A possible role for Vanin in the immunity and ageing of Drosophila melanogaster

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Abstract

The well-conserved Vanin family, which can be found in mice, humans and *Drosophila* Melanogaster, has already proven to play an important role in mice immunity, oxidative injury and inflammation. Vanin encodes an epithelial ectoenzyme with pantetheinase activity, which can generate cysteamine through the metabolism of pantothenic acid. Cysteamine is known to have an inhibitory effect on the production of reduced glutahione (GSH), a major cellular anti-oxidant. Vanin also inhibits the anti-inflammatory peroxisome proliferator-activated receptor-γ (PPARγ), making Vanin a pro-inflammatory protein. Although, the precise role of Vanin is not known yet, Vanin might be a pro-oxidant and play a role in immunity. This report focuses on the role of Vanin in *Drosophila Melanogaster*, more specifically on *Drosophila's* immunity and ageing.

Based on observations made by downregulating Vanin *in vivo* and overexpressing Vanin *in vitro*, and the effect it had on the immune response and ageing process of *Drosophila*, we report here that Vanin does play a role in *Drosophila* immunity and possibly in ageing.

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Introduction

Drosophila Melanogaster has proven to be a good model for human biology and has already been used to study a number of human diseases, including neurological disorders[1], cancer[2] and cardiovascular diseases[3]. Despite the obvious morphological differences between fruit flies and humans, they do share many molecular, cellular, and behavioral similarities, due to well-conserved genes[4].
Drosophila only possesses innate immunity, and no adaptive immune system. The two major immune pathways in the fruit fly are the Toll and the Imd pathway (figure 1). The Toll pathway mainly targets Fungi and gram-positive infections, through the activation of NFκB proteins, like Dorsal, which mediates the transcription of the antimicrobial peptide (AMP) *drocomycin*. Whereas the Imd pathway mainly targets Gram-negative bacterial infections through Relish activation, which mediates the transcription of the AMP, *diptericin*. These pathways are very relevant to humane biology because of the great homology in several proteins and enzymes, like the NFκB proteins, between the two species[5].

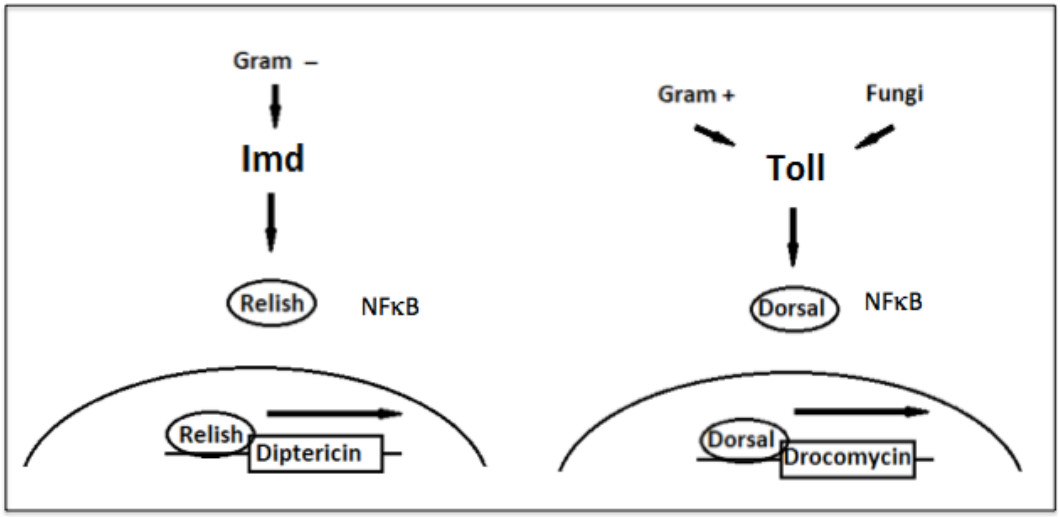


Figure 1. Schematic representation of the Imd and Toll signaling pathways in *Drosophila*. Upon infection the Imd and Toll signaling pathways activate humoral antimicrobial defenses in *Drosophila melanogaster*. Gram-negative bacteria activate the Imd signaling pathway, leading to the translocation of the NFκB Relish, which enters the nucleus and activates many genes including the anti-microbial peptide *diptericin*. Toll signaling is (mainly) activated by fungal and Gram-positive invaders, causing the translocation of the NFκB factor Dorsal to the nucleus, where it mediates the transcription of the anti-microbial peptide *drosomycin* and other genes.

Another well-conserved family of genes is the Vanin genes. Vanin stands for vascular non-inflammatory molecule, and was first identified to play a role in thymocyte homing in mice, in 1996[6]. Vanin encodes an epithelial ectoenzyme with pantetheinase activity, of which there are two in mice (Vanin-1 and Vanin-3), three in humans (VNN1, VNN2 and VNN3) and three in *Drosophila Melanogaster* (CG32750, CG32751 and CG32754)[7, 8, 9]. Vanin-1, as VNN2 and CG32750 contain a C-terminal GPI anchor. Vanin-1 is the isoform involved in thymus homing and cell adhesion in mice[6].
Vanin can generate cysteamine through the metabolism of pantothenic acid, better known as vitamin B5. The metabolite cysteamine seems to inhibit gamma-glutamylcysteine synthetase (γGCS)[10, 11]. γGCS is the rate limiting enzyme for synthesis of reduced glutathione (GSH), a major cellular anti-oxidant. This causes the glutathione store as well as the GSH/GSSG ratio to decrease, which subsequently intensifies the

oxidative stress. Considering "the Oxidative stress theory"[12, 13, 14], Vanin could play a role in the ageing process of *Drosophila.* It has already been shown that Vanin-1 -/- mice exhibit resistance to oxidative injury, together with reduced apoptosis and inflammation, suggesting that Vanin/Pantetheinase inhibitors could possibly be therapies for patients that have been treated with irradiation and or pro-oxidant inducers[15]. Vanin is also known as a pro-inflammatory protein by inhibiting peroxisome proliferator-activated receptor-γ (PPARγ) activity, which is an anti-inflammatory protein[16, 17] (figure 2).

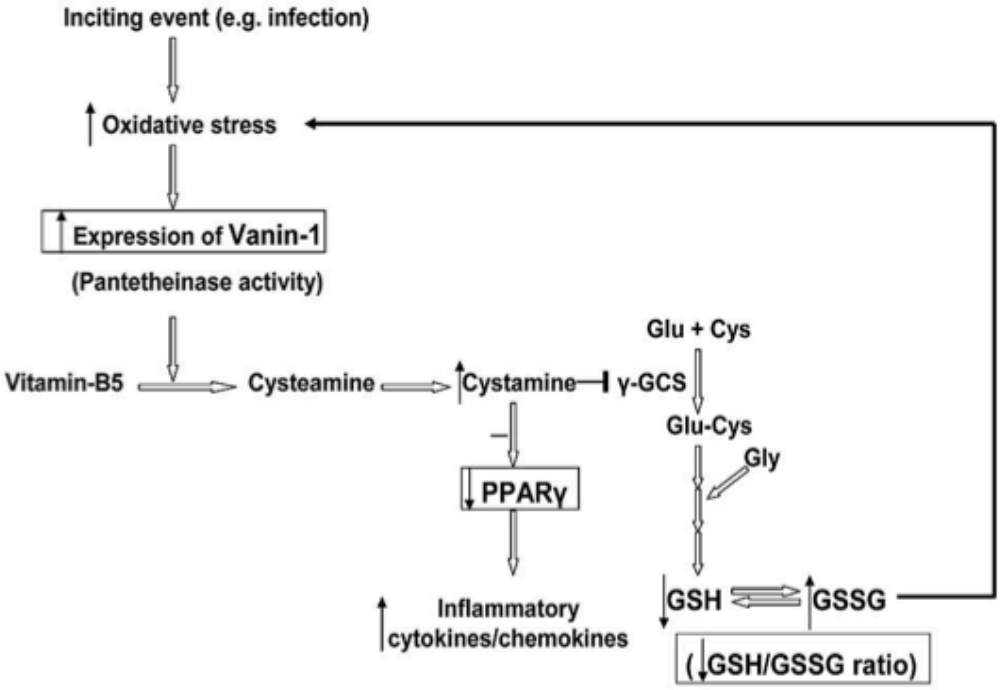


Figure 2. Schematic representation of the vanin-1 pathway. An inciting event can expose cells to oxidative stress, causing an upregulation of Vanin-1. Together with pantothenic acid (vitamin-B5), Vanin produces cysteamine. Cysteamine is converted to cystamine, which inhibits gamma-glutamylcysteine synthetase (γGCS), thereby inhibiting the synthesis of reduced glutathione (GSH). The anti-inflammatory checkpoint *PPAR*γ is also inhibited by cystamine, thereby lifting the inhibition on the production of inflammatory cytokines and chemokines and as a result, more inflammatory cytokines and chemokines are produced. (Zhang B. Et al. (2011) Blood 117(17): 4569-4597)[16]

In this report the role of the Vanin genes in the immunity of Drosophila melanogaster has been investigated, with tools as the *Drosophila* Schneider 2 (S2) cell line, Drosophila melanogaster (fruit fly) lines and the Gal4/Upstream activation system (UAS). The S2 cell line is one of the most commonly used *Drosophila Melanogaster* cell lines. S2 cells were derived from late stage (20-24 hours old) *Drosophila melanogaster* embryos, and have macrophage like properties[18]. The Gal4/UAS system is a commonly used two-component expression system in the fly where the tissue-specific expression of the yeast GAL4 transcription factor activates the expression of the transgene, or in this case the RNAi construct, via an upstream activator sequence[19]. The RNAi constructs have been used to knock down Vanin in fruit flies.

The aim of the project was to investigate the consequences of Vanin knockdown in *Drosophila Melanogaster*, focusing on immunity and ageing. Data from multiple experiments indicate that Vanin indeed plays a role in *Drosophila* immunity and possibly ageing.

Materials and methods

Fly stocks and Crosses

Homozygous RNAi lines were generated, combining two different RNAi constructs, with an RNAi construct targeting CG32750 on chromosome 2 (VDRC, Vienna, Austria, fly stock 52403 or 107171) and one targeting CG32754 on chromosome 3 (fly stock 50591). Fly line 50591 targets both CG32754 and CG32751. Therefore these homozygous RNAi lines downregulate all three Vanin genes and were given the name *Triple RNAi lines* (TR lines). All TR lines were made homozygous by appropriate crosses to balancer stocks in a W1118- background.

The GAL4/UAS system was used to drive expression of UAS transgenes in *Drosophila* lines in all tissue cells (Bloomington *Drosophila* Stock Center, DA-Gal4, 8641), Gut tissue (Bloomington, 25041-Gal4; *diptericin*-GFP) or hemocytes (Bloomington, Hml-Gal4 Delta, 30140). Females from these lines were collected and crossed against homozygous UAS-Vanin RNAi fly lines. All Vanin RNAi lines came from Vienna *Drosophila* RNAi Centre. The F1 generations were kept at 29 or 25°C. (See supplementary data 1 for all fly stock and lines)

Collecting larvae

Collecting larvae was accomplished by pouring a 20% sucrose (w/v) (Calbiochem, cat# 5737) solution into the food vials, where the larvae were growing. The larvae floated up, and were therefore easy to collect by a small spoon.

Infection studies

Having collected the Vanin RNAi larvae and control (W1118-) larvae, crossed with either daGal4:*dipt*GFP or 25041Gal4;*dipt*GFP, the larvae were then mixed with 400 mg of mashed banana and 200 µl of an overnight gram-negative Ecc15 bacteria culture. The overnight Ecc15 culture grew at 29 °C, at 200 rounds per minute (rpm) and was then centrifuged for 30 minutes at 3000 rpm. The bulk of supernatant was discarded and the pellet was resuspended in a minimum amount of supernatant, to obtain a highly concentrated bacteria culture. The larvae were left to eat the banana and bacteria mixture by pushing the larvae back, for 30-40 minutes. The larvae and the mixture were then put back into food vials, not containing any yeast and were left at 29 °C for either 6 or 16 hours, depending on future experiments, described in the next paragraph. The control group was fed only mashed banana.

After 6 hours of incubation, 20 larvae were collected for RNA isolation and cDNA synthesis purposes. Larvae collected after 16 hours of incubation were put into a glass dish, filled with demi water. To restrict their movement, the glass dish with the larvae was put on ice. A Leica DFC450 C microscope was used to visualize the immune induction/GFP-intensity in the larvae.

Bacteria intake was determined by serial dilutions of larva homogenates onto Luria Broth (LB) plates, consisting of 1.5% (w/v) Agar (Bacto, cat#214010), 0.5% (w/v) Yeast extract (Bacto, cat#212750) and 1% (w/v) Tryptone (Bacto, cat#211705), dissolved in demi water. After feeding the larvae for 30-40 minutes, they were collected and rinsed with PBS, then were briefly put into 70% (v/v) ethanol for external sterilization and were then put into a 1.5 ml microcentrifuge tube containing LB medium (0.5% Yeast

extract and 1% Tryptone). The larvae were homogenized by a pellet pestle motor (Kontes), spread onto LB plates and were left overnight, at 29 °C.

RNA isolation and cDNA synthesis

Collected larvae were collected in microcentrifuge tubes, on ice. Then were immediately frozen at -80 °C. After having collected and frozen the larvae, lysis buffer was added and the larvae were homogenized using a pellet pestle motor. RNA from the larvae was isolated using an Absolutely RNA miniprep kit (Agilent Technologies, cat# 400800). Next, cDNA was synthesized from the RNA samples by using M-MLV RT, from Invitrogen (Invitrogen, cat# 28025-013).

A polymerase chain reaction (PCR) was performed for the detection of the RP49 gene to verify the presence and quality of the cDNA. The primers were 5'-ATGACCATCCGCCAGCATA-3'as the forward primer and 5'-TTACTCGTTCTCTTGAGAAC-3' as the reverse primer. The PCR was performed with platinum PCR supermix (Invitrogen, cat#11306-016), to which 1 µM primers was added and 10% cDNA template. The PCR mixtures, were placed in a MJ Research Minicycler and a PCR program was initiated; consisting of a denaturation step of 95 °C for 30 seconds, annealing step of 55 °C for 30 seconds and an extension step of 72 °C for 30 seconds. This cycle was repeated 40 times.

Gel electrophoreses

All the PCR samples were mixed with 5x DNA loading buffer in a 4:1 ratio. After mixing the PCR samples with the dye, they were loaded on a 0.8% Agarose gel (Invitrogen, cat#16500-500) containing 0,5 µg/ml ethidium bromide, together with a 1 kb DNA ladder (NEB, cat#N0468L).

Cloning

Vanin (CG32750 and CG32754) constructs were generated by performing a PCR on cDNA collected from second and third-instar larvae. cDNA was synthesized as described in the previous section. The primers used for creating the CG32750 constructs were 5'-GGGGTACCATGGCTGTATTCCTCCGTCGA-3' as the forward primer and 5'-GCTCTAGAGCCTCCGAAATAGATGCATAAT-3' as the reverse primer. The forward primer has been designed with a kpnI site at the 5' end and the reverse primer has an XbaI site at the 5' end. The CG32754 construct was created by using 5'-TTGGTGATATCACCATGTCGAATACCTGGTGGTGG-3' as the forward primer, with an EcoRV site at the 5' end, and 5'-GCTCTAGAGCTGAACTCATTGTCCTGGCAAT-3' as the reverse primer with an XbaI site at the 5' end. PCR conditions were 1x Thermo buffer (NEB, cat#M0254L), 200 µM deoxynucleoside triphosphates (dNTPs) (Invitrogen, cat#18427-013), 1.5 mM Magnesium sulfate (MgSO4)(NEB, cat#M0254L), 0,2 mg/ml BSA (NEB, cat#B90015), 3% DMSO (NEB, cat#M05315), 1 u Vent DNA polymerase (NEB, cat# M0254L), 1 µM primers (Biolegio), 10% cDNA template and nuclease free water. The PCR mixtures were placed in a MJ Research Minicycler and a PCR program was initiated consisting of a denaturation step of 95 °C for 30 seconds, an annealing step of 55 °C for 35 seconds and an extension step of 72 °C for 110 seconds. This cycle was repeated 35 times, before mixing the samples with a 5x loading dye and loading them into a 0.8% agarose gel for gel electrophoresis. The amplified DNA fragments were then purified out of the gel with a High Pure PCR Cleanup micro kit (Roche, cat#04983912001).

The obtained constructs were then ligated with a blunt zero vector. The plasmids were then transformed into chemically competent DH5α Escherichia coli (E.coli) cells, following the protocol of the Zero blunt PCR cloning kit (Invitrogen, Cat# K2700-20). After up-scaling the amount of bacteria, the plasmids were purified out of the bacteria culture, and the Vanin inserts were then cut out of the Zero blunt plasmids through restriction digestion. KpnI (NEB, cat#R01425) and XbaI (Invitrogen, cat#15226012) were used to cut out CG32750 out of the zero blunt vector and EcoRV (Invitrogen, cat#15425010) and XbaI were used for CG32754. The digested products were run on a 0.8% agarose gel. After separation, the Vanin constructs were purified out of the agarose gel and were then ligated into a pAc5V vector (Invitrogen, cat# V4110-20), with a built-in C-terminal hemagglutinin (HA) tag. Ligation into the pAc5V vector was done via the same principle, with T4 ligase (Promega, cat#M1801) and buffer from Promega (Promega, cat#c1263). Prior to ligation, the pAc5V vector had been digested by the same two restriction enzymes that had been used to cut the Vanin construct.

Restriction digests

1 µg of plasmid was mixed with the appropriate restriction buffers to a final concentration of 1x, 10µg/µl BSA and nuclease free water and was then left to digest at 37 °C for 2 hours. The digested products were then separated on a 0.8% agarose gel by electrophoreses and visualized with ethidium bromide. When needed, the fragments were purified out of the 0.8% agarose gel by a High Pure PCR Cleanup micro kit.

Transformation

Chemically made competent DH5α E.coli cells were defrosted on ice and pipetted into 1.5 ml microcentrifuge tubes. Plasmid was added to the competent E.coli cells at a 1:30 ratio. The mixtures were then mixed by gently tapping the microcentrifuge tubes, followed by a heat shock of 1 minute at 42 °C. The E.coli/plasmid mixture was put on ice immediately, for 2 minutes. After the 2 minute incubation, sterile Luria Broth medium was added to the E.coli/plasmid mixture in a 3:1 ratio, where-after the samples were put at 37 °C for 30-60 minutes. After the incubation, the samples were spun for 2 min at 8000 rpm. Most of the supernatant was discarded, leaving approximately ¼. The pellet was then gently resuspended in the remaining supernatant and then plated onto LB agar plates containing 100 µg/ml kanamycin or ampicillin, depending on which vector was used. The plates were then put into a 37°C stove for overnight incubation.

Plasmid Purification

After having left the plates to grow overnight at 37 °C, a single colony was picked and put into a 15ml sterile tube containing LB media and 100 µg/ml antibiotic. The tubes were placed in a 37 °C shaker and left to grow for approximately 16 hours at 200 rpm. The plasmids were then purified out of the E.coli culture by a GeneJet plasmid miniprep kit (Fermemtas, cat#K0508). 200 µl of the culture was transferred to flasks containing 100 ml of LB media and 100 mg/ml antibiotic. The flaks were left to grow in a shaker for approximately 16 hours at 37 °C and 200 rpm. The plasmids were purified from the E.coli culture using a Pure Yield Plasmid midiprep system (Promega, cat#A2492). The plasmid concentration was measured by UV spectrophotometry at 260 nm, using a nanophotometer (Implen). The constructs were verified by sequencing.

Tissue culture
Drosophila Schneider 2 (S2) cells were used for *in vitro* studies. The cells were grown in Schneiders's *Drosophila* Medium (Gibco, cat# 11720034) containing 10% Fetal Bovine Serum (FBS) (Gibco, cat#10437028) and 100 µg/ml Penicillin/streptomycin (Invitrogen, cat#10378016), at their normal growth conditions (37°C and 5% CO2).

Transfection
S2 cells were transfected using Effectene transfection reagent (Qiagen, cat#301425). Cells from an exponentially growing cell culture were seeded into a 6 wells plate (2 ml per well) and were left at room temperature for 30 minutes. Meanwhile the transfection mixture was prepared; 0,6 µg plasmid DNA was added to 100 µl EC buffer, then 3,2 µl of enhancer was added and mixed by vortexing for 1 second. The mixture was left to incubate at room temperature for 4 minutes. Next, 10 µl of effectene was added to the mixture and was then mixed by gently pipetting up and down. The mixture was left to incubate at room temperature for 10 minutes. After the 10-minute incubation, 600 µl of pre-warmed growth medium was added. The mixture was again mixed by pipetting up and down and then added to the S2 cells in a dropwise manner. The 6 wells plate was then gently swirled to ensure uniform distribution of the transfection complexes and put into a 25 °C incubator for at least 48 hours, before performing experiments on them.

Immunofluorescence
After transfecting S2 cells with the HA-tagged Vanin constructs, slides were coated with concanavalin A, to which the cells were left to adhere on for approximately 30 minutes. The medium was then removed and the remaining cells were fixed with a 4 % formaldehyde solution in PBS (37% formaldehyde, Sigma Aldrich, cat#F8775) for 10 minutes. Next, the fixed cells were blocked with a 5% BSA solution in PBS-tween (PBST) for 1 hour. After removing the blocking solution, a 1:3000 anti-HA antibody solution (Invitrogen, cat#32-6700) was added to the cells and was left to incubate overnight at 4 °C. After the overnight incubation, the slides were washed 4x for 5 minutes in PBST and a last time with PBS. Next, a 1:1000 polyclonal rhodamine-conjugated anti-mouse antibody and 1:500 Hoechst staining solution (nuclear staining) was added to the slides, and left to incubate for 1 hour. The slides were washed 4x for 5 minutes with PBST and a final time with PBS. Finally the slides were mounted and photographed with a Leica DM6000 B microscope.

Luciferase assays
S2 cells were transfected with 400 ng Vanin construct, 200 ng Dorsal- or Relish-specific luciferase reporter construct[20] and 100 ng Renilla (Promega, cat# E1500). The luciferase reporter construct is based on the pBL3-Basic vector (Promega). Cells were harvested 2 days later, frozen at -80°C and processed. Samples were prepared according to the Luciferase assays promega protocol, and measurements were performed using a Berthold single tube luminometer detection systems (Sirius).

Hemocyte recruitment assays
Third-instar larvae expressing UAS-GFP, crossed with Hml-GAL4.Delta (Bloomington *Drosophila* stock center, 30140), were immobilized on double-sided tape, dorsal side up. Larvae were wounded in the middle of the A3/A4 segment with a pulled injection needle. The moment of injury was considered time point zero. Hemocyte recruitment

and segregation was tracked over a course of 2 hours, with capturing images every 10 minutes using a Leica DFC450 C microscope.

Lifespans

Fly stocks were maintained on sugar-agar medium. Flies with the desired phenotype were given time to mate and lay eggs over a period of two days. The flies were then removed and the vials containing the embryos were put in an incubator of either 25°C or 29°C. The F1 flies were collected 0 to 2 days post-eclosion, and this was considered time point zero. The adults were transferred to fresh vials every 2 to 3 days, and the number of dead flies was tracked. The flies remained at 25°C or 29°C, except when counting the flies, which was done at room temperature.

Western blot analysis

48 hours after transfecting S2 cells with CG32750 and CG32754 constructs, the cells were centrifuged 5 min at 1300 rpm. The supernatant was collected into a separate microcentrifuge tube and the cell pellet was resuspended in cold PBS, and centrifuged again for 5 min at 1300 rpm. PBS was removed and 1X sample buffer (Biorad, cat. no. 161-0791) was added to the cell pellet and supernatant. The samples were boiled at 95ºC for approximately 5 minutes before loading onto a 10% SDS-PAGE gel. Gels were run with 1x running buffer (10x Tris/Glycine/SDS) at 200 Volts (V) for approximately 1 hour. After running, the gels were soaked in 1x transfer buffer with 20% (v/v) methanol (TB) for approximately 15 min. Hybond PVDF membrane was activated by soaking in 100% methanol for a few seconds and then transferred to 1X TB buffer. 3mm Whatmann filter paper and the spongepads of the transfer apparatus were also equilibrated in 1X TB buffer. The spongepads, filter papers, gel and membrane were arranged in the right order and inserted into a Biorad wet transfer tank. The transfer was performed with 1X TB at 100 V for approximately 1 hour.

After transferring the protein from the gel to the membrane, the membrane was washed in MiliQ water and incubated for one hour shaking at room temperature with blocking solution consisting of 5% (w/v) skimmed dry milk in PBST. This was followed by overnight incubation at 4°C with primary anti-HA antibody diluted 500 times in blocking solution. Then the membrane was washed 4 times for 5 min in PBST, followed by a 1-hour incubation at room temperature with secondary anti-mouse antibody, diluted 5000 times in blocking solution. After incubation with the secondary antibody the membrane was washed again 4 times for 5 minutes in PBST.

The secondary antibody was detected by enhanced chemiluminescence (ECL). Solution A (luminol enhancer) was mixed with solution B (peroxide solution) in a 1:1 ratio. The ECL solution was then added to the membrane at 100µl/cm^2 and was left to incubate for 5 minutes at room temperature. Excessive ECL solution was drained and the membrane was put into transparent sheets ready for detection by film.

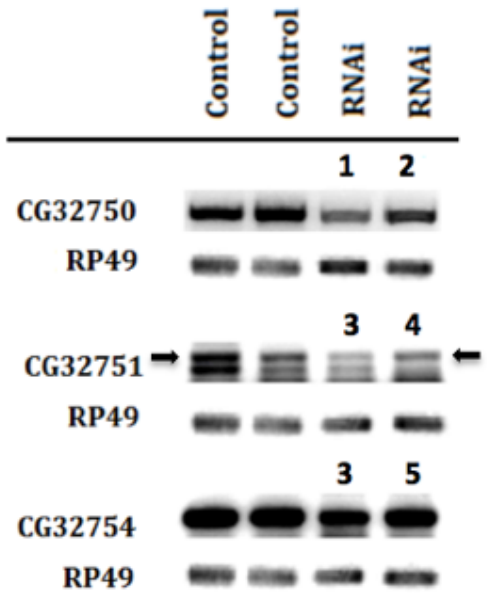
Results

Vanin knockdown verification in Vanin RNAi larvae

After crossing Vanin RNAi fly lines and the Vanin Triple RNAi (TR) fly lines against Da-Gal4 flies, cDNA was synthesized from RNA isolated out of third-instar larvae. Vanin expression was determined by PCR and visualized by gel electrophoreses. Figure 3A shows the difference in expression of the Vanin genes between different RNAi larvae and control larvae. The figure shows that RNAi lines 1 and 2 (targeting CG32750) do seem to have a lowered CG32750 expression compared to the control group. As do RNAi lines 3 and 4 (targeting CG32751), and to a lesser extent RNAi line 3 (targeting CG32754). However, there is no clear difference in expression of CG32754 between RNAi line 5 and the control. The figure also shows that RNAi line 3, which is specific for CG32754, also targets CG32751. Figure 3B show the total Vanin expression in TR larvae, compared to the control group. TR larvae are supposed to down regulate all three Vanin genes. Indeed CG32750 and CG32751 are lower in expression in TR lines 1, 2 and 3, compared to the control larvae. However, CG32754 knockdown isn't convincing in all three lines. Vanin expression of TR line 4 is shown separately, at the right hand side of figure 3B. The total amount of RNA isolated from TR 4 larvae was considerably lower then the other three lines, due to complications during the RNA isolation experiment. Therefore no control is shown for TR 4 in the figure. However, the control sample was diluted a 100 fold to theoretically match the cDNA levels of the TR 4 sample. Nevertheless, TR 4 does seems to have lowered Vanin expression of at least CG32751 and CG32750, although the differences in expression are not as evident as the other TR lines.

The quality of the synthesized cDNA was verified through the amplification of the household gene RP49. This also acted as a loading control for the amount RNA loaded into the cDNA synthesis, not including TR 4, and is shown under each band. Data shown in this figure indicate that at least two of the three isoforms of Vanin are being downregulated in third instar TR larvae. However, the results with CG32754 could be due to the oversaturation of the PCR. Therefore data with CG32754 are inconclusive.

A



B

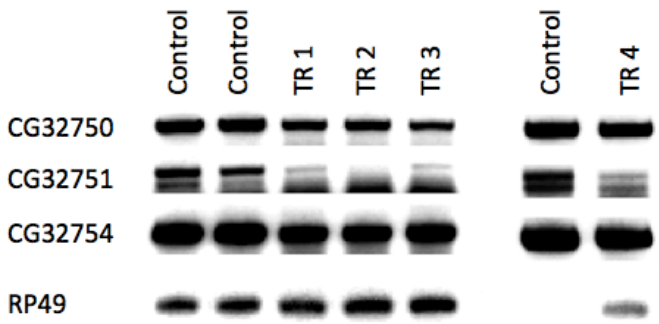


Figure 3. Vanin knockdown in *Drosophila Melanogaster* larvae, verified by RT PCR.
A) Vanin expression of Da-Gal4 larvae (control), compared to Vanin RNAi lines. Top: CG32750 double control, RNAi line 1 and 2. Middle: CG32751 double control, RNAi lines 3 and 4. Bottom: CG32754 double control, RNAi line 3 and 5. **B)** Total Vanin expression Da-Gal4 larvae (control) and TR lines. The first row shows expression CG32750 in control lines versus the TR lines. The second row shows CG32751 expression in control versus TR lines and third the row shows CG32754 expression in the controls versus TR lines. RP49 is the loading control for each sample and is shown under each band in A) and under each row in B) except the control for the comparison to TR 4.

Altered Immune response in Vanin RNAi larvae and Triple RNAi larvae

Larvae expressing the CG32750 RNAi constructs (RNAi line 1 with DA-Gal4) and containing a *diptericin*-GFP (Da-*dipt*-GFP) construct were fed a mixture of mashed banana and Ecc15 bacteria culture. Ecc15 is a gram-negative, non-pathogenic bacterium that activates the Imd pathway in flies, which leads to the transcription of the AMP *diptericin* (figure 1). In these lines, the GFP gene was put under control of the *diptericin* promotor. This way the Relish-specific immune induction can be visualized, using the GFP signal intensity as the read out for immune induction. Figure 4A shows the immune response of larvae expressing the CG32750 RNAi constructs, in comparison with the control larvae. Vanin has been described in literature as a pro-inflammatory protein[16, 17]. Therefore, one would expect that suppressing Vanin in flies or larvae would lead to a lowered immune response. Figure 4A indeed shows a lowered immune induction in larvae expressing the CG32750 RNAi constructs compared to control larvae. Although the number of responding larvae doesn't seem to differ between CG32750 larvae and the control larvae, the immune induction itself (intensity of the GFP signal) is greater in the infected control larvae then in the infected CG32750 downregulated larvae. This suggests that the immune response in fruit fly larvae is lowered when suppressing the CG32750 Vanin gene.
The experiment was repeated using two TR lines crossed against the 25041-Gal4 (Gut specific) *dipt*-GFP line. Vanin is most highly expressed in the gut tissue of second and third instar larvae. Also, gut tissue is known to play an important role in immunity and Vanin therefore doesn't need to be knocked down in other tissues. Knowing that this line also is expressed in the hemocytes of embryos, the hemocytes of the third instar larvae might also express the Vanin RNAi construct. TR larvae are larvae in which all three Vanins are knocked down (CG32750, CG32751 and CG32754). Figure 4B shows that infected TR 4 larvae do show a downregulation in immune response, compared to the control larvae. The lowered immune induction is comparable to the lowered immune induction in the larvae expressing the CG32750 RNAi constructs. However, infected TR 1 larvae show a considerably lowered immune induction, compared to the CG32750 downregulated larvae. The differences in immune response between the two TR lines is probably due to the different RNAi construct targeting the CG32750 gene, knowing that the RNAi construct targeting CG32751 and CG32754 is the same in both lines. Therefore, this data suggest that the CG32750 gene is more efficiently downregulated in TR 1 then in TR 4. This data also suggest that efficiently downregulating all three Vanin genes causes a more notable downregulation in the immune response then when only downregulating CG32750.
To confirm that the difference in immune induction is due to the downregulation of Vanin and not because of a difference in bacteria intake between the groups, 5 larvae from each group were harvested, after having eaten the bacteria mixture for 30 minutes, mashed up and spread out on LB agar plates. Next, the number of bacteria taken in by each group was established by counting the number of colonies on each plate. Figure 4C

shows that all three groups seem to have taking-in approximately the same amount of bacteria. Therefore the possibility of the food intake being responsible for the difference in immune induction can be excluded.

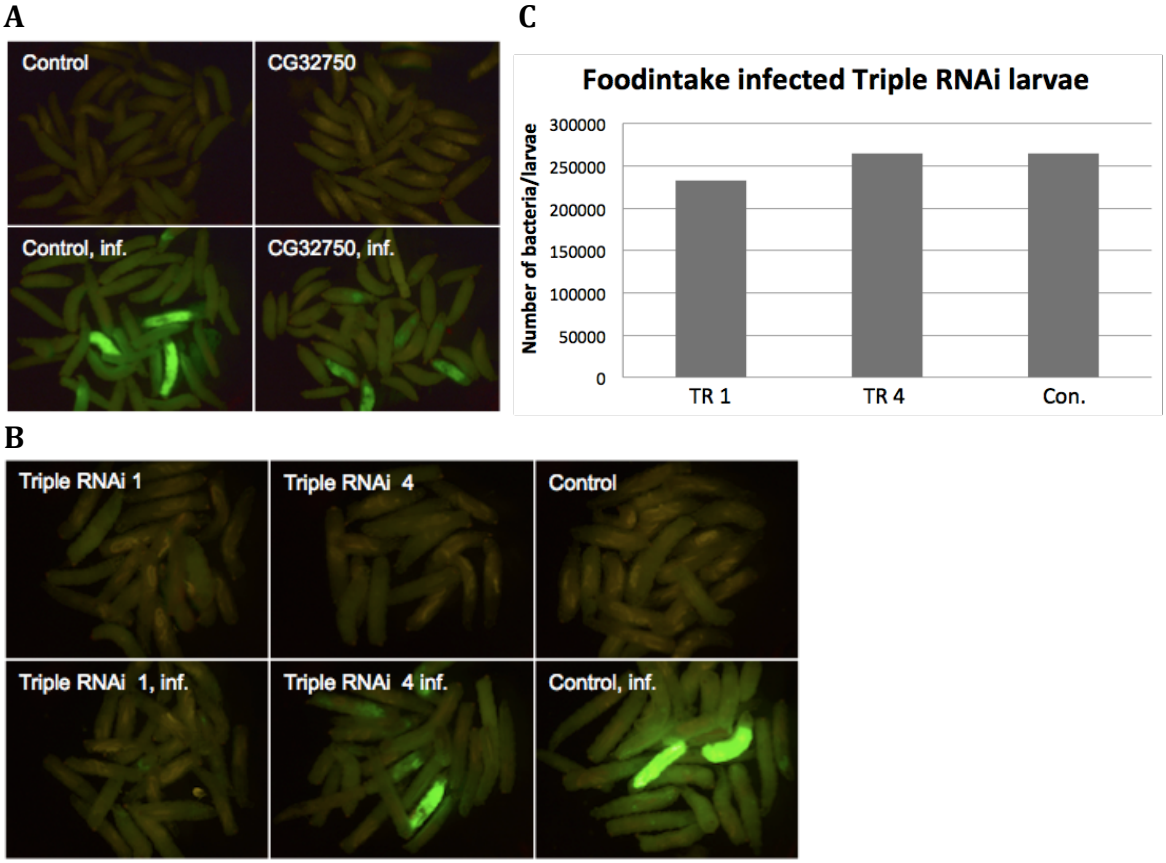


Figure 4. Altered relish specific immune induction in Vanin downregulated larvae.
A) - B) Immune induction of non-infected (top) and Ecc15 infected third-instar *dipt*-GFP larvae (bottom). The intensity of the GFP signal represents the intensity of the Imd pathway Immune induction. **A)** The CG32750 downregulated larvae are shown at the right and the control situation is shown at the left. **B)** Control larvae with no Vanin knockdown are show at the right, Vanin downregulated TR 1 larvae are shown at the left and TR4 larvae are shown in the middle. **C)** Number of bacteria taken-in per infected *dipt*-GFP larvae of the TR 1, TR 4 and the control (W1118-) groups.

The effect of infection on expression of Vanin and diptericin in third instar larvae

Literature shows that Vanin is upregulated upon infection, due to stress[16]. Because Vanin is a pro-inflammatory gene, knocking it down *in vivo* should cause a downregulation in immune response, upon infection (as was seen in figure 4). Thus *diptericin* expression should be lower in infected TR larvae versus the infected control larvae. *Diptericin* and Vanin expression in third instar larvae was established through RT PCR. However, figure 5A shows no downregulation in *diptericin* expression in the infected TR larvae. There also doesn't seem to be an upregulation in Vanin expression upon transfection (figure 5B), for reasons unknown.

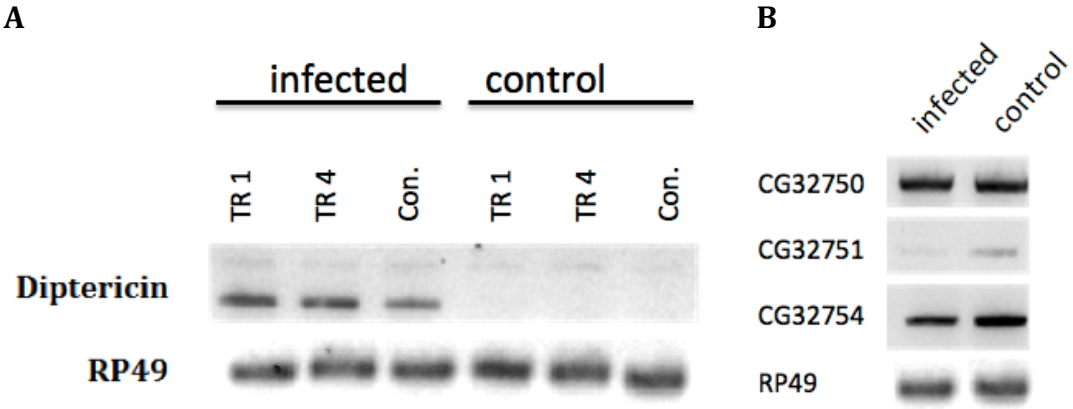


Figure 5. Diptericin and Vanin expression in infected and non-infected third instar larvae
A) *Diptericin* expression of infected and non-infected Vanin knockdown larvae (TR 1 and TR 4) and control larvae, verified by RT PCR. **B)** Vanin expression (CG32750, CG32751 and CG32754) in infected and non-infected control larvae.

Vanin localization and activity in CG32750 and CG32754 S2 transfected cells

To demonstrate the localization of Vanin *in vitro*, S2 cells (with low Vanin expression) were transfected with HA-tagged CG32750 and CG32754 constructs. The localization of Vanin inside the cells was visualized by immunofluorescence, using antibodies targeting the HA-tag attached on the C terminal of the Vanin proteins. Figure 6A shows that neither CG32750 nor CG32754 seems to be membrane bound, though more distributed in the cytosol and perhaps even in the endoplasmic reticulum (ER).
To determine the effects of overexpressing Vanin *in vitro* on the immune response, the effect of the Vanin proteins was established using luciferase assays. S2 cells were either transfected with the CG32750 or the CG32754 construct together with a luciferase construct with a Dorsal-specific AMP promoter or Relish-specific AMP promoter[20]. Control cells were transfected with the luciferase constructs and an empty vector. Figure 6B shows an increased immune induction in both the Imd and Toll pathway when overexpressing either of two Vanin genes. Overexpressing CG32750 causes an averaged 10 fold Dorsal- and an averaged 11 fold Relish immune induction, compared to the control cells. CG32754 overexpression also causes an averaged 10 fold Dorsal- and an averaged 7 fold Relish immune induction increase, compared to the control cells. These data indicate that overexpressing Vanin in S2 cells sufficiently increases the immune response.

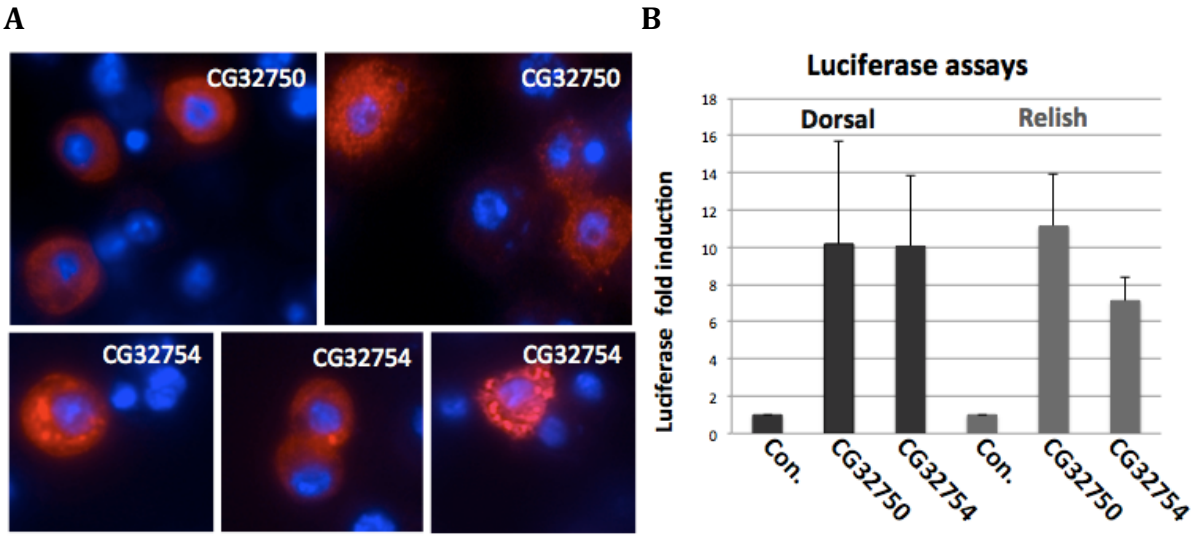


Figure 6. Vanin localization and activity in Vanin transfected S2 cells.
A) Immunofluorescence images of CG32750 and CG32754 transfected S2 cells. Hoechst coloring represent nuclei and the HA staining in red, representing the HA-tagged Vanin proteins. Top: S2 cells transfected with CG32750. Bottom: CG32754 transfected cells. **B)** Luciferase assays, detecting immune induction through both Toll (Dorsal) and Imd (Relish) pathway in S2 cells, upon Vanin transfection. Control cells (Con.) were not transfected with the Vanin constructs and do not overexpress either of the Vanin genes.

No difference in hemocyte recruitment in Vanin downregulated hemocytes

Drosophila melanogaster efficiently fights infection[21] and repairs damaged tissue[22, 23] inter alia, by the work of their blood cells (hemocytes), which play a crucial role in these responses. To further investigate the possible roles of Vanin in *Drosophila* we investigated the role of Vanin in hemocyte recruitment, knowing that Vanin was first discovered playing a role in thymocyte homing in mice[6]. Third-instar larvae, with GFP-positive hemocytes, wounded in the middle of the A3/A4 segment were tracked over a course of 2 hours. Hemocyte recruitment in TR larvae were compared to control larvae in witch Vanin was not downregulated in their hemocytes. Figure 7 shows no difference hemocyte recruitment between the TR larvae versus the control larvae. This suggests that Vanin knockdown in hemocytes doesn't influence hemocyte recruitment *in vivo*.

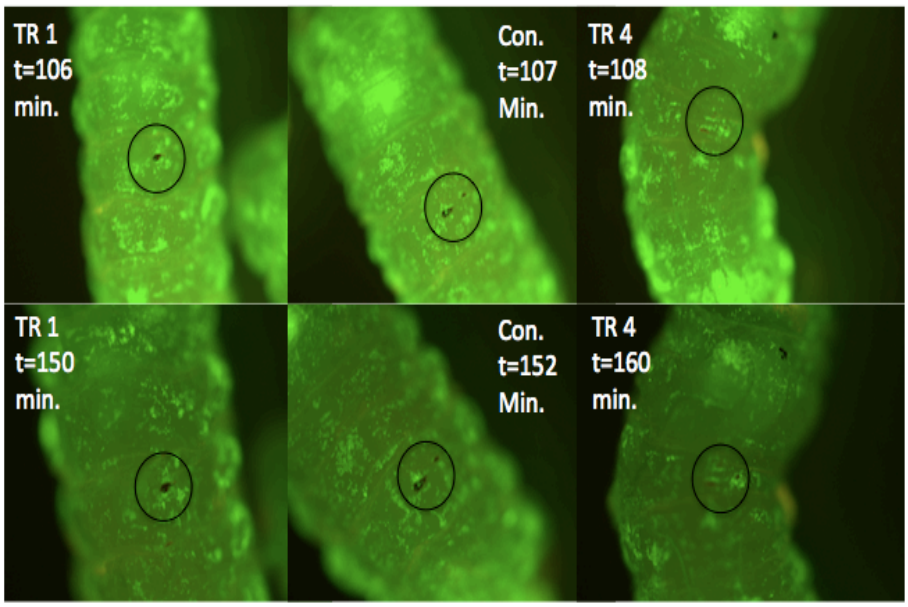


Figure 7. GFP-positive hemocytes recruitment after wounding Vanin TR third-instar larvae. The GFP-positive hemocytes of TR 1 and TR 4 third instar larvae were tracked over a course of over 2 hours, together with control larvae (W1118-). Images were tacking around 105 and 155 minutes upon wounding. Puncture sites are recognizeble as dark spots located withing circles, due to melanization.

Vanin downregulation influences fly lifespans

According to "the oxidative stress theory", oxidative damage contributes to the ageing process, causing impaired physiological function, increased incidence of disease and reduction in life span. Thus reducing oxidative damage, for example through the upregulation of anti-oxidants, could therefore possibly extend the lifespan of fruit flies[12, 13, 14]. Vanin is known to be not only pro-inflammatory but also pro-oxidant, by inhibiting the production of reduced glutathione, an important anti-oxidant. Considering "the oxidative stress theory", the expectations were that downregulating Vanin lifts the inhibitory effect of cystamine on GSH production, causing the glutathione store as well as the GSH/GSSG ratio to increase, thereby protecting the cells more efficiently against oxidative damage. The effect of Vanin knockdown on the lifespan of *Drosophila* was investigated by performing lifespan assays on different Vanin RNAi fly lines in parallel with the W1118- fly line and flies only containg Da-Gal4.

Figure 8A shows the life span of adult flies expressing Vanin RNAi (+RNAi) and flies not expressing Vanin RNAi (-RNAi). The –RNAi flies do not contain Da-Gal4 and therefore don't express the Vanin RNAi construct. Vanin is not suppressed in these fly lines, since these flies do not express the Gal4 transcription factor, in contrast to the +RNAi flies, which do suppress Vanin. Vanin RNAi line 4 is not inlcuded, for it would not eclose at 29 or at 25 °C. Lifespans of fly lines expressing Vanin RNAi, which did eclose, were tracked at 29 and 25°C. The flies not expressing Vanin RNAi, were also tracked at 29 °C. Figure 8B shows the life span of Da-Gal4 flies and W1118- flies, tracked at the same two temperatures. Because all Vanin RNAi flies were generated in a W1118- background, the expectations were that the flies not expressing Vanin RNAi (-RNAi lines) would live approximately the same length as the W1118- flies did at 29°C. The figure shows that the W1118- 29°C live to a maximum of 26 days (figure 8B), however the Vanin -RNAi fly lines (figure 8A) seem the have a somewhat longer lifespan, between 28 days (Vanin -RNAi 1) and 35 (Vanin -RNAi 5).

The graphs also show that +RNAi lines 1, 2 and 5 (figure 8A) have a longer life span compared to the non-activated (–) RNAi lines at 29°C. This could suggest that suppressing Vanin is indeed protecting the flies from oxidative damage. Vanin +RNAi line 3 seems to be the only RNAi fly line where suppressing Vanin seems to have a negative effect on the fly's lifespan. Fruit flies ideal living temperature is 25 °C, and explains why all Vanin +RNAi fly lines life longer at 25°C then at 29°C.

TR fly lines were also tracked at 29°C and are shown in figure 8C. These flies don't seem to have an extended lifespan compared to the control W1118- flies at 29°C. These flies actually show a more similar lifespan curve to the +RNAi line 3 (Figure 8A) at 29°C, which could be due to the fact that all TR fly lines contain the +RNAi 3 construct, targeting CG32754.

A

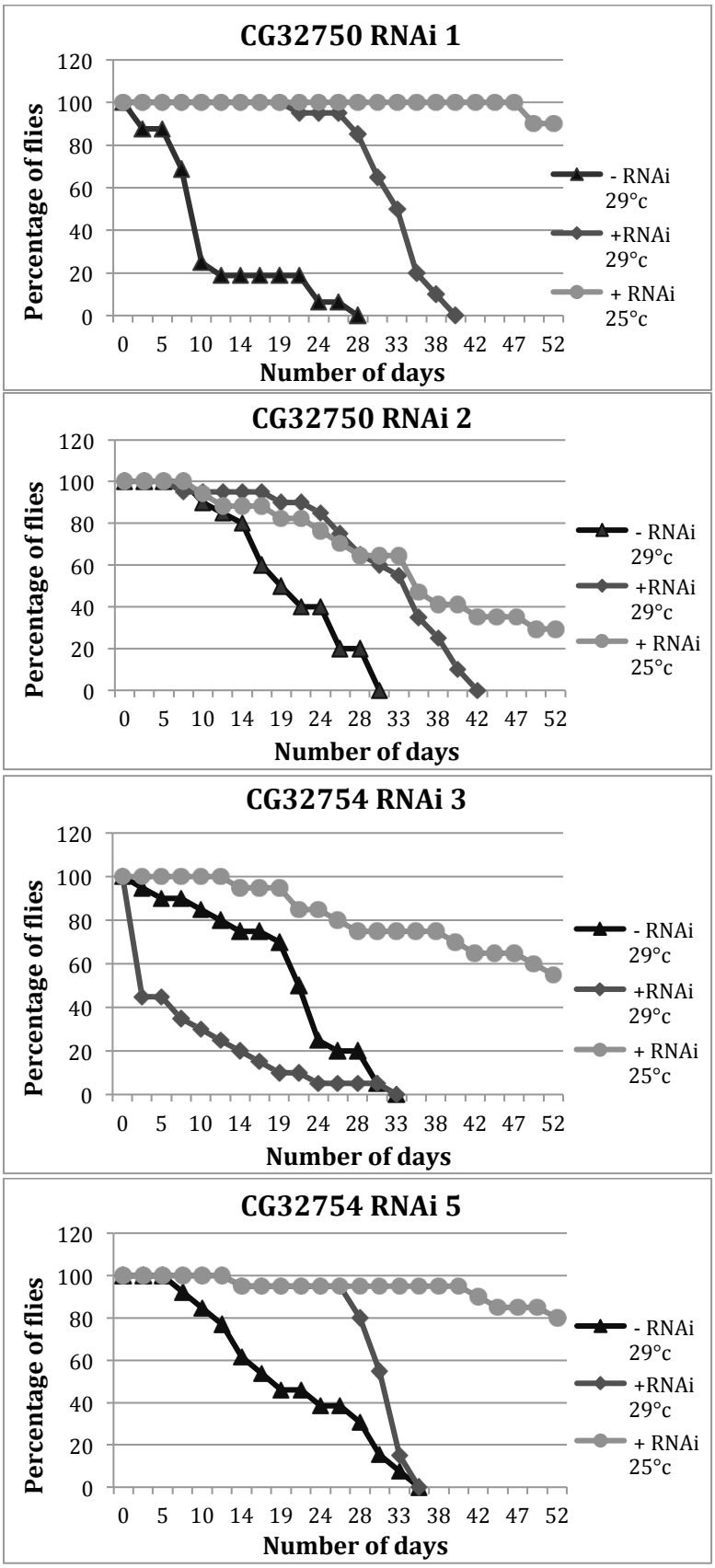
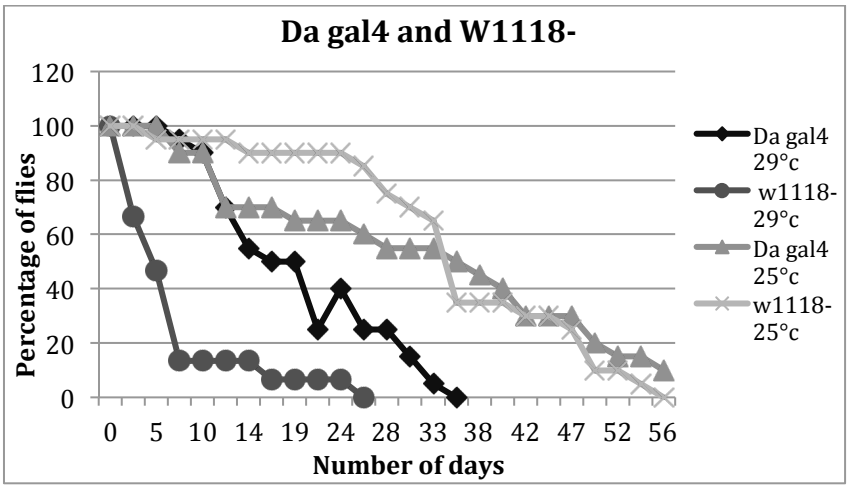
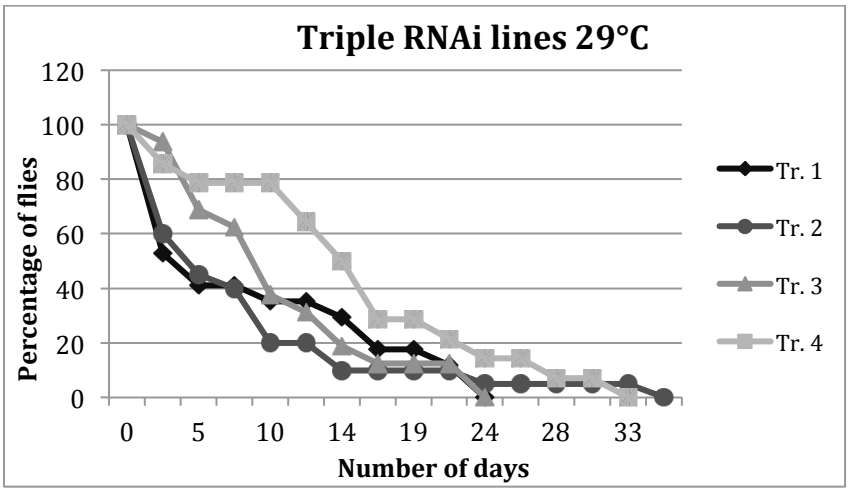


Figure 8. Lifespans of Vanin RNAi fly lines.
A) Vanin RNAi lines, targeting Vanin 50 or 54 and 51. Lifespans have been carried out at 29 and 25 °C for flies expressing the Vanin RNAi construct (+RNAi) and flies not expressing the Vanin RNAi construct (-RNAi). **B)** Da-Gal4 and W1118- flies life spans carried out at 29 and 25 °C. **C)** Triple RNAi (TR) fly lifespans carried out at 29 °C.

B


Da gal4 and W1118-
120
100
80
60
40
20
0
Percentage of flies
0 5 10 14 19 24 28 33 38 42 47 52 56
Number of days
Da gal4 29°c
w1118- 29°c
Da gal4 25°c
w1118- 25°c

C


Triple RNAi lines 29°C
120
100
80
60
40
20
0
Percentage of flies
0 5 10 14 19 24 28 33
Number of days
Tr. 1
Tr. 2
Tr. 3
Tr. 4

Discussion

Here we investigated the role of Vanin in the immunity and ageing process of fruit flies. Vanin is well conserved in mice, *Drosophila* and humans. The precise role of Vanin is not known yet. By performing experiments on *Drosophila*, we tried to better understand the role of Vanin in fly immunity. Because of the great homology that humans and *Drosophila* share in several proteins and enzymes of the immunity, findings might be translated to human biology. This report focused on the role of Vanin in the immunity and ageing of *Drosophila melanogaster*. The aim of the project was to investigate the consequences of Vanin knockdown in *Drosophila*, focusing on immunity and ageing.

We were able to find that the downregulation of Vanin affects the larvae their ability to provoke a proper immune response. The downregulation of CG32750 showed a decreased immune response, compared to control larvae. Downregulating all three Vanin genes (TR 1) had an even bigger impact on the larvae their immune response. Figure 3 shows that TR 1 larvae show less Vanin expression in of all the three isoforms, compared to the TR 4 larvae. Suggesting that TR 1 larvae are more efficient in downregulating Vanin then TR4 larvae, which correlates with the findings in the infection studied (figure 4). The data show that downregulating Vanin *in vivo* inhibits the immune response of at least the Imd/Relish pathway. Infection studies to investigate the affect of Vanin downregulation in the Dorsal pathway are yet to be performed. Expectations are that also this pathway might be affected.

Knowing the effect of downregulating Vanin in flies, the next step was to see the effects of overexpressing Vanin. This was done in S2 cells. First the localization of Vanin inside the cells was determined by transfecting S2 cells with HA-tagged CG32750 and CG32754 constructs. Knowing that CG32750 contains a GPI anchor, the expectations were to find CG32750 at the plasma membrane, though, they both seem to be located in the cytosol and maybe even the ER. However, the HA-tag is located at the C-terminal, as does the GPI anchor, and could therefore interfere with the anchor and change the protein's localization. The experiment should be repeated with constructs not containing a HA-tag, when one can label the Vanin protein directly.
Although the HA-tag might interfere with the localization of Vanin inside the cells, Vanin might still show activity. Luciferase assays show an increased immune response when overexpressing CG32750 or CG32754 in S2 cells. However, also this experiment should be repeated with constructs not containing the HA-tag, for it might also influence the activity of the protein. Still, this data confirms the conclusion that Vanin indeed seems to play a role in *Drosophila* immunity.

Knowing Vanin plays an important role in thymocyte homing in mice, Vanin might also play a role in hemocyte recruitment and thereby also take part in another aspect of *Drosophila* immunity. The downregulation of Vanin in the hemocytes self, did not affect the hemocyte recruitment. This doesn't mean that Vanin does not play a role in hemocyte recruitment at all; only that downregulating Vanin in the hemocytes doesn't influence their recruitment. Downregulation Vanin in other tissues, or in the whole body could have a different affect and should therefore also be investigated.

Vanin is known to inhibit the production of reduced glutathione, an important anti-oxidant, which subsequently intensifies the oxidative stress. According to "the oxidative

stress theory", oxidative damage contributes to the ageing process. The effect of Vanin knockdown on the flies lifespan suggest that Vanin knockdown indeed has a protective function, protecting the tissue cells from oxidative damage and thereby extending the lifespan of the fruit fly. However, not all Vanin knockdowns extended the lifespan. In fact, one fly line (Vanin RNAi line 4), which downregulates CG32751, seems to cause premature lethality. These flies did not eclose and died in their pupa at very advanced stages. Some even died while trying to crawl out their pupa. Suggesting that the flies did not have enough strength to come out of the pupa. However, TR fly lines, in which all three Vanins are downregulated, did eclose and produced adult flies. These flies did not show an extended lifespan and leads to the conclusion that although downregulation of Vanin might protect from oxidative damage, Vanin seems to be necessary for more important processes in the body.

Data shown in this report indeed suggest that Vanin plays a role in *Drosophila* immunity and possibly ageing. Downregulating Vanin seems to inhibit the immune response and in some cases extends the lifespan of *Drosophila*. Overexpressing Vanin causes an increase in immune response. This suggests that Vanin can possibly be used to modulate immune responses in certain situations, thereby making Vanin a possible target for treatment. For example, tissue specific downregulation of Vanin in certain types of inflammatory diseases could possibly help treating the disease or symptoms.

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Supplementary data 1

Fly lines and stocks

Vienna *Drosophila* RNAi Centre

	CG	Gene	Transformant ID	Construct ID	Inserted chromo-some	Off targets
1	CG32750	Vanin	52403	15946	2;2	CG17122; 1 19-mer
2	CG32750	Vanin	107171	104899	2;2	CG17122; 1 19-mer
3	CG32754	Vanin	50591	17655	3;3	3; CG32751 185 19-mers CG3599 5 19-3mers; CG32750s 5 19-mers
4	CG32751	Vanin	100206	105770	2;2	0
5	CG32754	Vanin	50592	17655	2;2	3; CG32751 185 19-mers CG3599 5 19-3mers; CG32750s 5 19-mers

Bloomington *Drosophila* stock centre

ID	Genotype	Expressed
53 8641	P{da-GAL4.w[-]}3	3 Expresses GAL4 in the pattern of the da gene.
495 30140	P{Hml-GAL4.Delta}2	2 Expresses GAL4 and GFP in lymph glands and circulating hemocytes.
49 25041	P{crq-GAL4}2	2 Expresses GAL4 in embryonic hemocytes **(Was however discovered to be a gut driver during experiments)**.

TR lines

Crosses of a CG32750 line with a CG32754 (with a double target effect in CG32751).

TR lines	Gene	Transformant IDs, Cross	CG, Chr. 2	CG, Chr. 3
1	Vanin	52403 x 50591	CG32750	CG32754 (+ CG32751)
2	Vanin	52403 x 50591	CG32750	CG32754 (+ CG32751)
3	Vanin	107171 x 50591	CG32750	CG32754 (+ CG32751)
4	Vanin	107171 x 50591	CG32750	CG32754 (+ CG32751)

Supplementary data 2

Additional experiment; Western blot

To confirm that S2 cells were succesfully transfected with the Vanin constructs, a western blot analysis was performed to show Vanin expression in these cells. Antibody targeting the HA-tag was used as the primary antibody, since there has yet to be an antibody produced that specifically targets *Drosophila* Vanin. However, multiple attemps to show Vanin expression via western blot failed. Immunefluorscence assays on the other hand, does show HA postive cells, at a low efficientcy. Therefore Westernblot might not be sensitive enough to detect the HA-tagged Vanin proteins.