이학박사 학위논문

초파리의 S2 세포에서 selenophosphate synthetase 1이 선천성 면역 신호전달과 ERK/MAPK 신호전달에 미치는 영향에 관한 연구

Studies on the effects of selenophosphate synthetase 1 on innate immune signaling and ERK/MAPK signaling in *Drosophila* S2 cell

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Ph.D. Dissertation

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College of Natural Sciences
Department of Biological Sciences
Seoul National University

Tack-Jin Yoo

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Advisor

Professor Jin-Hong Kim, Ph. D.

Submitting a Ph.D. Dissertation of Science

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College of Natural Sciences
Department of Biological Sciences
Seoul National University

Tack-Jin Yoo

Confirming the Ph.D. Dissertation written by
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Chair	김 종 서	(Seal)
Vice Chair	김 진 홍	(Seal)
Examiner	이 병 재	(Seal)
Examiner	김 락 균	(Seal)
Examiner	김 미 진	(Seal)

ABSTRACT

Selenophosphate synthetase 1 (*SPS1*) is an essential gene for the cell growth and embryogenesis in *Drosophila melanogaster*. We have previously reported that SPS1 deficiency stimulates the expression of genes responsible for the innate immune system, including antimicrobial peptides (AMPs), in *Drosophila* S2 cells. However, the underlying mechanism has not been elucidated. Here, we investigated the immune pathways that control the SPS1-deficiency–induced expression of AMPs in S2 cells. It was found that the activation of AMP expression is regulated by both immune deficiency (IMD) and the Toll pathway. Knocking down a member of each pathway along with SPS1 showed that the peptidoglycan recognition protein-LC (PGRP-LC) and Toll genes are targeted by SPS1 for regulating the IMD and Toll pathways, respectively. We also found that these two pathways regulate AMP expression by cross-talking. The levels of *PGRP-LC* and *Toll* mRNAs were upregulated upon *Sps1* knockdown, and overexpression of each protein upregulated AMPs. Interestingly, PGRP-LC overexpression upregulated AMP more than Toll overexpression did. These data strongly suggest that SPS1 controls the innate immune system of *D. melanogaster* through regulating PGRP-LC and Toll expression.

The primary effect of SPS1 deficiency is inhibition of vitamin B6 synthesis. While investigating this next mechanism in *Drosophila* S2 cells, we noted that SPS1 regulates cell growth. Therefore, it was hypothesized that SPS1 affects the ERK/MAPK signaling, and it was consequently revealed that SPS deficiency

decreases ERK phosphorylation. Surprisingly, while testing whether insulin treatment, which activates ERK, could prevent the cell growth retardation caused by SPS1 deficiency, it was observed that megamitochondria were not formed. Afterward, whether other phenotypes of SPS1 deficiency could be likewise suppressed was investigated, and both the accumulation of reactive oxygen species (ROS) and activation of innate immunity upon SPS1 deficiency were found suppressed by insulin treatment. These results suggest that SPS1 regulates cell growth, megamitochondria formation, ROS accumulation, and innate immunity through the ERK/MAPK signaling pathway.

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Keyword: selenium, SPS1, innate immunity, AMPs, PGRP-LC, Toll, ERK/MAPK signaling pathway, megamitochondria, reactive oxygen species

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LITERATURE REVIEW

1. SELENIUM BIOLOGY

1.1. Selenium and human health

Selenium is an essential trace element found in soil. The selenium content of plants depends on the selenium content of the soil they grow in, and animals acquire selenium by consuming plants or other animals. Selenium provides many health benefits. For example, it plays roles in the prevention of cancer and heart diseases, suppression of viral expression, male reproduction, anti-aging, and activating the immune system (Hatfield *et al.*, 2006). Most of the benefits of selenium are likely determined by selenoproteins, which comprise the amino acid selenocysteine (Sec) (Brigelius-Flohe, 2008; Hatfield & Gladyshev, 2002; Lu & Holmgren, 2009). Since selenium is important for living beings, fine regulation of its content is essential. Selenium deficiency or overdose induces many diseases. Keshan disease, which is endemic cardiomyopathy with high mortality rates, and Kashin-Beck disease, a chronic, endemic type of osteochondropathy, are caused by selenium deficiency. These diseases have been observed in China as well, where the selenium content of the soil is low (Burk, 1994). Conversely, selenium overdose induces selenosis, exemplified by alkali disease and blind stagger. Alkali disease is associated with impaired vision, depressed appetite, and wandering in circles. Blind stagger has symptom of several unrelated animal diseases; the affected animal walks with an unsteady, staggering gait and appears blind. Extreme overdose of selenium leads to acute selenosis and may result in death. Thus, the amount of selenium intake must be

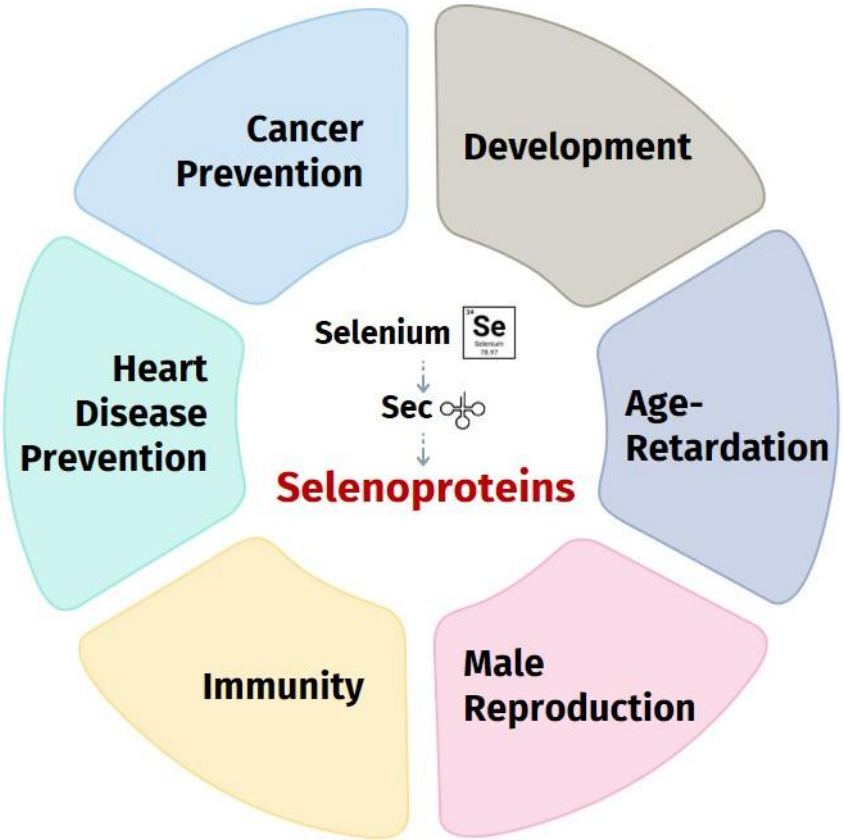

Figure 1.1. Roles of selenium in human health. (adopted from Bang, 2022).

regulated very precisely.

1.1.1. Selenium for cancer prevention

Among the roles of selenium, its function as a cancer chemopreventive agent has received the most attention. It has been known through several epidemiological studies that selenium suppresses cancer development. The inverse relationship between selenium supplementation and cancer mortality has been reported by Schrauzer *et al.* (Schrauzer *et al.*, 1977). Results from other studies also support the anti-cancer benefits of selenium. Notably, cancer mortality is significantly high in selenium-poor countries (Clark *et al.*, 1991).

The chemotherapeutic effect of selenium has been evidenced by comparing the incidence of cancer in people (Blot *et al.*, 1993) who daily consume a dietary supplement of 200 μg of selenium with those who do not (Clark *et al.*, 1996). Additionally, in a mouse xenograft model, sodium selenite or selenomethionine treatment significantly reduced cancer formation (Yang *et al.*, 2009). In addition, selenite is preferentially cytotoxic to various human glioma cells over healthy astrocytes through autophagic cell death, which is induced by superoxide anion generated by selenite (Kim *et al.*, 2007).

Several mechanisms have been proposed to mediate the anti-cancer effect of selenium. First, selenoproteins, such as glutathione peroxidase and thioredoxine reductase, remove reactive oxygen species, thereby providing an antioxidant effect and modulating the redox homeostasis. Second, the intermediates in selenium

metabolism, such as selenoglutathion, hydrogen selenide, and methylated metabolites of selenide, prevent the proliferation of cancer cells (Hatfield *et al.*, 2006).

However, the mechanism whereby selenium suppresses cancer is not well known, and there has been considerable debate in the field about whether it is selenoproteins or small molecule selenocompounds that suppress cancer (Hatfield *et al.*, 2009). Interestingly, some selenoproteins seem to be responsible to prevent and promote cancer. For example, TR1 and Sep15, and perhaps other selenoproteins, appear to perform in cancer prevention in healthy cells, but they promote malignancy in cancer cells (Hatfield *et al.*, 2009).

1.1.2. The effect of selenium on immunity

Immune-related organs, such as the liver, spleen, and lymph nodes, include significant amounts of selenium, suggesting the role of selenium in the immune system. In fact, selenium deficiency causes immunodeficiency and impairs both cell-mediated immunity and B-cell function (Spallholz *et al.*, 1990). Conversely, selenium supplementation has immunostimulatory effects, such as induction of the proliferation of activated T cells (Kiremidjianschumacher *et al.*, 1994), augmentation of the sensitivity of lymphocytes to antigen stimulation, and enhancement of the capacity of lymphocytes to differentiate into cytotoxic cells. Moreover, the activity of natural killer cells is also increased by 82% (Kiremidjianschumacher *et al.*, 1994).

Selenium supplementation can upregulate the receptors of the growth-regulatory cytokine interleukin-2 on the activated lymphocytes and natural killer cells.

Consequently, the augmented interaction of the receptors and interleukin-2 contributes to the clonal expansion and differentiation of T cells into cytotoxic T cells. Such enhancing activity of selenium on the immune system can be observed upon dietary supplement of selenium even in individuals with so-called "selenium-replete" levels (Kiremidjianschumacher *et al.*, 1994).

Another piece of evidence supporting the functional need for selenium in immune cells is the upregulated selenophosphate synthetase activity in activated T cells (Guimaraes *et al.*, 1996). The enhancement of selenophosphate synthetase activity facilitates the synthesis of selenocysteine, which in turn serves as the building block of selenoproteins.

1.2. Selenoprotein biosynthesis

Most of the characterized selenoproteins are enzymes involved in redox homeostasis and contain Sec in their active sites (Stadtman, 2000). Sec is structurally similar to serine and cytosine, except that it contains selenium instead of oxygen and sulfur, respectively. Most living beings, except higher plants and yeasts, have selenoproteins.

1.2.1. Mechanism of selenocysteine biosynthesis

Sec is the 21st amino acid in the genetic code and is incorporated into selenoproteins during translation in response to the UGA Sec codon (Birringer *et al.*, 2002; Hatfield & Gladyshev, 2002). Most amino acids are first synthesized or introduced into cells and then incorporated onto their cognate tRNAs by aminoacyl-tRNA synthetases. Sec

is distinct from the other 20 amino acids in that its biosynthesis occurs on its tRNA. The codon for this amino acid is UGA, also known as the stop codon.

The biosynthetic mechanism of Sec slightly differs between prokaryotes and eukaryotes. In prokaryotes, Sec is directly synthesized after serine is charged onto $tRNA^{[Ser]Sec}$ and the hydroxyl moiety of serine is converted with a selenium moiety (Forchhammer & Bock, 1991; Hatfield & Gladyshev, 2002). In eukaryotes, an additional enzyme is involved in Sec synthesis; Seryl-$tRNA^{[Ser]Sec}$ synthetase aminoacylates (SerS)$tRNA^{[Ser]Sec}$ with serine (SerS; Figure 1.2). Then, the seryl moiety of Seryl-$tRNA^{[Ser]Sec}$ is phosphorylated by O-phosphoseryl-$tRNA^{[Ser]Sec}$ kinase (PSTK) (Carlson *et al.*, 2004) to yield phosphoseryl-$tRNA^{[Ser]Sec}$, which is catalyzed to Sec-$tRNA^{[Ser]Sec}$ by selenocysteine-$tRNA^{[Ser]Sec}$ synthase (SEPSECS) (Yuan *et al.*, 2006). Sec-$tRNA^{[Ser]Sec}$ is used on the ribosome to incorporate Sec into a specific site in the nascent polypeptides of selenoproteins. The active donor of selenium in Sec biosynthesis is monoselenophosphate (Glass *et al.*, 1993), which is synthesized from selenite and ATP by selenophosphate synthetase (SPS) (Ehrenreich *et al.*, 1992). Later, SPS was officially renamed SEPHS (V. N. Gladyshev *et al.*, 2016). However, it is still called SPS in *Drosophila melanogaster*.

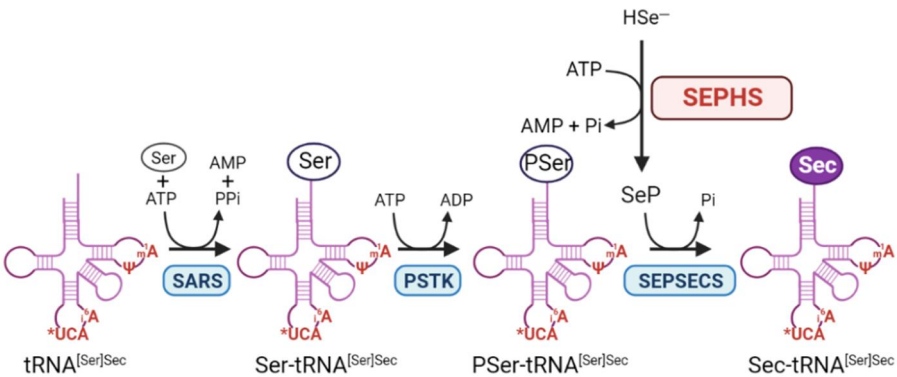

Figure 1.2. Sec biosynthesis in eukaryotes. The pathway of Sec biosynthesis. SARS, Seryl-tRNA$^{[Ser]Sec}$ synthetase; PSTK, O-phosphoseryl-tRNA$^{[Ser]Sec}$ kinase; and SEPSECS, selenocysteine-tRNA$^{[Ser]Sec}$ synthase. *U indicates mcm^{5}U or mcm^{5}Um (adopted from Bang, 2022).

1.2.2. Incorporation of selenocysteine into protein

For the UGA codon, known as the stop codon, to serve as the Sec codon, a distinct mechanism operates. In addition to the Sec-tRNA$^{[Ser]Sec}$, which decodes the UGA codon, several factors that are required in the re-coding of the UGA codon as Sec and incorporation of this amino acid into proteins have been identified. One of the novel features of selenoprotein mRNAs is the creation of a cis-stem-loop structure known as the Sec Insertion Sequence (SECIS) element or elements in the 3′- untranslated region (3′-UTR) of selenoprotein mRNAs (Low & Berry, 1996). SECIS elements recode the UGA codon to Sec and bypass the translational stop. In addition to these two cis-acting factors, there are several trans-acting factors involved in the incorporation of Sec into proteins. These factors include selenocystenyl-tRNA$^{[Ser]sec}$ specific elongation factor (EFsec) (Tujebajeva *et al.*, 2000), SECIS- binding protein (SBP2) (Copeland *et al.*, 2000), and L30 ribosomal protein (rpL30) (Chavatte *et al.*, 2005).

The mechanism of selenocysteine incorporation into proteins is summarized in Figure 1.3. Selenocysteyl-tRNA forms a complex with EFsec, SBP2, and the SECIS element to be transferred to the ribosomal aminoacyl site decoded by UGA. When the Sec-tRNA$^{[Ser]Sec}$ complex is transferred to the aminoacyl site, Sec-tRNA^{Sec} is transferred to the peptidyl site, and then Sec is incorporated into the nascent selenopeptide (Hatfield & Gladyshev, 2002).

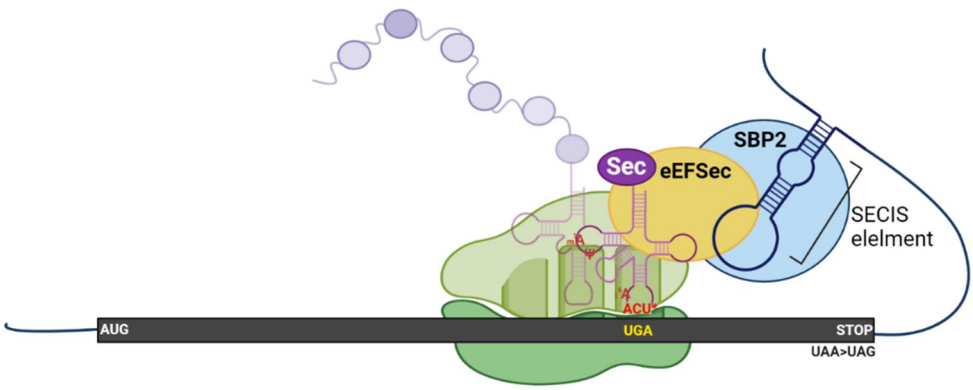

Figure 1.3. Selenoprotein biosynthesis. Selenocysteine is incoporated into protein at the UGA codons along with various factors that form the selenocysteine insertion machinery. SECIS, selenocysteine insertion sequence; SBP2, SECIS-binding protein 2; and eEFSec, eukaryotic elongation factor, selenocysteine-tRNA specific. *U indicates mcm^5U or mcm^5Um (adopted from Bang, 2022).

1.3. Selenoproteins

Selenoproteins play important roles in various biological processes, one of which is antioxidant defense. Glutathione peroxidases (GPx) reduce hydrogen peroxide and free fatty acid hydroperoxides to protect cells from peroxidative damage (Flohé & Brigelius-Flohé, 2006). Another GPx family member, phospholipid hydroperoxide glutathione peroxidase (PHGPx), decreases phospholipids, cholesterol, and cholesteryl ester hydroperoxides, thereby inhibiting cell membrane lipid peroxidation (Flohé & Brigelius-Flohé, 2006). In mammals, three distinct mammalian thioredoxin reductases function in cellular redox homeostasis by reducing thioredoxin and other substrates (Holmgren, 2006). Other oxidoreductases that include Sec contain the deiodinase family involved in thyroid hormone metabolism (Germain *et al.*, 2009), and selenophosphate synthetase 2 (SPS2), which synthesizes the selenium donor for Sec biosynthesis (Guimaraes *et al.*, 1996). An antioxidant function has also been proposed for the plasma protein selenoprotein P (SelP), which has been shown to be a methionine sulfoxide reductase (Kryukov *et al.*, 2003).

2. Selenophosphate synthetase (SPS)

Selenophosphate synthetase (SPS) catalyzes the production of monoselenophosphate, which is the active donor of selenium, from selenide and ATP (Glass *et al.*, 1993). Lower eukaryotes and bacteria have only one SPS protein, encoded by the *SelD* gene. However, higher eukaryotes have two isoforms of SPS—SPS1 and SPS2 (Guimaraes *et al.*, 1996; Leinfelder *et al.*, 1990). Phylogenetic analyses give insights into the evolution of SPSs (Figure 1.4). The fact that SPS1 and SPS2 cluster separately in vertebrates suggests that SPS1 and SPS2 most likely separated from their common ancestor before speciation. Interestingly, SPS2 from *Drosophila melanogaster* clusters with the prokaryotic SelD from *Caenorhabditis elegans* but not with SPS2 from vertebrates.

2.1. SPS in prokaryotes

Four mutants (SelA, SelB, SelC, and SelD) from *Eschericia coli* have been cloned and their functions have been identified using complementation tests. SelA is selenocysteine synthase, SelB is a eukaryotic elongation factor, SelC is Sec-tRNA$^{[Ser]Sec}$, and SelD is SPS (Forchhammer & Bock, 1991). SelD synthesizes monoselenophosphate, the selenium donor for Sec biosynthesis (Ehrenreich *et al.*, 1992). Gene disruption experiments showed that SelD is needed both for the incorporation of selenium into the modified nucleoside 5-methylaminiomethyl-2-

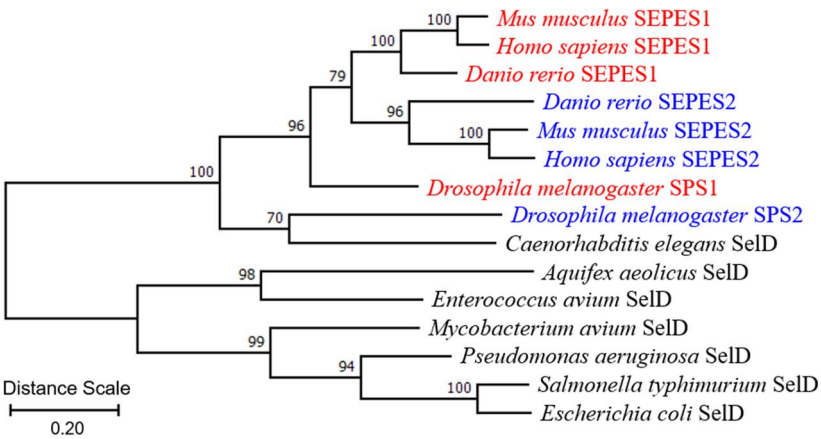

Figure 1.4. Phylogenetic profile of SPSs. A phylogenetic tree with the highest log likelihood (-16220.12) was inferred using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (adopted from Bang, 2022).

selenouridine of tRNA and for the biosynthesis of Sec from an L-serine residue ester-bonded to $tRNA_{UCA}$. In addition, selenocysteinyl-$tRNA_{UCA}$ could be sufficiently produced *In vitro* by using extracts of cells that overexpressed SelD (Leinfelder *et al.*, 1989).

2.2. SPS in eukaryotes

2.2.1. Structural characteristics of SPS

Although there are two isoforms of SPS (SPS1 and SPS2) in higher eukaryotes (Guimaraes *et al.*, 1996), the degree of homology between the two is species-specific. The amino acid sequence homology between human SPS1 and SPS2 is approximately 72% and between *Drosophila* SPS1 and SPS2 is approximately 45%. Several studies have shown that SPS1 is the only selenoprotein that plays a role in selenophosphate synthesis, whereas SPS1 is not a selenoprotein, because it does not have a Sec residue and is not involved in selenophosphate synthesis. It has been suggested that SPS1 is involved in recycling Sec through a selenium salvage system (Tamura *et al.*, 2004).

As shown in Figure 1.5, SPS1 and SPS2 contain two conserved domains. The first domain is the catalytic domain, which shows similarity with the consensus sequence of an ATP binding site, and it contains the well-conserved Cys 17 and Lys 20 residues. Cys 17 and Lys 20 residues are both essential active sites for the formation of selenophosphate from selenide and ATP (Kim *et al.*, 1992, 1993). The Cys 17 residue is substituted by threonine (in humans) and arginine (in *Drosophila*) SPS1. In contrast, SPS2 itself is a selenoprotein with Sec substituted at the same position (Guimaraes *et al.*, 1996). The second domain is a phosphate-binding domain that has an invariant asparagine starting a predicted beta/alpha turn neighboring ATP/GFP binding sequence which is conserved throughout ATP/GTP binding protein or protein kinase (Low *et al.*, 1995).

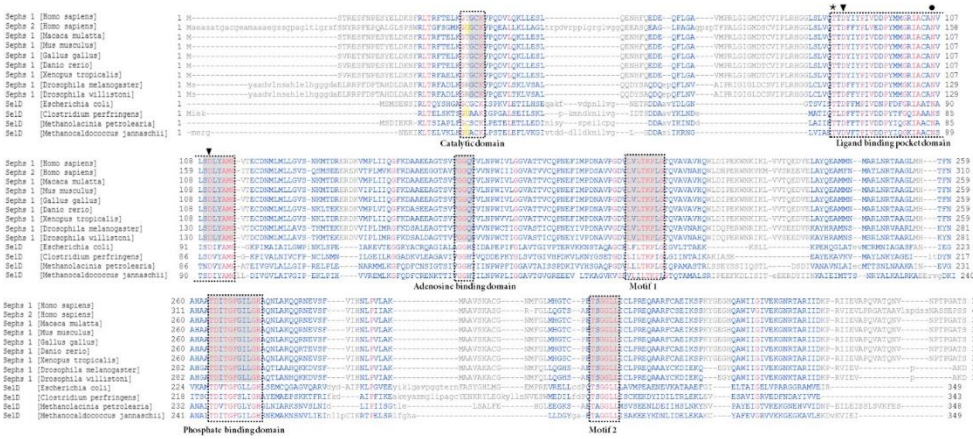

Figure 1.5. Alignment of SPS sequences. The amino acid sequences of SPS from 13 representative species (9 eukaryotes, 2 eubacteria, and 2 archaea) were aligned using the NCBI COBALT program. Residues that show identity are highlighted in red, and similarities shown in blue. Lowercase letters indicate sequences present in less than 50% of the 13 species. Dotted boxes represent conserved regions, and gray boxes represent homology regions in eukaryotes. Selenocysteine residues (U) are highlighted in yellow. All SPS sequences used for alignment were collected from the NCBI database (https://www.ncbi.nlm.nih.gov/gene). ★, ▼,and • denote conserved Thr, Asp, and Asn, respectively (Na *et al.*, 2018).

2.2.2. Function of SPS

Initially, both *SPSI* and *SPS2*, two homologs of *SelD*, were proposed to be involved in the synthesis of selenophosphate (Guimaraes *et al.*, 1996; Low *et al.*, 1995). In the earlier time, the study that both SPS1 and SPS2 were able to synthesize selenophosphate in different pathways. When cDNA of SPS1 from human lung adenocarcinoma was cloned and transformed into a SelD-deficient mutant of *E. coli*, the *SelD* mutation was not complemented when the cells were cultured in a selenite-containing medium, but complemented in the medium supplemented with L-selenocysteine (Tamura *et al.*, 2004). Based on these results, it was suggested that SPS1 can synthesize selenophosphate by recycling intracellular L-Sec via a salvage system. However, the detailed mechanism of L-Sec recycling and how SPS1 regulates this mechanism have not been elucidated.

There are some pieces of evidence that SPS2 has catalytic activity for selenophosphate synthesis, such as prokaryotic SelD. Both AMP and Pi, which are characteristic of SelD, are produced by *Drosophila* SPS2. SPS1, however, does not have any significant ATP hydrolysis activity to yield AMP and Pi (Persson *et al.*, 1997). When selenide and ATP were incubated *In vitro* with a mutant mouse SPS2 protein and Sec was replaced with Cys, the production of selenophosphate, AMP, and Pi was detected. However, mouse SPS1 did not exert this activity (Xu, Carlson, Mix, *et al.*, 2007). Knocking down *SPS2* in NIH3T3 cells severely reduced selenoprotein biosynthesis. However, SPS1 deficiency did not affect the biosynthesis of selenoproteins (Xu, Carlson, Irons, *et al.*, 2007). These experiments show that SPS2 is a prokaryotic SelD-like enzyme with the catalytic activity for selenophosphate

synthesis. SPS1, however, does not have the catalytic activity for selenophosphate synthesis (Figure 1.5).

The *SPS1* gene consists of several exons and introns (in Figure 1.6), but the *SPS2* gene has only one exon. In humans, SPS1 generates five splice variants encoding four different isoforms with unique dimerization patterns (Kim *et al.*, 2010). SPS1 not only forms homo- and hetero-dimers but also associates with many other proteins, containing those playing a role in selenoprotein biosynthesis, regulation of cell cycle, signal transduction, and regulation of transcription. However, the biological significance of the interaction between SPS1 and other proteins has not been elucidated.

2.2.2.1. SPS1 function in *Drosophila melanogaster*

Although SPS1 does not participate in the synthesis of selenophosphate, it has essential functions in the organism. Knocking out *SPS1*, also called *patufet*, leads to aberrant imaginal disc morphology and embryonic lethality in *Drosophila melanogaster* larva (Alsina *et al.*, 1999). The null mutation of *patufet* induces ROS accumulation, indicating that SPS1 plays a role in regulating intracellular ROS levels (Morey *et al.*, 2003). Haploinsufficiency of *patufet* dominantly suppresses the phenotypes caused by the Ras/mitogen-activated protein kinase (MAPK) cassette hyper-activation, and the activation of the *Drosophila melanogaster* epidermal growth factor receptor and Sevenless receptor tyrosine kinases (Morey *et al.*, 2001). Knocking down *SPS1* in S2 cells, a *Drosophila melanogaster* embryonic cell line, results in growth retardation, megamitochondria formation, and ROS accumulation

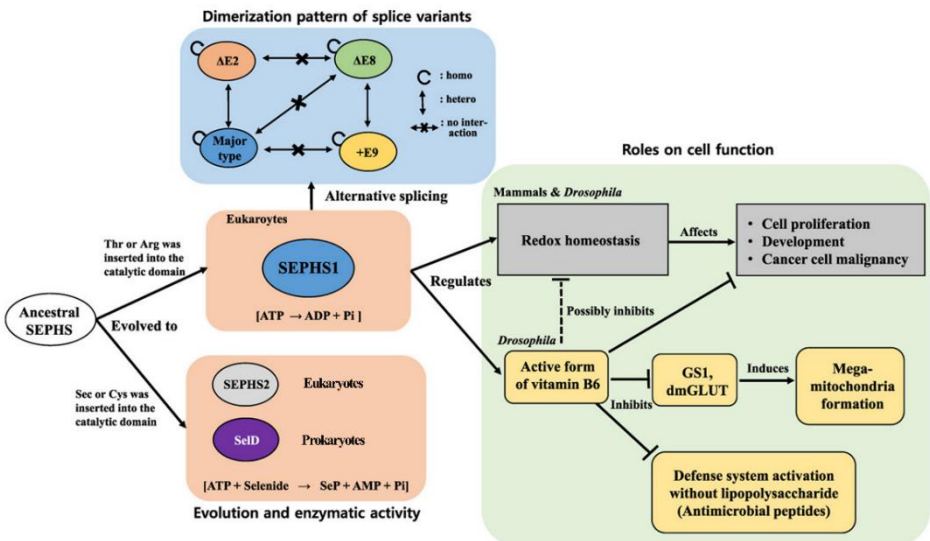

Figure 1.6. Selenophosphate synthetase 1 and its role in redox homeostasis, defense and proliferation (Na *et al.*, 2018)**.**

(Shim *et al.*, 2009). Interestingly, the primary effect of SPS1 deficiency in SL2 cells is the inhibition of the synthesis of pyridoxal phosphate, the active form of vitamin B6. Suppression of vitamin B6 synthesis upon SPS1 deficiency causes cell growth retardation and megamitochondria formation and activates innate immunity in these cells (Lee *et al.*, 2011).

2.2.2.1. SPS1 function in mice

Systemic *SPS1* knockout mouse embryos exhibited markedly underdeveloped by embryonic day (E)8.5 and virtually absorbed by E14.5 (Tobe *et al.*, 2016). Malignant characteristics of cancer cells, such as cell invasion and foci formation, were reversed by SPS1 deficiency in F9 cells, a mouse embryonic cancer cell line. In addition, although *SPS1* conditional knockout in the liver survived, transcriptome analysis showed that hepatic SPS1 deficiency significantly affected the expression of many genes involved in cancer development and redox regulation (Tobe *et al.*, 2016). Transcriptome analysis was performed in systemic *SPS1* knockout mice at E6.5, E7.5, and E8.5. Transcriptome analysis showed that SPS1-deficiency causes retardation of embryonic development and induction of oxidative stress. Additionally, SPS1 deficiency gradually increases the oxidative stress, which perturbs the signaling pathways during gastrulation, and then leads to apoptosis (Bang *et al.*, 2021). These observations indicate that SPS1 is responsible for the regulation of cellular redox homeostasis (Na *et al.*, 2018).

3. Innate immunity in *Drosophila* melanogaster

Metazoans have developed efficient mechanisms against infections. Innate immunity is the first line of defense that all metazoans have in common. Its hallmark is the recognition of microorganisms by germline-encoded, unrearranged receptors, and rapid effector mechanisms including phagocytosis, proteolytic cascades activation, and potent antimicrobial peptides synthesis. *Drosophila melanogaster* is particularly well-suited for studies on innate immunity because this model system lacks an adaptive immune response.

3.1. Antimicrobial defenses against bacteria and fungi

Immune responses in insects involved in localized lectin binding, melanization, and antimicrobial peptides (AMPs) induction through the immune deficiency (IMD) and Toll pathways activation. AMPs and complement-like proteins, humoral factors, are proposed to be key immune effector molecules. The representative AMPs in *Drosophila* are the Toll pathway readout AMPs (drosomycin, metchnikowin) and the IMD pathway readout AMPs (attacins, cecropins, diptericins, drosocin (Martinelli & Reichhart, 2005). AMPs are small and cationic. In addition, AMPs affect microbial membrane characteristics (Imler & Bulet, 2005).

3.1.1. Antimicrobial peptides

Although AMPs vary extremely in length and composition, they have some

common structural characteristics (Kosikowska & Lesner, 2016). The almost all AMPs are short polypeptides, and they are a cation with an average net charge of +3, and they are an average hydrophobic content of about 42%. The net positive charge and the hydrophobicity of these AMPs give rise to the observed amphipathic structure. This structure determines structural flexibility, which can acts as electrostatic attraction between these cationic peptides and the anionic bacterial membranes, and can penetration into bacterial cells leading to membrane disruption (Figure 1.7). However, cationic AMPs has no effect on the neutrally charged mammalian cells. The differences in composition between bacterial cell walls rich in phosphatidylglycerols and human cell walls dominated by zwitterionic phospholipids is thought to be the major cause of AMPs selectivity.

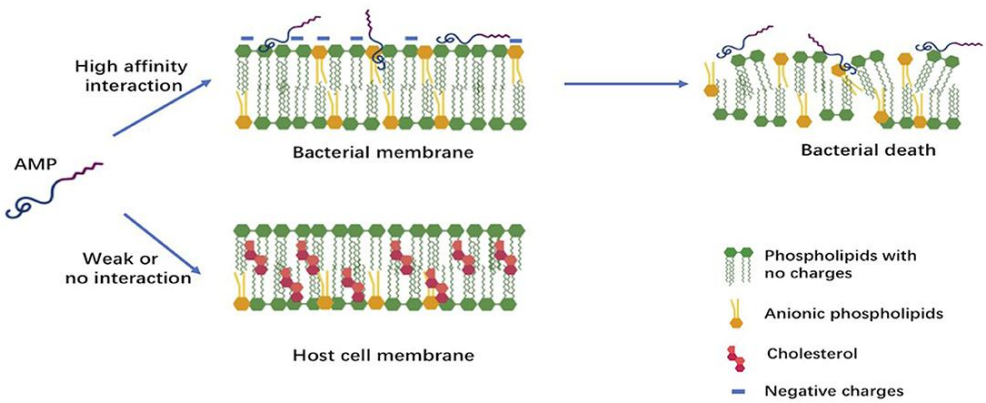

Figure 1.7. Interactions of cationic antimicrobial peptides with bacterial or host cell membrane (Geitani *et al.*, 2020).

3.2. The IMD pathway in *Drosophila melanogaster*

The IMD path is primarily implicated in defense against gram-negative bacteria, and most antimicrobial peptides (Attacins, Cecropins, Diptericins, and Drosocins) controlled by this pathway have an activity spectrum, mainly against Gram-negative bacteria.

3.2.1. Peptidoglycan-recognition proteins and activation of the IMD pathway

Microbial infection is the primary step in activating the innate immunity through the IMD signaling pathway. To detect pathogens, the innate immunity recognizes specific molecules that bind to conserved structures present in the pathogen but not in the host (Janeway, 2013). An example is peptidoglycan (PGN), which is recognized and bound by a host protein named a PGN recognition protein. (PGRPs). There are a family of four PGRPs in mammals, whereas there are more in insects. For example, *Drosophila melanogaster* has 13 genes coding for 19 proteins (Werner *et al.*, 2003; Werner *et al.*, 2000). The mammalian PGRPs are secreted proteins that associate with bacterial muramyl peptides. Some PGRPs in mammal have amidase activity to clear the pro-inflammatory PGN, while others are more divergent in the insect genes and function directly as bactericidal proteins (Dziarski & Gupta, 2006; Liu *et al.*, 2000; Swaminathan *et al.*, 2006). It reveals that mammalian PGRPs has no signaling activity.

Insect PGRP may act as an enzymatically active amidase that degrades PGN and activates signaling pathways. Insect PGRPs are assorted into short or long, depending

on their size. Short PGRPs can exist as extracellular proteins, while long PGRPs can exist as intracellular, extracellular and transmembrane proteins. The PGRP-SA in *Drosophila* is required to activating the Toll pathway (Michel *et al.*, 2001; Valanne *et al.*, 2011). Although PGRP-SB1 is strongly induced after infection and its bactericidal activity has been shown *In vitro* (Mellroth & Steiner, 2006; Paredes *et al.*, 2011; Zaidman-Remy *et al.*, 2011), immunological *In vivo* phenotypes for the amidases PGRP-SB1 and PGRP-SB2 have not been demonstrated. Different PGRPs are positively or negatively involved in the IMD pathway.

PGRP-SD acts upstream of PGRP-LC as an extracellular receptor. It promotes peptidoglycan localization to transmembrane receptor, and enhances IMD pathway (Iatsenko *et al.*, 2016). PGRP-LC is a transmembrane receptor protein that recognizes a mesodiaminopimelic acid–type PGN found in Gram-negative bacteria (Kaneko *et al.*, 2004; Leulier *et al.*, 2003). It functions as a major receptor mediating systemic infection and induction of the IMD pathway locally in the anterior-most part of the midgut (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002; Zaidman-Remy *et al.*, 2006). PGRP-LC has several isoforms. three of which there are three characterized isoforms. PGRP-LCx recognizes polymeric PGN and PGRP-LCa does not bind PGN, but works with PGRP-LCx at a co-receptor to bind to monomeric PGN fragments called tracheal cytotoxin (TCT) (Chang *et al.*, 2006; Kaneko *et al.*, 2004; Lim *et al.*, 2006). flies with mutation in PGRP-LC still recognizes TCT. This is due to the two forms PGRP-LE has (Kaneko *et al.*, 2006; Neyen *et al.*, 2012). The short form is secreted and binds PGN in the hemolymph (Takehana *et al.*, 2002) and is thought to aid IMD signaling by presenting PGN to PGRP-LC, but the detailed

mechanism of PGRP-LE has not been demonstrated. The full-length PGRP-LE remains in the cytoplasm, where it is thought to recognize cell-accessible TCT fragments. The binding of TCT induces the oligomerization of cytoplasmic PGRP-LE in a head-to-tail manner (Lim *et al.*, 2006). Ectopic expression of PGRP-LE in adipocytes is sufficient to activate expression of AMPs in a cell-autonomous process even in the absence of infection. It has also been shown that cytoplasmic PGRP-LE can activate the IMD pathway by interacting with IMD, independently of PGRP-LC (Kaneko *et al.*, 2006; Neyen *et al.*, 2012; Takehana *et al.*, 2002; Yano *et al.*, 2008). PGRP-LE is the only intracellular pathogen receptor found in *Drosophila*. The intracellular PGRP-LE can activate autophagy, whereas the transmembrane PGRP-LE is able to activate a prophenoloxidase cascade with PGRP-LC (Kurata, 2014; Schmidt *et al.*, 2008; Takehana *et al.*, 2002).

PGRP-LF is a transmembrane receptor similar to PGRP-LC but lacking an intracellular signaling domain and does not recognize to PGN. PGRP-LF inhibits IMD signaling pathway by binding to PGRP-LC and preventing its dimerization (Basbous *et al.*, 2011; Maillet *et al.*, 2008; Persson *et al.*, 2007). PGRP-LA is also not expected to bind to PGN and has been shown to be essential for systemic infections. However, consistent with the expression profile, PGRP-LA be likely to positively control the IMD pathway in barrier epithelia (Gendrin *et al.*, 2013). The amidase PGRP breaks down PGN into short, non-immunogenic or less immunogenic fragments, preventing or reducing activation of innate immunity (Paredes *et al.*, 2011; Zaidman-Remy *et al.*, 2011).

3.2.2. Regulation of the IMD signaling pathway

Activation of the IMD pathway requires activation of the transcription factor Relish (Dushay *et al.*, 1996). To activate Relish, it is thought that it probably masks the nuclear localization signal at the N-terminus of Relish and inhibits dimerization by the Relish homology domain. Thus, In addition to phosphorylation, cleavage of the inhibitory C-terminal portion is needed for Relish activation. This is likely achieved by the caspase Dredd, as it has been shown to cleave Relish *In vitro* (Stoven *et al.*, 2003).

As shown in Figure 1.8, PGN recognition induces the recruitment of IMD, the adapter protein dFadd, and Dredd (Choe *et al.*, 2005; Georgel *et al.*, 2001; Lemaitre, Kromer-Metzger, *et al.*, 1995; Leulier *et al.*, 2002). Dredd is activated by ubiquitination by the E3-ligase inhibitor of apoptosis 2 (Iap2) (Meinander *et al.*, 2012), associated with the E2-ubiquitin–conjugating enzymes UEV1a, Bendless (Ubc13), and Effete (Ubc5) (Zhou *et al.*, 2005). When activated, Dredd cleaves IMD, and produce a new binding site for Iap2, which is able to K63-ubiquitinate IMD (Meinander *et al.*, 2012; Paquette *et al.*, 2010). This induces activation of the Tab2/Tak1 complex, which manage the activation of the *Drosophila* IκB kinase (IKK) complex (Kleino *et al.*, 2005; Lu *et al.*, 2001; Rutschmann, Jung, Zhou, *et al.*, 2000; Silverman *et al.*, 2000). The phosphorylation of multiple sites at its N terminus by the IKK complex activates Relish (Silverman *et al.*, 2000). It has been reported that phosphorylation of serine residues is needed for efficient access RNA polymerase II to the promoter of the Relish target gene (Erturk-Hasdemir *et al.*, 2009; Silverman *et al.*, 2000). The C-terminal portion remains in the cytoplasm while the

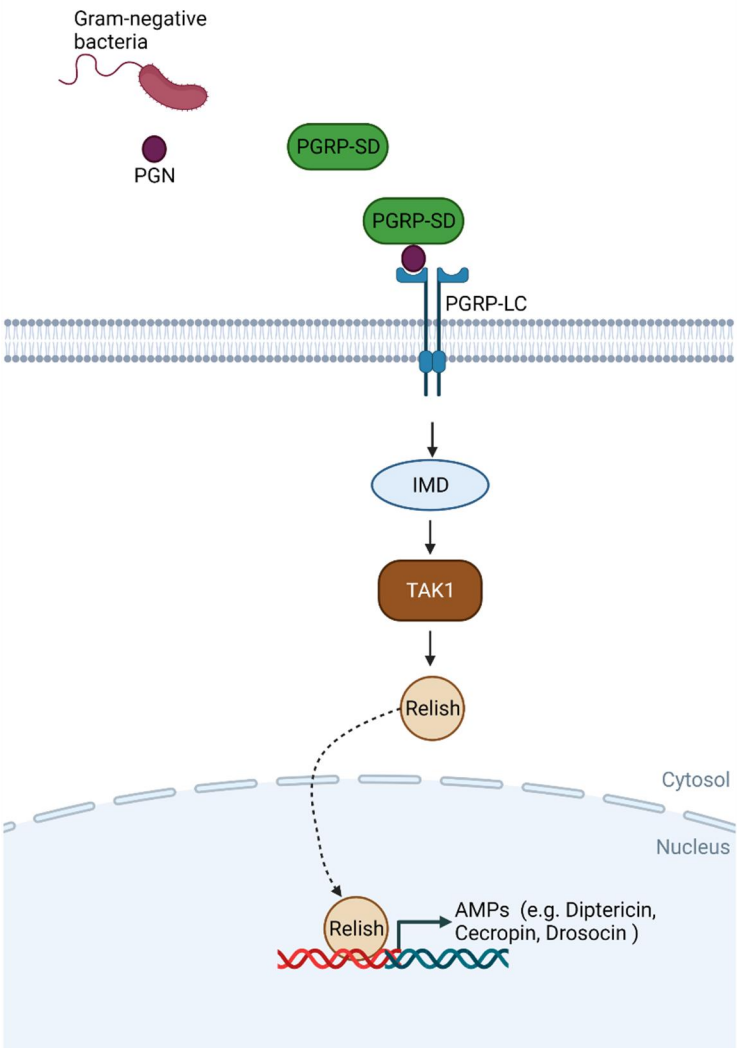

Figure 1.8. The *Drosophila* IMD pathway. .

active N-terminal portion translocates to the nucleus to activate transcription of the AMP genes, such as Attacins, Cecropins, Diptericins and Drosocins (Stoven *et al.*, 2000; Stoven *et al.*, 2003). The IMD pathway in *Drosophila* branchesinto the JNK pathway at the Tak1 and Tab2 levels (Delaney *et al.*, 2006; Silverman *et al.*, 2003; Valanne *et al.*, 2007).

The IMD signaling activity is finely regulated at multiple levels by molecules and mechanisms (Kleino & Silverman, 2014; Valanne, 2014; Valanne *et al.*, 2012). The E3-ligase Iap2 has been studied in many large screens for the IMD pathway regulators (Gesellchen *et al.*, 2005; Kleino *et al.*, 2005; Valanne *et al.*, 2012; Valanne *et al.*, 2010) and has also been shown to be essential for IMD pathway-mediated expression of AMPs and resistance to bacterial *In vivo* (Kleino *et al.*, 2005; Leulier *et al.*, 2006; Valanne *et al.*, 2007). In addition, numerous ubiquitination and deubiquitination enzymes are involved in the negative regulator for the signaling pathway. dUSP36/Scny, The ubiquitin-specific protease, inhibits the accumulation of the activated, K63-ubiquitinated IMD and induces K48-linked ubiquitination and degradation (Thevenon *et al.*, 2009). Fat is also a proposed ubiquitination enzyme to modulate the ubiquitination status of IMD (Yagi *et al.*, 2013). Ubiquitination and degradation by the RING-finger protein POSH are targets of Tak1 (Tsuda *et al.*, 2005). *Drosophila* CYLD interacts with Kenny, the IKK protein, and negatively modulates the IMD pathway (Trompouki *et al.*, 2003; Tsichritzis *et al.*, 2007). Activated Relish targets ubiquitination and proteasomal degradation by the SCF complex (Khush *et al.*, 2002), induced by dRYBP (Aparicio *et al.*, 2013). Dredd is repressed by the RING-domain including protein Dnr1 (Foley & O'Farrell, 2004;

Guntermann *et al.*, 2009), whereas Caspar prevents the Dredd-mediated cleavage of Relish (Kim *et al.*, 2006).

Mammalian NF-κB protein has its own inhibitor, IκB. It generates a negative feedback that is the main regulatory system for the signaling pathway (Sun *et al.*, 1993). Since Relish includes IκB, this regulation process is not practicable in *Drosophila*. Instead, the IMD pathway is affected by other negative feedback mechanisms, such as through the induction of a gene named pirk. (Kallio *et al.*, 2005; Kleino *et al.*, 2008). Expression of pirk is also known to be caused by the ERK/MAPK signaling pathway, suggesting that the ERK/MAPK signaling pathway may modulate immunity by negatively controlling the IMD pathway. (Ragab *et al.*, 2011). Pirk interacts with the PGRP-LC, PCRP-LE and IMD, interfering with signal transduction in the receptor complex (Aggarwal *et al.*, 2008; Kleino *et al.*, 2008; Lhocine *et al.*, 2008).

3.3. The Toll pathway in *Drosophila melanogaster*

A fungal or Gram-positive infection causes the activation of the Toll pathway, which induces the AMPs production such as Metchnikowins (Mtk) and Drosomycins (Drs) (Aggarwal & Silverman, 2008; Hetru & Hoffmann, 2009)

3.3.1. Toll receptors in *Drosophila melanogaser*

Drosophila has nine genes encoding Toll-associated receptors. Toll, or Toll-1, was initially identified as Toll and played role in induction of AMPs through the Toll

pathway. All Toll receptors in *Drosophila* have a similar molecular construction. Toll-5 is phylogenetically closest to Toll (Tauszig *et al.*, 2000). Unlike other Tolls, Toll-9 has one cystein-rich motif between its transmembrane domain and leucine-rich repeats, and its construction is similar to mammalian TLRs (Imler & Hoffmann, 2001). Tolls in *Drosophila* and the IL-1Rs in mammals have a homology domain called Toll/IL-1R (TIR) domain. These domains interact with adapter proteins to activate downstream proteins (Imler & Hoffmann, 2001).

Several Tolls may play an important roles in immunity. Toll-5 could lead to expression of *Drosomycin* and *Metchnikowin* (Imler *et al.*, 2000; Luo *et al.*, 2001; Tauszig *et al.*, 2000). It has also been shown that Toll-5 interacts with Toll and Pelle to induce the Toll pathway activation synergistically with Toll (Luo *et al.*, 2001). Additionally, Toll-9 has been reported to activate *Drosomycin* expression (Ooi *et al.*, 2002), for which Toll-9 could utilize the components of the Toll signaling pathway (Bettencourt, Tanji, *et al.*, 2004).

3.3.2. Activation of Spätzle

To activate the *Drosophila* Toll pathway during immune responses, extracellular recognition proteins start protease cascades inducing the activation of the Toll receptor ligand Spätzle (Spz) (Morisato & Anderson, 1994; Schneider *et al.*, 1994). Under non-signaling conditions, The hydrophobic C-terminal Spz region is masked by the prodomain of Spz. Activation leads to proteolysis, which results in a conformational changes that expose critical determinants for the binding of the Toll receptor (Arnot *et al.*, 2010). Interestingly, the prodomain exists bound to the C-

terminus and is only released when the extracellular domain of Toll binds to the complex (Weber *et al.*, 2007). There are two models of Spz-Toll binding. The first model is that one Spz dimer binds to two Toll receptors (Weber *et al.*, 2005). In a recent model, two Spz dimers, each binding to the N terminus of one of the two Toll receptors, have been proposed to cause a structural change in the Tolls to activate the downstream proteins (Gangloff *et al.*, 2008)

Spz is an inactive precursor composed of a prodomain and a C-terminal region (C-106) (DeLotto & DeLotto, 1998). In dorsoventral patterning, Spz is catalyzed to the active C-106 form by the serine protease cascade containg Defective, Easter, Nudel, and Gastrulation Snake (Chasan *et al.*, 1992; Hong & Hashimoto, 1995). Furthermore, the Pipe is needed to activate Easter (Cho *et al.*, 2010). When a microorganism is infected, Spz-processing enzyme (SPE) plyas role in Spz cleavage (Jang *et al.*, 2006). The current model for the SPE activation includes three cascades depending on activation. Two protease cascades inducing the gram-positive–specific serine protease (Grass) activation are started by cell membrane components of β-glucan and Lysine-type peptidoglycan (El Chamy *et al.*, 2008). Although Grass was originally known to be include the gram-positive bacteria recognition (Kambris *et al.*, 2006), it was later shown to be also important in the fungal components recognition (El Chamy *et al.*, 2008). In addition, four other serine proteases (sphinx1, sphinx2, spirit, and spheroide) have been found in response to fungi and gram-positive bacteria (Kambris *et al.*, 2006). Upstream signal of Grass, a modular serine protease (ModSP), conserved in insect immune reactions, is responsible to integrating the signals from the recognition molecules gram-negative binding protein (GNBP) 3 and PGN

recognition protein PGRP-SA to the Grass-SPE-Spatzle cascade (Buchon *et al.*, 2009). A third protease cascade inducing the SPE activation is coordinated by the Persephone, which is proteolytically complete by the secreted fungal virulence factor PR1 (Gottar *et al.*, 2006) and Gram-positive bacterial virulence factors (El Chamy *et al.*, 2008). A similar sensing mechanism has been proposed to rise in mammals, in which TLR or Nod-like receptors directly sense endogenous proteins or toxic factors released by damaged cells (Matzinger, 2002; Sansonetti, 2006).

3.3.3. Regulation of the Toll signaling pathway

After associating with the processed Spz, the Toll receptor is activated, and binds to the adaptor protein MyD88 through intracellular TIR domains (Horng & Medzhitov, 2001; Sun *et al.*, 2002; Tauszig-Delamasure *et al.*, 2002). Upon this interaction, MyD88, Tube, and Pelle are recruited to make a MyD88-Tube-Pelle complex via death domain (DD)-mediated interactions (Moncrieffe *et al.*, 2008; Sun *et al.*, 2002; Xiao *et al.*, 1999). MyD88 and Pelle are not adjacent to each other. Instead, two distinct DD of the adapter protein Tube assciate separately with MyD88 and Pelle, linking the two proteins (Sun *et al.*, 2002). Pellino interacing the highly conserved Pelle/IL-1R–associated kinase (IRAK) has been shown to positively modulate the Toll signaling pathway (Haghayeghi *et al.*, 2010). *Drosophila* Pellino mutants suppressed expression of *Drosomycin* and reduced viability against Gram-positive bacteria (Haghayeghi *et al.*, 2010). Since all Pellinos include a RING domain, it is speculated that *Drosophila* Pellino could ubiquitinate Pelle similarly to the mammalian Pellinos polyubiquitinate IRAK1 (Moynagh, 2009).

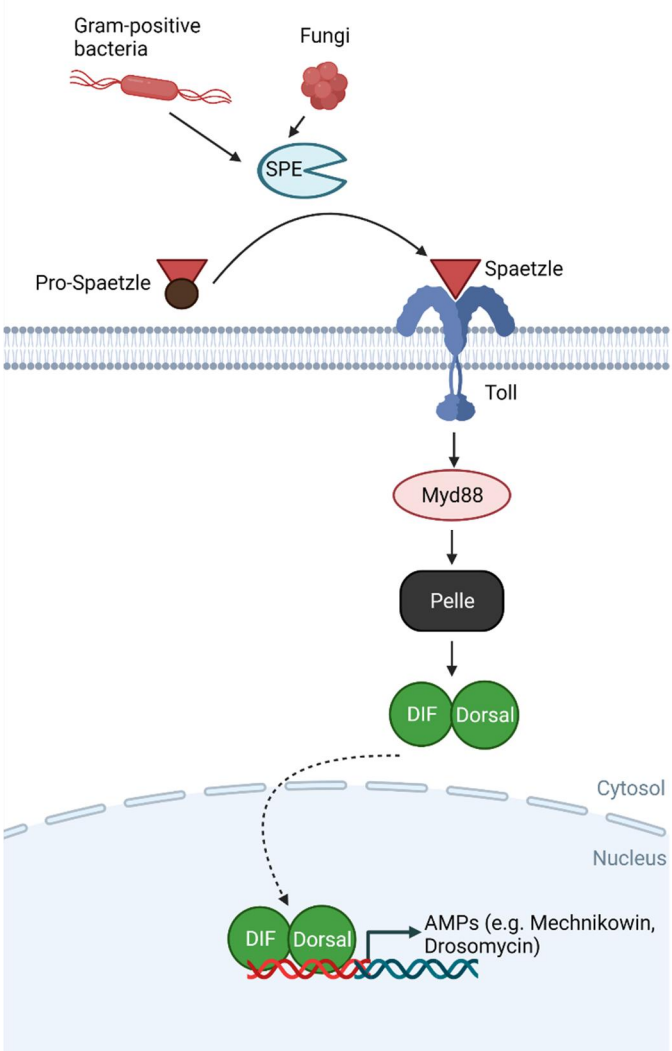

Figure 1.9. The *Drosophila* Toll pathway. .

The signal from the oligomeric MyD88-Tube-Pelle complex advances to the phosphorylation and degradation of the Cactus, *Drosophila* IκB factor. In non-signaling conditions, Cactus is contextually associated with the NF-κB transcription factor(s) Dorsal and/or Dif to prevent their activity and nuclear localization. Thus, the degradation of Cactus is needed for the nuclear translocation of Dorsal and Dif, and that is why Cactus must be phosphorylated (Wu & Anderson, 1998). Although not directly shown, Pelle may be responsible for this mechanism as it needs kinase activity for Cactus phosphorylation. (Towb *et al.*, 2001). Additionally, in a recent screening (Huang *et al.*, 2010) with 476 dsRNAs targeting all the known and predicted *Drosophila* kinases, Pelle was found to be the only kinase involved in Cactus phosphorylation. Cactus requires to be phosphorylated on two distinct N-terminal motifs (Fernandez *et al.*, 2001) that are similar to IκB kinase (IKK) targets, but the *Drosophila* IKK-β (Ird5) or IKK-γ (Kenny) are not implicated in the Toll pathway (Lu *et al.*, 2001; Rutschmann, Jung, Zhou, *et al.*, 2000). After phosphorylation, nuclear translocation of Dorsal/Dif induces the transcriptional activation of the *Drosophila* Dorsal. In larvae and adult *Drosophila*, Dorsal is existed in the fat body, and its mRNA levels (Reichhart *et al.*, 1993) and nuclear localization (Lemaitre, Meister, *et al.*, 1995) are elevated upon microbial infection. Dorsal interacts with Tube, Pelle, and Cactus (Edwards *et al.*, 1997; Kidd, 1992; Yang & Steward, 1997), and upon activation of the Toll pathway, Dorsal translocates to the nucleus and binds to the κB-related sequence of AMP genes promoter (Reichhart *et al.*, 1993). Dorsal can activate the diptericin promoter *In vitro* (Gross *et al.*, 1996), and bacterial culture supernatants can stimulate Dorsal nuclear translocation in

dissected fat bodies in a hemolymph-dependent manner (Bettencourt, Asha, *et al.*, 2004). Additionally, Dorsal activity is needed to limit the infectivity of *Pseudomonas aeruginosa* in adult *Drosophila*, and this finding gives evidence for Dorsal function in resistance to infections (Lau *et al.*, 2003).

Dif has been identified as a dorsal-related immunity factor that does not participate in dorsoventral patterning in *Drosophila*. Instead, it mediates the immune response of *Drosophila* larvae (Ip *et al.*, 1993) and interacts with Cactus *In vitro* (Tatei & Levine, 1995). Dif, but not Dorsal, mediates Toll-dependent transcriptional induction of the antifungal peptide gene *Drosomycin* in *Drosophila* adults (Lemaitre *et al.*, 1996; Rutschmann, Jung, Hetru, *et al.*, 2000), whereas Dorsal and Dif appear to overlap in larvae (Manfruelli *et al.*, 1999; Rutschmann, Jung, Hetru, *et al.*, 2000). Furthermore, Dif and Dorsal can form heterodimers *In vitro* (Gross *et al.*, 1996), and Dorsal appear to have a more important role in activation of Drosomycin promoter than Dif in a *Drosophila* S2 cell line (Valanne *et al.*, 2010).

3.4. Synergistic activation of the *Drosophila* immune-responsive pathways

The *Drosophila* Toll pathway plays a important role in gram-positive bacterial and fungal infections (Lemaitre & Hoffmann, 2007). The IMD pathway is started by the PGRP-LC–mediated recognition of predominantly a DAP-type PGN from gram-negative bacteria (Choe *et al.*, 2002; Ramet *et al.*, 2002). Activation of the IMD pathway finally induces the the NF-κB factor Relish activation (Dushay *et al.*, 1996; Hedengren *et al.*, 1999; Silverman *et al.*, 2000), its nuclear translocation, and the

transcriptional activation of target genes, including AMPs (Aggarwal & Silverman, 2008; Hetru & Hoffmann, 2009). Although the *Drosophila* immune pathways is selectively activated to a some extent (Lemaitre *et al.*, 1997), the synergistic interactions between the IMD and Toll pathways become increasingly evident (De Gregorio *et al.*, 2002; Kleino *et al.*, 2005; Tanji *et al.*, 2007; Valanne *et al.*, 2010). Moreover, in a *Drosophila* cell line, Relish RNAi downregulates $Toll^{10b}$-induced Drosomycin reporter gene (Kleino *et al.*, 2005), and the Drosomycin reporter is synergistically activated by $Toll^{10b}$ or gram-negative bacteria (Valanne *et al.*, 2010). Furthermore, the *Drosomycin* and *Defensin* expression is best caused by Relish/Dif and Relish/Dorsal heterodimers, respectively (Han & Ip, 1999). *In vivo*, after *Escherichia coli* infection, the double mutants for Dif and kenny die earlier than kenny mutants (Rutschmann *et al.*, 2002). The Relish/spz and Relish/Toll double mutants are identical compared to the Relish mutants (De Gregorio *et al.*, 2002).

4. The ERK/MAPK signaling pathway

Extracellular signal-regulated kinase 1/2 (ERK) is involved in the mitogen-activated protein kinase (MAPK) family, acts as a signaling cascades and transduces extracellular signals into cells. Thus, MAPK cascades are central signaling pathways that modulate basic processes, containing cell proliferation, stress responses, and differentiation (Keshet & Seger, 2010; Plotnikov *et al.*, 2011; Sabio & Davis, 2014).

4.1. ERK/MAPK structure and functions

Among the signaling pathways, the MAPK signal pathway plays an important role in regulating various physiological processes in cells, such as cell proliferation, death, division, and development. ERK belong to the MAPK family, and the ERK/MAPK signaling pathway is central to the signaling network implicated in regulating cell proliferation, division, and development. The basic signaling steps follow the MAPK tertiary enzymatic cascade, which consist of the upstream activator, MAP3K, MAP2K, and MAPK. In the ERK/MAPK signaling pathway, renin–angiotensin system (Ras) is the upstream activating protein, rapidly accelerated fibrosarcoma (Raf) is MAP3K, MAPK/ERK kinase (MEK) is MAPKK, and ERK is the MAPK. These proteins form the Ras-Raf-MEK-ERK pathway (Yang & Liu, 2017).

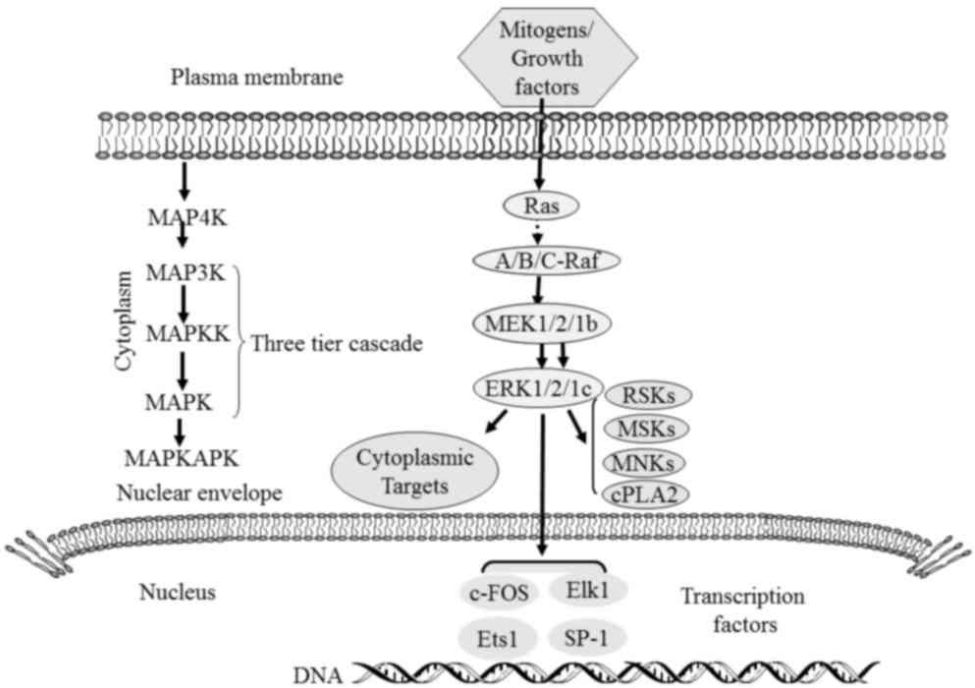

Figure 1.10. The MAPK cascades. MAPKs, which are present in the cytoplasm and can be translocated into the nucleus, catalyze the phosphorylation of dozens of cytoplasmic proteins and numerous nuclear transcription factors. MAPK, mitogen-activated protein kinase; MAP4K, MAPK kinase kinase kinase; MAP3K, MAPK kinase kinase; MAPKK, MAPK kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinases; MEK, Ras/Raf/MAPK; RSK, ribosomal s6 kinase; MSK, mitogen- and stress-activated protein kinases; MNK, MAP kinase-interacting serine/threonine-protein kinases; cPLA2, cytosolic phospholipase A2; c-FOS, proto-oncogene c-Fos; Elk1, ETS domain-containing protein Elk-1; Ets1, Protein C-ets-1; SP-1, transcription factor Sp1 (Guo *et al.*, 2020).

4.1.1. Members of the ERK family

Serine/threonine protein kinase ERK is a signaling protein that transduces mitogen signals (Anjum & Blenis, 2008). ERK is usually located in the cytoplasm. upon activation, ERK translocates the nucleus and regulates activation of transcription factor and gene expression (Boulton *et al.*, 1991). Through artificial cloning and sequencing analysis, ERKs are classified as ERKs 1, 2, 3, 5 and 6 (Plotnikov *et al.*, 2011) ERK1 and 2 are two important members of the MAPK/ERK pathway (Boulton *et al.*, 1991). The C-terminus of ERK5 includes a nuclear localization signal (NLS), two proline-rich regions, and activation of transcription.

4.1.2. The ERK/MAPK signaling pathway upstream protein and kinase activation mechanism

Various stimulants such as growth factors, G-protein-coupled receptor ligands, cytokines, oncogenes, and viruses activate the ERK/MAPK signaling pathway. The key molecules in the ERK/MAPK signaling pathway mainly contain the small G proteins Ras and the downstream Raf kinase, MEK1/2, and ERK1/2. ERK is activated by phosphorylation at two regulatory sites, Tyr 204/187 and Thr 202/185 (Chang & Karin, 2001). The ERK/MAPK signaling pathway is shown in Figure 1.10.

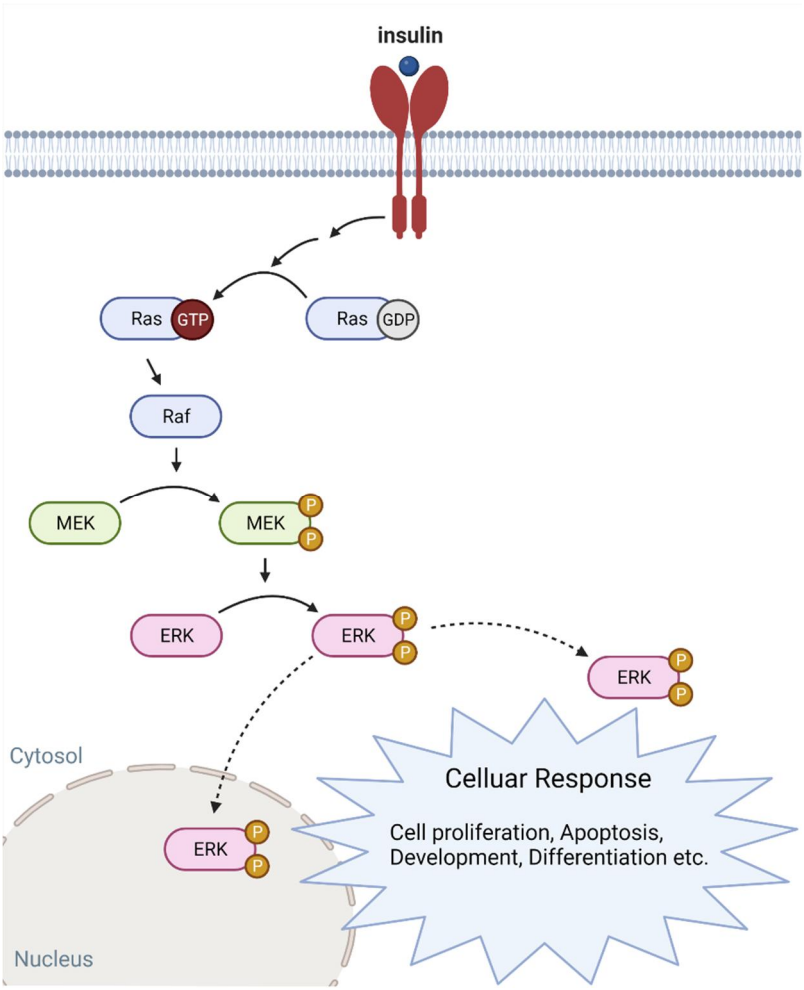

Figure 1.11. The ERK/MAPK signaling pathway. The ERK/MAPK signaling pathway consists of protein chains in the cell that carry extracellular information from receptors on the cell surface to DNA in the nucleus. When the ligand binds to the dimer transmembrane receptor, intracellular signaling in the middle and lower reaches of the Ras-Raf-MEK-ERK/MAPK pathway is triggered. Activation of the MAPK/ERK cascade leads to phosphorylation of ERK1/2, which consequently results in transcriptional induction of its target genes in the nucleus and cytoplasm .

4.1.3. Downstream of ERK1/2

In the absence of stimulation, ERK1/2 is located in the cytoplasm. Upon activation, ERK1/2 is phosphorylated and transmitted to the nucleus and modulates the various transcription factors activation, ultimately regulating cell metabolism and function and affecting the specific activities of cells. Cytoskeletal components, such as microtubule-associated protein (MAP) 1, MAP2, and MAP4, are phosphorylated in the cytoplasm and participate in controlling cell morphology and cytoskeletal redistribution. In the nucleus, the phosphorylation of nuclear transcription factors, such as ETS domain-containing protein Elk-1, cyclic AMP-dependent transcription factor ATF2, proto-oncogene c-Fos, proto-oncogene c-Jun, and proto-oncogene c-Myc. In the cytoplasm, ERK1/2 can phosphorylate a series of other protein kinases upstream of the ERK pathway, such as SOS, Raf-1, and MEK, in a negative feedback system. The ERK/MAPK signaling pathways activates other extracellular signaling pathways. Extracellular signals such as EGF, vascular endothelial growth factor (VEGF), and platelet-derived growth factor can be activated by receptor tyrosine kinase auto-phosphorylation of ERK/MAPK. Activated ERK can translocate the nucleus and bind to transcription factors that lead to gene expression in response to extracellular stimulation, and modulate cell growth, transcription, apoptosis, and differentiation (Deming *et al.*, 2008; Eblen, 2018; Kolch, 2000; O'Neill & Kolch, 2004; Schulze *et al.*, 2001).

4.1.4. Role of ERK/MAPK in cell proliferation

Unlimited cell growth, suppression of apoptosis, and dedifferentiation are important biological properties of tumors (Mader & Pantel, 2017). The ERK/MAPK signaling pathway activation causes cell growth and has an anti-apoptotic effect. Hypoxia-leaded to VEGF can prevent the apoptosis of serum-starved cells by activation of the ERK/MAPK signaling pathway (Baek *et al.*, 2000). preventing this pathway can restrain the growth of and lack of apoptosis in cancer cells, and accelerate their differentiation (Lefloch *et al.*, 2009). Gauthier *et al.* (Gauthier *et al.*, 2001) have found that the ERK1/2 signaling pathway is implicated in cell viability following intestinal injury, and block of this pathway can accelerate the apoptosis of intestinal injury cells. Huang *et al.* (Huang *et al.*, 2019) have found that inhibiting the ERK/MAPK signaling pathway prevents the growth of a diffuse large B cell lymphoma cell line and accelerate their apoptosis. Blocking the ERK/MAPK signaling pathway to prevent cancer cell growth could involve blocking of the cell cycle (Shah *et al.*, 2019). Sebolt-Leopold *et al.* (Sebolt-Leopold *et al.*, 1999) have reported that the use of MEK1/2 inhibitors to prevent activation of ERK1/2 in colon cancer cells inhibit the cells from entering the S phase from the G1 phase, and prevent the proliferation of adherent cells. Blocking of the ERK/MAPK signaling decreases the cell dedifferentiation and anti-apoptotic effect. Maemura *et al.* (Maemura *et al.*, 2009) have shown that the ERK/MAPK signaling pathway accelerate cell growth and prevents apoptosis by influencing the activity of the downstream cell cycle regulatory proteins, apoptosis-related proteins, and other effector molecules, such as the G1/S-specific cyclin D1. Ellipticine, an alkaloid with anti-tumor activity, leads to apoptosis

of the human endometrial cancer cell line RL95-2 by activating reactive oxygen species and ERK/MAPK (Kim *et al.*, 2011). Gonadotropin-releasing hormone activates the MAPK signaling pathway in healthy or carcinoma cells of the human ovary and placenta (Kang *et al.*, 2000). SPACRC-like protein 1 (SPARCL1) is overexpressed in ovarian cancer; prevention of the activation of the ERK/MAPK signaling downregulates SPARCL1 and blocks the growth and migration of ovarian cancer cells (Ma *et al.*, 2018).

5. Vitamin B6

Vitamin B6 is a water-soluble compound and is part of the vitamin B complex group (Combs, 2008). There are six forms of the vitamin B6, such as pyridoxamine (PM), pyridoxamine 5'-phosphate (PMP), pyridoxal (PL), pyridoxal 5'-phosphate (PLP), Pyridoxine (PN), and pyridoxine 5'-phosphate (PNP). There structures are summarized in Figure 1.11.

5.1. Function of vitamin B6

Among the several forms of vitamin B6, the metabolically active form is PLP. PLP is implicated in the variety of nutrient metabolism, including neurotransmitter, histamine and hemoglobin synthesis. Moreover, PLP generally act as a cofactor in many biological processes and could help to promte decarboxylation, racemization, transamination, replacement, and β–group interconversion reactions (Grogan, 1988; Mihara et al., 1997).

PLP is served as a cofactor for transaminases in amino acid metabolism (Dakshinamurti et al., 1990). It is an essential component of two enzymes implicated in cysteine and selenocysteine metabolism, or cystathionine β–synthase (CBS) and cystathionine β–lyase (CBL). In cysteine metabolism, CBS catalyzes the production of L–cystathionine, a precursor to L-cysteine, from L-homocysteine and L-serine as substrates (Oltean & Banerjee, 2005). CBL converts L-cystathionine to L–homocysteine in cysteine catabolism and also converts L-selenocystathionine to L–

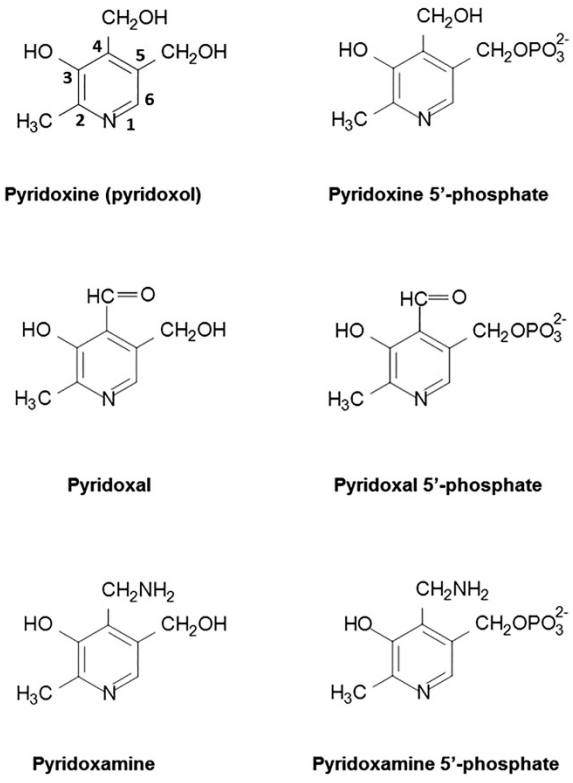

Figure 1.12. The chemical structures of vitamin B6 (Wilson et al., 2019).

selenohomocysteine in selenocysteine metabolism (Anderson & Thompson, 1979; Flavin & Slaughter, 1964; Mihara et al., 1997). Selenohomocysteine is then further converted to hydrogen selenide. Low vitamin B6 status reduces the activity of these enzymes. PLP is also required to convert tryptophan to niacin (Leklem et al., 1975) and used to yield the physiologically active amines. serotonin from tryptophan, histamine from histidine, dopamine from dihydroxyphenylalanine, and γ-aminobutyric acid (GABA) from glutamate (Lee et al., 1988; Schaeffer et al., 1998).

PLP is also participated in gluconeogenesis and lipid metabolism. In gluconeogenesis, PLP is an essential cofactor of glycogen phosphorylase required to initiate gluconeogenesis (Helmreich, 1992). In lipid metabolism, PLP plays an important role in the biosynthesis of sphingolipids, especially in the synthesis of ceramides. In this reaction, serine is decarboxylated and combined with palmitoyl-CoA to form sphinganine combined with a fatty acyl-CoA to form dihydroceramide, and then further desaturated to form ceramide.

5.2. Synthesis of vitamin B6

PLP can be synthesized by a salvage pathway (Figure 1.12) using the B6-vitamers pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) present in the growth medium (Yang et al., 1996). In this pathway, the substrates PL, PM and PN are phosphorylated by kinases to form PLP, PNP and PMP. Two other kinases with different substrate specificities have been confirmed in *E. coli*. The PL/PM/PN kinase PdxK (Yang et al., 1996) and the PL kinase PdxY (Yang et al., 1998) PMP and PNP are oxidized to PLP by the PdxH oxidase. Although PdxA.

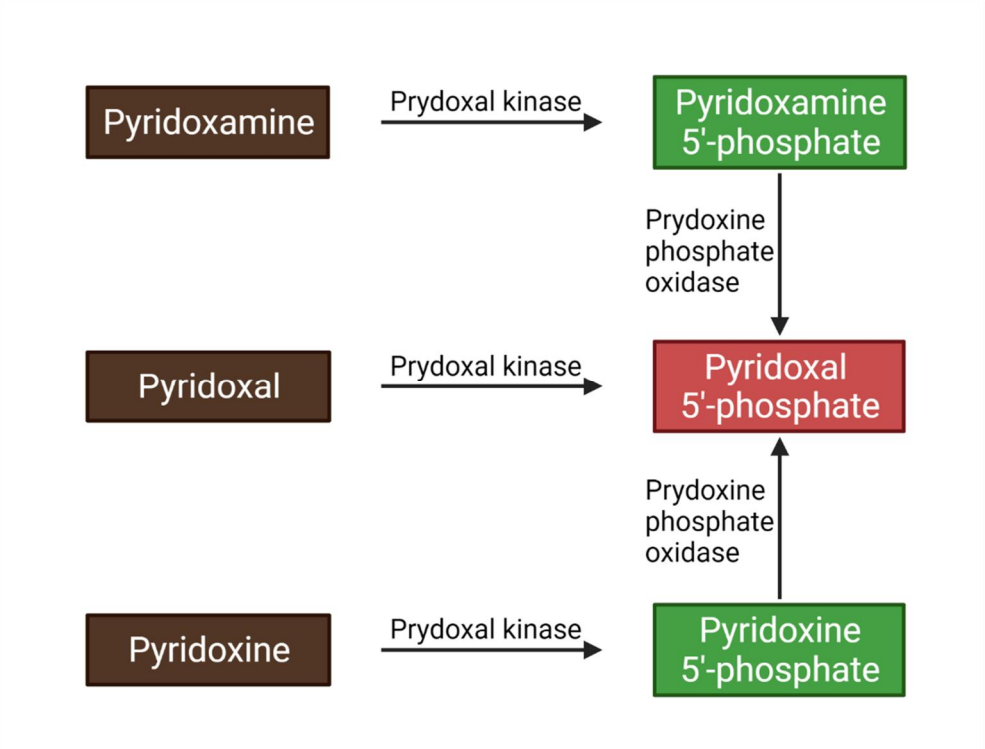

Figure 1.13. The pathway of vitamin B6 synthesis. .

and PdxJ have not been discovered in animals (Ehrenshaft et al., 1999), similar salvage pathways including oxidases and kinases are found in mammalian cells (McCormick & Chen, 1999). Therefore, species that synthesize PLP have one of the two PLP biosynthetic pathways

CHAPTER 2.

Selenophosphate synthetase 1 deficiency-triggered *PGRP-LC* and *Toll* expression controls innate immunity in *Drosophila* S2 cells

1. INTRODUCTION

Selenium is an essential trace element in the diet of humans and many other life forms. An appropriate amount of selenium offers many health benefits, such as preventing cancer and heart disease, acting as an antiviral agent, scavenging reactive oxygen species (ROS), and augmenting the immune system and male reproduction (Brigelius-Flohé & Sies, 2016; Vadim N. Gladyshev *et al.*, 2016; Na *et al.*, 2018). The many benefits of selenium may be due to its existence in selenoproteins in the form of the amino acid selenocysteine (Sec) (Brigelius-Flohe, 2008; Hatfield & Gladyshev, 2002; Lu & Holmgren, 2009). Sec is the 21st amino acid and enters into a growing peptide in response to the UGA codon during translation (Allmang *et al.*, 2009; Lee *et al.*, 1989; Leinfelder *et al.*, 1989; Longtin, 2004; Squires & Berry, 2008). The active donor of selenium in Sec biosynthesis is monoselenophosphate (Glass *et al.*, 1993). It is synthesized from selenide and ATP by the enzyme selenophosphate synthetase (SPS, also called *SelD* or *patufet*) (Ehrenreich *et al.*, 1992). Only one type of SPS exists in prokaryotes, including Archaea. However, there are two isoforms of SPS, SPS1 and 2, in eukaryotes (Guimaraes *et al.*, 1996). In higher animals, such as mammals, SPS is referred to as SEPHS, because sucrose-phosphate synthase is also designated as SPS. However, there is no sucrose-phosphate synthase in *Drosophila melanogaster*. The amino acid sequences of SPS1 and 2 are highly conserved. One of the main differences between the sequences of SPS1 and 2 is that they have an arginine and Sec, respectively, in a homologous region (Low *et al.*, 1995).

Additionally, only SPS2 has selenophosphate synthesis activity (Xu, Carlson, Mix, *et al.*, 2007).

In *Drosophila melanogaster*, SPS1 deficiency leads to aberrant imaginal-disc morphology and embryonic lethality (Alsina *et al.*, 1998). Furthermore, *Sps1* knockdown decelerates cell growth, activates the innate immunity by upregulating AMPs, increases ROS levels, and induces megamitochondria formation in *Drosophila* S2 cells (Lee *et al.*, 2011; Shim *et al.*, 2009). These phenotypes are caused through downregulation of pyridoxal phosphate (PLP), a biologically active form of vitamin B6 (Lee *et al.*, 2011). In mice, systemic knockout of *Sephs1* (*Sps1*) gradually increases the oxidative stress, thereby impairing gastrulation-related signaling pathways and causing embryonic lethality (Bang *et al.*, 2021; Tobe *et al.*, 2016). In 2H11 cells, SEPHS1 deficiency increases the superoxide level, thereby causing DNA damage, which suppresses the cell proliferation and impairs the cell functions (Jung *et al.*, 2021).

Innate immunity is an important defense system against infections in metazoans (Janeway & Medzhitov, 2002; Lemaitre & Hoffmann, 2007). As with all invertebrates, *Drosophila melanogaster* depends entirely on innate immunity to thwart infections (Lemaitre & Hoffmann, 2007). Antimicrobial peptides (AMPs) are one of the main effector molecules in the innate immune system. AMPs protect the host by destroying the cell wall of invading microorganisms with cationic and amphipathic peptides (Zhang & Gallo, 2016). The induction of AMP production upon infection is regulated via two distinct signaling pathways—the Toll and IMD pathways—in *Drosophila melanogaster* (Lemaitre & Hoffmann, 2007; Myllymaki *et al.*, 2014; Valanne *et al.*,

2011). The Toll pathway is used for activating the expression of *Drosomycin* (*Drs*) and *Metchnikowin* (*Mtk*), and these AMPs are required to protect cells from infections by fungi or Gram-positive bacteria. The active form of spätzle (Spz), cleaved by the spätzle-processing enzyme (SPE), activates the Toll signaling (Kanoh *et al.*, 2015) and finally induces the nuclear translocation of the proteins nuclear factor-kappa B (NF-κB), dorsal-related immunity factor (DIF), and dorsal, thereby activating the expression of AMP genes, including *Drs* and *Mtk* (Lindsay & Wasserman, 2014). The IMD pathway is activated upon detecting diaminopimelic acid (DAP)-type peptidoglycans, which are derived from Gram-negative bacteria, via the transmembrane receptor PGRP-LC (Kleino & Silverman, 2014). This transmembrane receptor transduces the signal to downstream factors, including the adaptor proteins (IMD) and NF-κB (relish), and eventually AMPs, such as Drosocin (Dro), Diptericin (Dpt), Attacin (Att), and Cecropin (Cec), are upregulated (Paquette *et al.*, 2010).

Although SPS1 is involved in the regulation of *Drosophila melanogaster* innate immune system, the mechanisms whereby SPS1 regulates the AMP production are elusive. In this study, we investigated the signaling components through which SPS1 modulates *Drosophila melanogaster* innate immunity and found that PGRP-LC and Toll, two genes of transmembrane receptors in the IMD and Toll pathways, respectively, are the primary targets in SPS1-deficiency–induced AMP production.

2. MATERIALS AND METHODS

2.1. Materials

Drosophila Schneider cell line 2 (S2) was purchased from Invitrogen. HyQ SFX-Insect medium was purchased from Hyclone, T3 Megascript kit was purchased from Ambion, PowerUpTM SYBRTM Green Master Mix was purchased from Thermo Fisher, TRIzol reagent was purchased from Invitrogen, Moloney murine leukemia virus reverse transcriptase and nPfu forte DNA polymerase were purchased from Enzynomics. Dimethyldioctadecyl ammoniumbromide was purchased from Sigma, and oligonucleotides were purchased from Cosmo Genetech. The sequences of oligos used for RT-PCR and dsRNA are listed in Supplementary data.

2.2. Vector Construction

pAcPA PGRP-LCa was produced by PCR amplification of *Bam*HI-PGRP-LCa-*Kpn*I from cDNA of S2 cells, cut and ligated into the pAcPA vector (Shim *et al.*, 2009), containing the actin 5C promoter, with *Bam*HI/*Kpn*I. pAcPA PGRP-LCx and pAcPA Toll were prepared in the same way.

2.3. Double-stranded RNA preparation *In vitro*

To prepare double-stranded RNA (dsRNA) of *Sps1*, *PGRP-SD*, *PGRP-LC*, *SPE* and *Toll*, each gene was amplified with a primer pair. The sequences of each primer are provided in Table S2. Each primer was fused with a T3 promoter sequence (5'-

Table 2.1. List of primers for qRT-PCR

Gene	Sequence (5'→3')	
	Forward	Reverse
RP49	CAGTCGGATCGATATGCTA	AATCTCCTTGCGCTTCTT
DptB	ATCCTGATCCCCGAGAGATT	TGAAGTGCCCTAAAACCTGAA
CecB	AATCCGATCGTAAGCCAACA	AGAGAAATGAGCGGGTCGAG
Dro	CATACCGCGGAGAAGTCATC	TTAGGGGACAAACCCATTCA
Mtk	CCACCGAGCTAAGATGCAA	TGTTAACGACATCAGCAGTGTG
Drs	GTACTTGTTCGCCCTCTTCG	ACAGGTCTCGTTGTCCCAGA
SPS1	AGGGGATGTACTGGTGCTAA	TCTTATTGCCCTTCTCAACG
PGRP-SD	CCTTTGCCGGTCCCAATAAC	GGAGTGGTCTTTACGTGTCG
PGRP-LC	AGGGTCTAACGGTGATCAGT	AACTGTTGTATCGTCACGGG
SPE	CCAATATGCCTTCCCACGGA	TCCAATCGATGAAAGCCCCC
Toll	GACCAGAGCTTCATTGAGGA	AATCGCAGCTTATCCCAGAA

Table 2.2. List of primers for dsRNA synthesis

Gene	Sequence (5'→3')	
	Forward	Reverse
SPS1	AATTAACCCTCACTAAAGGGATGAG CTACGCCGCTGATG	AATTAACCCTCACTAAAGGGAGTT CATCGCCCGGTGGTA
PGRP-SD	AATTAACCCTCACTAAAGGGGAATG ACTTGGATCGGTTTGCT	AATTAACCCTCACTAAAGGGCTCC AGGACTCTTGGTAGCAC
PGRP-LC [-exon E]	AATTAACCCTCACTAAAGGGGTCAG CCGTTTTGCCATACG	AATTAACCCTCACTAAAGGGGTGA ACTGTTGGGCGGGTAG
PGRP-LC [exon E]	AATTAACCCTCACTAAAGGGAACTG TCTGAATGGGGTGCA	AATTAACCCTCACTAAAGGGCCCA CGAGAAAGTTGTAGCCT
SPE	AATTAACCCTCACTAAAGGGGGGTT GAGGGCTTGGTGAAT	AATTAACCCTCACTAAAGGGCGCA CCAACGCAATGTCATT
Toll	AATTAACCCTCACTAAAGGGACTCT TTGCACATACCACCA	AATTAACCCTCACTAAAGGGTGCG TAATTTGAACTGCCTG

AATTAACCCTCACTAAAGGG-3') at its 5' end. *In vitro* transcription was performed using the T3 Megascript kit according to the manufacturer's protocols and then the dsRNAs were produced by annealing each complementary strand set.

2.4 S2 cell culture and RNA interference

S2 cell culture and RNA interference using dsRNAs were carried out as described previously with minor modification (Shim *et al.*, 2009). Briefly, for RNA interference, 2.5×10^5 cells were plated on a 24 well plate containing 0.5 ml of HyQ SFX-Insect medium. Four micrograms of dsRNAs were added directly to the medium and incubated for 48 hrs and cells were split into appropriate culture dishes for further incubation and other experiments.

2.5. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was carried out as described with minor modification (Shim *et al.*, 2009). Briefly, total RNA was isolated from the cells using the TRIzol reagent. cDNAs were synthesized from total RNAs with Moloney murine leukemia virus reverse transcriptase and oligo (dT) primers according to the manufacturer's protocols. RT-qPCR was carried out using an ABI 7300 real-time PCR system (Applied Biosystems) as follows. cDNAs were amplified using SYBR Green mix and specific primers for 40 cycles [initial incubation at 50°C for 2 min and then at 95°C for 10 min, and 40 cycles (95°C for 15 sec, 55°C for 1 min, and 72°C for 1 min)]. Output data was

obtained as Ct values using Sequence Detection Software (SDS) version 1.3 (7300 System, Applied Biosystems) and the differential mRNA expressions of each gene between control and knockdown cell were calculated using the comparative Ct method (Schmittgen & Livak, 2008). *rp49* mRNA, an internal control, was amplified along with the target genes, and the Ct value of *rp49* was used to normalize the expression of target genes.

2.6. DNA transfection

Vectors were transfected into S2 cells as described previously (Han, 1996) with minor modifications. Briefly, 2 μg of pAcPA (backbone vector), pAcPA PGRP-LCa, pAcPA PGRP-LCx, and pAcPA Toll were mixed with 100 μl of dimethyldioctadecyl ammonium bromide (125 μg /ml) and 200 μl of HyQ-SFX-Insect media. The mixture was incubated for 30 min at room temperature and then added into a well of a 6 well plate containing 2×10^6 cells.

2.7. Statistics

Each experiment was performed in biological triplicate for statistical analysis. Statistical analyses were performed using an unpaired Student's t-test or one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1. SPS1 deficiency activates innate immunity—the IMD and Toll pathways

We have previously reported that *Sps1* knockdown upregulates AMPs that are responsible for *Drosophila melanogaster* innate immunity (Lee *et al.*, 2011). To elucidate which immune pathways are regulated upon SPS1 deficiency, *Sps1* was knocked down in S2 cells, and the expression levels of AMPs were measured using RT-qPCR after 5 days. *DptB*, *CecB*, *Dro*, *Mtk*, and *Drs*, which are AMP genes widely used to assess whether the innate immune system is activated, were selected as AMP markers. Data analysis revealed that AMPs of both the IMD (*DptB*, *CecB*, and *Dro*) and Toll (*Mtk* and *Drs*) pathways were upregulated by 10–50 folds ($p < 0.001$) in SPS1-deficient cells (Figure 2.1A and 2.1B). These results indicate that SPS1 deficiency activates both the IMD and Toll pathways.

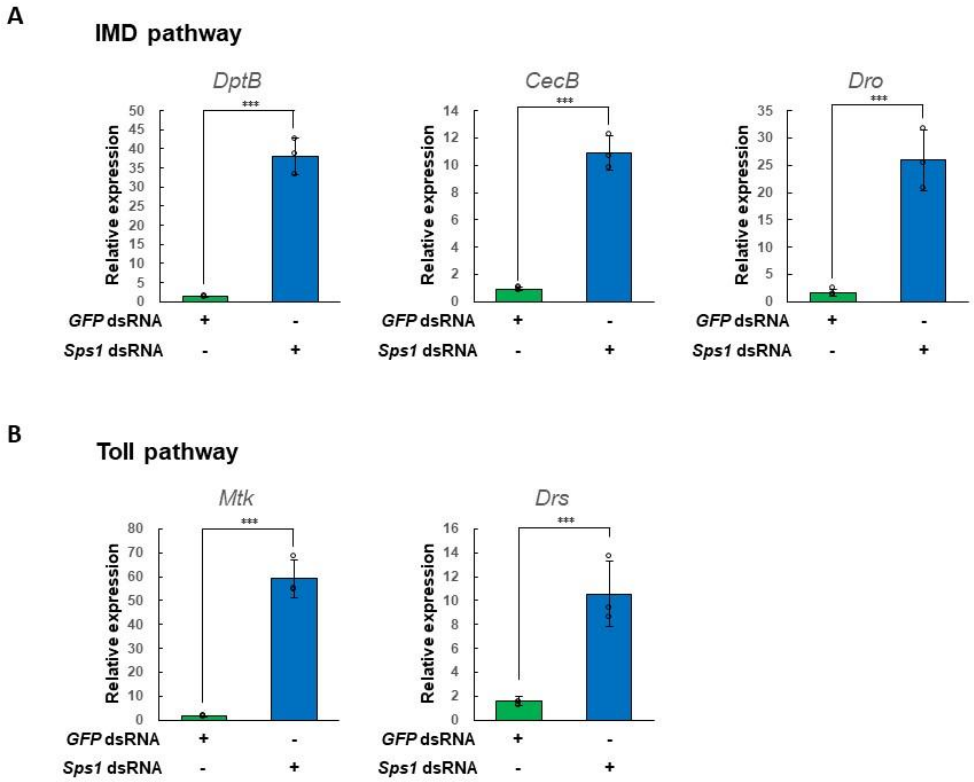

Figure 2.1. SPS1 deficiency upregulates the AMPs of the IMD and Toll pathways. Five days after adding the *Sps1* dsRNA, mRNA levels were measured via RT-qPCR using rp49 as a control for normalization. The y-axis represents the relative mRNA level of each gene in the cells treated with the *Sps1* dsRNA to that of no-dsRNA–treated cells. (A) Readout AMPs of the IMD pathway. (B) Readout AMPs of the Toll pathway. *** indicates p-value < 0.001, based on unpaired Student's t-test. *DptB*, *Diptericin B*; *CecB*, *Cecropin B*; *Dro*, *Drosocin*; *Mtk*, *Metchnikowin*; *Drs*, *Drosomycin*; *GFP*, *green fluorescent protein*.

3.2. SPS1 regulates innate immunity by targeting the transmembrane receptors PGRP-LC and Toll

To identify the target genes through which SPS1 regulates the expression of the AMPs in the IMD and Toll pathways, the upstream genes in each pathway were individually knocked down along with *Sps1*.

First, to investigate the IMD pathway, *PGRP-SD*, the most upstream gene in the IMD pathway and *Sps1* were co-knocked down. As shown in Figure 2.2A, *DptB* and *Mtk* were upregulated upon *Sps1* knockdown were not recovered upon *PGRP-SD*/*Sps1* co-knockdown. However, intriguingly, the expression levels of *DptB* and *Mtk* were significantly reduced to the background levels (GFP control) *upon PGRP-LC*/*Sps1* co-knockdown ($p < 0.001$) (Figure 2.2A). Other AMP genes (*CecB*, *Dro*, and *Drs*) showed similar results (Figure 2.3A). These results indicate that SPS1 deficiency affects the IMD pathway by regulating PGRP-LC.

Next, the Toll pathway was likewise investigated. No recovery was observed when *SPE* and *Sps1* were co-knocked down (Figure. 2.2B). However, when *Toll* and *Sps1* were co-knocked down, the expression of *DptB* and *Mtk* was decreased significantly ($p < 0.001$, Fig. 2.2B). Other AMP genes (*CecB*, *Dro*, and *Drs*) also showed similar results (Fig. 2.3B). The expression data for all the AMP genes analyzed in this study are summarized in Table 2.3. The knockdown efficiency was > 90% for all the genes. Interestingly, the readout AMPs of the Toll pathway were downregulated upon co-knocking down *PGRP-LC*, which participates in the IMD pathway, and vice versa, (the readout AMPs of IMD pathway were also downregulated by the co-knocking

down *Toll*). These data suggest that there is a crosstalk between the IMD and Toll pathways (Fig. 2.2). Taken together, it can be concluded that SPS1 regulates AMP expression through a crosstalk between the IMD and Toll pathways at the transmembrane receptor level, namely through *PGRP-LC* and *Toll*, respectively.

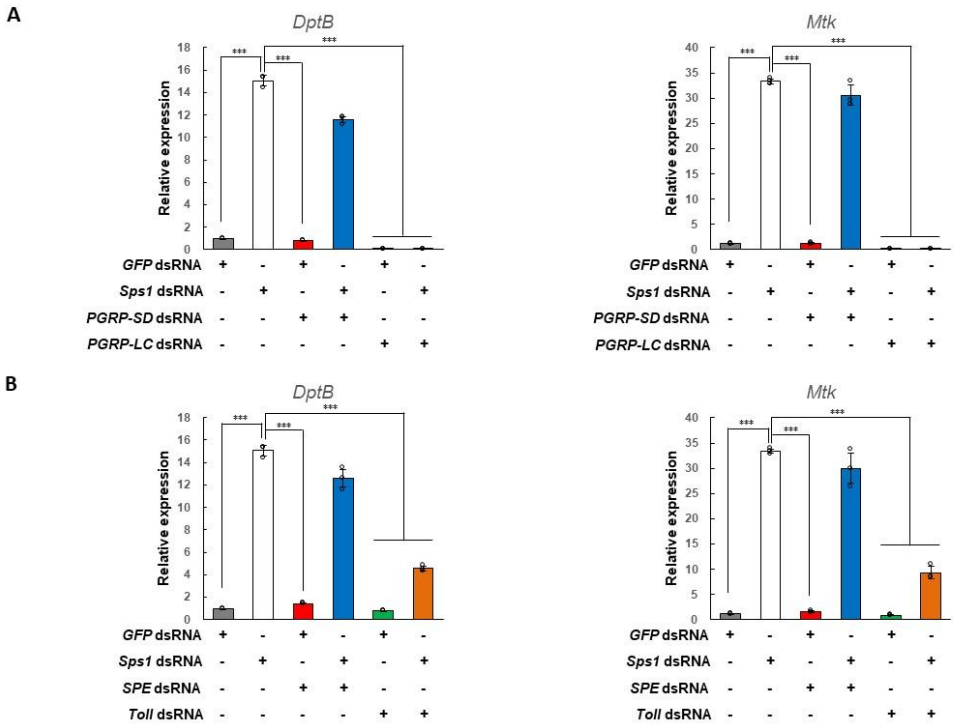

Figure 2.2. SPS1 deficiency upregulates the AMPs (*DptB*, *Mtk*) of the IMD and Toll pathways through the transmembrane receptors PGRP-LC and Toll, respectively. Five days after adding each dsRNA with *Sps1* dsRNA, the mRNA levels of AMP genes were measured via RT-qPCR using rp49 as a control for normalization. Relative expression levels of AMP genes after knocking down *Sps1* alongside *PGRP-SD* or *PGRP-LC* (A), and alongside *SPE* or *Toll* (B). *** indicates p-value < 0.001, based on one-way ANOVA with Tukey's multiple comparison test. *DptB*, *Diptericin B*; *Mtk*, *Metchnikowin*; *GFP*, *green fluorescent protein*.

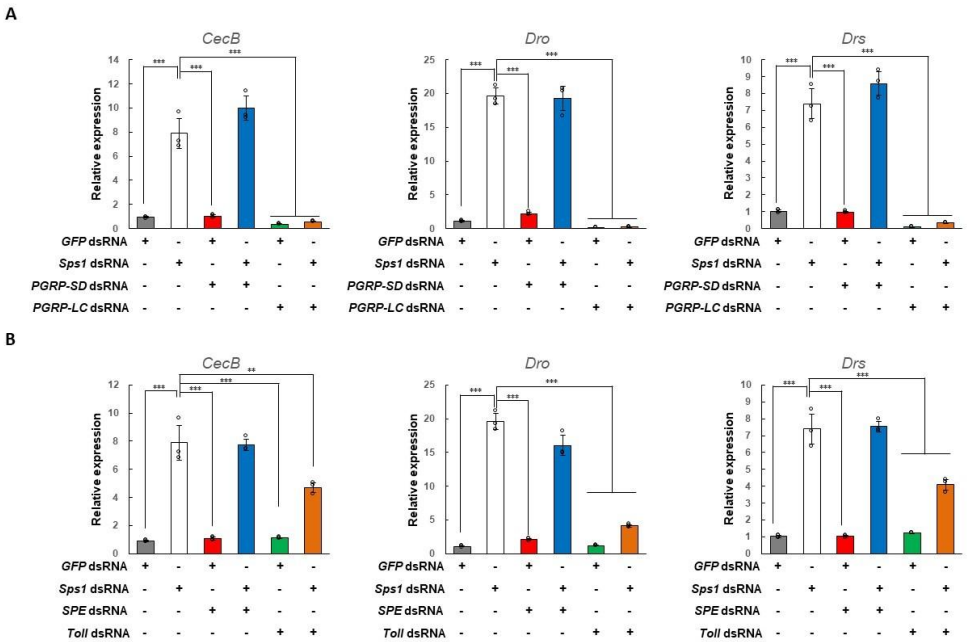

Figure 2.3. SPS1 deficiency upregulates the other AMPs (*CecB*, *Dro*, *Drs*) of the IMD and Toll pathways through the transmembrane receptors PGRP-LC and Toll, respectively. Five days after adding each dsRNA with *Sps1* dsRNA, the mRNA levels of AMP genes were measured via RT-qPCR using rp49 as a control for normalization. Relative expression levels of AMP genes after knocking down *Sps1* alongside *PGRP-SD* or *PGRP-LC* (A), and alongside *SPE* or *Toll* (B). *** indicates p-value < 0.001, based on one-way ANOVA with Tukey's multiple comparison test. *CecB*, *Cecropin B*; *Dro*, *Drosocin*; *Drs*, *Drosomycin*; *GFP*, *green fluorescent protein*.

Table 2.3. Effects of double knockdown on the relative expression of AMPs

Immune pathway	Gene[+]	*GFPi*	*SPS1i*	*GFPi +PGRP-SDi*	*SPS1i +PGRP-SDi*	*GFPi +PGRP-LCi*	*SPS1i +PGRP-LCi*	*GFPi +SPEi*	*SPS1i +SPEi*	*GFPi +Tolli*	*SPS1i +Tolli*
IMD pathway	*DptB*	0.96 ±0.02	15.06 ±0.45	0.81 ±0.03	11.58 ±0.29	0.02 ±0.00	0.03 ±0.01	1.43 ±0.04	12.59 ±0.81	0.80 ±0.01	4.55 ±0.21
	CecB	0.92 ±0.07	7.90 ±1.24	1.03 ±0.11	10.00 ±1.01	0.36 ±0.03	0.57 ±0.03	1.08 ±0.09	7.75 ±0.42	1.13 ±0.03	4.71 ±0.34
	Dro	1.08 ±0.08	19.62 ±1.16	2.22 ±0.20	19.26 ±1.82	0.13 ±0.03	0.20 ±0.02	2.14 ±0.16	16.04 ±1.47	1.22 ±0.03	4.14 ±0.18
Toll pathway	*Mtk*	1.17 ±0.07	33.36 ±0.46	1.23 ±0.13	30.56 ±2.01	0.03 ±0.00	0.07 ±0.00	1.55 ±0.18	29.99 ±3.02	0.91 ±0.10	9.34 ±1.21
	Drs	1.04 ±0.08	7.39 ±0.89	0.97 ±0.05	8.59 ±0.69	0.11 ±0.00	0.32 ±0.00	1.03 ±0.05	7.54 ±0.32	1.23 ±0.01	4.08 ±0.32

3.3. SPS1 regulates the transcription of *PGRP-LC* and *Toll*

Since PGRP-LC and Toll are the targets of SPS1, and it has been reported that ecdysone regulates the innate immune system by upregulating *PGRP-LC* (Rus *et al.*, 2013), we hypothesized that SPS1 deficiency upregulates *PGRP-LC* and *Toll*. As shown in Figure 2.4, *Sps1* knockdown significantly increased the levels of the *PGRP-LC* and *Toll* mRNAs (6.4 ± 0.36 and 3.2 ± 0.45-fold, respectively, n = 3), indicating that SPS1 regulates both *PGRP-LC* and *Toll* presumably at the transcription level. Interestingly, SPS1 deficiency upregulated *PGRP-LC* by approximately 2-fold ($p < 0.001$, n = 3) compared with that of *Toll*, suggesting that SPS1 deficiency has a stronger effect on PGRP-LC than on Toll.

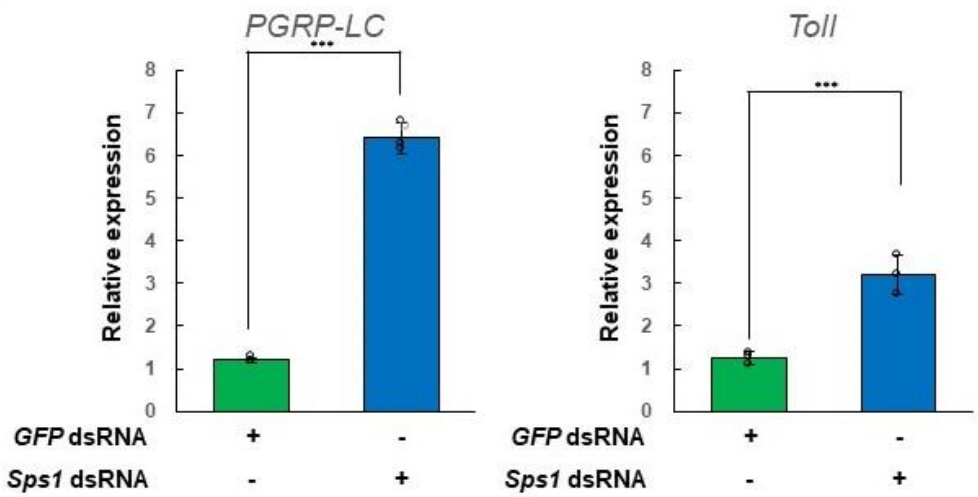

Figure 2.4. SPS1 deficiency upregulates PGRP-LC and Toll. Five days after adding the *Sps1* dsRNA, the mRNA levels of each gene were measured via RT-qPCR using rp49 for normalization. ** and *** indicate p-values < 0.01 and < 0.001, respectively, based on unpaired Student's t-test. *GFP*, *green fluorescent protein*.

3.4. Increased expression of *PGRP-LC* or *Toll* activates the innate immune system

To decipher whether upregulation of *PGRP-LC* or *Toll* upregulates AMPs, we overexpressed *PGRP-LCa*, *PGRP-LCx*, or *Toll* in S2 cells and examined the expression levels of AMPs. The overexpression of each protein upregulated *Mtk* (96.16 ± 12.71, 218.72 ± 10.08, and 3.04 ± 0.72-fold for PGRP-LCa, PGRP-LCx, and Toll, respectively, n = 3) and *DptB* (198.55 ± 18.95, 376.74 ± 5.33, and 2.28 ± 0.3-fold for PGRP-LCa, PGRP-LCx, and Toll, respectively, n = 3) (Figure 2.5A and 2.5B). The expression levels of AMPs upon *PGRP-LCa*, *PGRP-LCx*, or *Toll* overexpression is summarized in Table 2.4. Other AMPs (*CecB*, *Dro*, and *Drs*) were also likewise upregulated when *PGRP-LCa*, *PGRP-LCx*, or *Toll* was overexpressed (Figure 2.6). Notably, AMP production was induced more in cells overexpressing *PGRP-LC* than in those overexpressing *Toll* although the two proteins were overexpressed to a similar extent. In addition, *PGRP-LC* overexpression activated the expression of *Mtk*, which is a target of Toll. This result supports the crosstalk between the IMD and Toll pathways. Taken together, our observations indicate that SPS1 participates in the innate immune system by controlling the expression of the genes of two transmembrane receptors, *PGRP-LC* and *Toll*, and the amount of PGRP-LC affects the innate immune system more than the amount of Toll.

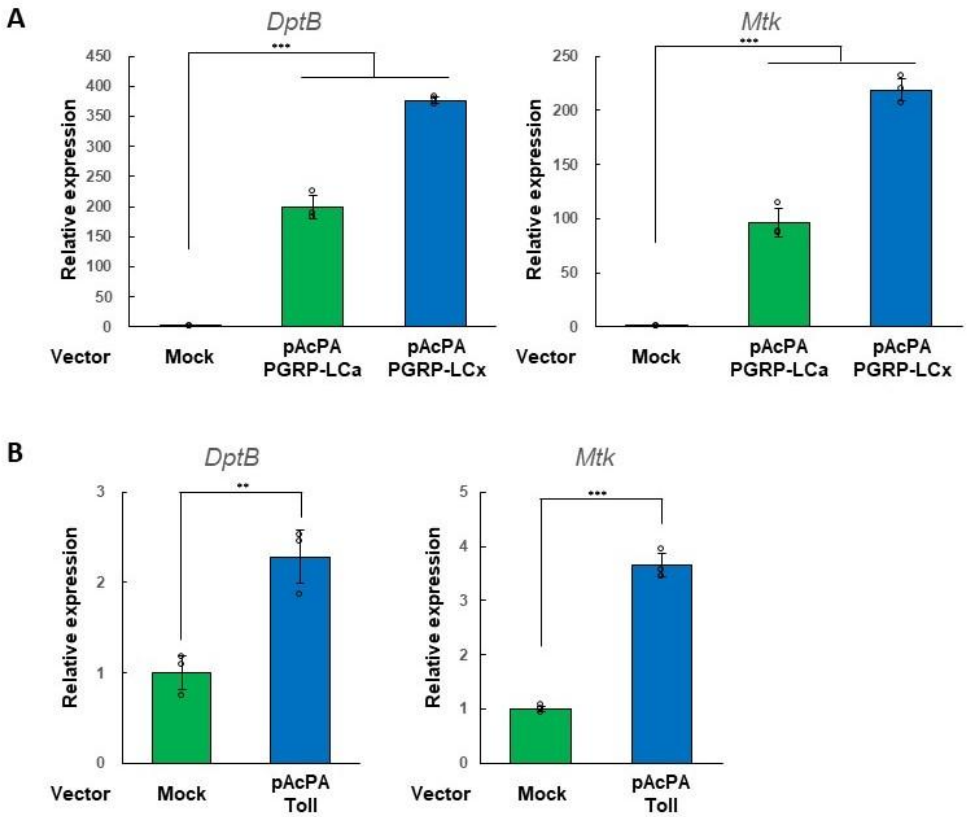

Figure 2.5. Overexpression of *PGRP-LC* or *Toll* induces AMPs (*DptB*, *Mtk*) expression. Three days after transfection of S2 cells with pAcPA-PGRP-LCa, pAcPA-PGRP-LCx, or pAcPA-Toll, the mRNA levels of AMP genes were likewise measured. ** and *** indicate p-values < 0.01 and < 0.001, respectively, based on unpaired Student's t-test. *Mtk*, *Metchnikowin*; *DptB*, *Diptericin B*; *GFP*, *green fluorescent protein*.

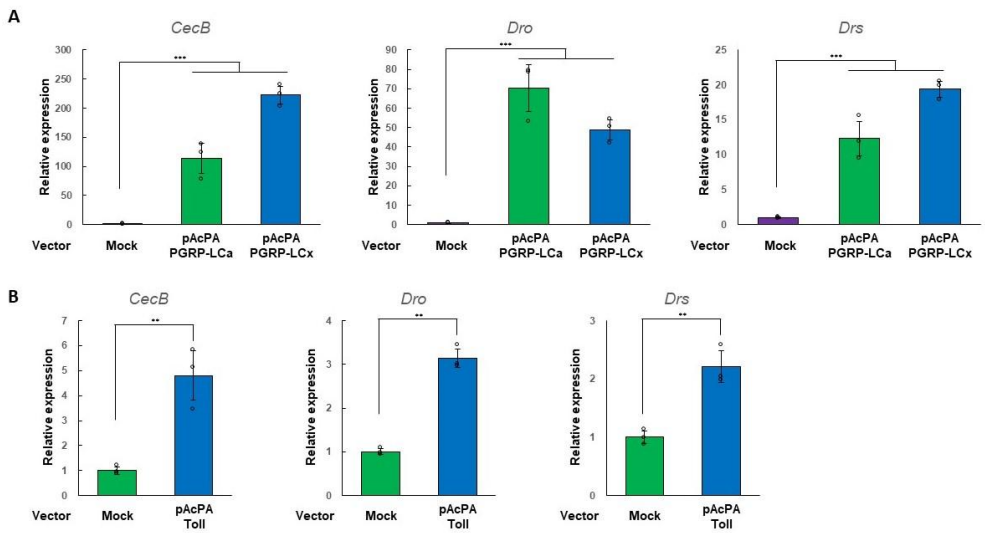

Figure 2.6. Overexpression of *PGRP-LC* or *Toll* induces AMPs (*CecB*, Dro, Drs) expression. Three days after transfection of S2 cells with pAcPA-PGRP-LCa, pAcPA-PGRP-LCx, or pAcPA-Toll, the mRNA levels of AMP genes were likewise measured. ** and *** indicate p-values < 0.01 and < 0.001, respectively, based on unpaired Student's t-test. *CecB*, *Cecropin B*; *Dro*, *Drosocin*; *Drs*, *Drosomycin*; *GFP*, *green fluorescent protein*.

Table 2.4. Relative AMPs expression in overexpression

Immune pathway	Gene[+]	Mock	pAcPA PGRP-LCa	pAcPA PGRP-LCx	pAcPA Toll
IMD pathway	*DptB*	1.00 ±0.18	198.55 ±18.95	376.74 ±5.33	2.28 ±0.30
	CecB	1.00 ±0.14	113.90 ±25.63	222.86 ±15.18	4.81 ±0.99
	Dro	1.00 ±0.07	70.40 ±12.21	48.95 ±5.18	3.14 ±0.21
Toll pathway	*Mtk*	1.00 ±0.06	96.16 ±12.71	218.72 ±10.08	3.64 ±0.22
	Drs	1.00 ±0.11	12.31 ±2.49	19.38 ±1.14	2.21 ±0.27

[+]*Mtk*, *Metchnikowin*; *Drs*, *Drosomycin*; *DptB*, *Diptericin B*; *CecB*, *Cecropin B*; *Dro*, *Drosocin*.

4. DISCUSSION

SPS1 is known to play an essential role in growth of cells, vitamin-B6 synthesis, and innate immunity in *Drosophila melanogaster* (Lee *et al.*, 2011; Shim *et al.*, 2009). Among these various functions of SPS1, we focused on how SPS1 affects the immune system, especially on the AMP overproduction upon SPS1 deficiency. Via *Sps1* knockdown in S2 cells, we found that SPS1 regulates both the IMD and Toll pathways in the innate immune system.

In this study, we identified PGRP-LC as the primary target gene of the IMD pathway. PGRP-LC is known as the most upstream gene in the sub-cellular IMD pathway (Buchon *et al.*, 2014). Recently, PGRP-SD was found to be involved in the IMD pathway outside the cell. It binds to peptidoglycans (PGNs) that are produced by digestion of bacterial cell walls, and the PGRP-SD/PGN complex helps re-localization of PGN to PGRP-LC on the cell surface (Iatsenko *et al.*, 2016). Our results from the co-knockdown experiments clearly revealed that SPS1 targets PGRP-LC, not PGRP-SD, to induce the IMD pathway upon SPS1 deficiency. Toll was also found to be the primary target of SPS1 to induce the Toll pathway. The induction of both the IMD and Toll pathways is triggered by the upregulation of transmembrane receptors－PGRP-LC and Toll for the IMD and Toll pathways, respectively.

Two different lines of evidence support our findings that the induction of PGRP-LC and Toll pathway triggers the activation of AMP expression. First, knocking down SPS1 (SelD) induced *Diptericin* expression, whereas knocking down PGRP-LC inhibited *Diptericin* expression (Foley & O'Farrell, 2004). Second, the activation of

PGRP-LC by ecdysone treatment upregulated AMPs (Rus *et al.*, 2013). Altogether, our results indicate that upregulation of PGRP-LC is sufficient to induce the AMP signaling pathway. Toll overexpression has been reported to slightly induce the expression of *Drosomycin* (approximately 5-fold) (Hu *et al.*, 2004). However, it is unclear why the upregulation of Toll had an insignificant effect on the activation of the innate immune pathways.

We found that the activation of AMP expression occurs as a result of a crosstalk between the IMD and Toll pathways. A crosstalk between these two innate immune pathways has been previously reported (Nishide *et al.*, 2019; Tanji *et al.*, 2007). Tanji *et al.* showed that overexpression of *PGRP-LC* or *Toll* activated both the IMD and Toll pathways. In our study, we also showed that overexpression of *PGRP-LC* or *Toll* induces both these pathways. In addition, our co-knockdown experiments showed that knockdown of *PGRP-LC* or *Toll* downregulates the AMPs in both the IMD and Toll pathways.

It was reported with microarray analysis that SPS1 deficiency upregulates the genes that participate in defense response, including *PGRP-SD*, *PGRP-LF*, and *pirk* (Lee *et al.*, 2011). In this study, we found that PGRP-SD, unlike PGRP-LC, did not affect the activation of the innate-immune signaling induced upon SPS1 deficiency. Since *PGRP-SD* itself is a target gene of the IMD pathway and is activated upon PGRP-LC upregulation (Iatsenko *et al.*, 2016), it is upregulated upon SPS1 deficiency presumably because the innate immune system is activated. PGRP-LF and Pirk suppress the IMD pathway and act as negative feedback regulators of this pathway

(Kleino *et al.*, 2008; Maillet *et al.*, 2008). Therefore, the upregulation of *PGRP-LF* and *pirk* might be due to the upregulation of AMP genes upon SPS1 deficiency.

Recently, SPS1 has been suggested to regulate redox homeostasis (Bang *et al.*, 2021; Jung *et al.*, 2021; Tobe *et al.*, 2016). To examine whether SPS1 affects the innate immune system through ROS in *Drosophila melanogaster*, we knocked down *Sps1* in S2 cells and then treated the cells with the antioxidant N-acetyl cysteine (NAC) to reduce ROS. We found that the expression levels of *PGRP-LC* and *Toll* were not consequently changed, and this result suggests that the regulation of the innate immune system through SPS1 is independent of the redox system (Figure 2.7).

In the previous study, we showed that the vitamin B6 metabolism was regulated by the intracellular SPS1 levels in *Drosophila* S2 cells (Lee *et al.*, 2011). We examined the effect of vitamin B6 on innate immunity in scrutiny in this study. When 4-deoxypyridoxine, an inhibitor of PLP biosynthesis, was administered to the cells without knockdown of *Sps1*, *PGRP-LC* and *Toll* were also upregulated, as in the case of *Sps1* knockdown (Figure 2.8). Therefore, it seems that SPS1 controls the expression of *PGRP-LC* and *Toll* by regulating the synthesis of vitamin B6. Like in *Drosophila melanogaster*, the effects of vitamin B6 on immunity were also examined in a mouse model. The deficiency of vitamin B6 that was achieved by feeding the mice a low vitamin B6 diet led to increased immunoglobulin E production, presumably by the upregulation of interleukin-4 (Doke *et al.*, 1997). The deficiency of vitamin B6, however, suppressed immunoglobulin G or immunoglobulin M (Kumar & Axelrod, 1968). Notably, Excess vitamin B6 levels affected immunity in the opposite manner (Inubushi *et al.*, 2000). Therefore, it seems that vitamin B6

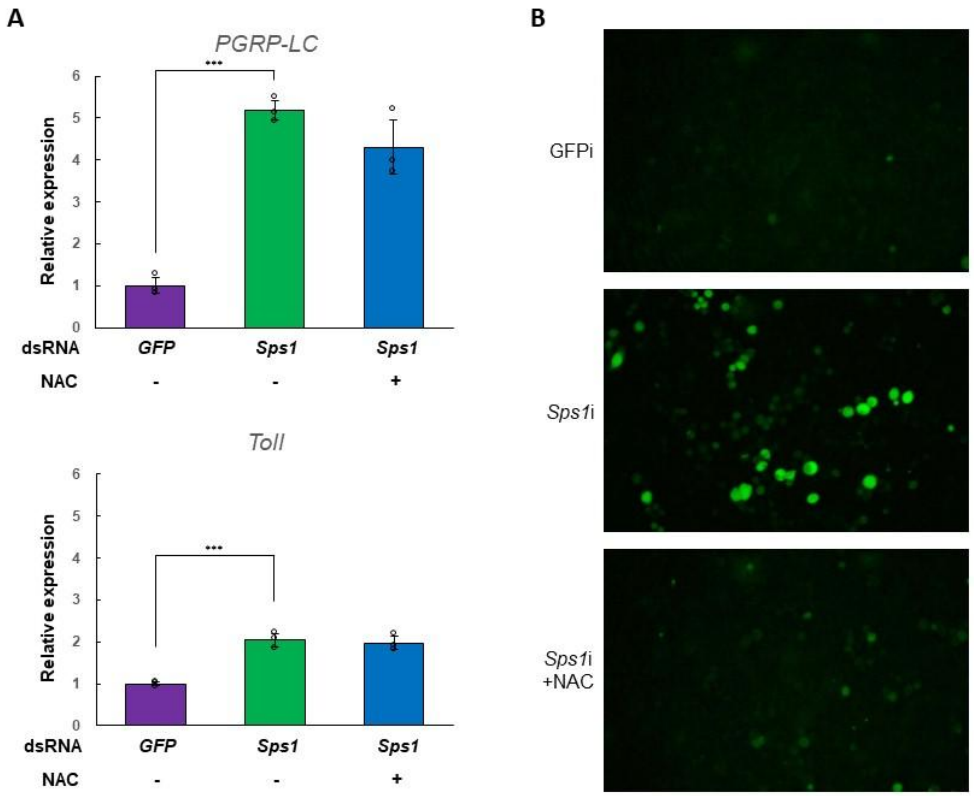

Figure 2.7. Upregulation of *PGRP-LC* and *Toll* by SPS1 deficiency is not recovered NAC treatment. (A) Five days after adding the *Sps1* dsRNA and NAC, the mRNA levels of each gene were measured via RT-qPCR using rp49 for normalization. ** and *** indicate p-values < 0.01 and < 0.001, respectively, based on unpaired Student's t-test. (B) Five days after adding the *Sps1* dsRNA and NAC, cells were stained with CM-H2DCFDA to detect intracellular ROS. The cells were photographed under a fluorescence microscope NAC, N-acetyl cysteine; *GFP*, *green fluorescent protein*.

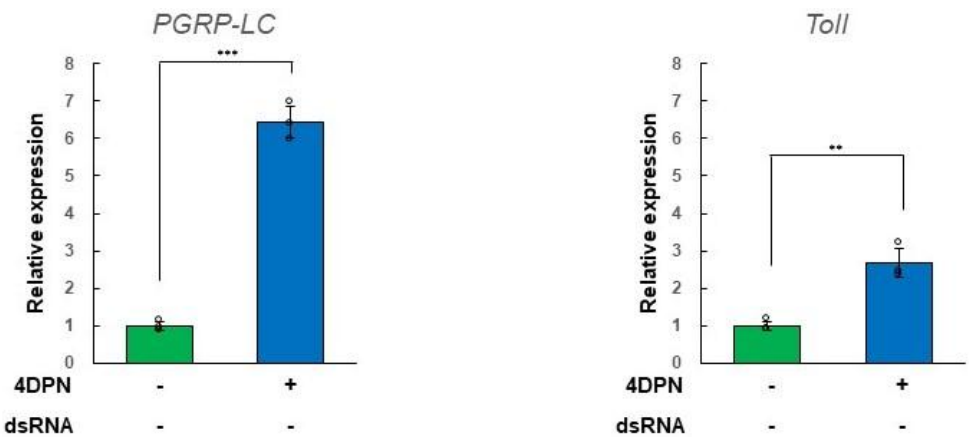

Figure 2.8. 4DPN treatment induces upregulation of *PGRP-LC* and *Toll*. Four days after adding the 4DPN, the mRNA levels of each gene were measured via RT-qPCR using rp49 for normalization. ** and *** indicate p-values < 0.01 and < 0.001, respectively, based on unpaired Student's t-test. 4DPN, 4-deoxypyridoxine.

regulates immune homeostasis in mammalian systems. However, it remains unclear whether vitamin B6 also regulates immune homeostasis in *Drosophila melanogaster*. An *In vivo* study may provide further insights into this issue.

A comparison of the mRNA levels of the AMPs induced upon *Sps1* knockdown with those induced upon immune stimulation would be interesting. Presumably, since SPS1 deficiency increases the expression of *PGRP-LC* and *Toll*, the increase in PGRP-LC and Toll will more efficiently recognize infection, and innate immunity will be more strongly activated. An *In vivo* study may also facilitate evaluation of the detailed relationship *Sps1* knockdown and immune stimulation. Therefore, to elucidate the function of SPS1 in more detail and to confirm the findings of our current *In vitro* study, an *In vivo* study using a fruit fly system may be helpful. As shown by Alsina *et al.*, SPS1-deficient fruit flies die in the late larval stage (Alsina *et al.*, 1998). Thus, it would be intriguing to examine the effects of SPS1 deficiency on the expression of *PGRP-LC* and *Toll*, upregulation of AMPs, response upon immune stimulation, and the mechanism how vitamin B6 regulates the innate immunity using *Sps1*-knockout *Drosophila melanogaster* larva.

Although our study has some pitfalls, we provide an important finding that SPS1 regulates the innate immune system of *Drosophila melanogaster* by controlling the expression of *PGRP-LC* and *Toll* without any other immune stimulation.

CHAPTER 3.

The ERK/MAPK signaling pathway inactivated by SPS1 deficiency induces cell dysfunction in *Drosophila* S2 cells.

1. INTRODUCTION

Selenium is an essential trace element necessary for many health benefits. For example, selenium has been implicated in immune system enhancement, cancer prevention, male reproduction, antiviral response, and embryonic development (Brigelius-Flohé & Sies, 2016; Vadim N. Gladyshev *et al.*, 2016; Na *et al.*, 2018). Selenium is the trace element required for the synthesis of selenocysteine (Sec), which is the 21st amino acid of the genetic code, and is incorporated into proteins during translation in response to the UGA codon (Lee *et al.*, 1989). Sec is produced by replacing the aminoacylated hydroxyl group of serine on $tRNA^{[Ser]Sec}$ with inorganic selenium (Hatfield & Gladyshev, 2002). During sec synthesis, selenophosphate behaves as a selenium donor. Selenophosphate synthase (SPS) is an enzyme that catalyzes the selenophosphate synthesis reaction by utilizing selenide and ATP as substrates (Glass *et al.*, 1993). In mammals, SPS was renamed SEPHS due to a similar gene called sucrose phosphate synthase (V. N. Gladyshev *et al.*, 2016). However, the SPS name remains in *Drosophila* because the sucrose phosphate synthase gene does not exist in this organism. In addition, only one type of SPS (SelD) is present in prokaryotes and Archaea. However, there are two types of SPSs (SPS1 and 2) in higher eukaryotes (Guimaraes *et al.*, 1996). Both isotypes have high amino acid sequence homology with each other, and both have ATP-binding and catalytic domains. One main difference between SPS1 and SPS2 is that SPS1 has an arginine residue in its catalytic domain corresponding to the selenocysteine location in SPS2

(Low *et al.*, 1995). Consequently, only SPS2 can synthesize selenophosphate, whereas SPS1 cannot (Xu, Carlson, Mix, *et al.*, 2007).

In *Drosophila*, SPS1 deficiency leads to embryonic lethality following aberrant imaginal disc morphology (Alsina *et al.*, 1998). In addition, SPS1 affects cell proliferation, innate immune system control, and glutamine level regulation in *Drosophila* S2 cells (Shim *et al.*, 2009). The primary target of SPS1 is vitamin B6 synthesis regulation, which affects several cellular processes (Lee *et al.*, 2011). Moreover, in *Sephs1* knockout mice, embryonic lethality occurs (Tobe *et al.*, 2016). This is due to changes in signaling pathways during gastrulation resulting from gradually increased oxidative stress levels (Bang *et al.*, 2021). A previous study showed the accumulation of superoxide in 2H11 cells generated by the *Sephs1* knockout leads to the inhibition of cell proliferation and angiogenic tube formation. (Jung *et al.*, 2021).

Extracellular signal-regulated kinase 1/2 (ERK), a member of the mitogen-activated protein kinase (MAPK) family, transfers cellular signals. MAPK cascades regulate cell growth, differentiation, and stress responses (Keshet & Seger, 2010; Plotnikov *et al.*, 2011; Sabio & Davis, 2014). Therefore, the MAPK cascades are a crucial signaling pathway. These cascades transduce extracellular signals through the sequential activation of MAPK kinase kinase kinase (MAP4K), MAPK kinase kinase (MAP3K), MAPK kinase (MAPKK), MAPK, and MAPK-activated protein kinases (MAPKAPK). The ERK cascades contain several kinases in the MAP3K layer, including renin–angiotensin system (Ras)/rapidly accelerated fibrosarcoma (Raf)/MAPK 1/2 at the MAPKK layer, ERK1/2 at the MAPK layer, and several

MAPKAPKs in the next layer (Eblen, 2018; Roskoski, 2012; Wortzel & Seger, 2011). The ERK cascades are regulated the pathways that control cellular processes, including cell growth and stress responses. These factors control the dynamic cellular localization of bispecific phosphatase (Patterson *et al.*, 2009), scaffolding of proteins (Kolch, 2005), signal duration and intensity (Marshall, 1995), and cascade components (Wainstein & Seger, 2016).

Previously, it was revealed that SPS1 affects cell growth, megamitochondria formation, innate immunity, and ROS formation in *Drosophila* through vitamin B6 synthesis regulation. Detailed studies on the phenotypes caused by SPS1 deficiency have not been elucidated. In this study, we found that SPS1 deficiency inactivates ERK/MAPK signaling, which results in SPS1 deficiency-associated phenotypes.

2. MATERIALS AND METHODS

2.1. Materials

Drosophila Schneider cell line 2 (S2) was purchased from Invitrogen. HyQ SFX-Insect medium was purchased from Hyclone, T3 Megascript kit was purchased from Ambion, PowerUpTM SYBRTM Green Master Mix was purchased from Thermo Fisher, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), and TRIzol reagent was purchased from Invitrogen, Moloney murine leukemia virus reverse transcriptase and nPfu forte DNA polymerase were purchased from Enzynomics. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and Insulin were purchased from Sigma, p-ERK and ERK antibody were purchased from Cell signaling and oligonucleotides were purchased from Cosmo Genetech.

2.2. double-stranded RNA preparation *In vitro*

To prepare dsRNA of *Sps1* and *ERK*, each gene was amplified with a primer pair with a T3 promoter sequence (5'-AATTAACCCTCACTAAAGGG-3') at the 5' end. *In vitro* transcription is performed using the T3 megascript kit according to the manufacturer's protocols and then completed by annealing.

Table 3.1. List of primers for qRT-PCR

Gene	Sequence (5'→3')	
	Forward	Reverse
RP49	CAGTCGGATCGATATGCTA	AATCTCCTTGCGCTTCTT
DptB	ATCCTGATCCCCGAGAGATT	TGAAGTGCCCTAAAACCTGAA
Mtk	CCACCGAGCTAAGATGCAA	TGTTAACGACATCAGCAGTGTG
SPS1	AGGGGATGTACTGGTGCTAA	TCTTATTGCCCTTCTCAACG
dmGlut	AGCGCGAGTATCTGGTCAAG	ACGGAAACGATCCACATCAT
GS1	GATCGCGTTTTGGACAAAGT	GACGTCCGTCCACGTCTAAT

2.3. S2 cell culture and RNA interference

SL2 cell culture and preparation of double-stranded RNAs were carried out as described previously (Shim *et al.*, 2009). Briefly, for RNA interference, 2.5×10^5 cells were plated on a 24 well plate containing 0.5 ml of HyQ SFX-Insect medium. Four micrograms of dsRNAs were added directly to the medium and incubated for 48 hrs and cells were split into appropriate culture dishes for further incubation and other experiments.

2.4. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was carried out as described (Shim *et al.*, 2009). Briefly, total RNA was isolated from the cells using the TRIzol reagent. cDNAs were synthesized from total RNAs with Moloney murine leukemia virus reverse transcriptase and oligo (dT) primers according to the manufacturer's protocols. RT-qPCR was carried out using an ABI 7300 real-time PCR system (Applied Biosystems) as follows. cDNAs were amplified using SYBR Green mix and specific primers for 40 cycles [initial incubation at 50°C for 2 min and then at 95°C for 10 min, and 40 cycles (95°C for 15 sec, 55°C for 1 min, and 72°C for 1 min)]. Output data was obtained as Ct values using Sequence Detection Software (SDS) version 1.3 (7300 System, Applied Biosystems) and the differential mRNA expressions of each gene between control and knockdown cell were calculated using the comparative Ct method (Schmittgen & Livak, 2008). rp49 mRNA, an internal control, was amplified along with the target genes, and the Ct value of rp49 was used to normalize the expression of target genes.

2.5. Western Blot Analysis

Western blot analysis was carried out as described previously (Kim *et al.*, 2013; Tobe *et al.*, 2016) with slight modifications. Briefly, cells were washed twice with PBS and harvested in ice-cold lysis buffer (PBS with 0.5% Triton X-100 and 0.1% PMSF cocktail). The protein concentrations of the resulting cell extracts were measured by Bradford dye-binding method and 25 μg of total protein from each sample were subjected to 10% SDS-polyacrylamide gel electrophoresis, then transferred to PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies against p-ERK (1:1000), ERK (1:1000), and alpha-tubulin (1:200000). Membranes were washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 and incubated with secondary antibodies for 30 min at room temperature. Immuno-labeling was detected using ECL reagent, and luminescence signal was detected using Chemi-Doc (Luminograph II, ATTO).

2.6. Measurement of ROS levels

The detection of intracellular ROS was carried out with CM-DCFDA as described previously (Shim *et al.*, 2009) with minor modifications. 2.5×10^5 cells were plated on a 24 well plate containing 0.5 ml of HyQ SFX-Insect medium. Four micrograms of dsRNAs were added directly to the medium and incubated. On day 5, the cells were incubated with 5 μM CM-DCFDA in HyQ SFX-Insect medium for 30 min,

washed twice with PBS, and then observed under fluorescence microscope (Nikon FL) at an excitation wavelength of 470 nm.

2.7. MTT Assay

The MTT assay was carried out as described previously (Kim *et al.*, 2003) with some modifications. Briefly, 24 h after dsRNAs treatment, 2×10^4 cells were seeded in a 96-well plate. Fifty microliters of 2 mg/ml MTT were added to each well and incubated for 4 h. After removing the supernatant, 200 μl of dimethyl sulfoxide were added to dissolve the formazan crystals that remained in the wells. Absorbance was determined using a microplate reader (Molecular Devices) at 540 nm. Wells without dsRNAs were used for control cell viability and wells without cells for blanking the spectrophotometer.

2.8. Statistics

Each experiment was performed in biological triplicate for statistical analysis. Statistical analyses were performed using an unpaired Student's t-test or one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1. SPS1 regulates ERK/MAPK signaling pathway activation

In a previous study, we reported that *Sps1* knockdown induced cell growth retardation in *Drosophila* S2 cells (Lee *et al.*, 2011). We hypothesized that SPS1 could regulate the ERK/MAPK signaling pathway, which is closely related to cell growth, because SPS1 also affects cell growth. SPS1 was knocked down in S2 cells and ERK phosphorylation levels were confirmed by western blot. As a result, ERK phosphorylation was reduced in SPS1-deficient cells (Figure 3.1). Since ERK phosphorylation increased when SPS1-deficient cells were treated with insulin, we investigated whether the *Sps1* knockdown-induced growth retardation recovered. MTT assay showed that cell growth decreased by *Sps1* knockdown was restored by insulin treatment (Figure 3.2). Therefore, these results indicate that SPS1 regulates the ERK/MAPK signaling pathway, and thus controls cell growth.

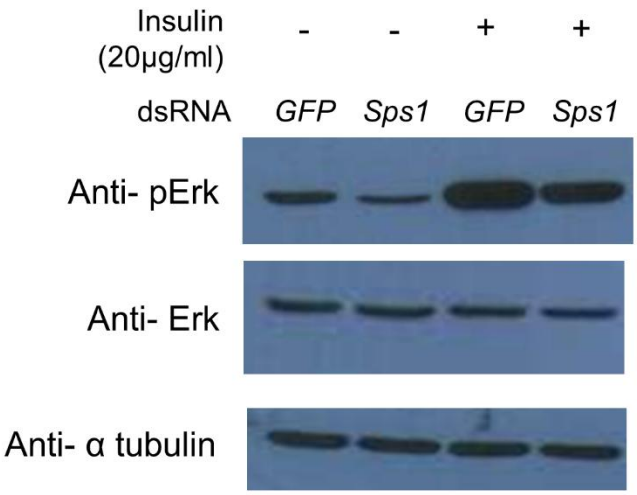

Figure 3.1. SPS1 deficiency reduces phosphorylation of ERK. Five days after adding the *Sps1* dsRNA and insulin was treated for 30mins, Western blot analyses to measureprotein the levels of p-ERK and ERK were measured Western blot analysis using α-tubulin for control.

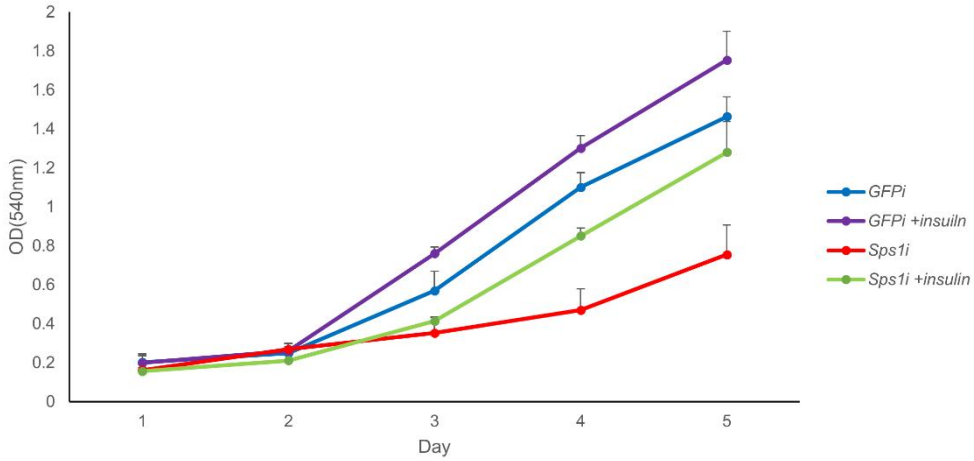

Figure 3.2. Cell proliferation inhibited by SPS1 deficiency is restored by insulin. Cell proliferation in GFP knockdown cells (blue line), GFP knockdown +insulin cells (purple line), *Sps1* knockdown cells (red line), and *Sps1* knockdown +insulin cells (green line) were measured by a MTT assay. Data shown are representative of at least three independent experiments.

3.2 Megamitochondria produced by SPS1 deficiency are regulated via the ERK/MAPK signaling pathway

In a previous study, we reported that SPS1 deficiency increases intracellular glutamine levels to form megamitochondria (Shim *et al.*, 2009). Surprisingly, we found that megamitochondria were not formed in experiments where growth recovery with insulin treatment was observed (Figure 3.3). Therefore, we investigated the mRNA levels of glutamine synthetase 1 (GS1) and dietary and metabolic glutamate transporter (dmGlut). However, the *Sps1* knockdown-induced increase in GS1 and dmGlut expression was not restored by insulin treatment (Figure 3.4), suggesting that there is an additional mechanism by which insulin blocks megamitochondria formation. These results indicate that megamitochondria generated by SPS1 deficiency are regulated through the ERK/MAPK signaling pathway, but the detailed mechanism requires further investigation.

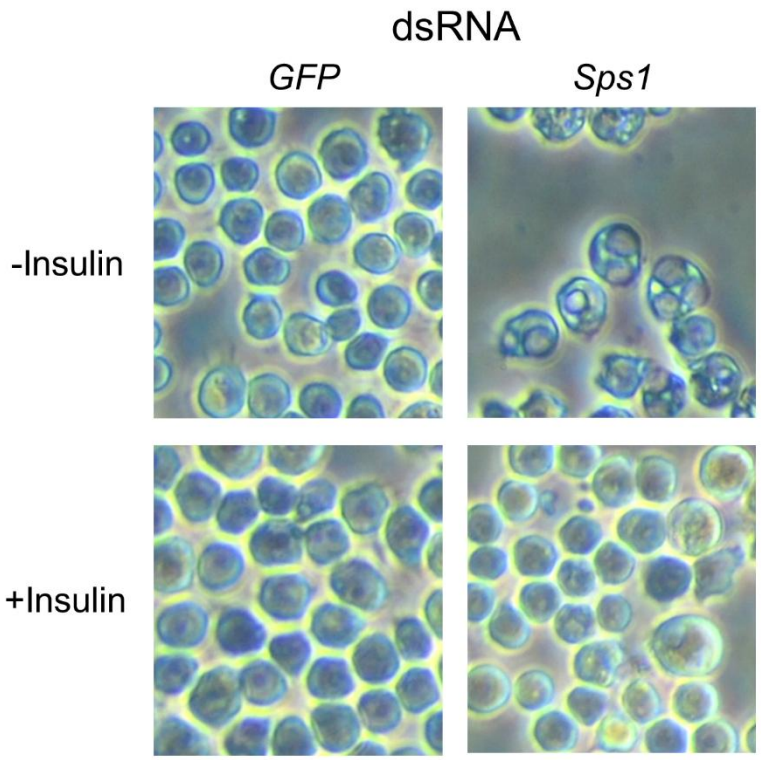

Figure 3.3. Megamitochondria produced by SPS1 deficiency are prevented by insulin. Two days after adding the *Sps1* dsRNA, insulin was treated. On day 4, the morphology of cells was examined under a light microscope.

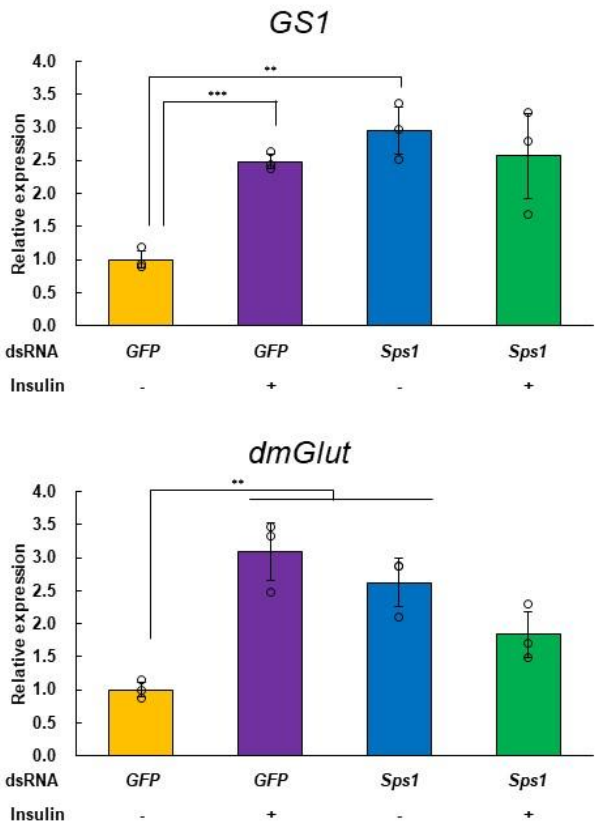

Figure 3.4. Insulin did not affect the expression of *GS1* and *dmGlut* increased by SPS1 deficiency. Five days after adding the *Sps1* dsRNA, the mRNA levels of each gene were measured via RT-qPCR using rp49 for normalization. ** indicate p-values < 0.01, based on unpaired Student's t-test.

3.3 SPS1 regulates ROS accumulation and innate immunity via the ERK/MAPK signaling pathway

Since it was confirmed by the above experiments that growth retardation and megamitochondria formation (representative SPS1 deficiency phenotypes) are regulated through the ERK/MAPK signaling pathway, we investigated whether ROS accumulation and innate immunity (other SPS1 deficiency phenotypes) are also regulated via this pathway. First, ROS accumulation induced by *Sps1* knockdown was restored by insulin treatment (Figure 3.5). Next, the SPS1-regulated innate immunity was investigated. SPS1 regulates the innate immune, immune deficiency (IMD), and Toll pathways through the expression of the membrane receptors peptidoglycan recognition protein LC (PGRP-LC) and Toll. Expression of *PGRP-LC*, *Toll*, and antimicrobial peptides (AMPs) (*Metchnikowin* [*Mtk*] and *Diptericin* B [*DptB*]) increased by *Sps1* knockdown were recovered by insulin treatment (Figure 3.6). These results indicate that ROS accumulation and innate immunity regulated by SPS1 are also controlled via the ERK/MAPK signaling pathway.

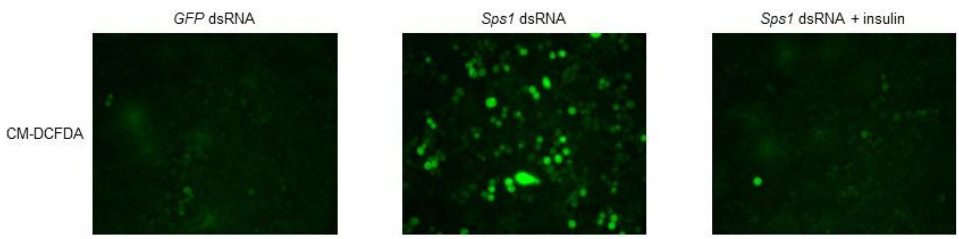

Figure 3.5. ROS production by SPS1 deficiency was recovered by insulin. Five days after adding the *Sps1* dsRNA, cells were stained with CM-H2DCFDA to detect intracellular ROS. The cells were photographed under a fluorescence microscope.

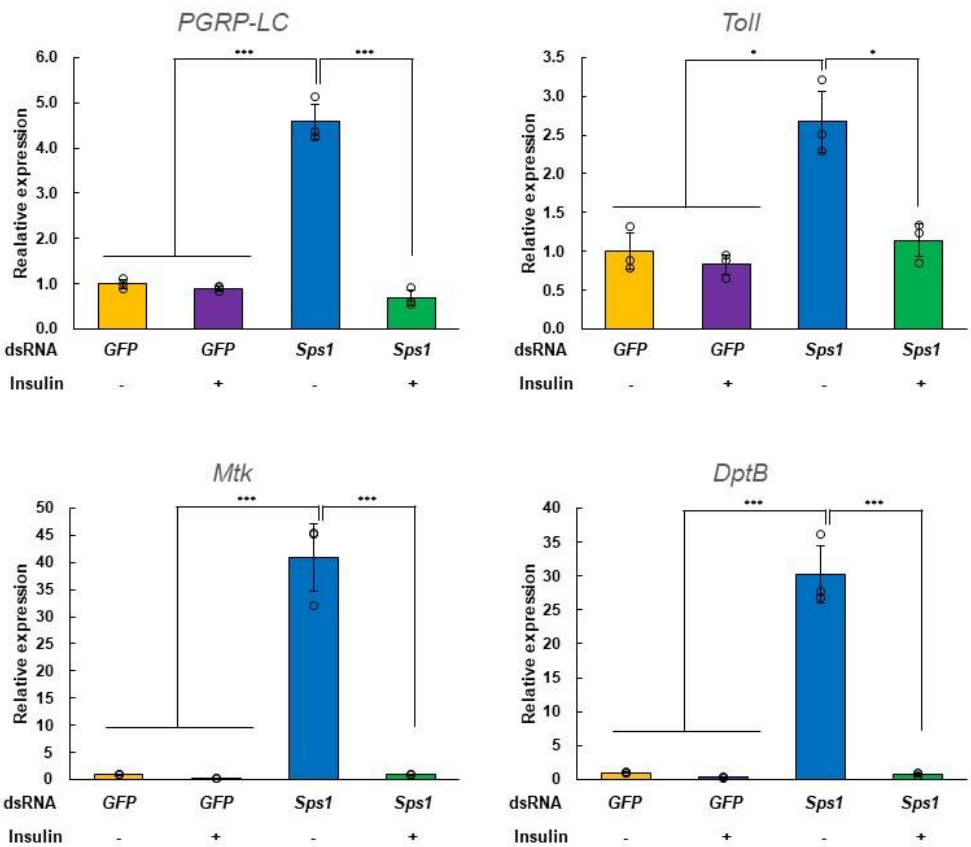

Figure 3.6. Innate immunity activated by SPS1 deficiency is restored by insulin. Five days after adding the *Sps1* dsRNA, the mRNA levels of each gene were measured via RT-qPCR using rp49 for normalization. *** indicate p-values < 0.001, based on one-way ANOVA with Tukey's multiple comparison test.

3.4 *ERK* knockdown induces growth retardation, megamitochondria formation, ROS accumulation, and innate immunity activation

To determine whether the ERK/MAPK signaling pathway regulates growth retardation, megamitochondria formation, ROS accumulation, and innate immunity, we investigated whether *ERK* knockdown results in the same phenotypes. First, *ERK* knockdown inhibited cell growth (Figure 3.7A). Second, *ERK* knockdown induced megamitochondria formation (Figure 3.7B). Third, *ERK* knockdown induced ROS accumulation (Figure 3.8A). Finally, *ERK* knockdown activated innate immunity by increasing the expression of *Mtk* (Figure 3.8B). These results confirmed that SPS1-regulated cell growth retardation, megamitochondria formation, ROS accumulation, and innate immunity are modulated via the ERK/MAPK signaling pathway.

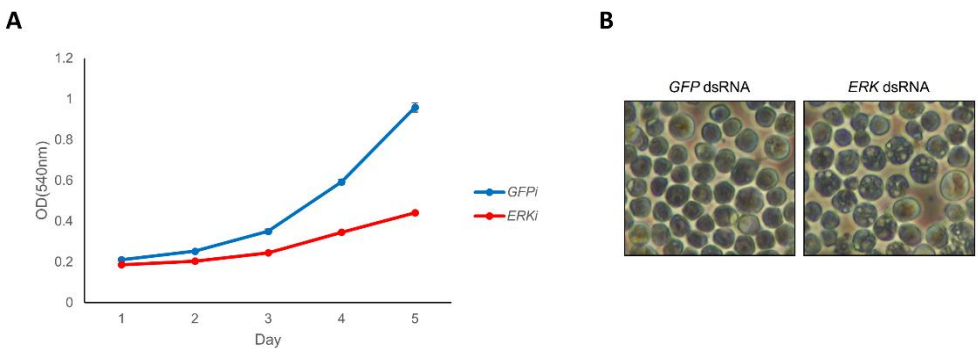

Figure 3.7. ERK deficiency induces growth retardation and megamitochondria formation. (A) Cell proliferation in *GFP* knockdown cells (blue line) and *ERK* knockdown cells (red line) were measured by a MTT assay. Data shown are representative of at least three independent experiments. (B) Five days after adding the *ERK* dsRNA, the morphology of cells was examined under a light microscope.

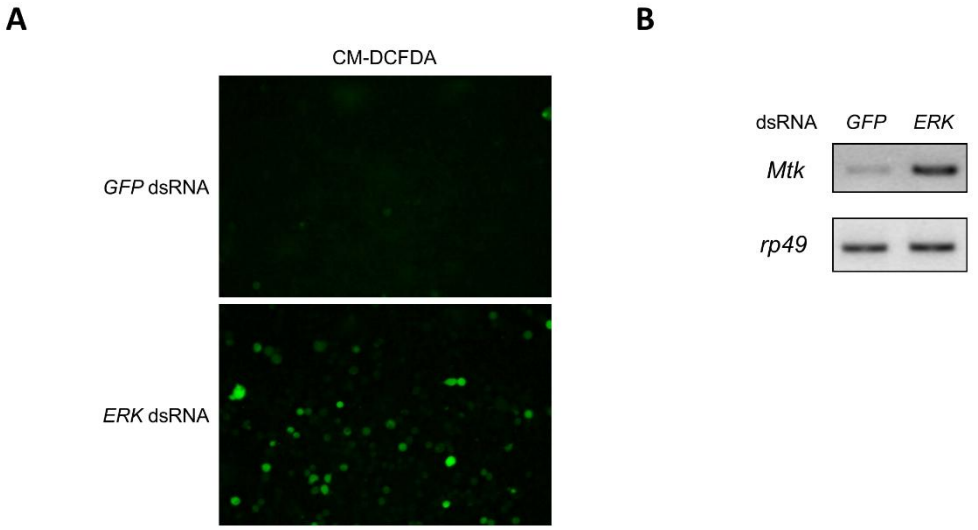

Figure 3.8. ERK deficiency produces ROS and increases the expression of AMP. (A) Five days after adding the *ERK* dsRNA, cells were stained with CM-H2DCFDA to detect intracellular ROS. The cells were photographed under a fluorescence microscope. (B) Five days after adding the *ERK* dsRNA, the mRNA levels of *Mtk* were measured via RT-PCR using rp49 for control.

3. Discussion

SPS1 is known to play an important role in growth regulation, ROS accumulation, and megamitochondria formation (Shim *et al.*, 2009). It has also been reported that SPS1 regulates megamitochondria formation and innate immunity by primarily targeting vitamin B6 synthesis (Lee *et al.*, 2011). However, the regulatory mechanism has not been studied in detail.

The ERK/MAPK signaling pathway plays a vital role in cell proliferation through its ability to regulate cell cycle entry (Brunet *et al.*, 1999; Chen *et al.*, 1992; Diehl *et al.*, 1998; Lenormand *et al.*, 1993), and the constitutive activation of the ERK/MAPK pathway has been identified as the driver of uncontrolled cancer cell growth (Roberts & Der, 2007). In this study, we focused on the ERK/MAPK signaling pathway's substantial effect on cell growth, and identified that it is regulated by SPS1. Also, when the inactivated ERK/MAPK signaling pathway was reactivated by insulin treatment, cell growth previously inhibited by *Sps1* knockdown was recovered.

We showed that the ERK/MAPK signaling pathway contributes to megamitochondria formation because the SPS1 deficiency-induced megamitochondria, which were accidentally observed through cell growth experiments, were not produced by insulin treatment. However, insulin treatment did not restore the expression of GS1 and dmGlut. This is probably because insulin acts beetween the upregulation of GS1 and dmGlut and the formation of megamitochondria. It would be interesting to study this detailed mechanism further.

We showed that ROS accumulation and innate immunity activation, which are other SPS1 deficiency phenotypes, are also regulated through the ERK/MAPK signaling pathway. In most recovery experiments, it was observed that SPS1-deficient cells treated with insulin showed greater recovery than control cells. This is because the ERK phosphorylation levels in SPS1-deficient cells treated with insulin were higher than in control cells.

In this study, increased ROS accumulation caused by SPS deficiency was restored with insulin, but another study found that insulin activates NADPH oxidase to generate ROS (Ma *et al.*, 2018). However, another study reported that ROS generated by glucose was recovered by insulin in the ischemia/reperfusion model (Chun *et al.*, 2015). Therefore, the relationship between insulin and ROS generation requires more detailed study, but ROS caused by SPS1 deficiency occurs through the ERK/MAPK signaling pathway, and it is thought that ROS was restored because the phosphorylation of ERK was increased by insulin.

It has been reported that the primary target of SPS1 deficiency modulates vitamin B6 levels (Lee *et al.*, 2011). As a result of examining the relationship between vitamin B6 synthesis and the ERK/MAPK signaling pathway, it was discovered that ERK activation decreased when vitamin B6 synthesis was inhibited by 4-deoxypyridoxine (4DPN) treatment (Figure 3.9A). Other phenotypes through 4DPN treatment were identified (Lee *et al.*, 2011) and ROS generation was investigated. As a result, inhibition of vitamin B6 synthesis resulted in ROS accumulation (Figure 3.9B). These results indicate that vitamin B6 synthesis regulates the ERK/MAPK signaling pathway.

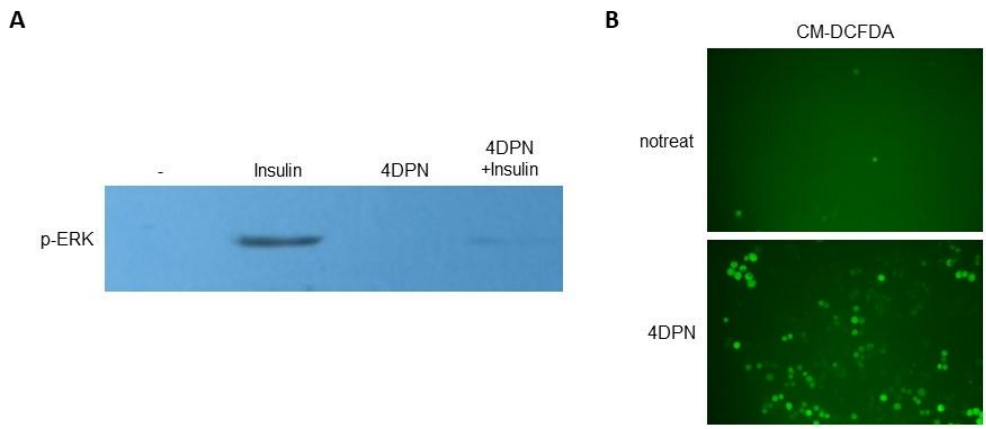

Figure 3.9. Vitamin B6 synthesis regulates the ERK/MAPK signaling pathway. (A) Five days after adding the Sps1 dsRNA and insulin was treated for 30mins, Western blot analyses to measureprotein the levels of p-ERK and ERK were measured Western blot analysis using α-tubulin for control. (B) Five days after adding the *ERK* dsRNA, cells were stained with CM-H2DCFDA to detect intracellular ROS. The cells were photographed under a fluorescence microscope.

Currently, there are no reports on how vitamin B6 affects the ERK/MAPK signaling pathway. By referring to evidence from the following two studies, we can speculate whether vitamin B6 regulates the ERK/MAPK signaling pathway. First, Qian *et al.* showed that vitamin B6 synthesis regulates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway (Qian *et al.*, 2017). Moreover, Winston *et al.* showed that JAK activates the ERK/MAPK signaling pathway (Winston & Hunter, 1996). This is probably because the vitamin B6 synthesis reduced by SPS1 deficiency affects the JAK/STAT signaling pathway, thereby also regulating the ERK/MAPK signaling pathway. Further studies are needed to deeply investigate the effects of vitamin B6 deficiency on the ERK/MAPK signaling pathway.

In this study, we only identified phosphorylation of ERK in the ERK/MAPK signaling pathway due to system limitations, but in future studies, it is necessary to investigate the regulation of other cascades *In vivo* or in other animal models. Although the detailed mechanism by which SPS1 regulates the ERK/MAPK signaling pathway is still unknown, we demonstrated that SPS1 regulates this pathway to affect cell growth, ROS accumulation, megamitochondria formation, and innate immunity.

CHAPTER 4.

DISCUSSION AND CONCLUSIONS

In contrast to SPS2, which can synthesize selenophosphate, SPS1 cannot synthesize it. However, SPS1 plays an essential role in the cell. Therefore, studies have actively investigated and revealed the function of SPS1. Although studies on the function of SPS1 have been conducted, studies on their mechanisms are lacking. The aim of these studies was to investigate the function and mechanism of *Drosophila* SPS1.

A study was conducted on SPS1 regulating innate immunity. To investigate the innate immune pathway regulated by SPS1, we measured the expression of readout AMPs in each pathway changed by *Sps1* knockdown. As a result, SPS1 deficiency increased the expression of readout AMPs in the IMD and Toll pathways. This result suggests that SPS1 regulates the IMD and Toll pathways. To study through which genes SPS1 regulates the IMD and Toll pathways, genes on each pathway and *Sps1* were co-knocked down. As a result, it was revealed that SPS1 regulates the immune pathway through PGRP-LC and Toll, the transmembrane receptors of each pathway. PGRP-LC and Toll mRNA levels increased in *Sps1* knockdown, and AMP expression increased as PGRP-LC and Toll overexpanded. This revealed that SPS1 regulates innate immunity through PGRP-LC and Toll expressions. Although further research is needed on how SPS1 regulates PGRP-LC and Toll expressions, this study revealed that SPS1 regulates innate immunity through the expression of transmembrane receptors PGRP-LC and Toll without any infection.

The next study investigated signaling regulated by SPS1. The function of SPS1 regulating cell proliferation was thought to be related to the ERK/MAPK signaling pathway. By reducing ERK activation through *Sps1* knockdown, we demonstrated that SPS1 regulates the ERK/MAPK signaling pathway. In addition, when insulin

treatment increased ERK activity reduced by *Sps1* knockdown, cell growth inhibition, megamitochondria formation, ROS accumulation, and activating innate immunity—which are cell dysfunctions caused by SPS1 deficiency—were recovered. In addition, when vitamin B6 synthesis was inhibited, ERK activity decreased, indicating that SPS1 regulates vitamin B6 synthesis and thus affects the ERK/MAPK signaling pathway. Further studies are needed to investigate how SPS1 regulates the synthesis of vitamin B6 and how the ERK/MAPK signaling pathway is affected by the regulated vitamin B6.

In fact, the detailed mechanism of how SPS1 regulates the synthesis of vitamin B6 has not been elucidated. However, through the report that SPS1 regulates the mRNA level of sugarlethal (Lee *et al.*, 2011), it was expected that SPS1 would regulate the transcription of pyridoxine phosphate oxidase (PNPO), and the expression of other PNPOs, CG31473 and CG15343, was also affected. Since the synthesis of vitamin B6 is predicted to be the primary target of SPS1, it is likely that SPS1 may play a role as a transcription factor regulating the expression of PNPO or interacting with the transcription factors.

These results revealed the sequence of signaling regulated by SPS1. The mechanism regulating innate immunity was revealed to be through PGRP-LC and Toll expressions. Additionally, SPS1 regulates the synthesis of vitamin B6 and, in doing so, affects the ERK/MAPK signaling pathway. The ERK/MAPK signaling pathway is regulated by vitamin B6 synthesis; this was found to regulate cell growth, megamitochondria formation, ROS production, and innate immunity. Although the mechanism by which each of the pathways regulated by SPS1 is not yet determined,

these studies provide data on the long journey of signal transduction regulated by SPS1 (Figure 4.1).

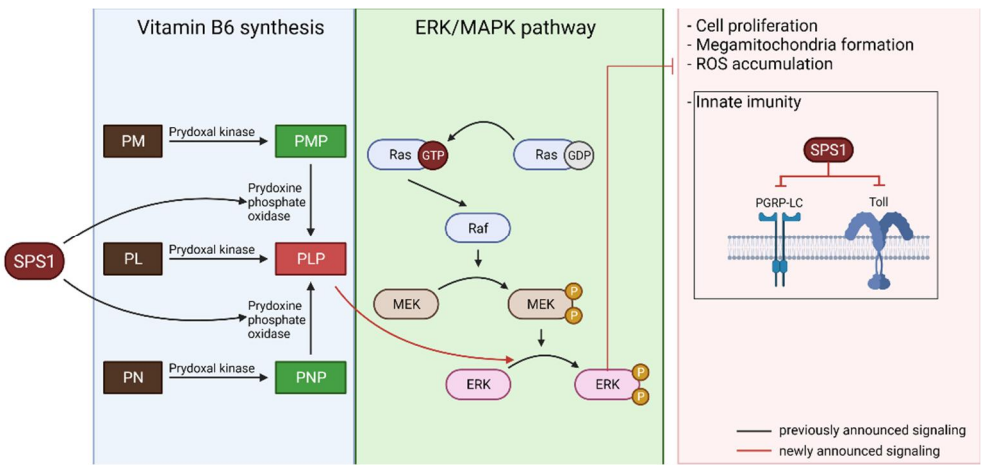

Figure 4.1. A schematic model for signal transduction regulated by SPS1.

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국문 초록

셀레노포스페이트 합성효소 1(Selenophosphate synthethase 1; SPS1)은 *Drosophila melanogaster* 의 세포 성장과 배아 발생에 필수적인 유전자이다. 이전 연구에서 SPS1 결핍이 *Drosophila* S2 세포에서 항균 펩타이드를 포함한 선천 면역 체계를 담당하는 유전자의 발현을 자극한다고 보고되었다. 그러나 그 근본적인 메커니즘은 밝혀지지 않았다. 따라서, 나는 S2 세포에서 SPS1 결핍으로 인한 AMP 의 발현을 조절하는 면역 경로를 조사했다. 항균 펩타이드 발현의 활성화를 통해 SPS1 이 IMD 와 Toll 경로 모두를 조절한다는 것을 밝혔다. 각 경로의 상위 단계 유전자와 SPS1 의 동시에 결손(knockdown)하는 실험을 통해 PGRP-LC 및 Toll 유전자가 각각 이러한 경로를 조절하기 위한 SPS1 에 의해 표적임을 보여주었다. 나는 또한 IMD 와 Toll 경로가 cross-talking 을 통해 항균 펩타이드의 발현을 조절한다는 것을 발견했다. PGRP-LC 및 Toll mRNA 의 수준은 SPS1 결손 시 상향 조절되었다. 각 단백질의 과발현 역시 항균 펩타이드를 상향 조절했다. 흥미롭게도 PGRP-LC 과발현은 Toll 과발현보다 항균 펩타이드를 더 많이 상향 조절했다. 이러한 데이터는 SPS1 이 PGRP-LC 및 Toll 발현 조절을 통해 *Drosophila melanogaster* 의 선천 면역 체계를 조절함을 강력하게 시사한다.

SPS1 결핍의 첫번째 표적은 비타민 B6 합성의 억제이다. 이후의 메커니즘을 조사하기 위해 나는 SPS1이 세포 성장을 조절한다는 사실에 주목했다. 따라서, SPS1 이 ERK/MAPK 신호전달에 영향을 미칠 것이라는 가설을 세웠고, 그 결과 SPS 결핍이 ERK 인산화를 감소시키는 것으로 밝혔다. 놀랍게도 ERK 를 활성화시키는 인슐린 처리를 통해 세포 성장 지연의 회복을 실험하는 중에 거대 미토콘드리아(megamitochondria)가 형성되지 않는 것이 관찰되었다. 인슐린 처리를 통해 SPS1 결핍의 다른 표현형도 회복되는지 조사한 결과 활성산소(ROS) 축적과 선천면역 활성화가 모두 회복되었다. 이러한 결과는 SPS1 이 ERK/MAPK 신호 전달 경로를 통해 세포 성장, 메가미토콘드리아 형성, ROS 및 선천 면역을 조절함을 시사한다.

핵심어: 셀레늄, 셀레노 포스페이트 합성효소 1, 선천 면역, 항균 펩타이드, PGRP-LC, Toll, ERK/MAPK 신호 전달 경로, 메가미토콘드리아, 활성 산소 종

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