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We previously carried out T2D linkage analysis in the families of many of our stage 1 cases (10). None of the 10 loci in Table 1 had large T2D logarithm of the odds (LOD) scores, although those for *FTO* and *TCF7L2* were 0.63 and 0.60 and so were nominally significant. LOD scores for six of the 10 loci were greater than 0.2, as compared to 2.2 that would be expected for random genome locations. This suggests enrichment for T2D-associated loci in regions with modest evidence of T2D linkage ($P = 0.01$) but that the power of the linkage approach was insufficient to distinguish these signals from background noise.

The ability to construct a list of ten robust and replicated T2D-associated loci (Table 1) represents a landmark in efforts to identify genetic variants that predispose to complex human diseases, although the specific predisposing variants and even the relevant genes remain to be defined. We examined the combined risk of T2D based on these 10 loci in our stage 1 + 2 sample by constructing a logistic regression model and predicting T2D risk for each person (8). We found a fourfold variation in T2D risk from the lowest to highest predicted risk groups, which is of potential interest for a personalized preventive-medicine program (Fig. 2). However, these predictions from our data may be biased as compared to predictions based on the general population, likely owing to the overestimation of ORs due to the “winner’s curse,” enrichment for familial T2D cases, and exclusion of individuals with impaired glucose tolerance or impaired fasting glucose.

Thirty years ago, James V. Neel labeled T2D as “the geneticist’s nightmare” (32), predicting that the discovery of genetic factors in T2D would be thoroughly challenging. Until recently, his prediction has proven true. Although large samples and collaboration among three groups were required, we can confidently state that new diabetes risk factors have been identified. Each gene discovery points to a pathway that contributes to pathogenesis, and all of these proteins and their relevant pathways represent potential drug targets for the prevention or treatment of diabetes. Based on the number of other interesting results observed in these studies, it is likely that there are additional T2D-predisposing loci to be found. Even though much remains to be done, we are at last awakening from Jim Neel’s nightmare.

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Complex I Binding by a Virally Encoded RNA Regulates Mitochondria-Induced Cell Death

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Human cytomegalovirus infection perturbs multiple cellular processes that could promote the release of proapoptotic stimuli. Consequently, it encodes mechanisms to prevent cell death during infection. Using rotenone, a potent inhibitor of the mitochondrial enzyme complex I (reduced nicotinamide adenine dinucleotide–ubiquinone oxidoreductase), we found that human cytomegalovirus infection protected cells from rotenone-induced apoptosis, a protection mediated by a 2.7-kilobase virally encoded RNA (β 2.7). During infection, β 2.7 RNA interacted with complex I and prevented the relocalization of the essential subunit genes associated with retinoid/interferon-induced mortality–19, in response to apoptotic stimuli. This interaction, which is important for stabilizing the mitochondrial membrane potential, resulted in continued adenosine triphosphate production, which is critical for the successful completion of the virus’ life cycle. Complex I targeting by a viral RNA represents a refined strategy to modulate the metabolic viability of the infected host cell.

During primary infection or reactivation of human cytomegalovirus (HCMV), especially in the immunocompromised, the virus is able to replicate in a number of cell types, often resulting in life-threatening disease (1). HCMV exhibits a relatively protracted life cycle (upwards of 5 days) and at early times of infection (12 to 24 hours) encodes a highly abundant 2.7-kb RNA transcript (β 2.7), accounting for >20% of total viral gene transcription (2, 3) of unknown function. The RNA may be associated with mitochondria (4), and no protein product of this RNA has ever been detected in

infected cells (3), suggesting that it functions as a noncoding RNA (5).

We investigated the possibility that β 2.7 could function as a noncoding RNA. A

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Northwestern screen of a human cDNA library with a $\beta 2.7$ probe identified potential cellular interaction partners for the $\beta 2.7$ RNA molecule. One of these proteins was a subunit of the mitochondrial enzyme complex I (reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase). Defective complex I activity has been implicated in numerous mitochondrial and genetic diseases, including Leigh's syndrome, Leber's hereditary optic neuropathy, and mitochondrial encephalopathy (6); and inhibition of complex I activity by reactive O or N species or by the direct binding of environmental toxins ultimately results in apoptosis (7, 8).

We first tested whether HCMV infection [multiplicity of infection (MOI) = 5] and, specifically, $\beta 2.7$ expression prevented cell death in neuronal U373 cells subjected to mitochondrial stress by treatment with rotenone—a highly effective complex I inhibitor (9, 10). As expected, the addition of rotenone promoted substantial cell death in U373 cells (70% in Fig. 1). We then compared the effect of preinfection of cells with the Toledo strain of HCMV and a recombinant Toledo virus, in which the $\beta 2.7$ gene had been deleted ($\Delta\beta 2.7$ Tol) (5). Toledo-infected U373 cultures showed profoundly ($P < 0.001$) reduced levels of apoptosis (3%), in contrast to $\Delta\beta 2.7$ Tol-infected cells (70% in Fig. 1A). Furthermore, the protective effect of $\beta 2.7$ could be restored with a revertant virus (Fig. 1A) and by transfection of a $\beta 2.7$ expression vector into U373 cells (Fig. 1B). Thus, HCMV-mediated protection of cells from rotenone-induced apoptosis correlated with expression of the viral $\beta 2.7$ gene. Although the consensus is that the $\beta 2.7$ transcript does not

encode a protein product (5), we also analyzed a clinical isolate of HCMV (HCMV-3157), which would produce a heavily truncated nonsense protein if $\beta 2.7$ were translated (5). HCMV-3157 was as efficient as Toledo at protecting cells from rotenone-induced death (fig. S1A). The protective effect of $\beta 2.7$ was observed only in Toledo-infected cells (fig. S1B), whereas $\Delta\beta 2.7$ Tol-infected cells routinely stained for both viral gene expression and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) (fig. S1B). The proportion of immediate-early (IE)—positivity in the $\Delta\beta 2.7$ Tol cells undergoing apoptosis was around 70% (fig. S1C). Given that only 45% of the whole population was infected, this finding implied that viral infection, in the absence of $\beta 2.7$, actually rendered cells more sensitive to rotenone-induced apoptosis.

One role of the HCMV $\beta 2.7$ transcript may be to mediate protection of the cell from apoptotic pathways activated by metabolic stress of complex I. Genes associated with retinoid/interferon-induced mortality (GRIM)-19 is a subunit of complex I that is essential for its assembly and function (11). HCMV infection up-regulates steady-state mRNA levels of some subunits of mitochondrial complexes I to V (12), but virus infection has no impact on GRIM-19 protein expression, up to 120 hours post infection (hpi) in U373 cells (fig. S2, A and B). We did, however, detect changes in GRIM-19 localization in response to rotenone and virus infection. In U373 cells, GRIM-19 expression appeared to be diffuse throughout the cytoplasm of the cell (Fig. 2A), and it be-

came relocalized into discrete perinuclear clumps after the addition of rotenone to uninfected U373 cells. However, preinfection with Toledo prevented this relocalization (Fig. 2A). GRIM-19 is known to localize to the nucleus under certain conditions (13), and it also interacts with the signal transducer and activator of transcription (STAT)-3 protein to prevent the nuclear import of STAT-3 in a perinuclear location (14). Although the GRIM-19 in Toledo-infected U373 remained predominantly cytoplasmic within the cell, it did exhibit a more punctuate pattern within the cytoplasm (Fig. 2A), perhaps suggesting more association with mitochondria. However, HCMV itself has profound effects on mitochondrial shape and localization after infection (15), and this may also partially account for the difference in GRIM-19 staining. In contrast, in $\Delta\beta 2.7$ Tol-infected cells, the rotenone-induced perinuclear relocalization of the GRIM-19 protein was still observed (Fig. 2A), identical to that observed with uninfected cells treated with rotenone (d in Fig. 2A). Thus, rotenone-induced mitochondrial stress promotes the relocalization of GRIM-19 within the cell, which can be abrogated by expression of the HCMV $\beta 2.7$ transcript.

We next tested whether this effect on GRIM-19 relocalization was due to a physical interaction between the GRIM-19 protein and $\beta 2.7$ RNA during infection. $\beta 2.7$ RNA was specifically immunoprecipitated from infected cells with an antibody to GRIM-19 (Fig. 2B). We also observed an interaction with native complex I, which is found only on the inner mitochondrial membrane, suggesting that the interaction between $\beta 2.7$ RNA and GRIM-19 targets complex I in mitochondria. No immunoprecipitation of a similarly abundant viral RNA (IE72 in Fig. 2, B and C) with GRIM-19, complex I, or complex V (Fig. 2C) was observed. As expected, analyses using the $\Delta\beta 2.7$ Tol virus showed no $\beta 2.7$ -specific polymerase chain reaction (PCR) band (Fig. 2D).

In a reciprocal analysis, we captured $\beta 2.7$ RNA with the use of biotin-labeled oligonucleotide probes (Fig. 2E). Immunoprecipitations (IPs) of the $\beta 2.7$ -captured complexes did contain GRIM-19 (Fig. 2F). Thus, $\beta 2.7$ RNA specifically interacts with GRIM-19, but with few other proteins in vivo.

The observed physical interaction with GRIM-19 and complex I was investigated further. Active complex I supports the formation of an electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membrane, which is imperative for the efficient production of adenosine triphosphate (ATP) (16). Expression of $\beta 2.7$ RNA during infection or transfection protected $\Delta\psi$ stability from rotenone (fig. S3, A and B), suggesting that the $\beta 2.7$ RNA interaction with complex I could affect mitochondrial energy production under oxidative stress after infection. Previous data have shown

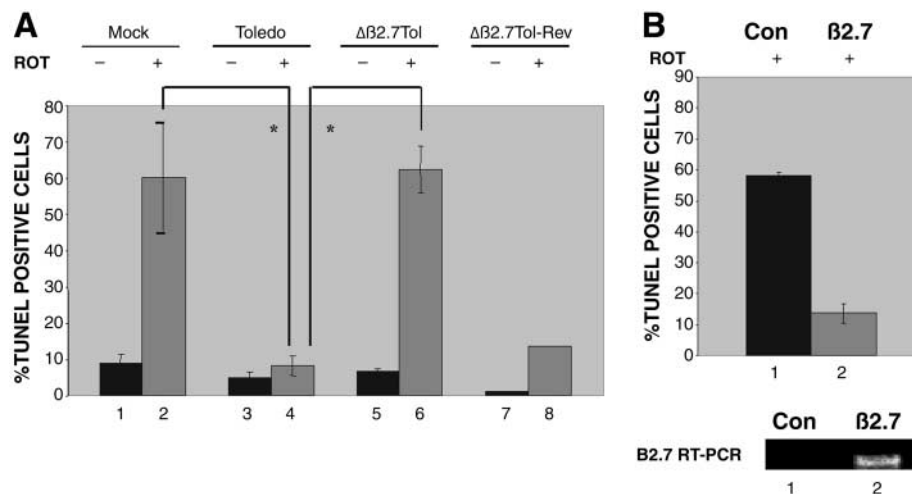


Fig. 1. HCMV protects cells from rotenone-induced apoptosis. **(A)** Percentage of TUNEL-positive cells in mock- (1 and 2), Toledo- (3 and 4), $\Delta\beta 2.7$ Tol- (5 and 6), and $\Delta\beta 2.7$ Tol-Revertant ($\Delta\beta 2.7$ Tol-Rev)-infected (7 and 8) U373 cells incubated with acetone (1, 3, 5, and 7) or rotenone (ROT) (2, 4, 6, and 8). $n = 3$ independent experiments. Asterisks denote $P < 0.01$ with Student's t test. Error bars indicate 1 SEM. **(B)** Percentage of TUNEL-positive pREP10- (Con) (1) and pREP10- $\beta 2.7$ -transfected (2) cells incubated with rotenone. from three independent analyses. Reverse transcription (RT)-PCR for $\beta 2.7$ expression in pREP10- (lane 1) and pREP10- $\beta 2.7$ -transfected (lane 2) cells is shown.

that, unlike in herpes simplex virus (17), ATP levels in HCMV-infected cells are maintained at 24 hpi (18), a requirement probably attributable to HCMVs' comparatively protracted growth phase (19). We therefore analyzed the role of $\beta 2.7$ in ATP production.

Rotenone substantially reduced ATP production in U373 cells (75% reduction, 1 and 2 in Fig. 3A). However, in Toledo-infected cells, only a 1.2-fold reduction was observed (84 to 67% in Fig. 3A), suggesting that HCMV protected ATP production in infected cells. In $\Delta\beta 2.7$ Tol-infected cells, rotenone treatment resulted in a 2.5-fold depletion of intracellular ATP (77 to 33% in Fig. 3A). At 6 hpi, before $\beta 2.7$ RNA expression, no protection from rotenone-induced ATP depletion occurred (fig. S4). Using a second strain of HCMV AD169 that encodes two copies of the

$\beta 2.7$ gene, we eliminated the possibility that green fluorescent protein (GFP) expression from the $\beta 2.7$ gene locus would have a phenotypic effect, because a recombinant AD169 virus still expressing GFP and one functional copy of the $\beta 2.7$ gene maintained ATP levels (Fig. 3A). Thus, the $\beta 2.7$ transcript is important for maintaining ATP production.

We hypothesized that the impact of $\beta 2.7$ expression on ATP production may be more profound at later times of infection. Analysis in the absence of rotenone showed that ATP levels in the $\Delta\beta 2.7$ Tol dropped significantly ($P < 0.01$) at 5 days post infection (dpi), in direct contrast with Toledo-infected cells. Confirmation that the difference was not an artifact of GFP production was performed with the AD169-GFP-infected cells, in which the levels of ATP were

comparable to those of the parent virus AD169 (Fig. 3B).

Deletion of the viral $\beta 2.7$ gene from HCMV has no significant effects on growth kinetics in fibroblasts (5) (Fig. 4A). At first, this appears to be contradictory; however, fibroblasts are particularly resistant to the induction of apoptosis by rotenone and oxidative stress (20), as compared with neuronal cells (21). However, a $\Delta\beta 2.7$ Tol growth defect was observed when compared with the Toledo virus (Fig. 4B), which was more profound in the presence of rotenone (Fig. 4B), as is entirely consistent with the $\beta 2.7$ transcript supporting virus production in times of metabolic stress. Tissue culture was performed in glucose-enriched media, and fibroblasts, particularly in times of diminished ATP production from the electron transport

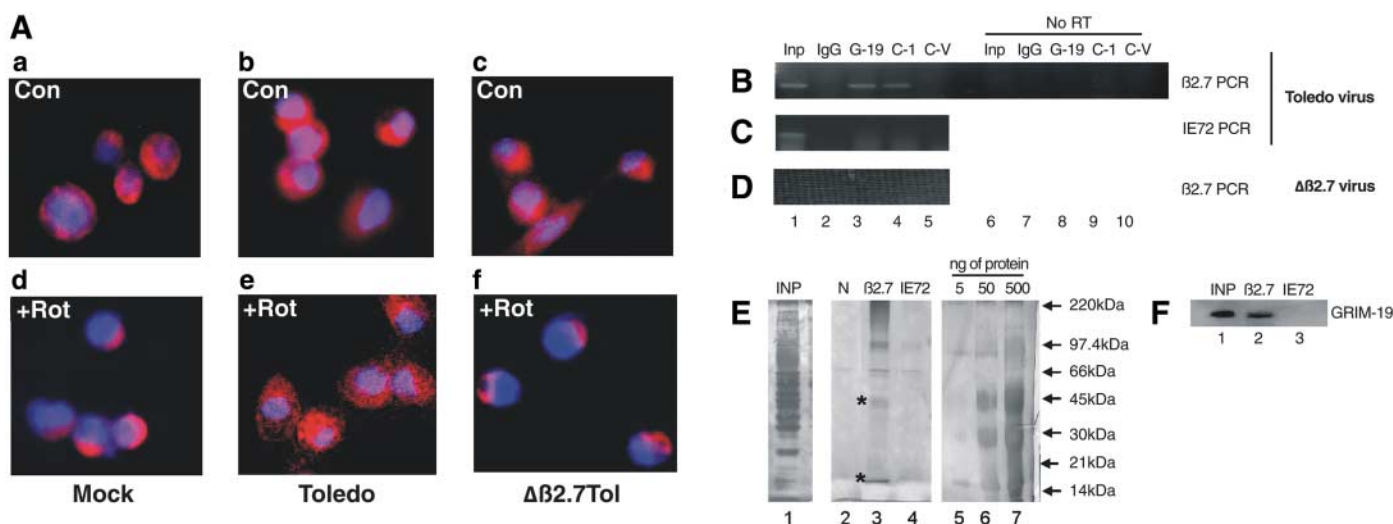


Fig. 2. HCMV interacts with and prevents the rotenone-induced relocalization of GRIM-19 in virally infected cells. (A) GRIM-19 localization (red) in mock- (a and d), Toledo- (b and e), and $\Delta\beta 2.7$ Tol-infected (c and f) U373 cells incubated with solvent control (Con) (a, c, and e) or rotenone (b, d, and f) 24 hpi. (B to D) Immunoprecipitation of control immunoglobulin G (lane 2), GRIM-19 (G-19) (lane 3), native complex I (C-1) (lane 4), and native complex V (C-V) (lane 5) from Toledo- (B and C) or $\Delta\beta 2.7$ Tol-infected (D) cells, and RT-PCR for $\beta 2.7$ [(B)

and (D), lanes 1 to 5], IE72 (C), or a $\beta 2.7$ PCR with no prior RT [(B), lanes 6 to 10]. Inputs are shown in lane 1. (E) IPs on Toledo-infected cells with anti-sense oligonucleotides to $\beta 2.7$ (lane 3) or IE72 (lane 4) or with no oligonucleotide (N) (lane 2). Silver stain of 1% input (INP) (lane 1), the immunoprecipitated proteins (lanes 2 to 4), and known protein loading controls (5 to 500 ng, lanes 5 to 7) is shown. (F) GRIM-19 expression in 10% input (lane 1) and the $\beta 2.7$ (lane 2) and IE72 (lane 3) RNA-IP samples.

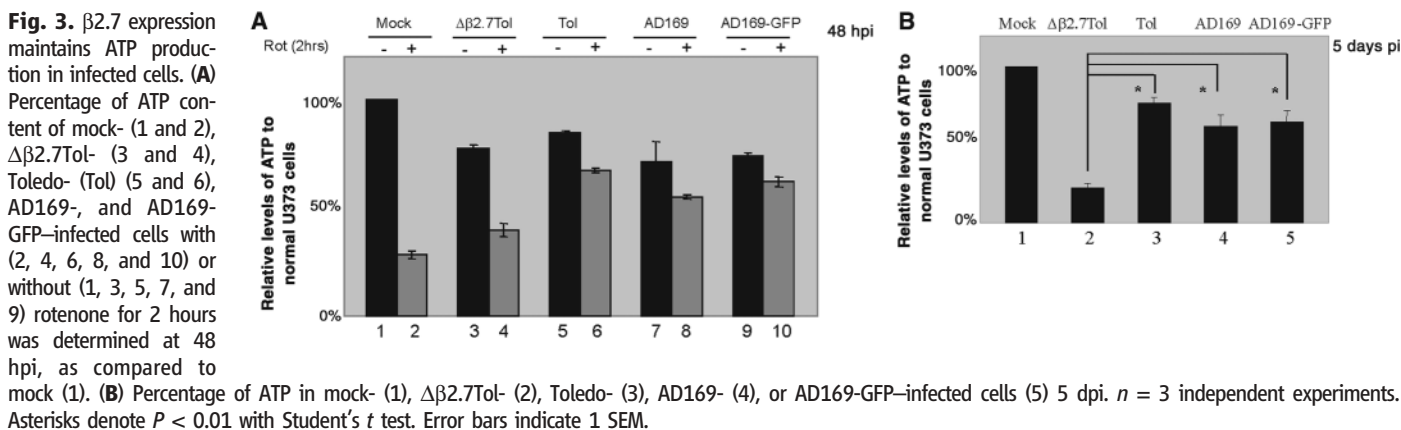


Fig. 3. $\beta 2.7$ expression maintains ATP production in infected cells. (A) Percentage of ATP content of mock- (1 and 2), $\Delta\beta 2.7$ Tol- (3 and 4), Toledo- (Tol) (5 and 6), AD169-, and AD169-GFP-infected cells with (2, 4, 6, 8, and 10) or without (1, 3, 5, 7, and 9) rotenone for 2 hours was determined at 48 hpi, as compared to mock (1). (B) Percentage of ATP in mock- (1), $\Delta\beta 2.7$ Tol- (2), Toledo- (3), AD169- (4), or AD169-GFP-infected cells (5) 5 dpi. $n = 3$ independent experiments. Asterisks denote $P < 0.01$ with Student's t test. Error bars indicate 1 SEM.

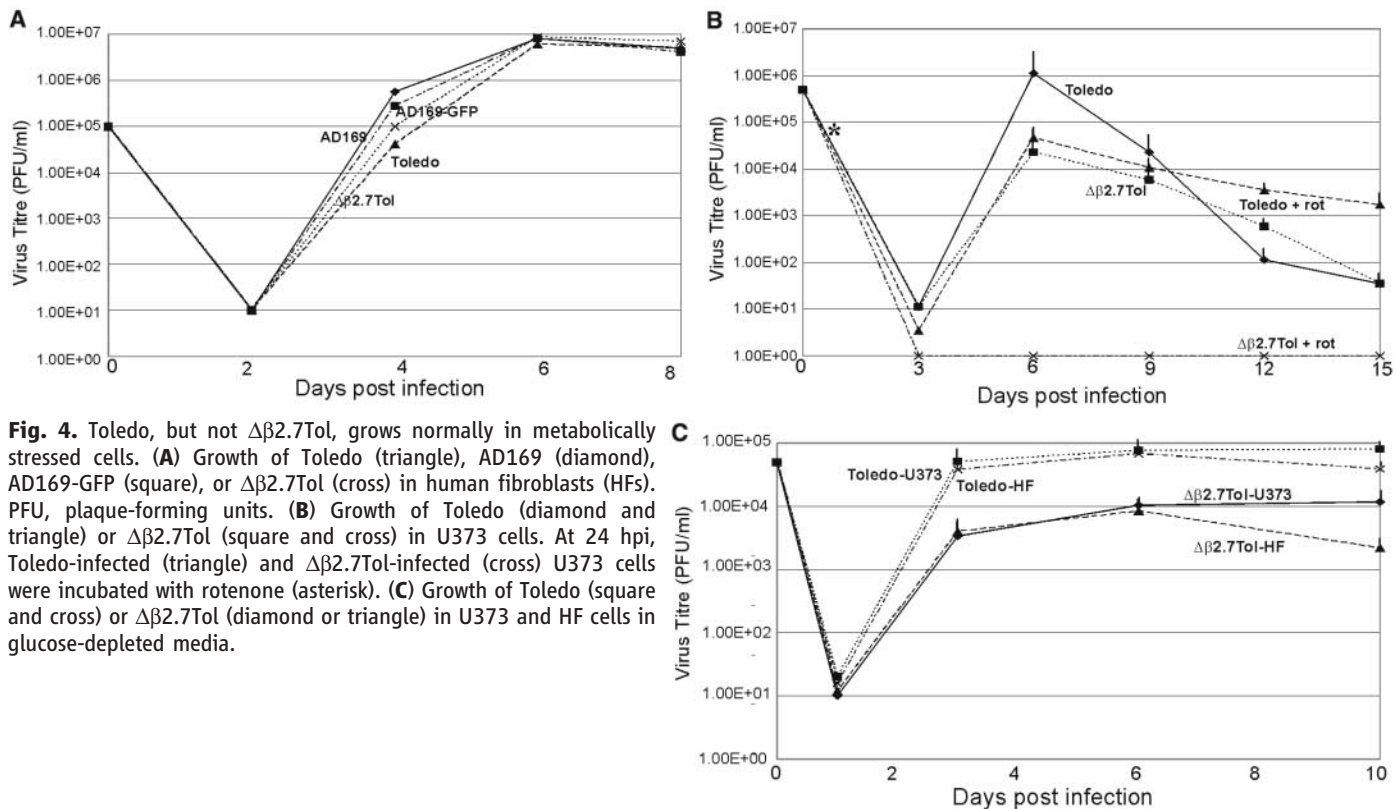


Fig. 4. Toledo, but not $\Delta\beta 2.7\text{Tol}$, grows normally in metabolically stressed cells. **(A)** Growth of Toledo (triangle), AD169 (diamond), AD169-GFP (square), or $\Delta\beta 2.7\text{Tol}$ (cross) in human fibroblasts (HF). PFU, plaque-forming units. **(B)** Growth of Toledo (diamond and triangle) or $\Delta\beta 2.7\text{Tol}$ (square and cross) in U373 cells. At 24 hpi, Toledo-infected (triangle) and $\Delta\beta 2.7\text{Tol}$ -infected (cross) U373 cells were incubated with rotenone (asterisk). **(C)** Growth of Toledo (square and cross) or $\Delta\beta 2.7\text{Tol}$ (diamond or triangle) in U373 and HF cells in glucose-depleted media.

chain (ETC), use this additional glucose to generate ATP via alternate pathways (22). We observed that glucose-depleted media impaired the growth of the $\Delta\beta 2.7\text{Tol}$ virus in U373 and fibroblast cells, as compared with Toledo (Fig. 4C). This finding correlated with a drop in ATP production in $\Delta\beta 2.7\text{Tol}$ -infected fibroblasts (fig. S5A) and the relocalization of GRIM-19 in $\Delta\beta 2.7\text{Tol}$ -infected, but not Toledo-infected, cells (fig. S5B). Because cells in vivo are not exposed to such artificially high levels of glucose and are more reliant on the ETC for ATP production, the effects of $\beta 2.7$ expression in vivo on metabolism are probably more overt than they are during tissue culture.

Metabolic dysfunction, the breakdown of mitochondrial integrity, and the release of pro-apoptotic stimuli are hallmarks of mitochondria-induced apoptosis (23). HIV-1 targets complex I for degradation promoting apoptosis (24), presumably by promoting the formation of reactive oxidative species. Because of the pivotal nature of mitochondria in cell death, it is not surprising that HCMV targets mitochondria function and makes a concerted effort to subvert the apoptotic response including UL36 (caspase inhibitor), UL37x1 (a B cell lymphoma 2 homolog with profound anti-apoptotic activity) (25, 26), and UL38, which protects infected cells from endoplasmic reticulum stress (27). UL37x1 is also known to promote mitochondrial membrane stability (26) and is predominantly active at late times of infection

(>48 hpi) (28). In contrast, the $\beta 2.7$ gene RNA is expressed much earlier during infection. Consequently, it is likely that HCMV has evolved multiple functions in order to hijack the mitochondria in the cell to enable continued energy production, as well as protection from cell death.

An intriguing aspect of this study is that an RNA molecule is used directly to exert these effects. Although at first appearing unusual, this may represent a highly refined viral strategy. First, because of the sheer numbers of mitochondria within the cell, the expression of a highly abundant RNA allows the virus to saturate these organelles effectively. Second, by disposing of the need to translate the super-abundant $\beta 2.7$ RNA, the virus can achieve this effect more quickly throughout the course of infection.

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