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K_v1.3 induced hyperpolarisation is required for efficient Kaposi's sarcoma-associated herpesvirus lytic replication

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Summary

Host factors critical for virus replication can identify new targets for therapeutic intervention. Pharmacological and genetic silencing approaches reveal that Kaposi's sarcoma-associated herpesvirus (KSHV) requires a B cell expressed voltage-gated K^+ channel, $K_v1.3$, to enhance lytic replication. The KSHV replication and transcription activator protein upregulates $K_v1.3$ expression, leading to enhanced K^+ channel activity and hyperpolarisation of the B cell membrane. Enhanced $K_v1.3$ activity promotes intracellular Ca^{2+} influx, leading to Ca^{2+} driven nuclear localisation of NFAT and subsequent NFAT1-responsive gene expression. Importantly, KSHV lytic replication and infectious virion production were inhibited by both $K_v1.3$ blockers and $K_v1.3$ silencing. These findings provide new mechanistic insight into the essential role of host ion channels during KSHV infection and highlight $K_v1.3$ as a potential druggable host factor.

Introduction

Ion channels are multi-subunit, pore-forming membrane proteins that control rapid and selective passage of ions across the plasma membrane and membranes of subcellular organelles [1]. As such, ion channels have roles in controlling the ion homeostasis of the cell and its organelles, action potential firing, membrane potential and cell volume. Given these various functions and their ubiquitous nature, ion channel dysregulation is implicated in multiple diseases, known as channelopathies [2] and may also enhance cell proliferation and invasion of tumour cells. Interestingly, several stages of virus replication, including virion entry, virus egress and the maintenance of an environment conducive to virus replication have been in-part, suggested to be dependent on virus-mediated manipulation of ion channel activity [2,3]. This is reinforced by observations that pharmacological modulation of ion channels can impede virus replication, highlighting ion channels as promising candidates for targeted anti-viral therapeutics. Importantly, some ion-channel blocking drugs are in widespread human use for ion channel-related diseases, highlighting potential for drug repurposing.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gamma 2-herpesvirus linked to the development of Kaposi's sarcoma (KS), a highly vascular tumour of endothelial lymphatic origin, and several other AIDs-associated malignancies [4-7]. KSHV exhibits a biphasic life cycle consisting of latent persistence or lytic replication. In contrast to other oncogenic herpesviruses in which latent gene expression drives tumorigenesis, both the latent and lytic replication phases are essential for KSHV-mediated tumorigenicity [8]. Latency is established in B cells and the tumour setting, where viral gene expression is limited to the latency-associated nuclear antigen (LANA), viral FLICE inhibitory protein, viral cyclin, kaposins and several virally-encoded miRNAs [9-11]. Upon reactivation, KSHV initiates lytic replication leading to the orchestrated expression of >80 viral proteins, sufficient for the production of infectious virions [12,13]. In KS lesions, most infected cells harbour the virus in a latent state. However, a small proportion of cells undergo lytic replication leading to the secretion of angiogenic, inflammatory and proliferative factors that act in a paracrine manner on latently-infected cells to enhance tumorigenesis [14]. Lytic replication also enhances genomic instability [15] and sustains KSHV episomes in latently-infected cells that would otherwise be lost during cell division [16]. The ability to inhibit lytic replication therefore represents a therapeutic intervention strategy for KSHV-associated diseases [17,18].

The transition from latent infection to lytic replication is controlled by host and viral factors [19,20]. They converge on the regulation of the latency associated nuclear antigen (LANA) and the master regulator of the latent-lytic switch, KSHV replication and transcription activator (RTA) protein [21]. Notably, agents

that mobilize intracellular calcium (Ca^{2+}) induce KSHV-RTA expression and enhance lytic replication [22], however this activity can be blocked with calcineurin-dependent signal transduction inhibitors [23]. Cytoplasmic Ca^{2+} concentrations are regulated by a network of ion channels and transporters [24]. To date, a specific role for host cell ion channels during KSHV lytic replication or any herpesvirus have yet to be fully defined. B lymphocytes, the primary site of KSHV latency, are regulated by a network of transporters and ion channels that control the cytoplasmic concentrations of Ca^{2+}, magnesium (Mg^{2+}) and zinc (Zn^{2+}), which act as important second messengers to regulate critical B cell effector functions [25]. The repertoire of ion channels in B cells include potassium (K^+) channels, Ca^{2+} channels, P2X receptors and transient receptor potential (TRP) channels, in addition to Mg^{2+} and Zn^{2+} transporters. To-date, a role for these channels during KSHV infection has not been described.

Combining electrophysiological and biochemical approaches, we show that KSHV activates a voltage-gated K^+ channel $K_v1.3$, the pharmacological and genetic silencing of which inhibits KSHV lytic replication. We further define the mechanism for this dependence by showing that $K_v1.3$ activation leads to hyperpolarisation-induced Ca^{2+} influx, enhancing the nuclear localisation of NFAT1, which is required to drive virus replication. We therefore reveal the essential role of $K_v1.3$ in KSHV lytic replication.

Results

K^+ channels are required for efficient KSHV reactivation

K^+ channels represent the largest family of ion channels with >70 genes identified in the human genome [26]. To determine if their activity is required for efficient KSHV lytic replication, virus reactivation assays were performed in the presence of potassium chloride (KCl) to collapse cellular K^+ channel gradients, or broad spectrum K^+ channel blockers, tetraethylammonium (TEA) and quinidine (Qn). All inhibitors were used at non-toxic concentrations measured by MTS and annexin V-based flow cytometry assays during both latent and lytic phases (**Figure S1**). KSHV reactivation was assessed in TREx BCBL1-RTA cells, a latently infected KSHV B-lymphocyte cell line expressing a Myc-tagged viral RTA under the control of a doxycycline-inducible promoter. TREx BCBL1-RTA cells reactivated for 24 h in the presence of each K^+ channel inhibitor showed a drastic reduction in the expression of early ORF57, delayed early ORF59 and the late minor capsid ORF65 proteins (**Figure 1A, Figure S2**). No such reduction was observed in the expression of Myc-RTA or GAPDH, highlighting specific effects on lytic replication as opposed to dox-induced induction. This indicates a requirement for K^+ channel function during KSHV lytic replication.

K^+ channel subfamilies include voltage-gated K^+ channels (K_v), calcium-activated K^+ channels (K_{Ca}), inwardly rectifying K^+ channels (K_{ir}) and two-pore domain K+ channels (K2P) channels. To identify the specific K^+ channel(s) required for KSHV lytic replication a more specific drug regime was utilised. 4-aminopyridine (4-AP), a non-selective K_v blocker, led to a concentration-dependent reduction in lytic replication (**Figure 1B, Figure S2**), suggestive of a role for K_v channels. Electrophysiological studies have identified an array of K_v channels expressed within B lymphocytes, with a member of the *Shaker* related family, $K_v1.3$, most extensively characterised [27]. Specific $K_v1.3$ blockers margatoxin (MgTX) and ShK-Dap[22] showed a concentration-dependent reduction of ORF57 protein production, implicating a role for $K_v1.3$ during KSHV lytic replication (**Figure 1C-D, Figure S2**). Similar inhibition of KSHV lytic replication was observed in KSHV infected BCBL-1 and iSLK cells in the presence of ShK-Dap[22] (**Figure S3**). In contrast, TRAM-34, a blocker of B lymphocyte $K_{Ca}3.1$ channels, showed no effect in TREx BCBL1-RTA cells (**Figure 1E, Figure S2**). ShK-Dap[22]-mediated $K_v1.3$ inhibition also lead to a dramatic reduction in a range of KSHV lytically expressed genes across the complete temporal cascade (**Figure 1F, Figure S4).**

To confirm a role for $K_v1.3$, TREx BCBL1-RTA cells were stably transduced with lentivirus-based shRNAs depleting $K_v1.3$ by >85% (**Figure 1G and 1H, Figure S2**). Reactivation assays showed that $K_v1.3$ silencing led to a significant reduction in ORF57 mRNA levels (**Figure 1I**) and lytic proteins compared to control (**Figure 1J and 1K, Figure S2**). $K_v1.3$ depletion also reduced infectious virus production by ~85%, measured by reinfection of naive cells with supernatants from $K_v1.3$ depleted cells and qRT-PCR of viral mRNA (**Figure 1L**) and LANA-immunostaining (**Figure 1M**). To ensure our $K_v1.3$ depletion studies were not due to off-target effects, complementation assays were performed using a lentivirus expressing a $K_v1.3$ shRNA-resistant expression construct. Results showed this rescued KSHV lytic replication and infectious virion production in the $K_v1.3$ depleted cell line, measured by ORF57 protein production (**Figure 1K, Figure S2**) and LANA immunostaining of reinfected supernatants (**Figure 1M**). Together, results show $K_v1.3$ channels are required for efficient lytic replication.

KSHV enhances $K_v1.3$ expression and activity

We next assessed whether KSHV modulated $K_v1.3$ activity. qRT-PCR and immunoblotting showed $K_v1.3$ expression increased in reactivated TREx BCBL1-RTA cells compared to latent cells (**Figure 2A, Figure S2**). To elucidate whether the increased $K_v1.3$ expression led to enhanced K^+ efflux during lytic replication, whole-cell patch clamp analysis was performed. Electrophysiological recordings revealed a voltage-gated outward K^+ current present in latent TREx BCBL1-RTA cells that was significantly enhanced in reactivated cells (**Figure 2B**). To conclusively determine that $K_v1.3$ channels were responsible, recordings were repeated in the presence of ShK-Dap^{22}, which led to a dramatic inhibition of the K^+ current (**Figure 2B**). A similar reduction was observed in $K_v1.3$ depleted cells, compared to controls (**Figure 2C**). We also observed that reactivated TREx BCBL1-RTA cells exhibited a significantly more hyperpolarised membrane compared to latent cells (**Figure 2D**), which was reversed upon $K_v1.3$ depletion (**Figure 2E**). Membrane hyperpolarisation was confirmed using a membrane potential-sensitive dye, bis (1,3-dibutylbarbituric acid) trimethine oxonol; $DiBAC_4(3)$. Results showed a time-dependent decrease in fluorescence intensity in control lytic cells, consistent with enhanced membrane hyperpolarization, whereas no reduction was observed in $K_v1.3$ depleted cells (**Figure 2F**). Conversely, addition of the calcium ionophore A23187, which induces depolarisation, enhanced DiBAC4(3) fluorescence (**Figure 2F**). Together results demonstrate that KSHV lytic replication increases $K_v1.3$ expression, resulting in enhanced $K_v1.3$ currents and hyperpolarisation.

KSHV RTA mediates the upregulation of $K_v1.3$ during lytic replication

Given that membrane hyperpolarisation was observed as early as 4 h post-reactivation, we examined whether any KSHV early proteins induced $K_v1.3$ expression. A549 and U87 cells were transiently transfected with control GFP, RTA-GFP or ORF57-GFP expression constructs and $K_v1.3$ transcript levels were assessed by qRT-PCR at 24 h post-transfection. RTA-GFP alone was sufficient to induce $K_v1.3$ expression in a dose-dependent manner (**Figure S5A-B**), confirming KSHV RTA as the direct inducer of $K_v1.3$ expression. Specificity Protein (Sp) 1 functions as a co-adapter for RTA-mediated transactivation and is known to regulate $K_v1.3$ expression [28]. We therefore examined a potential cooperative role for Sp1 during the upregulation of $K_v1.3$ during lytic replication. RTA-GFP transfections were performed in the presence of Mithramycin A, a selective Sp1 inhibitor that displaces Sp1 binding from its target promoter [29]. Mithramycin A suppressed RTA-mediated increase in $K_v1.3$ expression (**Figure S5C**), but had little effect on the upregulation of the IL-6 promoter, suggesting an in-direct mechanism whereby Sp1 recruits RTA to the $K_v1.3$ promoter. ChIP assays confirmed an association of both RTA and Sp1 with the $K_v1.3$ promoter, which significantly increased during lytic replication (**Figure S5D**). This reveals RTA as the driver of $K_v1.3$ expression during KSHV lytic replication.

$K_v1.3$ induced membrane hyperpolarisation drives Ca^{2+} influx required for KSHV reactivation

In B lymphocytes, $K_v1.3$ maintains a hyperpolarised membrane potential necessary to sustain the driving force for Ca^{2+} entry, which indirectly modulates an array of Ca^{2+}-dependent cellular processes. We therefore assayed Ca^{2+} influx into TREx BCBL1-RTA cells during KSHV lytic replication using the ratiometric Ca^{2+} dye Fura-Red. We observed an increase in cytoplasmic Ca^{2+} over a 24 h period of lytic reactivation, that was absent in $K_v1.3$-depleted TREx BCBL1-RTA cells (**Figure 3A, Figure S6A**). To determine whether Ca^{2+} influx defines the requirement of $K_v1.3$ for efficient lytic replication, we assessed what effect Ca^{2+} depletion, by EGTA chelation, had on lytic replication. Ca^{2+} depletion led to the cytoplasmic accumulation of KSHV RTA and a corresponding reduction in lytic gene expression across the temporal cascade, indicated by a reduction in ORF65 protein levels (**Figure 3B**) and KSHV-encoded transcripts (**Figure S6B**)**.** Conversely mimicking Ca^{2+} influx, by reactivating TREx BCBL1-RTA cells in the presence of the Ca^{2+} ionophore A23187, slightly enhanced ORF57 protein levels compared to control cells (**Figure 3C, Figure S2**), aligning with previous findings [22]. Notably, A23187 rescued KSHV lytic replication in $K_v1.3$ depleted cells, suggesting A23187 overrides the dependence of KSHV on $K_v1.3$ (**Figure 3D, Figure S2**).

To confirm an extracellular source of Ca^{2+} is required for KSHV lytic replication, we compared KSHV replication in TREx BCBL1-RTA cells cultured over 24 hours in normal growth media or calcium-free

media. Results showed that the lack of extracellular calcium dramatically reduced the levels of KSHV lytic replication. Importantly, lytic replication was rescued upon returning the cells to normal growth media (**Figure 3E, Figure S2**). Moreover, metallic divalent ions can inhibit Ca^{2+} permeation via voltage-dependent Ca^{2+} channels [30]. Therefore we assessed what affect the presence of Ni^{2+} had upon KSHV lytic replication. Results showed a significant reduction in virus replication, confirming extracellular Ca^{2+} is required (**Figure 3F, Figure S2**). In contrast Thapsigargin, which inhibits ER calcium pumps leading to depletion of ER Ca^{2+} stores [31], had little effect on KSHV lytic replication (**Figure 3G, Figure S2**). These data suggest extracellular Ca^{2+} influx is essential for KSHV lytic replication and is induced by $K_v1.3$-mediated hyperpolarisation.

To identify candidate Ca^{2+} channel(s) required for KSHV-mediated Ca^{2+} influx, lytic replication was assessed in presence of various Ca^{2+} channel modulating drugs at non-cytotoxic concentations (**Figure S1**). Incubation with either 2-APB, an inhibitor of intracellular calcium levels and transient receptor potential (TRP) channels, BTP2 a blocker of store-operated Ca^{2+} entry and Nifedipine, an L-type voltage-gated Ca^{2+} channel inhibitor, all showed little effect on KSHV lytic replication. In contrast, Mibefradil a specific inhibitor of T-type voltage-gated Ca^{2+} channels dramatically reduced the levels of KSHV lytic replication (**Figure S7**). This suggests that T-type voltage-gated Ca^{2+} channels may have a role in $K_v1.3$-mediated hyperpolarisation-induced calcium influx, although the exact channel is yet to be determined.

KSHV-mediated Ca^{2+} influx initiates NFAT1-mediated gene expression

Ca^{2+} influx initiates multiple signalling pathways, including the serine/threonine phosphatase calcineurin and its target transcription factor NFAT (nuclear factor of activated T cells) [24]. Dephosphorylation of cytoplasmic NFAT proteins by calcineurin unmasks their nuclear localization sequences, leading to nuclear translocation and NFAT-responsive gene expression. We therefore determined whether the calcineurin-mediated nuclear import of NFAT1 was important for KSHV lytic replication. In the presence of calcineurin/NFAT1 inhibitors, cyclosporin A (CsA) and VIVIT, a dose-dependent reduction in ORF57 protein production (**Figure 4A, Figure S2)** and a reduction in KSHV lytic genes was observed (**Figure S8**). To investigate whether KSHV-mediated hyperpolarisation and Ca^{2+} influx promoted the nuclear translocation of NFAT, the nuclear/cytoplasmic distribution was compared in latent versus lytic TREx BCBL1-RTA cells using immunofluorescence analysis. Results showed that NFAT1 translocates to the nucleus in lytic cells, but remains cytoplasmic during latency (**Figure 4B**). The nuclear localisation of NFAT1 was dependent on $K_v1.3$-mediated hyperpolarisation

and calcineurin activity, has it was prevented by ShK-Dap^{22} and CsA, respectively (**Figure 4B**). Consistent with enhanced NFAT nuclear localisation, we observed an increase in NFAT-responsive gene expression during KSHV lytic replication, which was reduced in the presence of ShK-Dap^{22} (**Figure 4C**), and upon K_v1.3 depletion (**Figure 4D**). Interestingly, a number of NFAT-responsive genes, such as COX-2 and ANGPT2 are upregulated during KSHV infection and implicated in KSHV-mediated pathogenesis [32-34]. Together, data suggest that KSHV-induced hyperpolarisation, mediated by K_v1.3, and the subsequent Ca^{2+} influx enhances NFAT1 nuclear localisation and NFAT-driven gene expression.

Discussion

Discovery of cellular determinants that control KSHV lytic induction can inform new therapeutic targets for anti-KSHV drug development. Ion channels control a range of cellular processes that are co-opted by viruses [2,3]. Accordingly, ion channels have emerged as druggable host targets to prevent both RNA and DNA virus replication. Given the known dependence of KSHV lytic replication on Ca^{2+} signalling [23], coupled to previous studies demonstrating VZV and HSV-1 activating Na^{+} and Ca^{2+} family members [35,36], we investigated the role of B-cell expressed ion channels during KSHV lytic reactivation. We reveal that KSHV requires a B-cell expressed voltage-gated K^{+} channel, $K_v1.3$, to enhance lytic replication and KSHV RTA protein upregulates $K_v1.3$ expression via indirect Sp1-mediated transactivation. Enhanced $K_v1.3$ expression and activity led to hyperpolarisation of B-cell membrane potential, initiating Ca^{2+} influx. At present the channel which drives the extracellular Ca^{2+} influx is unknown, however studies suggest a potential T-type channel. This Ca^{2+} elevation enhances the nuclear localisation of NFAT1 and KSHV RTA, which are both essential to drive KSHV lytic replication. At present the exact role of Ca^{2+} in RTA-mediated nuclear import is yet to be elucidated however potential mechanisms may involve enhanced recruitment of nuclear import proteins, or the unmasking of nuclear localisation signals [37]. Together, this reveals that $K_v1.3$-mediated hyperpolarisation and Ca^{2+} influx are direct contributors to KSHV lytic replication in B cells.

A striking feature of KSHV is the homology of its numerous ORFs to cellular genes [13]. These virus-encoded proteins contribute to KSHV-associated pathogenesis by subverting cell signalling pathways. Many viruses encode viroporins [38] that modulate the ionic milieu of intracellular organelles controlling virus protein stability and trafficking. However, no known viroporin exists amongst KSHV ORFs and it is likely that evolution has tailor-made KSHV proteins to regulate the expression of host ion channels to regulate Ca^{2+} signalling during infection. Tumorigenesis represents a by-product of this regulation, since enhanced $K_v1.3$ expression correlates with the grade of tumour malignancy in various cancers [39]. Notably, features of KS tumours mirror the phenotypic effects of $K_v1.3$ overexpression, including the enhanced expression of inflammatory and angiogenic cytokines and uncontrolled cell cycle progression. This suggests that KSHV-driven activation of $K_v1.3$ is similar to channelopathies, diseases characterised by altered function of ion channel proteins or their regulatory subunits.

Several ion channel inhibitors target $K_v1.3$, comprising small organic molecules such as quinine and 4AP or peptides purified from venom [28,40]. Venom-derived peptides are highly stable and resist denaturation due to the disulphide bridges formed within the molecules [40]. Like margatoxin, most are

derived from scorpion venom, such as agitoxins, kaliotoxin, maurotoxin and noxiustoxin, whereas ShK, is a peptide isolated from the sea anemone *Stichodactyla helianthus* [41]. Given the abundance of natural sources for K_v1.3-inhibition a safe, effective therapeutic based on these compounds is a promising target for prevention. Additionally, it is interesting to note that the CD20 monoclonal antibody rituximab, a known K_v1.3 inhibitor, substantially improves KSHV patients outcome [42].

Finally, K_v channels have been previously identified as a restriction factor to the entry of both Hepatitis C virus [43] and Merkel cell polyomavirus [44], through their abilities to inhibit endosome acidification-mediated viral membrane fusion. Whilst the inhibition of endosomal acidification reduces the entry and trafficking of KSHV virions, our electrophysiological analysis revealed enhanced cell surface K_v1.3 activity during lytic replication that directly contributed to the hyperpolarised membrane potential of cells required for efficient KSHV replication. Thus, whilst additional roles of K_v1.3 in endosomes cannot be excluded, our data suggest a divergent role of K_v1.3 during infection that may be cell-type and/or virus specific.

Limitations of the study. Although we reveal the requirement of K_v1.3 for KSHV reactivation it is important to note that compounds targeting K_v1.3 are only in preclinical development [45]. Moreover, although Ca^{2+} influx is essential for KSHV lytic replication and results suggest the involvement of a T-type voltage-gated Ca^{2+} channel, the exact channel is yet to be identified. Another key limitation of this study is that results are based on *in vitro* studies and further studies targeting these channels using appropriate *in vivo* models are now warranted.

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Author Contributions

Conceptualization (JM, AW); Data curation (HC, KLH, TJM, MLD, DJH, KLA, OM); Formal Analysis (HC, KLH, TJM, MLD, DJH, KLA, OM, JDL, JM, AW); Funding acquisition (AW, JM); Investigation (HC, KLH, MLD, DJH); Writing–original draft (HC, KLH, JM, AW); Writing–review & editing (All authors).

Competing interests: There are no financial and non-financial competing interests.

Figure Legends

Figure 1. $K_v1.3$ channels are required for efficient KSHV lytic replication.

(A-E) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-cytotoxic concentrations of (A) general K^+ inhibitors, 25 mM KCl, 100 µM Qn and 10 mM TEA for 45 minutes prior to reactivation with doxycycline hyclate for 48 hr, or increasing amounts of (B) 4AP (C) MgTX, (D) ShK-Dap^{22} and (E) TRAM34. Cell lysates were probed with ORF57-, ORF59- or ORF65-specific antibodies. GAPDH was used as a measure of equal loading.

(F) TREx BCBL1-RTA cells remained unreactivated or pre-treated with 100 pM ShK-Dap^{22} for 45 minutes prior to reactivation with doxycycline hyclate, samples were harvested at 0, 8, 16 and 24 hours post-reactivation, total RNA was extracted and relative ORF57 transcript levels were analysed by qRT-PCR using GAPDH as a reference. Fold change was determined by ΔΔCt and statistical significance analysed using a non-paired t-test, ** = $p<0.01$.

(G-J) Scramble and $K_v1.3$-depleted cells lines were reactivated with doxycycline hyclate for 24 hr. Total RNA was extracted and relative transcript levels of (G) $K_v1.3$ or (I) ORF57 were analysed by qRT-PCR using GAPDH as a reference. Fold change was determined by ΔΔCt and statistical significance analysed using a non-paired t-test, *** = $p<0.001$. Cell lysates were probed with (H) $K_v1.3$- or (J) ORF57-, ORF59- or ORF65-specific antibodies and GAPDH used as a measure of equal loading.

(K) Scramble, $K_v1.3$-depleted or $K_v1.3$-rescued cell lines were reactivated for 24 h, cell lysates were probed with ORF57 and $K_v1.3$-specific antibodies and GAPDH used as a measure of equal loading.

(L) Scramble and $K_v1.3$-depleted cell lines were reactivated for 72 hr, prior to the culture medium being incubated with HEK-293T cells. Total RNA was extracted and relative ORF57 transcript levels were analysed by qRT-PCR using GAPDH as a reference, results show the mean of three biological replicates with error bar as standard deviation, *** = $p<0.001$.

(M) Scramble, $K_v1.3$-depleted or $K_v1.3$-rescued cell lines were reactivated for 72 hr, prior to the culture medium being incubated with HEK-293T cells. Cells were then probed with a LANA-specific antibody and DAPI-stained before imaging on a Zeiss LSM880 confocal microscope.

Figure. 2. Increased K^+ currents during KSHV lytic replication is dependent on $K_v1.3$ expression.

(A) TREx BCBL1-RTA cells remained unreactivated or were reactivated with doxycycline hyclate for 24 hr. (i) Total RNA was extracted and relative $K_v1.3$ transcript levels were analysed by qRT-PCR using GAPDH as a reference. Fold change was determined by ΔΔCt and statistical significance analysed using a non-paired t-test, **** = $p<0.0001$. (ii) Cell lysates were probed with $K_v1.3$ and ORF57-specific antibodies and GAPDH used for equal loading.

(B-C) Mean current density voltage relationships for K^+ currents (n=5 for all populations, statistical significance *** = p<0.001, **** = p<0.0001) from (B) unreactivated and reactivated TREx BCBL1-RTA at 16 hr; cells were pre-treated for 24 hours with DMSO control or 100 pM ShK-Dap[22] and (C) Scramble and K_v1.3-depleted cells lines remained unreactivated or were reactivated with doxycycline hyclate for 24 hr.

(D-E) Pooled data highlighting resting membrane potentials in (D) latent and lytic TREx BCBL1-RTA cells or (E) Scramble and K_v1.3-depleted cells lines (statistical significance ** = p<0.01).

(F) Membrane polarisation of TREx BCBL1-RTA cells was measured by Flow cytometry after a 5 min incubation with DiBAC4(3) in unreactivated and reactivated control and K_v1.3-depleted TREx BCBL1-RTA cells (n=3, statistical significance * = p<0.05, ** = p<0.01, *** = p<0.001).

Figure 3. Ca^{2+} influx is essential during KSHV lytic replication and sufficient to override the effect of K_v1.3 knockdown.

(A) Fura Red staining of calcium ratios were measured in unreactivated and reactivated control and K_v1.3-depleted TREx BCBL1-RTA cells by Flow cytometry, the calcium ionophore A23187 was used as a positive control (n=3, statistical significance * = p<0.05, ** = p<0.01, *** = p<0.001).

(B) TREx BCBL1-RTA cells were pretreated with 1mM EGTA 45 minutes prior to reactivation (i) cells were probed with RTA-specific antibodies and DAPI-stained before imaging on a Zeiss LSM880 confocal microscope, (ii) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-cytotoxic concentrations of 1mM EGTA for 45 minutes prior to reactivation for 24 hr. Cell lysates were probed with ORF65-specific antibodies. GAPDH was used for equal loading.

(C-D) Unreactivated and reactivated (C) Control or (D) K_v1.3-depleted cells lines were assessed for levels of lytic replication in the presence of the calcium ionophore A23187, added to cells simultaneously to Dox. Cell lysates were probed with ORF57-specific antibodies and GAPDH used for equal loading.

(E) TREx BCBL1-RTA cells were incubated in normal or calcium free media for 24 hours prior to reactivation. Cells were also rescued from calcium free media for 12 hours prior to reactivation. Cell lysates were probed with ORF57-specific antibodies and GAPDH used for equal loading.

(F-G) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-cytotoxic concentrations of (F) Nickel chloride or (G) Thapsigargin for 45 minutes prior to reactivation for 24 hr. Cell lysates were probed with ORF57-specific antibodies. GAPDH was used for equal loading.

Figure 4. KSHV-mediated calcium influx initiates NFAT1 nuclear localisation and NFAT1-mediated gene expression.

(A) TREx BCBL1-RTA cells remained unreactivated or were pre-treated with non-cytotoxic dose-dependent concentrations of inhibitors (i) CsA and (ii) VIVIT for 45 minutes prior to reactivation with doxycycline hyclate for 24 hr and probed with ORF57-specific antibody, GAPDH was used for equal loading.

(B) TREx BCBL1-RTA cells remained unreactivated or were pre-treated with inhibitors and (i) probed with endogenous NFAT1 or ORF57-specific antibodies and DAPI-stained before imaging on a Zeiss LSM880 confocal microscope (ii) Quantification of NFAT1 immunofluorescence using Image J.

(C-D) Total RNA was extracted from unreactivated and reactivated (C) TREx BCBL1-RTA cells at 24 hr, or cells pre-treated with ShK-Dap^{22} and (D) Scrambled control and K_v1.3-depleted cells lines. Relative NFAT-responsive transcript levels were analysed by qRT-PCR using GAPDH as a reference. Fold change was determined by ΔΔCt and statistical significance analysed using a non-paired t-test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

384 **STAR METHODS**

385

386 **Key Resource Table**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GAPDH (60004-1)	Proteintech	60004-1-Ig
ORF57 (207.6)	Santa Cruz	sc-135746
ORF59	Britt Glaunsinger; University of California, Berkeley (gift)	
c-Myc (9E10)	Sigma-Aldrich	M4439-100UL
$K_V1.3$	Sigma-Aldrich	MFCD02097210
Sp1 (EPR22648-50)	Abcam	ab231778
ORF65	SJ Gao; University of Pittsburgh (gift)	
LANA	Sigma-Aldrich	MABE1109
NFAT1	Abcam	ab244310
Goat anti-mouse HRP	Agilent Technologies	P044701-2
Goat anti-rabbit HRP	Agilent Technologies	P044801-2
Alexa Fluor 488 anti-rabbit	ThermoFisher	A-11008
Alexa Fluor 568 anti-mouse	ThermoFisher	A-11004
Bacterial and virus strains		
BL21(DE3)	Thermo Scientific	C600003
Chemicals, peptides, and recombinant proteins		
2-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma-Aldrich	P1585
4-Aminopyridine (4AP)	Sigma-Aldrich	275875
Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC4(3))	ThermoFisher	B438
2-APD	Tocris	1224
BTP-2	Abcam	223499-30-7
Calcium ionophore A23187 (A23187)	Sigma-Aldrich	C7522
Cyclosporin A (CsA)	Generon	AOB5973
$DiBAC_4(3)$	ThermoFisher	B438
Doxycycline hyclate (Dox)	Sigma-Aldrich	D9891-1G
Fura Red	ThermoFisher	F3020
LunaScript RT SuperMix (5X)	New England Biolabs	M3010
Margatoxin (MgTX)	Sigma-Aldrich	M8437
Mithramycin A	Insight Biotechnology	sc-200909
Mibefradil (Mib)	Cambridge Bioscience	15037-5mg-CAY
Nickel Chloride	Sigma-Aldrich	654507
Nifedipine (Nif)	Sigma-Aldrich	N7634
Quinine hydrochloride dihydrate (Qn)	Sigma-Aldrich	Q1125
Puromycin	ThermoFisher	A1113803
SensiMix SYBR green master mix	Bioline	QT650-05
$ShK\text{-}Dap^{22}$ (ShK)	Bio-Techne	3220/100U
Sodium butyrate	Sigma-Aldrich	B5887
Thapsigargin	ThermoFisher	T7458

Tetraethylammonium chloride (TEA)	Sigma-Aldrich	T2265
TRAM-34 (TRAM)	Sigma-Aldrich	T6700
Vivit	Tocris	3930
Critical commercial assays		
EZ-ChIP	Merck Millipore	17–371
Pierce chromatin prep module	Thermo Scientific	26158
CellTiter 96 AQueous One Solution Cell Proliferation Assay	Promega	
Monarch® Total RNA Miniprep Kit	New England Biolabs	T2010S
Experimental models: Cell lines		
Human: A549	ATCC	CCL-185
Human: BCBL1	ATCC	RRID:CVCL_0165
Human: HEK-293T	ATCC	CRL-3216
Human: iSLK-BAC16	Prof. J. Jung; University of Southern California (gift)	
Human: TREx BCBL1-RTA	Prof. J. Jung; University of Southern California (gift)	
Human: U-87 MG	Prof. J. Ladbury; University of Leeds (gift)	HTB-14
Oligonucleotides		
See Supplementary Table 1 for Oligonucleotides	Integrated DNA Technologies	
Recombinant DNA		
Human Foetal Brain cDNA library	Invitrogen	D883001
pEGFP-N1	Clontech	CB2370178
pLENTI-CMV-Kv1.3-Zeo	This Paper	
pORF57-GFP	Detailed in Ref 49	
pRTA-EGFP	Detailed in Ref 49	
pVSV.G	Dr. E. Chen; University of Westminster (gift)	
psPAX2	Dr. E. Chen; University of Westminster (gift)	
pLKO.1 with KCNA3-targeting hairpin	Dharmacon	TRCN0000044121
Scramble shRNA	Addgene	Addgene plasmid # 1864
Software and algorithms		
ImageJ	https://imagej.nih.gov/ij/	https://imagej.nih.gov/ij/
Graphpad prism	Dotmatics	https://www.graphpad.com/scientific-software/prism/
Rotor-Gene Q	Qiagen	https://www.qiagen.com/gb/resources/
CytExpert Software for the CytoFLEX Platform	Beckman Coulter	https://www.beckman.com/flow-cytometry/research-flow-cytometers/cytoflex/software

Patchmaster	Heka	https://www.heka.com/downloads/downloads_main.html#down_patchmaster
Zen Microscopy software (Black and Blue)	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Adrian Whitehouse (a.whitehouse@leeds.ac.uk).

Materials availability

Plasmids and all unique reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Experimental model and subject details

Cells

TREx-BCBL-1-RTA cells (kindly provided by Prof. Jae Jung, University of Southern California) are a BCBL-1-based primary effusion lymphoma (PEL) B cell line engineered to express exogenous Myc-tagged RTA upon addition of doxycycline, triggering reactivation of the KSHV lytic cycle. BCBL1, A549 and HEK-293T cell lines were purchased from the American Type Culture Collection (ATCC). U-87 MG cells (kindly provided by Prof. J. Ladbury, University of Leeds) are a human brain glioblastoma astrocytoma cell line. iSLK-BAC16 cells (also provided by Prof. Jae Jung, University of Southern California) are a Caki1-derived renal carcinoma cell line, latently infected with bacterial artificial chromosome 16 (BAC16)-derived KSHV. A549, iSLK, U87 and HEK-293T cells were grown in DMEM (Life Technologies) supplemented with 10% foetal calf serum (FCS) (Life Technologies) and 1% penicillin/streptomycin (P/S). TREx BCBL1-RTA and BCBL1 cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS and 1% P/S or calcium free RPMI (Genaxxon Bioscience), TREx BCBL1-RTA were maintained under hygromycin B (Life Technologies) selection (100 µg/ml). Reactivation into the lytic cycle was induced using 2 µg/ml doxycycline hyclate, (Sigma) for TREx BCBL1-RTA or with 2 mM sodium butyrate and 20 ng/ml 2-O-tetradecanoylphorbol-13-acetate (TPA) (both Sigma). All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Method details

Lentivirus-based shRNA Knockdown and Rescue

Lentiviruses were generated by transfection of HEK-293T cells seeded in 12-well plates using a three-plasmid system[46]. Per 6-well, 4 µl of lipofectamine 2000 (Thermo Scientific) were used together with 1 µg of pLKO.1 plasmid expressing shRNA against the protein of interest (Dharmacon), 0.65 µg of pVSV.G, and 0.65 µg psPAX2. pVSV.G and psPAX2 were a gift from Dr. Edwin Chen (University of Westminster, London). Eight hours post-transfection, media was changed with 2 mL of DMEM supplemented with 10% (v/v) FCS. 500,000 TREx BCBL1-RTA cells in 6 well plates were infected by spin inoculation with the filtered viral supernatant for 60 min at 800 x g at room temperature, in the presence of 8 µg/mL of polybrene (Merck Millipore). Virus supernatants were removed 7 h post-spin inoculation and cells were maintained in fresh growth medium for 48 h prior to selection in 3 µg/mL puromycin (Sigma-Aldrich). Stable cell lines were generated after 8 days of selection. All shRNA plasmids were purchased from Dharmacon. Scramble shRNA was a gift from Professor David Sabatini (Addgene #1864). K_v1.3 codon exchange plasmids were generated via inverse PCR mutagenesis utilising a pLENTI-CMV-K_v1.3-ZEO plasmid generated via Gibson Assembly. The mutagenesis process involved exchanging the wobble base of each codon of the 20bp targeted by the shRNA constitutively expressed within the cells. Thus, the resulting K_v1.3 RNA transcripts show resistance to shRNA activity, restoring expression in transfected cells. The plasmids were transfected in to the ΔKv1.3 TREx-BCBL1-RTA cell line following the three-plasmid system described above, with the shRNA-resistant pLENTI-CMV-K_v1.3-ZEO plasmid replacing the pLKO.1 plasmid, and zeomycin used for selection at 250 µg/ml.

Transient Transfections

Plasmid transfections were performed using Lipofectamine 2000 (Life Technologies), at a ratio of 2 ug plasmid to 1 ul Lipofectamine in 100 ul opti-MEM. Transfection media was incubated at room temperature for 15 minutes before 1x 10^6 cells were treated, dropwise. Cells were harvested after 24 hours.

Immunofluorescence

Cells were cultured overnight on poly-L-lysine (Life Technologies) coated glass coverslips in 24-well plates. Cells were fixed with 4% paraformaldehyde (Calbiochem) for 10 min and permeabilised with 0.1% Triton X-100 for 20 min [47]. Cells were blocked in PBS containing 1% BSA for 1 h at 37°C and labelled with primary antibodies for 1 h at 37°C. Cells were washed five times with PBS and labelled with appropriate secondary antibodies for 1 h at 37°C. Cells were washed five times with PBS and

mounted in VECTASHIELD containing DAPI (Vector Labs). Images were obtained using a Zeiss LSM880 confocal microscope and processed using ZEN 2009 imaging software (Carl Zeiss) [48].

Electrophysiology

TREx BCBL1-RTA cells seeded onto poly-L-lysine (Life Technologies) coated glass coverslips and were transferred to a recording chamber, containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES-NaOH, pH 7.2, 2 mM $CaCl_2$, 10 mM glucose, and mounted on the stage of a Nikon Eclipse inverted microscope. Patch pipettes (5–8 MΩ) were filled with a solution consisting of: 140 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES KOH, pH 7.2, 10 mM glucose. Voltage-clamp recordings were performed using a HEKA EPC-10 integrated patch clamp amplifier controlled by Patchmaster software (HEKA). Series resistance was monitored after breaking into the whole cell configuration. To examine K^+ currents, a series of depolarizing steps were performed from −100 to +60 mV in 10 mV increments for 100 ms. Resting membrane potential was measured using the current clamp mode of the amplifier. Results are shown as the mean ± SEM of n number of individual cells. Statistical analysis was performed using an unpaired Student's T test. $p<0.05$ was considered statistically significant.

Flow Cytometry

Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol ($DiBAC_4(3)$) and Fura Red (both ThermoFisher) were added to cells at a final concentration of 1 µM in RPMI-media. Cells were incubated at 37°C with Fura Red for 30 min or $DiBAC_4(3)$ for 5 min and washed in PBS. Cells were analysed on a CytoFLEX Flow Cytometer (Beckman). Data were quantified using CytExpert software (Beckman).

Proliferation (MTS) assays

Cellular viability was determined using non-radioactive CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent (Promega), according to the manufacturer's recommendations [48,49]. TREx BCBL1-RTA cells (~20,000) were seeded in triplicate in a flat 96-well tissue culture plates (Corning) and treated with the indicated inhibitors for 24 h. CellTiter 96 AQueous One Solution Reagent was added to the cells for 1 h at 5% CO_2, 37^{O}C. Absorbances were measured at 490 nm using an Infinite plate reader (Tecan).

Two-step quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using the Monarch® Total RNA Miniprep Kit (New England Biolabs) as per the manufacturer's protocol. RNA (1 µg) was diluted in a total volume of 16 µl nuclease-free water, and 4 µl LunaScript RT SuperMix (5X) (New England Biolabs) was added to each sample. Reverse

transcription was performed using the protocol provided by the manufacturer. cDNA was stored at -20°C, RNA was stored at -80°C. Quantitative PCR (qPCR) reactions (20 µl) included 1X SensiMix SYBR green master mix (Bioline), 0.5 µM of each primer and 5 µl template cDNA (used at 1:200 dilution in RNase-free water). Cycling was performed in a RotorGene Q instrument (Qiagen) [48]. The cycling programme was a 10 min initial preincubation at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 20 sec. After qPCR, a melting curve analysis was performed between 65°C and 95°C (with 0.2°C increments) to confirm amplification of a single product. To assess primer amplification efficiency (AE), for each gene of interest a standard curve was constructed using a pool of cDNA derived from unreactivated and reactivated cells. At least four different dilutions of pool cDNA were quantified to generate a standard curve. The slope of the standard curve was used to calculate the AE of the primers using the formula: AE = (10−1/slope). For gene expression analysis all genes of interest were normalised against the housekeeping gene GAPDH (ΔCT) [50]. A summary of all the primers used in this study is provided in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP)

Formaldehyde-crosslinked chromatin was prepared using the Pierce Chromatin Prep Module (Thermo Scientific) following the manufacturer's protocol. Cells (2 x 10^6) were digested with six units of micrococcal nuclease (MNase) per 100 µl of MNase Digestion buffer in a 37°C water bath for 15 min. These conditions resulted in optimal sheared chromatin with most fragments ranging from 150–300 base pairs in size. Immunoprecipitations were performed using EZ-ChIP kit (Millipore) kits overnight at 4°C and contained 50 µl of digested chromatin (2 x 10^6 cells), 450 µl of ChIP dilution buffer and 1.5 µg of RNAPII antibody (clone CTD4H8) (Millipore) or isotype antibody, normal mouse IgG (Millipore). qPCR reactions were performed using either 2 µl of immunoprecipitated DNA or 2 µl of input DNA as template [51].

Immunoblotting

Protein samples were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham) via semi-dry transfer using a Trans-Blot® Turbo™ blotter (BioRad). Membranes were blocked in TBS + 0.1% Tween 20 and 5% dried skimmed milk powder and probed with relevant primary antibodies followed by horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse and polyclonal goat anti-rabbit secondary antibodies (Dako). Membranes were treated with EZ-ECL (Geneflow) and imaged using a G-Box (Syngene).

Quantification and statistical analysis

Statistical analysis as specified in figure legends were performed with Prism 9 (GraphPad software Inc., San Diego, California, United States). Graphical data shown represent mean ± standard deviation of mean (SD) using three or more biologically independent experiments. Differences between means was analysed by unpaired Student's t test, or distribution with two sample Kolmogorov-Smirnov test as detailed in the figure legends. Statistics was considered significant at $p < 0.05$, with *P<0.05, **P<0.01, ***P<0.001.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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