Peroxisomes: A possible association with the heat shock response.

Master thesis

Konstantinos Kounakis

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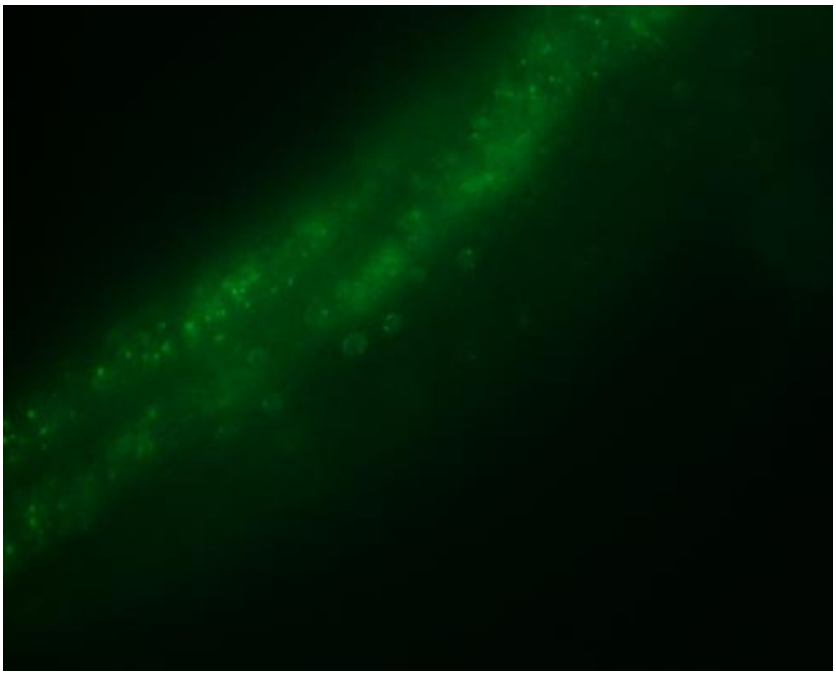

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Introduction

Peroxisomes are single membrane organelles that are almost ubiquitously present in eukaryotic cells. They are generally spherical, with a diameter of 0,1 to 1 micrometers and contain dense, sometimes even crystalloid, matrices of metabolic enzymes, substrates and co-factors. However, it should be noted that their exact morphology and contents can vary among different cells and conditions. [1]

Peroxisomes were first discovered in the 1950s in mouse renal cells and rat liver [2,3]. They were initially characterized as microbodies, due to their appearance as single membrane organelles containing a dense granular matrix. Later studies demonstrated that these new organelles contained a variety of oxidases that produce hydrogen peroxide (H_2O_2) and catalase, an enzyme critical for its catabolism [4,5]. Thus, the name "peroxisomes" was adopted.

Peroxisomal functions

Researchers over the last few decades have unveiled that peroxisomes can assume a quite diverse array of functions in cells, and be involved in several cellular pathways, both catabolic and anabolic [6,7].

Peroxisomes contain a fully functional fatty acid beta-oxidation pathway. This pathway is quite similar to the one used by mitochondria, and there are even instances of enzymes that are targeted to both organelles [8–12], but this instance differs in its specificity for Very-Long Chain Fatty Acids (VLCFAs, C22 +). These acids are generally only oxidized in peroxisomes, partially because the carnitine palmitoyltransferase 1 (CPT1) system cannot import them into mitochondria. Since peroxisomes lack the components of the citric acid cycle and the respiratory chain, most of the end products of peroxisomal beta-oxidation (acetyl-CoA, propionyl-CoA, other acyl-CoAs and NADH) are transported to mitochondria to complete oxidation to CO_2, H_2O and NAD^+, while the produced $FADH_2$ remains in peroxisomes and is reoxidized to produce H_2O_2 instead of ATP [7,13–15]. Interestingly, this pathway also has anabolic roles, since it contributes to the production of the primary bile acid components chenodeoxycholic acid and cholic acid from di- and trihydroxycholestanoic acid (DHCA and THCA), respectively (the final step in bile acid synthesis is also conducted in peroxisomes by bile acid-CoA:amino acid N-acyltransferase) [16–18] and the production of docosahexaenoic acid (DHA), a polyunsaturated fatty acid associated with several disorders and a source of inflammation mediators, from its predecessor tetracosahexaenoic acid [19,20].

Peroxisomes are also capable of alpha-oxidation, a process necessary for the handling of branched chain fatty acids (with a methyl group at the 3-position i.e. phytanic acid) before beta-oxidation can occur. This is achieved by the sequential action of phytanoyl-CoA 2-hyrdoxylase (PHYH), 2-hydrophytanoyl-CoA lyase and pristanal dehydrogenase, which shorten the fatty acid by one carbon, transforming it into a 2-methyl one that is accessible to the beta-oxidation pathway[7,15,21–23] . Additionally, they are responsible for the catabolism of long chain dicarboxylic acids [15,24], and they are equipped with additional enzymes for the oxidation of mono- and polyunsaturated fatty acids (including enoyl-CoA isomerase, and dienoyl-CoA reductase) as well as 2-methylacyl-CoA racemase (AMACR), an enzyme required for the conversion of (2R)-methyl branched-chain acyl-CoAs into the corresponding (2S)-methyl acyl-CoAs so that acyl-CoA oxidases can interact with them [15]. All in all, peroxisomes assume a role as specialized lipid processors, with capabilities far exceeding those of the mitochondria.

Peroxisomes contribute to the synthesis of ether phospholipids, a special phospholipid class characterized by the presence of an ether bond (as opposed to a usual ester bond) at the sn-1 position of the glycerol backbone. The formation of this bond occurs in peroxisomes, through the activity of glyceronephosphate O-acyl transferase (GNPAT) and alkylglycerone phosphate synthase (AGPS). Plasmalogens are the most prominent subgroup of ether lipids and they are regarded as a crucial component of the myelin sheath and most plasma membranes, as well as a constituent of lipid rafts, and a trap for reactive oxygen species. They are also a source of secondary messengers, since they are enriched in DHA, and arachidonic acid (AA) [25–27].

Reactive oxygen and nitrogen species (ROS / RNS) are very important to cellular survival since they are simultaneously signaling messengers (associated with gene expression, cell proliferation, angiogenesis, innate immunity, programmed cell death and senescence) and dangerous reagents that can cause oxidative damage to biological macromolecules and disrupt the cellular redox balance. Disturbances in ROS/RNS homeostasis have been associated with diseases such as atherosclerosis, diabetes, neurodegeneration and cancer [28–31]. Cells employ a large array of mechanisms to control ROS/RNS production and detoxification and peroxisomes are one of them [32].

Peroxisomes generate significant amounts of ROS/RNS as part of their normal metabolism (as both by-products and endpoints) to the point that a long-term administration of peroxisome proliferators to rodents induces oxidative stress in liver cells (possibly because the associated increase in H_2O_2 production exceeds the increase in catalase activity). These species can have signaling functions by interacting with receptors and modifying the properties of macromolecules both within and outside the peroxisomes [32]. For instance, in human cells ROS can activate the peroxisomally localized tumor suppressors ataxia telangiectasia mutated (ATM) and tuberous sclerosis complex 2 (TSC-2), which subsequently repress mTORC1 signaling, increasing general autophagic flux, and phosphorylate PEX-5, inducing peroxisomal specific autophagy (pexophagy) [33]. Reactive species may even modulate transcription in the nucleus by affecting factors such as NFκB [34,35].

However, peroxisomes also have a protective role. They contain several anti-oxidant mechanisms, which include enzymes such as catalase, superoxide dismutase 1, peroxiredoxin 5, glutathione S-transferase kappa, 'microsomal' glutathione S- transferase, and epoxide hydrolase 2 and non enzymatic compounds such as plasmalogens, glutathione and ascorbic acid. Defects in these mechanisms are associated with apoptosis during mouse development and increased risk of developing age related diseases such as diabetes, atherosclerosis and cancer [32].

Peroxisomes also exhibit a variety of organism-specific roles. For instance, human peroxisomes are capable of detoxifying glyoxylate produced in the liver, thus preventing the possible negative effects of its oxidization to oxalate, through the activity of peroxisomal alanine-glyoxylate aminotransferase (AGT). A deficiency of this enzyme leads to the disorder known as hyperoxaluria type 1 [7,15,36]. Peroxisomes have been associated with the antiviral response in mammals as they carry MAVS, an adaptor protein that can induce the interferon-independent production of short-term protective factors (interestingly MAVS is also localized on mitochondria and that instance is responsible for the induction of an interferon-dependent delayed response that provides long-term protection) [37]. In *C. elegans* [38,39] and *L. longipalis* [40] peroxisomes produce, through the beta-oxidation pathway, pheromones important to the organisms development. In *P. chrysogenum* peroxisomes contain enzymes crucial to the synthesis of penicillin [41,42], while in other fungi they are responsible for producing toxins that facilitate host invasion [43,44]. In the multicellular filamentous fungus *N. crassa* peroxisomes can differentiate into Woronin bodies, a specialized organelle that contains a crystalline hexagonal core of self-assembling HEX proteins and acts as a plug on exposed open intercellular channels during wound repair [1,45,46]. Plant peroxisomes have a high degree of tissue specialization and can adopt three additional forms: glyoxysomes (enriched with enzymes of the fatty acid oxidation and the glyoxylate cycle), leaf peroxisomes (specialized in glycolate metabolism and hosting part of the photorespiration mechanism) and root nodule peroxisomes [47]. They are also involved in the metabolism of compounds such as purines, branched-chain amino acids and polyamines and the production of plant specific signaling molecules [48].

Peroxisomal biogenesis and maintenance.

Peroxisomal biogenesis has been a cause of dispute in the scientific community for years, since some proposed that peroxisomes act as autonomous organelles like mitochondria and chloroplasts and are generated via the growth and fission of pre-existing ones, while others believed that peroxisomes originate from vesicles budding from the ER. Today, it is generally accepted that both biogenesis pathways co-exist in parallel and their contribution may vary based on current cellular conditions [49–51]. The main proteins that facilitate peroxisomal biogenesis are called peroxins (pex or *prx* genes, depending on the organism), and their defects in humans are linked with the aptly called peroxisome biogenesis disorders (PBDs), characterized by severe developmental issues and typically death during early childhood [1]. Here we will provide a general overview of how peroxisomes obtain their membrane proteins (Peroxisome Membrane proteins –PMPs), their matrix proteins, and their lipids, as well as how existing organelles can proliferate. Generally the pathways demonstrate significant conservation, although variations among different organisms do exist (especially since some lack specific peroxins). We will also discuss the known mechanisms of organelle quality control.

Peroxisomal membrane proteins can follow one or more of three different pathways: one dependent on Peroxin 19, one independent from it, and one specific to tail-anchored (TA) PMPs. All peroxisomal proteins are synthesized by cytosolic ribosomes. The peroxin 19 independent pathway involves proteins that are introduced directly to the membrane of the ER through its typical import pathways and are then sorted into specific pre-peroxisomal domains. These domains bud into pre-peroxisomal vesicles (ppVs) that can either fuse to form new peroxisomes *de novo* or merge into existing organelles to supply them (fig. 1). This fusion requires peroxins 1 and 6. The recruitment of these types of PMPs (most prominently Peroxin 3) seems to require the function of Peroxin 16. The peroxin 19 dependent pathway involves proteins attaching to it while it's predominantly cytoplasmic. After the Peroxin 19 – cargo PMP complex is formed, Peroxin 19 interacts with Peroxin 3 on the surface of the peroxisomes. This interaction causes the release of the PMP from Peroxin 19 and its integration onto the peroxisomal membrane, although it is still not entirely clear how (fig. 2b). Finally, TA PMPs (for instance Peroxin 15) are typically characterized by a single Transmembrane Segment (TMS) that is near the C-terminus of the protein and therefore inaccessible for ER import before the termination of translation. Thus they are imported post translationally with the help of the Hsp40/70 machinery, SRP or the Get-machinery (Get3 and its receptors Get1 and Get2 in yeast, ASNA-1 and its receptors WRB/CAML in mammals). Of course TA PMPs may still use the Peroxin 19 pathway in some cases [47,49–53].

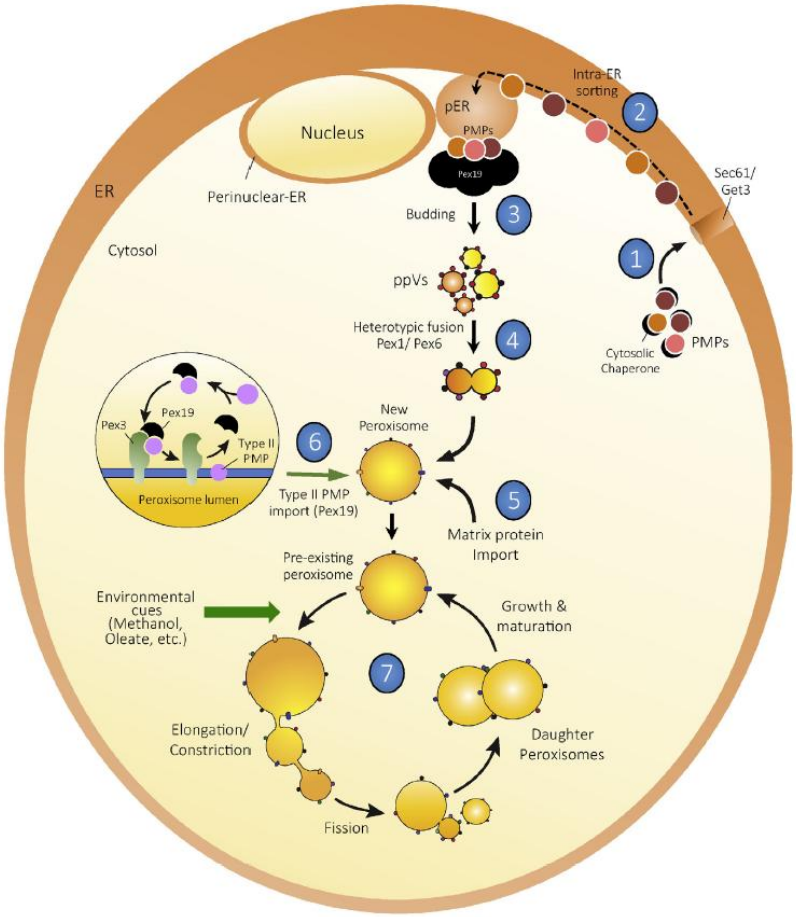


Figure 1: The two pathways of peroxisomal biogenesis. Adapted from [49].

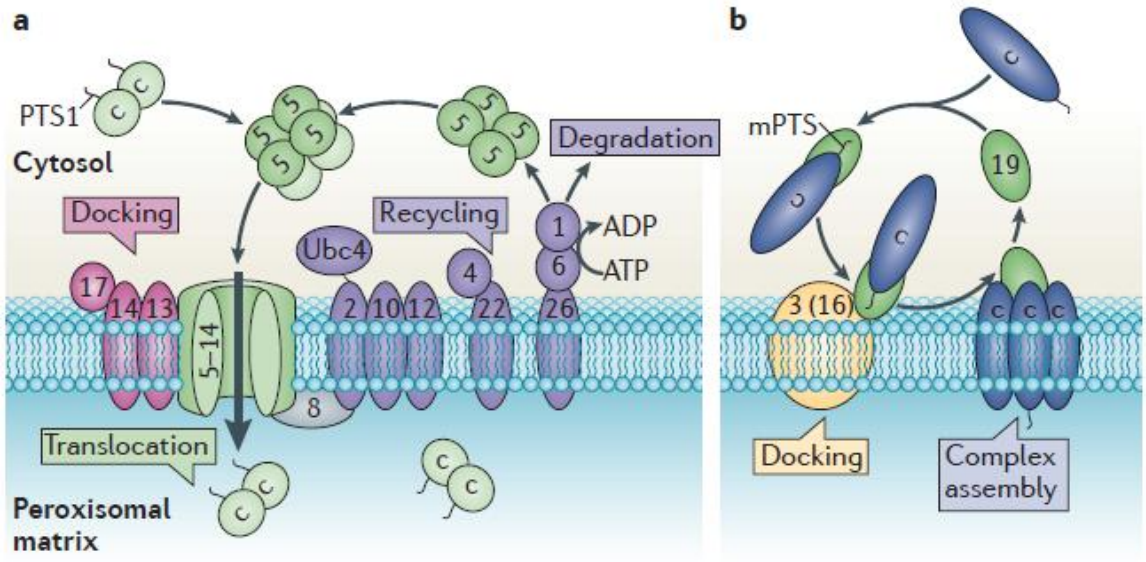


Figure 2: a) Summary of the PTS1 peroxisomal matrix import pathway. **b)** Summary of the PMP import pathway. Adapted from [1].

Peroxisomal matrix proteins are similarly synthesized by cytosolic ribosomes and subsequently imported into the organelles. Interestingly they are capable of import even when they are folded or oligomerized, according to some published data. At first, the imported cargo proteins are recognized in the cytosol by a soluble receptor, usually through a specific targeting signal they carry. The two general receptors are peroxins 5 and 7 (although 7 and the associated pathway and signals may not be present in every organism). Peroxin 5 recognizes the C-terminal peroxisomal targeting signal type 1 (PTS1), which is composed of the non-cleavable tripeptide SKL or conserved variants [54,55] After the recognition, the protein-cargo complex docks onto a complex that generally includes peroxins 13, 14 and 17 and peroxin 5 adopts a transmembrane form. This is believed to be the main cause for the formation of a transient translocation channel that allows the cargo protein to enter the peroxisomal matrix. After the translocation peroxin 5 is ubiquitinated through the activity of the ring finger complex on the peroxisomal membrane (which consists of the ubiquitin ligases peroxin 2, 10 and 12) and other ubiquitin conjugating enzymes such as peroxin 4. The ubiquitination is subsequently recognized by the soluble AAA-type ATPases peroxin 1 and peroxin 6 which associate with the peroxisomes (via PMPS such as peroxin 15 and 26) and extract peroxin 5 (fig. 2a). Interestingly the type of ubiquitination determines the fate of the molecule. Mono-ubiquitination induces simple export and receptor recycling for further use, while poly-ubiquitination marks the factor for proteasomal degradation. Furthermore, the extraction of the receptor from the membrane is the only part of the import cycle that requires ATP. Peroxin 7 recognizes proteins bearing the peroxisomal targeting signal type 2 (PTS2), a nonapeptide with the consensus sequence (R/K)-(L/V/I/Q)-XX-(L/V/I/H)- (L/S/G/A)-X-(H/Q)-(L/A) that is located near the N-terminus of matrix proteins [56] Peroxin 7 acts in conjunction with other co-receptors (peroxins 18, 20 and/or 21 depending on the species) and its import pathway follows a process similar to that of peroxin 5. Finally, there are instances of proteins that have neither type of PTS but can enter the peroxisomal matrix via piggybacking on other cargo or via PTS1 independent interaction with peroxin 5. [1,57–59]

Despite their general role in lipid metabolism, peroxisomes are incapable of synthesizing all the types of phospholipids required for the extension of their membrane during growth and division. This means that they must obtain them from other organelles. The ER can obviously contribute in this through ppVs, however there are reports of a possible non-vesicular pathway that can transfer lipids, although it may require close proximity between the two organelles [51,60,61]

As mentioned above, peroxisomes are capable of proliferating via growth and fission and can be removed via autophagy. This ability can be very relevant when specific environmental conditions demand an increase or decrease in peroxisomal activity. The main regulators of peroxisomal growth and proliferation are the proteins of the PPAR family (Peroxisome Proliferator-Activated Receptor). This family includes three members, PPRAα, PPRAβ/δ and PPRAγ, which have different tissue specific expression, different interaction partners and different targets. PPARs regulate gene transcription in response to environmental and endogenous stimuli via binding ligands, translocating to the nucleus, heterodimerizing with a retinoid-X-receptor and then binding to peroxisome proliferator response elements, thus recruiting co-activators or co-repressors and altering gene expression. PPREs generally contain

tandem repeats of the consensus motif TGACC separated by one base pair and can be found upstream of a number of genes, for instance those of the peroxisomal b-oxidation pathway. PPARs can control peroxisomal function in a quite specific manner: they may only affect some of the enzyme pathways of the organelle (i.e. in humans PPARα doesn't affect catalase expression, while PPARγ does) and may or may not induce actual peroxisome abundance through proliferation. Peroxisome numbers may also be affected in PPAR independent manners, for instance by external chemical compounds or growth factors, or even by DHA, notably produced by the peroxisomes themselves [62–64].

The process of peroxisomal proliferation involves membrane remodeling via elongation, constriction, and fission. Peroxin 11, a PMP, guides the peroxisomal membrane into bending and elongating via the insertion of amphipathic, N-terminal helices into one leaflet of the lipid bilayer causing membrane asymmetry and bending. The growing membrane extension recruits other proteins (Fis1, Mff, and DLP1, factors notably involved in mitochondrial proliferation as well) that form a constricting ring around it. Finally, peroxin 11 acts as a GTPase activating protein on DLP1 causing the ring to constrict completely and conclude membrane scission (fig. 3). Dysfunction of DLP1 and loss of mitochondrial fission-fusion balance has been implicated in several neurological and cardiovascular diseases [62–64].

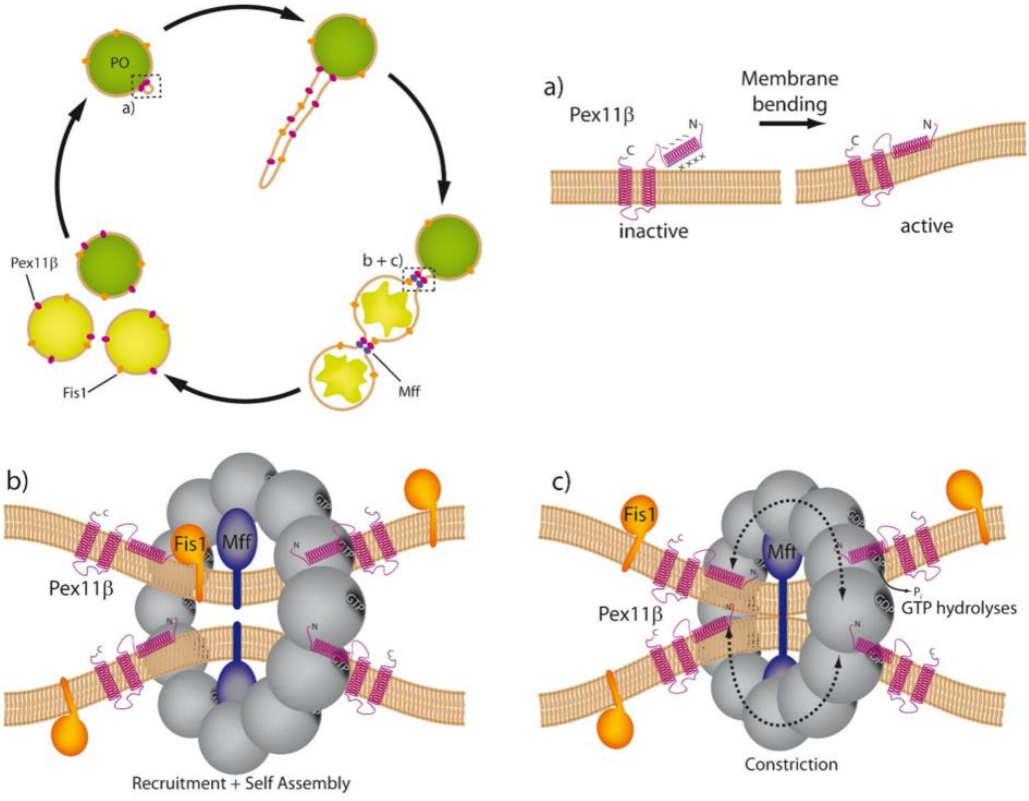


Figure 3: Summary of peroxisomal growth and fission. Adapted from [62].

In addition to the formation of new peroxisomes when the need arises, cells utilize a few maintenance pathways that discard defunct peroxisomal proteins or entire organelles. Firstly, peroxisomal matrix proteins can be degraded by Lon protease 2. Lon proteases are homo-oligomeric ATP-dependent proteases with chaperone-like activity present in eukaryotic mitochondria, chloroplasts and peroxisomes [65,66] . Peroxisomal lon can degrade defective enzymes before they can damage the organelle or the cell. Secondly, whole peroxisomes can be eliminated through 15-lipoxygenese (LOX15)-mediated autolysis. LOX15 can integrate onto organelle membranes and convert poly-unsaturated fatty acids into conjugated hydroperoxides. This destabilizes the membarane, causing the release of peroxisomal matrix and membrane proteins into the cytosol, where they are promptly disposed of by proteases. Finally, unwanted peroxisomes can be degraded via autophagy (pexophagy) [52,63,67–70]. Interestingly the maintainance pathways seem to have some degree of regulatory interaction. For instance, a knockout of *LON2* in *Arabidopsis thaliana* enhances the pathway of peroxisomal destruction-pexophagy, probably to compensate for the organelle's inability to degrade specific matrix proteins [71,72].

The main pathway of pexophagy is macroautophagic. The organelle is encapsulated into a specialized autophagosome which is subsequently transported to endosomes/lysosomes (via the cytoskeleton) for fusion and

degradation. This process utilizes receptors and adaptors that recruit the main autophagic factors (LC3/GABARAP families) specifically on peroxisomal targets. Known receptors include *P. pastoris* Atg30, *S. cerevisiae* Atg36 and mammalian NBR1 and p62. Atg30 and Atg36 bind non-ubiquitinated PMPs (for instance peroxins 3 and 14) and seem to be regulated mostly via phosporylation. On the other hand NBR1 and p62 recognise ubiquitinated substrates, such as PMP3, peroxin 3 and possibly even peroxin 5 (as mentioned above, the latter can be induced via ROS through ATM kinase). Notably peroxin 14 has been suggest to be capable of direct interaction with activated LC3-II under starvation conditions, acting as another possible receptor without a need for a mediating adaptor [33,52,63,67–70].

Furthermore, yeasts seem to be capable of eliminating peroxisomes through microautophagy. This is achieved through the formation of a planar autophagic membrane structure called the micropexophagy-specific apparatus (MIPA) on the organelle surface. This allows for direct fusion with endosomal / lysosomal vacuole membrane and provides a different pathway for peroxisomal engulfment [73].

Peroxisomal dysfunction and disease

Due to the critical contribution of peroxisomes to normal human metabolism, they are indispensible for normal and healthy survival. Thus, defects in genes involved in their biogenesis and / or function can have grave consequences. Disorders related to peroxisomal dysfunction are generally categorized into two classes: Peroxisome Biogenesis Disorders (PBDs), with issues primarily in the import of PMPs or matrix proteins, and single Peroxisomal Enzyme Deficiencies (PEDs), with defunct matrix enzymes or metabolite transporters [7].

PBDs are genetically heterogeneous group of autosomal recessive disorders with a defect in peroxisomal biogenesis. The majority of cases are caused by a by-allelic mutation in a peroxin gene involved in PMP or matrix import. The clinical presentation of these diseases can be quite varied and has to led to the classification of three PBD subtypes: the Zellweger spectrum disorders (ZSDs), RCPD type 1 and type 5, and peroxisomal fission defects [7].

ZSDs include the three historically defined disorders known as Zellweger syndrome, neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). All three are believed to be different cases of defects in the same biochemical pathways with phenotypes of different severity. Mutations in peroxins 1, 2, 3, 5, 6, 10, 12, 13, 14, 16, 19, and 26 have all been associated with ZSD emergence. Zellweger syndrome typically presents severe hypotonia, ocular abnormalities, seizures, renal cysts, hepatic dysfunction and characteristic craniofacial malformations. Patients typically cannot survive beyond the first year of their life. NALD and IRD symptoms may include developmental delay, hypotonia, liver dysfunction, sensorineural hearing loss, retinal dystrophy and vision impairment. NALD patients can reach adolescence, while IRD patients may even reach adulthood [7,74].

Rhizomelic chondriodysplasia punctata (RDCP) is a condition characterized by congenital contractures, cranial abnormalities, severe hypotonia, cataract, dwarfism, pronounced symmetric proximal shortening of the extremities (rhizomelia), skeletal deformities, and non-homogeneous calcification of cartilage tissue (chondrodysplasia punctata). There are 5 distinct subtypes of RDCP. RDCP type 1 is caused by mutations in the PEX7 gene, which facilitates PTS2 matrix import, thus hindering plasamalogen synthesis and a-oxidation. RDCP type 5 is associated with a frame shift mutation in PEX5, which leads to a similar phenotype. There are three more types of RDCP which are however classified as PEDs instead of PBDs, since they involve mutations in individual enzymes of ether phospholipid biosynthesis [7,74].

Peroxisomal division disorders (involving DLP1, Mff and PEX11) have only been recognized recently. They generally demonstrate little biochemical abnormalities, but patients can still have quite severe symptoms, similarly to Zellweger syndrome [7].

X-linked adrenoleukodystrophy (X-ALD) is the most common type of PED, with an occurrence rate of about 1:17000. As the name suggests, it is an X-linked disorder and therefore significantly more frequent on males rather than females [7]. The disorder is caused by mutations on ABCD1, one of transporters involved in ATP-driven import of fatty acids into peroxisomes. This renders cells incapable of metabolizing VLCFAs and leads to cerebral white matter inflammation, cerebral demyelination, adrenocortical insufficiency, and progressive behavioral and cognitive decline culminating to total disability and death within a few years after disease onset, which can typically occur within the first 10 years of life (childhood ALD) or past 30 (adrenomyeloneuropathy) depending on the severity. [75,76]

Acyl-CoA oxidase deficiency is associated with mutations in the ACOX1 gene, rendering patients incapable of metabolizing very long straight fatty acids. Symptoms can range from early fatal onset, with moderate neonatal hypotonia, frequent seizures and impaired hearing, vision and motor capabilities to a late onset with cerebellar and brainstem atrophy. [77–80]

D-bifunctional protein deficiency is caused by mutations in the HSD17B4 gene encoding 17-beta-estradiol dehydrogenase, an enzyme catalyzing the second and third step of peroxisomal beta-oxidation. The disorder typically has similar clinical presentation to Zellweger syndrome. [81]

Classical (adult) Refsum disease is caused either by mutations in the PHYH gene encoding the enzyme that initiates peroxisomal a-oxidation or by mild mutations in the PEX7 gene, since PHYH is imported to the matrix via the PTS2 pathway. The disease presents symptoms typically after childhood. Those include progressive retinitis pigmentosa, anosmia, deafness, polyneuropathy and/or ichthyosis. [82–85]

Primary hyperoxalurias involve defects in the pathways that eliminate glyoxylate. This can occur through defects in the aforementioned peroxisomal enzyme AGT (primary hyperoxaluria type 1). Clinical onset varies from a fatal neonatal presentation to adulthood and is characterized by progressive oxalosis and renal failure. [86]

Acatalasamia is caused by mutations in the catalase gene and is typically symptom free. However, there have been reports of increased susceptibility to oral ulcers and gangrenes, as well as diabetes mellitus and age-related diseases. [87–89]

C. elegans as a research model for peroxisomal biology

The nematode worm *C. elegans*, since its original implementation for developmental biology studies, has proven itself as an invaluable model that is useful in the study of various fields. Its versatility can be attributed to some extent to the unique combination of beneficial properties the organism possesses. First and foremost, its small and transparent body renders it ideal for the *in vivo*, non-invasive and real-time monitoring of processes through simple optical and fluorescent microscopy. Furthermore, the organism can be manipulated genetically with relative ease, thus facilitating the dissection of molecular pathways through forward and reverse genetics. This endeavour is further supported by the invariant number and lineage of the organism's cells and the complete knowledge of its neuronal connectome. Finally, its short base lifespan and its abundant reproduction make it ideal for the study of processes related to ageing [90]. All in all, the organism provides an ideal setup for studying the biology of all organelles, and peroxisomes are no exception.

Peroxisomes in *C. Elegans* have been reported to be mostly present in the epithelial cells of the gut and in the pharyngeal gland. Their number and size fluctuates with aging, and generally decreases past the larval stages [91,92]. *C. elegans* possesses only 11 peroxin genes: *prx-1*, *prx-2*, *prx-3*, *prx-4*, *prx-5*, *prx-6*, *prx-10*, *prx-11* (only one isoform), *prx-12*, *prx-13*, *prx-14* and *prx-19*. Homologues of peroxins 7 and 16 are notably absent. This of course means that the worm lacks a PTS2 matrix import pathway and may possess some differences in the PMP recruitment pathway. [93,94]

In addition to the typical functions, worm peroxisomes undertake a unique additional role by being responsible for the production of the dauer pheromones (daumones), a mixture of ascarosides that block larva development under hostile conditions and induce the transformation of the worm to the dauer, a resilient alternative developmental phase [90]. Daumone synthesis occurs through the b-oxidation pathway and requires the function of *daf-22*, *dhs-28* and *acox-1* [38,95,96].

A series of genetic experiments on *C. elegans* have made clear that peroxisomes are crucial to larval development. Silencing peroxin genes typically leads to developmental delay and larval arrest at early stages [91,93,97,98] The exact mechanism that causes this is still unclear, however it has been observed that *prx-5* (RNAi) can prevent the initiation of post embryonic cell divisions and normal cell migrations [93]. It has also been observed that developmental delay phenotypes can be reproduced by inhibiting ether lipid synthesis or beta oxidation [91,94]

On the other hand, silencing *prx* genes after development seems to be harmless or even beneficial under normal lab conditions. Silencing of *prx-5* in L4 larvae in an RNAi sensitive strain (*eri-1(mg366),* used to ensure maximum RNAi

effectiveness) leads to lifespan extension. A possible explanation could be that the reduced production of ROS in life stages when peroxisomal function is less relevant might be beneficial to the organism [99].

Peroxisomes and HSF-1: A possible link?

HSF-1 is the master regulator of the heat shock response, a widely conserved system that responds to sources of protein damaging stress (such as heat shock, oxidative stress, infection and inflammation) and induces the expression of cytoprotective factors known as heat shock proteins. Upon induction, HSF-1 trimerizes, accumulates in the nucleus and binds specific response elements (Heat Shock response Elements –HSEs), activating transcription [100].

HSF-1 is conserved in *C. elegans* and is similarly critical to the heat shock / proteotoxic response [101]. It is also necessary for proper larval development, implicated in the regulation of adult lifespan and crucial to the innate immunity response [102–108]. Even though it is generally agreed that upon heat shock HSF-1 is modified post-translationally, trimerizes and binds DNA in the nucleus, its exact localization is under dispute. Some report that HSF-1 is evenly distributed between the cytoplasm and the nucleus before heat shock, and that it acquires strong nuclear localization after it [109], while others claim that factor distribution between the cytosol and the nucleus is unaffected by the shock which instead redistributes the already nuclear HSF-1 to form distinct granules (notably co-localized with sites of active transcription) [110].

There is evidence that HSF-1 is related to peroxisomal function. Experiments in human embryonic kidney cells demonstrated that peroxisomally targeted luciferase can refold after heat shock in an HSF-1 dependent manner [111]. Peroxisomes can modify the activities of their pathways to adapt to different organismal temperature [95] Finally, hsf-1 is known to act protectively against oxidative stress [100,112] All this data led me into researching the possible role of HSF-1 in the regulation of peroxisomal biogenesis and function. The goal of this thesis is to present all the relevant data I have acquired up to this point as well as describe the procedures that I gradually developed to ensure experimental accuracy.

Experiments / Results

1) Examination of the role of HSF-1 on peroxisomal biogenesis.

In order to identify if HSF-1 has a regulatory role on peroxisomal biogenesis, RNAi silencing experiments were performed on a worm strain expressing GFP targeted to the matrix of gut peroxisomes (VS15: WT; *Is*[p_{ges-1}GFP::PTS1]). Photographs were taken (using a 4x lens) from day 4 adult VS15 worms treated (from stage L4) with either *hsf-1*(RNAi) or an empty RNAi vector (pL4440). Whole body GFP fluorescence intensity was measured in each worm and the two samples were compared statistically. A significant reduction of GFP fluorescent emission was observed in worms treated with *hsf-1*(RNAi) (fig. 4A-C).

In order to exclude the possibility that this effect was due to inhibition of the *ges-1* promoter, its activity was measured with RT-qPCR. Whole worm mRNA was isolated from day 4 adult N2 worms treated from stage L4 with *hsf-1*(RNAi) or an empty RNAi vector (pL4440). Reverse transcription was performed and real time PCR was used to quantify the produced cDNA. Comparing the two samples demonstrated that *hsf-1*(RNAi) has no effect on *ges-1* transcription in WT worms (fig. 4D).

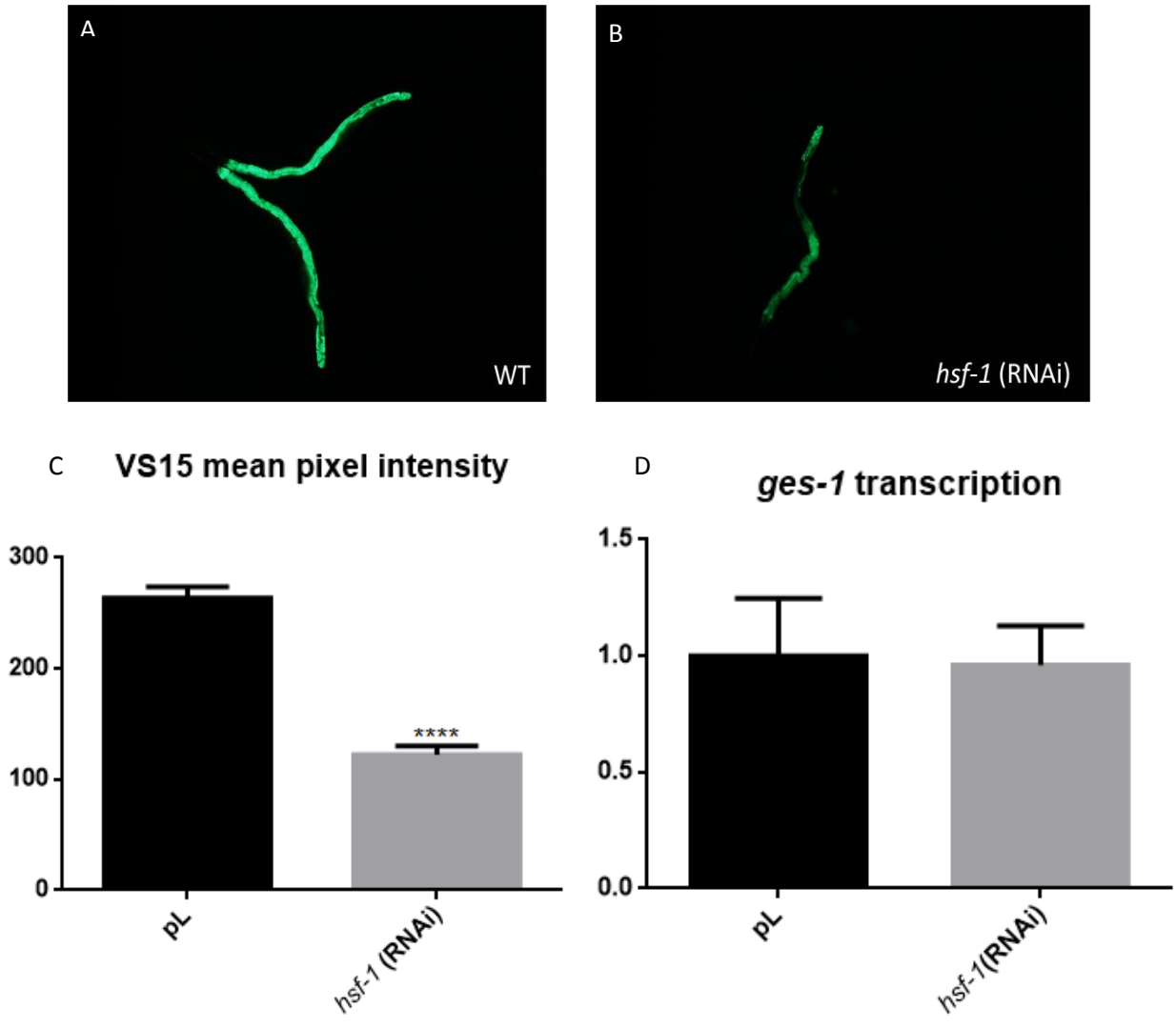


Figure 4: A-C) Assessment of peroxisomal import via the VS15 (WT; *Is*[p_{ges-1}GFP::PTS1]) reporter strain. The levels of imported GFP are reduced under *hsf-1* RNAi. **D)** Confirmation via qPCR that the observed GFP reduction is not a result of *ges-1* promoter inhibition.

2) Acute heat shock resistance assay.

An acute heat shock survival assay was performed to examine the possibility of peroxisomes being involved in the heat shock response. Wild type worms (N2 strain) and worms with defective hsf-1 (PS3551: *hsf-1*(sy441)) were submitted (from stage L4) to RNAi for three peroxisomal genes (*prx-5*, *prx-19* and *lonp-2*) or RNAi control (empty pL4440 vector). When the animals reached 4 days of adulthood they were exposed to acute heat shock for 9 hours, and their survival was measured. RNAi inhibition of *prx-19* and *lonp-2* significantly reduced the resistance of N2 worms to HS and further augments the sensitivity of *hsf-1*(sy441) worms. RNAi for *prx-5* had no effect on the survival of either strain (fig. 5).

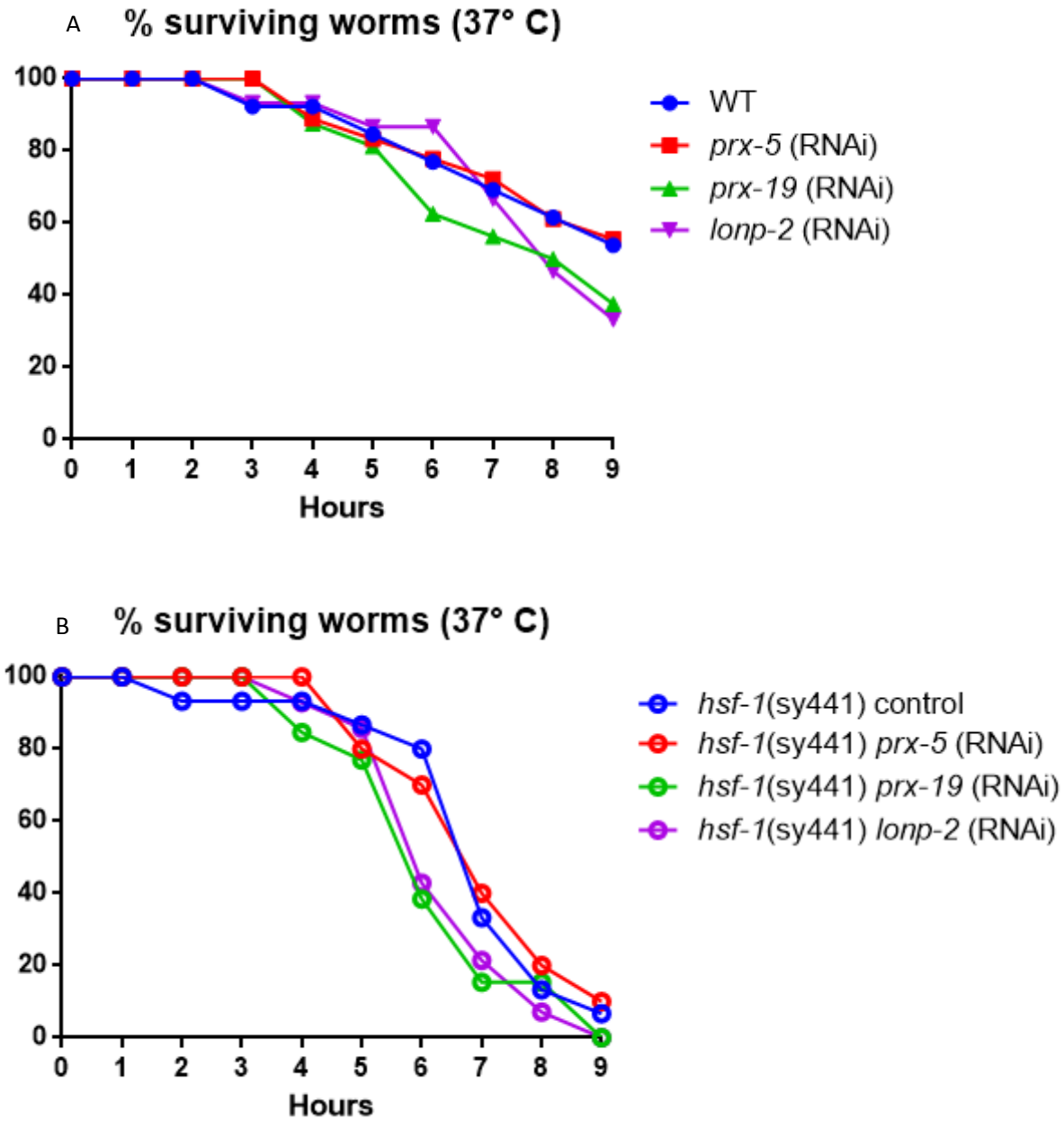


Figure 5: Assessment of survival under combination of acute heat shock (37°) and RNAi treatment for peroxisomal genes in WT (**A**) and *hsf-1* defective (**B**) worms.

3) High temperature lifespan assay

In addition to the short term, acute heat stress survival assay, I conducted a long term, mild heat stress lifespan assay. Wild type worms were submitted (from the egg stage) to RNAi for three peroxisomal genes (*prx-5*, *prx-19* and *lonp-2*) or RNAi control (empty pL4440 vector) and their ability to survive in conditions of mild heat stress (25°) was measured and compared to normal growth conditions (20°). RNAi for *prx-19* negatively affected worm survival at

both temperature conditions. RNAi for *lonp-2* had a negative impact on lifespan, but only under normal temperature. RNAi for *prx-5* had no significant effect in either case (fig. 6).

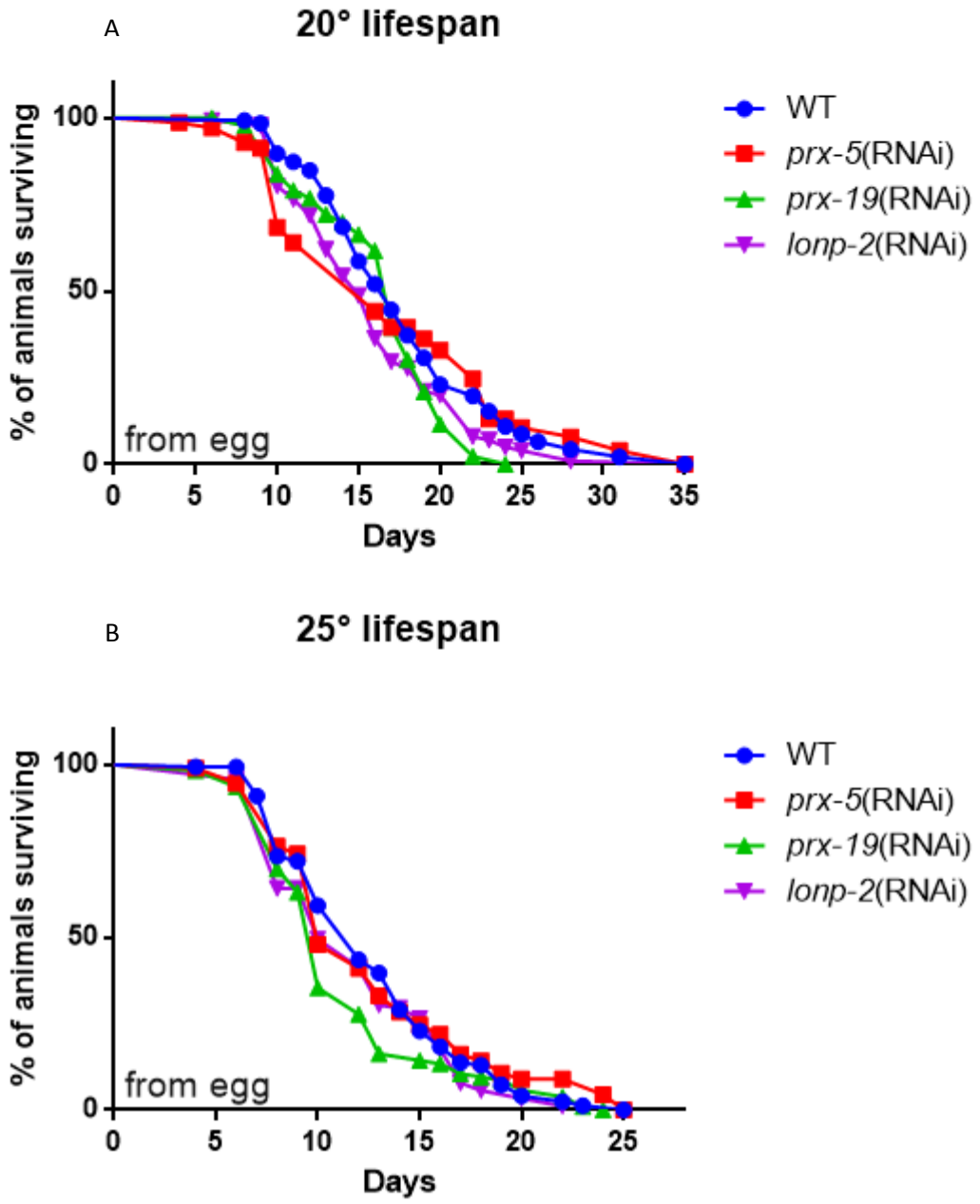


Figure 6: N2 worm lifespan assessment under RNAi for different peroxisomal genes at 20° (**A**) and 25° (**B**)

4) Heat shock response activation assay

In order to further examine the possible relation of peroxisomal genes to the heat shock response I performed RNAi experiments on two different fluorescent reporter systems.

The first was OG497, a transgenic strain ([*hsf-1*p::*hsf-1*::GFP::*unc-54*utr]) expressing GFP-tagged HSF-1. The strain was produced by Morton *et. al.* [110] and, according to their published data, exhibits induction of HSF-1::GFP granulization under heat stress or oxidative stress (conditions known to induce the *hsf-1* response pathway). Initial attempts to examine the effect of RNAi for peroxisomal genes on HSF-1 induction were a failure, as control strains demonstrated significantly more granulization than reported in the original data. Thus a series of experiments was conducted to identify possible external factors with an effect on the HSF-1::GFP system so that they can be properly controlled in future experiments.

First and foremost, I confirmed that the system worked properly at experimental conditions matching those used in the original publication (D1 adult worms on NGM medium, 20' HS at 37°) (fig. 7A-C). I then proceeded to examine if

worm age had any effect on the system by measuring granulization in D1 and D4 adult worms, with and without HS. D4 animals demonstrate elevated baseline granulization compared to D1 worms, but the effect of response induction persists (fig. 7D-F).

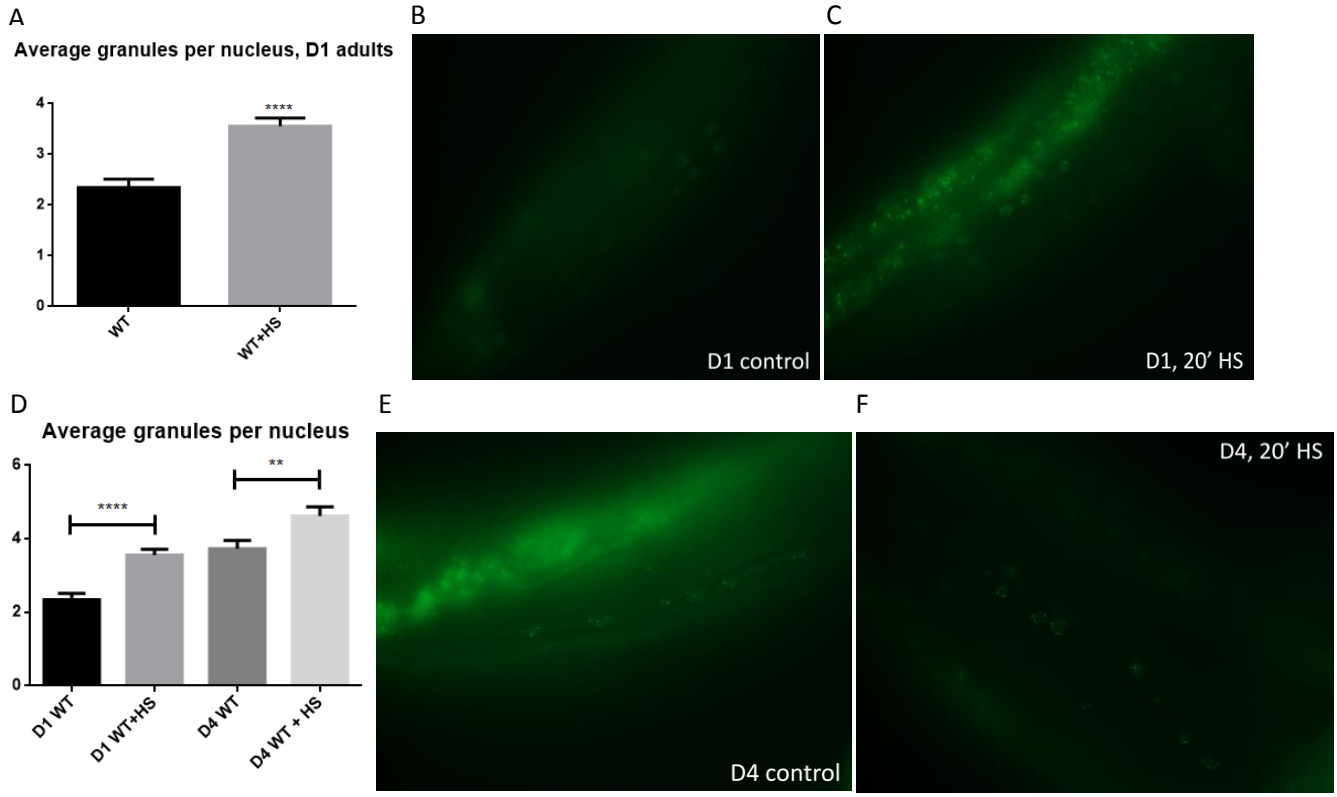


Figure 7 A-C) Assessment of heat shock induced HSF-1::GFP nuclear granulization in D1 animals. **D)** Assessment of the effect of animal age on heat shock induced HSF-1::GFP nuclear granulization. **E-F**) Assessment of heat shock induced HSF-1::GFP nuclear granulization in D4 animals.

Since Morton et. al. didn't perform RNAi experiments with their worm model, there was a possibility that the RNAi feeding process itself has an effect on HSF-1 granulization. Indeed feeding worms with HT115 bacteria containing an empty RNAi vector (pL4440) is sufficient to induce granulization in D1 adult worms (fig. 8A), however further HS induction effects persist (fig. 8B).

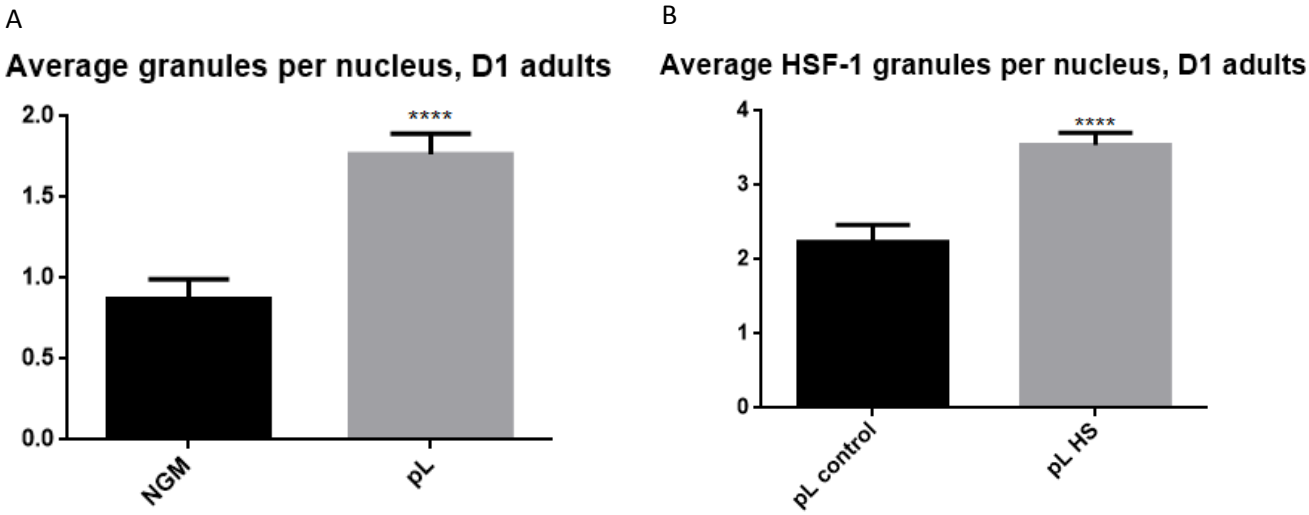


Figure 8: A) Assessment of food type effect on baseline HSF-1::GFP nuclear granulization in D1 animals. **B)** Assessment of heat shock induced HSF-1::GFP nuclear granulization in D1 animals fed with HT115 bacteria expressing empty pL4440 vector.

Finally, I performed a delayed observation test. I submitted OG497 animals to 20' 37° HS and prepared both the stressed and control samples for microscopy immediately after the shock. However, I delayed observation for 20 minutes, to emulate the typical delay caused by the preparation of additional samples in an experiment with more conditions. This led to the loss of the induction phenotype, as even unstressed worms exhibit elevated granulization (fig. 9). This indicates that each sample should be observed immediately after preparation. Thus I decided to alter my typical experimental methodology (heat shock each sample simultaneously, prepare all of them for microscopy and observe them sequentially) to accommodate for this need. The new method involves submitting each sample individually to heat shock, preparing it for microscopy and immediately observing it for a maximum of 15-20 minutes before repeating the procedure for the next sample.

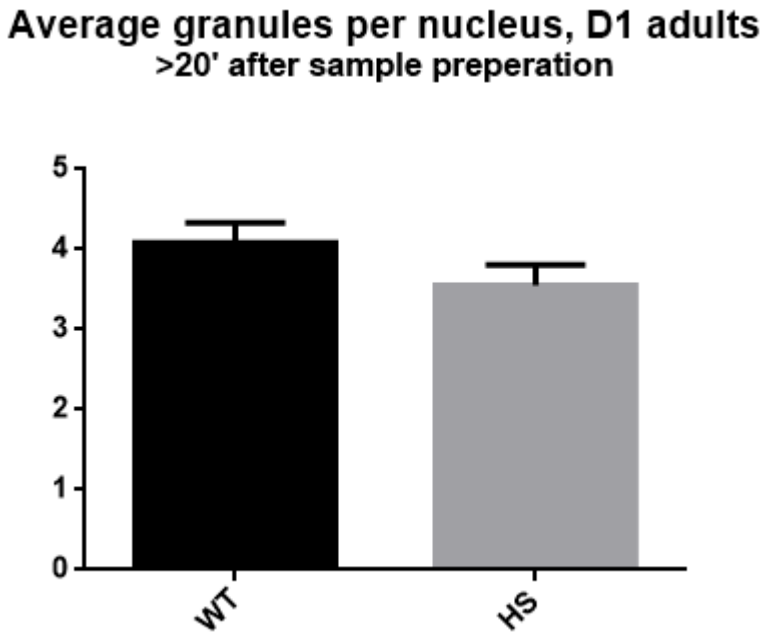


Figure 9: Assessment of the effect of sample preparation for microscopy on HSF-1::GFP nuclear granulization in D1 animals.

Following this new method, I submitted OG497 worms to RNAi for *prx-5, prx-19* and *lonp-2* and compared HSF-1::GFP granulization with worms fed with a control vector, both stressed and unstressed. The first iteration of this experiment demonstrated that RNAi for *prx-5* reduces granulization in D1 adult worms, while RNAi for *lonp-2* induces it to levels similar to those caused by heat shock (fig. 10).

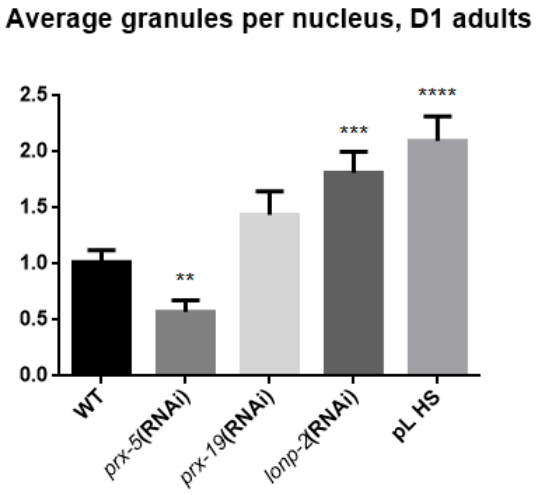


Figure 10: Assessment of the effect of peroxisomal gene RNAi on HSF-1::GFP nuclear granulization in D1 animals.

The second reporter is CL2070, a strain expressing GFP under the control of the promoter of *hsp-16.2.* The gene is a constituent of the HS response and a known transcriptional target of HSF-1. Therefore measuring GFP emission from worms of this strain allows for relative quantification of HSF-1 transcriptional activity.

Performing RNAi for peroxisomal genes (*prx-5, prx-19* and *lonp-2*) has no effect on the reporter in the absence of HS in D4 adult animals (fig. 11A). Under heat shock however (20 minutes at 37°, followed by an hour of recovery at 20°), the results are not so definitive. RNAi for *prx-5* reduced induction levels consistently. RNAi for *prx-19* had no significant effect but demonstrated trends towards both induction and inhibition. RNAi for *lonp-2* exhibited contradictory significant effects in different iterations of the experiment (fig. 11D-E).

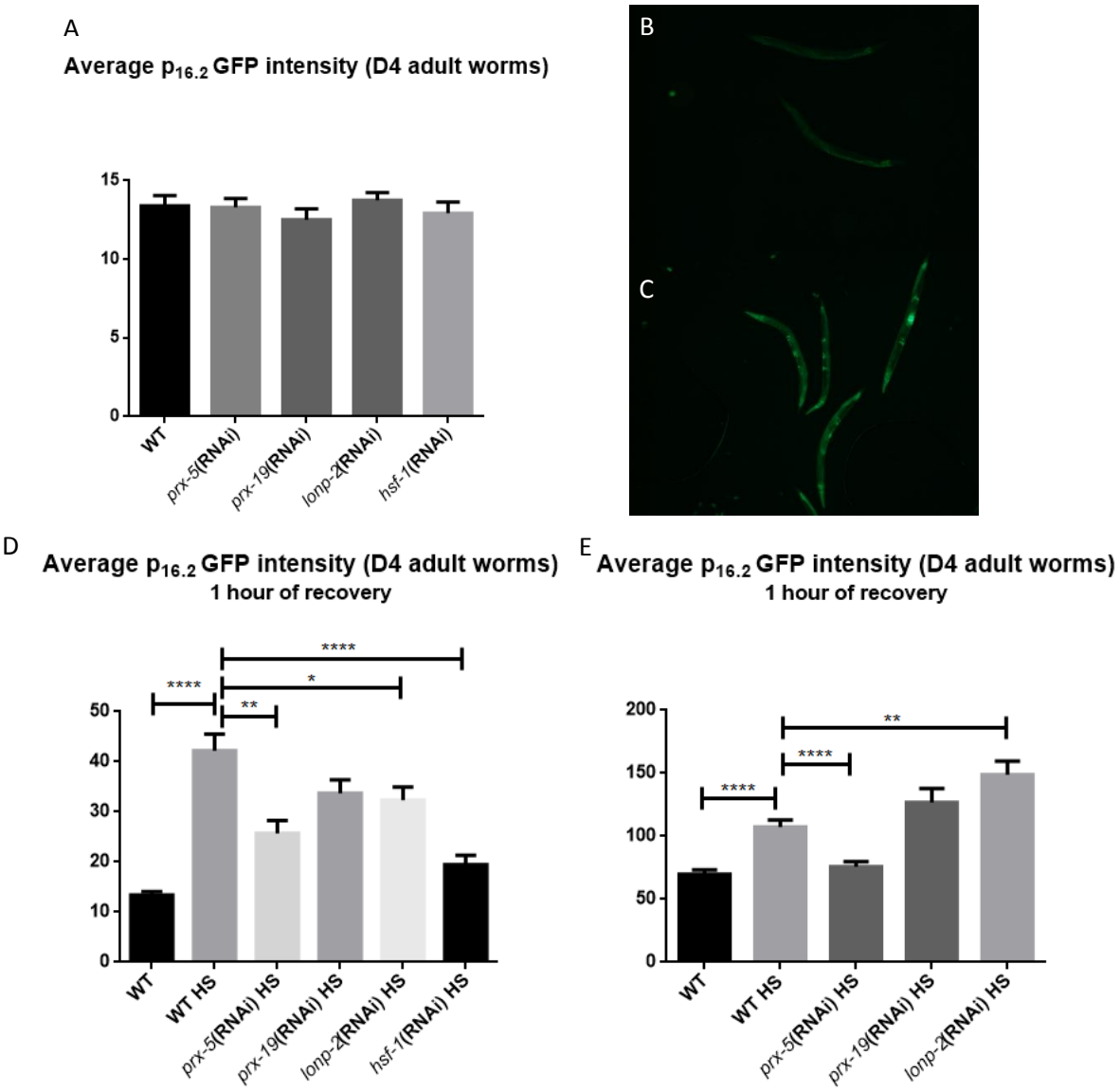
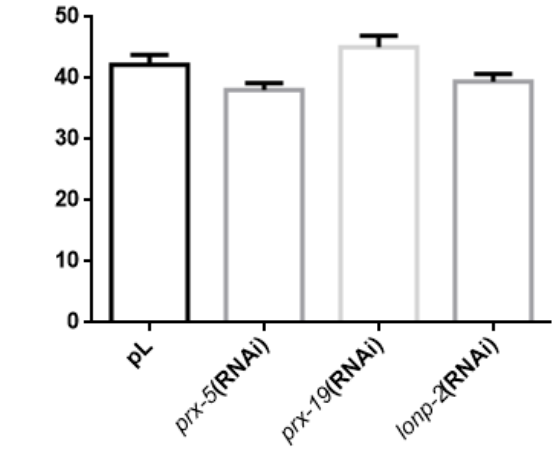


Figure 11: A) Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D4 adult worms. **B)** $p_{hsp-16.2}$ GFP fluorescence in normal conditions. **C)** $p_{hsp-16.2}$ GFP fluorescence induced by 20' 37° heat shock after 1 hour of recovery. **D-E)** Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D4 adult worms after 20' 37° heat shock and 1 hour of recovery.

Similar experiments were performed on D1 adults. The first iteration demonstrated that, similar to D4 worms, none of the RNAi treatments affected the reporter in the absence of heat shock (fig. 12A). After HS and 1 hour of recovery, both *prx-5* and *prx-19* RNAi augmented *hsp-16.2* promoter induction, while *lonp-2*(RNAi) had no significant effect (fig. 12B).

A



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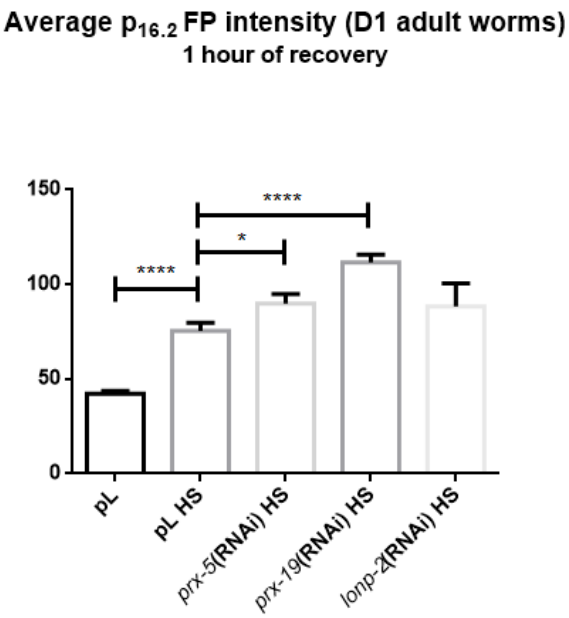
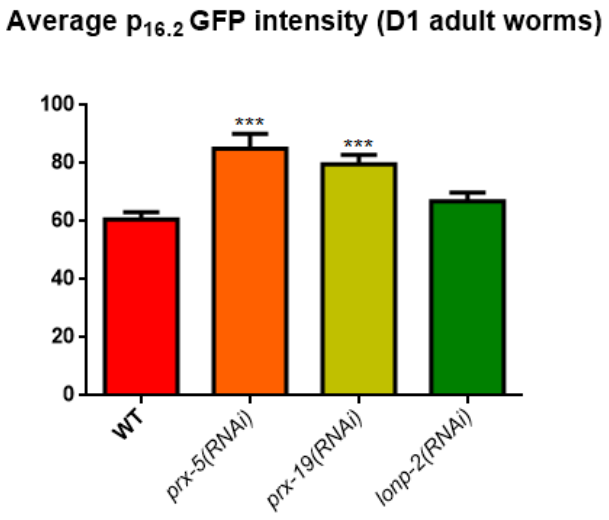


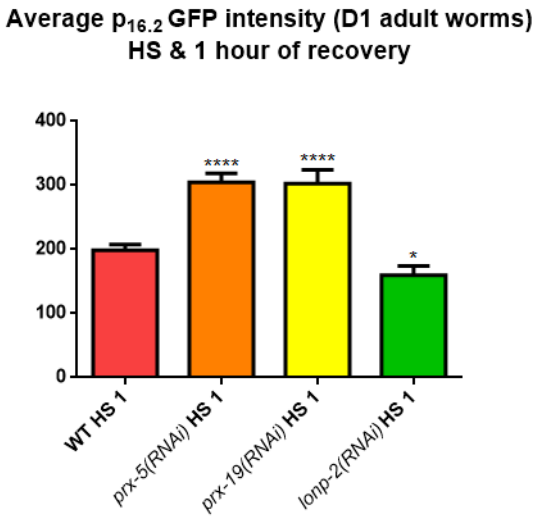
Figure 12: A) Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D1 adult worms. **B)** Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D1 adult worms after 20' 37° heat shock and 1 hour of recovery.

The second iteration demonstrated the only case of significant *hsp-16.2* promoter induction under regular conditions, in worms subject to RNAi for *prx-5* or *prx-19* (fig. 13A). RNAi for these genes also augments reporter activity after HS and 1 hour of recovery, while RNAi for *lonp-2* inhibits it (fig. 13B). In this instance, I also observed shocked worms after 2 hours our recovery, which led to a surprising discovery: the augmenting effect of *prx-5* RNA seems to disappear, as *hsp-16.2* promoter induction seems to pause. All other samples exhibit increased induction after the additional recovery, which allows control worm reporter activity to reach levels equal to the *prx-5* RNA population (fig. 13C-D).

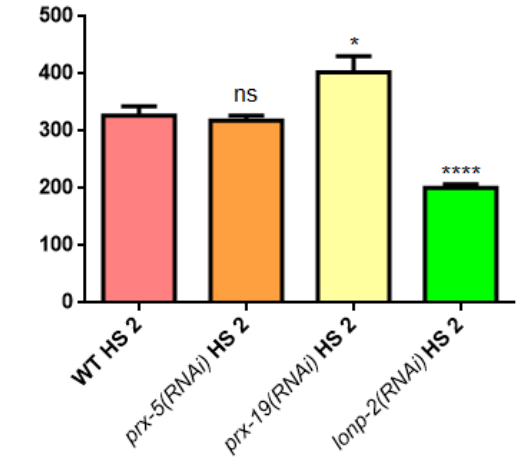
A



B



C



D

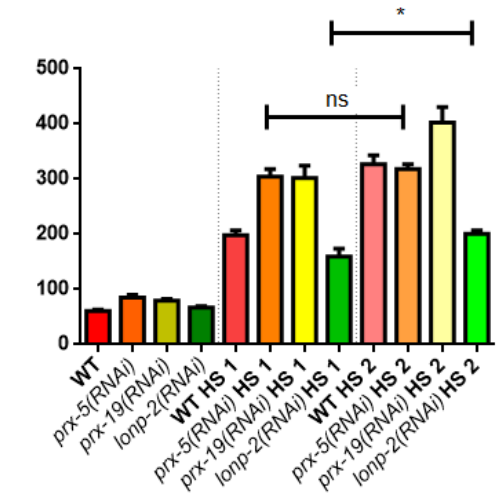


Figure 13: A) Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D1 adult worms. **B)** Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D1 adult worms after 20' 37° heat shock and 1 hour of recovery. **C)** Assessment of *hsp-16.2* promoter activity under RNAi for peroxisomal genes in D1 adult worms after 20' 37° heat shock and 2 hours of recovery. **D)** Summary of A-C.

Discussion

1) HSF-1 affects peroxisomal dynamics.

The RNA inhibition of *hsf-1* causes a reduction of peroxisomal matrix GFP in a promoter independent way. There are several possible explanations for this phenomenon. First, the RNAi treatment could simply inhibit peroxisomal matrix protein import, thus preventing GFP from entering the organelle. There is evidence that *hsf-1* inhibition may affect the expression of components of the matrix import pathway, particularly *prx-5*[113]. Alternatively, it could cause a significant effect on peroxisomal membrane biogenesis, leading to a reduction in total peroxisomal quantity. There is also the possibility that the effect is a result of increased protein turnover in the peroxisomes (for instance by the worm homologue of LON protease 2) or increased organelle degradation via pexophagy or the 15-LOX pathway. All these possibilities are going to be addressed in subsequent experiments.

2 & 3) Peroxisomal genes and heat shock resistance.

Contrary to previously published data [114], RNAi for *prx-5* has no effect on the ability of worms to survive heat shock. Inhibition of *prx-19* and *lonp-2* negatively affects heat resistance in cases of acute heat shock. However since both genes seem to be somewhat necessary for survival under normal conditions, and *lonp-2* RNAi worms live as long as WT controls at 25°, it is likely that the observed effects represent a role in a survival related mechanism that is not heat-shock specific. Nevertheless, more experimental iterations are required before we can safely draw any conclusions and hypothesize what that mechanism (if it exists) might be.

4) Peroxisomal genes and the heat-shock resistance pathway

The results obtained from experimentation with the CL2070 reporter are somewhat contradictory. Peroxin 5 seems to be necessary for the induction of the HS response in D1 adults, while it seems to inhibit the response (at least during the first hour of recovery) in D4 animals. Peroxin 19 has an unclear, if any effect in D1 adults, yet consistently seems to inhibit pathway activity in D4 animals. RNAi for *lonp-2* causes contradictory but significant effects in D1 animals and may repress the pathway in D4 ones. Inconsistency between animals of different ages is not necessarily unexpected, as aging can severely affect cell metabolism and alter gene expression, however it is probably better to avoid drawing any conclusions before more experimental iterations clarify what happens in each individual age group.

The different effect of *prx-5* RNAi in D4 adult worms after 1 and 2 hours of recovery is particularly notable, as it reveals that *hsp-16.2* promoter activation may not always follow similar kinetics. In worms fed with control vector or RNAi for *prx-19 / lonp-2*, the reporter seems to follow a linear activation pattern (fig. 13D), as its activity gradually increases over time after HS (of course the rate of this increase differs among the samples, as *prx-19* RNAi augments it and *lonp-2* RNAi reduces it compared to control worms). In worms fed with *prx-5* RNAi the reported is activated quickly but also seems to reach a maximum activation level. A possible explanation for this is that the heat shock response is biphasic; if it involves a fast short term component and a slow long term component then *prx-5* might interact only with the first, leading to quick induction that cannot be maintained for prolonged periods of time. To address this possibility, I believe that all new experiments involving this reporter system should assess kinetics by observation at multiple time points.

As far as OG497 is concerned, it is clear that the system is quite sensitive to factors beyond heat shock. The elimination of the delay between shock and observation under the microscope seems to have solved prior issues with seemingly stressed controls, meaning that the reporter is definitely usable and could provide us with valuable data after a few experimental repeats.

Concluding remarks

All in all, despite being somewhat inconclusive, the data presented in this thesis supports the idea of a relation between peroxisomes and the heat shock response pathway. I believe that the further investigation of this relation and its mechanistic details will be a worthwhile endeavor in our attempt to further our understanding of peroxisomal and cellular biology.

Materials and methods

Worm feeding and handling

During regular strain maintenance and any experiment that didn't involve RNAi, worm strains were fed with OP50 bacterial food (OP50 *E. coli* strain grown on Normal Growth Medium Petri dishes). The worms were manipulated under a dissecting microscope with the help of picks made out of platinum wire or eyelash hair. Due to the nature of the investigated pathways, all manipulations (when possible), including sample preparation for microscopy, were conducted at a temperature of 20° to prevent accidental induction of the heat shock response.

Strains were maintained every 4 days on average (with the exception of *hsf-1*(sy441) worms, which grow slower than normal) by moving a few L4 worms from the latest generation into a Petri dish with fresh bacterial food. All experiments were performed using strains that had been well fed and unstressed for at least two generations beforehand, to prevent any transgenerational effects from affecting the outcome of the experiments.

RNA inhibition

All RNA experiments were performed by feeding worms with *E. coli* (strain HT115) carrying a vector (pL4440) with the ability to express double stranded RNA (with the help of two flanking T7 promoters) for an insert of choice after induction with IPTG. Experiments used at least 20 worms per strain.

In order to ensure all animals were of the same age (and thus prevent any age-related factors from skewing the end results), worms were typically synchronized either during the L4 stage (by selecting worms based on vulval morphology), or during the egg stage via egg-laying (moving adult worms to an empty dish and collecting all eggs laid within a 3-4 hour window). The exception was the lifespan experiment, where worms were synchronized during the egg stage with hypochloride treatment.

Unless stated otherwise, worms observed at the D4 adult stage were submitted to RNAi from the L4 stage, while worms observed at the D1 adult stage were submitted to RNAi from the egg stage. This was done to maximize the effectiveness of the treatment.

Fluorescent microscopy

Worms were immobilized with the use of a 20µl drop of 20mM tetramisole for imaging. All imaging experiments presented here were performed under a Zeiss Axio Imager Z2 Epifluorescence/DIC Microscope. Imaging conditions (i.e exposure, contrast) remained consistent throughout each experiment to ensure different samples were actually comparable. All image analysis was conducted with the help of the ImageJ software platform.

Granulization in OG497 worms was assessed by counting the number of distinct high intensity granules in every observable nucleus of the worm gut and hypodermis and extracting the mean granule per nucleus ratio for the entire sample population (multiple photographs were taken with a 63x lens for each section to ensure granules from all possible focus layers were accounted for).

The activation of the *hsp-16.2* promoter in CL2070 worms was assessed by measuring mean GFP fluorescence in the front third of worm bodies (to avoid GFP emission from eggs within the gonad) in photographs taken with a 4x lens.

All statistical analysis of the acquired data (t-tests and ANOVA) was performed with the help of the Graphpad Prism software platform.

Heat shock

Heat shock was induced by moving worm plates to a 37° incubator for an appropriate amount of time (20' unless mentioned otherwise). All worm plates were in contact with the same surface in the incubator to ensure equal heating. For recovery (which is necessary for *hsp-16.2* promoter activation), worm plates were moved to 20° immediately after the end of HS exposure.

The acute heat shock survival experiment involved submitting 25 worms from each sample to continuous heat shock for up to 9 hours. Worm survival was assessed after every hour by briefly removing the animals from the incubator and examining their mobility.

Lifespan assay

The long term lifespan assay involved measuring the survival of worms every day until every single one was dead. Each sample population included ~100 worms at the beginning. Worms food was renewed every 2 days, to prevent starvation and ensure consistent RNA inhibition throughout the whole experiment.

Lifespan curve statistical analysis was performed with the help of Graphpad Prism.

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