Peer-reviewed version available at Viruses 2018, 10, 349; doi:10.3390/v10070349

Review

Molecular aspects of Varicella-Zoster Virus latency

Daniel P. Depledge [1,*], Tomohiko Sadaoka [2] and Werner J. D. Ouwendijk [3]

[1] Department of Microbiology, New York University School of Medicine, NY 10016, USA. E-mail: daniel.depledge@nyumc.org. ORCID ID: https://orcid.org/0000-0002-4292-0599.
[2] Division of Clinical Virology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan. E-mail: tomsada@crystal.kobe-u.ac.jp. ORCID ID: https://orcid.org/0000-0001-7951-6904.
[3] Department of Viroscience, Erasmus Medical Centre, 3015 CN, Rotterdam, The Netherlands. E-mail: w.ouwendijk@erasmusmc.nl. ORCID ID: https://orcid.org/0000-0001-8393-296X.
* Correspondence: daniel.depledge@nyumc.org; Tel.: +1-212-263-0445

Abstract: Primary varicella-zoster virus (VZV) infection causes varicella (chickenpox) and the establishment of a lifelong latent infection in ganglionic neurons. VZV reactivates in about one-third of infected individuals to cause herpes zoster, often accompanied by neurological complications. The restricted host range of VZV and, until recently, the lack of suitable *in vitro* models to study VZV latency have seriously hampered molecular studies of viral latency. Nevertheless, recent technological advances facilitated a series of exciting studies that resulted in the discovery of a VZV latency-associated transcript (VLT) and have redefined our understanding of VZV latency and factors that initiate reactivation. Together, these findings pave the way for a new era of research that may finally unravel the precise molecular mechanisms that govern latency. In this review, we will summarize the implications of recent discoveries in the VZV latency field from both a virus and host perspective and provide a roadmap for future studies.

Keywords: varicella-zoster virus, latency, reactivation, sensory ganglia, VZV latency-associated transcript, open reading frame 63, RNA-sequencing, epigenetics, immunity.

1. Introduction

Most adults worldwide are infected with the neurotropic human alphaherpesvirus varicella-zoster virus (VZV) [1]. VZV is the causative agent of two distinct diseases: a generalized vesicular skin rash referred to as varicella (chickenpox), and a localized dermatomal skin rash referred to as herpes zoster (HZ; shingles) [2]. Although varicella and HZ were known to be related [3], it was not until 1965 that the British general practitioner Dr Robert E. Hope-Simpson suggested that "*herpes zoster is a spontaneous manifestation of varicella infection*". This observation was based on a careful examination of ~3,500 patients, including 192 HZ cases, who visited his practice over a 16-year period, combined with cautious reading of the available anatomical and epidemiological literature regarding HZ. This led to his famous hypothesis that: "*Following the primary infection (chickenpox), virus becomes latent in the sensory ganglia, where it can be reactivated from time to time (herpes zoster)*" [4]. Eighteen years later, this hypothesis was proven by Dr Donald Gilden's crucial discovery of VZV DNA in latently infected human ganglia [5]. Although we have learned much about VZV biology in general over the last decades, the mechanisms underlying VZV latency and reactivation have remained enigmatic, at least in part because VZV is a human pathogen that does not cause disease in experimental animal models. However, recent insights into the VZV latency program and newly developed *in vitro* models using human embryonic stem cell (hESC)-derived neurons for viral

latency and reactivation now set the stage to unravel the molecular mechanisms that regulate VZV latency. In preparing this review we sought to be inclusive of as many view points as possible and to consider only human-derived cell or tissue based systems (animal models utilised for VZV studies are the subject of another review in this issue). At the outset, we therefore limit this review to only include studies of VZV latency in which there is a demonstrable lack of infectious virus production and/or any observable pathology.

2. Molecular biology of VZV

2.1 Structure and genomic organization of VZV

VZV particles are pleomorphic to spherical in shape, ~150–200 nm in diameter and composed of a nucleocapsid containing the viral double stranded DNA (dsDNA) genome, which is surrounded by a layer of tegument proteins of both viral and host origin, all contained within an envelope comprising a host-derived lipid bilayer inserted with viral glycoproteins (Figure 1A-B) [6–8]. Upon entry of a VZV virion into the host cell, tegument proteins are released into the newly infected cell, altering the host environment to inhibit antiviral responses and influencing the fate of virus program i.e. a lytic or latent infection (reviewed in [9]). The VZV dsDNA genome is about 125 kilo base pairs (kbp) in size, has a G+C content of 46% and is composed of two unique segments, termed unique long (U_L) and unique short (U_S), that are flanked by inverted terminal repeat (TR) and internal repeat (IR) structures with high G+C content (68% for the TR_L/IR_L and 59% for the IR_S/TR_S) (Figure 1C) [10–19]. The very short (~88 bp) TR_L and IR_L sequences flank the U_L region, while the long (7,319 bp) IR_S and TR_S sequences flank the U_S region. The composite structure generally allows for two isomeric configurations [17] that differ only in whether the U_S region is inverted. The absence of structural isoforms with inverted U_L regions is attributed to a unique DNA sequence at the extreme 5′ end of U_L region that is required for viral DNA cleavage during packaging [20]. Five regions of the genome contain tandem direct reiterations (R1, R2, R3, R4, and R5) of short repeat sequences, one of which is located in the IR_S/TR_S. All except R5 are G+C rich, and all are subject to length and structural polymorphisms that vary both within and between strains [21–25]. Three of these reiterative regions (R1, R2, and R3) are located within the coding portion of VZV genes [open reading frame (ORF) 11, ORF14, and ORF20, respectively] and may therefore exert an affect over protein function.

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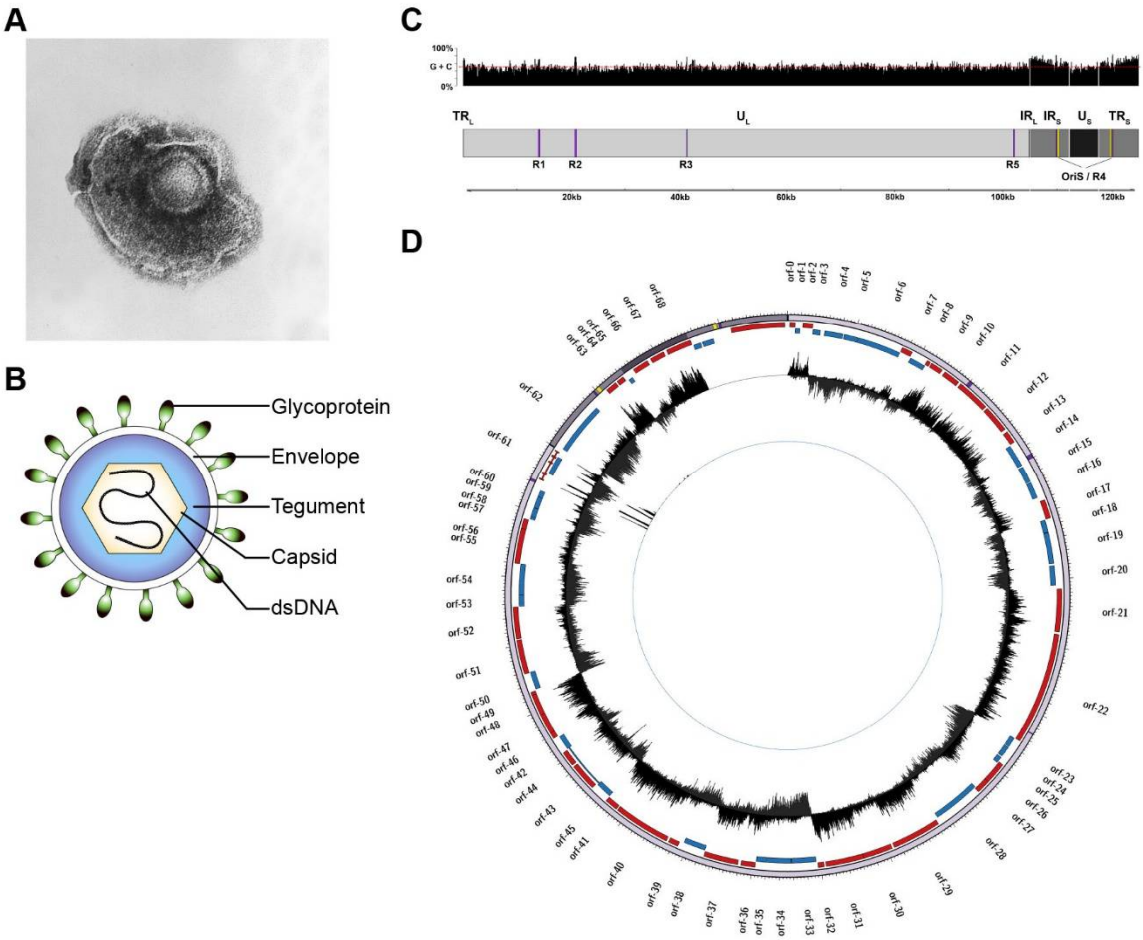

Figure 1. Structure of VZV particles and genome. (**A**) Electron microscopy image of VZV (obtained from the CDC/Dr Erskine Palmer; B.G. Partin. [26]). (**B**) Schematic representation of the VZV virion. (**C**) Schematic representation of the VZV genome structure. (**D**) VZV transcriptome profile during lytic infection of ARPE-19 cells (outer track) and latent infection of human TG (inner track). Circos plots of the VZV genome (grey band; sense and antisense ORFs indicated as blue and red blocks, respectively). Data represent strand-specific VZV-enriched mRNA-sequencing with peaks facing outward from the centre indicating reads mapping to the sense strand, while peaks facing inward originate from the antisense strand. The *y* axis is scaled to the maximum read depth per library in all cases. dsDNA, double-stranded DNA; UL, unique long; Us, unique short; TR, terminal repeat; IR, internal repeat; R, reiterative region; Ori, origin of replication; ORF, open reading frame.

2.2 Coding potential of the VZV genome

The VZV genome was originally reported to encode 68 unique viral genes, three of which are located in the duplicated IRs/TRs region [19]. Four additional VZV genes have since been identified including ORF0 [27], ORF9A [28], ORF33.5 [29], and the newly discovered VZV latency-associated transcript (VLT) [30] (Figure 1D). An underappreciated feature of VZV is that transcription of several genes (ORF0, ORF42/45, ORF50 and VLT) require host-splicing machinery to remove introns from pre-mRNA and show evidence of alternative splicing, perhaps dependent on host cell type or time after infection, resulting in synthesis of alternative proteins [19,30–33]. It thus seems likely that the full transcriptional potential of VZV remains to be elucidated and we predict that the latest technological advances (e.g. long-read direct sequencing of RNA) will yield further novel discoveries. It is also worth noting that the encoding of additional RNA types, including

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microRNAs and small non-coding RNAs, remains an area of active study with contrasting results [30,34,35]. How these studies might impact on our understanding of viral latency remains to be seen.

2.3 VZV gene expression during productive infection

Based on limited experimental data [36,37], and by analogy to other alphaherpesviruses, VZV genes are classed as immediate-early (IE) genes, early (E) genes, and late (L) genes, with each ordered wave of expression dependent on the previous classes. Proteins encoded by IE genes act as transcriptional regulators, those produced by E genes are mainly involved in DNA replication, and L genes encode structural proteins that are crucial for virion formation and egress. VZV encodes at least five transcriptional regulatory proteins specified by four IE genes ORF4, ORF61, ORF62 and ORF63, and one L gene, ORF10. All except the ORF61 protein, IE61 are part of the VZV virion [38,39]. Our understanding of the transcriptional regulation of VZV genes remains incomplete, in part due to the highly cell-associated nature of VZV that precluded synchronized infections using cell-free virus. VZV IE62 appears to be the major viral transactivator protein that can activate all three kinetic classes of VZV genes in the absence of other viral proteins (reviewed in [40]). Unfortunately, the function of VZV transactivators other than IE62 are ill-defined and additional research is needed to define their effects on virus and host gene expression. Host transcription factors, either by themselves or through interactions with viral transcriptional regulatory proteins, also contribute to viral gene expression (reviewed in [40]). VZV virion proteins delivered into newly infected cells upon entry are not absolutely required to initiate VZV gene expression - evidenced by the resulting VZV replication upon transfection of cells with viral DNA (reviewed in [41]). Notably, near identical VZV transcriptomes are detected during productive infection of diverse cell types, including neurons [30,42–45], suggesting a prominent role for either commonly expressed cellular transcription factors, or viral proteins in coordinating VZV gene expression. Importantly, these findings also imply that viral latency does not result from an intrinsic inability of neurons to support lytic VZV gene expression (Figure 1D) [42,43,45] and produce infectious VZV [46].

2.4 Stability of the VZV genome

Recent advances in the fields of genomics and computational biology have led to an explosion in high-throughput VZV genome sequencing studies [24,47–52], with multiple studies focused on exploring the evolution of VZV [49,53,54]. Perhaps the most pertinent observations made by these studies are that (i) the VZV genome is very stable with over 98% sequence conservation between the most distant strains sequences to date, and (ii) the evolutionary history of VZV, like other herpesviruses, is shaped by extensive recombination [49], the latter requiring that two or more viral genomes occupy the same cell nucleus at some stage in their life cycles. Intriguingly, the live-attenuated nature of the VZV vaccine enabled comparative analyses of vaccine-induced varicella and HZ isolates, showing that the VZV genome remains highly stable during latency [48,51].

3. Location of latent VZV

3.1 Sites of VZV latency

During primary infection, VZV infects and establishes lifelong latency in sensory neurons located in dorsal root ganglia (DRG) and trigeminal ganglia (TG), and most likely enteric ganglia.

Specifically, while VZV DNA has been detected in various sensory ganglia (DRG, TG, geniculate, vestibular and spiral ganglia) [5,55–57] and autonomic ganglia (nodose, enteric and thoracic sympathetic ganglia) [58–60] of individuals latently infected with VZV, it remains unclear whether the virus can establish latency and reactivate from all of these sites. The only confirmed sites of VZV latency are the DRG and TG, where the latent viral DNA is maintained as a circular episome in ~1–10% of sensory neurons at approximately 5–7 genome copies per neuron [30,61–66]. Additionally, VZV DNA and a restricted number of viral transcripts are detected in intestine biopsies of naturally infected and vaccinated individuals [58,67–69]. Although *in situ* demonstration of VZV DNA or RNA in human enteric neurons is lacking, clinical evidence suggests that VZV reactivation from the enteric nervous system could be associated with gastrointestinal dysfunction [70–72].

3.2 Entry of VZV into the peripheral nervous system

Two non-mutually exclusive routes have been proposed by which VZV infects sensory ganglion neurons (Figure 2A). First, VZV could enter nerve endings innervating the epidermis at sites of cutaneous lesions and gain access to ganglia by retrograde axonal transport. This route is supported by the detection of viral antigens in Schwann cells and peripheral nerve axons in the dermis of varicella patients [73], and observations that HZ occurs at the site of varicella vaccine inoculation [74], or sites most affected by varicella [4]. More recently, VZV infection of axons and retrograde axonal transport to neuronal cell bodies was formally demonstrated in cell culture [75,76], possibly involving fusion between VZV-infected non-neuronal cells and neuronal axons [77]. Notably, nerve endings are located in close proximity to the cutaneous vasculature at the dermal-epidermal junction and hair follicles [78], supporting a model in which VZV may concurrently infect epidermal or hair follicle keratinocytes and neurons via local cell-to-cell spread. Second, VZV-infected lymphocytes, most likely T-cells, could disseminate virus to ganglia during varicella-associated viremia. This is supported by detection of VZV DNA in ganglia obtained at autopsy from patients in the prodromal stage of varicella [7]. Moreover, localized injection of live-attenuated VZV vaccine virus results in establishment of viral latency in bilateral DRGs and enteric ganglia [67]. VZV infects T-cells, prolongs their survival via STAT3 phosphorylation and induction of survivin [79], and modulates their phenotype to induce an activated skin-tropic memory T-cell [80–82]. While the prerequisites for T-cell entry into ganglia are unknown, intravenously injected VZV-infected tonsillar T-cells transfer virus to human foetal DRG xenografts implanted under the kidney capsule of severe combined immunodeficiency (SCID) mice [83]. Moreover, T-cells infected with simian varicella virus (SVV), the closest relative to VZV and natural cause of varicella and HZ in nonhuman primates, are detected in ganglia of nonhuman primates during primary infection [84]. Thus, the definitive route(s) by which VZV infects ganglionic neurons with or without cutaneous innervation needs to be investigated in future studies.

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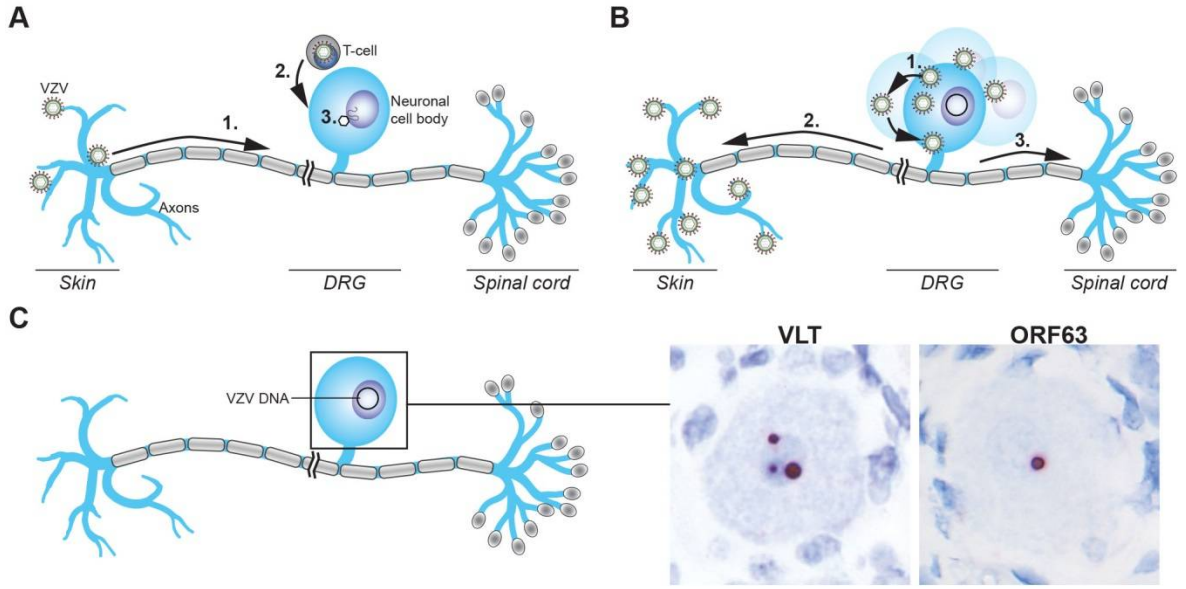

Figure 2. Schematic representation of establishment of and reactivation from VZV latency in sensory neurons. (**A**) VZV gains access to sensory ganglion neurons via infection of nerve endings in skin and retrograde axonal transport to the neuronal cell body (1) or direct infection of cell bodies via VZV-infected T-cells. (**B**) VZV reactivation results in virus replication and spread in the cell body (1), followed by transaxonal spread to the skin to cause HZ (2), possibly involving concordant virus spread to the spinal cord (3). (**C**) VZV latently infected sensory neurons contain viral episomal DNA in their nucleus and express VLT and/or ORF63 RNA, as shown by RNA *in situ* hybridization on human TG (red signal).

4. Transcriptional repression of latent VZV genomes

In the absence of robust animal models, VZV latency studies have been dominated by the use of naturally VZV-infected cadaveric human ganglia, whether snap frozen at autopsy [85] or explanted into short term cultures (reviewed in [86]). More recently, the use of *in vitro* hESC-derived neurons has provided a more accessible approach to latency studies [44,87].

4.1 VZV transcription in human ganglia

The difficulty of obtaining and working with post-mortem human ganglia resulted in discrepant numbers and identities of viral transcripts, and in some cases the corresponding proteins, detected [88–92]. The latter was confounded by the use of ascites-derived murine and rabbit antibodies that also contained endogenous antibodies shown to react with human blood type A antigens expressed by sensory neurons [93,94]. Consequently, VZV protein expression in human ganglia appears to be absent or extremely rare, most likely associated with virus reactivation [88,90]. The role of cellular dysregulation following death is undoubtedly a major influence [95], evidenced by post-mortem interval (PMI) being a major factor in determining the number and identities of viral transcripts present [96]. Most recently, unbiased virus nucleotide-enriched ultra-deep RNA-sequencing confirmed that viral transcription in short-PMI human ganglia is highly restricted and limited to VLT, frequently accompanied by co-expression of ORF63 RNA, albeit at lower quantities than VLT [30] (Figure 2C). Although both VLT and ORF63 RNA retain the potential to be translated during latency, we were not able to detect these viral proteins in latently infected human ganglia by immunohistochemistry [30]. However, given the very low abundance of VLT and ORF63

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RNAs, alternative approaches with higher sensitivity, such as screening for the loading of VLT and ORF63 RNA onto polysomes, combined with targeted enrichment for viral RNAs, could provide conclusive data. Thus, VZV latency in naturally infected human ganglia is characterized by the exclusive expression of VLT and often ORF63.

4.2 Comparison of VZV latency in vivo and in vitro

Although naturally VZV-infected short-PMI human cadaveric ganglia most closely resemble *in vivo* VZV latency, these individuals are likely to have been latently infected for many decades. By contrast, latency is usually profiled within 14 days using *in vitro* hESC-derived neuronal models. Although transcriptome-wide profiling by RNA-sequencing of *in vitro* "latent" VZV infections reveals a marked reduction in viral gene expression, all viral genes are detectably expressed in the absence of detectable levels of viral proteins [44,87]. No particular enrichment for classical VZV latency-associated ORF63 RNA was observed and VLT was not yet investigated [44,87]. This has led to the introduction of new terminology such as non-productive or quiescent infections, and questions over the relative merits of *in vitro* models. As a counterpoint, these models may in fact be highly informative of early events during VZV infection of neurons and the establishment of latency. Moreover, *in vitro* models remain the only system in which the establishment of latency and subsequent reactivation can be studied and these studies as a whole are benefitting from continual improvements in culture longevity and axonal infection protocols.

4.3 Epigenetic silencing of the latent VZV genome

The molecular mechanisms by which VZV latency is established are largely unknown and much of what is assumed is actually informed by studies of the related herpes simplex virus type 1 (HSV-1). Here, the key idea is that during traversal of neuronal axons, the genome-containing nucleocapsid is delivered to the nucleus in the absence of functional tegument proteins for IE gene transcription (i.e. VZV ORF10 – the homolog of HSV-1 virion protein 16 [VP16]) and this allows loading and maintenance of repressive chromatin upon the viral episome which inhibits viral transcription ([97] and reviewed in [98]). Whether this is a rapid or slow process is not known and data from VZV *in vitro* models may provide evidence for the latter, i.e. viral transcription in newly infected neurons is gradually suppressed over time, eventually leading to the transcriptional profile we correlate with latency in human ganglia (i.e. expression of VLT and, frequently, ORF63 RNA). Either way, the remarkable switch between lytic and latent transcriptional states is best explained by the assembly of repressive chromatin upon the viral episome. Indeed, studies using late-PMI VZV-latently infected human ganglia demonstrated euchromatic (H3K9ac) chromatin modifications on promotors of latency-associated ORF63 and ORF62, a gene frequently expressed in post-mortem deregulated ganglia [99]. By contrast, promotors of viral genes ORF14 and ORF36, which are not expressed during latency nor detected in late-PMI ganglia, were not associated with H3K9ac [99]. The observation of increased viral transcription following increased post-mortem intervals suggests these studies bear extending to shorter PMI and a genome-wide analysis. Indeed, one would speculate that all but VLT and ORF63 loci would be bound by repressive chromatin and it will be important to determine whether the VLT locus remains active due to the presence of flanking chromatin insulators such as CCCTC-binding factor (CTCF), as has been observed for HSV-1 [100].

5. The varicella zoster virus latency-associated transcripts: VLT and ORF63

The identification of VLT represents a major advance in studies of VZV latency. VLT is encoded antisense to the ORF61 and it is remarkable to note that all known alphaherpesvirus latency-associated transcripts (LATs) originate from genomic regions encoding infected cell polypeptide 0 (ICP0) homologs (Figure 3). This is indicative of significant evolutionary conservation, although the encoding of LATs and ICP0 homologs antisense to each other makes it harder to assess their individual contributions to maintaining this locus. Notably, VZV is the only alphaherpesvirus analysed in detail to date that expresses an additional latency transcript, ORF63.

5.1 Comparison of VLT and LATs of related alphaherpesviruses

VLT is a poly-adenylated RNA comprising at least five distinct exons and is encoded antisense to VZV ORF61, a homolog of the alphaherpesvirus *RL2* gene (encoding ICP0). During latency, a single isoform is expressed by neurons of virtually all analysed VZV-infected human TGs [30]. Notably, VLT appears to be strikingly more complex compared to LATs of HSV-1, pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV-1), which are composed of only two exons and a single intron (Figure 3). The possible exception is the closest relative of VZV, the nonhuman primate SVV, which expresses a transcript mapping to the same region as VLT during latency [101,102], although more detailed studies are needed to define the exact location and structure of the SVV VLT homolog. Unlike the stable LAT introns of HSV-1 that accumulate to high abundancies in latently infected human TG [30,103], VLT appears to be expressed at relatively few copies per neuron [30]. Furthermore, while LATs of HSV-1, PRV and BHV1 encode viral miRNAs [104–106], we did not find evidence for VZV miRNA expression within VLT – or expressed anywhere else in the VZV genomes – in latently-infected human ganglia [30]. Thus, while all alphaherpesviruses express LATs, their size, coding potential, functions and mechanisms of action may vary.

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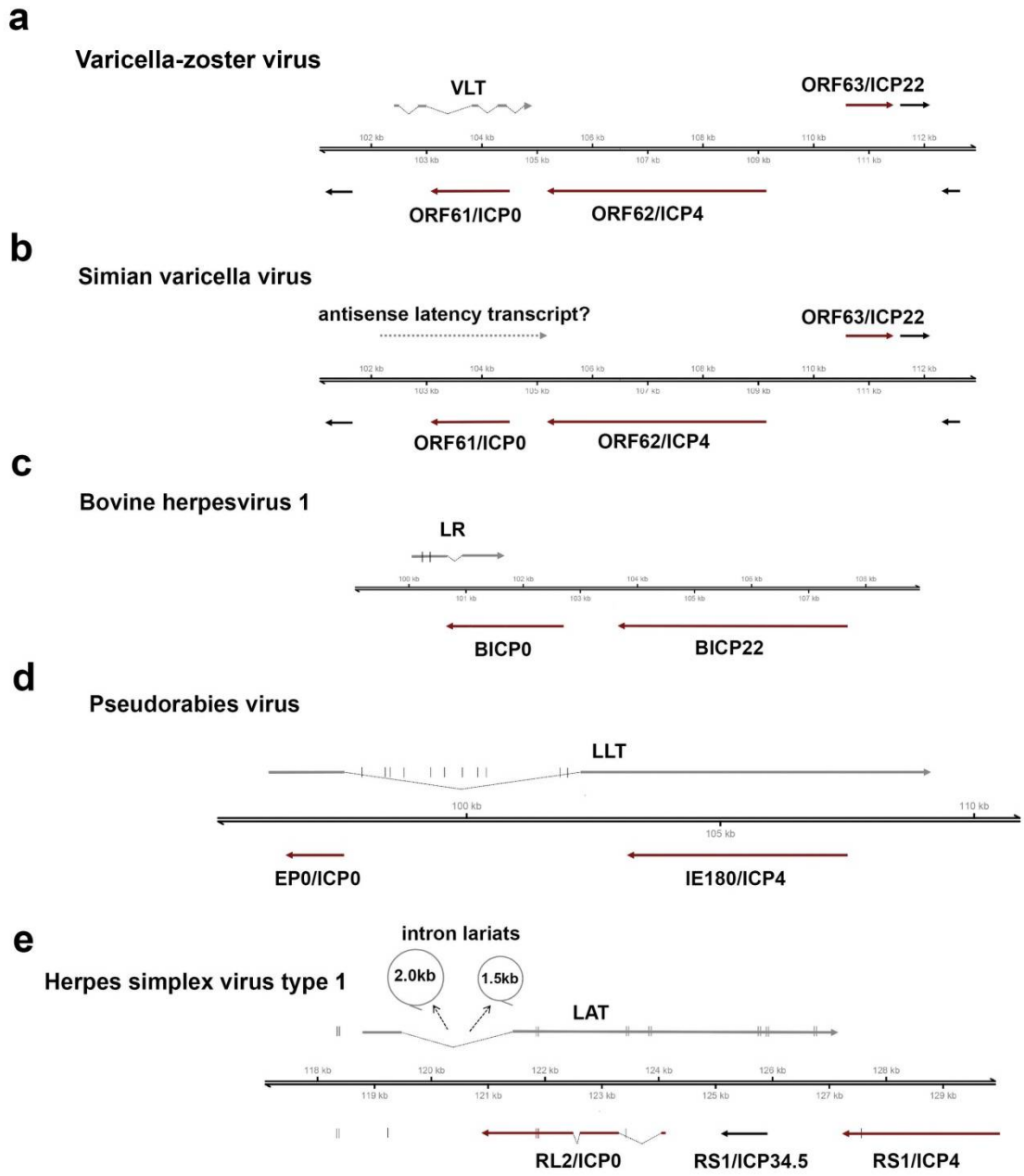

Figure 3. Comparison of latency-associated transcripts among alphaherpesviruses. All alphaherpesvirus latency-associated transcripts (LATs) are located antisense to the ICP0 locus, encoding a conserved major immediate-early transactivator (ICP0 or homologs). (**A**) VZV latency transcript (VLT) is a 496-nucleotide multi-exon mRNA and partially antisense to ORF61 coding region via exon 3 and 4. (**B**) Transcripts mapping antisense to simian varicella virus ORF61 are expressed during latency, but their identity has not yet been defined. (**C**) Bovine herpesvirus 1 encodes a 2.2 kb latency related (LR) RNA and encodes two miRNAs in exon 1 (**D**) The pseudorabies virus large latency transcript (LLT) is the largest characterized alphaherpesvirus latency transcript and encodes eleven distinct miRNAs within the spliced intron. (**E**) The 8.2 kb herpes simplex virus type 1 (HSV-1) LAT undergoes splicing that yields two highly stable intron lariats approximately 1.5 and 2.0 kb in size (shown as circles). Note that latency transcripts are shown as grey arrows, immediate early viral transactivators as dark red arrows, and encoded miRNAs as short vertical lines. Scaling across all schematics is equal.

Although VLT is the predominant transcript expressed during latency, its expression is not restricted to the latent phase. During VZV lytic infections of epithelial cell or melanoma cell cultures, multiple alternatively spliced VLT isoforms were identified [30]. Termed VLT_{ly}, these lytic isoforms are more complex than the 'core' latent VLT isoform detected during latency. Exon skipping, intron retention and additional upstream exons are all features of VLT_{ly}, with most variation apparently occurring between 'core' exons 3 and 4. Notably, the core latent VLT isoform has never been observed in lytic infections which may suggest the core isoform is driven by a cell type (i.e neuron) specific promoter and/or the expression of the core VLT decides the fate of infection program, lytic or latent. During productive infections, VLT_{ly} is transcribed with true-late kinetics, defined as being absolutely dependent on viral replication, and translated into protein (pVLT) in cell cultures and zoster skin lesions [30]. Although the expression and function of LATs during lytic alphaherpesvirus infection is incompletely understood, above features of VLT fit the general picture that emerges. During lytic infection LATs appear to be expressed with late kinetics [30,107], alternative LAT isoforms are produced using different transcription start sites or alternative splicing [30,108,109], and some LATs encode proteins [30,110]. Given that hESC neuronal models support both lytic and quiescent VZV infections [44,87], these could be particularly useful to investigate regulation of lytic and latent isoforms of VLT in the same cell type.

5.2 Function(s) of VLT

Functional characterisation of VLT, during lytic and latent stages, is now a priority. Early investigations using transfection assays showed that VLT selectively represses ORF61 transcription when co-expressed with multiple VZV coding IE genes (ORF61, ORF62 and ORF63) in ARPE-19 epithelial cell cultures, resulting in diminished expression of ORF61 protein, IE61 [30]. This effect is not pVLT-specific as mutation of the initiating *ATG* start codon (to *ATA*) showed a similar effect – suggesting this effect is mediated at by the RNA itself. Whether this observation translates into other experimental systems is now an area of active study. While the specific functions and requirements of VLT and VLT_{ly} isoforms during lytic and latent infections remain unknown, these data allow us to speculate that VLT may function to maintain latency by repressing transcription of ORF61, a promiscuous transcriptional regulator during lytic infections.

5.3 Function(s) of ORF63

Intriguingly, VZV expresses two latency-associated transcripts: VLT and ORF63 RNA. We showed that VLT and ORF63 RNA levels correlate significantly in human TG, independent of latent viral DNA load [30], suggesting that expression of both transcripts is linked. It is unclear whether VLT and ORF63 RNA are produced by the same or distinct populations of neurons, and how this may influence the ability of the virus to reactivate. ORF63 is essential for VZV replication [111] and its encoded protein, IE63 functions not only as a transcriptional regulator activating E genes [112] but also modifies neuronal susceptibility to apoptosis and functions as a immunoevasin that blocks type I interferon (IFN) signalling [113–115]. As such, we speculate that ORF63, once translated as IE63, could be more important for the initiation of reactivation, which is supported by the correlation between ORF63 RNA abundance and PMI [96]. The observation that IE61 is required for nuclear entry of IE63 in guinea pig enteric neurons [116], suggests that VLT-mediated repression of ORF61

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transcription and translation may also function to retain IE63 in the cytoplasm, if expressed, and prevent transactivation of lytic viral promotors (Figure 4A).

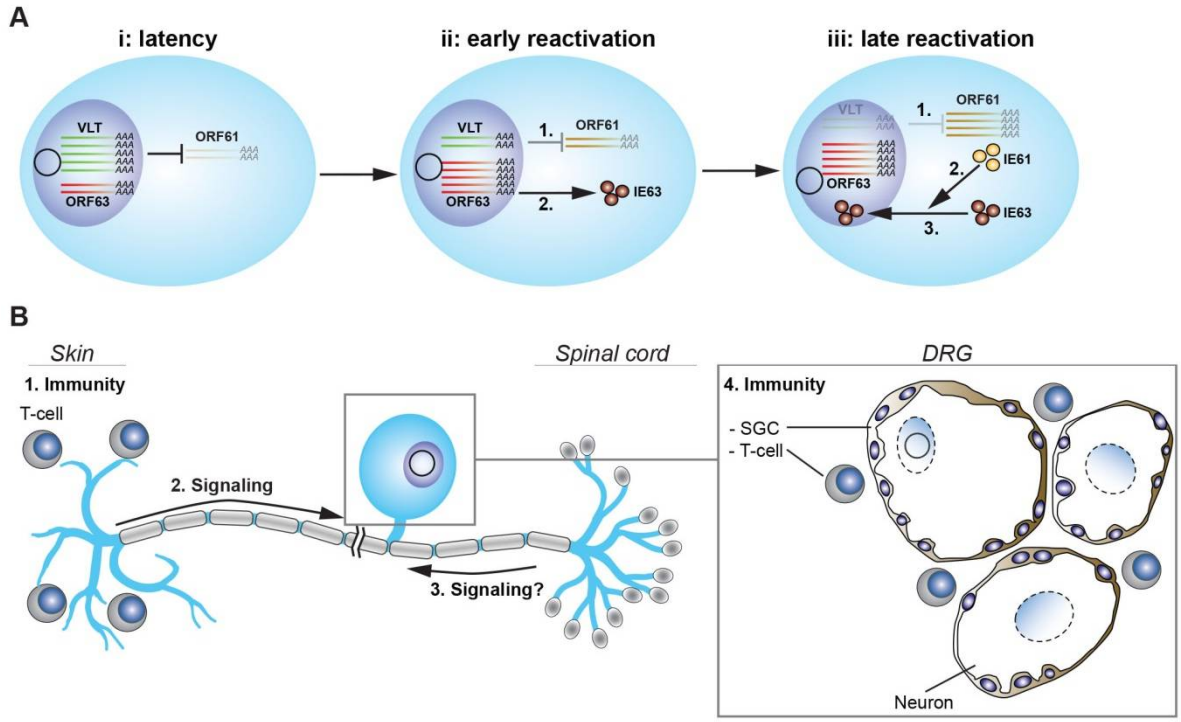

Figure 4. Model of VZV latency and reactivation. (**A**) Hypothesized roles for VLT and ORF63 during VZV latency and reactivation. i) During latency VLT and ORF63 RNA are expressed, and VLT represses expression of ORF61. ii) Upon initiation of reactivation we hypothesize that VLT expression levels are reduced, enabling low-level ORF61 expression (1), and ORF63 transcription is increased, enabling low-level ORF63 protein expression (IE63). However, in the absence of IE61, VZV IE63 is retained in the cytoplasm. iii) At later stages of reactivation even lower levels of VLT allow for more ORF61 expression (1), resulting in IE61 production (2) and nuclear translocation of IE63 (3). Nuclear IE63 is anticipated to contribute to VZV reactivation e.g. by regulating viral and host transcription and modifying the neuron's susceptibility to apoptosis and interferon responsiveness. (**B**) Schematic representation of the regulation of VZV latency and reactivation. Activation of neuronal signaling pathways in response to stimuli at the periphery (2), and possibly within the spinal cord (3), may induce VZV reactivation. At the same time, adaptive T-cell-mediated immune responses in skin (1) and ganglia (4), and local ganglionic innate immunity provided by neuron-interacting satellite glial cells (SGC) are believed to prevent symptomatic VZV reactivation.

6. VZV reactivation: restarting lytic gene expression

Symptomatic VZV reactivation, which leads to HZ and/or associated pathologies, typically occurs just once or twice in an infected individual's lifetime. By contrast, asymptomatic reactivation is thought to occur more frequently [2,117]. Possibly, the phase of intra-ganglionic virus replication that is presumed to occur upon VZV reactivation [118,119] (Figure 2B) before virus descends down sensory axons to the skin, provides the host immune system more time to control most reactivation events before becoming symptomatic. The environmental factors and molecular mechanisms that underpin VZV reactivation in humans remain poorly understood. Clinical observations of HZ after trauma [120] or neurosurgical treatments [121,122] suggest that signalling events from the periphery

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may induce VZV reactivation in the soma of sensory neurons. Two main signalling pathways have been implicated in VZV reactivation: the phosphatidylinositol-3 kinase (PI3K)-Akt pathway and the mitogen-activated protein kinase (MAPK) pathway. The nerve growth factor (NGF) receptor, TrkA, is expressed by a subpopulation of sensory neurons and signals via the PI3K-Akt and MAPK pathways [123]. Depletion of NGF using anti-NGF antibody treatment of VZV-latently infected neurons leads to the reactivation of VZV in an *in vitro* latency system [44]. The role of NGF signalling in contributing to the maintenance of VZV latency was subsequently confirmed in VZV-latently infected human TG removed within 24 hours after death [124]. Intriguingly, the effect of chemical PI3K blockade on VZV reactivation appears to be dependent on the experimental conditions, as PI3K inhibition did not reactivate VZV in *ex vivo* cultures of naturally infected human TG neurons [124] and variable effects are obtained in hESC-derived neurons [44,87]. More recently, NGF depletion was shown to result in phosphorylation and activation of the MAPK family member c-Jun N-terminal kinase (JNK), which was critical for efficient VZV replication in hESC neurons. Selective JNK inhibition limits VZV reactivation in the *in vitro* VZV latency system, suggesting JNK signalling plays important role for VZV reactivation in neurons [125].

To reactivate from latency, the latent viral episome needs to be de-repressed and viral gene expression has to occur as downstream events of PI3K-Akt and/or JNK pathways. The mechanisms underlying reversal of repressive chromatin on the VZV genome and the viral gene expression machinery required for VZV reactivation are poorly understood. Unlike HSV-1, inhibition of histone deacetylases did not reactivate VZV from latently infected hESC neurons [44]. Possibly, additional chromatin modifications like methylation of histone H3 lysine 4 (H3K4) or demethylation of H3K9 need to take place before VZV reactivation can occur [126]. Alternatively, JNK signalling may induce a methyl/phospho switch on promoters of VZV lytic genes to facilitating their expression and virus reactivation [127]. Clearly, additional environmental factors and neuronal signalling pathways are involved, and the overall outcome of VZV infection will be determined by the combined integration of all these pathways.

7. Intrinsic, innate and adaptive immunity to VZV infection in ganglia.

The intrinsic properties of neurons and innate and adaptive immune responses mounted by ganglion-resident or infiltrating cells that contribute to control VZV replication in ganglia are largely unknown. VZV infection of human foetal DRG xenografts in SCID mice produces transient virus replication and spread, followed by a persistent lower levels of viral DNA and limited viral transcription after 4–8 weeks [83], suggesting that adaptive immune responses are not essential to control VZV replication in ganglia. Intrinsic differences in neuronal subpopulations affect the outcome of VZV infection, as virus replication is blocked in neurons expressing the mechanoreceptive marker RT97, but not in neurons expressing the nociceptive marker peripherin [128]. Furthermore, neurons may restrict VZV replication by sequestering nucleocapsids in promyelocytic leukaemia protein nuclear bodies [129], and/or, by analogy to HSV-1, use autophagy to inhibit virus replication [130].

7.1 Satellite glial cells and innate immunity

Although sensory neurons produce little type I IFNs α/β, neurons are sensitive to IFN-α/β signalling as exposure to IFN-α/β at nerve endings prevents retrograde axonal transport of alphaherpesvirus

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virions [131]. Notably, various cytokines/chemokines are produced in VZV-infected foetal DRG, including IFN-α, interleukin 1 alpha (IL-1α), IL-6, CXCL10 and transforming growth factor beta [128]. While the cellular source of these cytokines is unknown, satellite glial cells (SGC) are a prime candidate. SGC are a specialized ganglionic cell type that shares phenotypic and functional features with professional antigen presenting cells like macrophages and dendritic cells [132,133]. SGC completely enwrap the neuronal cell body and provide physical and nutritional support to the neuron, but also function as tissue-resident innate immune cells that express pattern recognition receptors (e.g., Toll-like receptors), phagocytose, produce inflammatory mediators and potentially regulate local T-cell responses [132–134]. Moreover, SGC surrounding HSV-1 infected neurons produce IL-6 and tumour necrosis factor α [135], and we have shown that SGC are activated in response to primary SVV infection in monkeys [136]. However, more detailed studies on the role of SGC in response to ganglionic VZV infected are warranted.

7.2 T-cell immunity

VZV reactivation, clinically presenting as HZ, is associated with waning of systemic VZV-specific T-cell immunity, but not humoral immunity, suggesting a critical role of VZV-specific T-cell memory in preventing virus reactivation [137,138]. However, a large fraction of pathogen-specific memory T-cells are retained in organs, referred to as tissue-resident memory T-cells (T_{RM}), and provide swift protective immunity upon pathogen re-exposure [139,140]. By analogy to HSV-1 mouse models [141,142] and human TG [143], VZV-specific T_{RM} are expected to home to sites of latency and reactivation: ganglia and skin. Indeed, recent studies showed VZV-specific T_{RM} cells persist in healthy skin of latently VZV-infected adults decades after primary infection [144]. Profound T-cell responses detected in ganglia of HZ patients hint at the presence of VZV-specific T_{RM} in ganglia [145,146]. While we previously could not detect VZV-specific T-cells in latently infected human TG, this study analysed only a restricted panel of VZV proteins (IE4, ORF29p, IE62 and IE63) [147]. Thus, more detailed studies are needed to determine the presence, specificity and function of VZV-specific T_{RM} in latently VZV-infected human ganglia in contribution for VZV latency and reactivation control.

8. Future perspectives

VZV evolved the ability to establish lifelong latent infection in neurons, enabling the virus to reactivate later in life and cause recrudescent disease accompanied by VZV spread to naïve individuals. Throughout these decades of latency, the virus must ensure survival of the infected neuron, avoid irreversible silencing of the viral episome and prevent elimination by host immune responses. Although the mechanisms that coordinate the interplay between virus and host remain largely unknown, the many recent exciting advances in the field provide us with the questions to be addressed and tools (*ex vivo* and *in vitro* latency models) that can be used to answer them. Thirty-five years on from the discovery of latent VZV residing in sensory ganglia [5], the identification of VLT and the latent VZV transcriptome in naturally infected short-PMI TG provides us with an updated perspective on VZV latency [30]. With this advancement, we would also propose to enhance the definition of VZV latency by requiring: i) the presence of the viral genome as an episome in the nucleus of host cells without production of infectious progeny, ii) the capacity of latent VZV to

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reactivate and produce infectious virus, and iii) a distinct and restricted pattern of viral gene transcription, i.e. exclusive expression of VLT and/or ORF63 RNA.

It is now critical to determine the roles of (and potential interplay between) VLT and ORF63 RNA in latently infected short-PMI human TG [96], and to analyse their function in the establishment of quiescence in human pluripotent stem cell (hPSC; hESC/hiPSC)-derived neuronal models [44,46,87,148] or reactivation from latency using these same models and/or *ex vivo* cultures of dissociated naturally VZV-infected human ganglia [124]. Human TG are composed of diverse subtypes of neurons [149] and not all neuronal subpopulations may be equally susceptible to VZV infection [128], as shown for HSV-1 [150,151]. Therefore, *in situ* analyses are required to determine whether VLT and ORF63 RNA are co-expressed by the same or distinct neurons, and define the subtypes of neurons harbouring the latent VZV genome. Recent developments in the generation of recombinant VZV (reviewed in [41]) will facilitate functional analysis of VLT/pVLT and ORF63 RNA/IE63 using hPSC-derived neuronal systems. Current evidence suggests that both pVLT and IE63 are not expressed, or expressed below detectable levels, in latently infected ganglia [30,93,94]. While we have identified one possible function of the VLT RNA [30], no functions are currently attributed to ORF63 RNA or pVLT.

Transcriptional regulation of VZV gene expression during lytic and latent infection is poorly defined. By analogy to HSV-1 [152], reactivation of latent VZV is presumed to follow a regulated cascade of gene expression and more detailed analyses of lytic VZV transcriptional regulation is warranted to provide insights into the requirements of VZV reactivation. Recent developments in next-generation sequencing facilitate detection of low-abundant VZV genomes in latently infected human TG [47,48], and will open up new avenues to unravel epigenetic modifications of latent VZV genomes using chromatin immunoprecipitation sequencing (ChIP-seq) for histone modifications or CTCF binding. Furthermore, *in vitro* quiescence models could be used to prospectively map epigenetic chromatin modifications on the VZV genome during quiescent and reactivated stages of infection. Of particular interest will also be the relative expression levels of VLT and ORF63 RNA, and IE63 and IE61 during the initiation of virus reactivation (Figure 4A).

The factors triggering and barriers restricting VZV reactivation are poorly understood, and will be of great interest for future research. The infrequent clinical reactivation of latent VZV suggests that either virus reactivation is rare or that most reactivation events are cleared before HZ can develop. Local immune responses mediated by VZV-specific T_{RM} in skin [144,153] or ganglion, or neuron-interacting SGC [133,134] are likely to be pivotal factors controlling VZV reactivation, e.g. by secreting IFNs to block early reactivation attempts [154]. The recently developed *in vitro* and *ex vivo* models to study VZV reactivation [44,46,87,124,148] facilitate systematic analysis of signalling pathways modulating viral latency, and could be used to investigate roles of non-neuronal cells and their secreted antiviral factors in this process.

Post-mortem acquired human samples are extremely informative, if handled precisely, especially for investigating human-restricted pathogens like VZV, but are also restrictive in that the resulting datasets only represent a 'snapshot' of latency. To overcome this, we hope that technological advances in specific subtypic differentiation methodology for human sensory/sympathetic neurons or organoid culture system from hPSC, combined with single cell sequencing methodologies targeting transcriptomes and epigenomes, will provide novel ways to

examine the dynamics of VZV latency and provide new insights into the molecular mechanisms that control this.

Finally, the discovery of VLT and its potential role in governing VZV latency and/or reactivation also opens up the tantalising possibility re-engineering current VZV vaccines within a view to eventually eradicating VZV entirely. While a significant amount of work is required to get from here to there, not least determining whether VLT is truly required for latency and/or reactivation, pursuing this long term goal gives new impetus to the VZV research field.

Author Contributions: All authors contributed to the writing, editing and contents of this manuscript.

Funding: This work was supported in part by Public Health Services grant AG032958 from the National Institutes of Health (W.J.D.O.) and by the Takeda Science Foundation, Daiichi Sankyo Foundation of Life Science, Japan Society for the Promotion of Science (JSPS KAKENHI JP17K008858, JP16H06429 and JP16K21723) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT KAKENHI JP17H05816) (T.S.).

Acknowledgments: The authors would like to thank Drs. Georges Verjans and Angus Wilson for critical reading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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