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Differential gene expression related to Nora virus infection of *Drosophila melanogaster*

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Abstract

Nora virus is a recently discovered RNA picorna-like virus that produces a persistent infection in *Drosophila melanogaster*, but the antiviral pathway or change in gene expression is unknown. We performed cDNA microarray analysis comparing the gene expression profiles of Nora virus infected and uninfected wild-type *D. melanogaster*. This analysis yielded 58 genes exhibiting a 1.5-fold change or greater and *p*-value less than 0.01. Of these genes, 46 were up-regulated and 12 down-regulated in response to infection. To validate the microarray results, qRT-PCR was performed with probes for *Chorion protein 16* and *Troponin C isoform 4*, which show good correspondence with cDNA microarray results. Differential regulation of genes associated with Toll and immune-deficient pathways, cytoskeletal development, Janus Kinase-Signal Transducer and Activator of Transcription interactions, and a potential gut-specific innate immune response were found. This genome-wide expression profile of Nora virus infection of *D. melanogaster* can pinpoint genes of interest for further investigation of antiviral pathways employed, genetic mechanisms, sites of replication, viral persistence, and developmental effects.

Keywords

Nora virus; RNA viruses; cDNA microarray; Gut immunity

1. Introduction

Nora virus is a recently discovered picorna-like virus that establishes persistent infections in natural and laboratory populations of *Drosophila melanogaster*. It is similar to other members of the *Picornaviridae* superfamily, including poliovirus, with the exception of a relatively large (~ 12 kb) genome with 4 open reading frames (ORFs) (Habayeb et al., 2006). The site of localization and replication is hypothesized to be the gut through fecal-oral transmission without exhibiting pronounced pathology (Habayeb et al., 2009a).

A pattern of titer dependent persistence or clearance exists, although immunological interaction experiments utilizing RNA interference (RNAi), Janus Kinase-Signal Transducer and Activator of Transcription (Jak-STAT), and Toll pathway mutant flies have produced results similar to wild type infection (Habayeb et al., 2009b). These pathways, in addition to the immune deficiency (Imd) pathway, are used to control viral infections in *Drosophila*. The Toll pathway is capable of recognizing pathogen associated molecular patterns (PAMPs) from Gram-positive bacteria, fungi, and viruses in *D. melanogaster*. Pathogen recognition molecules, called peptidoglycan recognition proteins (PGRPs), set a serine

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protease cascade into motion with the terminal transcription of antimicrobial peptides (AMPs) (Kambris et al., 2006). The Imd pathway primarily combats Gram-negative bacteria through recognition of peptidoglycan, which is specifically mediated through the binding of peptidoglycan recognition protein-LC (PGRP-LC) (Gottar et al., 2002). It acts by controlling the downstream activation of the NF-κβprotein Relish (Choe et al., 2002), which is important for antibacterial peptide synthesis (Rämet et al., 2002). The *Drosophila* Jak-STAT pathway is thought to promote cellular immune processes such as phagocytosis and production of reactive oxygen species (ROS) by hemocytes after septic injury (reviewed in Hoffman, 2003). The RNAi pathway recognizes foreign dsRNA and destroys it to prevent virus gene expression. A systemic method for viral dsRNA uptake is also essential for *D. melanogaster* antiviral immunity, where dsRNA is released from lysis or budding and taken up into uninfected cells by an uncharacterized mechanism to induce RNAi mediated immunity (Saleh et al., 2009).

The lack of measurable response to established anti-viral pathways suggests a different mechanism for clearance of Nora virus (Habayeb et al., 2009b). Microarray analysis provides a genome wide approach to identify transcriptional responses to Nora virus infection. The goal of this study was to determine the *D. melanogaster* genes that are affected by the establishment of Nora virus infection. Efforts can then be focused towards characterization of these genes and their potential roles in innate anti-viral immunity.

2. Materials and methods

2.1. D. melanogaster culture, husbandry, and infection

Canton S wild type flies (Carolina Biological Supply, Burlington, NC) and Nora virus infected flies (received as a kind gift from Dr. Dan Hultmark, Umeå, Sweden) were maintained on standard cornmeal, molasses, and torula yeast medium at 25 °C with diurnal light. For the initial cDNA microarray experiment and qRT-PCR validation, one hundred Nora virus infected *D. melanogaster* males were placed in each of 6 bottles with standard cornmeal–molasses–yeast food. Six replicate bottles with one hundred Canton S uninfected males served as the control. The males were allowed to defecate on the food for 6 days to ensure adequate transfer of Nora virus to the surface of the food in the experimental bottles. After 6 days, the males were removed and killed and 25 female and 25 male Canton S wild type uninfected flies were added to each of the 6 Nora virus infected and Canton S uninfected bottles. The flies were allowed to mate, lay eggs for 5 days, and removed and killed. The F1 generation was allowed to emerge and age for approximately 4 days to allow for productive infection (Habayeb et al., 2006). They were collected in aliquots of 5 male and 5 female *D. melanogaster*, and frozen at −80 °C. This infection protocol was repeated for the subsequent validation of gene expression levels of the cDNA microarray results in independently infected and uninfected (control) *D. melanogaster*.

2.2. RNA extraction and RT-PCR

RNA extraction was performed using TRIzol® per manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA was cleaned using an RNeasy mini kit (Qiagen, Germantown, MD) per manufacturer's instructions. The samples were analyzed for the presence of Nora virus via RT-PCR using *Nora ORF 1* 54–844 (forward 5′-TGGTAGTACGCAGGTTGTGGGAAA-3′ reverse 5′-AAGTGGCATGCTTGGCTTCTCAAC-3′) primers and Promega Access Quick RT-PCR master mix and reverse transcriptase according to manufacturer's instructions (Madison, WI). Reactions were carried out in duplicate under the following conditions: 45 °C for 45 min, 94 °C for 2 min (94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min) 30 cycles, 68 °C for 10 min, and hold at 4 °C. A positive reaction yielded a product at approximately 790 bp.

2.3. RNA cleanup and cDNA microarray hybridization

Ten micrograms from each RNA sample for microarray analysis was cleaned with an RNeasy cleanup kit (Qiagen Inc.) according to manufacturer's instructions (Germantown, MD). One microgram of each sample was used for cDNA microarray hybridization and analysis. Three arrays comparing *D. melanogaster* Nora virus infected vs. uninfected cDNA were used, with one dye swap.

All cDNA hybridizations were performed at the University of Nebraska Medical Center (UNMC) Microarray Core Facility by Dr. Jim Eudy. Glass microarrays containing 14,593 70-mer oligonucleotides corresponding to 13,664 *D. melanogaster* genes and were obtained from Microarray Inc. (Nashville, TN). Prior to hybridization, the glass microarrays were prehybridized in a solution of 5× saline sodium phosphate EDTA (ethylene diamine tetra-acetic acid) (SSPE), 1% bovine serum albumin (BSA), and 0.1% sodium dodecyl sulfate (SDS) for 45 min at 42 °C to prevent nonspecific binding.

To generate fluorescent probes for hybridization, 1 μg of total RNA from each of the control and test samples were reverse transcribed using the Ambion MessageAmp II kit per manufacturers' recommendations (Applied Biosystems, Ambion, Austin, TX) to generate amino-allyl aRNA. Five micrograms of aRNA from each of the test and control samples was coupled to either CY3 or CY5 (GE Healthcare, Pittsburgh, PA) and probes were purified by column chromatography using the kit reagents. CY3 and CY5 fluorescently labeled control and test aRNA samples, respectively, were eluted from the columns, mixed (30 μl total) and fragmented to a size range of 60–200 bp by treatment with 1 μl Ambion Fragmentation Reagent at 70 °C for 15 min (Ambion, Austin, TX). Fragmented probes were added to 40 μl of Ambion Microarray Hybridization Buffer II and denatured by heat at 95 °C for 5 min and kept at 60 °C until hybridization. Probe solutions were placed upon the slides, covered with a cover slip, placed in hybridization chambers and hybridized 16 h at 42 °C. Following hybridization, the slides were washed in a solution of Wash I (2.0× SSPE, 0.1% SDS) for 2 min (42 °C) followed by a solution of Wash II (0.1× SSPE, 0.1% SDS) for 2 min (42 °C).

The slides were scanned with an Axon 4000B scanner at a resolution of 5 μm (Molecular Devices, Sunnyvale, CA). During the scanning procedure, the photomultiplier tubes (PMTs) were balanced to achieve an overall approximate CY5/CY3 ratio of 1. The images were gridded using GenePix 6.0 software (Molecular Devices). Problematic spots due to any local hybridization artifacts were flagged as "bad" for resultant removal from the final dataset.

Data from the microarray results were analyzed by Lynette Smith in the UNMC Department of Biostatistics. Normalization of gene expression data was done using Biometric Research Branch (BRB) ArrayTools (Simon et al., 2007). Prior to analysis, the median background was subtracted, Log_2 transformation was applied to all ratios, normalization was done to "center" each array using Lowess smoother, empty and control spots were excluded, and duplicate spots were not averaged but treated as separate genes for analysis. Random-variance paired *t*-tests were used to determine the genes that were differentially expressed between Nora virus infected and uninfected samples. An initial significance level of 0.001 was selected to limit the false discovery rate (FDR) of multiple comparisons.

2.4. Validation of cDNA microarrays by qRT-PCR

Reverse transcription was performed using TaqMan Gene Expression Assay® kits and the 7500 Real Time PCR® system (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. For the initial validation using the same RNA as was used in the cDNA microarrays, the primer and probe sets used were *Troponin C isoform 4* (*TpnC4*; assay #Dm01815264 n1), *Chorion protein 16* (*Cp16*; assay #Dm01822369 g1), and *Ribosomal protein 49* (*Rp49*; endogenous control; assay #Dm02151827 g1). Reactions were

carried out in quadruplicate and performed in a 50 μl volume utilizing 200 ng total RNA sample and TaqMan® One-Step RT-PCR Mix (Applied Biosystems). Negative controls without RNA for each primer/probe set were also run. Cycling parameters included 48 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR products were analyzed in the linear range for amplification with *Rp49* using the 7500 Real Time PCR System Sequence Detection Software® (Applied Biosystems). The relative quantitative results were used to determine changes in gene expression on a Log_2 scale via the Δ ΔCT method (Livak and Schmittgen, 2001). For the subsequent qRT-PCR validation using RNA from independently infected or uninfected flies, the protocol was the same as above with the exception that the samples were run in triplicate and the primer probe sets used were *TpnC4*, *Cp16*, *Rp49*, *Femcoat*, *follicle cell protein 3C* (*Fcp3c*; assay #Dm01845104_g1), *BTB-protein-VII* (*BtbVII*; assay #Dm01821984_m1), and *CG6639* (assay #Dm01792031_g1). For all qRT-PCR experiments, the $n = 3$, which represents the number of biological replicates used in the experiment.

2.5. Gene analysis

After the initial filtering of the data, only those genes changed 1.5-fold or more with a *p*-value of 0.01 or less were selected for further study. From this analysis, there were fifty-eight differentially regulated genes selected and submitted to Flybase, a *Drosophila* gene database (www.flybase.org), and GenBank: genetic sequence database at the National Center for Biotechnology Information (NCBI) Gene (http://www.ncbi.nlm.nih.gov/gene) for identification of gene names, gene identification numbers (Gene ID #), and experimentally tested for putative biological functions. The identified differentially expressed genes were subjected to gene ontology analyses using PANTHER (Protein ANalysis THrough Evolutionary Relationships; http://www.pantherdb.org; Thomas et al., 2003).

3. Results

3.1. Nora virus infection confirmation via RT-PCR

To determine whether the *D. melanogaster* were successfully infected with Nora virus, we examined the presence of Nora virus ORF1 by RT-PCR. Four samples each of five male and female *D. melanogaster* were collected at day 4 post-eclosion on either Nora virus contaminated or Nora virus free food. Total RNA was extracted from eight biological replicates (uninfected controls A–D; Nora virus infected A–D) and subjected to RT-PCR for Nora virus ORF1. The Nora virus infected *D. melanogaster* total RNA RT-PCR reaction produced a 790 bp product in each of the groups, indicating successful infection. Control *D. melanogaster* reactions did not amplify Nora virus RNA, thus confirming a lack of infection (Fig. 1). This was also repeated for the *D. melanogaster* for the independent validation of the cDNA microarray results. Total RNA was extracted from three samples each of six biological replicates (uninfected controls A1–3, B1–3, and C1–3; Nora virus infected A1–3, B1–3, and C1–3) and subjected to RT-PCR for Nora virus ORF1. The Nora virus infected *D. melanogaster* had a product at 790 bp, whereas the uninfected controls did not (data not shown).

3.2. cDNA microarray analysis

To begin to elucidate the effects of Nora virus infection on gene expression in *D. melanogaster*, cDNA microarray analyses were performed comparing three of the Nora virus infected samples with the three replicate uninfected controls previously tested with conventional RT-PCR for Nora virus status. On the array, there were 15,409 genes included. After filters and normalization were applied, 14,888 genes were left for analysis. The majority of genes examined showed only small differences that were not considered statistically significant and were not included in further analyses (data not shown). The fifty-

eight genes differentially regulated (1.5-fold or more with a *p*-value less than 0.01) were submitted to Flybase and NCBI Gene for identification of biological functions. Of these, 46 were significantly up-regulated (Table 1) and 12 were significantly down-regulated (Table 2). Numbers in parentheses denote genes in the category. Up-regulated genes include metabolic processes (16), morphogenesis (8), eggshell chorion formation (16), and ovarian follicle cell development (4). The most numerous category of down-regulated gene biological functions was the chitin metabolic process with 2 genes belonging to this group (Table 2). The fifty-eight differentially regulated genes were submitted to the PANTHER Classification System, which is a unique resource that classifies genes by their functions, using published scientific experimental evidence and evolutionary relationships to predict function even in the absence of direct experimental evidence (Thomas et al., 2003). Of the twelve down-regulated genes, 5 of the IDs were unrecognized (Gene ID #s *CG10332*, *CG32302*, *CG7298*, *flightin [fln]*, and *TpnC4*). Of the remaining 7 genes, these were grouped into the selected annotated biological functions (gene ontology accession number; total # genes; percent of gene hit against total # genes; percent of gene hits against total # of process hits; Gene ID #s) that included various roles in cell communication (GO:0007154; 1; 14.3; 8.3; *Rhodopsin 5 [Rh5]*), cellular processes (GO:0009987; 2; 28.6; 16.7; *CG10910* and *Rh5*), transport (GO:0006810; 2; 28.6; 16.7; *CG15096* and *Niemann-Pick type C-2d [Npc2d]*), cellular component organization (GO:0016043; 1; 14.3; 8.3; *CG10910*), system processes (GO:0003008; 1; 14.3; 8.3; *Rh5*), generation of precursor metabolites and energy (GO:0006091; 1; 14.3; 8.3; *CG15434*), and metabolic processes (GO:0008152; 4; 57.1; 33.3; *CG6639*, *CG10910*, *CG15096*, and *Npc2d*). Of the forty-six up-regulated genes, 19 were not mapped (Gene ID #s *anastral spindle 2 [ana2]*, *CG1648*, *CG11079*, *CG13084*, *CG13309*, *CG14187*, *CG14957*, *CG14963*, *CG40337*, *Chorion protein 16 [Cp16]*, *Cp18*, *Cp19*, *Femcoat*, *follicle cell protein 3C [Fcp3C]*, *Glutathione S transferase E7 [GstE7]*, *Mucin 11A [Muc11A]*, *Mucin related 11Da [Mur11Da]*, *Sarcoplasmic calcium-binding protein 2 [Scp2]*, and *Vago*). Of the remaining twenty-seven genes that could be mapped, these were again grouped into the selected annotated biological functions (gene ontology accession number; total # genes; percent of gene hit against total # genes; percent of gene hits against total # of process hits; Gene ID #s) that included various roles in cell communication (GO:0007154; 2; 7.4; 6.7; *CG7763* and *CG13650*), cellular processes (GO: 0009987; 3; 11.1; 10.0; *CG7763*, *CG13650*, and *Huntingtin interacting protein 1 [Hip1]*), transport (GO:0006810; 1; 3.7; 3.3; *Npc2e*), cellular component organization (GO:0016043; 1; 3.7; 3.3; *Hip1*), apoptosis (GO:0016043; 1; 3.7; 3.3; *Hip1*), developmental processes (GO:0032502; 4; 14.8; 13.3; *BTB-protein-VII [BtbVII]*, *CG329*, *CG15534*, and *Hip1*), generation of precursor metabolites and energy (GO:0006091; 1; 3.7; 3.3; *CG11257*), metabolic processes (GO:0008152; 16; 59.3; 53.3; *Amyrel*, *BtbVII*, *Maltase A4 [Mal-A4]*, *Mal-B2*, *CG1600*, *CG7059*, *CG10588*, *CG10814*, *CG11257*, *CG13650*, *CG15534*, *CG31089*, *CG31926*, *CG31928*, *CG32523*, and *Npc2e*), and immune system (GO:0002376; 1; 3.7; 3.3; *CG7763*).

3.3. qRT-PCR validation of cDNA microarray analysis

To confirm and validate the results of our cDNA microarray analysis, we initially examined the levels of one significantly up-regulated gene (*Cp16*) and one significantly down-regulated gene (*TpnC4*) from the 58 differentially regulated genes. The reactions were carried out in quadruplicate for each of the three biological replicates for the Nora virus infected and uninfected control RNA samples that were used for the cDNA microarray analyses. Therefore, the RNA that was used in the microarray and qRT-PCR analyses was the same. As shown in Fig. 2, the qRT-PCR yielded good correspondence with the cDNA microarray, with up-regulation of 8.95-fold in *Cp16* compared to 3.695 fold in the cDNA microarray, and down-regulation of 1.60 fold in *TpnC4* compared to 3.3 fold in the cDNA microarray.

To further confirm and validate the cDNA microarray data, we performed an independent infection experiment to determine if the host gene response was repeatable. The two genes previously tested, *TpnC4* and *Cp16*, were used again for consistency between experiments. In addition, two more up-regulated genes, *Fcp3c* and *BtbVII*, and one down-regulated gene, *CG6639*, were examined. The reactions were carried out in triplicate for the three biological replicates of Nora virus infected samples and uninfected controls (i.e., 18 determinations for infected and 18 determinations for uninfected for each gene tested). Four Nora virus infected determinations of the eighteen tested for the *CG6639* analysis were removed as outliers as assessed by the Grubbs test (2.2 standard deviations) (Grubbs, 1969; Burns et al., 2005). As shown in Fig. 3, the qRT-PCR once again yielded good correspondence with the cDNA microarray results.

4. Discussion

The current study provides a starting point for selecting candidate genes to be further characterized to gain a better understanding of Nora virus infection in *D. melanogaster*. The mode of transmission of Nora virus is hypothesized to be fecal-oral (Habayeb et al., 2009a), therefore the up-regulation of *Femcoat* and *Cp16*, *18*, and *19* in this study was interesting as it may suggest that vertical transmission could be occurring. These genes encode proteins that are involved in eggshell chorion assembly and chorion structure (Kim et al., 2002). Results from a cDNA microarray study of *D. melanogaster* infection with Sigma virus (SIGMAV) indicate up-regulation of *Cp16*, as well as *Cp15* and *38*. Chromosomal copies of chorion proteins can increase by 10 fold during oogenesis, and further up-regulation may relate to SIGMAV infection via vertical transmission (Carpenter et al., 2009). A similar up-regulation of chorion proteins and related factors in Nora virus infected *D. melanogaster* suggests that Nora virus may be vertically transmitted. Habayeb et al., stated that Nora virus is not vertically transmitted after dechorionating eggs from infected flies, placing them on uninfected food, and failing to detect viral RNA via RT-PCR (Habayeb et al., 2009a). Therefore, the results seen in the current study may actually be due to an indirect effect on the females that could not be controlled for in the study. To confirm that vertical transmission of Nora virus is not occurring, immunohistochemical staining of eggs for capsid protein needs to be performed. To date, an antibody to Nora virus does not exist, therefore these types of analyses do not exist. Efforts in our lab are underway to produce monospecific antibodies for Nora virus detection.

Differential regulation of immune genes is of interest, as RNAi experiments suggest the antiviral pathways currently described had no impact on Nora virus titer. Of particular interest is the Toll pathway antiviral response, which was tested with the loss-offunction *pelle* gene, pll^2, and found to have no effect on viral titer (Habayeb et al., 2009b). Our results suggest Nora virus infection may affect transcription of genes in the Toll pathway, although up-regulation of AMPs did not result. *D. melanogaster* gene *CG6639* is characterized as having serine-type endopeptidase activity in the Toll pathway, which responds to Gram-positive bacterial infection. An RNAi knockout experiment of *CG6639* did not result in increased Gram-positive bacterial growth (Kambris et al., 2006), suggesting knockout of *CG6639* did not inhibit Toll pathway activity. Therefore, the down-regulation of *CG6639* in our experiment may not affect activation of the Toll pathway if it responded to Nora virus infection.

Nora virus infection may differentially regulate potential constituents of the Imd pathway. Cytoskeletal factors *TpnC41C* and *fln* were induced in TI^{10b} (constitutive and global up-regulation of immune genes) mutants, but not *cactus* mutants (Boutros et al., 2002), which suggest additional signaling branches to traditional NF-κβ. Two genes from our study, *fln* and *TpnC4*, were downregulated. The parallel differential regulation of *fln* and two related

genes of the *Troponin C* family in separate studies may indicate an immune response function, although the nature of the response is not fully understood. *Npc2d* (down-regulated) and *Npc2e* (up-regulated) were cataloged in this study into the GO groups of transport and metabolic processes, specifically Npc2d has a role in lipid metabolism. A bovine homolog of *npc2* is related to MD-2, a lipid recognition protein thought to play a part in stimulation of signal transduction in the Toll-like receptor TLR-4 (Gangloff and Gay, 2004). In a recent study, overexpression of Npc2e in *Drosophila* S2 cells activated *diptericin* by binding two meso-diaminopimelic acid (DAP)-type peptidoglycan (PG) receptors. This suggests Npc2e may function as a co-receptor for DAP-type PG to stimulate the Imd pathway (Shi et al., 2012). Therefore, up-regulation of Npc2e may be a way to modulate the host response to infection to Nora virus or a host defense to the Nora virus infection.

Muc11A and *Mur11Da* were also up-regulated in this study and found to be interesting when taking into account the hypothesized fecal-oral mode of transmission. *Drosophila* Muc11A is expressed in the developing salivary gland at the egg stage, midgut/proventriculus in the larval stage, and in unidentified tissues midway through the pupal stage and throughout adulthood, while Mur11Da is expressed in unidentified tissues throughout adulthood. *Drosophila* mucins function as glycosylated proteins in a gel matrix and bind to chitin to protect cuticle free organ epithelia and may serve as a scaffold for lumen formation during early development (Zulfeqhar et al., 2008). Up-regulation of these genes during Nora virus infection may be in response to viral entry through the gastrointestinal tract after consumption of feces laden food.

Nora virus was found almost exclusively in the intestinal tract, detected via qRT-PCR (Habayeb et al., 2006). The gut is constantly exposed to commensal microbes that produce PAMPs capable of eliciting an immune response. To avoid constitutive expression of AMPs from the Imd pathway in epithelial cells, several negative regulatory mechanisms have recently been described. Alternative Imd regulation and stimulation of the *duel oxidation* (*duox*) gene to produce ROS provides innate immunity within the gut (Ryu et al., 2010). Our results indicate differential regulation of genes involved in calcium ion binding, including *TpnC4* down-regulation and *gomdanji* up-regulation. Mammalian epithelial cells demonstrate calcium signaling via binding intracellular uptake is essential for DUOX activation during immune response (Ryu et al., 2010). Also, several genes involved in redox reactions (*CG10814*, *CG1600*, and *CG11257*) were up-regulated, possibly during the production or removal of ROS.

Although no observable increase in viral titer was derived from knockout of highly characterized constituents of antiviral pathways (Habayeb et al., 2009b), differential regulation of genes thought to play a part in these pathways may suggest alternate branches are stimulated upon Nora virus infection. Elucidation of the potential antiviral role of the Imd pathway and ROS production in the gut may provide a mechanism for Nora virus clearance. Many questions remain to be answered concerning Nora virus infection of *D. melanogaster*. The data presented is a starting point for determining candidate genes that may have antiviral properties, but this remains to be evaluated. It is likely that some of the genes discussed were induced in response to Nora virus infection, but it is also likely that some of the genes may be responding to stress and/or tissue damage. The results from this study provide evidence that an alternative pathway for viral clearance may be at work, and remains to be fully characterized. To test the antiviral properties and assess increased viral titers of the candidate genes described in this study, publicly available RNAi lines and the UAS-Gal4 system can be employed. The production of monospecific antisera will allow for detection of Nora virus replication within specific tissues and vertical transmission can be investigated.

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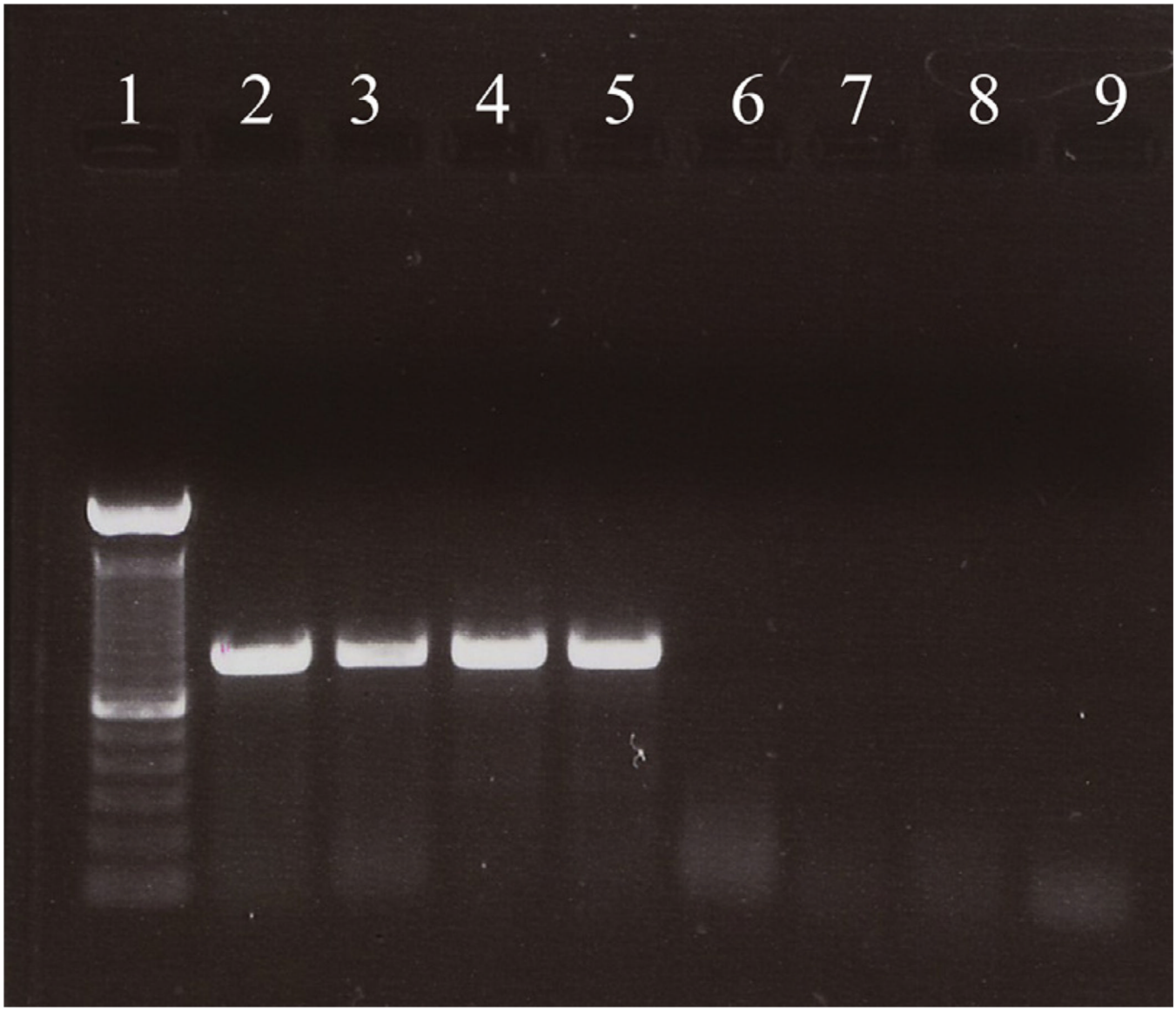

Fig. 1.

RT-PCR verification of presence of Nora virus infection of Canton S wild type *D. melanogaster*. Lane 1: 100 bp DNA marker; lanes 2–5: RNA from Nora virus infected *D. melanogaster* A–D; lanes 6–9: RNA from uninfected *D. melanogaster* A–D. The presence of a PCR product at 790 bp demonstrates a positive result of Nora virus infection in lanes 2–5, whereas, no evidence of a PCR product demonstrates no infection in lanes 6–9.

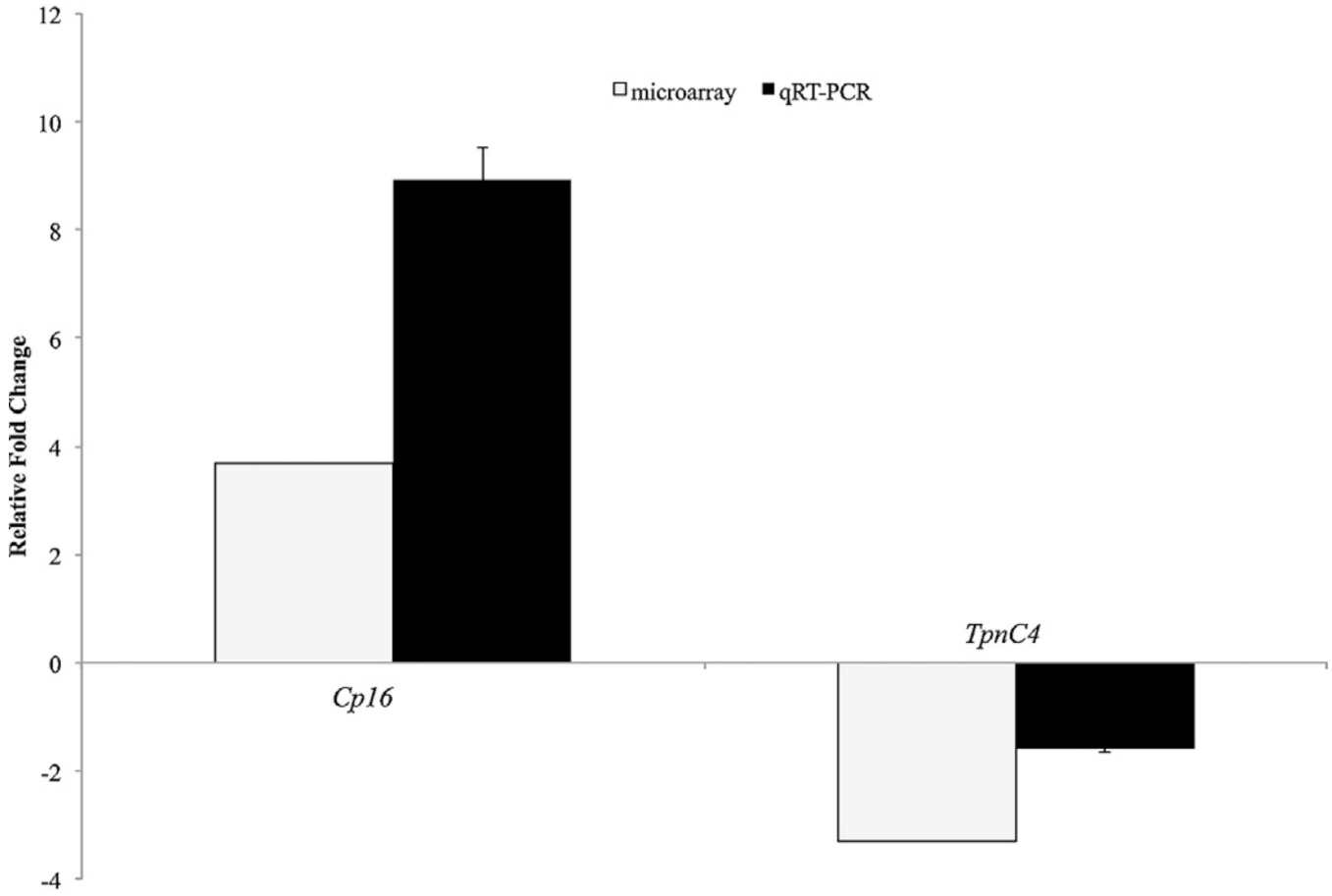

Fig. 2.

Initial validation of microarray data by qRT-PCR analysis of one up-regulated (*Cp16*) and one down-regulated (*TpnC4*) gene. The light gray bars represent the mRNA levels quantified by cDNA microarray and the black bars represent the mRNA levels quantified by qRT-PCR analysis. The data from both analyses is consistent. The error bars represent the standard error of the mean and $n = 3$.

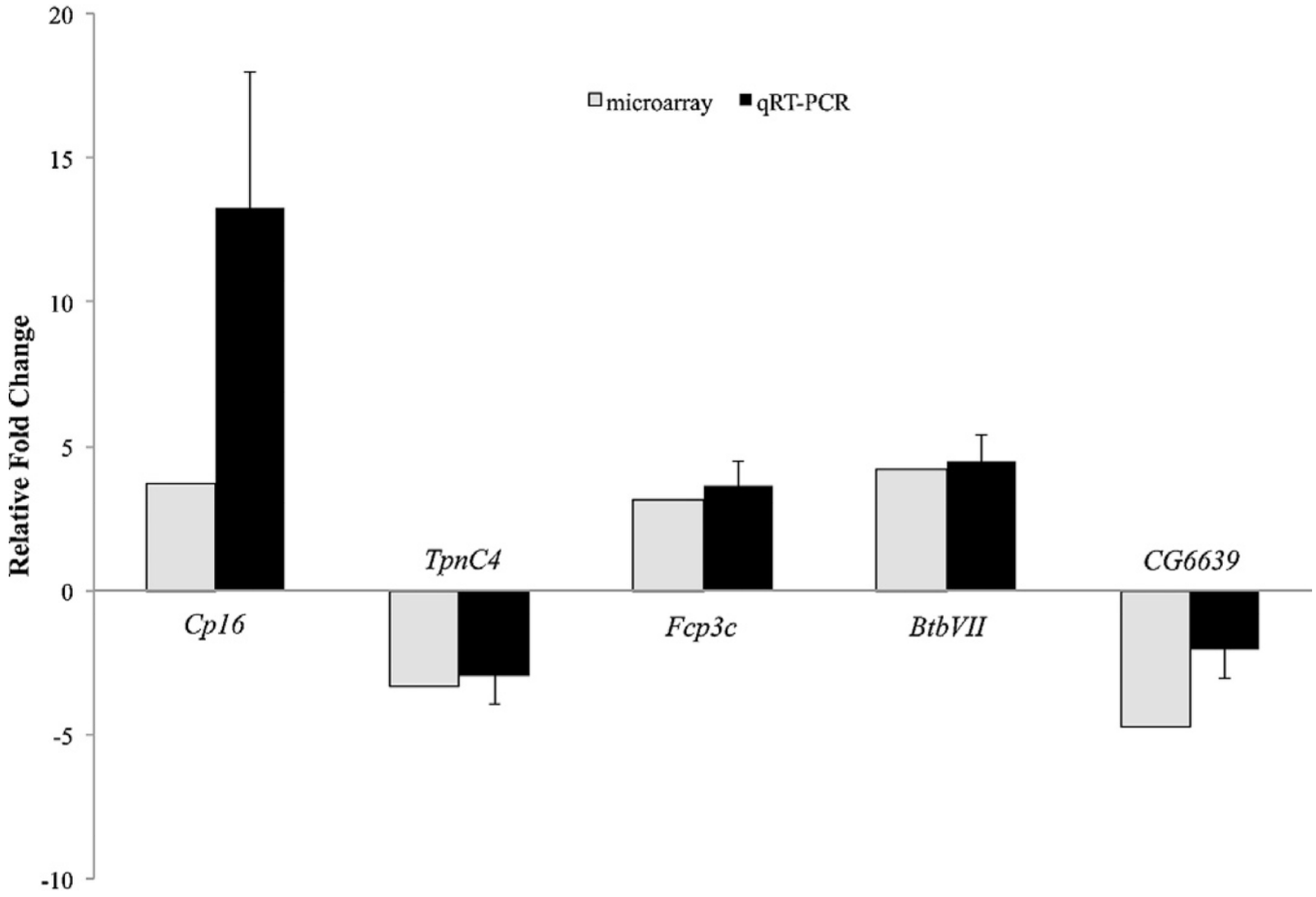

Fig. 3.

Independent infection experiment to validate microarray data by qRT-PCR analysis of three up-regulated (*Cp16*, *Fcp3c*, and *BtbVII*) and two down-regulated (*TpnC4* and *CG6639*) genes. The light gray bars represent the mRNA levels quantified by cDNA microarray and the black bars represent the mRNA levels quantified by qRT-PCR analysis. *Cp16* and *TpnC4* are shown first because they were used in the initial validation and in this experiment to provided consistency and direct data comparison. The data from the cDNA microarray and qRT-PCR analyses is consistent, as are the results between the initial validation and this subsequent experiment. The error bars represent the standard error of the mean and $n = 3$.

Table 1

Genes identified by cDNA microarray analysis as up-regulated by 1.5 fold or greater in Nora virus infected flies as compared to uninfected flies with a *p*-value of less than 0.01. The fold change was determined based on the average results of 3 microarrays and the gene names, gene ID #, and gene biological functions were identified using Flybase and NCBI GenBank.

Gene	Gene ID #	Biological function	Fold Δ
Scp2	42015	GTPase activity	1.505
Vago	32040	Response to virus	1.507
ana2	35878	Mitotic spindle organization; centriole replication; centrosome duplication	1.526
CG6830	41408	Unknown	1.533
CG17906	34224	Unknown	1.534
CG9119	38124	Hydrolase activity; acting on ester bonds; zinc ion binding	1.536
CG10621	35152	Selenocysteine methyltransferase activity	1.568
Muc11A	32174	Chitin binding	1.569
CG7059	42626	Glycolysis; phosphoglycerate mutase activity	1.578
CG7206	32788	Unknown	1.584
CG11257	37227	Heme binding; oxidoreductase activity	1.593
gomdanji	43934	Calcium binding and mediated signaling, courtship behavior;	1.599
CG11079	5740313	Unknown	1.606
CG10814	36481	Gamma-butyrobetaine dioxygenase activity; oxidase reduction	1.607
CG10588	40316	Proteolysis, metalloendopeptidase activity	1.624
Mur11Da	318139	Unknown	1.631
CG14963	38383	Unknown	1.646
CG18607	37213	N-acetyltransferase activity; metabolic processes	1.648
CG13650	43014	Proteolysis; metalloendopeptidase activity	1.648
Mal-B2	34598	Carbohydrate metabolic process; alpha-glucosidase activity	1.668
CG31089	43135	Lipid metabolic process; triglyceride lipase activity	1.672
Hip1	39450	Cytoskeleton organization; actin binding	1.695
GstE7	37112	Glutathione transferase activity	1.702
Cpr67Fb	39225	Structural constituent of chitin-based cuticle	1.708
Mal-A4	35827	Carbohydrate metabolic process; alpha-glucosidase activity	1.729
CG1600	35687	Hypoxia response; oxidoreductase activity, zinc ion binding	1.743
CG32523	318071	Proteolysis; serine-type endopeptidase activity	1.757
CG7763	36235	Binding	1.760
CG15534	43613	Sphingomyelin metabolic process; sphingomyelin phosphodiesterase activity	1.764
CG13084	35229	Unknown	1.791
CG14187	40199	Unknown	1.801
CG1648	36009	Unknown	1.804
CG3290	37539	Metabolic process; alkaline phosphatase activity	1.812
CG31926	326174	Proteolysis; aspartic-type endopeptidase activity	1.861
Amyrel	36863	Carbohydrate metabolic process, oligo-1,6-glucosidase activity; alpha-amylase activity	1.897
CG13309	39012	Unknown	1.902
CG31928	326175	Proteolysis, aspartic-type endopeptidase activity	1.906

Gene	Gene ID #	Biological function	Fold Δ
CG14957	38386	Chitin binding	1.950
Npc2e	326136	Unknown	2.011
CG40337	3354920	Unknown	2.457
Femcoat	31367	Eggshell chorion assembly; structural constituent of chorion	2.659
Cp18	38998	Eggshell chorion assembly; structural constituent of chorion	3.015
BTBVII	38376	DNA binding	3.162
Cp19	39000	Eggshell chorion assembly; structural constituent of chorion	3.593
Cp16	39001	Eggshell chorion assembly; structural constituent of chorion	3.695
Fcp3C	31294	Unknown	4.198

Table 2

Genes identified by cDNA microarray analysis as down-regulated by 1.5 fold or greater in Nora virus infected flies as compared to uninfected flies with a *p*-value of less than 0.01. The fold change was determined based on the average results of 3 microarrays and the gene names, gene ID #, and gene biological functions were identified using Flybase and NCBI GenBank.

Gene	Gene ID#	Gene function	Fold Δ
CG6639	35049	Serine-type endopeptidase activity; proteolysis	−4.739
TpnC4	6539120	Calcium ion binding	−3.3
CG15434	50178	NADH dehydrogenase activity; mitochondrial electron transport-NADH to ubiquinone	−2.445
fln	40185	Contractile fiber	−2.288
CG10332	3771883	Similar to immune induced molecule 18; function unknown	−2.183
CG10910	37052	Unknown	−2.105
Rh5	34615	G protein coupled photoreceptor activity	−1.689
CG15096	37170	Transmembrane transport; high affinity inorganic phosphate: sodium symporter activity	−1.582
CG15818	34019	Unknown; binding	−1.58
CG7298	40210	Structural constituent of peritrophic membrane; chitin metabolic process	−1.538
CG32302	38293	Chitin metabolic process	−1.534
Npc2d	41232	Unknown	−1.52