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ABSTRACT

Bovine Herpesvirus Type 1 (BoHV-1) infection causes infectious bovine rhinotracheitis and genital disease in cattle, with significant economic and welfare impacts. However, the role of cellular host factors during viral replication remains poorly characterised. A previously performed genome-wide CRISPR knockout screen identified pro- and antiviral host factors acting during BoHV-1 replication. Herein we validate a pro-viral role for a candidate from this screen: the cellular protein tetracopeptide repeat protein 4 (TTC4). We show that TTC4 transcript production is upregulated during BoHV-1 infection. Depletion of TTC4 protein impairs BoHV-1 protein production but does not reduce production of infectious virions, whereas overexpression of exogenous TTC4 results in a significant increase in production of infectious BoHV-1 virions. TTC4 itself is poorly characterized (especially in the context of virus infection), but is a known co-chaperone of heat shock protein 90 (HSP90). HSP90 has a well-characterized pro-viral role during the replication of diverse herpesviruses, and we therefore hypothesized that HSP90 is also pro-viral for BoHV-1. Drug-mediated inhibition of HSP90 using geldanamycin at sub-cytotoxic concentrations inhibited both BoHV-1 protein production and viral genome replication, indicating a pro-viral role for HSP90 during BoHV-1 infection. Our data demonstrates pro-viral roles for both TTC4 and HSP90 during BoHV-1 replication; possibly, interactions between these two proteins are required for optimal BoHV-1 replication, or the two proteins may have independent pro-viral roles.

KEYWORDS

Bovine herpesvirus 1; tetracopeptide repeat protein 4 (TTC4); heat shock protein 90 (HSP90); geldanamycin

1. INTRODUCTION

Bovine Herpesvirus Type 1 (BoHV-1) of the *Alphaherpesvirinae* subfamily has a global distribution in cattle [1]. BoHV-1 infection can cause rhinotracheitis, abortion, conjunctivitis, vulvovaginitis or encephalitis, and can be transmitted horizontally through the respiratory route or mucosal secretions, or vertically [2]. As is characteristic of alphaherpesvirus life cycles, BoHV-1 undergoes initial lytic replication of mucous membranes, following which it establishes latency in neuronal ganglia. Reactivation of the virus from latency can lead to recrudescence of lytic virus infection and disease [3] [4, 5].

While vaccines licensed to prevent disease due to BoHV-1 infection are in widespread use [6], recombination between a live attenuated vaccine strain and wild type virus has been reported [7], and reversion to virulence of live attenuated vaccines has been described on several occasions [8, 9]. No antivirals are licenced for treatment of BoHV-1 infection, meaning management is dependent on vaccination, regulation of environmental factors, and treatment of symptoms - which currently includes the use of antibiotics [10]. Despite these limitations to disease management and the resultant high economic burden attributable to BoHV-1, little is known about the role of host cell factors in BoH'V-1 replication. Recently, promising insights into understanding BoHV-1 interactions with its host have been delivered by genome-wide CRISPR knockout (GeCKO) screens that were performed to identify host cell factors augmenting or restricting BoHV-1 replication [11, 12].

In this screen, host cell protein tetracopeptide repeat protein 4 (TTC4) was among the genes that were found to be most strongly pro-viral for BoHV-1 replication [12]. As far as we are aware, only one report had previously identified a role for TTC4 during viral infection, finding that TTC4 was potently antiviral for Sendai virus, an RNA virus of the *Paramyxoviridae* family [13]. The physiological function(s) of TTC4 remain poorly defined, but it is known that TTC4 is a HSP90 co-chaperone in human cells [14]. HSP90 assists the proper folding of cellular proteins [15] and is known to be pro-viral for other alphaherpesviruses including herpes simplex virus 1 (HSV-1), acting to support viral protein expression and translocation, and virion

egress [16-19]. We therefore sought to confirm a pro-viral role for TTC4 during BoHV-1 replication, and test whether HSP90 plays a role during virus replication.

2. METHODS

2.1 Cell lines and virus stocks.

Wildtype (WT) and Cas9-expressing (Cas9+) Madin Darby Bovine Kidney (MDBK) cells (WT cells from ATCC batch CCL-22; Cas9+ cell generation was described previously [11]) were cultured in Dulbecco's Modified Eagles Medium (DMEM, Merck: D5796) supplemented with 5% horse serum (FHS), penicillin at 100u/ml and streptomycin at 100 µg/ml (5% FHS-DMEM). HEK293FT cells and HEK293T cells (both Sigma, Glasgow, UK) were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) with penicillin and streptomycin (10% FBS-DMEM). Cells were maintained at 37°C, 5% CO_2 and with twice weekly passage. BoHV-1 with recombinant GFP (BoHV-1-GFP) tagged VP26 was a kind gift from Dr Kurt Tobler, Institute of Virology, University of Zurich [20]. Herpesviruses express mRNAs and consequentially proteins in three sequential classes: immediate early, early and late. In BoHV-1-GFP, GFP is tagged to the C-terminus of VP26, which is a non-essential protein expressed late during the viral replication cycle.

2.2 BoHV-1-GFP infections.

MDBK cells at 80-90% confluency in 96 well/ 6 well plates were inoculated with BoHV-1-GFP at a multiplicity of infection (MOI) of 0.1 (low MOI) or MOI of 10 (high MOI). After 1 hour the medium was aspirated, cells were washed with PBS and 100 µl/ 500 µl 5% FHS-DMEM was added. HEK293T cells at 70-80% confluency in a 24 well plate were washed with PBS and 100 µl of 10% FBS-DMEM with BoHV-1-GFP at a MOI of 10 was added. After 1 hour, medium was aspirated, cells were washed with PBS and 500 µl of 10% FBS-DMEM was added. GFP fluorescence was measured using a CLARIOstar® Plus (BMG LabTech), at 10 minute intervals for 24 hours (high MOI) or 72 hours (low MOI), with cells maintained at 37°C, 5%

87 CO_2. At 24 hours (high MOI) or 72 hours (low MOI), supernatant was collected and stored at
88 -80°C.

89 **2.3 Quantitative (q)PCR**.

90 MDBK cells at 90% confluency were infected with BoHV-1-GFP at an MOI of 10 as described
91 above. At 4, 8 and 16 hours post infection (hpi), medium was removed and cells were lysed
92 in 350 µl of RLT cell lysis buffer (QIAGEN). RNA was extracted using an RNeasy Mini Kit
93 (74104, QIAGEN) according to the manufacturer's instructions. In order to remove co-
94 extracted DNA, the extracted RNA was treated using the RQ1 DNAse according to
95 manufacturer's instructions (M6101, Promega). cDNA was produced using GoScript Reverse
96 Transcriptase (A5000, Promega) using oligo-dT primers. qPCR was then performed for TTC4
97 and GAPDH using Brilliant III Ultra-Fast SYBR® GreenQPCR Master Mix (600882, Agilent),
98 specific primers **(Table 1)** and 1 µl cDNA according to the manufacturer's instructions. The
99 qPCR was conducted in a QIAGEN Rotor-Gene Q using reaction conditions of 95ºC for 3
100 minutes and then 40 cycles of 95ºC for 10 seconds /60ºC for 20 seconds. After amplification,
101 a melting curve analysis was performed increasing from 50ºC to 90ºC at 1ºC/ second.

102 **Table 1. PCR/ qPCR/ Sequencing primers used in this study.**

Primer	Target	Sequence
sgRNA 1 site fwd	TTC4 gDNA	CTGGCTGGTAGCTGGTTGAA
sgRNA 1 site rev	TTC4 gDNA	GTCCCTACCCATGAGGCTCT
sgRNA 2 site fwd	TTC4 gDNA	GGTGGGTTGCCTGCTATTCT
sgRNA 2 site rev	TTC4 gDNA	TCAGCAGCAACATGCCATTT
CMV Forward	PCDNA3.1(+)	CGCAAATGGGCGGTAGGCGTG
BGH Reverse	PCDNA3.1(+)	TAGAAGGCACAGTCGAGG
M13 Forward	PCR Blunt II	TGTAAAACGACGGCCAGT
M13 Reverse	PCR Blunt II	CAGGAAACAGCTATGAC
LKO1.5	PKLV2-2a	GACTATCATATGCTTACCGT
GAPDH qPCR fwd	GAPDH cDNA	GGTGATGCTGGTGCTGAGTA
GAPDH qPCR rev	GAPDH cDNA	TCATAAGTCCCTCCACGATG
TTC4 qPCR fwd	TTC4 cDNA	ACTATACTGCGTGCCTCCCA
TTC4 qPCR rev	TTC4 cDNA	CAGCATGGCCCAGTCACTTT

103

104 **2.4 Generation of PKLV2-2A- sgRNA 1/2 plasmid constructs.**

Lentivirus vector plasmid PKLV2-2a was modified to express small guide (sg) RNAs targeting TTC4 under the control of a CMV promoter [11]. PKLV2-2A was digested using BbsI (R0539S, New England Biolabs) according to the manufacturer's instructions, and complete digestion was confirmed by agarose gel electrophoresis. Sense and antisense DNA oligonucleotides (**Table 2)** encoding the TTC4 sgRNA 1 or sgRNA 2 sequence template, as used previously [12] and the BbsI restriction site were annealed. 100 nm each of sense and antisense DNA oligonucleotides in 1 x NEBuffer 2.1 in a final volume of 50 µl were heated to 95 °C for 5 minutes before being allowed to cool slowly. 100 ng of digested plasmid and 4 µl annealed oligonucleotides were ligated using T4 DNA ligase (M0202S, NEB) according to manufacturer's instructions. Annealed products were transformed into DH5α competent *Escherichia coli* bacteria and plated onto agar plates containing 100 µg/ml ampicillin. Plates were incubated at 37°C overnight, and colonies were picked and amplified in 100 ml LB broth containing 100 µg/ml ampicillin overnight at 37°C. Plasmids were purified using a Plasmid Midiprep kit (12143, QIAGEN) according to the manufacturer's instructions. Insertion of oligos into the PKLV2-2A vector was confirmed by Sanger sequencing (Genewiz) using the LKO1.5 primer **(Table 1)**.

Table 2. TTC4 sgRNA Template DNA Oligonucleotides used to make sgRNA 1 and 2 PKLV2-2a TTC4 sgRNA plasmids.

Guide	Sense/antisense	Sequence
sgRNA1	Sense	CACCGCTGTGAACTGGTGCGATGAG
	Antisense	AAACCTCATCGCACCAGTTCACAGC
sgRNA2	Sense	CACCGTTCTTCTCTCAATGATGTGA
	Antisense	AAACTCACATCATTGAGAGAAGAAC

2.5 Lentivirus production.

4 µg of vector plasmid (empty PKLV2-2a, or PKLV2-2a sgRNA 1 or PKLV2-2a sgRNA 2), with 2 µg of pMDG2 and 6 µg of pPAX2 were transfected into 80-90% confluent HEK293FT cells in a 10 cm dish using 36 µl Lipofectamine 2000 (11668019, Invitrogen) according to the

manufacturer's instructions. After 24 hours, the medium was removed and replaced with 10 ml of 10% FBS-DMEM (HEK293FT cells do not tolerate the substitution of FBS for FHS in medium). After 48 hours, the supernatant was harvested and filtered (45 µm filter), aliquoted and stored at -80°C as lentivirus stock.

2.6 Production of TTC4 -/- cells.

1 ml of PKLV2-2a Control, PKLV2-2a sgRNA1 or PKLV2-2a sgRNA2 lentivirus and 1 ml of 5% FHS-DMEM were added along with polybrene (8 µg/ml) to 70-80% confluent Cas9+ MDBK cells per well of a 6 well plate. After 24 hours the inoculum was removed, cells were washed with PBS and 5% FHS-DMEM was added. Puromycin (1.8 µg/ml) was added 24 hours later. After 4 days, populations of Cas9+ MDBK PKLV2-2a control, sgRNA 1 or sgRNA 2 (control cells, sgRNA 1 cells and sgRNA 2 cells) were seeded into a 96 well plate for subsequent infections.

2.7 Analysis of knockdown efficiency.

Mixed populations of Cas9+ MDBK control, sgRNA 1 cells and sgRNA 2 cells were analysed for TTC4 editing efficiency. DNA was extracted from 1.2 x 10^6 cells using the DNeasy blood and tissue extraction kit (69504, QIAGEN). A 467 bp (sgRNA 1) or 418 bp (sgRNA 2) region covering the sgRNA target sites was amplified using specific primers **(Table 1)** and Q5 high fidelity DNA polymerase (M0491L, New England Biolabs). Cycling conditions were 98ºC for 30 seconds, followed by 35 cycles of 98ºC for 10 seconds, 62ºC for 30 seconds, and 72ºC for 45 seconds, then a final elongation of 72 ºC for 120 seconds. PCR amplicons were purified using the MinElute PCR purification kit (28004, QIAGEN) and sent for Sanger sequencing (Genewiz) with their respective forward primers. Chromatograms were analysed using Synthego ICE analysis (Synthego Performance Analysis, ICE Analysis. 2019. v3.0. Synthego; [13/05/22]) to characterise editing events.

2.8 TTC4 overexpression.

As there is no commercially available bovine TTC4 antibody, and a polyclonal human TTC4 antibody did not cross react (LS-C187352, Life Span Biosciences, data not shown), the coding sequence (CDS) of *Bos taurus* TTC4 (NM_001015554.1) with an in-frame Flag tag at the C terminus was cloned into a pcDNA3.1 (+) backbone. A gene block was ordered from IDT (5' -BmtI site > TTC4 CDS with no stop codon >Flag tag >stop codon>NotI site-3'). The gene block was cloned into the PCR blunt II vector using the Zero Blunt TOPO cloning kit (K275040, Thermo-Fisher UK) and transformed into DH5α competent *E. coli* bacteria as per manufacturer's instructions. Cloning of the TTC4 CDS into PCR Blunt II was confirmed with Sanger sequencing (Genewiz) using M13 forward and reverse primers **(Table 1).** The PCR Blunt II TTC4-Flag construct and pcDNA 3.1 (+) were both digested with BmtI and NotI in 1x NEB buffer 3.1 at 37ºC. The pcDNA backbone and TTC4-Flag insert were gel extracted using the MinElute Gel Extraction Kit (28604,QIAGEN). 500 ng of both TTC4-Flag insert and pcDNA 3.1(+) backbone were annealed using T4 DNA Ligase (M0202S, NEB), as per manufacturer's instructions. Annealed products were transformed into DH5α competent *E. coli* bacteria and plated onto agar plates containing 100 µg/ml kanamycin. Plates were incubated at 37°C overnight, and colonies were picked and amplified in 100 ml LB broth containing 100 µg/ml kanamycin overnight at 37°C. Plasmids were purified using a Plasmid Midiprep kit (12143, QIAGEN) according to the manufacturer's instructions. Insertion of the TTC4-Flag CDS into the pcDNA3.1 (+) vector was confirmed by Sanger sequencing (Genewiz) with CMV forward and BGH reverse primers **(Table 1).**

To generate a cell line stably expressing the TTC4-Flag fusion protein, HEK293T cells at 70% confluency in a T25 flask were transfected as follows. 10 µg of pcDNA 3.1(+) empty or pcDNA 3.1(+) TTC4-Flag was transfected into cells using 12 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours, medium was replaced with 10% FBS-DMEM supplemented with 5 µg/ml G418 (13200-1g-CAY, Cambridge Biosciences). After ten days, G418-containing medium was replaced with fresh 10% FBS-DMEM. The next day,

cells were seeded in a 24 well plate. At 70-80% confluency, cells were then infected with BoHV-1-GFP at MOI of 10 as described above.

2.9 Virus quantification.

BoHV-1-GFP was titred by plaque assay. Virus stocks and supernatants were serially diluted in 5% FHS-DMEM in 10-fold increments. Wildtype (WT) MDBK cells at 70-80% confluency in 6 well plates were inoculated with 400 µl of diluted virus for 1 hour, after which cells were overlaid with 2 ml of 0.6% Avicel in FHS-DMEM (25% Avicel (FMC Biopolymer), (2.4% in H_2O)/ 75% 5% FHS-DMEM). After 4 days, cells were fixed with 1 ml of neutral buffered formalin (10%)/ well and stained with toluidine blue. Plaques were counted by eye.

2.10 Fluorescence-activated cell sorting (FACS).

MDBK Cas9+ cells were transduced with PKLV2-2A control or PKLV2-2A sgRNA1 lentivirus and selected as described above (control cells and sgRNA1 cells). At ten days post-transduction, control and sgRNA1 cells were seeded into 6 well plates. At 90% confluency, cells were infected with BoHV-1-GFP at an MOI of 10 as described above. At 10 hpi, cells were detached with 0.5 ml trypsin-EDTA (25200056, Life Technologies)/ well, pelleted by centrifugation and resuspended in 1 ml of PBS. Cells were sorted using the BD FACSAria™ III Cell Sorter (BD Biosciences) to give 10,0000 cells in a population of single cells with normal morphology. Sorted cells were further divided into 3 subpopulations – No GFP, Low GFP and High GFP, correlating with the levels of VP26 protein production.

2.11 Western blotting.

HEK293T cells transfected with pcDNA 3.1(+) +/- TTC4-Flag as described above were lysed in 100 ul of Laemmli buffer (1610737, Bio-Rad) containing 10% β-mercaptoethanol. Samples were run on a 10% Mini-PROTEAN® TGX™ Precast Protein Gel (4561036, Bio-Rad) for 90 minutes at 100 V. Protein was then wet transferred to a nitrocellulose membrane at 100 V for 90 minutes at 4°C. The membrane was blocked in 5% BSA/ PBS for 1 hour. Primary antibodies targeting Flag (SAB4301135, Sigma) or α-tubulin (NB600-506, Novus Bio) were

added at 1:1000 or 1:5000, diluted in blocking buffer, and incubated overnight or for 1 hour respectively with agitation. Membranes were washed 3 times in Tris-Buffered Saline (TBS)/ 0.1% Tween-20. Secondary antibodies, goat anti-rat Alexa Fluor 680 (926-68076, LI-COR) or donkey anti-rabbit Alexa Fluor 800 antibodies (926-32213, LI-COR) were added to the membranes at 1:5000 and incubated for 90 minutes at room temperature with agitation. Membranes were washed 3 times in TBS/ 0.1% Tween-20 and then imaged using the LI-COR FC Odyssey system.

2.12 Geldanamycin cytotoxicity assay.

WT MDBK cells in 96 well plates (70-80% confluency) were washed with PBS and medium was replaced with 100 µl of 5% FHS-DMEM containing geldanamycin (Cambridge Biosciences, SM55-2) at concentrations ranging from 0 – 500 µM or equivalent concentrations of DMSO (used to resuspend the drug). After 72 hours, cell viability was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega G7570) as per the manufacturer's instructions. Luminescence was read using a BioTek Cytation 3 plate reader. Cell viability was calculated relative to untreated WT MDBK cells at 72 hours.

2.13 Data analysis and statistics.

To examine for statistical significance, two-way T tests or Fisher's Exact tests were performed in Graph Pad Prism 8 with a threshold of $p < 0.05$ used (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA). Figures were created in GraphPad Prism 8 and BioRender (BioRender.com).

3. RESULTS

3.1 TTC4 mRNA production increases during BoHV-1 infection.

As a first step towards validating a role for TTC4 in BoHV-1 replication, we assessed whether TTC4 expression was altered by BoHV-1 infection. Direct evaluation of protein expression was

not possible due to the lack of a commercially available antibody against bovine TTC4. Therefore we used qPCR to quantify TTC4 mRNA production during virus infection. RNA from MDBK cells infected with BoHV-1-GFP at MOI 10 and uninfected MDBK cells was extracted at 4, 8 and 16 hpi. At 4 hpi, there was no significant difference in TTC4 transcript levels in infected versus uninfected cells. However at 8 hpi and 16 hpi there was a significant 2-fold increase in TTC4 transcript levels in BoHV-1-GFP infected cells **(Fig. 1A).** This shows that TTC4 transcript levels were significantly increased within the timeframe of one BoHV-1-GFP replication cycle.

3.2 TTC4 depletion inhibits BoHV-1 protein production but not generation of viral progeny.

To directly validate the results of the previous GeCKO screen [11], we repeated the GeCKO methodology with a single sgRNA targeting TTC4 in place of an sgRNA library. Cas9+ MDBK PKLV2-2a control or PKLV2-2a sgRNA1 or sgRNA2 cells were produced (control, sgRNA1 and sgRNA2 cells). sgRNA1 and sgRNA2 were chosen due to high editing efficiency coupled with low off target editing [11]. Control, sgRNA1 and sgRNA2 cells were infected with BoHV-1-GFP at an MOI of 10. At 10 hpi, cells were sorted into 3 populations; no GFP, low GFP and high GFP. 22.0% of control cells were sorted into the high GFP population indicating high BoHV-1 protein expression, in comparison to 8.5% and 7.1% of sgRNA1 cells respectively. Similarly, 14.9% of control cells were in the low-GFP population compared to 11.4% of sgRNA1 and 10.2% of sgRNA2 cells **(Fig. 1B)**. The trend to reduced GFP expression in sgRNA1 and sgRNA2 cells validates the GeCKO screen finding that reduced VP26 production occurs in Cas9+ MDBKs expressing sgRNAs that target TTC4, indicating that depleting TTC4 inhibits viral protein production.

The GeCKO screen methodology correlates viral protein production with sgRNA expression to identify cellular genes that affect virus replication; however, sgRNA expression does not guarantee KO of the target gene (for example, in-frame deletions or insertions may occur, preserving the target protein open reading frame). Here, using a single sgRNA allowed direct

correlation of VP26 expression with TTC4 KO. Levels of editing resulting in TTC4 KO were quantified in unsorted sgRNA1 cells (used as a representative population) and the No GFP, Low GFP and High GFP populations by Sanger sequencing and ICE analysis. 40% of the unsorted sgRNA1 cells were TTC4 -/-. If TTC4 editing had no impact on BoHV-1 protein production there would be equivalent levels of TTC4 -/- cells in each of the subpopulations. No GFP cells, which were exposed to virus but were not infected with BoHV-1, contained 46% TTC4 -/- cells. Reduced percentages of TTC4 -/- cells were found in the GFP Low (36%) and GFP High (33%) cell populations, indicating that VP26 production was higher in cells with an intact TTC4 gene **(Fig. 1C).**

To determine whether TTC4 is needed for the production of infectious BoHV-1 progeny and not just VP26 protein production, control, sgRNA1 and sgRNA2 MDBK cells were infected with BoHV-1-GFP at an MOI 0.1 (low MOI) or 10 (high MOI) for 72 hours and 24 hours respectively. During low MOI infections, sgRNA1 cells had significantly reduced GFP fluorescence relative to control cells indicating reduced expression of VP26, whereas sgRNA2 reduced GFP signal albeit not significantly (**Fig. 2A**). At high MOI, both guides resulted in a significant reduction in GFP signal (**Fig. 2B**). Endpoint BoHV-1 titres were not significantly different upon editing with either guide at low or high MOI (**Fig. 2C and D**). The efficiency of TTC4 knockdown in the sgRNA1 cells used for these infections was determined for each repeat by Sanger sequencing and ICE analysis. Generally, sgRNA1 edited more efficiently than sgRNA 2 **(Fig. 2E)**, in keeping with the greater inhibition of GFP production by sgRNA1.

In summary, while TTC4 depletion reduced viral late protein expression, this did not translate to diminished production of infectious progeny virions.

3.3 TTC4 overexpression significantly increases BoHV-1 virion production.

As TTC4 expression is increased during BoHV-1 replication, we next tested the effect of TTC4 overexpression on BoHV-1 infection. Despite multiple attempts with several methods including transfection, electroporation and lentivirus transduction, consistent expression of Flag-tagged

TTC4 protein could not be achieved in MDBK cells (data not shown). Therefore HEK293T cells, which are susceptible to BoHV-1-GFP infection and are highly amenable to plasmid transfection were used instead. HEK293T cells were transfected with pcDNA3.1 (+) empty or pcDNA3.1 (+) TTC4-Flag and transfected cells were selected using G418. Expression of TTC4-Flag was confirmed by western blotting **(Fig. 3A).** Empty vector and TTC4-Flag vector-incorporating HEK293T cells were infected with BoHV-1-GFP at an MOI of 10 for 48 hours. No significant difference in VP26 production was seen in the TTC4-Flag expressing cells relative to cells transfected with empty control vector **(Fig. 3B).** However, a significant increase in viral titre, of approximately three-fold was observed in the TTC4 overexpressing cells **(Fig. 3C).** Thus, TTC4 overexpression did not affect late viral protein production, but did improve viral yield.

3.4 HSP90 inhibition blocks BoHV-1 replication.

TTC4 is known to interact with HSP90 [14], and we therefore hypothesised that HSP90 is also pro-viral for BoHV-1 replication. Due to the essential role for HSP90 in cell homeostasis, we tested this using chemical inhibition. Geldanamycin specifically inhibits HSP90 through steric inhibition of its ATP binding site [21]. The cytotoxic threshold of geldanamycin in MDBK cells was established at 8 μM (**Fig. 4A**).

To determine the amount of drug required to block infection we performed limiting dilution growth curves. At low MOI, only the highest concentration of geldanamycin used of 0.027 μM abrogated GFP signal (**Fig. 4B**). Correspondingly, virus replication was limited at this drug concentration (**Fig. 4C**). At high MOI, a dose-dependent effect of drug treatment was observed, with 0.01 μM failing to inhibit replication, 0.03 μM partially inhibiting and 0.09 μM completely abrogating GFP signal (**Fig. 4D**). At 0.03 μM, the production of infectious virus was not blocked (**Fig. 4E**), indicating a minimal requirement of HSP90 to support production of infectious virus progeny. We therefore determined that the amount of drug required to block viral replication gives an *in-vitro* therapeutic index of 267 (**Fig. 4A**). The antiviral effects of geldanamycin in BoHV-1 infection strongly suggests a pro-viral role for HSP90.

4. DISCUSSION

In this study we have confirmed a pro-viral role for TTC4 during BoHV-1 replication, and uncovered parallel pro-viral activity of HSP90. TTC4 knockdown reduced VP26 expression but not new virion production *in-vitro*. This agrees with the previous GeCKO screen, which assessed viral replication solely on VP26-GFP fluorescence [11, 12]. VP26 is expressed late during viral replication, and is therefore a good proxy to indicate that the virus has progressed most of the way through a replication cycle. However, VP26 is non-essential in BoHV-1 *in vitro* replication [22], and so findings made using a VP26 reporter may not necessarily reflect the impact on production of BoHV-1 virions. VP26 is, however, crucial for the neurovirulence of HSV-1 in mouse models [23]. Therefore a reduction in VP26 production may reduce new virion production *in vivo* or in neuronal cell lines, and could impact viral reactivation from latency.

Mixed populations of TTC4 -/- cells were used in this project, meaning that unedited cells or cells with in-frame edits were included in the KO cell population. Single cell cloning could be used to establish a stable TTC4 -/- MDBK cell line. However, transduced KO cells had slower growth kinetics than their wild type counterparts, indicating that TTC4 KO may be toxic to cells. Despite these limitations, we were able to validate the result that TTC4 KO impairs viral protein production.

Conversely, TTC4 overexpression significantly increased BoHV-1 virion production but not VP26 production in HEK293T cells. HEK293T cells, whilst susceptible and permissive to BoHV-1 replication, are human kidney cells and therefore a less ideal model than MDBK cells, which are bovine kidney cells, for studying BoHV-1 replication.

In this work we uncoupled VP26 expression and BoHV-1 virion production during TTC4 KO and overexpression. Whilst VP26 is commonly targeted for fluorescent protein fusion, variable levels of incorporation into BoHV-1 virions may explain the disparity between its expression and new virion production [12, 20, 24]. GFP fusion to another viral capsid protein UL25 has

been used as a reporter system for other alphaherpesviruses, with more consistent protein incorporation into the capsid [25], indicating that this may provide a better proxy to viral replication. Tagging of BoHV-1 UL25, expressed early during infection [26] and required for optimal viral packaging [27], would overcome the possibility of variable reporter protein incorporation into new virions. This would further allow characterisation of additional steps in the viral replication cycle.

The finding that geldanamycin inhibits BoHV-1 replication provides the first evidence that HSP90 is likely to be pro-viral in BoHV-1 infection. This was as expected; geldanamycin has a well-described antiviral effect, *in vitro* and *in vivo*, for both DNA and RNA viruses [28, 29]. The antiviral effect of geldanamycin strongly suggests that HSP90 is pro-viral for BoHV-1 as well. However, geldanamycin has more off target effects than its derivatives and targets all four isoforms of HSP90 non-selectively. To validate the role of HSP90 in BoHV-1 replication, and better characterise the role of individual isoforms, siRNAs could be used to reduce protein expression of specific HSP90 isoforms [29]. KO would be impractical, as HSP90 is essential in eukaryotic cells [16]. Future investigations of the role of HSP90 during BoHV-1 infection may be guided by previous studies of HSP90 in HSV-1 infection, for example through identification of viral products that HSP90 interacts with [18, 30], and subcellular localisation studies [16, 17].

In vivo validation of the pro-viral roles of TTC4 and HSP90 would be challenging to perform as small animal BoHV-1 model are limited to rabbits or immunocompromised mice [31, 32] and large animal models are taxing for practical, financial, and ethical reasons. However, bovine lung and reproductive tract explants and *in vitro* neuronal models have previously been used to study BoHV-1 [24, 28, 33, 34], and this would be valuable future work. Another barrier to the therapeutic use of HSP90 inhibitors is the development of a product accessible to the veterinary market. Despite multiple clinical trials of HSP90 inhibitors, none have gained FDA approval [35, 36]. However, there is significant interest in their development due to suggested therapeutic value in several malignant, infectious and neurodegenerative disorders [37].

HSP90 is a chaperone protein that assists cellular protein folding [15]. It is a component of a heterogeneous population of complexes that exist in a highly dynamic state of flux, interacting with HSP70, HSP40 and a range of other co-chaperones including TTC4. While HSP90 and HSP70 play a central role in protein folding complexes, co-chaperones are predicted to be more client specific, and therefore may only be needed to assist the folding of a smaller subset of cellular proteins [38, 39]. Our proposed model for the roles of HSP90 and TTC4 during BoHV-1 replication is summarised (**Fig. 5**). Possibly, TTC4 is required for optimal pro-viral activity of HSP90. HSP90 interacts with glucocorticoid receptors, increasing cellular cortisol and thereby activating stress responses that in turn stimulate herpesvirus replication [31, 40-42]. Members of the tetratricopeptide repeat protein family (though not specifically TTC4) have been shown to promote cellular stress responses [43-45]. Whether TTC4 and HSP90 together synergise to increase stress responses, and thereby viral replication, should be investigated in the future as a potential mechanism to explain the pro-viral activity of TTC4.

Alternatively, TTC4 and HSP90 may have independent proviral roles. While knockdown of TTC4 typically resulted in ~2-fold reductions in VP26-GFP detection and no reduction in viral progeny production (Fig. 2), chemical inhibition of HSP90 resulted in reduction of VP26-GFP expression several-fold and in viral progeny production by several $\log_{10}$s. Although by necessity different mechanisms of inhibition were used to target the two proteins, the different effects on progeny virion production are striking and may hint at independent pro-viral mechanisms, or that TTC4 may facilitate HSP90 pro-viral activity but is non-essential for this. HSP90 is thought to be essential, and poor toxicity profiles/ severe side effects, including hepatotoxicity have prevented HSP90 inhibitors from become licenced for use in humans. Co-chaperone proteins are considered potential drug targets for cancer therapeutics [46].

GeCKO screens have become widely popular as high throughput methods for identifying novel antiviral and pro-viral cellular proteins. Due to the nature of these whole genome screens, post screen validation of candidate genes is essential. Here we have validated the screen result that TTC4 KO reduced production of the VP26 protein **(Fig. 1)**, thereby showing that the

readout from the screen [11] was accurate. However, TTC4 KO did not significantly alter BoHV-1 virion production **(Fig. 2)**. Nevertheless, we have shown that TTC4 mRNA expression is increased during viral replication **(Fig. 1)**, and that overexpression of exogenous TTC4 significantly increases BoHV-1 replication **(Fig. 3).** We further demonstrated that its co-factor HSP90 is also pro-viral **(Fig. 4)**, though it is unknown whether the two pro-viral factors are acting together. Further work is needed to determine the mechanism(s) underpinning the pro-viral roles of these interacting cellular proteins **(Fig. 5)**.

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COMPETING INTERESTS

None declared.

Figure 1. During BoHV-1 infection, TTC4 transcription increases, and TTC4 KO reduces VP26-GFP protein production but does not impact new virion production. A.TTC4 mRNA and GAPDH mRNA transcript levels were measured in uninfected and MOI 10 infected MDBK cells at 4, 8 ($p = 0.028$) and 16 hpi ($p = 0.036$). **B-C.** MDBK cas9+ PKLV2-2a Control or PKLV2-2a sgRNA1 cells (control and sgRNA1 cells) were infected with BoHV-1 VP26-GFP at MOI 10. At 10 hpi, cells were sorted into 3 subpopulations according to GFP fluorescence. **B.** Proportion of the cells in each of the 3 GFP (No GFP, Low GFP and High GFP) groups in control, sgRNA1 ($p = 0.016$) and sgRNA2 ($p = 0.0053$) cells. **C.** Proportion of TTC4 KO in each cell population was analysed using PCR and ICE analysis for unsorted sgRNA1 cells and the 3 GFP subgroups (no GFP vs low GFP $p = 0.20$; no GFP vs high GFP $p = 0.082$).

Figure 2. Knockdown of TTC4 using CRISPR-cas9 editing impairs viral protein production but not viral replication. BoHV-1 replication was compared in Cas9+ MDBK cells, PKLV2-2a control, sgRNA1 or sgRNA2 cells. Cells were infected at low MOI for 72 hours and high MOI for 24 hours. GFP fluorescence as a proxy for VP26 expression and end point viral titres were used to evaluate BoHV-1 replication. **A**. GFP fluorescence during low MOI BoHV-1-GFP infection. **B**. GFP fluorescence during high MOI BoHV-1-GFP infection. **C**. Viral titres at 72 hpi low MOI BoHV-1-GFP infection. **D**. Viral titres at 24 hpi high MOI BoHV-1-GFP infection. **E.** The efficiency of TTC4 gene editing in control, sgRNA1 and sgRNA2 cells was evaluated using Sanger sequencing and synthego ICE analysis.

* $p < 0.05$.

Figure 3. Overexpression of TTC4 significantly increases BoHV-1 replication but does not alter VP26 expression. HEK293T cells transfected with pcDNA 3.1 (+) empty or pcDNA 3.1 (+) TTC4-Flag were infected with BoHV-1 at an MOI of 10 for 48h. GFP fluorescence as a proxy for VP26 expression and end point viral titres were used to evaluate BoHV-1 replication. **A.** Western blot showing expression of Flag-tagged TTC4 in HEK293T cells. **B**. GFP fluorescent during high MOI BoHV-1-GFP infection. **C.** Viral titres at 48hpi High MOI BoHV-1-GFP infection.

* $p < 0.05$

Figure 4. TTC4 co-factor HSP90 is required for optimal BoHV-1 replication. A. Geldanamycin cytotoxicity in MDBK cells. Cytotoxicity of geldanamycin in WT MDBK cells was evaluated at concentrations from 0 - 500µm. The blue dashed line indicates 0.27µm geldanamycin, the therapeutic dose, at which VP26 expression and virion production were blocked at MOIs of both 0.1 and 10. The red dashed line indicates 8 µm geldanamycin (the minimum cytotoxic dose in MDBK cells). **B-E**. MDBK cells were infected with BoHV-1 at high and low MOI, with geldanamycin at concentrations from 0-0.27 µm. GFP fluorescence as a proxy for VP26 expression and end point viral titres were used to evaluate BoHV-1 replication. **B.** GFP fluorescence during high MOI BoHV-1-GFP infection. **C.** Viral titres at 24hpi high MOI BoHV-1-GFP infection. **D.** GFP fluorescence during low MOI BoHV-1-GFP infection. **E.** Viral titres at 72hpi low MOI BoHV-1-GFP infection.

*$p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$

Figure 5. Illustration of the alphaherpesvirus life cycle including a proposed pro-viral mechanism for HSP90 and TTC4 during BoHV-1 replication. Here we have established a

role for both HSP90 and TTC4 in BoHV-1 replication. We have shown that inhibition of HSP90 using geldanamycin inhibits VP26 expression and new virion production. TTC4 overexpression significantly increases new BoHV-1 virion production, and TTC4 knockdown inhibits VP26 expression. Whilst there has previously been no established roles for TTC4 in herpesvirus replication, its co-factor HSP90 has a well stablished broad pro-viral role, including during replication of the human alphaherpesvirus. Herpes Simplex Virus -1 (HSV-1) is an alphaherpesvirus which infects humans, with a very similar replication cycle to BoHV-1. The roles of HSP90 in HSV-1 replication include stabilisation of the VP16 and UL42 viral proteins [18, 30]. HSP90 inhibitors are antiviral in HSV-1 infection, with AT-533 blocking HSV-1 nuclear egress and assembly [17]. HSP90 is also required for the nuclear transport of HSV-1 DNA polymerase [16] and the ICP5 capsid protein [19]. TTC4 is a HSP90 co-chaperone [14] and was identified as pro-viral in the BoHV-1 GeCKO screen [12]. HSP90 interacts with glucocorticoid receptors that translocate from the cytoplasm to the nucleus to activate stress responses that enhance herpesvirus replication [41-44]. Members of the tetratricopeptide repeat protein family (to which TTC4 belongs) promote cellular stress responses [43-45]; we hypothesise that interactions between TTC4 and HSP90 enhance the glucocorticoid receptor-mediated cellular stress response and so viral replication. Created using BioRender.com.

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