015).

1.8 Other osmoprotectants (osmolytes)

Proteins are between folding and unfolding equilibrium in aqueous solution and the equilibrium can be revised by osmolytes. Osmolytes are small molecules that are synthesized to maintain protein stability and turgor pressure. They shift the protein folding equilibrium toward the folded side and support their refolding. They prevent cellular damage by keeping proteins in their native state acting as “chemical chaperones.” It has been shown (Hammer & Baltz, 2002) that mammalian embryos accumulate osmolytes in the early stages of development.

1.8.1 Proline

Proline is a water soluble amino acid which is also an effective osmolyte in many organisms. Proline protects membranes and proteins against high concentration of inorganic ions and high temperature differences (Larosa, Rhodes, Rhodes, Bressan, & Csonka, 1991). In bacteria, exogenous proline works as an osmolyte under highly saline culture conditions (Larosa, Rhodes, Rhodes, Bressan, & Csonka, 1991).

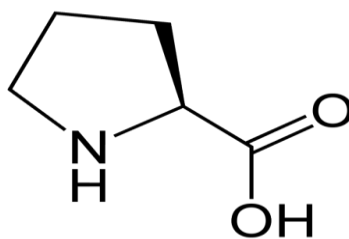


Figure 1.22: Structure of proline.

In plants, proline is synthesized by glutamic acid and Δ^1 -proline-5-carboxylate. Proline comprises the 5% of total level of amino acids. This level may increase to 80% during stress conditions. In addition, proline can also act as antioxidant. Cells treated with proline are protected against hydrogen peroxide and other ROS production (C. Chen & Dickman, 2005).

Proline breakdown takes place in mitochondria. Proline dehydrogenase (ProDH) and Δ^1 -proline-5-carboxylate dehydrogenase together convert proline into glutamate. Their misregulation is related some diseases. For instance; ProDH upregulation causes proline related ROS formation and leads to apoptosis in human colon cancer cell line. In addition, ProDH loss of function is related to hyperprolinemia which has been linked to schizophrenia (C. Chen & Dickman, 2005).

In bacteria, exogenously supplied proline works as an osmoprotectant under highly saline culture conditions (Csonka, 1989, Csonka and Hanson 1991). Accumulation of proline is facilitated when the concentrations of less compatible solutes are reduced and cytosolic water volume is increased (Cayley et al, 1992). When proline has been introduced exogenously to immature maize embryos, it stimulated somatic embryogenesis (Hayat et al., 2012).

Baltz and his coworkers have shown that mammalian embryos utilize osmolytes during early stages of development. The tolerance of preimplantation mouse embryos to increased osmolarity *in vitro* was increased in the presence of proline in the culture medium (Anas, Hammer, Lever, Stanton, & Baltz, 2007).

1.8.2 Methyl amines and flavonoids

Methylamines are small cellular compounds that have roles related to stress responses in plants. Methyl amines accumulate under several abiotic stress conditions including salt and drought. Trimethylamine N-oxide (TMAO) is an important methylamine in plants. TMAO serves as a crowding factor for proteins to help to form more stable native state. Dominic Lambert has demonstrated that TMAO reduces the overall dimensions of unfolded RNAs by altering the distribution of monovalent ions around RNA (Lambert et al., 2010) (Harmon & Patel, 2004).

Flavanoids are plant metabolites found in fruits and herbs. They are widely used for pharmaceutical aspects because of their antioxidant capacity. Naringin (4', 5, 7-trihydroxyflavanone 7-rhamnoglucoside) is one of the flavonoids which found in *Citrus* fruits such as pomelo (*C. grandis*) (Lin, Hou, Tsai, Wang, & Chao, 2014). There are several studies establishing the beneficial effects of naringin in microbial diseases and cancer (Harmon & Patel, 2004; Heo et al., 2004).

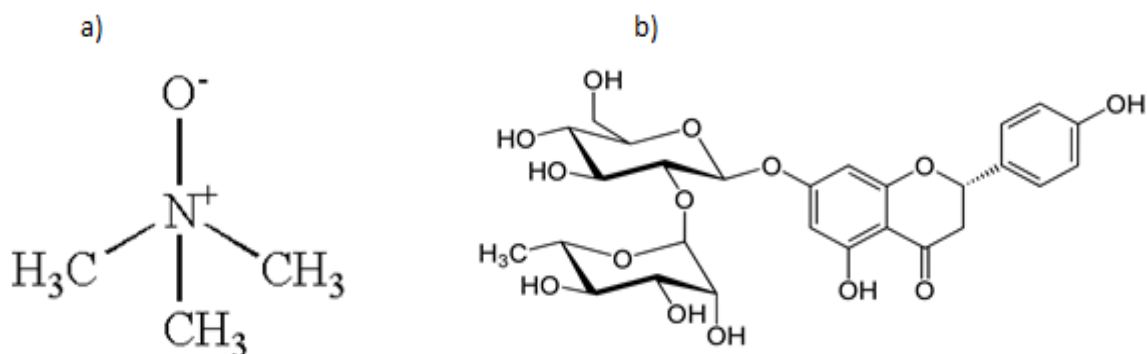


Figure 1.23: Structure of a) TMAO and b) Naringin.

1.9 Other autophagy inducers

Autophagy is a process of cell degradation. It induces formation of autophagosomes, membrane structures consists of cytosol and organelles that fuses with lysosome (Yorimitsu & Klionsky, 2005). There are several individual miRNAs that regulate mTOR pathway. *mir-193* is one of the important miRNAs that inhibits mTOR signaling by suppressing ERBB4 and S6K2 (Yu et al., 2015).

1.9.1 Rapamycin

Target of rapamycin (TOR) is a highly conserved serine/threonine kinase that is an important member of cell proliferation and autophagy pathways. It has two complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC 2). Rapamycin induces autophagy by inhibiting TORC1.

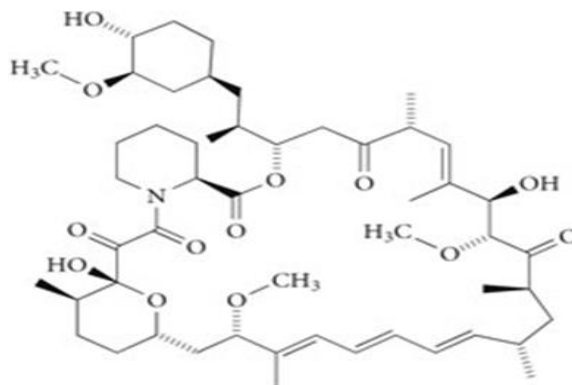


Figure 1.24: Structure of Rapamycin.

Sarkar and his colleagues (Sovan Sarkar, Huntington) showed that inhibition of mTOR by rapamycin results in cell cycle arrest and defects in wound healing when used as a treatment. Use of alternative autophagy inducers such as trehalose can help eliminate these side-effects. Sarkar and his colleagues also have demonstrated that rapamycin and trehalose together decrease aggregation at highest amount compared their single treatments.

Treatment with rapamycin causes some changes in miRNA expression and these changes correspond to rapamycin resistance. It has been demonstrated that mTOR induction upregulates Mdm2 which is an E3 ligase of DROSHA and this causes inhibition of miRNA biogenesis by ubiquitinylation of DROSHA (Ye et al., 2015) .

Rapamycin has been also found to extend lifespan in *C. elegans* and mice by inducing autophagy (Kapahi & Zid, 2004).

1.9.2 Spermidine

Spermidine is polyamine that is isolated from semen. It has a triamine structure. Spermidine synthase (SpdS) catalyzes monoalkylation of putrescine (1, 4-diaminobutane)

and decarboxylated S-adenosylmethionine 3-aminopropyl. This reaction produces spermidine. Spermidine is found mostly in ribosomes.

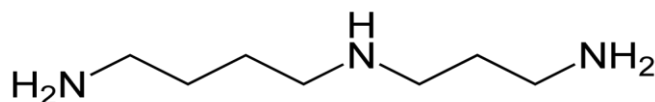


Figure 1.25: Structure of Spermidine.

Spermidine induces histone acetylation at the ATG7 gene's promoter region in yeast. This results in upregulation of ATG7 gene expression that mediates autophagy. It has been also shown that spermidine prolongs lifespan in yeasts, *C. elegans* and mice, similar to rapamycin (Eisenberg et al., 2009).

Seminal plasma of infertile men contains deficient level of spermidine compared to the control group which supported the findings that showed polyamines are crucial for mammalian reproduction in spermatogenesis, embryogenesis and implantation (Calandra, Rulli, Frungieri, Suescun, & Gonzalez-Calvar, 1996).

1.9.3 3-methyladenine (3- MA)

3-MA is inhibitor of phosphatidylinositol-3- kinase and it suppresses cellular autophagy. It inhibits PI3-kinase activity and autophagosome formation (Petiot, Ogier-Denis, Blommaert, Meijer, & Codogno, 2000). 3-MA is a potential chemotherapy drug because of it inhibitory effect on autophagy. It has been shown that treatment with 3-MA increases the apoptosis in colon cancer cells by enhancing the autophagy and inhibiting tumor growth (Li et al., 2009).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Sigma or Carbosynth.

Chemicals used in the study:

Table 2.1: Chemicals used in the study.

Chemical	Concentration	Manufacturer
α , α Trehalose	5mM	Carbosynth (Berkshire, UK)
α , β Trehalose	5mM	Carbosynth (Berkshire, UK)
Proline	5mM	Sigma (St. Louis, MO, USA)
Serine	5mM	Sigma (St. Louis, MO, USA)
Spermidine	5mM	Sigma (St. Louis, MO, USA)
3-methyladenine	10 μ M	Sigma (St. Louis, MO, USA)
Naringin	5mM	Sigma (St. Louis, MO, USA)
Rutin	5mM	Carbosynth (Berkshire, UK)
Rapamycin	0.2 μ M	Sigma (St. Louis, MO, USA)

2.1.2 *C. elegans* strain list

A list of *C. elegans* strain used in this study is list in the table below:

Table 2.2: Strains used in the study.

STRAINS	GENOTYPE	RESOURCES
N2	<i>wild type</i>	Purchased from CGC
SX1137	<i>pash-1 ts</i>	Gift from Miska Lab
SX1359	<i>pash-1 rescue</i>	Purchased from CGC
CB1402	<i>unc-15(e1402)I.</i>	Purchased from CGC
DG1701	<i>cgh-1(tn691) III.</i>	Purchased from CGC
MT14119	<i>nDf50 II</i>	Purchased from CGC
MT17143	<i>nDf67 mir-52(n4114) IV/nT1[qIs51] (IV;V); nDf58 X.</i>	Purchased from CGC
AR1	<i>hcp-6(mr17) I</i>	Purchased from CGC
ML691	<i>vab-10(ok817)/unc-75(e950) unc-101(m1) I.</i>	Purchased from CGC
ML653	<i>vab-10(mc44)/unc-75(e950) unc-101(m1) I.</i>	Purchased from CGC
CB698	<i>vab-10(e698)</i>	Purchased from CGC
VC117	<i>vab-10(gk45)</i>	Purchased from CGC
NL1888	<i>pash-1(pk2083)/+</i>	Purchased from CGC
SX1483	<i>pash-1(mj100) I ; mjEx365</i>	Purchased from CGC
SX1749	<i>pash-1(mj100) I ; mjEx423</i>	Purchased from CGC
SX1501	<i>pash-1(mj100) I ; mjEx383</i>	Purchased from CGC

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DR721	<i>lin-4(e912)</i>	Purchased from CGC
MT5790	<i>lin-4(e912); nIs2</i>	Purchased from CGC
SP231	<i>let-7(mn112)</i>	Purchased from CGC
GR1432	<i>let-7 (mg279)</i>	Purchased from CGC
SX1858	<i>pash-1(mj129)</i>	Gift from Miska Lab
JM144	<i>mnDp1 /+ ; lin-15B&lin-15A(n765) gob-1(ca17)</i>	Purchased from CGC
RB760	<i>tps-2(ok526)</i>	Purchased from CGC
RB728	<i>tre-1(ok327)</i>	Purchased from CGC
RB806	<i>tre-5(ok612)</i>	Purchased from CGC
RB919	<i>snf-1(ok790)</i>	Purchased from CGC
RB1400	<i>tre-3(ok394)</i>	Purchased from CGC
IG130	<i>tol-1(nr2013)</i>	Purchased from CGC
MT12955	<i>mir-1(n4102)</i>	Purchased from CGC
TX189	<i>P(oma-1)::oma-1::gfp</i>	Purchased from CGC

2.2 Methods

2.2.1 Bacterial food source and NGM petri plates preparation

A glycerol stock of *E. coli* HB101 was streaked on to the LB agar plate using aseptic technique and incubated overnight at 37°C. On the next day, a single colony was inoculated aseptically in to 100 ml B-Broth medium. The inoculated culture was grown overnight at 37°C with 250 *g* shaking. The *E. coli* HB101 culture was then stored at 4°C till its use in seeding NGM plates. When seeding, 50 or 100 µl *E. coli* HB101 liquid culture was applied the 60mm or 90mm NGM plates using sterile technique, respectively. The bacteria lawn was allowed to grow overnight at room temperature before placing worms.

2.2.2 Maintenance of the strains

C. elegans are maintained on the NGM plates pre-seeded with *E. coli* HB101 bacteria as food source. Temperature sensitive mutant strains were grown at 15°C or at 25°C as required. Worms were transferred to new plates by sterile platinum wire and chunking agar parts with many worms to a new plate. Worms were sealed with parafilm to minimize drying and evaporation.

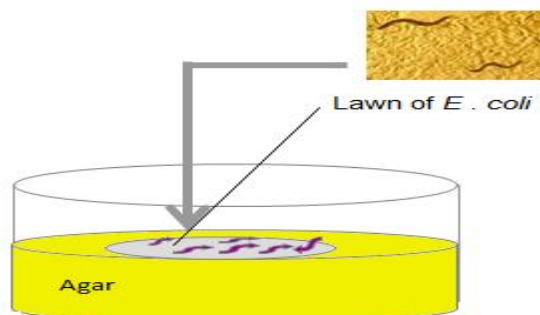


Figure 2.1: Illustration of *C. elegans* habitat in a Petri dish on the surface of agar seeded with *E. coli*.

MATERIALS AND METHODS

Strains carrying fluorescent markers were checked and kept using a Nikon Intenseilight C-HGFI Leica MS7.5 microscope with GFP and mCherry filters.

2.2.3 Preparation of the reagents and buffers

NGM + Agar (Nematode Media): 1L double distilled H₂O is measured in 1L bottle with a magnetic stirrer. 3g NaCl, 2.5g bacto peptone and 17g agar is added and is stirred until all solutes are dissolved, labeled and autoclaved.

M9 Buffer: 12g Na₂HPO₄, 6g KH₂PO₄ and 10g NaCl are dissolved in 1.5L double distilled water. Volume is adjusted to 2L using double distilled water. After autoclaving, 100ul 1M MgSO₄ is added per 100ml. 1M MgSO₄ at 1ul/1ml in the M9 buffer gives a final concentration of 1mM.

KH₂PO₄: 272 g KH₂PO₄ (potassium dihydrogen orthophosphate) is dissolved in 1L double distilled water in 2L measuring cylinder. pH is adjusted to 6.0 by gradually adding KOH (potassium hydroxide) pellets. Volume is adjusted to 2L and autoclaved.

B-Broth: 20 gram Bacto tryptone and 10 gram NaCl are dissolved in 1L double distilled H₂O in a 2L measuring cylinder. Final volume is adjusted to 2 L using double distilled H₂O and autoclaved.

Cholesterol (Nematode Media): 1 gram cholesterol was initially dissolved in 100% ethanol while stirring. Using graduated cylinder, the volume is completed to 200 ml with ethanol and it is filter sterilized in a laminar flow hood.

LB: 10 g tryptone, 5 g yeast extract and 10 g NaCl are dissolved in 900 ml double distilled H₂O. pH is adjusted to 7.4. Final volume is adjusted to 1 L using double distilled H₂O and autoclaved.

Superbroth: 5.4L of double distilled H₂O is dissolved with 72 g bacto-tryptone, 144 g yeast extract and 24 ml glycerol. The solution is poured into six 1L bottles as 900 ml to each bottle and they are autoclaved.

MgSO₄: 492.96 g MgSO₄ · 7H₂O (magnesium sulphate 7-hydrate) is dissolved in 1.5L double distilled H₂O. The volume is adjusted to 2 ml with double distilled H₂O and autoclaved.

2.2.4 Cleaning the strains

2.2.4.1 Double Bleach Protocol:

Strains were grown on 90 mm plates. Gravid adults were washed with 15 ml M9 buffer and placed into 15 ml sterile canonical tube. Tubes were centrifuged for 2 minutes at 2000 rpm at room temperature. Supernatant was removed and washing was repeated 2 more times with M9 buffer. After last wash, supernatant should be removed as much as possible without disturbing the pellet. 6 ml NaOCl (3.5 ml NaOCl+ 4 ml 10M NaOH and completed up to 50 ml with double distilled H₂O) was added. The worms in NaOCl solution were vortexed for 6 minutes at maximum speed. ~8 ml of sterile M9 was added under aseptic conditions. Embryos were spinned down and supernatant is discarded with a disposable pipette. Sterile M9 was added up to 15 ml for wash and centrifuged at 2K for minutes. The last step was repeated 3 times. Supernatant was discarded and only ~100 µL M9 was left. 90 mm plates are spotted with bleached embryos. After the plates were dried at room temperature (~10 minutes), they were parafilmed and placed at 15°C or 25°C.

2.2.4.2 Spot bleach Protocol

In a bacteria spotted 60mm NGM plate, one drop of bleach (3.5 ml NaClO, 4ml 10M NaOH, completed up to 50 ml with double distilled H₂O) was spotted onto the out of the bacterial spotted area. 15 gravid adults (adults with embryos) were put onto the bleach spot. To make sure if they are all dissolved in bleach, worms were observed couple of minutes. The plate was parafilmed. Since only embryos should be survived after bleach exposure, L1 worms will move to the bacterial spotted area. Next day, bleach spot was removed with the help of a sterile spatula.

2.2.5 Freezing the strains

Worms were starved in 90mm NGM plates. 2, 5 ml of freezing buffer was put into 15 ml falcon tube. Worms were collected with 3 ml of M9 buffer and placed into 15 ml falcon tube containing freezer buffer. 2, 5 ml of it was put into 15 ml falcon with freezer buffer and mixed. 1 ml of solution was individually placed into 5 cryo tubes and placed at -80°C in a freezing box. Next day, the quality of freezing was examined by checking the survival of the animals after thawing one of the aliquots.

2.2.6 Embryo hatching assay

All worms were grown on standard NGM plates seeded with *E. coli* HB101. Plates were sealed with parafilm to minimize evaporation and contamination.

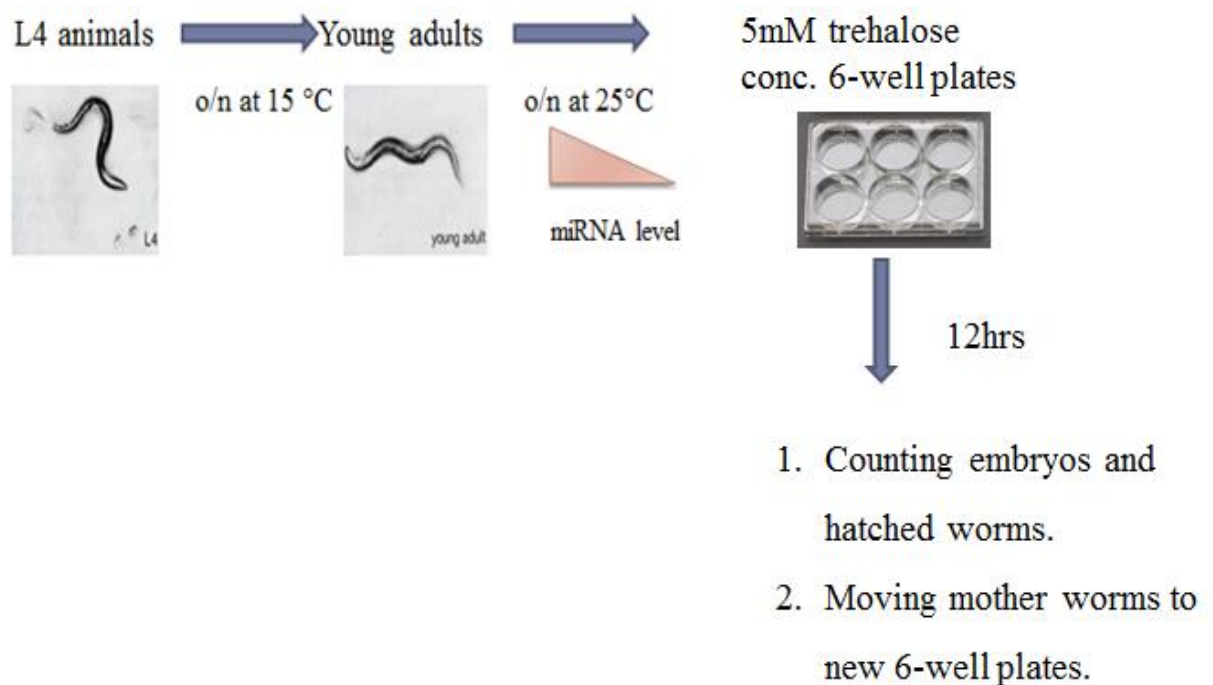


Figure 2.2: Illustration of embryo hatching assay.

MATERIALS AND METHODS

L4 stage hermaphrodite worms were picked by stereomicroscope and kept overnight at 15°C to allow them to complete their development and become young adults. When the worms were reached to young adult stage, they were shifted to 25°C incubation temperature and grown overnight. Next, the worms were individually transferred 6-well NGM plates containing chemicals seeded with UV killed *E. coli* HB101 and incubated at 25 °C. Every 12 hours, embryos and hatched worms were counted in each well and the mothers are transferred to new 6-well plates. This procedure continued for approximately 3 days every 12 hours until reproduction ceases.

2.2.7 Survival assay

All worms were grown on standard NGM plates seeded with *E. coli* HB101. Plates were sealed with parafilm to minimize evaporation and contamination. L4 stage hermaphrodite worms were picked as described above and grown overnight at 15°C. When the worms were reached to young adult stage, they were shifted to 25°C incubation temperature and grown overnight. Next, the worms were transferred to NGM plates containing 5mM trehalose seeded with UV killed *E. coli* HB101 and incubated at 25 °C (n= 30 for each genotype). Survival assay begins when worms were placed on trehalose containing plates until they die. Worms were scored as dead if they fail to respond to mechanical stimuli and scored as touch responsive if they begin to move after mechanical stimuli.

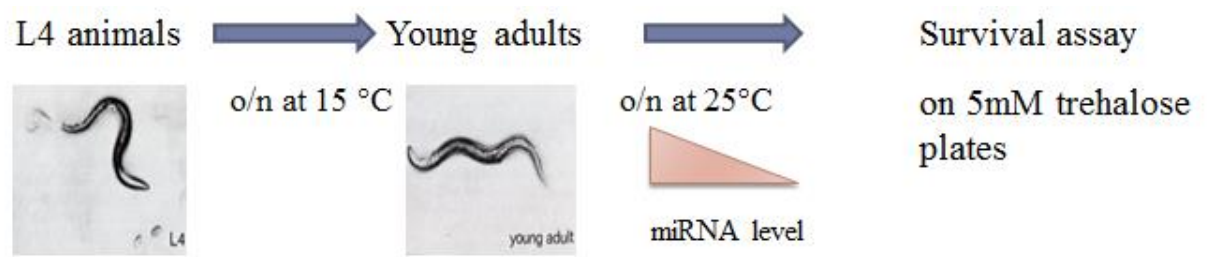


Figure 2.3: Illustration of survival assay.

2.2.8 Carminic acid staining

Carminic acid was added to a final concentration of 1 mg/ ml into sterile B-Broth culture which was then spotted onto NGM plates. For all assays synchronized L1 larvae were grown on the carminic acid containing NGM plates and transferred to the trehalose and control plates when they reach to young adult stage, later assayed as one-day old adults. Worms were placed on a 2% agarose pad containing 0.2 mM levamisole in M9. Worms were examined using a Nikon 80i fluorescence microscope; image acquisition was done using NIS-Elements Viewer 4.20. For all imaging analysis, exposure was identical (100 ms). For each condition, 15 worms were imaged and fluorescence intensity was quantified.

2.2.9 Microscopy

2.2.9.1 Imaging Setup

Reagents:

Agarose (2% w/v)

M9 Buffer with 0.2 mM Levamisole

MATERIALS AND METHODS

Vaseline

Poly-L-lysine

Materials:

Cover slips (18x18mm)

Microscope Slides

Platinum wire pick

Syringes

Agarose pad preparation for imaging embryos laid on the plates:

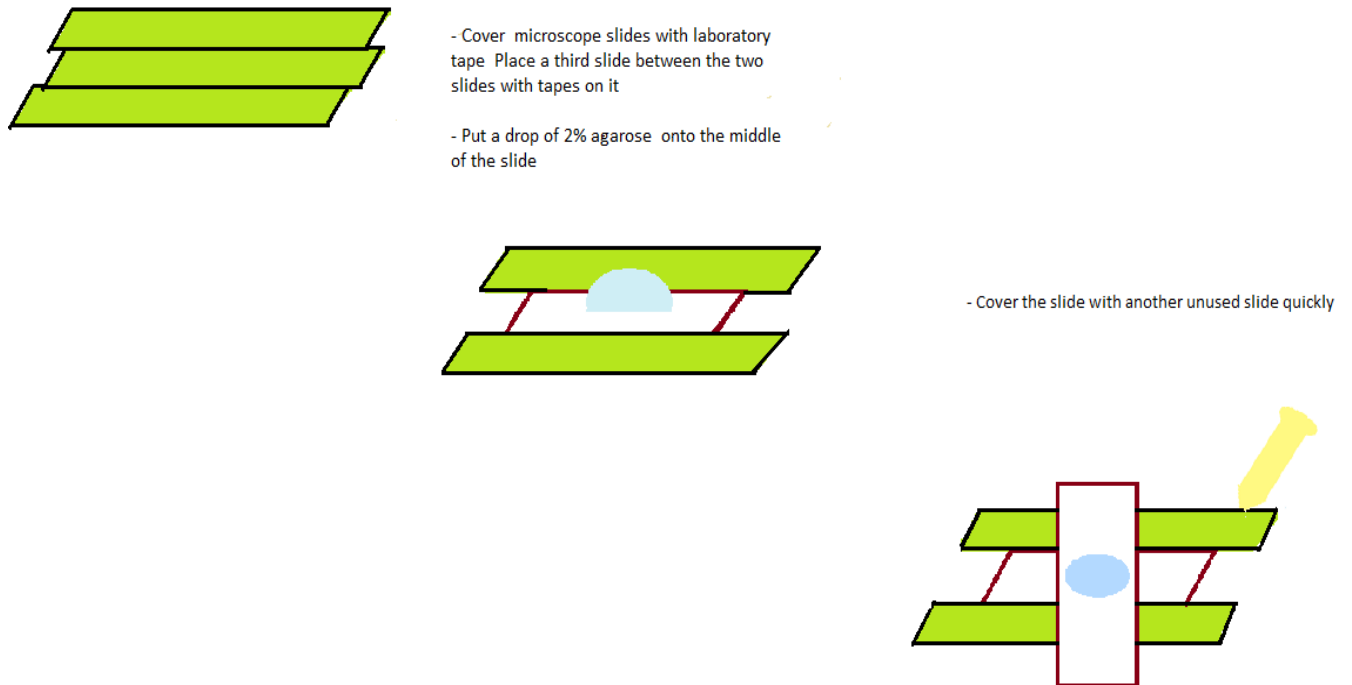


Figure 2.4: Illustration of slide preparation.

MATERIALS AND METHODS

Onto two microscope slides a layer of laboratory tape was placed. In between the two slides with tapes on it, a third slide was placed. A drop of 2% agarose was put onto the middle of the slide and quickly covered with another unused slide like a “sandwich”. This way, the thickness of agarose pad will be the same as the thickness of the laboratory tape. After agarose is set up, untapped slide was sliced apart.

Embryos were picked from NGM plates to the microscope slide with 2% agarose pad containing 3-5 μ l M9 Buffer. Cover slip was placed onto the slide carefully. Edges of the cover slip were sealed with melted Vaseline and imaged under DIC or fluorescence microscope.

For imaging unlaidd embryos inside the worms:

1 mg/ml Poly-L- lysine was poured onto microscope slides, and air-dried for 1 hour. 15 gravid adult hermaphrodites were picked from NGM plate to the poly-L-lysine coated microscope slide containing M9 Buffer with 1 M Levamisole. With the help of a syringe, the worms were cut in half. The embryos were released from the worms onto the slide. It is important to cut close to the vulva. Cover slip was laid upon the slide carefully. Edges of the cover slip were sealed with melted Vaseline and imaged under DIC or fluorescence microscope.

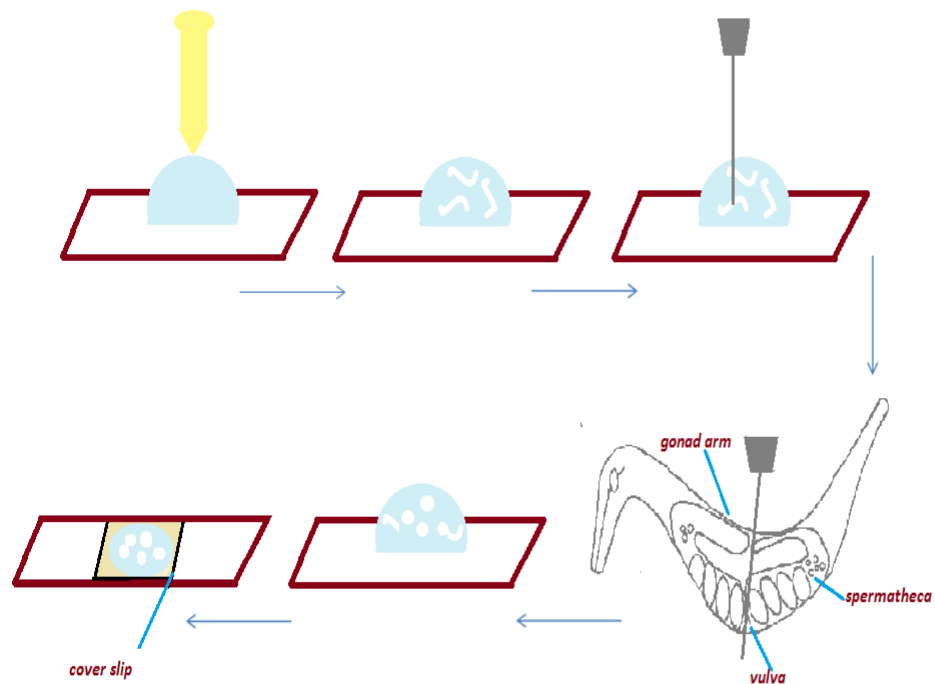


Figure 2.5: Dissection method for embryo analysis.

Time-lapse microscopy

Wild-type and mutant *mir-35(ndf50)* and *pash-1(mj100)* strains adult hermaphrodites were dissected and embryos were imaged every 10 minutes for 14 hours. The pictures were converted to a time-lapse movie with 16, 7 frames per seconds with NIS ELEMENTS 4.20.

2.2.10 Data Analysis

All test statistics were calculated using Origin Pro and Excel.

CHAPTER 3

RESULTS

3.1 *pash-1(mj100)* embryos and *mir-35(ndf50)* embryos are arrested at later stages of embryogenesis at restrictive temperature, 25°C.

pash-1(mj100) animals maintain sufficient levels of miRNAs at 15°C; however miRNA synthesis is impaired at 25°C. 100% *pash-1(mj100)* mutant embryos fail to hatch at 25°C. It is known that *mir-35* family of miRNAs is also required for embryogenesis. Embryonic lethal *mir-35* family contains 7 miRNA genes, *mir-35* to *mir-42*. There is a conditional allele of *mir-35* family which lacks 6 out of 7 members and *ndf50* allele also shows temperature dependent embryonic lethality phenotype. *mir-35(ndf50)* mutant embryos die after 2- fold stage (Alvarez-Saavedra, E., and H. R. Horvitz, 2010). When *pash-1 (mj100)* embryos die hasn't been examined in details.

Firstly we wanted to investigate when *pash-1 (mj100)* and *mir-35(ndf50)* embryos die at restrictive temperature, 25°C during embryogenesis. Embryos of 2 embryonic lethal mutants; *mir-35(ndf50)* family (ts) and *pash-1(mj100)* (ts) were analyzed with DIC after 24 hours of laying. Their developmental stages were characterized. Although the embryos died at various stages during embryogenesis, almost all embryos died after gastrulation in later stages of embryonic development which was close to hatching process (%93, %92 percent respectively). For example, 55% of the *mir-35(ndf50)* embryos mostly died at 3/ 3, 5 fold stages while 37% of them dies at 2/2,5 fold stage and 34% of them died at 3/3,5 fold stage. A small number of embryos of both *mir-35(ndf50)* and *pash-1(mj100)* were arrested in comma and gastrulation stages. 8% of *mir-35(ndf50)* embryos died at gastrulation stage while 7% of *pash-1(mj100)* embryos died at gastrulation stage and 14% of *pash-1(mj100)* at comma stage.

RESULTS

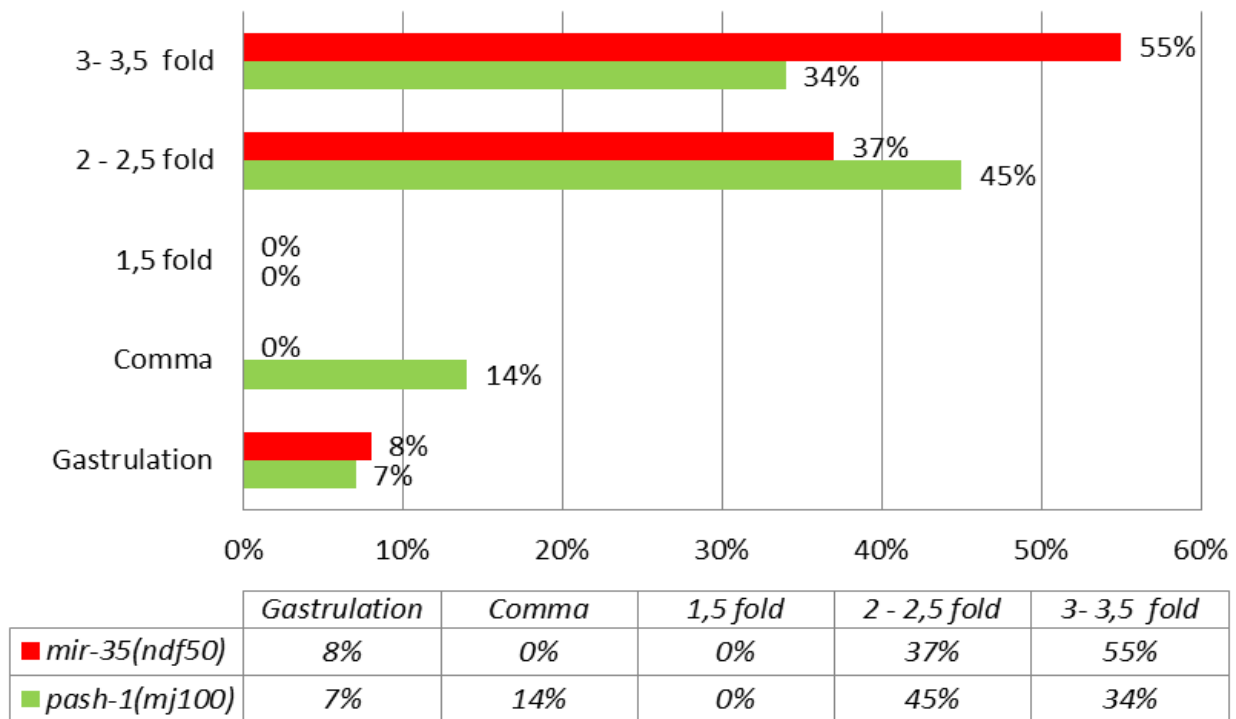


Figure 3.1: Embryonic stages of arrested embryos in *pash-1(mj100)* and *mir-35(ndf50)* at 25°C. 230 embryos for *mir-35(ndf50)* and 290 embryos for *pash-1(mj100)* were imaged 24 hours later after they were laid by the hermaphrodites.

3.2 Both *pash-1(mj100)* and *mir-35(ndf50)* have egg laying defects at restrictive temperature, 25°C.

We observed both *mir-35(ndf50)* family (ts) and *pash-1(mj100)* (ts) mutant animals fail to lay embryos and retain some embryos in their uterus and may have egg laying defects. In order to understand egg laying defects of embryonic lethal mutants; *mir-*

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35(ndf50) family (ts) and *pash-1(mj100)* (ts), we visualized unlaidd embryos inside the one day old adult worms with DIC microscopy. Previously, it has been shown that wild-type worms lay their embryos after 200 minutes after fertilization which is the mid-gastrulation stage. Similarly, we observed that wild-type unlaidd embryos were in gastrulation stage (74%). In embryonic lethal mutants; *mir-35(ndf50)* and *pash-1(mj100)*, unlaidd embryos were found frequently in post-gastrulation stage. In comma stage, both wild-type and mutant embryos were observed in similar percentages, in wild-type 26%, in *mir-35(ndf50)* 28% and in *pash-1(mj100)* 22%. Surprisingly, some of *mir-35(ndf50)* and *pash-1(mj100)* unlaidd embryos reached to 2-2, 5 fold and 3-3, 5 fold stage.

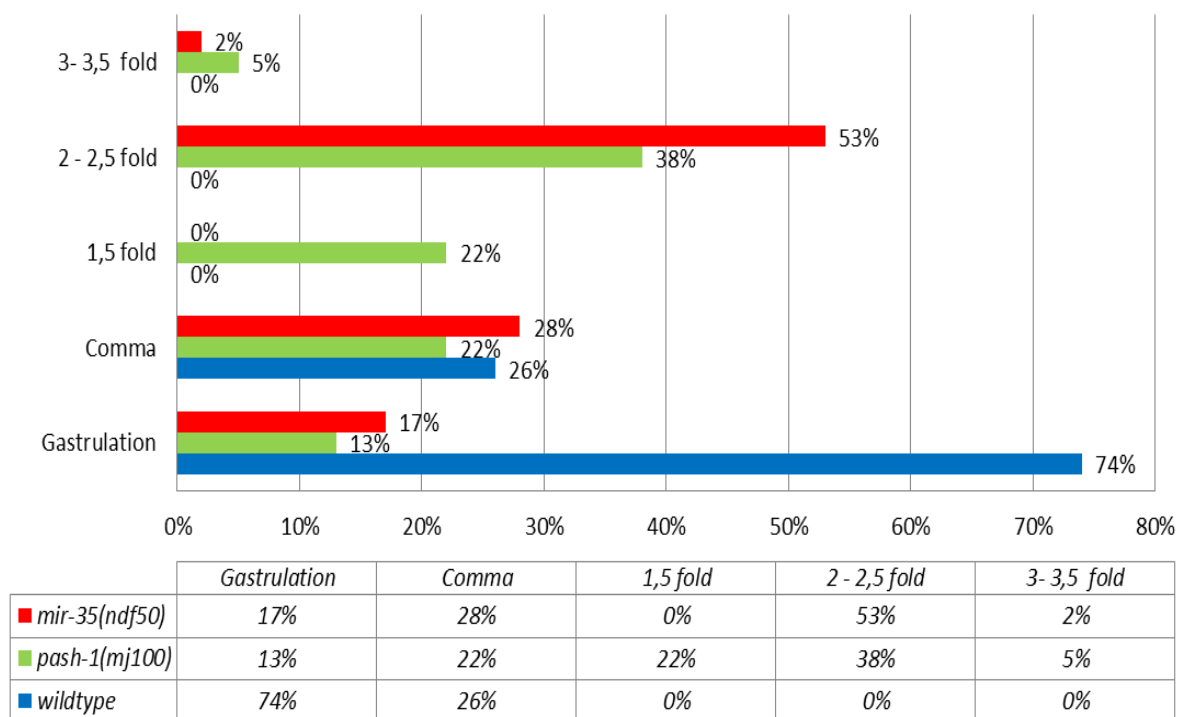


Figure 3.2: Embryonic stages of unlaidd embryos in uterus. 214 embryos for *mir-35(ndf50)* and 153 embryos for *pash-1(mj100)*, 283 embryos for wild-type were imaged dissected from one day adult hermaphrodites.

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Especially, there is an ineligible amount of 2-2,5fold stage embryos for both of the mutants, in *mir-35(ndf50)* 53% and in *pash-1(mj100)* 38%. This result enlightens that these mutants are deficient to lay the embryos in gastrulation stage and embryos continue their development in uterus.

3. 3 *pash-1(mj100)* embryos and *mir-35(ndf50)* embryos are arrested at later stages of embryogenesis with various abnormal morphologies.

Embryos of embryonic lethal mutants; *mir-35(ndf50)* and *pash-1(mj100)* were analyzed after 24 hours of laying. Their developmental stages were imaged and characterized. Embryos show various morphological defects.

Images in **Figure 3.3** show the dead embryos of *pash-1(mj100)* 24 hours later after they were laid at 25°C. Body malformations in these embryos were due to the formation of large vacuoles and apoptotic cells in some areas. Their pharynx and tail movement was also slow to follow up elongation stage and hatch from their eggshells.

RESULTS

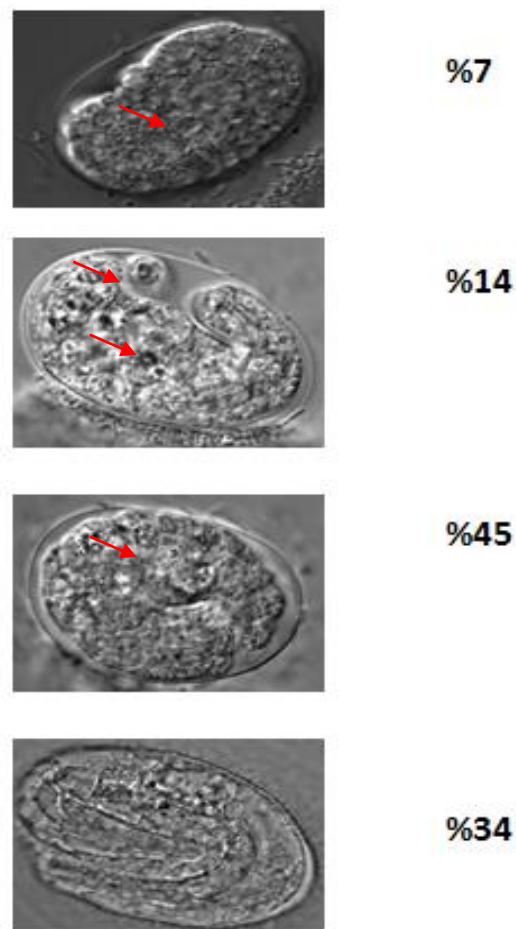


Figure 3.3: DIC images of embryonic stages of arrested embryos in *pash-1(mj100)* 24 hours later at 25°C. Red arrows show apoptotic cells.

Figure 3.4 demonstrates the dead embryo images of *mir-35(ndf50)* 24 hours later after they were laid at 25°C. There was large vacuole formation in arrested *mir-35(ndf50)* embryos. Different from *pash-1(mj100)* embryos, they were more transparent. Their pharyngeal pumping was defected.

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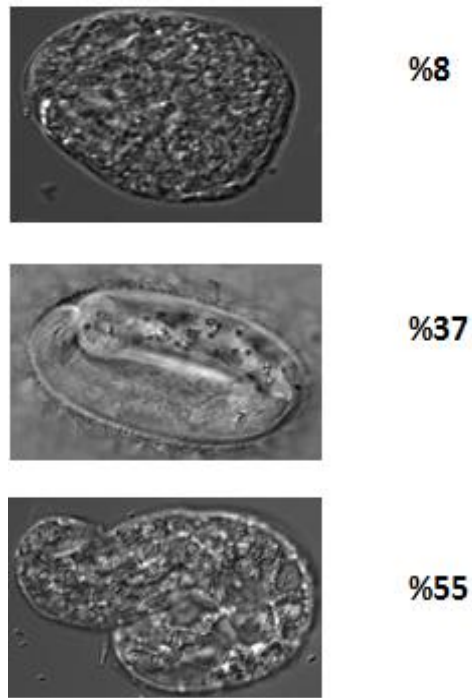


Figure 3.4: DIC images of embryonic stages of arrested embryos in *mir-35(ndf50)* 24 hours later at 25°C.

3.5 Laying deficient *pash-1(mj100)* embryos inside mother hermaphrodites already contain some misshaped morphology before laying.

To understand whether eggs laying deficient *pash-1(mj100)* embryos look normal inside mother and they begin to show abnormalities after they hatched, we visualized unlaidd embryos of 1 day old adult worms.

Figure 3.5 shows the unlaidd embryos of *pash-1(mj100)* inside one day old mothers at 25°C. Although most of them look normal developing embryos in gastrulation, comma and 1, 5 fold stages; still late stage embryos, 2, 5 and 3 fold embryos, contained vacuoles and apoptotic cells.

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Even unlaidd *pash-1(mj100)* late stage embryos in uterus of mother hermaphrodites contain some misshaped morphology before laid from the uterus.

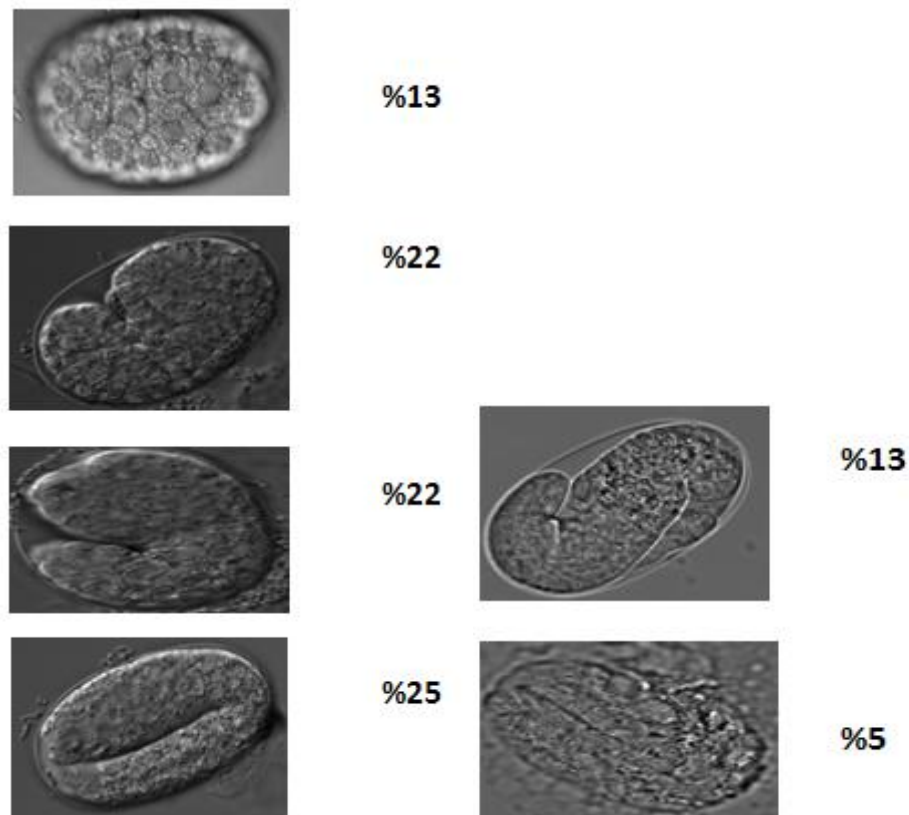


Figure 3.5: DIC images of embryonic stages of unlaidd embryos in uterus of *pash-1(mj100)* animals.

3.6 Trehalose increases lifespan of *pash-1(mj100)* and *mir-52(ndf58)*.

Previous studies (Honda et al., 2010) have shown that trehalose increases lifespan of *C. elegans* 30% by reducing polyglutamine (polyQ) and poly alanine (polyA) mediated protein aggregation. To validate this result and search if trehalose also increases lifespan of miRNA deficient mutants, *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*, we

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performed lifespan assay in the presence of trehalose. Lifespan assays can be influenced by environmental and experimental conditions. NGM growth plates and feeding conditions were kept similar in all lifespan analysis. As all the tested mutants are temperature sensitive, temperature was controlled carefully throughout the analysis.

Both wild-type and *pash-1 eft-3::pash-1::gfp::unc-54* animals, which carry an extrachromosomal array transgene containing *eft-3::pash-1::gfp::unc-54*, were used as control in the experiments. We referred this line as *pash-1* rescue line in further parts.

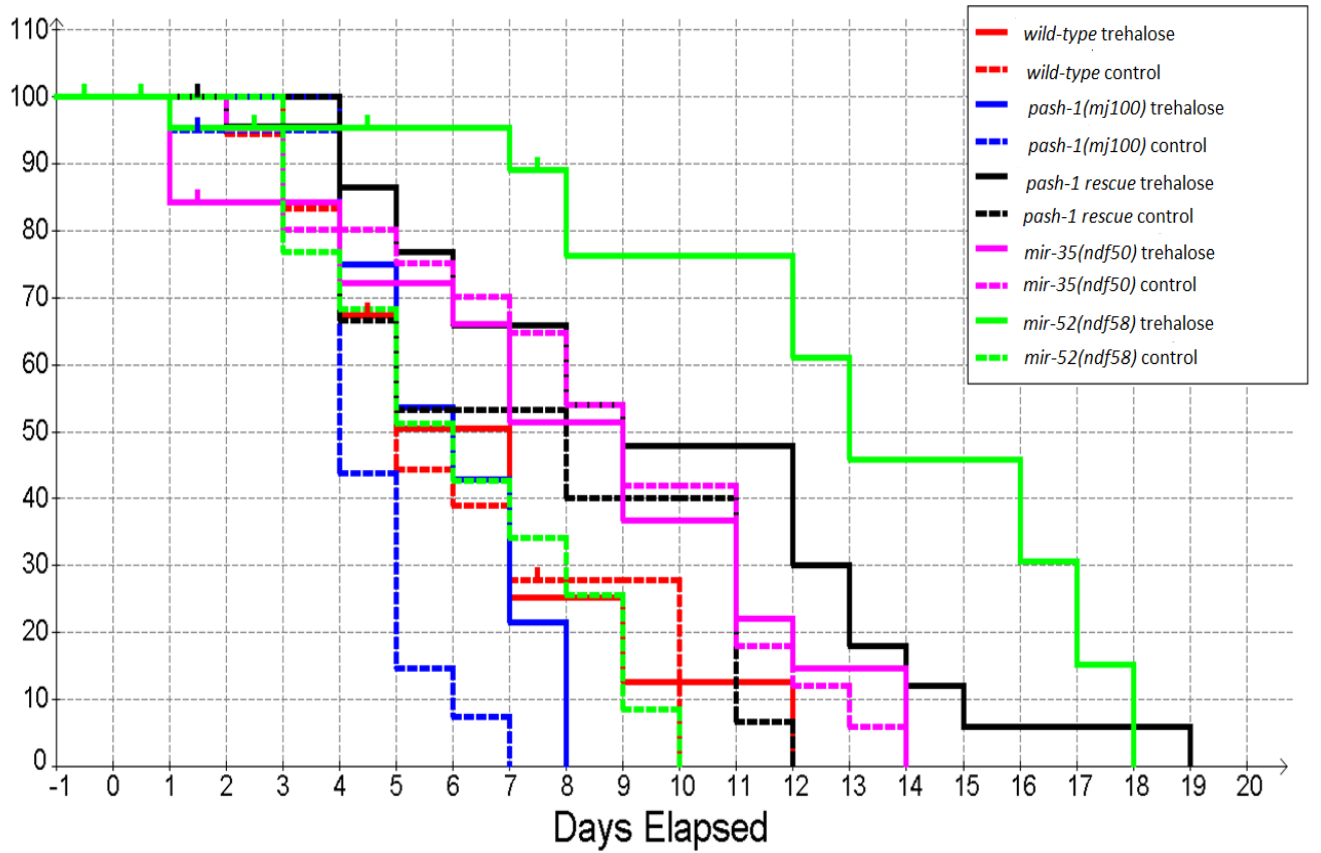


Figure 3.6: Lifespan of wild-type, *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)* with or without trehalose. L4 animals grown at 15°C were placed at 25°C for overnight and transferred to control and trehalose NGM plates, then checked daily for survival. The results are representative of 2 biological replicates with 30 worms in each experiment.

RESULTS

As shown in **Figure 3.6** and **Table 3.1**, we validated the outcome of Honda et al with 20% increase in lifespan of wild-type. Trehalose increased half-life of *pash-1(mj100)* by 50% and maximum lifespan of *pash-1(mj100)* by 14%. Trehalose didn't change half-life or maximum life of *mir-35(ndf50)* mutant animals. Strikingly, trehalose increased half-life of *mir-52(ndf58)* by 160% and maximum lifespan of *mir-52(ndf58)* by 80%. Up to date, this is the highest increase in lifespan analysis ever reported in the literature.

Table 3.1: Maximum and half-life of the genotypes based on the Figure 3.6.

Genotype	Half-life	Maximum Lifespan
<i>pash-1</i>	50%	14%
<i>pash-1 rescue</i>	13%	58%
<i>wild-type</i>	0%	20%
<i>mir-35(ndf50)</i>	0%	0%
<i>mir-52(ndf58)</i>	160%	80%

3.6 Trehalose increases chronological healthspan of *pash-1(mj100)* and *mir-52(ndf58)*.

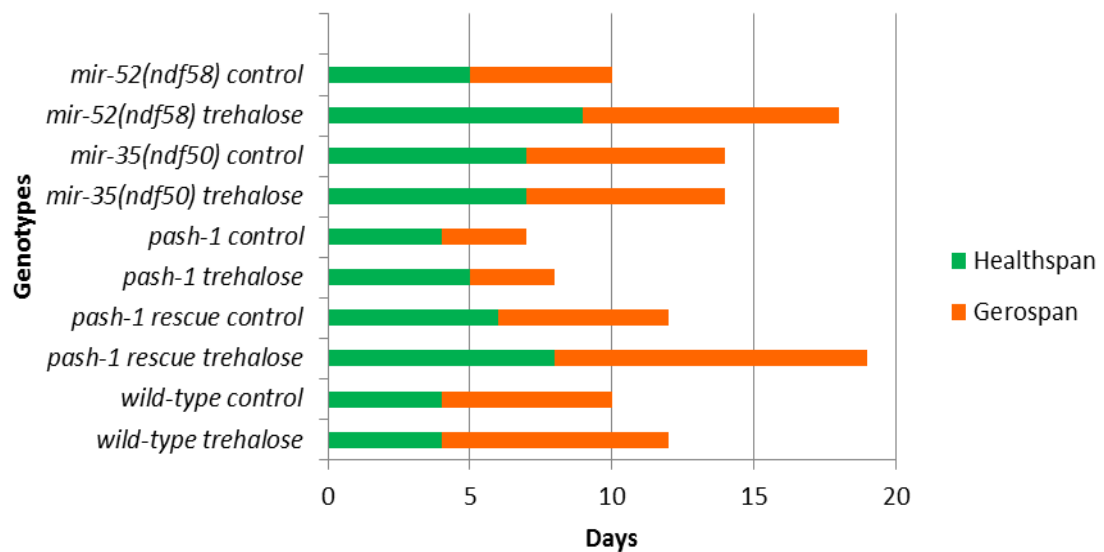


Figure 3.7: Chronological healthspan and gerospan of *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)* in the presence of trehalose. L4 animals grown at 15°C were placed at 25°C for overnight and transferred to control and trehalose containing NGM plates. Worms were checked daily for survival and locomotion. The results are representative of 2 biological replicates with 30 worms in each experiment. The results were compared for each day depends on the maximum lifespan of each genotype.

Healthspan is the time point where worms achieve larger than 50% of the “maximal functional capacity of wild-type. Gerospan is, on the other hand, the time point where the worms are less than 50% of the “maximal functional capacity” of wild-type (Bansal, 2015). Healthspan and gerospan levels are important, because they indicate of healthy and unhealthy periods of lifespan, basically quality of their lifespan.

RESULTS

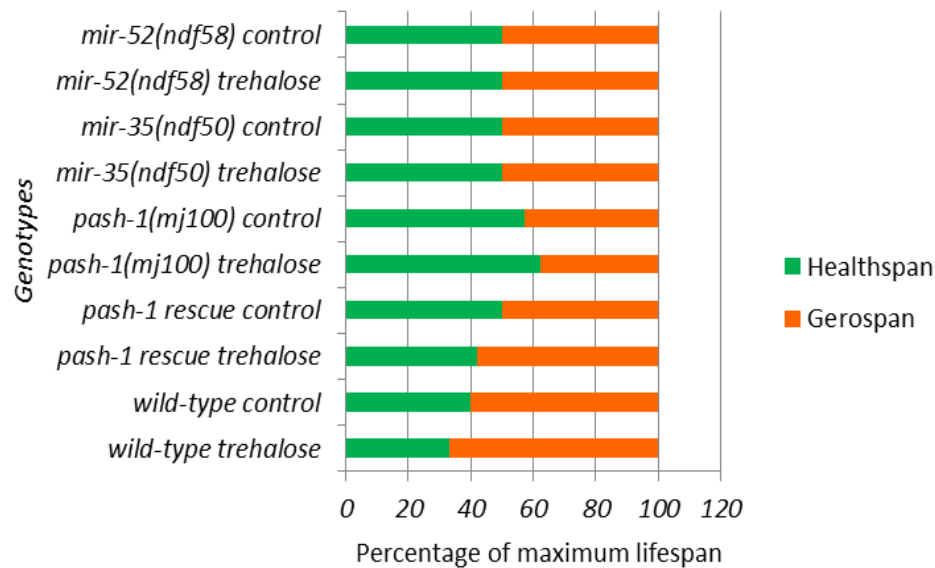


Figure 3.8: Physiological healthspan and gerospan of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)* in the presence of trehalose. L4 animals grown at 15°C were placed at 25°C for overnight and transferred to control and trehalose NGM plates, then checked daily for survival and locomotion. The results are representative of 2 biological replicates with 30 worms in each experiment. The maximal lifespan of all strains were normalized to 100%.

Results in **Figures 3.7** and **Figure 3.8** show that trehalose enhanced the chronological healthspan of *pash-1(mj100)* and *mir-52(ndf58)*, but it did not improve physiological healthspan of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*. We have shown that trehalose extends actual days (chronological) lifespan of animals, but does not improve the healthy periods of their lifespan.

3.7 Trehalose improves locomotion of *pash-1(mj100)*.

pash-1(mj100) animals start to display locomotion defects two days after temperature up-shift. They become uncoordinated and paralyzed on the third day of their adulthood at 25°C. This phenotype can be rescued by the transgene expressing the wild-

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type PASH-1 protein, suggesting that these abnormal locomotion phenotypes are due to *pash-1* loss of function (Lehrbach et al., 2012).

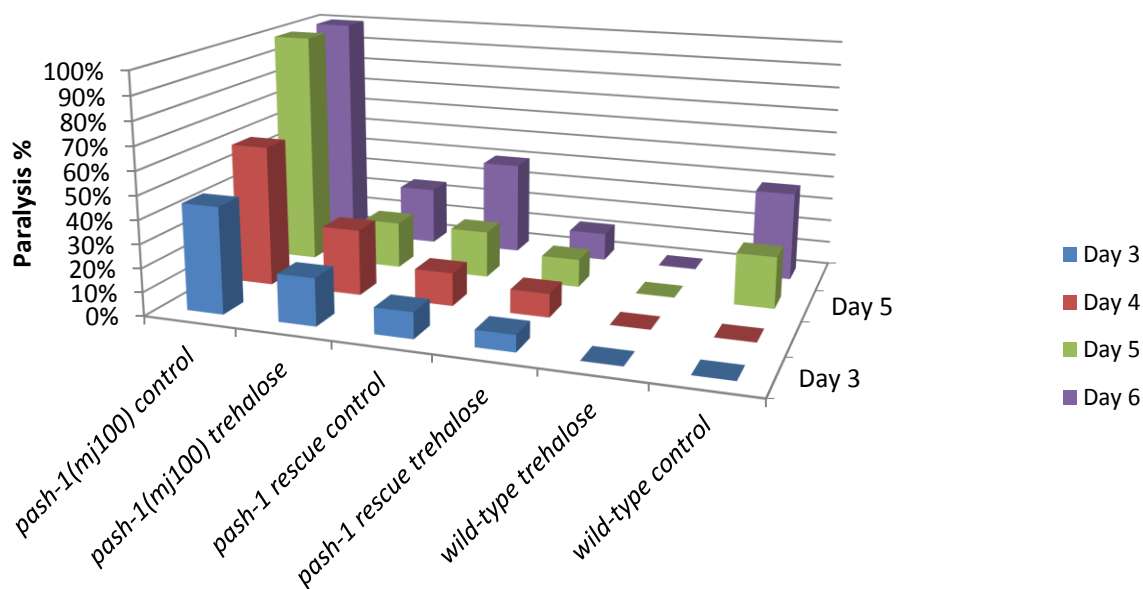


Figure 3.9: Trehalose rescues the aging related locomotion defects of wild-type, *pash-1(mj100)*, *mir-35(nDf50)* and *pash-1* rescue. L4 animals grown at 15°C were placed at 25°C for overnight and transferred to control and trehalose containing NGM plates, then checked daily for locomotion. The results are representative of 2 biological replicates with 30 worms in each experiment.

As seen in **Figure 3.9**, paralysis of *pash-1(mj100)* declines from 40% to 20% on the third day, from 55% to 25% on the fourth day, from 97% to 15% on the fifth day and from 98% to 22% on the sixth day of adulthood in the presence of trehalose. Trehalose decreases paralysis of *pash-1* rescue from 10% to 6% on the third day, from 30% to 10% on the fourth day, from 35% to 12% on the fifth day and from 50% to 15% at sixth day. Wild-type animals start to paralyze on the fifth day and trehalose decreases paralysis percentage of

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wild-type from 30% to 0% on the fifth day and from 50% to 0% at sixth day. These data suggest that trehalose rescues motility defects caused by miRNA loss and helps adult worms to maintain normal locomotion.

3.8 Trehalose partially rescues the embryonic lethal phenotype of *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)*.

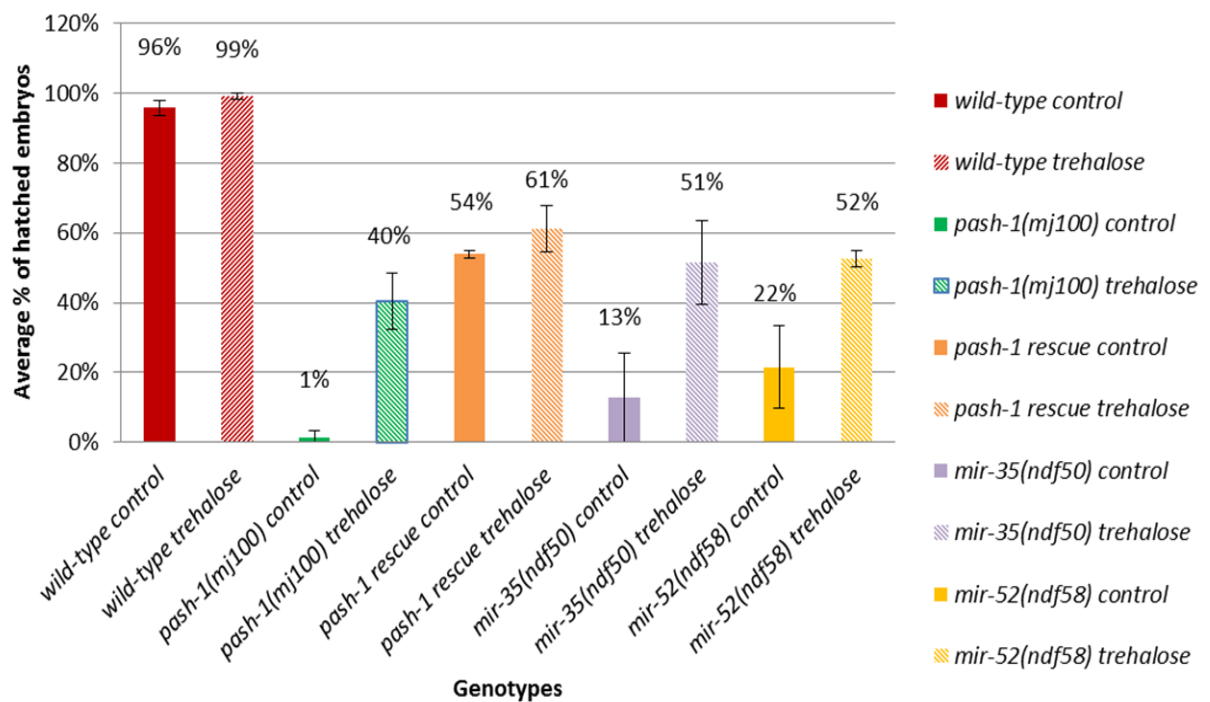


Figure 3.10: Trehalose partially rescues embryonic lethality of *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)*. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. Every 12 hours, embryos and hatched worms were counted in each well and the mothers are transferred to new 6- well plates every 12 hours until reproduction ceases. The results are representative of 3 biological replicates with 6 worms in each experiment. Error bars refer standard deviation.

RESULTS

In lifespan analysis, we observed live and hatched embryos, while treating the *pash-1(mj100)* worms with trehalose, which normally has 100% embryonic lethal phenotype at 25°C. In order to understand and quantitate this observation, we treated embryonic lethal *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)* worms with 5mM trehalose. Results in **Figure 3.10** shows the effect of trehalose on miRNA related embryonic lethal phenotype of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*. 40% of *pash-1(mj100)* embryos hatched in the presence of trehalose. We have seen the similar observation with *mir-35(nDf50)* and *mir-52(nDf58)*. Hatching of *mir-35(nDf50)* mutant embryos were increased from 13% to 51%, while that of *mir-52(nDf58)* was increased from 22% to 52% when worms were grown with trehalose. As expected, 100% of wild-type embryos were hatched in both control and trehalose plates. When wild type copy of *pash-1* gene was re-introduced into the *pash-1(mj100)* background (*pash-1* rescue strain), the embryonic lethality was rescued by 54%. Trehalose did not significantly rescue the embryonic lethality of *pash-1* rescue strain in this and also in further experiments. These data suggest that trehalose's effect is miRNA dependent since trehalose could not increase hatching of *pash-1* rescue adults, which is able to express mature miRNAs. Therefore, we concluded that trehalose can rescue the embryonic lethality as good as the wild type copy of the *pash-1* gene, suggesting that trehalose's effect is miRNA dependent since trehalose could not increase hatching of *pash-1* rescue adults and rescuing effect of trehalose in miRNA deletion mutants is almost as hatching percentage of *pash-1* rescue.

Although trehalose rises the percent of the hatched embryos of *pash-1(mj100)* animals almost to 40%, hatched L1s maintain several abnormal phenotypes and never reach L2 stage, because trehalose is unable to rescue miRNA function required for L1 to L2 stage transition. We made brief characterization of hatched L1s after treated with trehalose.

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Figure 3.11 shows the different body parts of *pash-1(mj100)* L1 larva treated with trehalose. They contained large vacuoles near the gonad and tail. Vacuole formation is commonly observed in *pash-1(mj100)* embryos, dead embryos of *pash-1(mj100)* contained vacuoles after 2 fold stage of embryogenesis. Hatched L1s were thicker compared to wild-type L1s because of the sugar effect of trehalose. Their pharynx movement was very slow, although morphology of their pharynx looked normal.

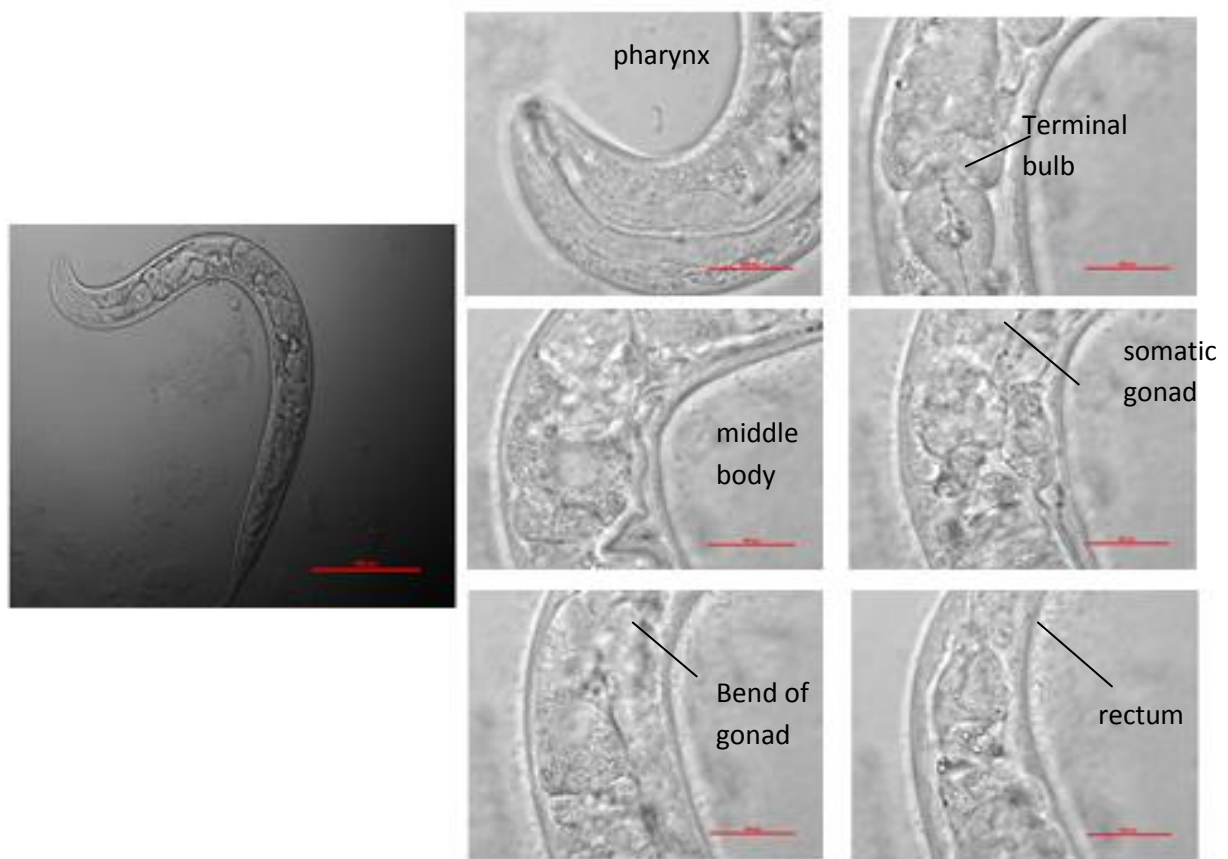


Figure 3.11: Imaging of *pash-1(mj100)* L1 larva hatched in the presence of trehalose. DIC images show a representative L1 animal whose mother was treated with trehalose.

RESULTS

In contrast to *pash-1(mj100)*, hatched L1s of *mir-35(ndf50)* mutant animals were viable after trehalose treatment. They were able to complete their larval development and become fertile adults if they were continuously fed with trehalose. The progeny of these fertile adults also requires trehalose for the viability.

3.9 Trehalose does not affect brood size of wild-type, *pash-1(mj100)*, *pash-1 rescue (eft-3::pash-1::gfp::unc-54)* and *mir-35(ndf50)*.

Embryo laying capacity of *C. elegans* hermaphrodites peaks approximately 20 hours after they reach adulthood at 25°C. It is the time point when we put the worms to the trehalose plates after their miRNA production was diminished at restrictive temperature, 25°C. In the following days, their embryos were counted on the plates. The question was whether trehalose affects the brood size of *pash-1(mj100)* and *mir-35(ndf50)* beside its rescuing effect on embryonic lethality. However, the results shown in **Figure 3.12** demonstrates that trehalose does not change the brood size of wild-type, *pash-1(mj100)*, *pash-1 rescue (eft-3::pash-1::gfp::unc-54)* and *mir-35(ndf50)* animals.

RESULTS

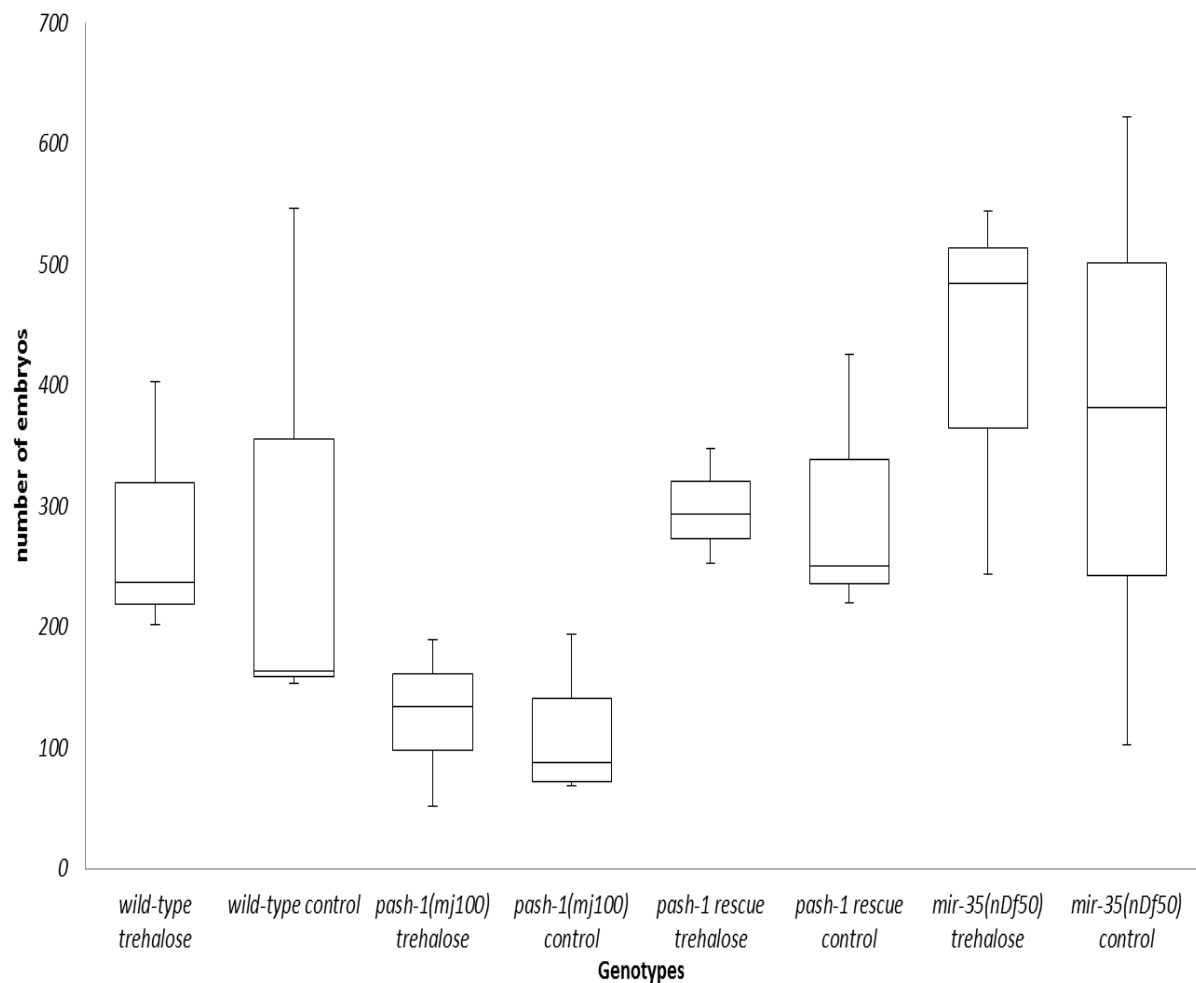


Figure 3.12: Effects of trehalose to brood size of wild-type, *pash-1(mj100)*, *pash-1 rescue (eft-3::pash-1::gfp::unc-54)* and *mir-35(ndf50)* animals. Four biological replicates with 6 worms were carried out. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. Every 12 hours, the mothers are transferred to new 6- well plates and embryos were counted in each well until reproduction ceases.

3.10 α,β -trehalose like α,α -trehalose partially rescues the embryonic lethal phenotype of *pash-1(mj100)* and *mir-35(ndf50)*.

α,α -trehalose is the only version of trehalose that has been shown to exist in various organisms and in our previous experiments we used α,α -trehalose. However, trehalose has other stereoisomeric forms such as α,β -trehalose. To investigate whether α,β stereoisomer of trehalose can rescue the miRNA related embryonic lethality, we treated *pash-1(mj100)* and *mir-35(ndf50)* worms with α,β -trehalose which is 12% lower than the α,α -trehalose treatment. 23% hatching of *mir-35(ndf50)* embryos were also increased by α,β -trehalose up to 47%, which was comparable to the α,α -trehalose treatment (~44%).

RESULTS

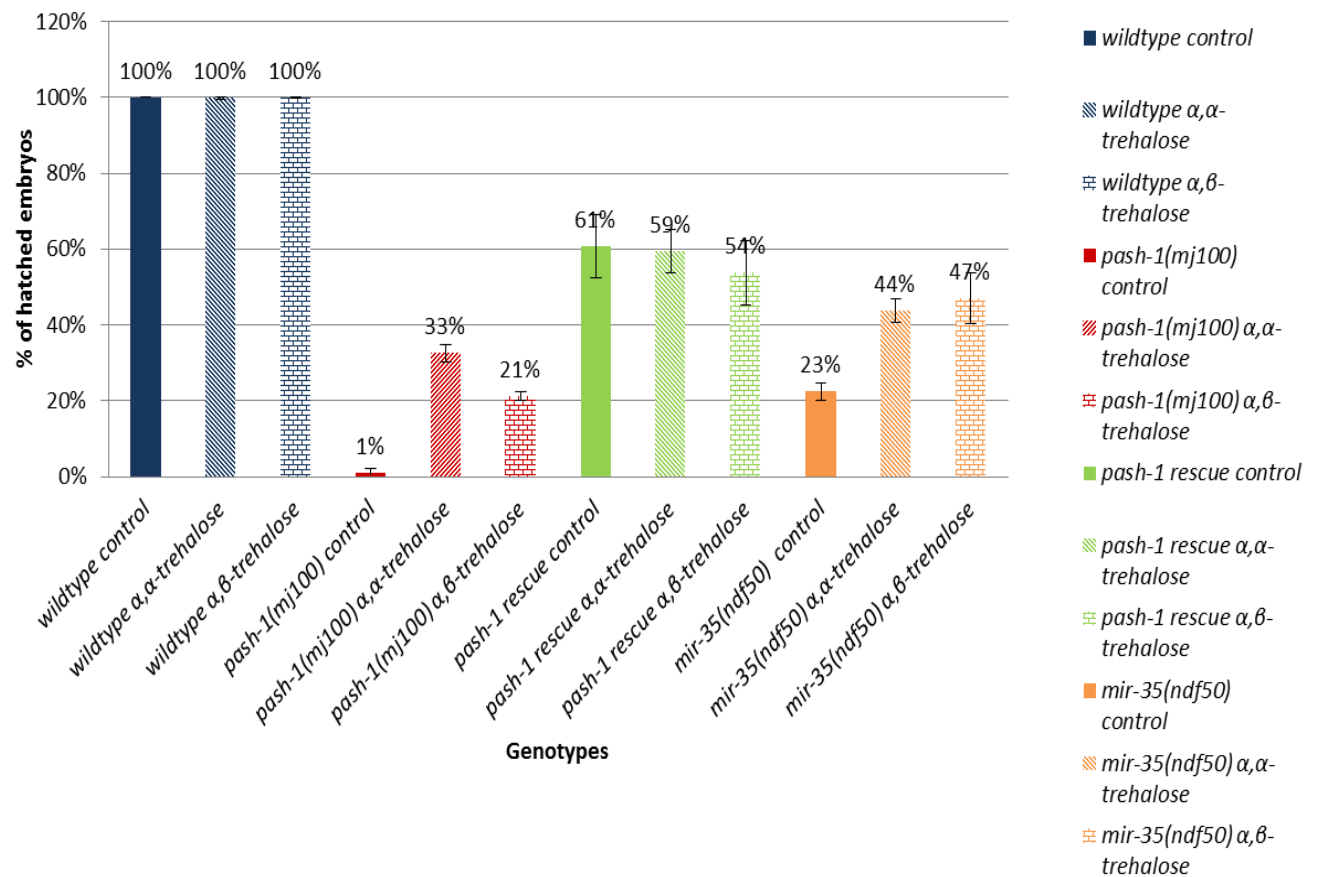


Figure 3.13: Comparison of the effects of α,α - and α,β -trehalose in *pash-1(mj100)* and *mir-35(ndf50)* embryonic lethality. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation.

RESULTS

3.11 Intensity of carminic acid staining is higher in trehalose treated *pash-1(mj100)*, *pash-1 rescue*, *mir-35(ndf50)* and wild-type concentrated largely in pharynx, tail and embryos.

Carminic acid is a fluorescent dye which binds trehalose and glycogen. We used carminic acid to visualize the uptake of trehalose to quantify trehalose levels and determine the location of trehalose within the body of the worms.

Figure 3.14 shows carminic acid staining image of one day old wild-type adult animal treated with or without trehalose. **Figure 3.15** shows carminic acid staining of one day old *pash-1(mj100)* adult animal not treated and treated with trehalose. **Figure 3.16** shows carminic acid staining of one day old *mir-35(ndf50)* adult animal not treated with trehalose and treated with trehalose.

RESULTS

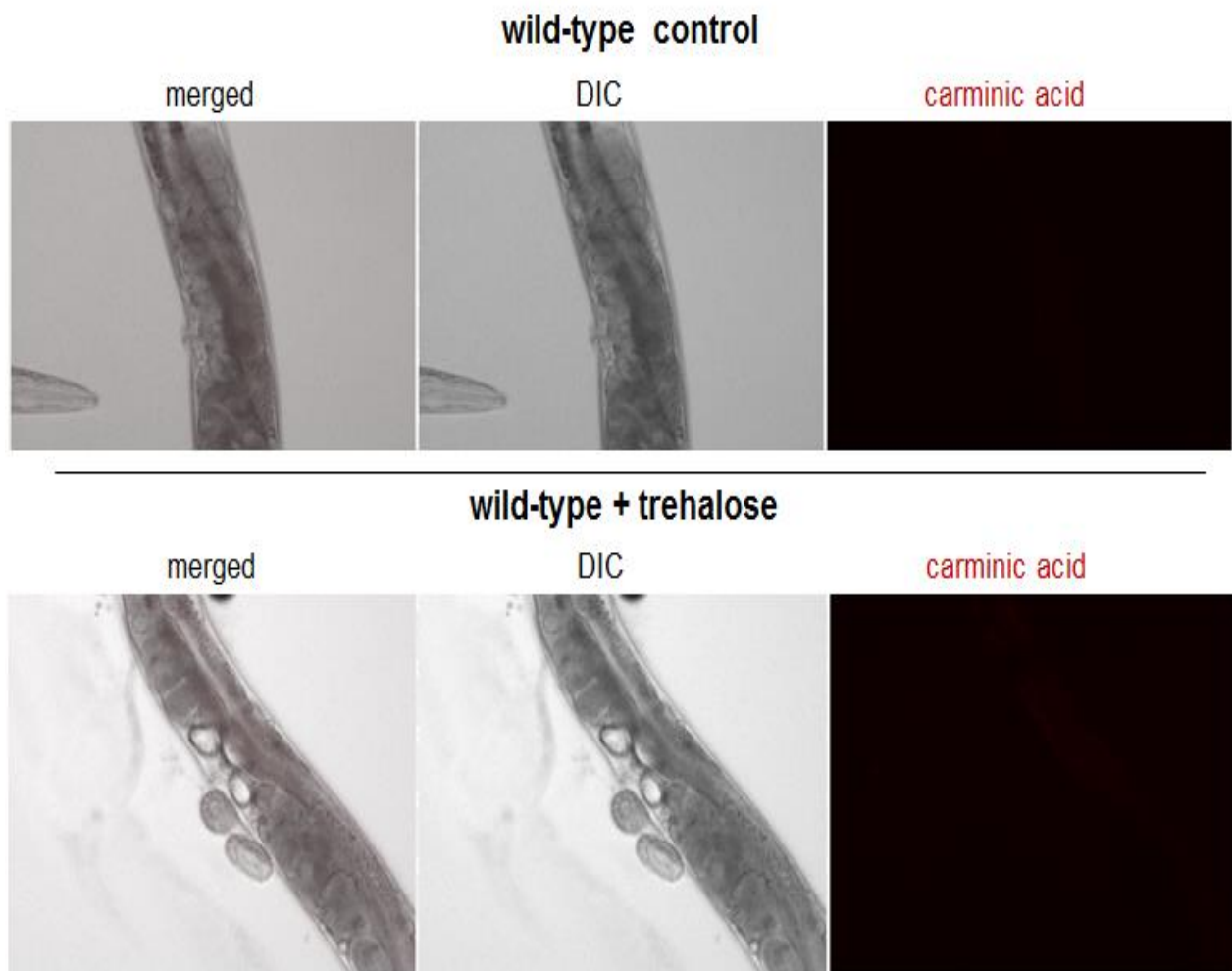


Figure 3.14: Determination of trehalose levels in wild type animals with carminic acid staining. L1 worms were grown on the carminic acid (1mg/ml) containing NGM plates and transferred to the trehalose and control, assayed as one-day old adults. Worms were imaged on a 2% agarose pad containing 0.2 mM levamisole in M9. The results are representative of two biological replicates with 15 worms. For all imaging analysis, exposure was 100 ms.

RESULTS

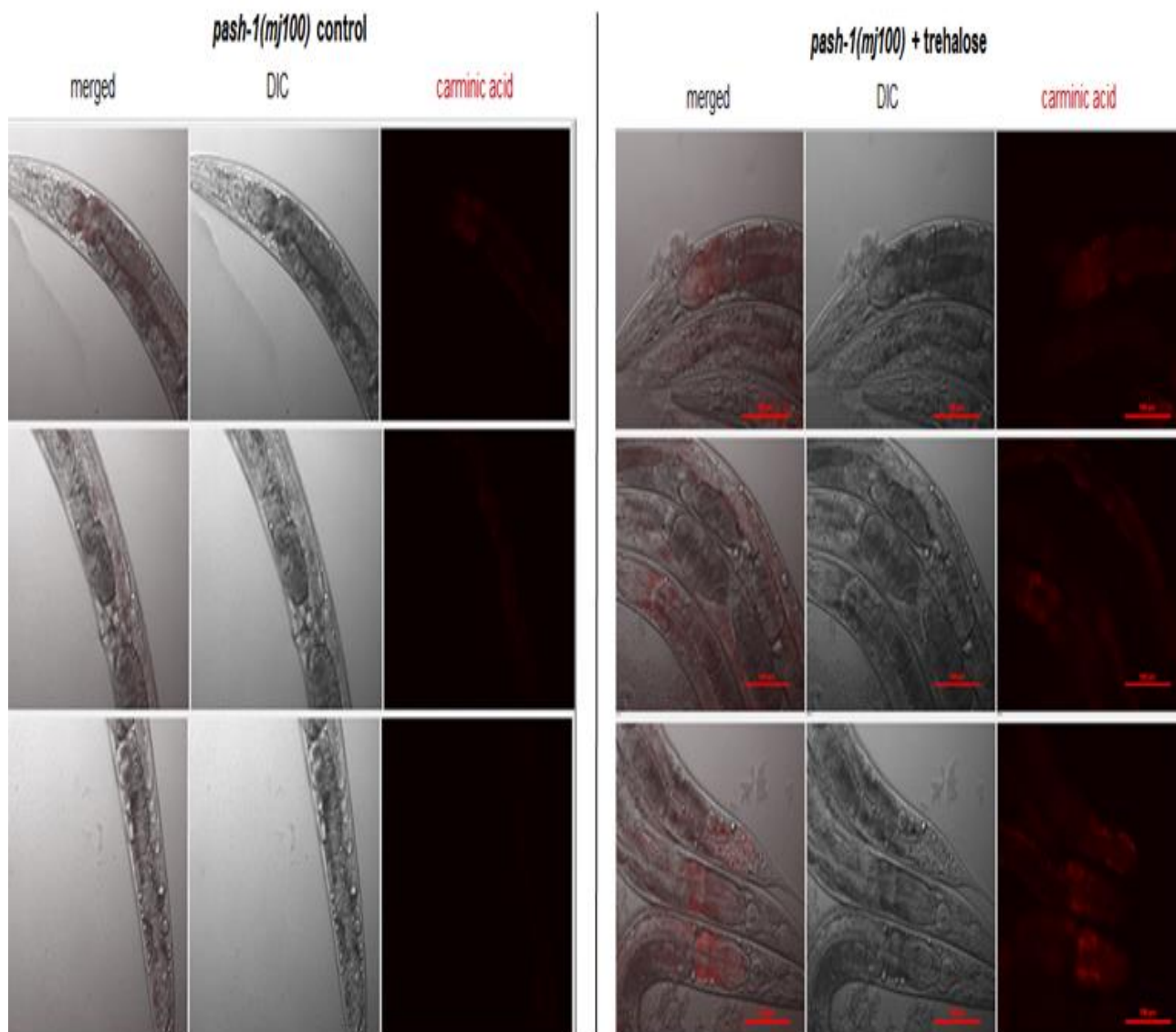


Figure 3.15: Determination of trehalose levels in *pash-1(mj100)* animals with carminic acid staining. L1 worms were grown on the carminic acid (1mg/ml) containing NGM plates and transferred to the trehalose and control, assayed as one-day old adults. Worms were imaged on a 2% agarose pad containing 0.2 mM levamisole in M9. The results are representative of two biological replicates with 15 worms. For all imaging analysis, exposure was 100 ms.

RESULTS

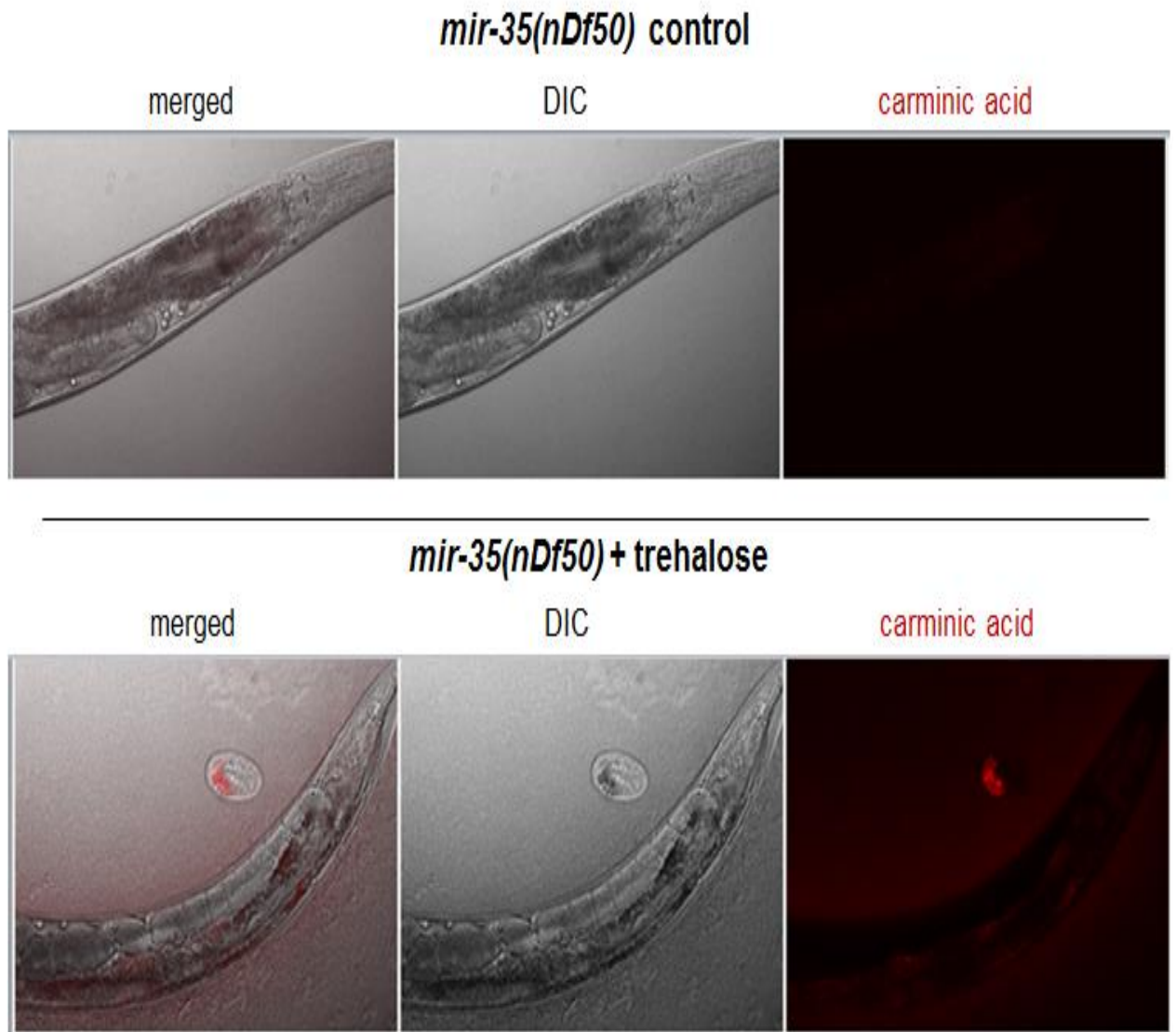


Figure 3.16: Determination of trehalose levels in *mir-35(ndf50)* animals with carminic acid staining. L1 worms were grown on the carminic acid (1mg/ml) containing NGM plates and transferred to the trehalose and control, assayed as one-day old adults. Worms were imaged on a 2% agarose pad containing 0.2 mM levamisole in M9. The results are representative of two biological replicates with 15 worms. For all imaging analysis, exposure was 100 ms.

RESULTS

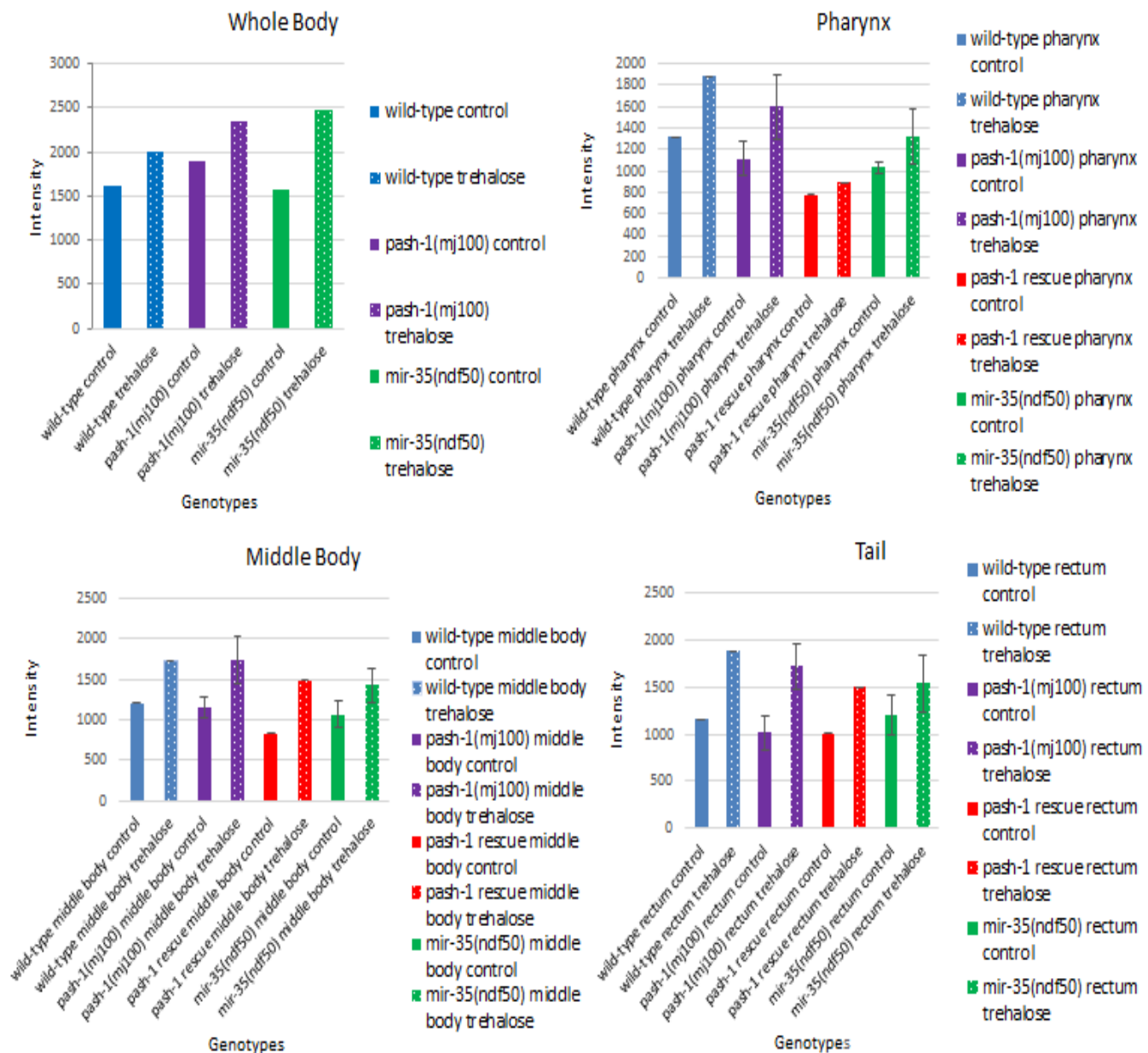


Figure 3.17: Quantification of fluorescence intensity of carminic acid treated animals. Image acquisition was done using NIS-Elements Viewer 4.20. For each condition, 15 worms were imaged and fluorescence intensity was quantified. Error bars represent standard deviation.

RESULTS

As seen in **Figure 3.17**, trehalose treated animal of all genotypes, wild-type, *pash-1(mj100)*, *pash-1 rescue* and *mir-35(ndf50)* had larger carminic acid intensity. Intensity and difference between with and without trehalose was higher in pharynx and tail parts of body.

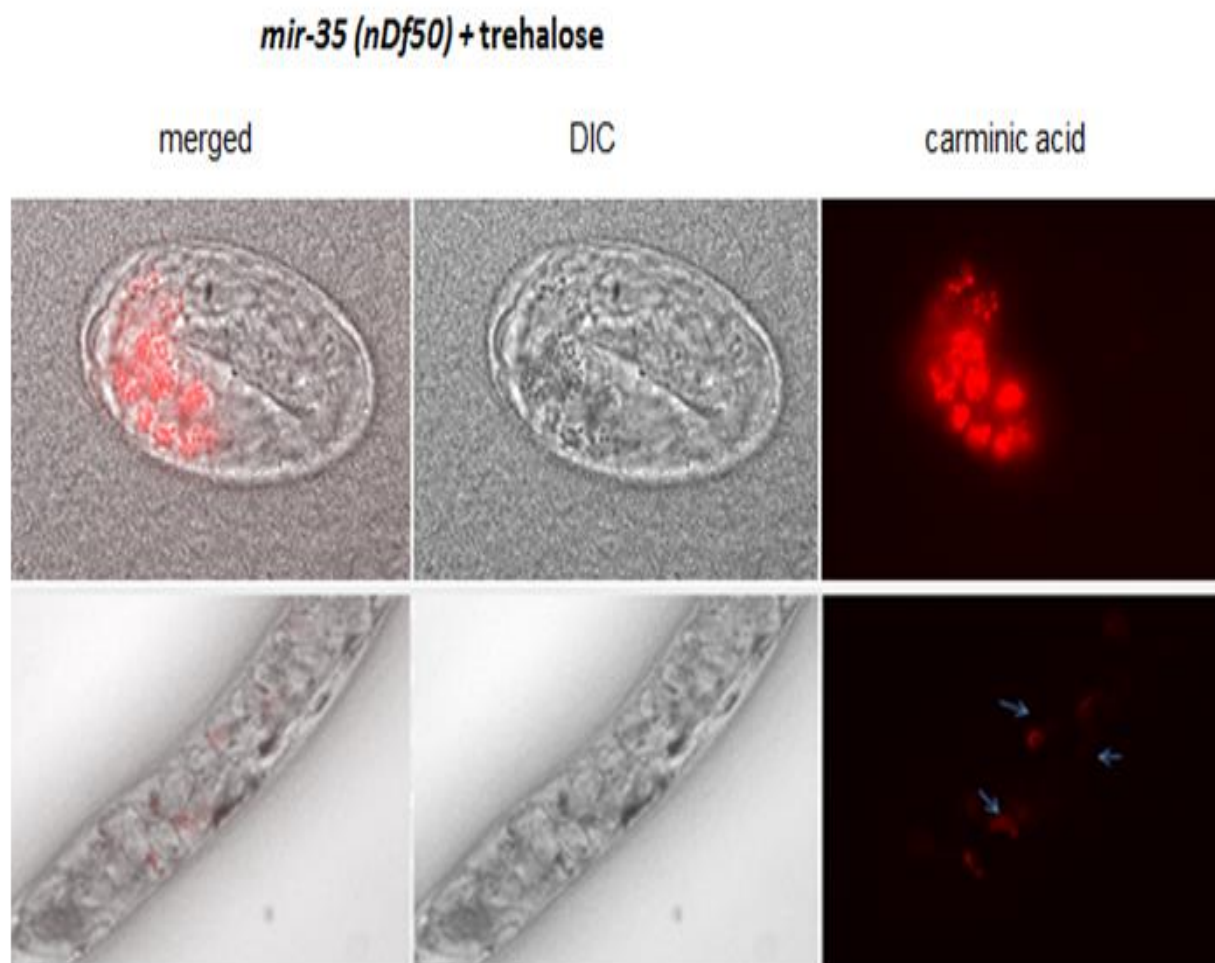


Figure 3.18: Carminic acid staining of *mir-35(ndf50)* embryos treated with trehalose. Blue arrows show embryos inside the uterus. L1 worms were grown on the carminic acid (1mg/ml) containing NGM plates and transferred to the trehalose and control, assayed as one-day old adults. Worms were imaged on a 2% agarose pad containing 0.2 mM levamisole in M9. For all imaging analysis, exposure was 100 ms. The results are representative of two biological replicates with 15 worms.

RESULTS

Intensity of carminic acid is very high in embryos of *mir-35(ndf50)*, although carminic acid intensity of *mir-35(ndf50)* animals was in average levels. In **Figure 3.18**, embryos inside the *mir-35(ndf50)* animal stained with carminic acid and a *mir-35(ndf50)* laid embryo stained with carminic acid were visualized. This data supports that worms could exogenously take trehalose and incorporate trehalose.

Higher carminic acid intensity could prove the fact that trehalose is found in embryos with large amount and exogenously given trehalose can be incorporated into the embryos.

3.12 Trehalose doesn't rescue the embryonic lethal phenotypes of miRNA independent embryonic lethal mutants.

To investigate whether trehalose specifically rescue miRNA related embryonic lethality, we included miRNA independent embryonic lethal mutants to our analysis. *cgh-1(tn691)* is temperature-sensitive embryonic lethal mutant, which encodes RNA helicase in embryonic cytokinesis. *hcp-6(mr17)* is as well temperature-sensitive embryonic lethal mutant and *hcp-6* encodes a holocentric binding protein taking part in the attachment of microtubules to kinetochores. *unc-15(e1402)* is a gene encoding myosin heavy chain protein and the corresponding allele mutation causes temperature-sensitive embryonic lethality and paralyzed, uncoordinated phenotype.

Results in **Figure 3.19** showed the three miRNA independent embryonic lethal mutants exposed with trehalose. Embryonic lethality of none of the mutants was rescued by trehalose. Only hatching of *unc-15(e1402)* slightly increased from 6% to 14%, but the increment is not significant. This result suggested that trehalose specifically affect miRNA regulated pathway involved in the embryonic development.

RESULTS

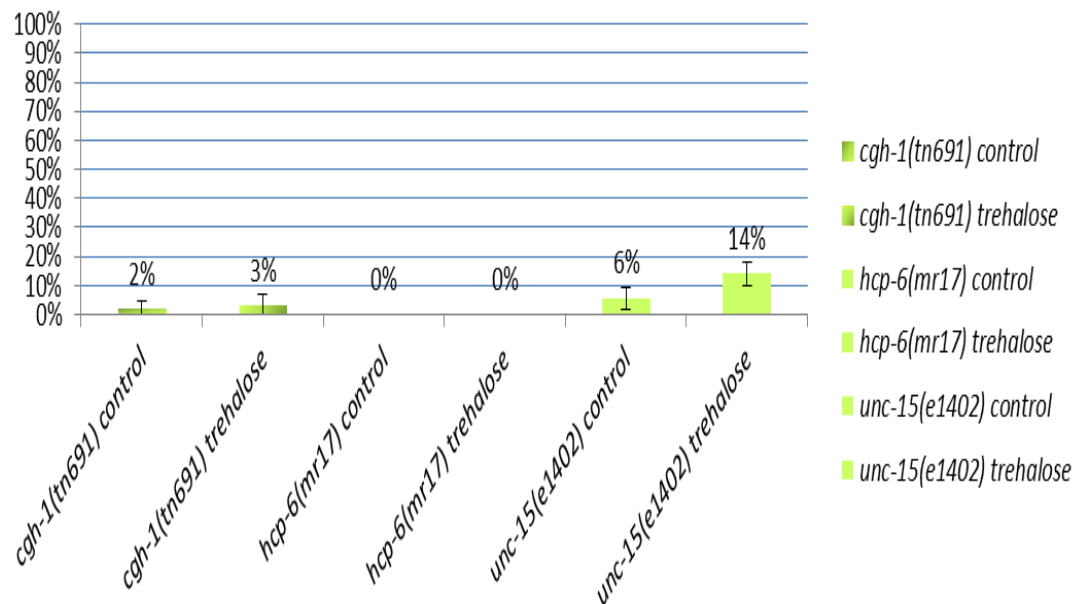


Figure 3.19: Trehalose doesn't rescue the embryonic lethal phenotypes of miRNA independent embryonic lethal mutants. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. Every 12 hours, embryos and hatched worms were counted in each well and the mothers are transferred to new 6-well plates every 12 hours until reproduction ceases. Error bars represent standard deviation. The results are representative of two biological replicates with 6 worms in each experiment.

3.13 Trehalose only rescues embryonic lethality of body wall muscle rescue line of *pash-1(mj100)* suggesting that trehalose could act in cytoskeleton- muscle interaction of embryogenesis.

RESULTS

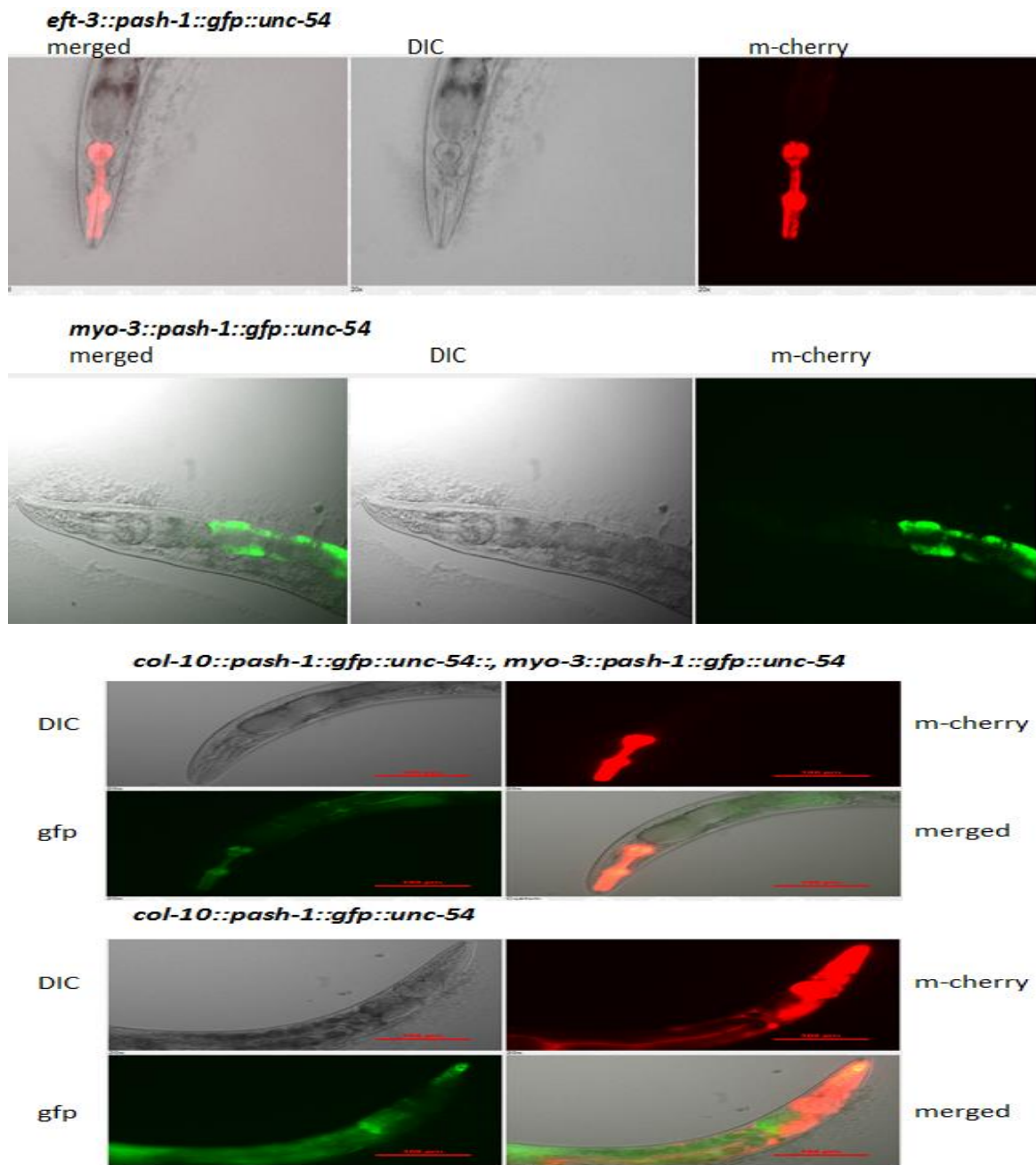


Figure 3.20: Fluorescence imaging of tissue specific *pash-1* rescue constructs.

RESULTS

Table 3.2: Tissue specific *pash-1* rescue constructs.

Strain Name	Rescue Construct	Expression Pattern
SX1359	<i>eft-3::pash-1::gfp::unc-54</i> (<i>pash-1</i> rescue)	All tissues
SX1501	<i>col-10::pash-1::gfp::unc-54</i> (<i>pash-1</i> hypodermis rescue)	hypodermis
SX1486	<i>myo-3::pash-1::gfp::unc-54</i> (<i>pash-1</i> muscle rescue)	Body wall muscles
SX1483	<i>col-10::pash-1::gfp::unc-54::, myo-3::pash-1::gfp::unc-54</i> (<i>pash-1</i> muscle and hypodermis rescue)	Both body wall muscles and hypodermis

Figure 3.20 and **Table 3.2** above show the fluorescence images and genotypes of tissue specific *pash-1* rescue constructs. So far from earlier experiments, we know that *pash-1* rescue animals has still embryonic lethality with approximately 54% hatching, although it is *pash-1::gfp* rescued in all tissues. Furthermore, trehalose does not enhance the situation significantly. On the other hand, 0% hatching of *pash-1(mj100)* was rescued to 40% with trehalose. Bringing up these two outcomes, we wanted to search the effect of trehalose to the rescue constructs which restore expression of wild-type PASH-1 protein fused to GFP under the control of different promoter elements (**Table 3.2**). These are the constructs expressed in all tissues, only in the body wall muscles, only in the hypodermis and in the body wall muscle and hypodermis. We did not obtain neuronal rescue for the assay.

RESULTS

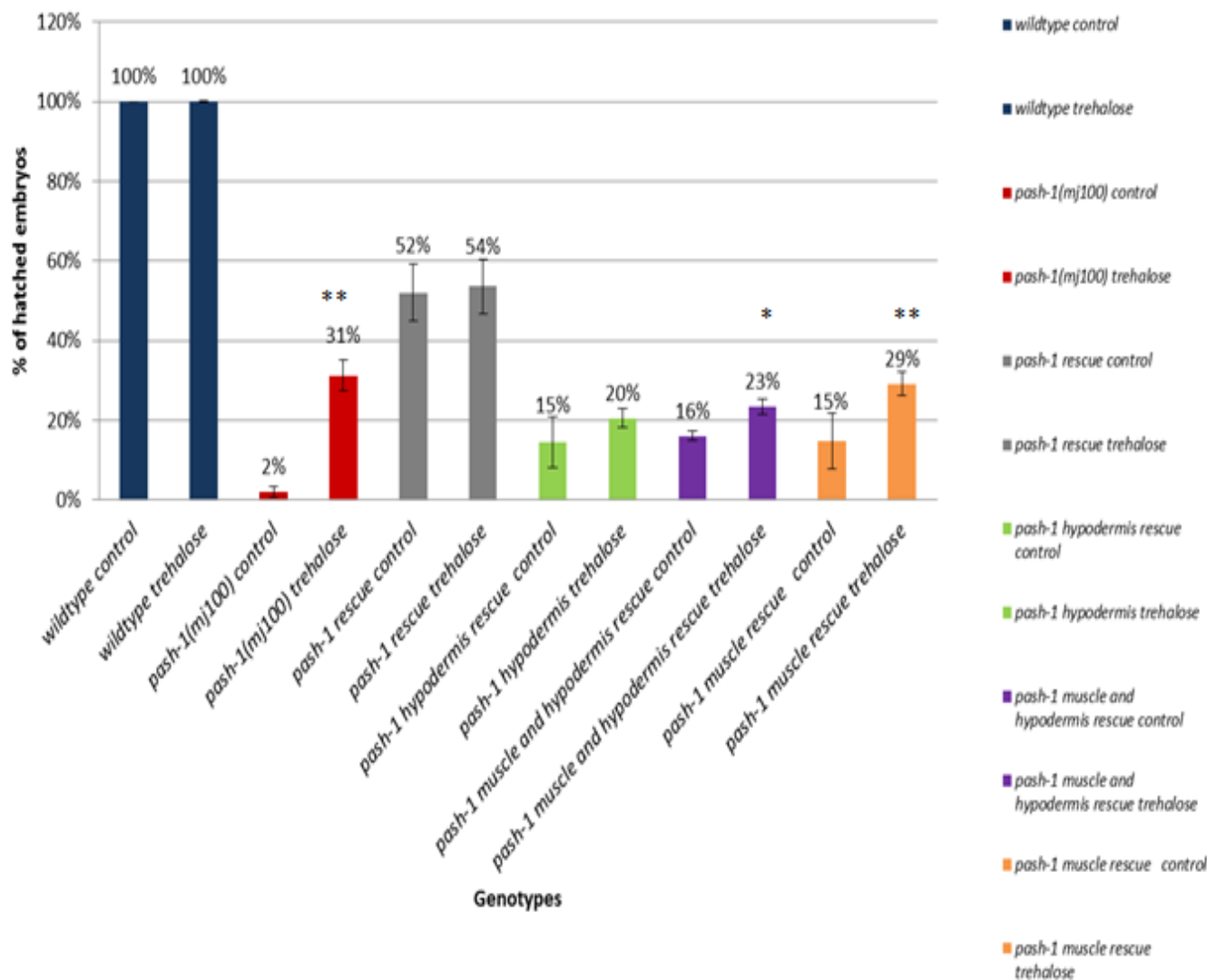


Figure 3.23: Effects of trehalose in embryonic lethality of tissue specific *pash-1* rescue constructs. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

Figure 3.21 shows the results of *pash-1* rescue constructs. Trehalose did not change the embryonic lethality of *pash-1* hypodermis rescue. Hatching of *pash-1* muscle rescue embryos increased from 16% to 23%. The change is slight but significant. Hatching of *pash-1* hypodermis and muscle rescue embryos also rose from 15% to 29% with trehalose treatment. The increase of hatching in *pash-1* hypodermis and muscle rescue embryos can be explained by the increase of hatching in *pash-1* muscle rescue.

From this result, we could conclude that trehalose might act in a pathway related to muscle formation and elongation in embryogenesis.

3. 14 Other disaccharides, maltose and sucrose do not rescue embryonic lethality of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*.

Trehalose is a disaccharide which could act as carbon source, autophagy inducer and osmolyte. Maltose is simple disaccharide of glucose like trehalose and sucrose is a disaccharide of glucose and fructose. Maltose is a reducing sugar while both trehalose and sucrose are non-reducing sugars. Maltose consists of two α -D-glucose molecules with the alpha bond at carbon 1 of one molecule attached to the oxygen at carbon 4 of the second molecule. This is called a $1\alpha\rightarrow4$ glycosidic linkage. Trehalose has also two α -D-glucose molecules connected through carbon number one in a $1\alpha\rightarrow1$ linkage. Sucrose is formed by one α -D-glucose and one β -D-fructose molecule with a carbon number one in a $1\alpha\rightarrow2$ linkage.

For all the genotypes, *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)* trehalose was the only disaccharide which increased the hatching percentages. Trehalose rescued embryonic lethality in *pash-1(mj100)* from 2% to 42%, in *mir-35(nDf50)* from 17% to 45% and in *mir-*

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52(nDf58) from 35% to 49% significantly. Maltose did not affect the hatching of *pash-1(mj100)* at a significant level.

Interestingly, maltose increased the embryonic lethality of *mir-35(nDf50)*. Hatching of *mir-35(nDf50)* decreased from 17% to 6%. Similarly, maltose also caused toxicity in in wild-type animals. There was a slight decrease in embryonic hatching from 97% to 91%. On the other hand, sucrose increased the hatching of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)* embryos about 2-5%. Although sucrose has a small effect on embryonic hatching, the change is very small. This could be related to high sucrose levels related to high T6P level. T6P is regulator protein both in sucrose and trehalose circulatory pathway. Sucrose and T6P levels were correlated and T6P is formed by degradation of sucrose and leads to formation of trehalose (Henry et al., 2014).

RESULTS

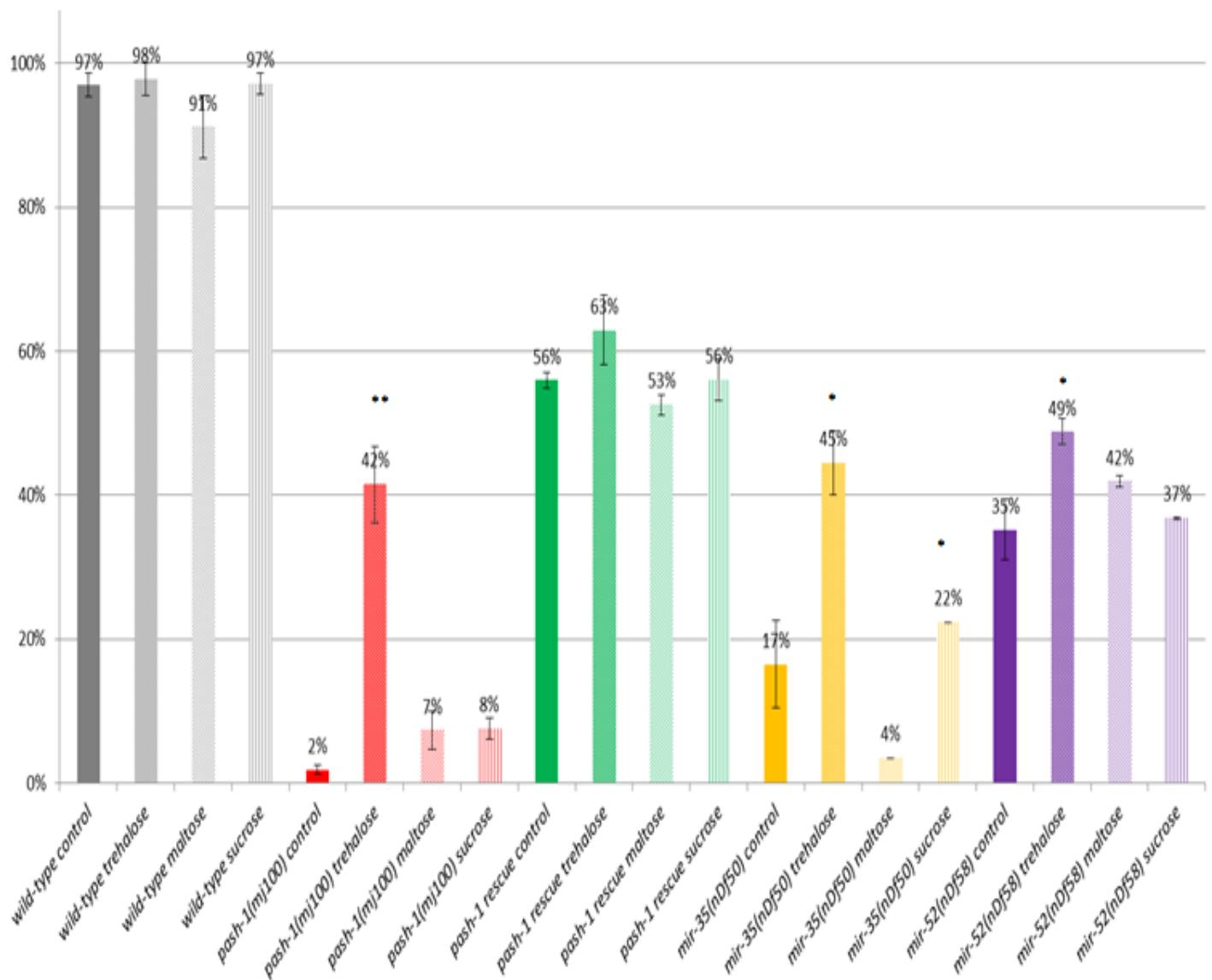


Figure 3.22: Other disaccharides do not rescue embryonic lethal phenotypes of *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

3.15 Other osmolytes, proline and serine and flavonoid, naringin have similar rescuing effect in *pash-1(mj100)* and *mir-35(ndf50)* embryonic lethal genotypes.

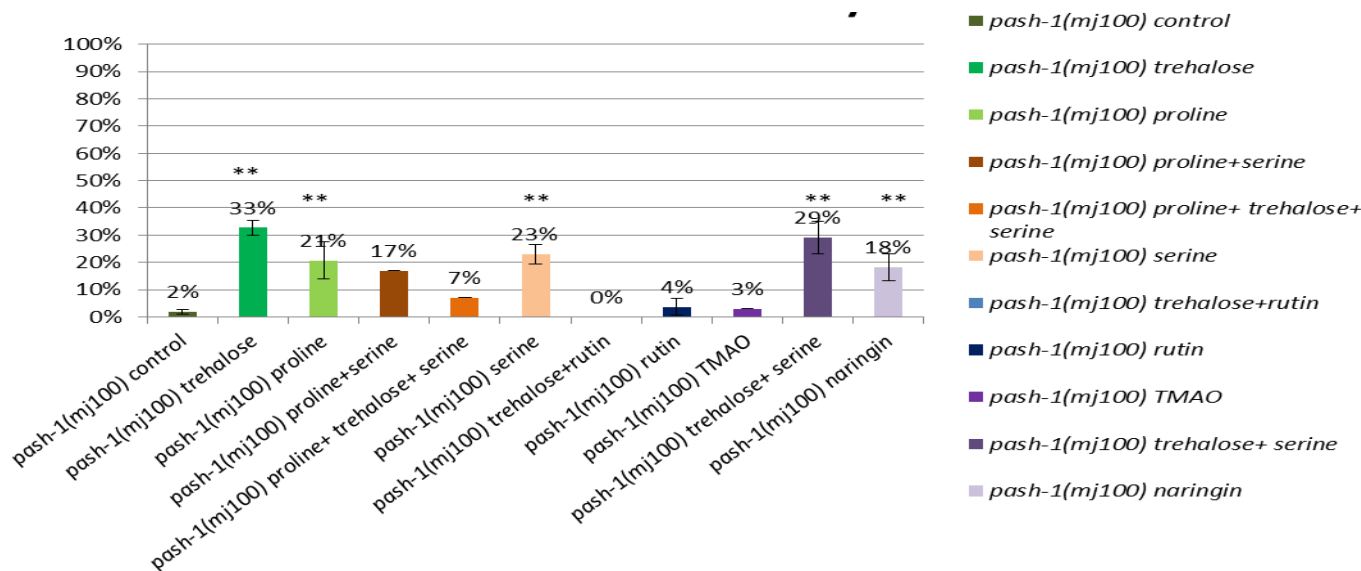
Trehalose is a disaccharide which could act as osmolyte, autophagy inducer and carbon source. After the observation of approximately 40% hatching of *pash-1(mj100)* treated with trehalose, we focused on which of these capacities of trehalose is the reason of this phenotype. Because trehalose has been mostly investigated in plants to be an important regulator of growth, we focused on other important osmolytes in plants. Proline and serine are amino acids which are shown to be an essential stress protectants and embryogenesis (Hayat et al., 2012). Trimethylamine N-oxide (TMAO) is an important methylamine in plants and its structural properties are similar to trehalose. Naringin and rutin are essential flavonoids with their antimicrobial pharmaceutical usages (Harmon & Patel, 2004; Heo et al., 2004).

As seen in **Figure 3.23**, proline and serine (with same concentration of trehalose 5mM) increased the hatching of *pash-1(mj100)* embryos to 21% and 23%, respectively. Naringin (with the same concentration of trehalose 5mM) raised the hatching of *pash-1(mj100)* embryos to 18%. On the other hand, rutin and TMAO did not change the embryonic lethal phenotype of *pash-1(mj100)*.

Since proline and serine had a positive effect on hatching, we wanted to examine whether combining proline or serine with trehalose have a better effect on the survival of the embryos. For that reason, serine and trehalose were used together, each with 2,5mM concentration. Combination of trehalose and serine increased hatching of *pash-1(mj100)* to 29%. Combination of trehalose and serine increased hatching of *pash-1(mj100)* to 29%. Although trehalose and serine combination shows the most effective result among trehalose and serine, it is not sufficient amount to conclude that the effect is additive. Combination of three chemicals together (each with 2,5 mM concentration); trehalose, serine and proline

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together did not impact on embryonic lethality of *pash-1(mj100)* with 7%. It could be explained by increased osmolarity with total 7,5mM concentration.



Chemicals	% of hatched animals in <i>pash-1(mj100)</i>	Standard deviation	Significancy
control	2%	0,013058946	
trehalose	33%	0,049206726	1,91327E-07
TMAO	3%	0,0459	0,038598114
proline	21%	0,117993093	0,001972639
serine	23%	0,061441029	0,055287338
naringin	18%	0,085080994	0,000810856
rutin	4%	0,052769163	0,192035081
trehalose+proline	17%	0,098994949	0,055287338
trehalose+ serine	29%	0,101566247	0,000120376
trehalose+proline+serine	7%	0,061685006	0,187178185

Figure 3.23: Effects of the osmolytes in *pash-1(mj100)* embryonic lethality. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Four biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

pash-1 rescue allele was examined with trehalose, proline, serine, TMAO, naringin and rutin. None of these chemicals significantly rescue the embryonic lethality of *pash-1* rescue.

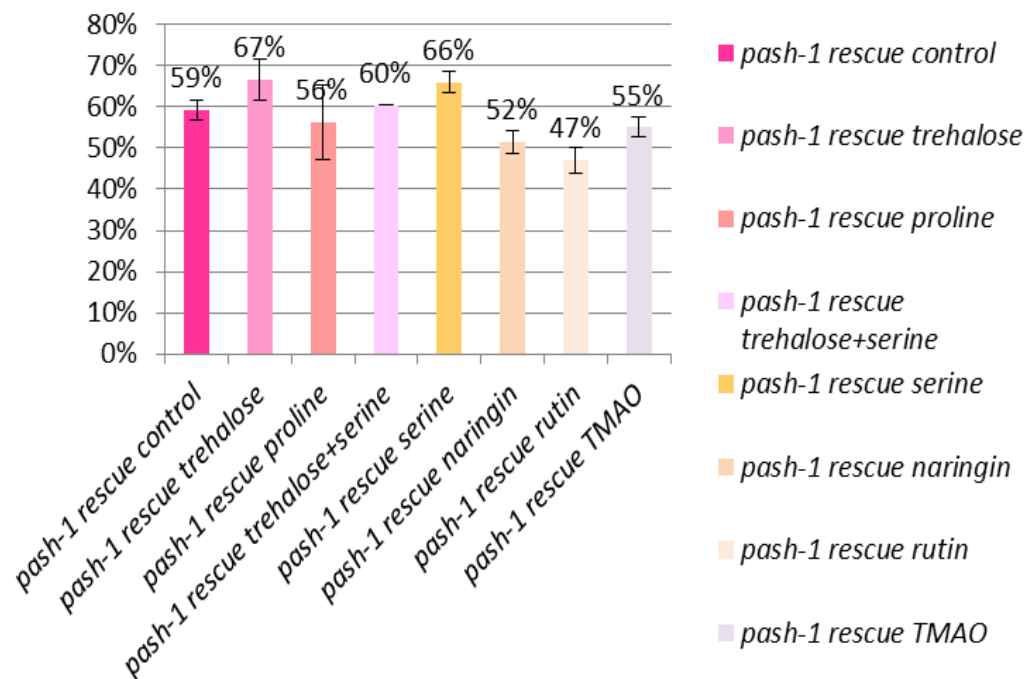


Figure 3.24: Effects of the osmolytes in *eft-3::pash-1::gfp::unc-54* embryonic lethality. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation.

RESULTS

pash-1(mj100) is the strongest allele of *pash-1*. Using this allele, it is possible to stop miRNA production; therefore it allows to investigate roles of miRNAs in post-developmental stages.

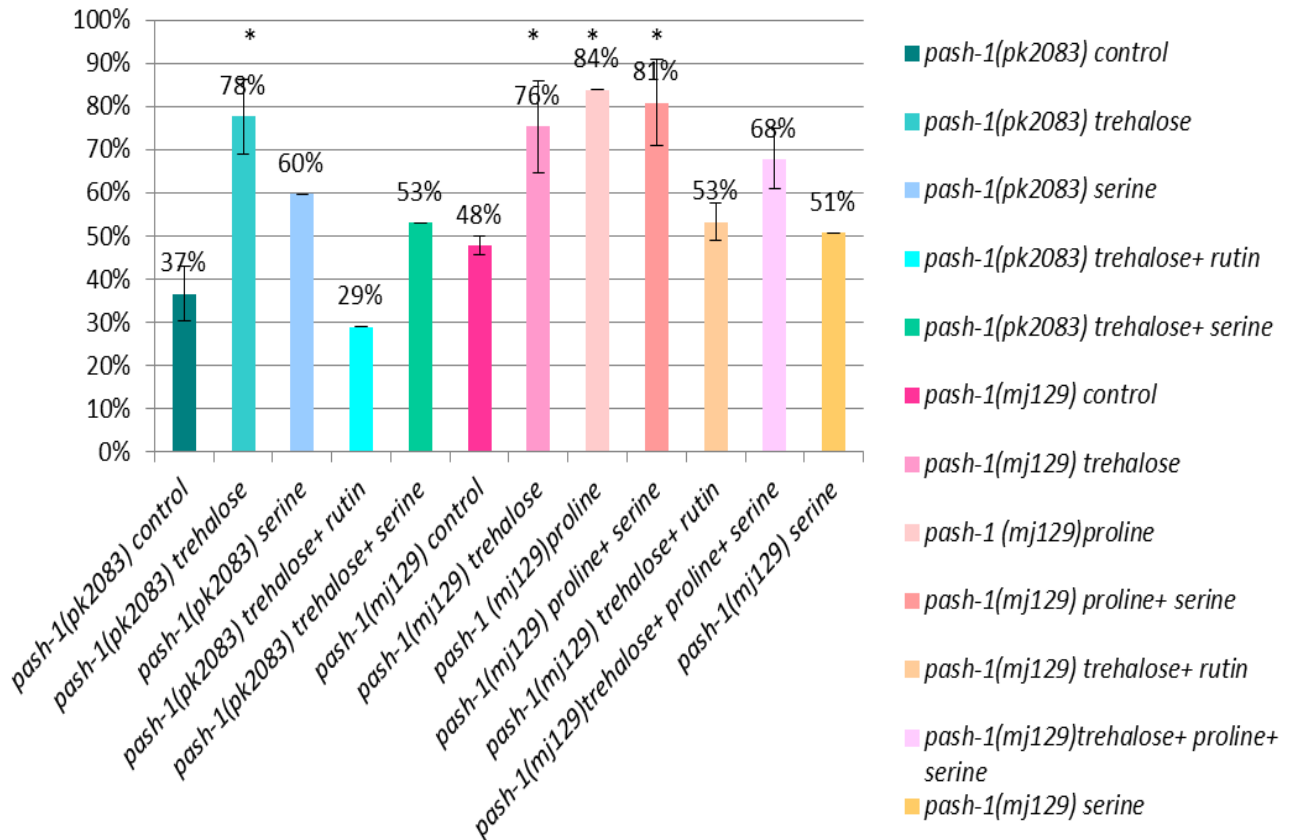
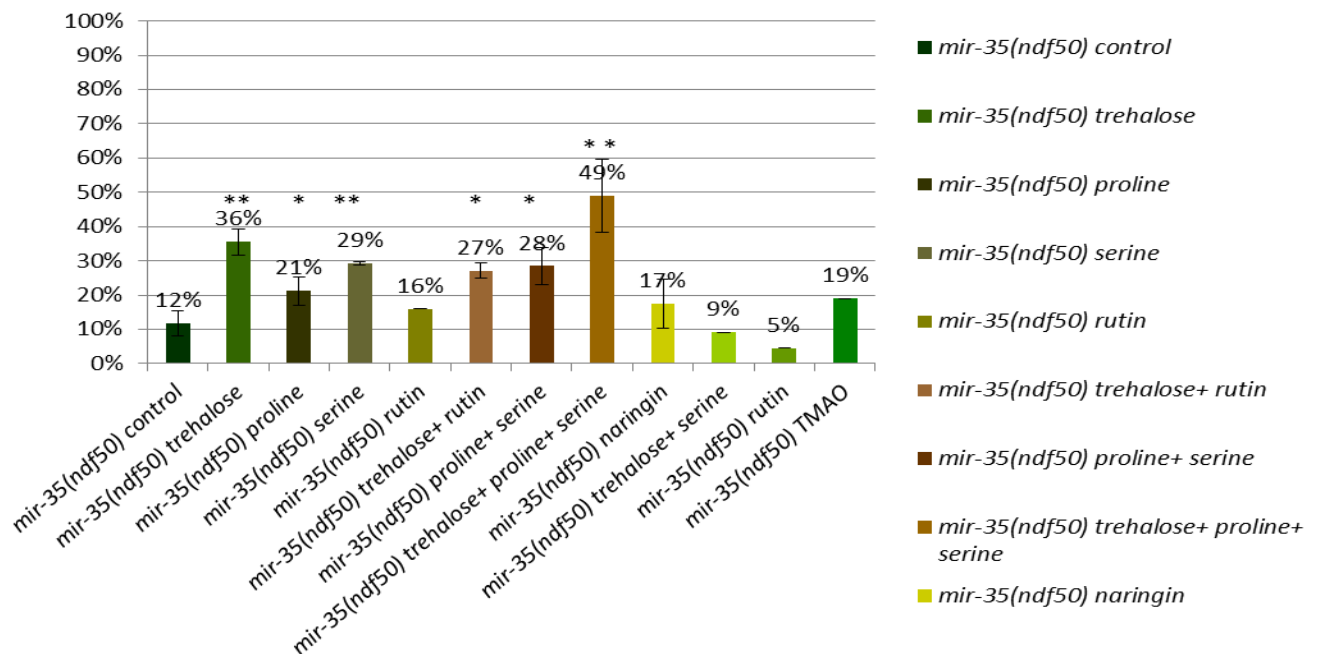


Figure 3.25: Effects of the osmolytes in embryonic lethality of weak alleles of *pash-1*. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated from Student's t-test.

RESULTS

Albeit weaker, there are additional viable *pash-1* alleles, *pk2083* and *mj129*. Mutant animals of these alleles also die at embryonic stages and few animals that survive become sterile adults. Therefore, it is maintained as heterozygotes. First, we determined if these alleles of *pash-1* gene have embryonic lethality and whether this phenotype can be rescued by trehalose, proline or serine treatments. Similar to *pash-1(mj100)*, Trehalose, serine, trehalose and serine together increased hatching of *pash-1(pk2083)* from 37% to 78%, 60% and 53% respectively. Trehalose, proline, proline and serine together significantly increased hatching of *pash-1(mj129)* embryos from 48% to 76%, 84% and 81% respectively.

RESULTS



Chemicals	% of hatched animals in <i>mir-35(ndf50)</i>	Standard deviation	Significancy
control	12%	0,061690989	
trehalose	36%	0,066148625	0,000409632
TMAO	19%	0,121600939	0,031054797
proline	21%	0,071299446	0,034532209
serine	29%	0,009007246	0,000432628
naringin	17%	0,123115475	0,211539739
rutin	10%	0,081285428	0,469301638
trehalose+proline	35%	0,092807765	0,016927138
trehalose+ serine	28%	0,021856028	0,050426839
trehalose+proline+serine	49%	0,183847763	0,003190643

Figure 3.26: Effects of the osmolytes in *mir-35(ndf50)* embryonic lethality. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Four biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated from Student's t-test.

RESULTS

As seen in **Figure 3.26**, proline and serine increased hatching of *mir-35(ndf50)* embryos to 21% and 29%, respectively. However, rutin, naringin and TMAO did not significantly rescue the embryonic lethal phenotype of *mir-35(ndf50)*. Combination of proline/ serine and trehalose/ rutin increased hatching of *mir-35(ndf50)* to 27% and 28% respectively. Combination of three chemicals together; trehalose, serine and proline each with 2,5mM concentration has the most robust effect on embryonic lethality of *mir-35(ndf50)* with 49% different from *pash-1(mj100)*. Corresponding chemicals did not cause variation on hatching of wild-type embryos.

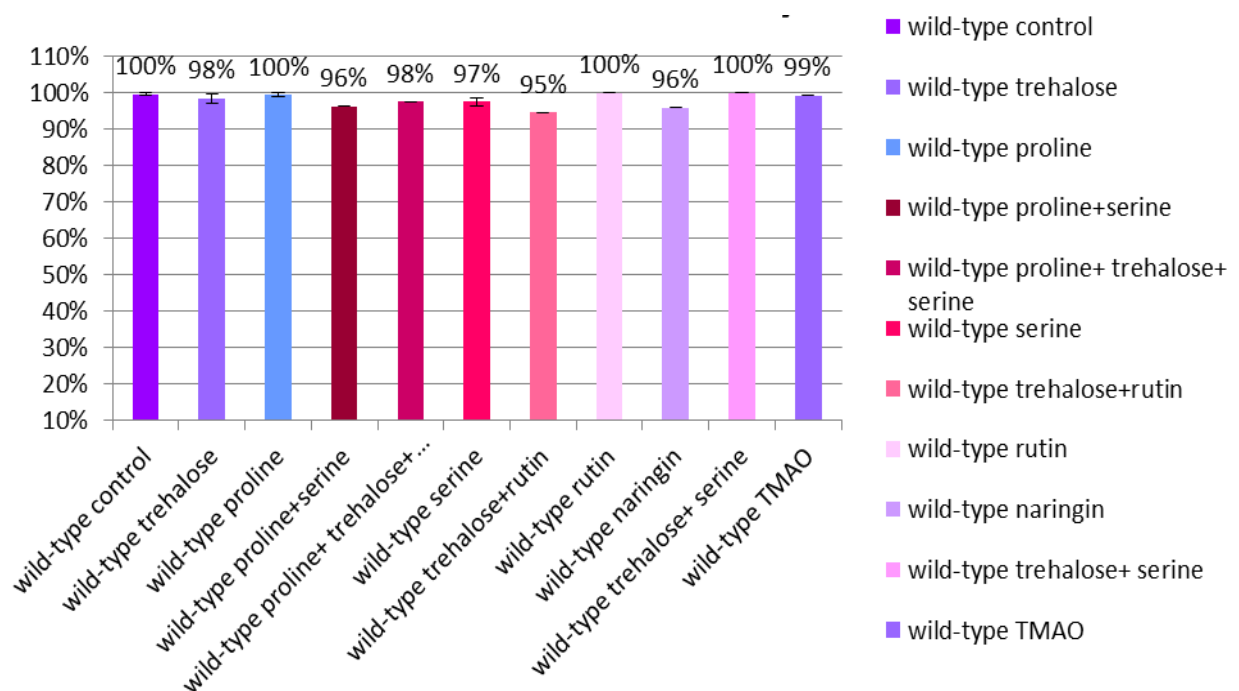


Figure 3.27: Effects of the osmolytes to hatching of wild-type embryos. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Four biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

Wild-type animals were also tested with trehalose, proline, serine, TMAO, naringin and rutin. None of these chemicals significantly change the 100% of hatching of wild-type adults.

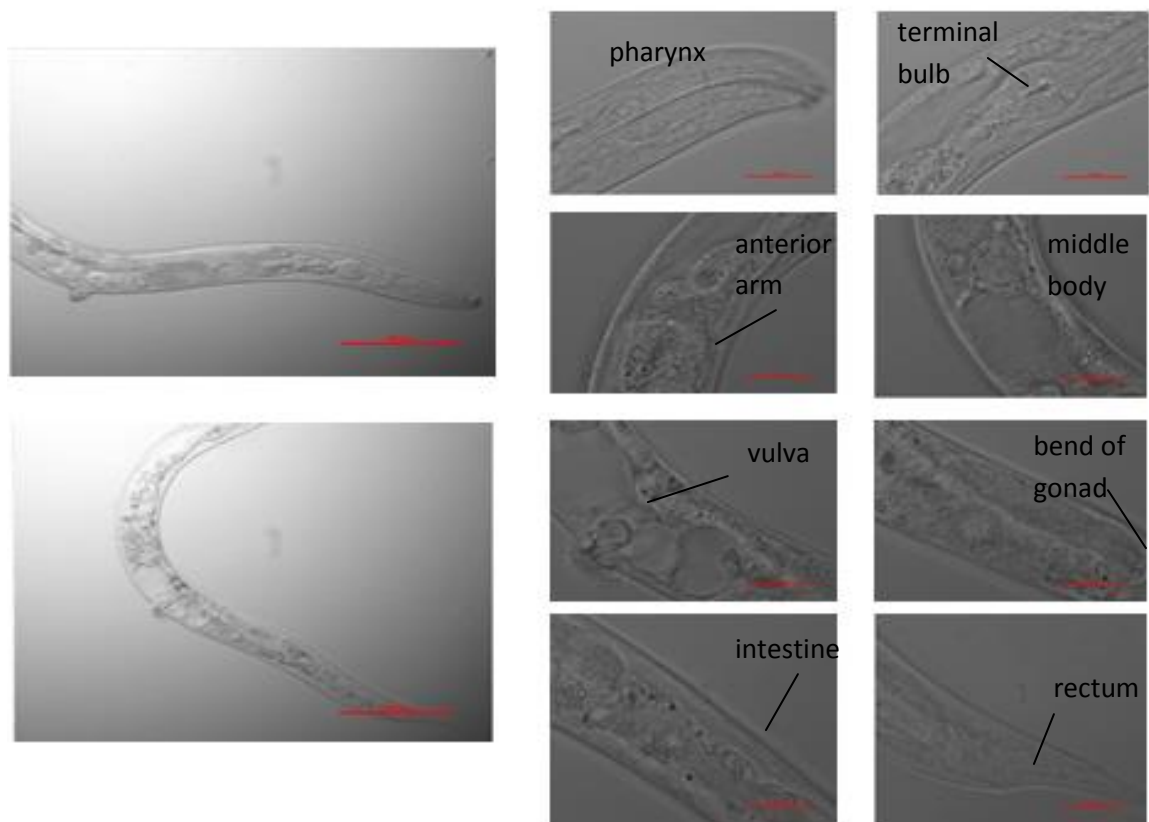


Figure 3.28: Serine treated hatched *pash-1(mj100)* L1. DIC images show an L1 animal whose mother was treated with serine.

3.16 Trehalose and other osmolytes proline and serine enhance the embryonic development defects of heterochronic gene deletions, *lin-4* and *let-7*.

lin-4 and *let-7* are important heterochronic genes in *C. elegans*. *lin-4* controls cell-fate decisions from L1 stage to L2 stage transition, while *let-7* regulates the transition from the L4 to the adult stage. In the assay, weak alleles of *lin-4* and *let-7* were used, since their null mutants are not viable. Although both *mn112* and *e912* are weak alleles, several developmental defects were observed in these mutant animals such as small brood size, partial embryonic lethality. *e912* has additional defects leading to uncoordinated locomotion, failure to vulva formation and clear body.

Results in **Figure 3.29** show the effect of trehalose and other osmolytes on miRNA related heterochronic gene mutants *let-7(mn112)* and *lin-4(e912)*. 38% hatching of *let-7(mn112)* was increased significantly to 56% with trehalose and to 66% with proline/serine and proline/serine/trehalose. Naringin and serine alone didn't improve the hatching of *let-7(mn112)*. We have seen the similar improvement with *lin-4(e912)*. *lin-4(e912)* had 91% embryonic lethality. Embryonic survival was increased to 79% with trehalose treatment. Hatching of *lin-4(e912)* also increased to 55% with trehalose/proline/serine combination. Interestingly, naringin and serine alone made embryonic lethality of *lin-4(e912)* worse. *lin-4(e912)* worms treated with naringin and serine become sterile in all 3 biological replicates. Trehalose increases brood size and rescues embryonic lethality of *lin-4(e912)* animals, while trehalose has no effect on brood size of *let-7(mn112)* animals and slightly increased percentage of their hatching.

RESULTS

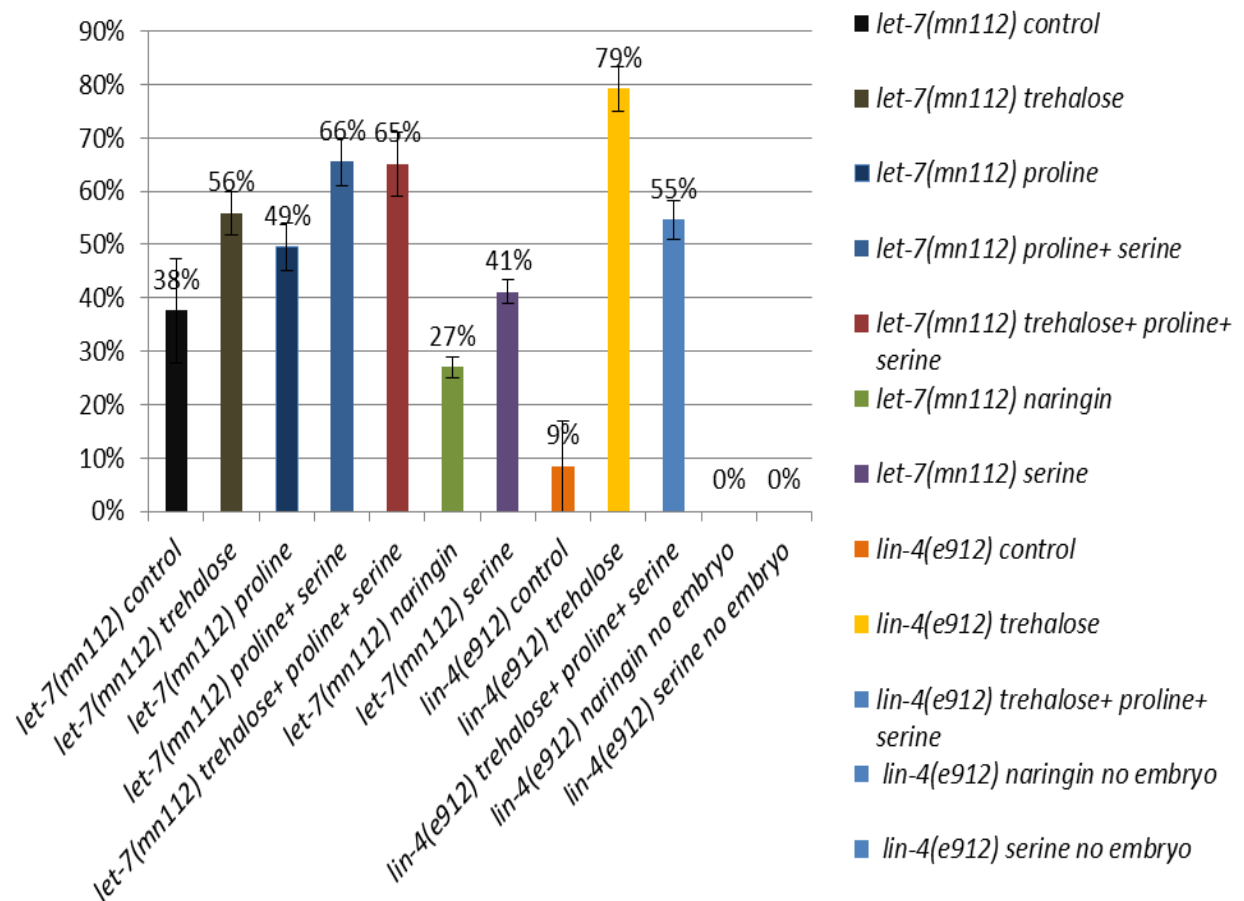


Figure 3.29: Effects of trehalose and other osmolytes in *lin-4* and *let-7* mutants' embryonic hatching. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Concentration of the each chemical was 5mM for single treatments and 2.5 mM for combination treatments. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

One of the phenotypes of *let-7(mn112)* is enlarged lumen. As seen in **Figure 3.30**, the most significant change after *let-7(mn112)* worms were treated with trehalose since they reached adulthood is that lumen size of *let-7(mn112)* returned to normal size with the influence of trehalose.

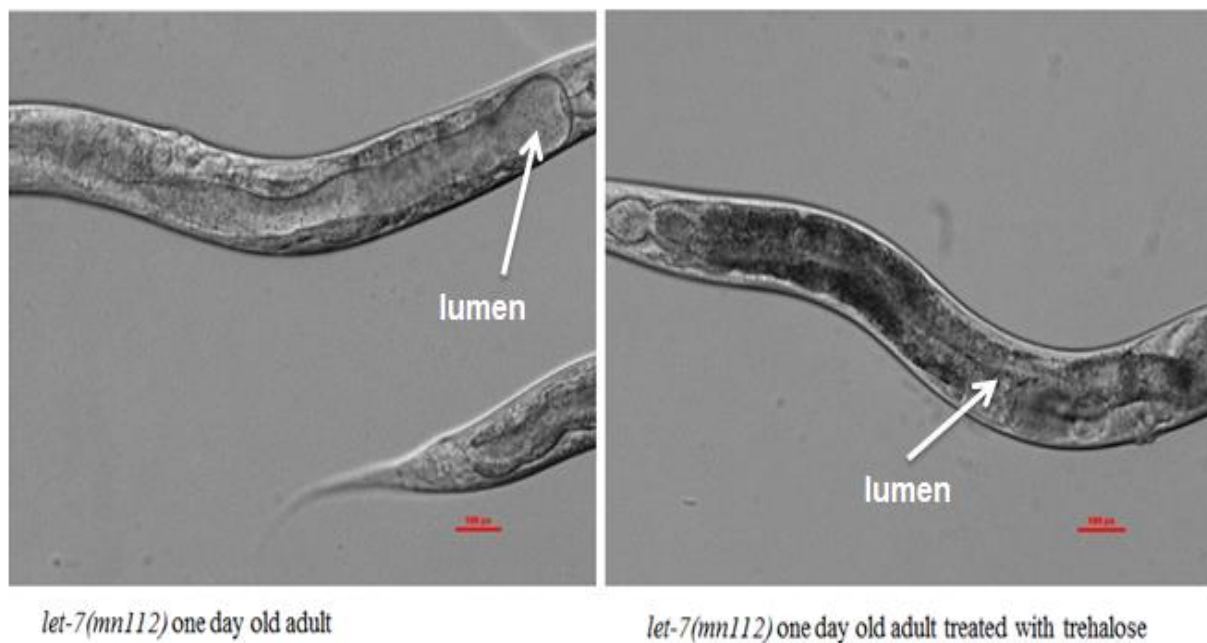


Figure 3.30: Trehalose reduces the size of *let-7(mn112)* enlarged lumen. Three biological replicates were carried out with 6 worms in each replicate. DIC images show 1 day old adults treated and not treated with trehalose.

3.17 Trehalose does not rescue embryonic lethality of *pash-1(mj100)* and *mir-35(ndf50)* acting as autophagy inducer.

Trehalose has been demonstrated to act as an mTOR-independent autophagy inducer. Autophagy can be inhibited by 3-methyladenine (3-MA) and enhanced by rapamycin, an mTOR-dependent autophagy inducer (Aguib et al., 2009). In this assay, we wanted to investigate whether rapamycin and spermidine, other autophagy inducers have similar effect like trehalose and 3-MA could reduce this effect.

Trehalose was the only autophagy inducer, which significantly increased the hatching percentages of *pash-1(mj100)* and *mir-35(ndf50)*. Trehalose increased embryonic lethality in *pash-1(mj100)* from 2% to 32% and in *mir-35(ndf50)* from 21% to 42% as also similarly observed in previous experiments. Rapamycin also enhanced hatching of *pash-1(mj100)* and *mir-35(ndf50)* to a lesser extent than the trehalose treatment. Rapamycin increased hatching of *pash-1(mj100)* from 2% to 11% and in *mir-35(ndf50)* from 21% to 37%. Rapamycin was dissolved in DMSO, therefore DMSO was used as a control in the experiment, interestingly DMSO also influences embryonic lethality of the *mir-35(ndf50)* mutants (compare 21% to 46%) and it does not show any effect on *pash-1* mutants. For this reason, slight enhancement in embryonic hatching in rapamycin treatment could be attributed to the effect of DMSO. . Spermidine has no significant effect on hatching of the *pash-1(mj100)* (compare 2% to only 4%) and of the *mir-35(ndf50)* embryos (from 21% to 28%). Inhibition of autophagy also didn't cause any changes in both *pash-1(mj100)* and *mir-35(ndf50)* mutant embryos.

We concluded that trehalose does not rescue embryonic lethality of *pash-1(mj100)* and *mir-35(ndf50)* acting as autophagy inducer.

RESULTS

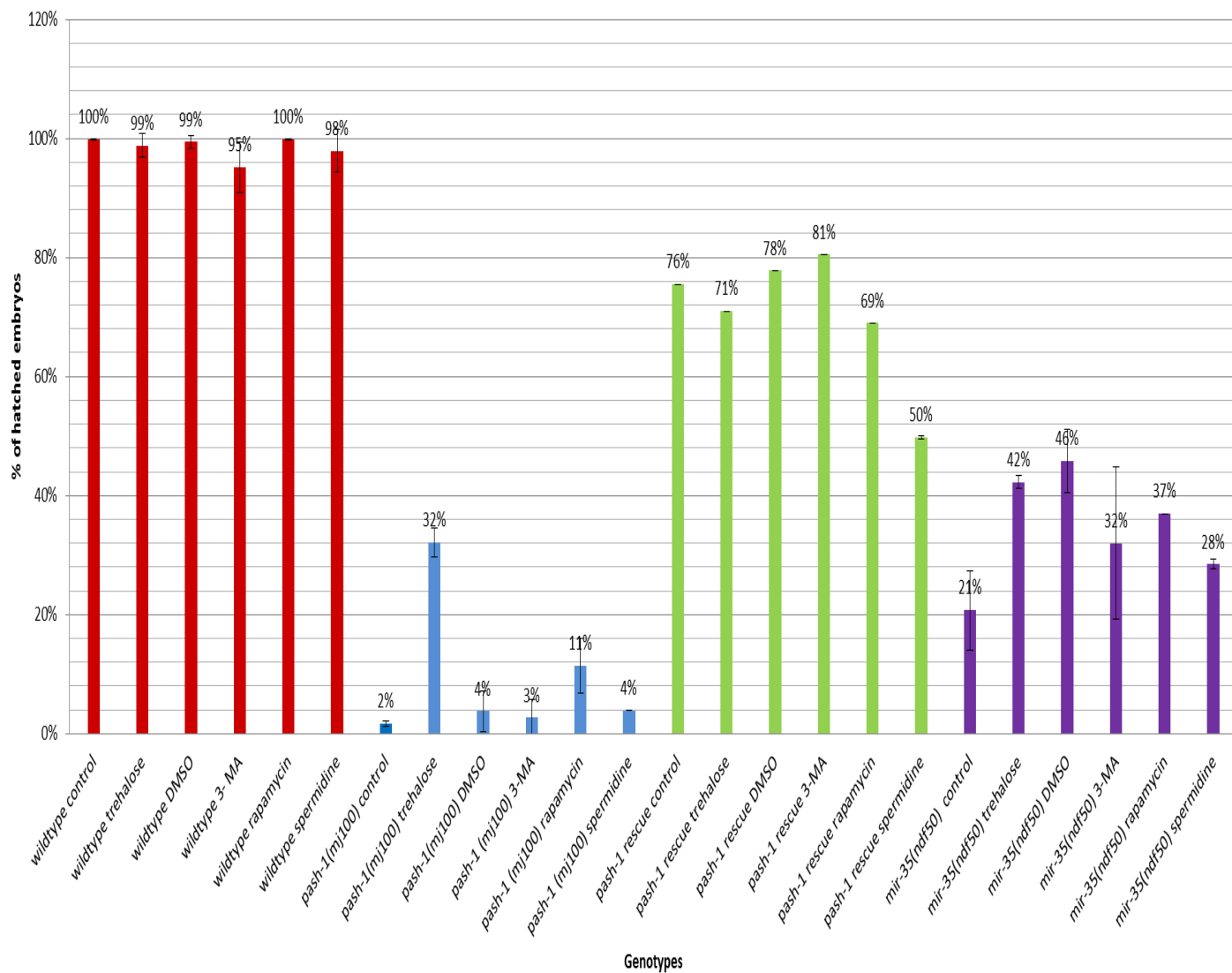


Figure 3.31: Autophagy inducers do not rescue embryonic lethal phenotypes of *pash-1(mj100)* and *mir-35(ndf50)*. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

3.18 Trehalose rescues the embryonic lethal phenotypes of VAB10 mutant alleles.

Since we found some clues that trehalose could rescue embryonic lethality of miRNA related deletions which are mostly died at later stages of embryogenesis, we wanted to try other genes with late-embryonic defects. *vab-10* is a FO gene that encodes spectraplakins cytoskeleton crosslinking protein. FOs are attached to cuticle and muscle cells.

Figure 3.32 illustrates the *vab-10* gene location on the genome. *vab-10* encodes a large protein consisting of with two isoforms, namely VAB10A and VAB10B. VAB10A is plectin that is required for IF attachment, while VAB10B is plakin that connects muscle to epidermis.

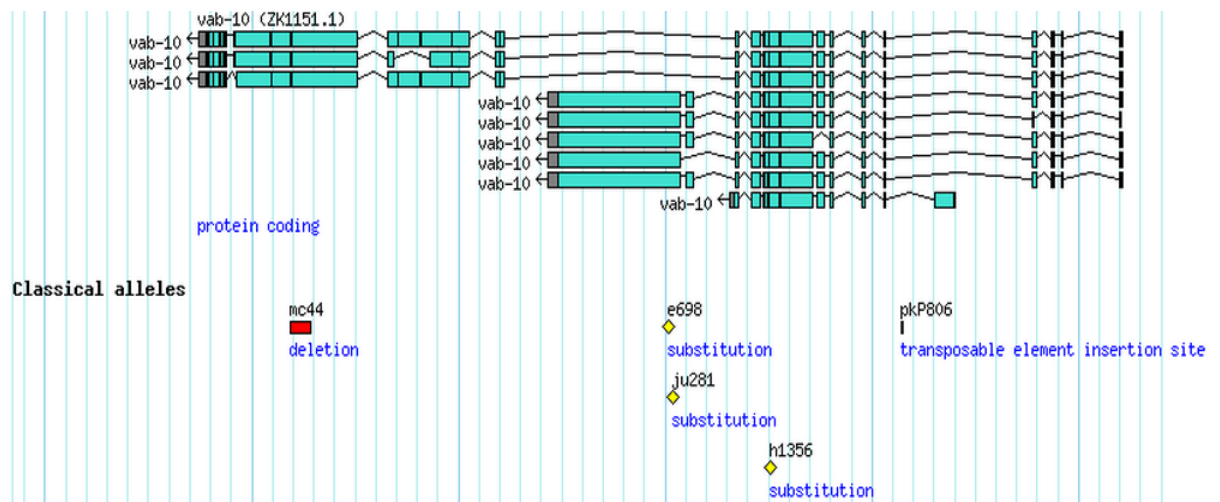


Figure 3.32: *vab-10* gene deletion placed on the genome. (From www.wormbase.org)

RESULTS

VAB10A deletion causes epidermal detachment from the cuticle and VAB10B deletion resulted with epidermal thickness during embryonic development (Bosher JM, Hahn B-S, Legouis R, et al., 2003).

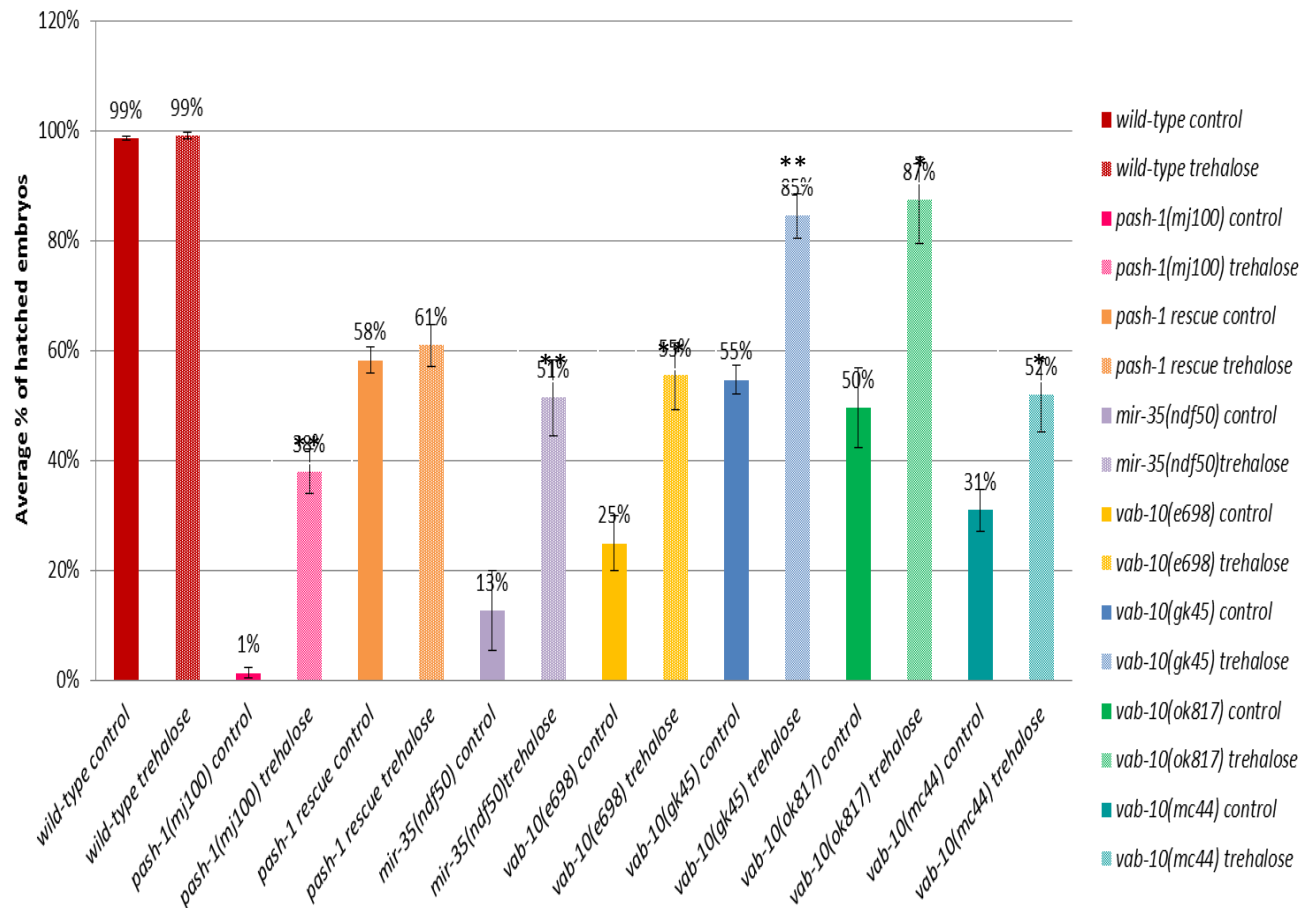


Figure 3.33: Effects of trehalose and other osmolytes in embryonic lethality of different *vab-10* alleles deletions. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

Results in **Figure 3.33** shows different *vab-10* alleles treated with trehalose. 25% hatching of *vab-10a(e698)* was increased to 55%. Embryonic hatching of *vab-10a(gk45)* was rescued from 55% up to 85% treated with trehalose. 50% hatching of *vab-10b(ok817)* increased to 87% and 31% hatching of *vab-10b(mc44)* increased to 52% with trehalose treatment.

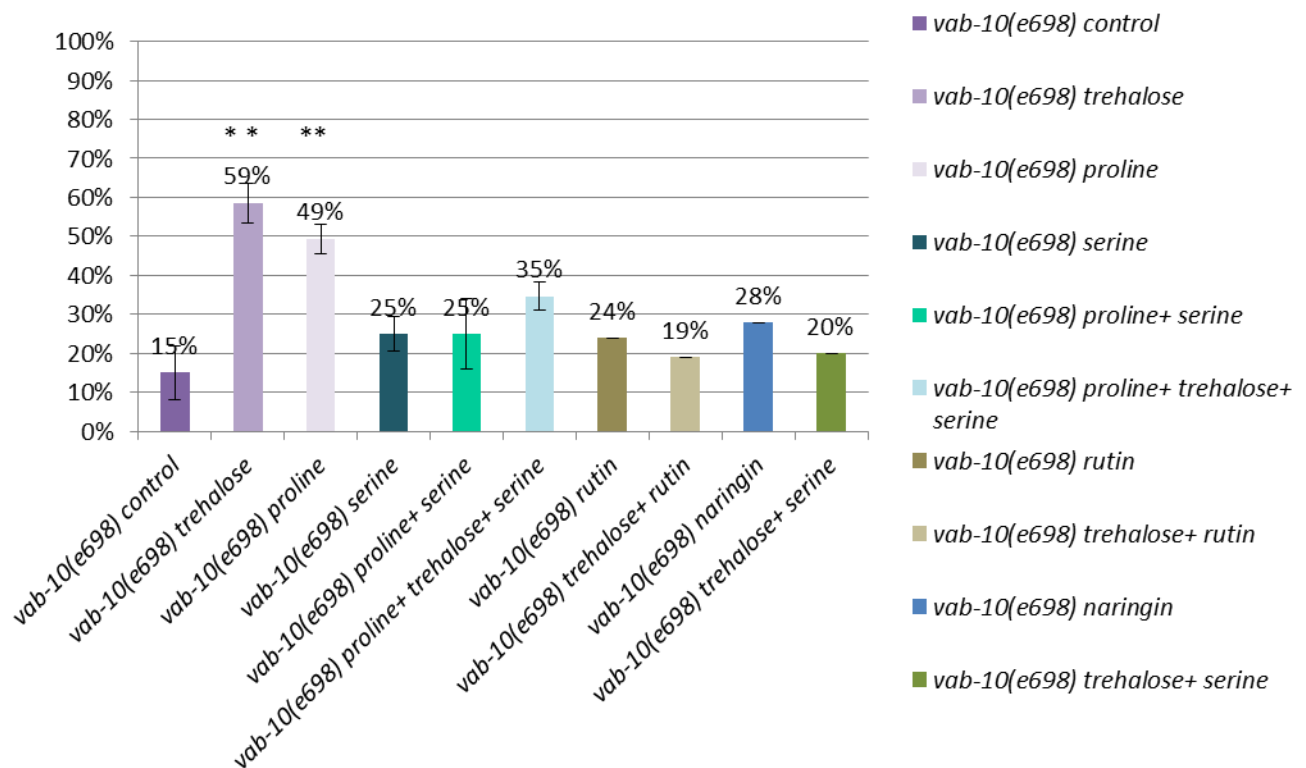


Figure 3.34: Effects of trehalose and other osmolytes in embryonic lethality of *vab-10(e698)*, an allele of VAB10A locus. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

Figure 3.34 demonstrates effects of other osmolytes in embryonic lethality of *vab-10a(e698)*. Hatching of *vab-10a(e698)* was increased from 15 to 49% with proline treatment. Combination of trehalose/proline/serine also improves hatching of *vab-10a(e698)* to 35%. Other osmolytes; serine, rutin, naringin and their combination with trehalose didn't cause any significant change in hatching percentage of *vab-10a(e698)*.

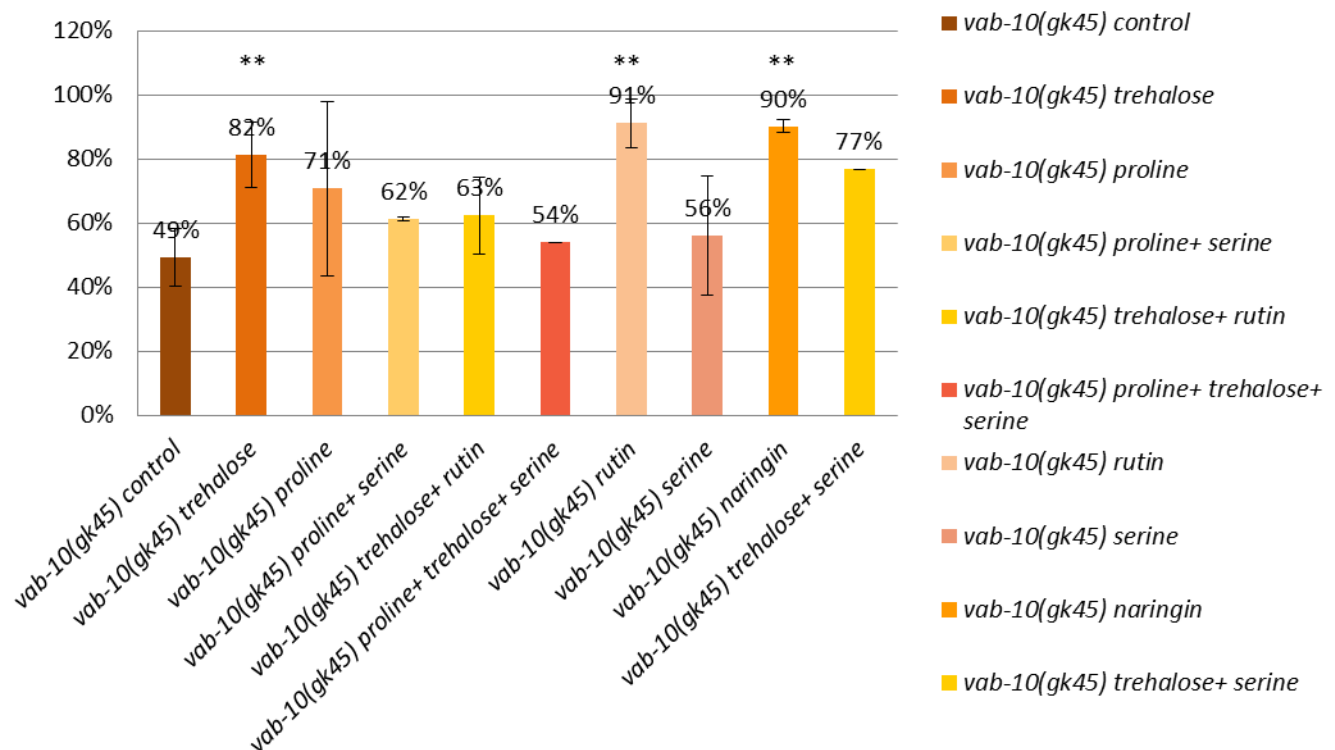


Figure 3.35: Effects of trehalose and other osmolytes in embryonic lethality *vab-10(gk45)*, an allele of *VAB10A* locus. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

RESULTS

Results in **Figure 3.35** demonstrate effects of the osmolytes in embryonic lethality of *vab-10a(gk45)*. Interestingly, hatching of *vab-10a(gk45)* was increased only treated with proline from 49% to 72% and treated with rutin to 91%. Other osmolytes; serine, naringin and their combination with trehalose didn't cause any significant change in hatching percentage of *vab-10a(gk45)*.

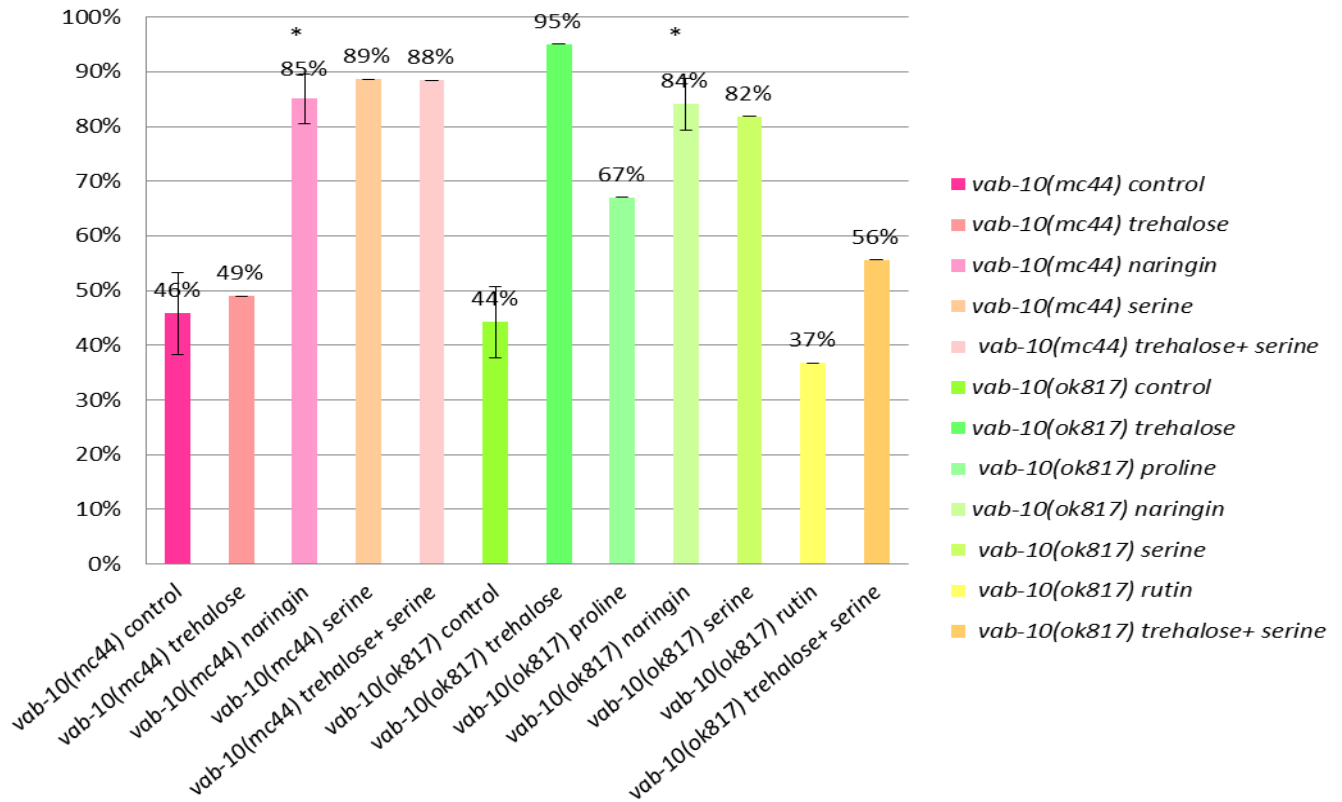


Figure 3.36: Effects of trehalose and other osmolytes in embryonic lethality *vab-10(mc44)* and *vab-10(ok817)*, alleles of VAB10B locus. L4 worms were kept overnight at 15°C, they were then shifted to 25°C. Next, worms were individually transferred to 6-well NGM plates seeded with UV killed *E. coli* HB101. The mothers are transferred to new 6-well plates every 12 hours and embryos and hatched worms were counted in each well until the reproduction ceases. Three biological replicates were carried out with 6 worms in each replicate. Error bars represent standard deviation. p-Values were calculated by Student's t-test.

Figure 3.36 shows the percentages of embryonic hatching of *vab-10b(mc44)* and *vab-10b(ok817)* treated with osmolytes. Hatching of *vab-10b(mc44)* was increased from 46% to 85% treated with naringin, to 78% treated with serine. %. Embryonic hatching of *vab-10b(ok817)* was rescued from 44% to 69% treated with proline, to 84% treated with naringin and to 80% treated with serine. Rutin did not change percentage of hatching.

CHAPTER 4

DISCUSSION

In this study, we examined the role of trehalose in miRNA dependent embryonic development of *C. elegans*. We used a temperature sensitive allele *mj100* of *pash-1* gene in order to study miRNAs in our lab. Because *pash-1* gene loss of function mutation consists of complete deletion of miRNAs, we could examine the roles of miRNAs conditionally using temperature sensitive property of the allele. As expected, *pash-1(mj100)* mutant animals involve many abnormal phenotypes such as short lifespan, locomotion defects, small brood size and mating defects. Embryonic lethality is also one of these phenotypes and *pash-1(mj100)* animals are 100% embryonic lethal at restrictive temperature, 25°C. As far as we know, there is no treatment that can reverse this phenotype. In the present study, it is shown that trehalose actually can rescue embryonic lethality of *pash-1(mj100)* animals to 35-40%. We added other embryonic lethal miRNA family mutants, *mir-35(ndf50)* and *mir-52(ndf58)* to the experiments. As well, we have observed that trehalose increases hatching of *mir-35(ndf50)* from 15-20% to 45-50% and hatching of *mir-52(nDf58)* from 20-25% to 50 %. 40% increase of hatching in *pash-1(mj100)* worms is very promising recovery, since *pash-1* rescue embryos are also 45% embryonic lethal. It means that trehalose can rescue embryonic lethality of *pash-1(mj100)* almost as *pash-1* all tissues rescued construct.

Honda et al have shown that trehalose increases lifespan of *C. elegans* 30% by reducing polyglutamine (polyQ) - and poly alanine (polyA) - mediated protein aggregation level(Honda et al., 2010). To validate the result and investigate whether this finding is adapted in miRNA deficient mutants, *pash-1(mj100)*, *mir-35(nDf50)* and *mir-52(nDf58)*,

DISCUSSION

we performed lifespan assay with trehalose. We validated the outcome of Honda et al with 20% increase in lifespan of wild-type. Trehalose additionally increased half-life of *pash-1(mj100)* 50% and maximum lifespan of *pash-1(mj100)* 14% and half-life of 5 *mir-52(ndf58)*, 160% and maximum lifespan of *mir-52(ndf58)* 80%. In addition to that, trehalose decreases paralysis of *pash-1(mj100)* from 40% to 20% at third day, from 55% to 25% at fourth day, from 97% to 15% at fifth day and from 98% to 22% at sixth day of adulthood. Both lifespan extension and improvement of motility of *pash-1(mj100)* treated with trehalose confirmed that trehalose helps to recover embryonic developmental and post-developmental defects of miRNA deletion mutants.

One of the phenotypes of *pash-1(mj100)* animals is small brood size. Next we examined brood size of wild-type, *pash-1(mj100)*, *pash-1 rescue* and *mir-35(ndf50)* animals treated with trehalose. The results illustrate that trehalose does not change the brood size of *pash-1(mj100)*, and *mir-35(ndf50)* animals proving that trehalose specifically enhances quality of the embryos not their quantity.

Alvarez et al showed that *mir-35(ndf50)* embryos die after 2-fold stage (Alvarez-Saavedra, E., and H. R. Horvitz, 2010). To validate this finding and find out whether *pash-1(mj100)* embryos also die at similar stages, we characterized developmental stages of dead *mir-35(ndf50)* and *pash-1(mj100)*. The results indicate that both *mir-35(ndf50)* and *pash-1(mj100)* embryos die mostly in later stages of embryonic development which were close to hatching after 2-2,5 fold stages. Arrest of the embryos of both *mir-35(ndf50)* and *pash-1(mj100)* might be due to large vacuole formation and apoptosis during embryogenesis. Development of their pharynx and tail is also altered because of loss of miRNAs. The molecular mechanisms underlying these defects were not characterized in this thesis. However, it will be of a great interest to characterize the morphological defects of these

DISCUSSION

mutant animals to gain better understanding of the molecular mechanisms that gets dysregulated in the absence of miRNAs.

Next, we examined whether *mir-35(ndf50)* and *pash-1(mj100)* have egg laying defects. While wild type animals lay eggs that are in gastrulation stage, animals with egg laying defects accumulates embryos in their uterus. These embryos are usually found in late stages of development. We visualized unlaidd embryos inside the one day old adults of *mir-35(ndf50)* and *pash-1(mj100)* animals, embryos in uterus were predominantly found in post-gastrulation stages, such as 28% of *mir-35(ndf50)* and 22 % of *pash-1(mj100)* embryos were in comma stage, while 74% of wild-type embryos in the uterus of wild type animals were in gastrulation stage . Even a significant percent of the *mir-35(ndf50)* and *pash-1(mj100)* embryos also reached to late embryonic stages. Furthermore, these late stage embryos inside mother contained large vacuoles and morphological defects. Based on these data, we concluded that *mir-35(ndf50)* and *pash-1(mj100)* animals have egg laying defects.

In the presence of trehalose, we saw that ~44% of the embryos survived. Then, we asked if we can increase the survival of embryos if we have wild type *pash-1* expression in a subset of tissues. There are various tissue specific *pash-1* rescue strains. These strains contain *pash-1(mj100)* mutation in the background and also have extrachromosomal arrays that express wild type copy of *pash-1* under the control of tissue specific promoters. To investigate specifically related to which tissue trehalose can act, we used three tissue specific *pash-1* rescue constructs in which *pash-1::gfp* is rescued in all tissues; hypodermis, body wall muscles and both body wall muscles and hypodermis. We did not test neuronal rescue in this assay. Only hatching of muscle rescue embryos increased from 16% to 23% and hatching of hypodermis and muscle rescue embryos increased from 15% to 29% with trehalose.

DISCUSSION

To further understand whether trehalose specifically rescue miRNA related embryonic lethality, we used three miRNA independent temperature-sensitive embryonic lethal mutants; *cgh-1(tn691)* encoding RNA helicase in embryonic cytokinesis, *hcp-6(mr17)* that encodes a holocentric binding protein taking part in the attachment of microtubules to kinetochores and *unc-15(e1402)* which encodes myosin heavy chain protein. Trehalose could not rescue embryonic lethality of these three mutants. It only slightly increased hatching of *unc-15(e1402)* from 6% to 14%. These findings suggest that trehalose acts in specifically miRNA related pathway of embryonic development.

Trehalose is an important disaccharide of glucose accumulated in many organisms with various different properties. Its physical and chemical properties allow it to serve as carbon source, an autophagy inducer and an osmolyte. To understand how trehalose rescues miRNA related embryonic lethality, trehalose effect was compared with

1. Maltose and sucrose, other disaccharides
2. Rapamycin, spermidine and 3-MA, other autophagy inducers and inhibitor
3. Proline, serine, TMAO, naringin and rutin, other osmolytes and flavonoids used for conservation of proteins.

Firstly we examined the effects of maltose, a reducing disaccharide of glucose and sucrose, a non-reducing disaccharide like trehalose on embryonic lethality of miRNA deletions, *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)*.

We found out that only trehalose decreased the embryonic lethality of *pash-1(mj100)*, *mir-35(ndf50)* and *mir-52(ndf58)*. Additionally, sucrose slightly increased the hatching of *pash-1(mj100)* from 2% to 8% and hatching of *mir-35(ndf50)* from 17% to 22%. This small change could be explained by the fact that sucrose and trehalose take place

DISCUSSION

in similar pathways and related pathways. High sucrose levels have been related to high T6P level in a previous study. T6P is regulator protein both in sucrose and trehalose circulatory pathway. T6P is formed by degradation of sucrose and leads to formation of trehalose (Henry et al., 2014).

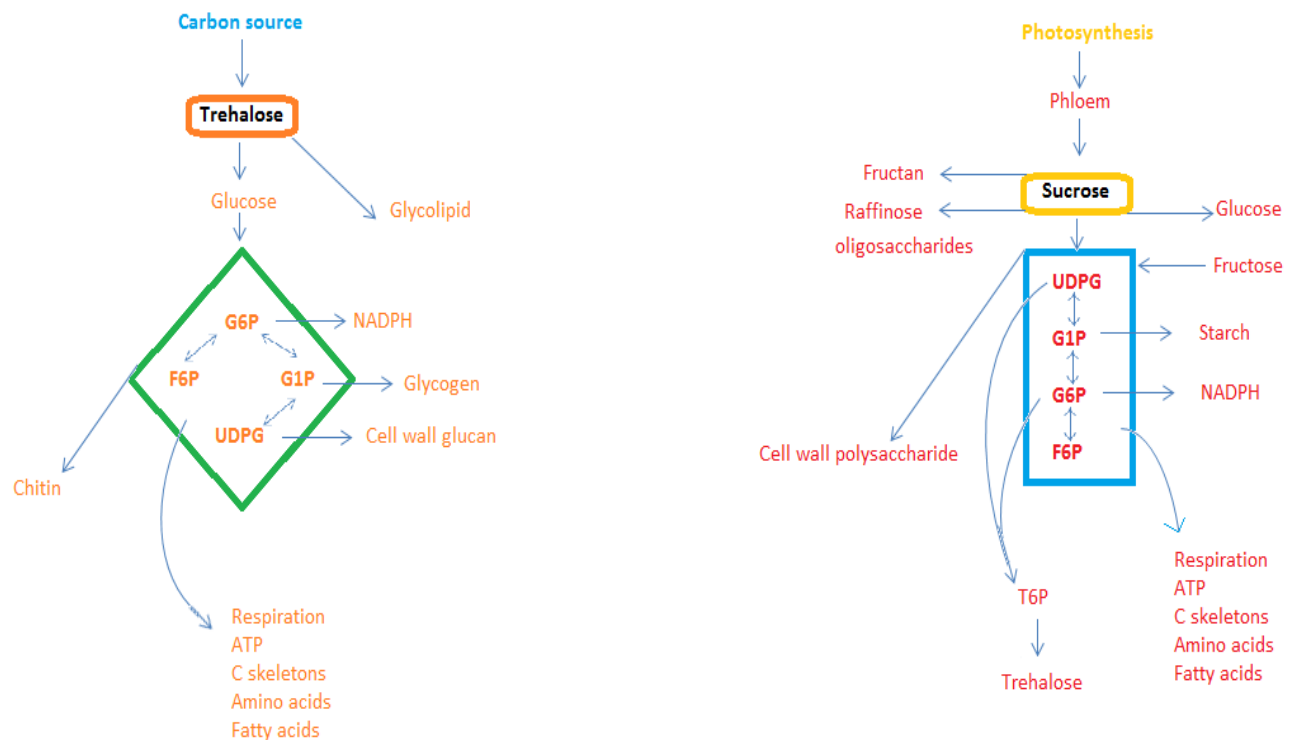


Figure 4.1: Trehalose (in anthropods, fungi, bacteria, archaea and nematodes) and sucrose circulatory pathways. (Adapted from Henry et al., 2014)

Secondly, other autophagy inducers beside trehalose had been examined to understand if trehalose acts as autophagy inducer while rescuing embryonic lethality of miRNA mutants. From previous works, we know that trehalose is an mTOR-independent autophagy inducer and it has parallel impact with rapamycin, an mTOR-dependent

DISCUSSION

autophagy inducer and opposite impact with 3-methyladenine, autophagy inhibitor (Aguib et al., 2009). Here, our focus was to investigate whether rapamycin and spermidine, other autophagy inducers also rescue embryonic lethality like trehalose.

From the results of the assay, trehalose and rapamycin increased the hatching percentages of *pash-1(mj100)* and *mir-35(ndf50)*. Rapamycin's effect was very small compared to trehalose (hatching of *pash-1(mj100)* increased from 2% to 11% and *mir-35(ndf50)* from 21% to 37%). In addition to that rapamycin rescuing effect in embryonic hatching could be because of DMSO which we used to dissolve rapamycin, because DMSO itself already changes hatching of *pash-1(mj100)* from 2% to 4% and in *mir-35(ndf50)* from 21% to 46%. As spermidine, rapamycin or 3MA treatments did not affect the survival of the embryos, we concluded that trehalose does not rescue embryonic lethality of *pash-1(mj100)* and *mir-35(ndf50)* acting as autophagy inducer.

Lastly, we checked another possibility whether trehalose acts as an osmolyte in miRNA dependent embryogenesis of *C. elegans*. For this purpose, we added different osmolytes into the assay. These were proline and serine, amino acids important for stress protection and embryogenesis in plants (Hayat et al., 2012), TMAO, an important methylamine in plants; naringin and rutin, crucial flavonoids (Harmon & Patel, 2004; Heo et al., 2004). Results have shown that proline, serine and naringin increase hatching of *pash-1(mj100)* to 21%, 23% and 18%, respectively. However, rutin and TMAO have no effect on embryonic lethality of *pash-1(mj100)*. Treatment of *mir-35(ndf50)* with the same osmolytes supported the results of *pash-1(mj100)*. Proline and serine also increased hatching of *mir-35(ndf50)* to 21% and 29%, respectively. On the other hand; rutin and TMAO did not enhance the embryonic hatching of *mir-35(ndf50)*. Taken together, these findings may indicate that trehalose could rescue miRNA dependent embryonic lethality acting as both an osmolyte and metabolite, since TMAO, an osmolyte acting similar to

DISCUSSION

trehalose has no effect and amino acids like proline and serine also have similar effects. This could be explained by the fact that trehalose, proline and serine take part differently in the same metabolic pathway of the animals related to embryogenesis.

In the experiments we used α,α -trehalose stereoisomeric form found in organisms. There is an additional isoform, α,β trehalose. To examine if α,β -trehalose has similar effect like trehalose, we used α,β -trehalose with miRNA dependent embryonic lethal genotypes *pash-1(mj100)* and *mir-35(ndf50)*. As a conclusion, α,β -trehalose has rescuing effect like α,α -trehalose albeit at a smaller extent.

We also examined other *pash-1* alleles treated with trehalose. As all these alleles were independently obtained, they are expected to have different background mutations. If the trehalose rescues a *pash-1* dependent phenotype, it should have similar effects on the other alleles of *pash-1*. This will ensure the effect we see is due to rescue of loss of *pash-1* function and not a rescue of a background mutation. We demonstrated that trehalose rescued the embryonic lethality of other alleles of *pash-1(mj100)* at a similar level. Trehalose and serine increased hatching of *pash-1(pk2083)* from 37% to 78% and 60% respectively. Trehalose and proline also increased hatching of *pash-1(mj129)* from 48% to 76% and 84% respectively.

Interestingly, we found out that trehalose and other osmolytes proline and serine alleviated some of the embryonic development defects of heterochronic miRNA gene deletions, *lin-4* and *let-7*. 38% hatching of *let-7(mn112)* was increased to 56% with trehalose and to 66% with proline/serine and proline/serine/trehalose. *lin-4(e912)* 9% hatching was increased to 79% with trehalose treatment. Additionally, trehalose returned enlarged lumen of *let-7(mn112)* to its normal size. These findings also support the fact that trehalose acts to alleviate several miRNA dependent developmental defects. It will be

DISCUSSION

exciting to determine the commonalities between these phenotypes to understand how trehalose acts to reduce the severity of the phenotypes.

Lastly, we assayed *vab-10* alleles with embryonic defects. Because we found some clues that trehalose could rescue embryonic lethality of miRNA related deletions which are mostly died at later stages of embryogenesis, we wanted to try other genes unrelated to miRNAs with late-embryonic defects. *vab-10* is a FO gene that encodes spectraplakins cytoskeleton crosslinking protein with two isoforms, VAB10A (plectin in IF attachment) and VAB10B (plakin between muscles and epidermis).

Previous studies (Bosher JM, Hahn B-S, Legouis R, et al., 2003) have shown that VAB10A deletion causes in epidermal detachment from the cuticle and VAB10B deletion resulted with epidermal thickness during embryonic development. Deletion of *vab-10a* and *vab-10b* cause late embryonic lethality, frequently in elongation stage of embryogenesis. From the assay, we found that trehalose and other osmolytes proline and serine increase embryonic hatching of *vab-10a(e698)*, *vab-10a(gk45)*, *vab-10b(mc44)* and *vab-10b(ok817)*. Because *vab-10* alleles are the only one whose embryonic lethality was partially rescued by trehalose beside miRNA deletion alleles, it could be possible that trehalose may act in later stages of embryonic development of *C. elegans*.

Our major finding is that trehalose helps *pash-1(mj100)* embryos to hatch but hatched embryos treated with trehalose still cannot process their development because of the absence of essential miRNAs for development. We further analyzed the hatched L1s treated with trehalose. These L1s are viable for a limited duration with vacuoles, which could indicate moulting defects related to high protein aggregation in mutant animals and slow pharynx movement (pumping bacteria into the gut slower than wild-type).

DISCUSSION

This study is mainly focused on influence of trehalose in embryonic development of *C. elegans*, but how trehalose exogenously taken from the worms and where it is localized in the body of worms are important questions. We used carminic acid, a fluorescent dye that binds to trehalose and glycogen. Carminic acid staining allowed us to determine the uptake and location of trehalose and to quantify its levels in the adults and embryos. The results of the staining showed that trehalose can be exogenously incorporated from animals. In addition to that, trehalose treated *pash-1(mj100)*, *pash-1 rescue*, *mir-35(ndf50)* and wild-type animals contain higher amount of trehalose in their body, especially around their pharynx and tail. Trehalose accumulation in the embryos of *mir-35(ndf50)* is also very higher compared to wild-type embryos which could be related to trehalose's role in embryogenesis.

CHAPTER 5

CONCLUSION

miRNAs are small non-coding RNAs that regulate post-transcriptional gene regulation. They have important roles in many biological aspects such as development, aging, apoptosis and proliferation. The objective of this study was to identify the role of trehalose, a disaccharide of glucose in embryogenesis of *C. elegans* using an embryonic lethal mutant of miRNA defective *pash-1(mj100)*. For this purpose, embryonic lethal mutants related to miRNAs *mir-35(ndf50)* and *mir-52(ndf58)* and other biological pathways were used with the presence of trehalose. It was shown that trehalose can rescue miRNA dependent embryonic lethality. To better understand the functions of trehalose, other similar types of chemicals were used and examined whether they have similar effects like trehalose. Osmolytes like proline and serine seemed also to have similar rescuing phenotypes.

The thesis focused on the identification of trehalose's roles in miRNA dependent embryonic development of *C. elegans* and the outcomes of the study provide a new aspect that miRNA related defects can be recovered by a simple chemical like trehalose. Future studies are needed to understand molecular mechanism of trehalose in miRNAs in embryogenesis.

CHAPTER 6

FUTURE DIRECTIONS

Future work is going to involve functional characterization of trehalose's effect on embryogenesis. We are going to focus on understanding molecular mechanism of trehalose in miRNAs in embryogenesis. we firstly want to do metabolite analysis of trehalose in mutant and wildtype animals. We are planning to sorting embryos to obtain a synchronized population using a microfluent microfluidic system designed by Dr. Aşkın Kocabaş and use sorted embryos for metabolite analysis and gene expression analysis in order to find effects of trehalose. measuring trehalose contents of the worms would be nice to compare the level of trehalose contents of wild-type, *pash-1(mj100)* and *mir-35(ndf50)* animals in order to answer the question how loss of miRNAs affect trehalose metabolism of the worms and investigate whether loss of miRNAs decreases trehalose levels. Performing RT-PCR would be also valuable to determine PASH-1 protein levels in the presence of trehalose indicating the role of trehalose in miRNA related pathways.

Since we explored that trehalose rescues embryonic lethality of *mir-35(ndf50)* and these mutant animals are able to complete their larval development and become fertile adults in the presence of trehalose, RNAi using potential targets of *mir-35* family could be performed to explore a target gene, which is completely rescued by trehalose. There are ~100 predicted targets of *mir-35* family. RNAi knockdown of its targets do not rescue the lethality. It would be interesting to see if RNAi knockdown of its targets can change the percent of hatched animals in the presence of trehalose. This will also help us to understand how trehalose acst to rescue embryonic lethality. Potential target genes caught by RNAi could help to find out in which pathway trehalose acts on miRNA dependent embryonic lethality of *C. elegans*.

APPENDIX I

Strains	Average of # of embryos
<i>pash-1 control</i>	101
<i>pash-1 trehalose</i>	154
<i>pash-1 maltose</i>	309
<i>pash-1 sucrose</i>	195
<i>pash-1 rescue control</i>	251
<i>pash-1 rescue trehalose</i>	348
<i>pash-1 rescue maltose</i>	308
<i>pash-1 rescue sucrose</i>	343
<i>nDf50 control</i>	103
<i>nDf50 trehalose</i>	244
<i>nDf50 maltose</i>	59
<i>nDf50 sucrose</i>	306
<i>mir-52 control</i>	432
<i>mir-52 trehalose</i>	396
<i>mir-52 maltose</i>	426
<i>mir-52 sucrose</i>	445

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<i>wildtype control</i>	541
<i>wildtype trehalose</i>	555
<i>pash-1 hypodermis rescue control</i>	231
<i>pash-1 hypodermis trehalose</i>	247
<i>pash-1 muscle and hypodermis rescue control</i>	281
<i>pash-1 muscle and hypodermis rescue trehalose</i>	286
<i>pash-1 muscle rescue control</i>	154
<i>pash-1 muscle rescue trehalose</i>	392
<i>wildtype control</i>	541
<i>wildtype α,α-trehalose</i>	555
<i>wildtype α,β-trehalose</i>	421
<i>pash-1(mj100) control</i>	157
<i>pash-1(mj100) α,α-trehalose</i>	143
<i>pash-1(mj100) α,β-trehalose</i>	112
<i>pash-1 rescue control</i>	278
<i>pash-1 rescue α,α-trehalose</i>	112
<i>pash-1 rescue α,β-trehalose</i>	247
<i>mir-35(ndf50) control</i>	446

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<i>mir-35(ndf50) α,α-trehalose</i>	646
<i>mir-35(ndf50) α,β-trehalose</i>	409
<i>wildtype control</i>	437
<i>wildtype trehalose</i>	116
<i>wildtype DMSO</i>	567
<i>wildtype 3- MA</i>	482
<i>wildtype rapamycin</i>	383
<i>wildtype spermidine</i>	686
<i>pash-1(mj100) control</i>	291
<i>pash-1(mj100) trehalose</i>	244
<i>pash-1(mj100) DMSO</i>	327
<i>pash-1 (mj100) 3-MA</i>	301
<i>pash-1 (mj100) rapamycin</i>	258
<i>pash-1 (mj100) spermidine</i>	287
<i>pash-1 rescue control</i>	433
<i>pash-1 rescue trehalose</i>	452
<i>pash-1 rescue DMSO</i>	550
<i>pash-1 rescue 3-MA</i>	559
<i>pash-1 rescue rapamycin</i>	361

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<i>pash-1 rescue spermidine</i>	121
<i>mir-35(ndf50) control</i>	923
<i>mir-35(ndf50) trehalose</i>	682
<i>mir-35(ndf50) DMSO</i>	436
<i>mir-35(ndf50) 3-MA</i>	703
<i>mir-35(ndf50) rapamycin</i>	333
<i>mir-35(ndf50) spermidine</i>	61
<i>wildtype glucN</i>	191
<i>pash-1 (mj100) glucN</i>	225
<i>pash-1 rescue glucN</i>	365
<i>pash-1(pk2083) glucN</i>	321
<i>ndf50 glucN</i>	493
<i>pash-1(pk2083) control</i>	124
<i>pash-1(pk2083) trehalose</i>	311
<i>pash-1 (pk2083)DMSO</i>	256
<i>pash-1(pk2083) 3- MA</i>	29
<i>pash-1(pk2083) rapamycin</i>	102

APPENDIX II

Carminic Acid Staining

Genotypes	Carminic acid average intensity
<i>wild-type pharynx control</i>	1314
<i>wild-type pharynx trehalose</i>	1877,46
<i>pash-1(mj100) pharynx control</i>	1222,49
<i>pash-1(mj100) pharynx trehalose</i>	1698,42
<i>pash-1 rescue pharynx control</i>	778,05
<i>pash-1 rescue pharynx trehalose</i>	893,39
<i>mir-35(ndf50) pharynx control</i>	996,13
<i>mir-35(ndf50) pharynx trehalose</i>	1035,18
<i>wild-type middle body control</i>	1205
<i>wild-type middle body trehalose</i>	1728,07
<i>pash-1(mj100) middle body control</i>	1236,09
<i>pash-1(mj100) middle body trehalose</i>	1752,33
<i>pash-1 rescue middle body control</i>	829,95
<i>pash-1 rescue middle body trehalose</i>	1487,26

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<i>mir-35(ndf50) middle body control</i>	1193,06
<i>mir-35(ndf50) middle body trehalose</i>	1282,32
<i>wild-type rectum control</i>	1156
<i>wild-type rectum trehalose</i>	1888,49
<i>pash-1(mj100) rectum control</i>	1143,2
<i>pash-1(mj100) rectum trehalose</i>	1474,38
<i>pash-1 rescue rectum control</i>	1007,88
<i>pash-1 rescue rectum trehalose</i>	1506,04
<i>mir-35(ndf50) rectum control</i>	1051,79
<i>mir-35(ndf50) rectum trehalose</i>	1322,55

APPENDIX III

RNAi library of *mir-35* targets

Gene symbol	Gene ID	Library	Plate	Reagent	Genes
lin-59	WBGene00003040	JA	3 D03	sjj_T12F5.4	T12F5.4
snf-1	WBGene00004900	JA	5 H05	sjj_W03G9.1	W03G9.1
bli-4	WBGene00000254	JA	9 C02	sjj_K04F10.4	K04F10.4
D2092.4	WBGene00017065	JA	9 H11	sjj_D2092.4	D2092.4
ser-3	WBGene00004778	JA	10 E03	sjj_K02F2.6	K02F2.6
C30F12.2	WBGene00016261	JA	10 G03	sjj_C30F12.2	C30F12.2
F26A3.4	WBGene00009142	JA	12 B10	sjj_F26A3.4	F26A3.4
ztf-11	WBGene00009939	JA	16 H04	sjj_F52F12.6	F52F12.6
F25H2.5	WBGene00009119	JA	18 C09	sjj_F25H2.5	F25H2.5
ced-1	WBGene00000415	JA	20 G11	sjj_Y47H9C.4	Y47H9C.4
nlp-15	WBGene00003753	JA	23 E03	sjj_CC4.2	CC4.2
W05B5.1	WBGene00012274	JA	23 E05	sjj_W05B5.1	W05B5.1
bath-40	WBGene00013689	JA	25 G11	sjj_Y105E8A.e	Y105E8B.4
ptr-23	WBGene00004237	JA	26 F07	sjj_ZK270.1	ZK270.1
Y71F9B.8	WBGene00022129	JA	29 E08	sjj_Y71F9B_297.c	Y71F9B.8
T05C1.1	WBGene00020248	JA	42 D08	sjj_T05C1.1	T05C1.1
tsn-1	WBGene00006626	JA	42 H01	sjj_F10G7.2	F10G7.2
rig-6	WBGene00016354	JA	43 B08	sjj_C33F10.5	C33F10.5
dsh-1	WBGene00001101	JA	44 C02	sjj_C34F11.9a	C34F11.9
F13H8.5	WBGene00017438	JA	46 F06	sjj_F13H8.5	F13H8.11
lin-23	WBGene00003009	JA	46 H06	sjj_K10B2.1	K10B2.1
cex-2	WBGene00023408	JA	50 D08	sjj_T09A5.1	T09A5.1
dab-1	WBGene00000894	JA	51 C04	sjj_M110.5	M110.5
cutl-15	WBGene00011888	JA	52 H11	sjj_T21B10.6	T21B10.6
mab-3	WBGene00003100	JA	54 H05	sjj_Y53C12B.5a	Y53C12B.5
ZK1321.2	WBGene00014261	JA	54 H08	sjj_ZK1321.2	ZK1321.2
T06D8.1	WBGene00011522	JA	57 H01	sjj_T06D8.1	T06D8.1
lin-29	WBGene00003015	JA	59 D08	sjj_W03C9.4	W03C9.4

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cyd-1	WBGene00000870	JA	61 C05	sjj_Y38F1A.5	Y38F1A.5
C13B4.1	WBGene00007545	JA	63 G01	sjj_C13B4.1	C13B4.1
unc-52	WBGene00006787	JA	64 A10	sjj_ZC101.2	ZC101.2
H10E21.5	WBGene00019185	JA	66 A06	sjj_H10E21.5	H10E21.5
gei-16	WBGene00001573	JA	67 A01	sjj_T17H7.4	T17H7.4
toh-1	WBGene00006591	JA	69 F06	sjj_T24A11.3	T24A11.3
msi-1	WBGene00003423	JA	69 H11	sjj_R10E9.1	R10E9.1
sup-26 (tag-310)	WBGene00011199	JA	70 E09	sjj_R10E4.2	R10E4.2
nhl-2	WBGene00003598	JA	72 C04	sjj_F26F4.7	F26F4.7
mig-10	WBGene00003243	JA	79 G05	sjj_F10E9.6	F10E9.6
F42H10.3	WBGene00018367	JA	80 B09	sjj_F42H10.3	F42H10.3
C06E1.3	WBGene00015519	JA	80 D02	sjj_C06E1.3	C06E1.3
hlh-11	WBGene00001955	JA	82 F09	sjj_F58A4.7	F58A4.7
unc-49	WBGene00006784	JA	84 E11	sjj_T21C12.1	T21C12.1
T28D6.4	WBGene00012124	JA	86 A03	sjj_T28D6.4	T28D6.4
T28D6.5	WBGene00012125	JA	86 A04	sjj_T28D6.5	T28D6.5
nas-1	WBGene00003520	JA	88 F09	sjj_F45G2.1	F45G2.1
ATPase	WBGene00019875	JA	94 E08	sjj_R05C11.3	R05C11.3
Y24D9A.1	WBGene00021281	JA	97 B08	sjj_Y24D9A.i	Y24D9A.1
F29B9.8	WBGene00017923	JA	97 F05	sjj_F29B9.8	F29B9.8
Y11D7A.14	WBGene00012437	JA	107 B01	sjj_Y11D7A.14	Y11D7A.14
wrt-3	WBGene00006949	JA	107 F04	sjj_F38E11.7	F38E11.12
dnj-1	WBGene00001019	JA	112 C06	sjj_B0035.14	B0035.14
F07C6.4	WBGene00008555	JA	115 C01	sjj_F07C6.4	F07C6.4
C39E9.8	WBGene00008032	JA	115 H09	sjj_C39E9.8	C39E9.8
cho-1	WBGene00000501	JA	116 B12	sjj_C48D1.3	C48D1.3
Y55F3AM.10	WBGene00021927	JA	122 G10	sjj_Y55F3A_748.e	Y55F3AM.10
mca-3	WBGene00003153	JA	123 B04	sjj_Y67D8A_367.a	Y67D8C.10
F13H6.1	WBGene00017430	JA	139 G09	sjj_F13H6.1	F13H6.1
spn-4	WBGene00004984	JA	140 H03	sjj_ZC404.8	ZC404.8
F09G2.1	WBGene00017310	JA	141 F12	sjj_F09G2.1	F09G2.1
egl-3	WBGene00001172	JA	149 D01	sjj_C51E3.7	C51E3.7
D1054.9	WBGene00008376	JA	150 G09	sjj_D1054.9	D1054.9
F32D8.1	WBGene00009326	JA	150 H12	sjj_F32D8.1	F32D8.1
K03B8.6	WBGene00010524	JA	152 B02	sjj_K03B8.6	K03B8.6

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snb-2	WBGene00004898	JA	154 B05	sjj_F23H12.1	F23H12.1
uig-1	WBGene00009337	JA	156 B06	sjj_F32F2.1	F32F2.1
letm-1	WBGene00010279	JA	157 A09	sjj_F58G11.1	F58G11.1
F58E10.1	WBGene00010259	JA	158 A03	sjj_F58E10.1	F58E10.1
par-1	WBGene00003916	JA	158 C03	sjj_H39E23.1	H39E23.1
mrp-7	WBGene00003413	JA	172 B06	sjj_Y116F11A.zz5	Y43F8C.12
Y44A6D.3	WBGene00012854	JA	173 H06	sjj_Y44A6D.3	Y44A6D.3
F53B1.3	WBGene00018736	JA	178 H11	sjj_F53B1.3	F53B1.3
ceh-18	WBGene00000441	JA	181 H01	sjj_ZC64.3	ZC64.3
uvt-2	WBGene00006861	JA	183 G05	sjj_C24H10.5	C24H10.5
egl-13	WBGene00001182	JA	184 E11	sjj_T22B7.1	T22B7.1
rgs-6	WBGene00004349	JA	184 G03	sjj_C41G11.3	C41G11.3
tag-275	WBGene00016423	JA	185 D10	sjj_C34H3.b	C34H3.1
tnt-2	WBGene00006587	JA	189 G12	sjj_F53A9.10	F53A9.10
ZK899.1	WBGene00014140	JA	190 H10	sjj_ZK899.1	ZK899.1
C11E4.4	WBGene00007519	JA	191 B08	sjj_C11E4.4	C11E4.4
alr-1	WBGene00044330	JA	193 E05	sjj_R08B4.2	R08B4.2
tsp-20	WBGene00006646	JA	195 A09	sjj_B0198.1	B0198.1
cab-1	WBGene00000277	JA	195 C08	sjj_C23H4.1	C23H4.1
lin-2	WBGene00002991	JA	195 G05	sjj_F17E5.1	F17E5.1
C04C11.2	WBGene00007296	JA	198 C12	sjj_C04C11.2	C04C11.2

REFERENCES

- Aguib, Y., Heiseke, A., Gilch, S., Riemer, C., Baier, M., Schatzl, H. M., & Ertmer, A. (2009). Autophagy induction by trehalose counteracts cellular prion infection. *Autophagy*, 5(3), 361-369.
- Alvarez-Peral, F. J., Zaragoza, O., Pedreno, Y., & Arguelles, J. C. (2002). Protective role of trehalose during severe oxidative stress caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*. *Microbiology*, 148(Pt 8), 2599-2606.
- Anas, M. K., Hammer, M. A., Lever, M., Stanton, J. A., & Baltz, J. M. (2007). The organic osmolytes betaine and proline are transported by a shared system in early preimplantation mouse embryos. *J Cell Physiol*, 210(1), 266-277. doi: 10.1002/jcp.20872
- Arguelles, J. C., Rodriguez, T., & Alvarez-Peral, F. J. (1999). Trehalose hydrolysis is not required for human serum-induced dimorphic transition in *Candida albicans*: evidence from a *tps1/tps1* mutant deficient in trehalose synthesis. *Res Microbiol*, 150(8), 521-529.
- Avonce, N., Mendoza-Vargas, A., Morett, E., & Iturriaga, G. (2006). Insights on the evolution of trehalose biosynthesis. *BMC Evol Biol*, 6, 109. doi: 10.1186/1471-2148-6-109
- Behling, C. A., Perez, R. L., Kidd, M. R., Staton, G. W., Jr., & Hunter, R. L. (1993). Induction of pulmonary granulomas, macrophage procoagulant activity, and tumor necrosis factor-alpha by trehalose glycolipids. *Ann Clin Lab Sci*, 23(4), 256-266.
- Behm, C. A. (1997a). The role of trehalose in the physiology of nematodes. *Int J Parasitol*, 27(2), 215-229.

REFERENCES

- Behm, C. A. (1997b). The role of trehalose in the physiology of nematodes. *Int J Parasitol*, 27(2), 215-229. doi: Doi 10.1016/S0020-7519(96)00151-8
- Bergsma, J. C., Kasri, N. N., Donaton, M. C., De Wever, V., Tisi, R., de Winde, J. H., . . . Wera, S. (2001). PtdIns(4,5)P(2) and phospholipase C-independent Ins(1,4,5)P(3) signals induced by a nitrogen source in nitrogen-starved yeast cells. *Biochem J*, 359(Pt 3), 517-523.
- Bernstein, E., Kim, S. Y., Carmell, M. A., Murchison, E. P., Alcorn, H., Li, M. Z., . . . Hannon, G. J. (2003). Dicer is essential for mouse development. *Nat Genet*, 35(3), 215-217. doi: 10.1038/ng1253
- Bohnsack, M. T., Czaplinski, K., & Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*, 10(2), 185-191.
- Braendle, C., & Felix, M. A. (2006). Sex determination: ways to evolve a hermaphrodite. *Curr Biol*, 16(12), R468-471. doi: 10.1016/j.cub.2006.05.036
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1), 71-94.
- Cai, X., Hagedorn, C. H., & Cullen, B. R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, 10(12), 1957-1966. doi: rna.7135204 [pii]
- 10.1261/rna.7135204
- Calandra, R. S., Rulli, S. B., Frungieri, M. B., Suescun, M. O., & Gonzalez-Calvar, S. I. (1996). Polyamines in the male reproductive system. *Acta Physiol Pharmacol Ther Latinoam*, 46(4), 209-222.
- Carmell, M. A., & Hannon, G. J. (2004). RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol*, 11(3), 214-218. doi: 10.1038/nsmb729
- nsmb729 [pii]

REFERENCES

- Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda, N., . . . Hayashizaki, Y. (2005). The transcriptional landscape of the mammalian genome. *Science*, 309(5740), 1559-1563. doi: 309/5740/1559 [pii]
10.1126/science.1112014
- Castillo, K., Nassif, M., Valenzuela, V., Rojas, F., Matus, S., Mercado, G., . . . Hetz, C. (2013). Trehalose delays the progression of amyotrophic lateral sclerosis by enhancing autophagy in motoneurons. *Autophagy*, 9(9), 1308-1320. doi: 10.4161/auto.25188
- Chen, C., & Dickman, M. B. (2005). Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. *Proc Natl Acad Sci U S A*, 102(9), 3459-3464. doi: 10.1073/pnas.0407960102
- Chen, Q., & Haddad, G. G. (2004). Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *J Exp Biol*, 207(Pt 18), 3125-3129. doi: 10.1242/jeb.01133
- Colaco, C., Kampinga, J., & Roser, B. (1995). Amorphous stability and trehalose. *Science*, 268(5212), 788.
- Colaco, C., Sen, S., Thangavelu, M., Pinder, S., & Roser, B. (1992). Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. *Biotechnology (N Y)*, 10(9), 1007-1011.
- Crowe, J. H., Crowe, L. M., & Chapman, D. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*, 223(4637), 701-703. doi: 10.1126/science.223.4637.701
- Eastmond, P. J., van Dijken, A. J., Spielman, M., Kerr, A., Tissier, A. F., Dickinson, H. G., . . . Graham, I. A. (2002). Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. *Plant J*, 29(2), 225-235.

REFERENCES

- Eisenberg, T., Knauer, H., Schauer, A., Buttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., . . . Madeo, F. (2009). Induction of autophagy by spermidine promotes longevity. *Nat Cell Biol*, *11*(11), 1305-1314. doi: 10.1038/ncb1975
- Erkut, C., Penkov, S., Khesbak, H., Vorkel, D., Verbavatz, J. M., Fahmy, K., & Kurzchalia, T. V. (2011). Trehalose renders the dauer larva of *Caenorhabditis elegans* resistant to extreme desiccation. *Curr Biol*, *21*(15), 1331-1336. doi: S0960-9822(11)00770-6 [pii]
- 10.1016/j.cub.2011.06.064
- Fernandez, O., Bethencourt, L., Quero, A., Sangwan, R. S., & Clement, C. (2010). Trehalose and plant stress responses: friend or foe? *Trends Plant Sci*, *15*(7), 409-417. doi: 10.1016/j.tplants.2010.04.004
- Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., & Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature*, *432*(7014), 235-240. doi: nature03120 [pii]
- 10.1038/nature03120
- Guo, N., Puhlev, I., Brown, D. R., Mansbridge, J., & Levine, F. (2000). Trehalose expression confers desiccation tolerance on human cells. *Nat Biotechnol*, *18*(2), 168-171. doi: 10.1038/72616
- Hammer, M. A., & Baltz, J. M. (2002). Betaine is a highly effective organic osmolyte but does not appear to be transported by established organic osmolyte transporters in mouse embryos. *Mol Reprod Dev*, *62*(2), 195-202. doi: 10.1002/mrd.10088
- Harmon, A. W., & Patel, Y. M. (2004). Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation. *Breast Cancer Res Treat*, *85*(2), 103-110. doi: 10.1023/B:BREA.0000025397.56192.e2

REFERENCES

- Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., & Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signal Behav*, 7(11), 1456-1466. doi: 21949 [pii]
- 10.4161/psb.21949
- Henry, C., Bledsoe, S. W., Siekman, A., Kollman, A., Waters, B. M., Feil, R., . . . Lagrimini, L. M. (2014). The trehalose pathway in maize: conservation and gene regulation in response to the diurnal cycle and extended darkness. *J Exp Bot*, 65(20), 5959-5973. doi: eru335 [pii]
- 10.1093/jxb/eru335
- Heo, H. J., Kim, D. O., Shin, S. C., Kim, M. J., Kim, B. G., & Shin, D. H. (2004). Effect of antioxidant flavanone, naringenin, from Citrus junoson neuroprotection. *J Agric Food Chem*, 52(6), 1520-1525. doi: 10.1021/jf035079g
- Herndon, L. A., Schmeissner, P. J., Dudaronek, J. M., Brown, P. A., Listner, K. M., Sakano, Y., . . . Driscoll, M. (2002). Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature*, 419(6909), 808-814. doi: 10.1038/nature01135
- nature01135 [pii]
- Honda, Y., Tanaka, M., & Honda, S. (2010). Trehalose extends longevity in the nematode *Caenorhabditis elegans*. *Aging Cell*, 9(4), 558-569. doi: ACE582 [pii]
- 10.1111/j.1474-9726.2010.00582.x
- Hulme, S. E., & Whitesides, G. M. (2011). Chemistry and the worm: *Caenorhabditis elegans* as a platform for integrating chemical and biological research. *Angew Chem Int Ed Engl*, 50(21), 4774-4807. doi: 10.1002/anie.201005461

REFERENCES

- Iordachescu, M., & Imai, R. (2008). Trehalose biosynthesis in response to abiotic stresses. *J Integr Plant Biol*, 50(10), 1223-1229. doi: 10.1111/j.1744-7909.2008.00736.x
- Iturriaga, G., Suarez, R., & Nova-Franco, B. (2009). Trehalose metabolism: from osmoprotection to signaling. *Int J Mol Sci*, 10(9), 3793-3810. doi: 10.3390/ijms10093793
- Jannot, G., Boisvert, M. E., Banville, I. H., & Simard, M. J. (2008). Two molecular features contribute to the Argonaute specificity for the microRNA and RNAi pathways in *C. elegans*. *RNA*, 14(5), 829-835. doi: rna.901908 [pii]
- 10.1261/rna.901908
- Kapahi, P., & Zid, B. (2004). TOR pathway: linking nutrient sensing to life span. *Sci Aging Knowledge Environ*, 2004(36), PE34. doi: 10.1126/sageke.2004.36.pe34
- Kim, H. S., Murakami, R., Quintin, S., Mori, M., Ohkura, K., Tamai, K. K., . . . Nishiwaki, K. (2011). VAB-10 spectraplakins act in cell and nuclear migration in *Caenorhabditis elegans*. *Development*, 138(18), 4013-4023. doi: dev.059568 [pii]
- 10.1242/dev.059568
- Kormish, J. D., & McGhee, J. D. (2005). The *C. elegans* lethal gut-obstructed *gob-1* gene is trehalose-6-phosphate phosphatase. *Dev Biol*, 287(1), 35-47. doi: 10.1016/j.ydbio.2005.08.027
- Larosa, P. C., Rhodes, D., Rhodes, J. C., Bressan, R. A., & Csonka, L. N. (1991). Elevated Accumulation of Proline in NaCl-Adapted Tobacco Cells Is Not Due to Altered Delta-Pyrroline-5-Carboxylate Reductase. *Plant Physiol*, 96(1), 245-250.
- Lee, Y., Jeon, K., Lee, J. T., Kim, S., & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, 21(17), 4663-4670.
- Lehrbach, N. J., Castro, C., Murfitt, K. J., Abreu-Goodger, C., Griffin, J. L., & Miska, E. A. (2012). Post-developmental microRNA expression is required for normal

REFERENCES

- physiology, and regulates aging in parallel to insulin/IGF-1 signaling in *C. elegans*. *RNA*, 18(12), 2220-2235. doi: 10.1261/rna.035402.112
- Leyman, B., Avonce, N., Ramon, M., Van Dijck, P., Iturriaga, G., & Thevelein, J. M. (2006). Trehalose-6-phosphate synthase as an intrinsic selection marker for plant transformation. *J Biotechnol*, 121(3), 309-317. doi: 10.1016/j.jbiotec.2005.08.033
- Li, J., Hou, N., Faried, A., Tsutsumi, S., Takeuchi, T., & Kuwano, H. (2009). Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. *Ann Surg Oncol*, 16(3), 761-771. doi: 10.1245/s10434-008-0260-0
- Lin, S. P., Hou, Y. C., Tsai, S. Y., Wang, M. J., & Chao, P. D. (2014). Tissue distribution of naringenin conjugated metabolites following repeated dosing of naringin to rats. *Biomedicine (Taipei)*, 4, 16. doi: 10.7603/s40681-014-0016-z
- Liu, M., Liu, P., Zhang, L., Cai, Q., Gao, G., Zhang, W., . . . Fan, Q. (2011). mir-35 is involved in intestine cell G1/S transition and germ cell proliferation in *C. elegans*. *Cell Res*, 21(11), 1605-1618. doi: 10.1038/cr.2011.102
- Matsuda, H., Yamada, T., Yoshida, M., & Nishimura, T. (2015). Flies without Trehalose. *Journal of Biological Chemistry*, 290(2), 1244-1255. doi: DOI 10.1074/jbc.M114.619411
- McJunkin, K., & Ambros, V. (2014). The embryonic mir-35 family of microRNAs promotes multiple aspects of fecundity in *Caenorhabditis elegans*. *G3 (Bethesda)*, 4(9), 1747-1754. doi: 10.1534/g3.114.011973
- Pellerone, F. I., Archer, S. K., Behm, C. A., Grant, W. N., Lacey, M. J., & Somerville, A. C. (2003). Trehalose metabolism genes in *Caenorhabditis elegans* and filarial nematodes. *Int J Parasitol*, 33(11), 1195-1206. doi: S0020751903001735 [pii]
- Petiot, A., Ogier-Denis, E., Blommaert, E. F., Meijer, A. J., & Codogno, P. (2000). Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem*, 275(2), 992-998.

REFERENCES

- Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., . . . Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, 403(6772), 901-906. doi: 10.1038/35002607
- Riddle, D. L., Swanson, M. M., & Albert, P. S. (1981). Interacting genes in nematode dauer larva formation. *Nature*, 290(5808), 668-671.
- Romero, C., Belles, J. M., Vaya, J. L., Serrano, R., & Culianez-Macia, F. A. (1997). Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, 201(3), 293-297. doi: 10.1007/s004250050069
- Sarkar, S., Davies, J. E., Huang, Z., Tunnacliffe, A., & Rubinsztein, D. C. (2007). Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem*, 282(8), 5641-5652. doi: 10.1074/jbc.M609532200
- Skiba, F., & Schierenberg, E. (1992). Cell lineages, developmental timing, and spatial pattern formation in embryos of free-living soil nematodes. *Dev Biol*, 151(2), 597-610. doi: 0012-1606(92)90197-O [pii]
- Sontheimer, E. J., & Carthew, R. W. (2005). Silence from within: endogenous siRNAs and miRNAs. *Cell*, 122(1), 9-12. doi: S0092-8674(05)00649-5 [pii] 10.1016/j.cell.2005.06.030
- Sussman, A. S., & Lingappa, B. T. (1959). Role of Trehalose in Ascospores of *Neurospora Tetrasperma*. *Science*, 130(3385), 1343. doi: 10.1126/science.130.3385.1343
- Thompson, S. N., & Lee, R. W. (1994). Glucose metabolism in an insect *Manduca sexta* and effects of parasitism. *Biochim Biophys Acta*, 1200(3), 322-330.
- Vasquez-Rifo, A., Jannot, G., Armisen, J., Labouesse, M., Bukhari, S. I., Rondeau, E. L., . . . Simard, M. J. (2012). Developmental characterization of the microRNA-specific

REFERENCES

- C. elegans* Argonautes *alg-1* and *alg-2*. *PLoS One*, 7(3), e33750. doi: 10.1371/journal.pone.0033750
- Wahl, V., Ponnu, J., Schlereth, A., Arrivault, S., Langenecker, T., Franke, A., . . . Schmid, M. (2013). Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science*, 339(6120), 704-707. doi: 10.1126/science.1230406
- Ward, S., & Carrel, J. S. (1979). Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. *Dev Biol*, 73(2), 304-321.
- Wightman, B., Ha, I., & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*, 75(5), 855-862.
- Wyatt, G. R., & Kale, G. F. (1957). The chemistry of insect hemolymph. II. Trehalose and other carbohydrates. *J Gen Physiol*, 40(6), 833-847.
- Ye, P., Liu, Y., Chen, C., Tang, F., Wu, Q., Wang, X., . . . Zheng, P. (2015). An mTORC1-Mdm2-Drosha axis for miRNA biogenesis in response to glucose- and amino acid-deprivation. *Mol Cell*, 57(4), 708-720. doi: 10.1016/j.molcel.2014.12.034
- Yorimitsu, T., & Klionsky, D. J. (2005). Autophagy: molecular machinery for self-eating. *Cell Death Differ*, 12 Suppl 2, 1542-1552. doi: 10.1038/sj.cdd.4401765
- Yu, T., Li, J., Yan, M., Liu, L., Lin, H., Zhao, F., . . . Yao, M. (2015). MicroRNA-193a-3p and -5p suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway. *Oncogene*, 34(4), 413-423. doi: 10.1038/onc.2013.574
- Zhao, Y., Deng, C., Wang, J., Xiao, J., Gatalica, Z., Recker, R. R., & Xiao, G. G. (2011). Let-7 family miRNAs regulate estrogen receptor alpha signaling in estrogen receptor positive breast cancer. *Breast Cancer Res Treat*, 127(1), 69-80. doi: 10.1007/s10549-010-0972-2