Supplementary Material

Ecdysone inducible ORF63 stable cell line construction.

Following previously described methods, we constructed an ORF63 ecdysone inducible stable cell line (Stolarov *et al.*, 2001). The ecdysone inducible system requires two inducible receptor proteins (VgEcR and RXR) and an ecdysone-sensitive response element (EcRE) positioned upstream of a minimal promoter directing expression of a target gene. In the presence of hormone (Muristerone A), VgEcR and RXR heterodimerize to form a functional ecdysone receptor that activates transcription of the target gene from the EcRE containing promoter. Following previously described methods, an ORF63 ecdysone inducible stable cell line was constructed (Stolarov *et al.*, 2001). MeWo cells were infected with replication deficient retroviruses encoding VgEcR and RXR and selected with puromycin and G418. Stable clones (MeWo-ECD) were isolated, transformed with a plasmid expressing green fluorescent protein (GFP) under an ecdysone inducible promoter, treated with 1 μM Muristerone A (Invitrogen) and a clone expressing high levels of GFP was selected. An N-terminal FLAG-tagged ORF63 with a C-terminal Myc/His tag was cloned into pI-TKHygro (Stolarov *et al.*, 2001) using *Pme*I and *Xho*I restriction sites to generate pI-FlagORF63Myc/His. This construct expresses ORF63p from an ecdysone inducible promoter. Replication deficient retroviruses were made using pI-FlagORF63Myc/His and a retrovirus packaging cell line system (Invitrogen). MeWo-ECD cells were infected with replication deficient retroviruses, selected on hygromycin and an ecdyone inducible ORF63p expressing stable cell line (ORF63-ECD) was selected after screening.

Immunoprecipitation.

For immunoprecipitations, 15×150 cm dishes of MeWo-ECD and ORF63-ECD were induced with 1 μM Muristerone A. Seventy-two hours post-induction cells were washed three times with cold PBS, scraped into radioimmunoprecipitation lysis buffer (RIPA) (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.5% NP-40, 50 mM NaF) plus Complete protease inhibitor cocktail (Roche) and Halt Phosphatase Inhibitor cocktail (Pierce) and incubated on ice for 30 min. Lysates were clarified by centrifugation for 10 min in a Tomy MX-160 high-speed refrigerated microcentrifuge at 22500 ***g***. Total protein concentration was measured using a Bio-Rad protein assay kit. Equal amounts of total protein were incubated overnight (at 4 °C with rotation) with EZview Red Anti-FLAG M2 affinity gel (Sigma) prepared using RIPA buffer plus inhibitors to the manufacturer's guidelines. Following overnight incubation, beads were collected by centrifugation at 400 ***g*** at 4 °C in a Tomy MX-160 high-speed refrigerated microcentrifuge and washed three times with RIPA buffer plus inhibitors. After washing, bound complexes were eluted from the beads using FLAG peptide (Sigma) and analysed by SDS-PAGE. Gels were stained with EZBlue Gel Staining Reagent (Sigma), followed by excision of ORF63p protein bands that were subsequently sent for MS analysis.

Mueller, N. H., Walters, M. S., Marcus, R. A., Graf, L. L., Prenni, J., Gilden, D., Silverstein, S. J. and Cohrs, R. J. (2010). Identification of phosphorylated residues on varicella-zoster virus immediate-early protein ORF63. *J Gen Virol* **91**, 1133–1137.

Indirect immunofluorescence.

Microscopy was done as previously described (Walters *et al.*, 2008). Briefly, cells grown on glass coverslips were washed twice with PBS, fixed for 20 min with 3.7% formaldehyde in PBS, washed two more times in PBS and permeabilized with PBS plus 0.2% Triton (Sigma) for 10 min at room temperature. Permeabilized cells were washed twice with PBS, blocked with 10% normal goat serum (Roche) in PBS plus 0.1% Tween 20 (Sigma) (PBST) for 30 min. Cells were incubated with primary antibody (1:100) in 10% normal goat serum in PBST for 1 h, washed three times for 5 min in PBST, incubated for 1 h with Alexa Fluor-conjugated secondary antibody (1:1000) in 10% normal goat serum in PBST and washed twice with PBST. After washing, cells were incubated for 30 min with PBST plus 0.5 μg Hoechst (Sigma) ml^{-1}, washed twice for 5 min with PBST and once with PBS. Coverslips were subsequently mounted with GEL/MOUNT (Biomeda). All samples were visualized with a Zeiss Axiovert 200M inverted microscope using a ×63 objective (Carl Zeiss Microimaging Inc) and images were acquired with a Hamamatsu C4742-80-12AG Digital CCD Camera (Hamamatsu Photonics) using Openlab 5 software (Improvision). Images were analysed using Openlab 5 and assembled in Photoshop CS3 (Adobe Systems).

Supplementary Fig. S1. Mass spectral analysis of ORF63p peptide (aa 150–179). (a) Peptides generated by trypsin digestion of OFR63p purified from transformed HEK 293 cells were analysed by MS. A parental peptide ion at charge-to-mass ratio (*m*/*z*) of 1643.44 was selected for further analysis. (b) The selected peptide, SALEC$_{me}$DVSDDGGEDDSDDDGST*PSDVIEFR, where C_{me} is a carbamidomethylation of Cys163 due to iodoacetamide treatment, and T* is a putative phosphorylated Thr171 (Mascot database search score of 99.5), was subjected to MS/MS analysis. The dominate green peak at ~1575 *m*/*z* represents a neutral loss of phosphoric acid from the parent peptide ion formed by collision induced fragmentation in the mass spectrometer. The neutral loss peak suggests the phosphorylation of an amino acid within the parental peptide. While this spectrum indicates phosphorylation at Thr171, other observed spectra (not shown) indicate phosphorylation at Ser170 or 173. (c) The neutral loss peak in panel (b) (green) was subjected to further MS analysis. The resulting MS/MS/MS fragmentation spectrum independently confirms the identity of the unphosphorylated peptide SALEC*DVSDDGGEDDSDDDGSTPSDVIEFR (Mascot database search score of 59).

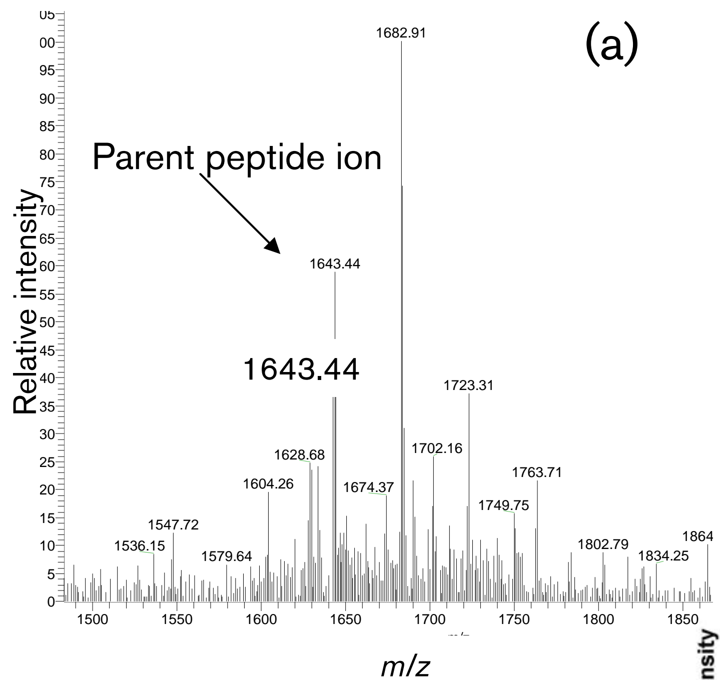
Supplementary Fig. S2. ORF63p expression analysis in ecdysone inducible cell line (ORF63-ECD). (a) ORF63-ECD cells were induced with 1 μM Muristerone A and harvested at 0, 24, 48 and 72 h post-induction. The negative control, MeWo-ECD cells, was induced and harvested 72 h post-induction. Equal amounts of total protein (20 μg) were analysed by Western blot using antisera

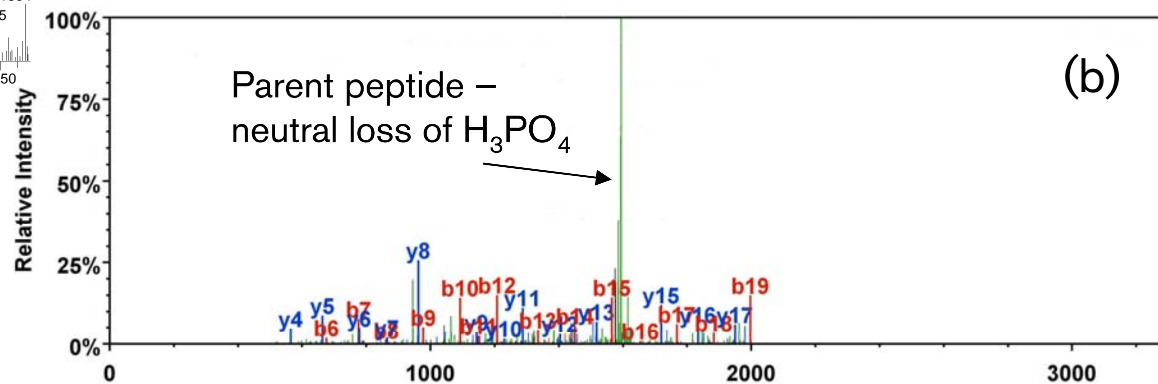
specific for ORF63 and HSP90. (b) Localization of ORF63p expressed following induction of ORF63-ECD cells. Seventy-two hours post-induction the cells were fixed, stained using antisera specific for FLAG, ORF63 or Myc and observed by immunofluorescence microscopy. Images were taken with a ×63 objective. (c) Isolation of ORF63p for MS analysis. MeWo-ECD and ORF63-ECD cells were induced with 1 μM Muristerone A, harvested at 72 h post-induction. ORF63p was immunoprecipitated using Anti-FLAG M2 affinity gel, bound complexes were eluted using FLAG peptide, and precipitated protein analysed by 10% SDS-PAGE gel stained with Coomassie brilliant blue. The protein band corresponding to ORF63p was excised from the gel and sent for MS analysis.

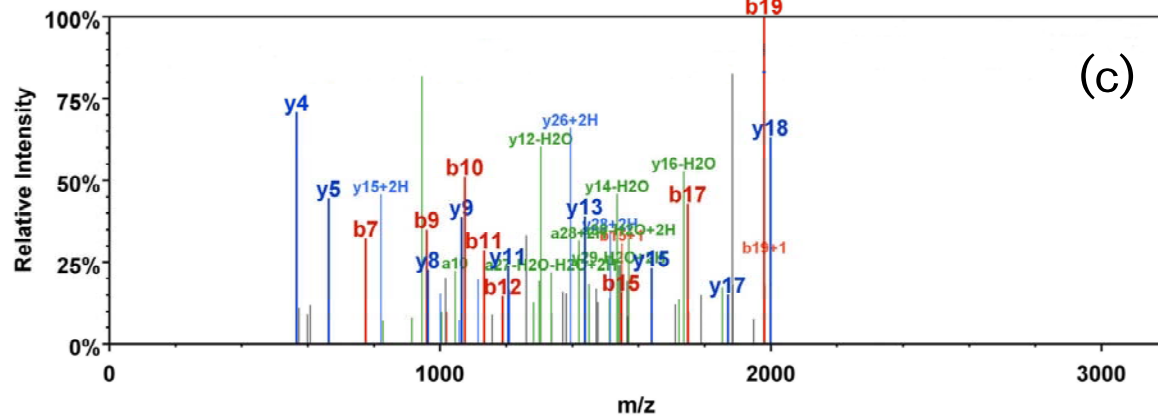
References

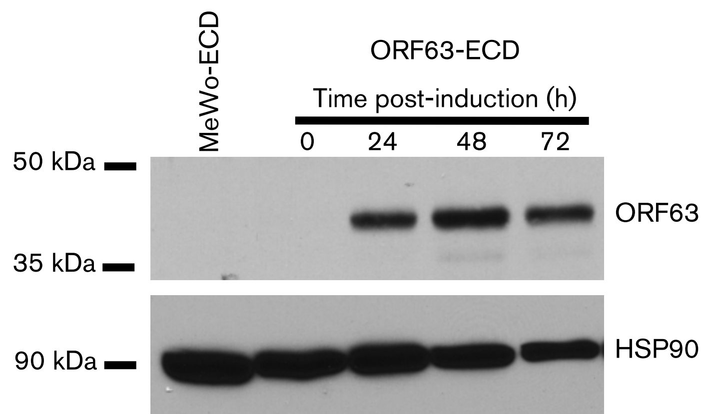
Stolarov, J., Chang, K., Reiner, A., Rodgers, L., Hannon, G. J., Wigler, M. H. & Mittal, V. (2001). Design of a retroviral-mediated ecdysone-inducible system and its application to the expression profiling of the PTEN tumor suppressor. *Proc Natl Acad Sci U S A* **98**, 13043–13048. Medline

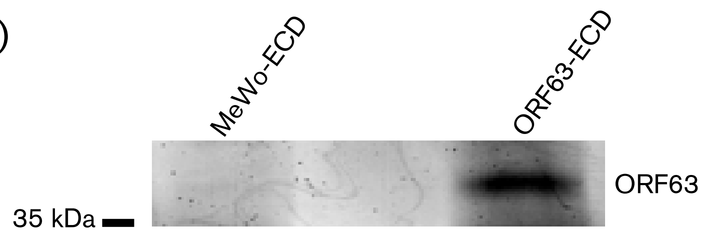
Walters, M. S., Kyratsous, C. A., Wan, S. & Silverstein, S. (2008). Nuclear import of the varicella-zoster virus latency-associated protein ORF63 in primary neurons requires expression of the lytic protein ORF61 and occurs in a proteasome-dependent manner. *J Virol* **82**, 8673–8686. Medline

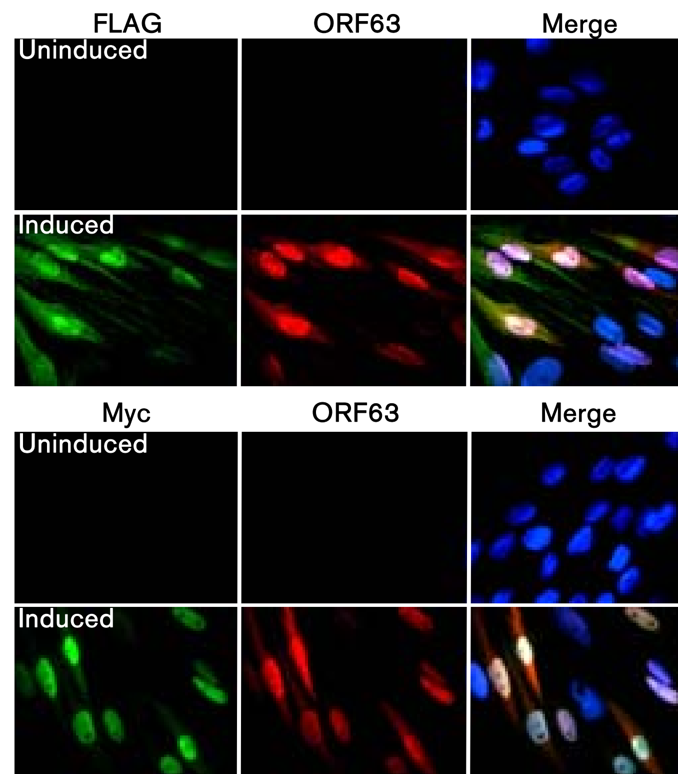

(a)
Parent peptide ion
1643.44
1682.91
1723.31
1702.16
1628.68
1604.26
1674.37
1763.71
1749.75
1547.72
1536.15
1579.64
1802.79
1834.25
1864
Relative intensity
m/z


(b)
Parent peptide –
neutral loss of H_3PO_4
Relative Intensity


(c)
Relative Intensity
m/z


(a)
MeWo-ECD
ORF63-ECD
Time post-induction (h)
0
24
48
72
50 kDa
35 kDa
90 kDa
ORF63
HSP90


(c)
MeWo-ECD
ORF63-ECD
35 kDa
ORF63


(b)
FLAG
ORF63
Merge
Uninduced
Induced
Myc
ORF63
Merge
Uninduced
Induced