RESEARCH ARTICLE

Characterization of gene expression regulated by human *OTK18* using *Drosophila melanogaster* as a model system for innate immunity

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Abstract

OTK18 is a human transcriptional suppressor implicated in the regulation of human immunodeficiency virus type-one infection of mononuclear phagocytes. It is ubiquitously expressed in all normal tissues, but its normal homeostatic function is yet to be characterized. One hypothesis is that OTK18 aids in the regulation of the innate immune system. To test this hypothesis, cDNA microarray analysis was performed on the total RNA extracted from *Drosophila melanogaster* embryonic Schneider 2 (S2) cells transfected with either *pEGFP-OTK18* (enhanced green fluorescent protein) or empty vector controls (*pEGFP-N3*) for 6, 12 and 24 h. cDNA microarray analysis revealed differential expression of genes known to be important in regulation of *Drosophila* innate immunity. The expression levels of two genes, *Metchnikowin* and *CG16708* were verified by quantitative real-time reverse transcription PCR. These results suggest a role for OTK18 in innate immunity.

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Introduction

OTK18 is a novel transcriptional suppressor with 13 zinc finger motifs and both Krüppel-associated boxes A and B (Saito *et al*. 1996). It has a specific role in the regulation of human immunodeficiency virus type-one (HIV-1) infection of mononuclear phagocytes (MP) by suppressing viral replication in monocyte-derived macrophages (MDM) (Carlson *et al*. 2004a), being up-regulated in MP of human brain tissue with severe HIV encephalitis (Carlson *et al*. 2004b), and being regulated by interactions with the Tat protein (Carlson *et al*. 2004a). In contrast, *OTK18* is ubiquitously expressed in all normal human tissues, and *OTK18* expression in HIV-1 infected MDM is not significantly different at day 9 post-infection when compared to uninfected controls (Carlson *et al*. 2004b). These contrasting results suggest that OTK18 not only serves a specific role as an antiretroviral molecule, but may also have multiple generalized roles within human development, such as in the innate immune response.

Many model systems are used to study cellular and transcriptional regulation of HIV-1 infection, replication, dissemination, persistence and the elicitation of the immune response. Of these systems *D. melanogaster* provides an excellent opportunity to study the multitudes of issues that surround the development, immune response, human pathologies and neurodegenerative disorders, including prion disease (Raeber *et al*. 1995), polyglutamine-mediated neurodegeneration (Warrick *et al*. 1998), Parkinson's disease (Feany and Bender 2000), Huntington's disease (Sipione and Cattaneo 2001) and Alzheimer's disease (Jackson *et al*. 2002). This has been shown to be true, even if an ortholog of a human gene does not exist in *Drosophila*, as is the case for *OTK18*. In fact, Battaglia *et al*. (2001) used *Drosophila* to characterize Tat-mediated HIV pathogenicity, even though this is strictly a viral gene not found in this organism. Utilization of this system for mechanistic insights into human neurodegenerative disorders and therapeutic interventions is becoming more accepted with the discovery that the mechanisms of neuronal cell death and innate immunity are conserved from invertebrates to humans (Aguzzi and Raeber 1998; Dushay and Eldon 1998; Bonini and Fortini 2003; Driscoll and Gerstbrein 2003; Bilen and Bonini 2005; Tountas and Fortini 2007). In fact, much of what we know about

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innate immunity in vertebrates comes from *Drosophila* studies (Kimbrell and Beutler 2001). When considering the immune response, the major difference between *Drosophila* and vertebrates is the lack of an adaptive immune response in *Drosophila*. This does not diminish the importance of using this model system to study innate immunity, as it is now understood that the innate reactions trigger the adaptive immune responses and orient effector mechanisms of these responses (Fearon and Locksley 1996).

The *Drosophila* Schneider 2 (S2) cell line is ideal for immune studies, because they recapitulate the key features of innate immunity, suggesting, that they were derived from a macrophage-like lineage (Echalier 1997). Exposure of these cells to Gram-negative bacteria results in activation of the immune deficiency (Imd) pathway resulting in a rapid transcription of antimicrobial peptides. Further, S2 cells have the ability to phagocytose both in Gram-positive bacteria and Gram-negative bacteria. Finally, transfection of these cells with a constitutively active form of Toll, *Toll10b*, will fully stimulate the Toll pathway of *Drosophila* immunity (Kleino *et al*. 2005). The strong link of mammalian immunity to *Drosophila*, coupled with the close ties of S2 cells to the *Drosophila* immune response, make them an excellent model to study the regulation of human immunity genes. Overall, the similarities between the pathways regulating immune responses in both *Drosophila* and humans demonstrate that this model system could effectively be used to characterize both the functional and mechanistic roles of *OTK18*.

To gain more insight into the possible functions of *OTK18*, we evaluated the effect of up-regulation of this gene by transfection of S2 cells and subsequent cDNA microarray analysis. Verification of the cDNA microarray results was done by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). cDNA microarray results showed significant down-regulation of *Metchnikowin (Mtk)* and *Cecropin A1* and *A2* (*CecA1* and *CecA2*) genes, responsible for *Drosophila*'s antifungal and anti-Gram negative bacterial responses, respectively (Hoffmann 2003). In addition, up-regulation of the candidate apoptotic gene, *CG16708*, was found (Gorski *et al*. 2003). The qRT-PCR analysis validated these findings by showing down-regulation of *Mtk* and up-regulation of *CG16708*. The data indicates the role for OTK18 in regulation of the innate immune response that complements its previously characterized role as an anti-retrovirus molecule.

Materials and methods

S2 cell culture and transfection

S2 cells (Drosophila Genomics Resource Center, Bloomington, USA) were cultured in T-75 flasks in M3+BPYE media (3.94% Shields and Sang M3 powdered medium; Sigma Aldrich, St Louis, USA), 0.05% potassium bicarbonate (BD Biosciences, San Jose, USA), 0.1% yeast extract (Fisher Scientific, Hampton, USA), 0.25% bactopeptone (Fisher Scientific, Hampton, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco Invitrogen, Grand Island, USA) and 1% penicillin/streptomycin (American Type Culture Collection, Manassas, USA) at 24°C. For transfection, S2 cells were plated at a density of 3×10^6 cells/ml in T-25 flasks for 48 h at 24°C. Later, 250 μl of transfection mix was added to each flask. The mix consisted of M3+BPYE-lacking serum containing 25 μl of GenePORTER 2 reagent (Gelantis, San Diego, USA), and 5 μg of either *pEGFP-N3* (plasmid containing the enhanced green fluorescent promoter, empty vector; Clontech, Palo Alto, USA) or *pEGFP-OTK18* (Carlson *et al*. 2004a) incubated at room temperature for 10 min before adding to the T-25 flask containing the S2 cells. Transfection was carried out for 6, 12, and 24 h. The efficiency of transfection and gene expression was determined by viewing the cells using an inverted fluorescent microscope. After the appropriate length of transfection, the supernatant fraction and cells were collected, and these cells pelleted by centrifugation. The cells were lysed by re-suspending the pellet in either RNAwiz (cDNA microarrays) or TRI Reagent (qRT-PCR; Ambion, Austin, USA), and the lysate frozen at −80°C.

cDNA microarrays

Total RNA samples were sent to the University of Nebraska Medical Center (UNMC), microarray core facility, for processing and analysis. From each sample, 5 μg of total RNA were reverse transcribed using Superscript II (Invitrogen, Carlsbad, USA) to generate single stranded cDNA. The single stranded cDNA was subsequently converted to double stranded cDNA as per the manufacturer's instructions. *In vitro* transcription to generate biotinylated cRNA was performed using T7 RNA polymerase (Invitrogen, Carlsbad, USA). Fifteen μg of fragmented cRNA was hybridized for 16 h to *Drosophila* genome array chips (Affymetrix, Santa Clara, USA) at 45°C using the Affymetrix 640 hybridization oven. These arrays were stained and scanned using a high resolution 3000 scanner as per suggested by standard Affymetrix protocols. Images were analysed with GCOS (GeneChip Operating Software, Affymetrix, Santa Clara, USA). All the images were passed through a standard quality control parameters as defined by Affymetrix. All the comparison analyses were performed using GCOS software.

cDNA microarray analysis

Analyses were conducted with BRB ArrayTools (Simon and Peng Lam 2006). Low level analysis which converts probe level data to gene level expression data was done using robust multiarray average (RMA). RMA was implemented using the *rma* function of the *affy* package of the Bioconductor project (http://www.bioconductor.org/) in the R programming language (Irizarry *et al*. 2004; R development core team 2004). RMA does background correction, normalization and summarization of probe-level data. The background

correction method corrects the perfect match (PM) probe intensities by using a model based on the assumption that the observed intensities are the sum of signal and noise. Quantile normalization is used to normalize the PM probes, and the calculation of summary expression measures was done using the median polish method, which fits a multichip linear model to the data, and gives the expression on the $\log_2$ scale (Irizarry *et al*. 2002). A gene filter was applied prior to the analysis to set a minimum-fold change. A gene was excluded from the analysis if none of the sample expression data values (over all time points) had at least a 1.5-fold change in either direction from the gene's median value. The *pEGFP-OTK18* group was compared to the *pEGFP-N3* group at each of the three time points. For each gene, a student's *t*-test with a random variance model was used to determine if there was a significant difference in expression between the groups for that gene. To help them control the false discovery rate, an alpha level of 0.001 was used for comparison.

Validation of cDNA microarrays by qRT-PCR

Frozen lysates were thawed, and the RNA was extracted using a RiboPure kit (Ambion Austin, USA), according to manufacturer's instructions. Reverse transcription was performed using TaqMan Gene Expression Assay kit and the 7500 Real Time PCR system (Applied Biosystems, Foster City, USA) according to manufacture's instructions. The primer and probe sets used were *Mtk* (assay #Dm01821460_s1), *CG16708* (assay #Dm02151827_g1), *OTK18* (assay #Hs00232535_m1), and *Rp49* (*Ribosomal protein 49*; endogenous control; assay #Dm02151827_g1). Reactions were carried out in triplicate and performed in a 50 μl volume utilizing 200 ng total RNA samples and TaqMan One-Step RT-PCR mix (Applied Biosystems, Foster City, USA). 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 analysed in the linear range for amplification with *Rp49* using the 7500 RT-PCR System Sequence Detection Software (Applied Biosystems, Foster City, USA). The relative quantitative results were used to determine changes in gene expression in *pEGFP-OTK18* and *pEGFP-N3* transfected S2 cells on a $\log_2$ scale. For each gene tested, a one-tailed student's *t*-test for unpaired data with an alpha level of 0.05 was performed.

Results

cDNA microarray analysis

cDNA microarray analysis was performed at 6, 12, and 24 h posttransfection with either *pEGFP-OTK18* or *pEGFP-N3*. After a minimum-fold change filter was applied, 310 genes were available for analysis from all three time points. At 6 h, no genes were up-regulated by *OTK18* expression at the 0.001 level. However, three genes were significantly suppressed at the 0.001 level. *Smrter* was down-regulated 1.3 fold (P = 0.0004), *Hormone receptor-like in 38 (HR38)* was down-regulated 1.3 fold (P = 0.0005), and *mastermind (mam)* was down-regulated 1.3 fold (P = 0.0006). At 24 h, there were no genes significantly down-regulated by *OTK18* expression at the 0.001 level. However, one gene, *slamdance (sda)* was significantly up-regulated (1.4 fold, P = 0.00007) at the 0.001 level.

At 12 h, 53 genes were significantly up-regulated or down-regulated at the 0.001 level. Of these, 31 were significantly up-regulated (table 1) and 22 were significantly down-regulated (table 2). The biological function of the up-regulated and down-regulated genes are shown in tables 3 and 4, respectively. The up-regulated genes (the numbers in parentheses are number of genes in group) include cell death or proteolysis (2), metabolism (7), cation, phospholipids, or electron transport (5), DNA replication or methylation (2), development (2), tRNA modification (1), gene transcription regulation (1), nuclear envelope reassembly (1), metal ion binding (1), anterior–posterior axis specification (1), and unknown function (7). The biological functions of the down-regulated genes (the numbers in parentheses are number of genes in group) include immune/defense response (3), regulation of gene transcription or signal transduction (8), transport (2), chaperone or metal ion binding (2), proteolysis or endocytosis (2), DNA replication (1), protein targeting (1), and unknown function (4).

Table 1. Gene profiles activated at 12 h posttransfection with *pEGFP-OTK18*[a].

Gene symbol	*pEGFP-OTK18*[b]	*pEGFP-N3*[c]	Fold activation[d]
CG32649	334.8	187.1	1.8
ScpX	411.5	242.5	1.7
Orct	1714.3	980.4	1.7
CG15897	694.0	413.6	1.7
CG16708	308.3	189.2	1.6
CG9666	331.3	210.9	1.6
Orct2	990.6	632.0	1.6
CG10575	114.9	71.6	1.6
CG9006	578.2	371.0	1.6

Table 1 *(contd.)*

Gene symbol	*pEGFP-OTK18*[b]	*pEGFP-N3*[c]	Fold activation[d]
CG9743	556.6	373.4	1.5
CG2669	452.1	292.8	1.5
Gfat2	1739.5	1126.9	1.5
CG4045	1186.5	786.0	1.5
CG10962	141.4	92.1	1.5
CG14777	396.4	258.7	1.5
CG11788	302.9	198.3	1.5
CG17209	146.8	97.9	1.5
CG5325	2235.7	1473.2	1.5
CG9581	678.1	465.3	1.5
Tam	161.9	110.3	1.5
Cyp9c1	251.8	181.9	1.4
Pi3K59F	186.5	135.3	1.4
Ote	969.7	681.5	1.4
Mcm3	377.5	274.9	1.4
Regucalcin	2066.1	1515.0	1.4
CG1516	735.0	541.9	1.4
Noa36	402.4	284.9	1.4
CG4095	1474.1	1028.9	1.4
CG7530	796.1	623.7	1.3
CG5412	397.9	299.2	1.3
AcCoAS	472.4	359.2	1.3

[a]The ratio of *pEGFP-OTK18/pEGFP-N3* is shown as fold activation. *pEGFP-OTK18*[b] and *pEGFP-N3*[c] are the geometric mean of intensities on a $\log_2$ scale ($n = 3$). [d]$P \leq 0.001$

Table 2. Gene profiles suppressed at 12 h posttransfection with *pEGFP-OTK18*[a].

Gene symbol	*pEGFP-OTK18*[b]	*pEGFP-N3*[c]	Fold suppression[d]
Mtn A	791.4	1319.9	1.7
Mtk	762.2	1202.6	1.6
CG11279	131.7	206.7	1.6
Maf-S	448.1	729.7	1.6
CG6770	410.2	616.8	1.5
CG10635	341.1	500.2	1.5
CG18619	394.3	596.2	1.5
CG8498	785.6	1191.9	1.5
Zfh2	126.2	185.7	1.5
CecA2	1281.1	1894.7	1.5
CG5896	219.9	334.9	1.5
CG18081	247.7	380.4	1.5
Sun	891.4	1336.5	1.5
CecA1	1994.3	2717.5	1.4
MED26	132.7	182.3	1.4
CG14464	525.0	751.3	1.4
HmgZ	225.8	326.8	1.4
p120ctn	382.4	532.5	1.4
Mes4	122.7	167.9	1.4
CG11825	17.0	22.3	1.3
Ank	117.8	153.3	1.3
Prx2540-1	443.1	590.8	1.3

[a]The ratio of *pEGFP-N3/pEGFP-OTK18* is shown as fold suppression. *pEGFP-OTK18*[b] and *pEGFP-N3*[c] are the geometric mean of intensities on a $\log_2$ scale ($n = 3$). [d]P value ≤ 0.001

Table 3. Biological function of genes activated at 12 h posttransfection with *pEGFP-OTK18*.

Gene symbol	FlyBase ID no.	Selected annotated biological function[a]
CG32649	FBgn0052649	Coenzyme metabolism and mitochondrial electron transport
ScpX	FBgn0015808	Phospholipid transport
Orct	FBgn0019952	Cation transport
CG15897	FBgn0029857	Unknown
CG16708	FBgn0037315	Autophagic cell death
CG9666	FBgn0036856	DNA methylation
Orct2	FBgn0029146	Cation transport
CG10575	FBgn0035632	Unknown
CG9006	FBgn0021848	Acyl-CoA dehydrogenase
CG9743	FBgn0039756	Fatty acid biosynthesis and metabolism
CG2669	FBgn0037316	Unknown
Gfat2	FBgn0039580	Monosaccharide and carbohydrate metabolism
CG4045	FBgn0025629	tRNA modification
CG10962	FBgn0030073	Metabolism
CG14777	FBgn0026872	Multiple roles in development
CG11788	FBgn0034495	Unknown
CG17209	FBgn0030687	Regulation of gene transcription
CG5325	FBgn0032407	Unknown
CG9581	FBgn0031093	Proteolysis
Tam	FBgn0004406	DNA replication
Cyp9c1	FBgn0015040	Electron transport
Pi3K59F	FBgn0015277	Protein targeting, endocytosis, and phosphoinositide phosphorylation
Ote	FBgn0003022	Nuclear envelope reassembly
Mcm3	FBgn0024332	Prereplicative complex formation and maintenance, and roles in development
Regucalcin	FBgn0030362	Anterior–posterior axis specification
CG1516	FBgn0027580	Fatty acid biosynthesis, gluconeogenesis, and pyruvate metabolism
Noa36	FBgn0026400	Metal ion binding
CG4095	FBgn0029890	Fumarate metabolism and tricarboxylic acid cycle
CG7530	FBgn0038256	Unknown
CG5412	FBgn0038806	Unknown
AcCoAS	FBgn0012034	Fatty acid metabolism

[a]Gene names and biological functions were obtained from FlyBase (Crosby *et al*. 2007).

Table 4. Biological function of genes suppressed at 12 h posttransfection with *pEGFP-OTK18*.

Gene symbol	FlyBase ID no.	Selected annotated biological function[a]
Mtn A	FBgn0002868	Metal ion binding
Mtk	FBgn0014865	Gram positive antibacterial and antifungal immune responses
CG11279	FBgn0036342	Unknown
Maf-S	FBgn0034534	Regulation of gene transcription
CG6770	FBgn0032400	Unknown
CG10635	FBgn0035603	Chaperone binding
CG18619	FBgn0032202	Regulation of gene transcription
CG8498	FBgn0031992	Acyl-CoA homeostasis and lipid transport
zfh2	FBgn0004607	Regulation of gene transcription

Table 4 *(contd.)*

Gene symbol	FlyBase ID no.	Selected annotated biological function[a]
CecA2	FBgn0000277	Gram negative antibacterial and antifungal immune responses
CG5896	FBgn0039494	Proteolysis
CG18081	FBgn0036537	Regulation of gene transcription
Sun	FBgn0014391	ATP synthesis coupled proton transport
CecA1	FBgn0000276	Gram negative antibacterial and antifungal immune responses
MED26	FBgn0039923	Regulation of gene transcription
CG14464	FBgn0033000	Unknown
HmgZ	FBgn0010228	Regulation of gene transcription
p120ctn	FBgn0015587	Cell adhesion and signal transduction
Mes4	FBgn0034726	DNA replication
CG11825	FBgn0033519	Unknown
Ank	FBgn0011747	Cytoskeletal anchoring and signal transduction
Prx2540-1	FBgn0033520	Oxygen and reactive oxygen species metabolism and defense response

[a]Gene names and biological functions were obtained from FlyBase (Crosby *et al*. 2007).

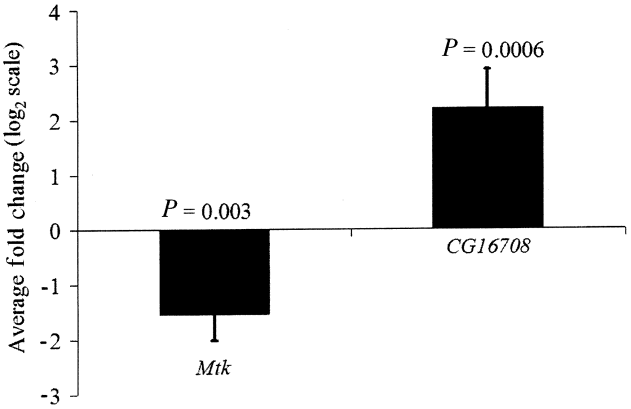

Figure 1. qRT-PCR of *Mtk* and *CG16708* at 12 h posttransfection of S2 cells transfected with *pEGFP-OTK18*. *Mtk* is significantly down-regulated in *pEGFP-OTK18* transfected cells versus *pEGFP-N3* controls, whereas *CG16708* is significantly up-regulated in *pEGFP-OTK18* transfected cells versus *pEGFP-N3* controls. $n = 9$.

qRT-PCR analysis validation of cDNA microarrays

To validate the microarray results seen at 12 h posttransfection, qRT-PCR was performed. We chose one up-regulated gene (*CG16708*) and one down-regulated gene (*Mtk*) for which premade TaqMan expression kits Applied Biosystems, Foster City, USA were available to validate results. *Mtk* was selected because it is immune inducible, and *CG16708* was chosen because it is a candidate apoptotic gene. Human *OTK18* was assayed with a premade TaqMan expression kit to assess up-regulation in the *pEGFP-OTK18* transfected S2 cells and was significantly up-regulated ($P = 9.0 \times 10^{-12}$) versus *pEGFP-N3* transfected cells and the levels were similar to those published for HIV-1 infected human monocytes (Carlson *et al*. 2004a). The *Mtk* was significantly suppressed 1.5 fold ($P = 0.003$), and *CG16708* was significantly activated 2.2 fold ($P = 0.0006$) in *pEGFP-OTK18* transfected cells versus *pEGFP-N3* controls (figure 1).

Discussion

In this paper, we report the characterization of differential gene expression of the human gene, *OTK18*, via transfection of *Drosophila* S2 cells and cDNA microarray analysis. The effects of *OTK18* up-regulation on various immune components, including *Mtk, CecA1* and *CecA2*, and *CG16708*, an apoptotic gene candidate, were determined. The cDNA microarray results were validated with a confirmatory qRT-PCR study. Our results suggest a potential role for *OTK18* in the human innate immune response, as the production of *Drosophila* anti-microbial peptides (AMPs), such as *Mtk*, *CecA1* and *CecA2*, are an important aspect of host defense in this organism. AMPs play a role in adaptive immunity in invertebrates as well as in vertebrate (Bulet *et al*. 2004). A role for *OTK18* in the immune response by showing suppression of retroviral replication due to *OTK18* up-regulation in HIV-1 infected MDM has been reported (Carlson *et al*. 2004a). Interestingly, our results also suggest a possible role for *OTK18* in apoptotic regulation, due to the up-regulation of *CG16708*. Apoptosis is important in various immune responses, such as, those involved in viral infections and tumorogenesis. Studies in multiple organisms, including *Drosophila*, indicated that the apoptotic molecular machinery is conserved between all metazoan cells (Renehan *et al*. 2001).

In this study, cDNA microarray analyses were performed at 6, 12, and 24 h posttransfection with either *pEGFP-OTK18* or *pEGFP-N3* to determine the length of exposure needed to achieve differential gene regulation. At 6 h, no genes were

significantly up-regulated; however, three genes were significantly suppressed. The small number of genes differentially regulated at this time point indicates that at 6 h the level of *OTK18* being produced by the S2 cells was probably not sufficient to initiate an advanced immune response. The observation that the three genes found were down-regulated and not up-regulated is expected since *OTK18* has been characterized to have transcriptional suppressive activity (Carlson *et al*. 2004a). Of these, *Smrter* and *mam* are of interest, because of their roles in the Notch signaling pathway (Helms *et al*. 1999; Tsuda *et al*. 2002). The other gene, *HR38*, is involved in regulation of gene transcription and ecdysteroid signalling (Kozlova *et al*. 1998). The Notch signaling pathway is involved in many developmental processes including stem cell selfrenewal, proliferation, specification, differentiation, and apoptosis (Lasky and Wu 2005). It was originally discovered in *Drosophila*, but is found in many organisms including humans (Artavanis-Tsakonas *et al*. 1999). Dysregulation of the Notch pathway is also implicated in human diseases including, T-cell leukemia, cortical dysplasia, and brain tumorgenesis (Lasky and Wu 2005). Both *Smrter* and *mam*, have orthologs in humans. *Smrter* produces a corepressor that complexes with suppressor of Hairless (Su(H)) in the Notch pathway (Tsuda *et al*. 2002). Loss of Notch function causes these cells to assume the default neuronal fate rather than an epidermal fate (Artavanis-Tsakonas *et al*. 1999). The *mam* gene product is one of the key elements in the Notch pathway. Mam proteins bind to and also stabilize the DNA-binding complex of the intracellular domain of Notch and Su(H). In mammals, Mam-1 is essential for development of marginal zone B cells and partially required for the development from $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ thymocytes (Oyama *et al*. 2007). In human HIV-1 infected MDM, overexpression of *OTK18* led to a significant down-regulation of *CD4* expression (Carlson *et al*. 2004a). These results suggest that *OTK18* may play a role in the specification and differentiation of cell lineages, specifically $CD4^{+}$ cells, by down-regulation of both *Smrter* and *Mam* expression. The outcome of this dysregulation is entirely dependent upon OTK18's specific role in this pathway, which has yet to be characterized.

At 24 h, no genes were significantly down-regulated by *OTK18* expression at the 0.001 level. However, *sda*, a gene involved in proteolysis was significantly up-regulated. The *sda* gene codes for *Drosophila aminopeptidase N (APN)*, a homolog to human *APN* (Zhang *et al*. 2002). Human APN has a role in antigen processing and presentation (Falk *et al*. 1994), cell differentiation of monocytes to macrophages, and mitogenic activation of lymphocytes (Kido *et al*. 1999). This suggests that *OTK18* up-regulation may be functioning in an alternate pathway suppressing the normal repressor of *sda* in an attempt to curtail cell death. Further, suggesting that *OTK18* may play a role in protecting the cell from apoptosis, a function that needs to be further characterized.

The results of this study demonstrate that 12 h posttransfection was the optimal time point for differential expression of genes due to *OTK18* overexpression. At 12 h, 31 genes were significantly up-regulated (table 1) and 22 genes were significantly down-regulated (table 2). The biological function of these differentially regulated genes was diverse (tables 3 and 4). From the cDNA microarray data, we chose two candidate genes, one down-regulated immune inducible gene (*Mtk*) and the other an up-regulated apoptotic gene candidate (*CG16708*) to verify and validate by qRT-PCR. We chose these two genes because premade TaqMan gene expression kits were available. The qRT-PCR analyses revealed the same results that were found in the cDNA microarray analysis at this time point, therefore validating the cDNA microarray results. Human *OTK18* was also assayed with a premade TaqMan gene expression kit to assess up-regulation in the *pEGFP-OTK18* transfected S2 cells. The results demonstrate a significant increase ($P = 9.0 \times 10^{-12}$) in *OTK18* expression in *pEGFP-OTK18* transfected cells versus *pEGFP-N3* transfected cells. This verified that the S2 cells were producing *OTK18* message at a high level and that there was no contamination of the negative controls (*pEGFP-N3*).

The down-regulation of *Mtk, CecA1*, and *CecA2*, is consistent with *OTK18* induced down-regulation of immune response genes in human HIV-1 infected MDM found previously (Carlson *et al*. 2004a). These three genes do not have homologs in humans, but AMPs demonstrate ubiquitous distribution throughout the animal kingdom. Molecular characterizations of the regulatory pathways of AMPs in *Drosophila* have provided insight into innate immunity in mammals. For example, the role of the *Drosophila* Toll receptor was paramount in uncovering the related molecule in humans (Tzou *et al*. 2000). AMPs are as important in host defense in humans as in *Drosophila*. Humans express a number of AMPs, including human α-defensins HD-5 and HD-6 (Mallow *et al*. 1996), two β-defensins (Goldman *et al*. 1997; Bals *et al*. 1998a), the cahelicidin LL-37 (Bals *et al*. 1998b), and histatins secreted in the saliva (Edgerton *et al*. 1998). Overall, this suggests that *OTK18* may play a role in the regulation of immune response-related genes or AMPs and in the innate immune response as a whole. This is an avenue of study that deems further intensive investigation.

Lastly, *CG16708*, a candidate apoptotic gene (Gorski *et al*. 2003), was found to be up-regulated at 12 h, suggesting a role for *OTK18* in regulation of cell death. OTK18 blocks HIV-1 tat long terminal repeat (LTR) promoter activity by binding to two distinct regions, the negative-regulatory element at $-255/-238$ and the Ets-binding site (EBS) at $-150/-139$. This reduces the production of progeny viruses in infected macrophages (Horiba *et al*. 2007). Further, up-regulation of the proapoptotic candidate gene *CG16708* could be another anti-viral defense mechanism as one of the primary methods to control the viral infections is apoptosis induced via natural killer cells and cytotoxic T-lymphocytes. The data uncovered in this experiment warrants further research into the possibility of a carryover for the role of *OTK18* in retroviral apoptosis along with the potential for

discovering a new apoptotic pathway or better defining an existing one. Our results support the role for *OTK18* as a generalized transcriptional suppressor with possible roles in the innate immune response, Notch signaling, and apoptosis, to name a few. The up-regulation and down-regulation of different classes of genes demonstrates the duality of *OTK18*. It may directly induce transcriptional suppression of specific candidate genes (*Mtk*), or working through other cell factors and components of signalling pathways, may suppress the transcriptional suppressor of other important developmental genes resulting in up-regulation (*CG16708*).

The potential implications shown in this study are not only for immunoprotective, but also for immunocompromising roles for OTK18 in human infections should be evaluated. In addition, a role for *OTK18* regulation of innate immune response genes and AMPs should be studied. Our data provides support for the hypothesis that *OTK18* has a role in the functioning of the innate immune system.

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