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On Fidelity, Adaptation and Reproduction: A Study of Hypermutation in Herpes Simplex Virus 1

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Abbreviations

(-)ssRNA	Negative-sense single stranded RNA
(+)ssRNA	Positive-sense single stranded RNA
ACV	Acyclovir
ANOVA	Analysis of variance
aphAI	Aminoglycoside phosphotransferase
ATCC	American type culture collection
BAC	Bacterial artificial chromosom
BER	Base excision repair
bp	Base pairs
BWA	Burrow-Wheeler aligner
CCL	Cellular clone
CD	Codon deoptimized
CMV	Cytomegalovirus
cPCR	Colony PCR
CPD	Codon pair deoptimized
CPE	Cytopathic effect
CpG	C-G dinucleotide
D301A	Polymerase aspartate 301 to alanine mutant (HCMV)
D358A	Polymerase aspartate 358 to alanine mutant (MDV)
D368A	Polymerase aspartate 368 to alanine mutant (HSV-1)
DBH	Drift-barrier hypothesis
DIG	Defective interfering genome
DIP	Defective interfering particle
DMEM	Dulbecco's modified eagle's medium
dN/dS	Non-synonymous to synonymous substituion rate
DNA	Desoxribonucleic acid
dpi	Days post infection
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTDS	Polymerase functional modif
dTTP	Deoxy thymidine triphosphate
dUTP	Deoxy uridine triphosphate
dUTPase	Deoxy uridine triphosphatase
E303A	Polymerase glutamate 303 to alanine mutant (HCMV)
E370A	Polymerase glutamate 370 to alanine mutant (HSV-1)
EBV	Epstein-Barr virus
EDTA	Ethylendiamintetraacetat
ESCRT	Endosomal sorting complex required for transport
Exo	3'-5' Exonuclease

FASTQ	Nucleotide sequence data format
Fc	Antibody constant region
FCS	Fetal calf serum
FFU	Focus forming unit
FOS	Foscarnet
GaAHV-2	Gallid alphaherpesvirus 2 (MDV)
GCV	Ganciclovir
GFP	Green fuorecense protein
H_2O	water
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HFF	Human foreskin fibroblasts
HHV-1/2	Human herpesvirus 1/2 (formal name for HSV-1/2)
HHV-6AB	Human herpesvirus 6AB
HHV-7	Human herpesvirus 7
HIV	Human immunodeficency virus
hpi	Hours post infection
HR	Homologous recombination
HSV-1/2	Herpes simplex Virus 1/2
IC_{50}	Half inhibitory concentration
ICP	Infected cell protein
ICTV	International Committee for Taxonomy of Viruses
IU	International Unit
Kan	Kanamycin
kb	Kilobase
KSHV	Kaposi's sarcoma herpesvirus
LAT	Latency associated transcript
LoFreq	Low frequency variant caller
MAFFT	Multiple Alignment using Fast Fourier Transform
Mb	Megabase
MDV	Marek's disease virus
microRNA	Small non-coding RNA
Mini-F	Minimal replicative unit of the bactirial F plasmid
mL	Milliliter
MLH	MutL homolog
MMR	Missmatch repair
MNR	Protein complex consistend of Mre11, Rad50 and Nbs1; involved in recombination
MOI	Multiplicity of infection
MRC-5	Human lung fibroblasts
mRNA	Messenger RNA
MSH	MutS homolog
NEB	New England Biolabs
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NIH	National institutes of health
nm	Nanometer
Opti-MEM	Fetal calf serum reduced minimal essential medium for transfection
ORF	Open reading frame

oriL	Origin of replication long
oriS	Origin of replication short
PBS	Phosphate buffered saline
PCA	principal component analysis
PCNA	prolifering cell nuclear antigene (sliding clamp)
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pfu	Plaque forming units
PILRα	Paired immunoglobin like type 2 receptor alpha
PIPES	Piperazine-N,N"-bis(2-ethanesulfonic acid)
PMSF	Phenylmethylsulfonyl fluoride
Pol	DNA-Polymerase
PyMOL	Structural data viewer, Python integrated, Molscript based
qPCR	quantitative PCR
RBD	Receptor binding domain
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RQC	Red Queen conflict
RQH	Red Queen hypothesis
RT	Room temperature
RVKNL	Origin binding protein functional modif
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
ShoRAH	Short read assembly into haplotypes
SIE	Superinfection exclusion
SIFT	Sorting intolerant from tolerant
SNP	Single nucleotide polymorphism
SSA	Single strand annealing
ssDNA	Single stranded DNA
T	Triangulation number
TEN	Tris, EDTA and NaCl buffer
TET	Tris, EDTA and tween buffer
TK	Thymidine kinase
tRNA	Transfer RNA
UL	Unique long (for HSV-1 genes)
UNG	Uracil-nucleotide glycosidase
US	Unique short (for HSV-1 genes)
VILOCA	Virus haplotype locater
ViReMa	Virus recombination mapper
VirGA	Virus *de novo* assembly pipeline
VP	Viral protein
WHO	World health organisation
WT	Wild type
wt	Wild type
XRCC	X-ray repair cross-complementing protein
Y547S	Polymerase tyrosine 547 to serine mutant (MDV)
Y557S	Polymerase tyrosine 557 to serine mutant (HSV-1)
Y567F	Polymerase tyrosine 567 to phenylalanine mutant (MDV)
Y577F	Polymerase tyrosine 577 to phenylalanine mutant (HSV-1)

YS	Polymerase tyrosine 557 to serine mutant (HSV-1)
ZEDAT	Zentraleinrichtung für Datenverarbeitung
µm	Micrometer

Chapter 1
Introduction

1.1 Viruses

"People and gorillas, horses and duikers and pigs, monkeys and chimps and bats and viruses: We're all in this together." David Quammen, Spillover: Animal Infections and the Next Human Pandemic, 2012

Among biological agents, viruses hold a special status. Not alive in a strict sense, but still replicating, propagating and evolving, they are among the simplest subjects to study evolutionary questions while being highly relevant in everyday live (Flint, et al. 2015a; Howley and Knipe 2020).

1.1.1 Definition and Classification

"We demand rigidly defined areas of doubt and uncertainty!" Douglas Adams, The Hitchhikers Guide to the Galaxy, 1979

Viruses were initially defined by what they lack more so than by what they are. They are no cells, they do not employ any sort of energy metabolism, they do not encode ribosomes and they could not be observed with light microscopes. So, what are they and what do they do? Viruses are obligatory intracellular parasites of their respective hosts (Flint, et al. 2015a). They contain DNA or RNA genomes protected by protein shells called capsids and are membrane enveloped or naked. Attachment and entry into host cells is mediated by binding cellular surface structures, called receptors. Viral particles are incredibly small, so tiny in fact that being filterable with 0.2 µm pores was early on one of their defining attributes (van der Want and Dijkstra 2006). Nowadays, after the discovery of giant viruses, size parameters are less strict and span from 40 nm up to 1 µm (Aherfi, et al. 2016). Genome sizes scale similarly from few kilobases (kb) to megabases (Mb) (Aherfi, et al. 2016; Sanjuan and Domingo-Calap 2016). Most importantly, they reproduce in infected host cells by expressing and replicating their genomes, which leads to the assembly of infectious particles (called virions), that are released and start the infectious cycle all over again (Flint, et al. 2015a).

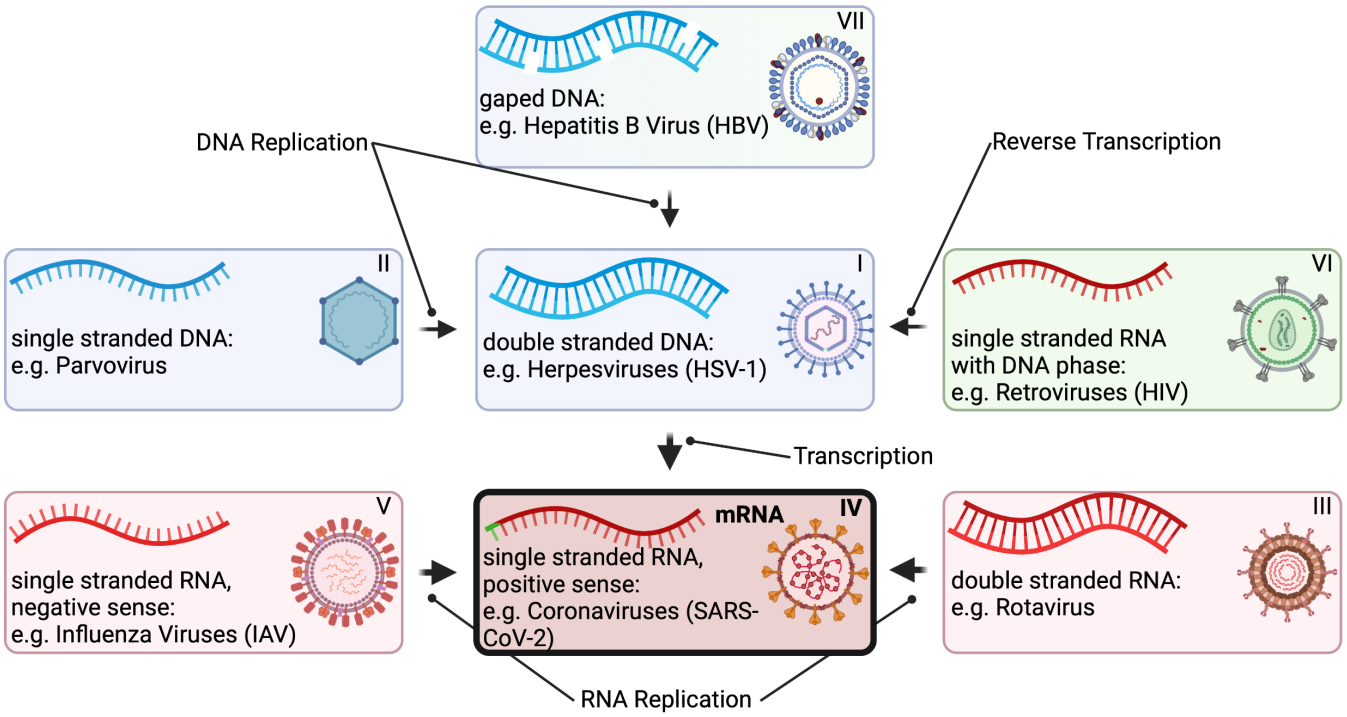

Figure 1.1: Baltimore scheme. Classification of viral genome types according to David Baltimore's Scheme. Central is the production of mRNA (class IV) and every possible genome type is arranged as to how mRNA is produced. The system recognizes 7 genome types: dsDNA (class I), ssDNA (class II), dsRNA (class III), (+)ssRNA (class IV), (-)ssRNA (class V), ssRNA with DNA intermediate (class VI) and gaped DNA with RNA intermediate (class VII).

Genome Types (Baltimore Scheme)

Classifying viruses is incredibly difficult, as they differ enormously in structure, genome composition, size and replication strategies. However, the Baltimore Scheme, introduced by David Baltimore, the co-discoverer of the reverse transcriptase, classifies viral groups very reliably (Baltimore 1971; Koonin, et al. 2021). The system is based on how different genome types produce messenger RNA (mRNA; Figure 1.1). There are 7 classes of genomes in the scheme: double stranded (ds) DNA, single stranded (ss) DNA, dsRNA, (+)ssRNA, (-)ssRNA, ssRNA viruses with DNA phases (retroviruses) and dsDNA viruses with RNA phases (gaped DNA viruses).

1.1.2 Herpesviruses

"Remember, what happens in Vegas stays in Vegas. Except for herpes. That shit'll come back with you." Todd Phillips & Daniel Goldberg, Hangover, 2009

The order *Herpesvirals* contains three families of enveloped large dsDNA viruses: *Malacoherpesviridae*, *Alloherpesviridae* and *Herpesviridae* (ICTV and King 2011). Members of the *Malacoherpesviridae* and *Alloherpesviridae* infect bivalves and fish or amphibia respectively. Both families are severely understudied. In contrast, species in the *Herpesviridae* infect mammals, birds and reptiles (Figure 1.2A).

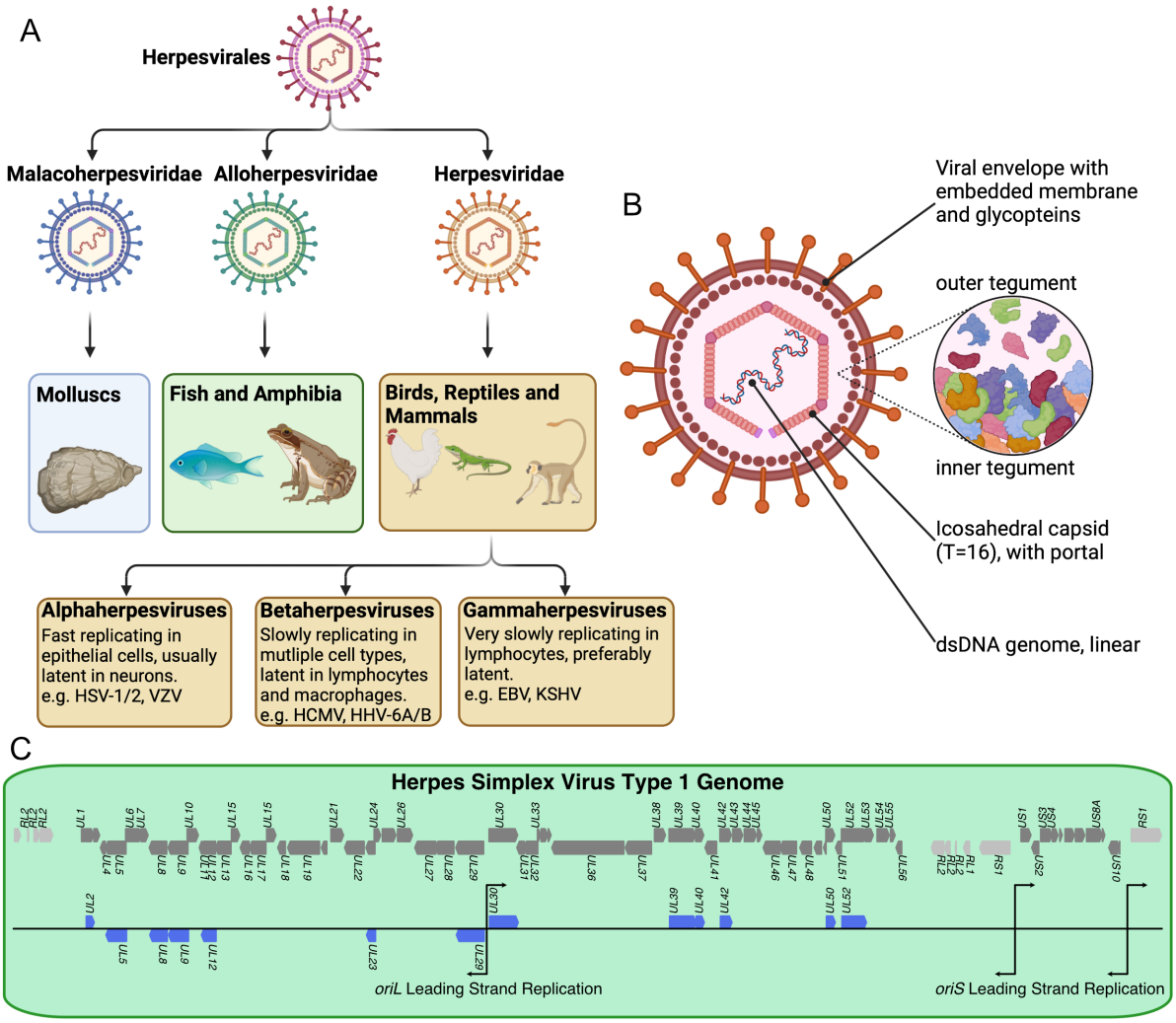

Figure 1.2: Herpesviruses. A) classification of families in the order *Herpesvirales*. Herpesviruses that infect birds, reptiles and mammals (*Herpesviridae*) are further divided in subfamilies. B) Cartoon representation of the herpesvirus particle. A linear copy of the double stranded DNA genome is packaged into an icosahedral capsid with a triangulation number of T=16. One of the capsid vertices features a portal for DNA. The capsid is surrounded by a protein layer called tegument. The inner tegument is more tightly structured than the outer layer. A lipid membrane decorated with multiple glycoproteins envelopes the particle. C) Genome structure of HSV-1. The upper representation shows all major open reading frames, while beneath only genes and DNA regions involved in nucleic acid metabolism are depicted.

Herpesviridae

Herpesviruses are incredibly diverse, almost every animal species hosts at least one herpesvirus, many multiple (Damania 2004). The *Herpesviridae* family features three subfamilies of alpha-, beta- and gammaherpesviruses, first based on biological properties like cell tropism and further on confirmed by the International Committee for Taxonomy of Viruses (ICTV) based on DNA sequences (Roizman, et al. 1981; Gatherer, et al. 2021) (Figure 1.2A).

Alphaherpesviruses reproduce relatively fast in epithelial cells by lytic replication and establish latency in sensory ganglia. So far, only alphaherpesviruses are known to infect birds and reptiles, this though could be attributed to easier detection. The prototype virus for this group is herpes simplex virus (HSV). Betaherpesviruses replicate slower and infected cells usually enlarge (cytomegalia). They have broad tissue tropism but narrow host ranges and establish latency in multiple cell types including salivary glands, kidneys, lymph cells and more. Human viruses from

this group include human cytomegalovirus (HCMV), human herpesvirus (HHV) 6A and B as well as HHV-7. Gammaherpesviruses usually replicate very slowly in lymphoblasts (B or T-cells) and persist latently. Their multiple forms of latency are epigenetically regulated. Well characterized members include Epstein-Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV).

Evolutionary Origin

Viruses infecting eukaryotes originated from prokaryotic viruses (Koonin, et al. 2015; Krupovic, et al. 2023). For herpesviruses, the prokaryotic progenitor was most likely an ancestor of the *Caudovirales*, in contrast to the *Tectiviridae* ancestor of other eukaryotic dsDNA viruses. However, recent discovery of mirusviruses argues for a more complex evolutionary relationship between DNA viruses (Gaïa, et al. 2023). These relations are based on homology between multiple viral genes and on structural similarity of capsid proteins.

Diversification of herpesviruses is mostly attributed to co-speciation with their respective hosts due to their close association and narrow host range (Azab, et al. 2018). However, recent studies highlight the importance of intra-host speciation and host spillovers (Brito, et al. 2021). Apart from single nucleotide mutations, which occur with rather moderate frequency (Sanjuan, et al. 2010; Jaramillo, et al. 2013), recombination also plays a major role, especially between viruses from closely related strains or species (Loncoman, et al. 2017). Furthermore, gene deletions or duplications as well as capture of host genes allows herpesviruses to increase evolutionary rates beyond their mutation frequencies, raising some very interesting research questions (Schumacher, et al. 2012; Schönrich, et al. 2017).

1.1.3 Herpes Simplex Virus

"If you love something set it free, but don't be surprised if it comes back with herpes." Chuck Palahniuk

Prevalence of HSV in human populations differs from around 55% in America to about 80% in Africa (WHO 2023). Two closely related viruses called HSV-1 and 2 (formally known as HHV-1 and 2) are infecting about 4 billion people under the age of 50 worldwide.

Clinical Manifestations

Initially, HSV-1 was attributed for oral and HSV-2 for genital infections. However, more recent research observes a shift with HSV-1 now causing more primary genital herpes (Xu, et al. 2006; Ayoub, et al. 2019; Rathbun, et al. 2022). Both viruses are very capable to infect both areas. Primary HSV-1 infection can cause fever, body ache, sore throat and headache, while reactivation from the trigeminal ganglia, triggered by stress, illness or other external factors, causes cold sores on lips and mouth (WHO 2023). More severe complications, like encephalitis or keratitis, occur

in immunocompromised patients. Especially devastating is neonatal herpes when mothers suffer acute HSV (commonly HSV-2) episodes during birth, of which neurological damage or death are frequent consequences (Cherpes, et al. 2012). Fortunately, antiviral therapy against HSV is available and effective (Gnann, et al. 1983; Zeichner 1998; Kukhanova, et al. 2014; Whitley and Baines 2018; Klysik, et al. 2020)

Virion Structure

Same as all herpesviruses, HSV virions consist of a linear dsDNA genome, packaged into an icosahedral capsid, surrounded by the loose tegument protein layer and a membrane envelope (Figure 1.2B). HSV capsids are built from four protein subunits VP5, VP26, VP23 and VP19c encoded by *UL19*, *UL35*, *UL18* and *UL38* respectively (Howley, et al. 2021). Structurally, the capsid consists of 150 hexons and 11 pentons of VP5. Additionally, hexamers of VP26 sit on top of the hexons which are linked to pentons via 320 triplexes of VP23 and VP19c in a $VP23_2VP19c$ conformation. Furthermore, one of 12 vertices incorporates a portal for DNA packaging and release, built from 12 copies of UL6 (McElwee, et al. 2018). Scaffold proteins (encoded by *UL26* and *UL26.5*) are required to chaperone structural proteins into the nucleus as well as to assemble and mature them into functional capsids (Loret, et al. 2008; Maier, et al. 2016). This yields about 125 nm big, icosahedral capsids with a triangulation number of T=16 (Howley, et al. 2021).

Preassembled capsids are packaged with concatemeric dsDNA (Deiss, et al. 1986). Packaging is dependent on several proteins (UL6, UL15, UL17, UL25, UL28, UL32 and UL33) (Baines 2011; Conway and Homa 2011). UL17 and UL25 are outer capsid proteins which are included within the tegument of mature virions (Newcomb, et al. 2000). UL15, UL28 and UL33 make up the DNA terminase complex which recognizes packaging sites within the viral genome (*pac1* and *pac2*, located within a-like sequences in the repeats) and cleaves the concatemeric DNA (Nadal, et al. 2010). After the nuclear egress complex (encoded by *UL31* and *UL34*) mediated transport of nucleocapsids to the cytoplasm (Chang, et al. 1997; Roller, et al. 2000; Reynolds, et al. 2004), tegument proteins get assembled, dependent on protein-protein interactions between UL17/25 and UL36 (Owen, et al. 2015). The inner tegument layer also includes UL37 and is more structed than the outer layer, which in addition to viral tegument proteins also captures several host factors like kinesin (Pegg, et al. 2021).

Tegumented, cytoplasmic nucleocapsids get engulfed into trans-Golgi vesicles embedded with viral membrane and glycoproteins, resulting in exosomes filled with enveloped virions (Sugimoto, et al. 2008; Henaff, et al. 2012). The endosomal sorting complex required for transport (ESCRT) pathway transports exosomes to the cell membrane and mediates release of mature virions into the extracellular environment (Kharkwal, et al. 2014; Arii, et al. 2018; Ahmad and Wilson 2020). There are 12 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN) and 5 membrane proteins (UL20, UL56, US9, UL24 and UL43) featured in the viral envelope (Loret, et al. 2008; Hilterbrand and Heldwein 2019). Essential for receptor recognition and membrane fusion are

gD, gB, gH and gL (Eisenberg, et al. 2012; Agelidis and Shukla 2015). gD receptors include nectin-1 and 2, herpes virus entry mediator and 3-O-sulfonated heparan sulfate (Spear, et al. 2000). Receptor binding triggers gH/L to activate gB and mediate membrane fusion (Atanasiu, et al. 2010). Interactions with cellular membrane proteins like a_V integrins or PILRα influence membrane fusion efficiency by an, as of now, unknown mechanisms (Satoh, et al. 2008).

Tropism and Latency

Different glycoproteins allow HSV to infect multiple cell types (Eisenberg, et al. 2012; Hilterbrand and Heldwein 2019; Hilterbrand, et al. 2021). In general, primary infections initiate at mucosal sites and progress to sensory ganglia in proximity to the site of infection. There HSV genomes persist in a latent fashion. However, tissue tropism extents from the nervous system to eyes, liver and kidneys (Howley, et al. 2021).

Fundamentally, herpesviruses are very host specific. However, in the case of HSV multiple instances of non-human infections have been documented. These occur mostly in other primates, especially great apes like chimpanzees, gorillas or orangutans but also in more distantly related new world monkeys (Eberle and Hilliard 1989). Interestingly, even a case of HSV-1 infection in a chin-chilla was observed (Wohlsein, et al. 2002). Host spillover also led to the emergence of HSV-2, as suggested by genomic analysis of HSV-1, HSV-2 and chimpanzee herpesvirus (Wertheim, et al. 2014).

Retrograde axonal transport delivers HSV to neuronal cell bodies (Howley, et al. 2021). After release into the nucleus, viral DNA circularizes and associates with histones to form epigenetically controlled viral chromatin (Rock and Fraser 1983; Deshmane and Fraser 1989). ICP0, encoded by *RL2*, plays an important role for the switch between latent or lytic replication (Raja, et al. 2016). As a E3 ubiquitin ligase, it influences host shut-off, immune evasion and epigenetic regulation (Rodríguez, et al. 2020). Absence of ICP0 facilitates silencing of early and late genes and therefore promotes latency (Smith, et al. 2011; Wang, et al. 2012). Latently infected neurons harbor about 10-30 HSV genomes, indicating co- and superinfection or some early DNA replication (Rock and Fraser 1983; Rathbun and Szpara 2021). The only major transcript present in latently infected cells is *LAT*, the latency associated transcript (Stevens, et al. 1987). *LAT* is 8.3-9 kb long and gets processed into a mature RNA that is transported into the cytoplasm while introns accumulate in the nucleus due to very stable tertiary structures. Other transcripts include several microRNAs and extremely low levels of lytic genes (Kramer and Coen 1995; Whitley and Baines 2018). Upon reactivation, viral particles are transported to axonal ends where they bud and re-infect mucosal sites (Howley, et al. 2021).

Nucleic Acid Metabolism

The HSV-1 genome encodes about 80-200 genes (Whisnant, et al. 2020), tightly organized within the 152 kbp genome. Many of those genes are involved in nucleic acid metabolism, like DNA replication, recombination and nucleotide metabolism (Weller and Coen 2012; Packard and Dembowski 2021) (Figure 1.2C).

DNA Replication. Upon infection, viral DNA circularizes in the nucleus (Strang and Stow 2005). However, whether HSV DNA replication follows a rolling-circle or recombination dependent model is not fully understood. Regardless, the end product is packageable concatemeric genomic DNA (Howley, et al. 2021). DNA replication initiates at one of the three origins of replication within the viral genome (Figure 1.3A). Two of these are located within the repeats flanking the unique short region (*oriS*), whereas the third is between *UL29* and *UL30* in the unique long part (*oriL*; Figure 1.2C). UL9, the origin binding protein, specifically interacts with the origin as a dimer in a cooperative fashion (Elias, et al. 1990; Fierer and Challberg 1992; Weller and Coen 2012). It exhibits multiple biochemical functions including helicase and nucleoside triphosphatase activity as well as unspecific ssDNA and specific dsDNA binding, which requires a RVKNL motif within the carboxy terminus (Fierer and Challberg 1992; Marintcheva and Weller 2003; Olsson, et al. 2009). After binding, UL9 recruits the helicase/primase complex (encoded by *UL5/8/52*), the single strand binding protein (ICP8, encoded by *UL29*) and the DNA polymerase (Pol, encoded by *UL30*, complexed with the processivity factor UL42) (Weller and Coen 2012; Packard and Dembowski 2021). Once initiated, elongation continues independent of UL9.

Pol is essential for DNA replication. Together with UL42, Pol follows a dimer of the helicase/primase complex at the replication fork (Weller and Coen 2012). Folding into the usual B-family polymerase structure, Pol resembles a cupped right hand with fingers, palm and thumb domains respectively (Figure 1.3A) (Knopf and Weisshart 1988; Takemura, et al. 2015). The finger domain interacts selectively with incoming nucleotides, increasing replication fidelity (Tian, et al. 2009). Template DNA is contacted via the thumb which guides it toward the catalytic center located within the palm (Knopf 1987; Knopf and Weisshart 1988; Cannistraro and Taylor 2004). Like all B-family polymerases, the catalytic core contains the highly conserved DTDS motif important for the “two-metal-ion-catalysis”. Additionally, to further increase replication fidelity, Pol also features a 3’-5’ exonuclease domain (Hwang, et al. 1997; Hwang, et al. 1999; Hwang and Hwang 2003). This proofreading subunit allows wrongly paired nucleotides to be excised and therefore increases replication fidelity beyond thermodynamic base pairing. Domains are based on tertiary structures, however, on sequence level seven conserved polymerase and three exonuclease features are recognized according to their level of similarity (I being the most and VII the least conserved) (Knopf and Weisshart 1988). Multiple studies have shown that mutations within the exonuclease region increase mutations rates, however most of the resulting viruses are not viable (Kuhn and Knopf 1996; Hwang and Hwang 2003). In other herpesviruses, like Marek’s disease virus (MDV) or HCMV, exonuclease mutants are more frequently found to yield viable,

though impaired viruses (Chen, et al. 2014; Trimpert, et al. 2019; Trimpert and Osterrieder 2019; Xing, et al. 2022). Interestingly, only exonuclease mutations seem to confer low-fidelity genome replication. Consequently, no high-fidelity exonuclease mutation has been characterized so far.

HSV DNA replication also depends on host factors (Packard and Dembowski 2021). PCNA (the human DNA sliding clamp) interacts with viral DNA and replication forks, as do mismatch repair (MMR) proteins and topoisomerases (Ebert, et al. 1990; Dembowski, et al. 2017; Dembowski and DeLuca 2018). As HSV-1 does not encode its own topoisomerases, subjugating host enzymes is essential for productive DNA replication, especially for covalently bound circular DNA templates. Similarly, host ligases are also needed for DNA-replication, recombination and repair (Dembowski, et al. 2017). Translesion polymerases play essential roles for HCMV genome replication and integrity (Zeltzer, et al. 2022), which argues for a similar involvement in HSV replication.

Recombination and Repair. If replication stops, whether because of DNA damage or replication fork collisions, restarting is nearly always mediated by recombination (Figure 1.3B). One particularly important recombination system encoded by HSV utilizes a 5'-3' exonuclease (UL12) and the single strand binding protein ICP8 to mediate single strand annealing (SSA) (Schumacher, et al. 2012). Interestingly, other forms of recombination, like non-homologous end joining (NHEJ) or homologous recombination (HR), are decreased in cells infected with HSV (Schumacher, et al. 2012). UL12 binds double strand breaks and starts degrading single strands of DNA in a 5'-3' direction. ICP8 binds the exposed single strand and, either by itself or in concert with cellular Rad52, mediates strand invasion. This mechanism repairs double strand breaks which occur regularly during DNA metabolism (Ranjha, et al. 2018). Furthermore, it could occasionally lead to the capture of host genes or recombination between two genomes (Loncoman, et al. 2017; Schönrich, et al. 2017).

HSV also encodes an uracil-DNA glycosidase (UNG) encoded by *UL2* (Bogani, et al. 2009; Bogani, et al. 2010). Oxidative deamination of cytosine bases, which turn cytosine into uracil, occurs frequently under aerobic conditions (Figure 1.3C). As uracil is not featured in DNA and also does not pair with guanine, this sort of damage results in structural differences in dsDNA which can be detected by DNA repair systems (Biechele-Speziale, et al. 2022). UNG can excise uracil from its deoxyribose backbone by mediating base excision repair (BER). Baseless sugar-phosphates are substrate for AP endonucleases, which remove the backbone in order for polymerases to fill the gap and ligases to rejoin the strands (Bogani, et al. 2009; Bogani, et al. 2010).

Again, several host factors also play a role for recombination and DNA repair in HSV infections. NHEJ proteins XRCC5 and XRCC6 both bind viral DNA, while the MNR complex interacts with the viral replication fork as well as UL12 and ICP8 (Packard and Dembowski 2021). Single nucleotide mis-parings can be resolved by BER (viral or cellular) or MMR.

Nucleotide Metabolism. HSV encodes at least three proteins important to make nucleotides available (Figure 1.3C). Best known of those is the thymidine kinase (TK), an enzyme encoded

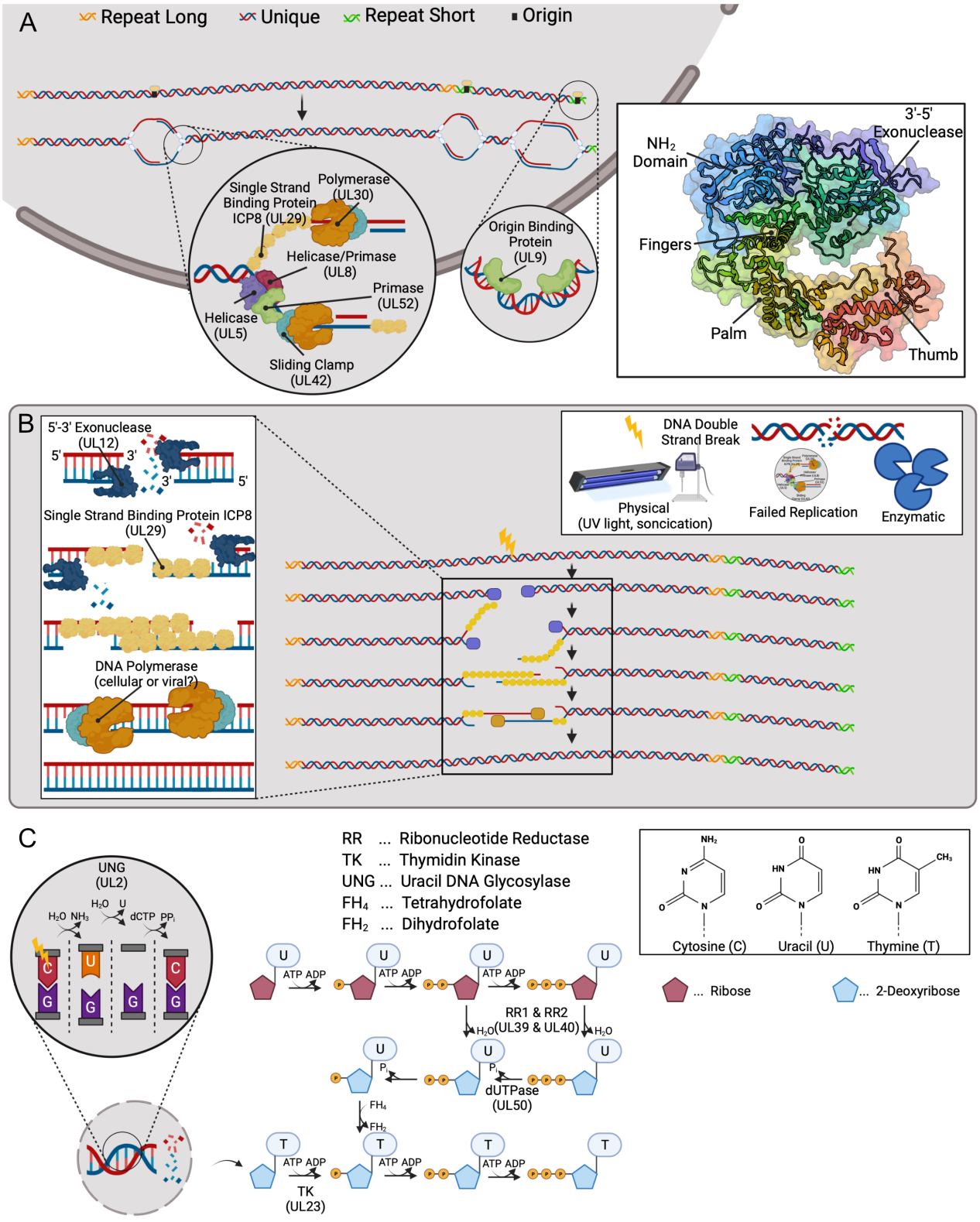

Figure 1.3: Nucleic acid metabolism in HSV-1. A) DNA Replication in HSV-1. Replication requires origin binding and initiation by UL9 as well as elongation by viral factors at the replication fork. The right side shows a structural representation of the viral DNA polymerase with labeled domains. B) Single strand annealing recombination mediated by UL12 and ICP8. Double strand breaks in DNA, which occur after physical or enzymatic causes, initiate exonucleolytic digestion by UL12, while exposed single strands recruit ICP8 and cellular single strand binding proteins which mediate strand exchange. C) Further nucleic acid metabolism in HSV-1. The left side shows base excision repair by UL2. The middle part gives an overview of nucleotide biosynthesis and recycling. Viral proteins involved in those are labeled at the respective step. Structural differences between cytosine, uracil and thymine are shown on the right.

by *UL23*. Deoxy thymidine triphosphates (dTTPs) are essential substrates for DNA-syntheses but are biochemically more demanding to synthesize than other nucleotides (Hu and Chang 2007). Intracellular dTTP concentrations can be increased by recycling from degraded DNA. The initial step is catalyzed by TK and proceeds with cellular enzymes (Howley, et al. 2021). Apart from recycling, deoxy ribose nucleotides can also be produced *de novo* from ribose nucleotides by reducing the sugar backbone. HSV encodes a ribonucleotide reductase, a two-subunit enzyme encoded by *UL39/40* (Averett, et al. 1983; Nikas, et al. 1986). Furthermore, HSV destroyes deoxy uridine triphosphates (dUTPs) enzymatically with its dUTPase encoded by *UL50* (Kato, et al. 2014). As dUTPs can be incorporated into DNA and overall decrease genomic stability, destroying existing pools of the molecule ensures proper and faithful DNA replication.

1.2 Viral Evolution and Ecology

"Nothing in biology makes sense except in the light of evolution" Theodosius Dobzhansky, 1973

Evolution, as described by Charles Darwin and Alfred Russel Wallace in the 19th century, challenged the preexisting notion of divine creation and revolutionized the way biologist observed the living world (Darwin and Wallace 1858; Darwin 1859). While evolution describes how species and higher orders of taxa are related, ecology defines the environment and interactions between and within species. Viruses are special among biological entities which influences how they evolve. Consequently, there are some caveats to viral evolution (Holmes 2009; Flint, et al. 2015b; Howley and Knipe 2020)

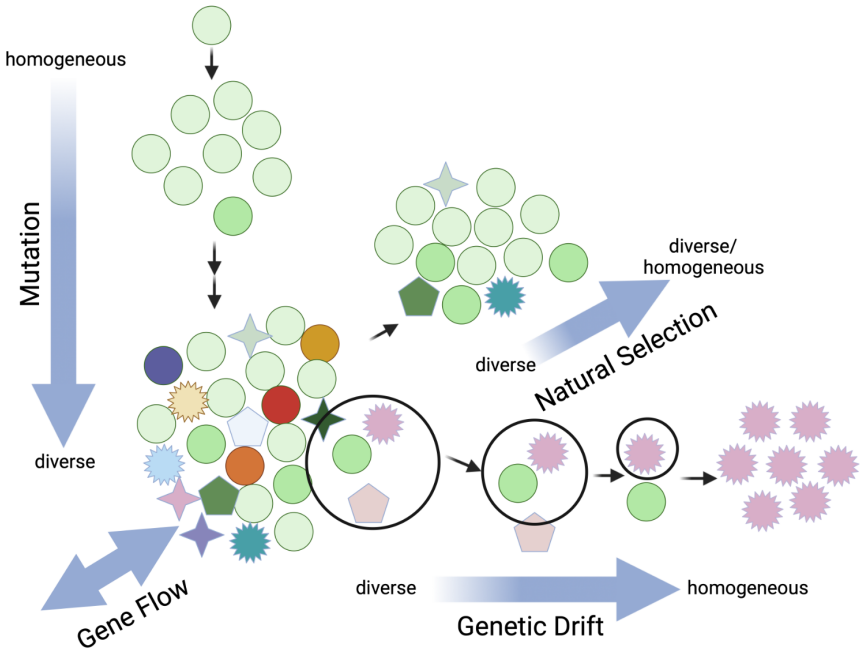

Figure 1.4: Evolutionary forces. Genetic diversity is produced by mutation and lost by genetic drift. Natural selection acts on genetic diversity to mediate adaptation to specific environments. Furthermore, gene flow between subpopulations can change individual allele frequencies and overall genetic diversity.

1.2.1 Population Genetics

"'Don't tell me from genetics. What've they got to do with it?' said Crowley. 'Look at Satan. Created as an angel, grows up to be the Great Adversary. Hey, if you're going to go on about genetics, you might as well say the kid will grow up to be an angel. After all, his father was really big in Heaven in the old days. Saying he'll grow up to be a demon just because his dad _became_ one is like saying a mouse with its tail cut off will give birth to tailless mice. No. Upbringing is everything. Take it from me.'" Terry Pratchett & Neil Gaiman, Good Omens: The Nice and Accurate Prophecies of Agnes Nutter, Witch, 1990

The field of population genetics aims to describe the genetic structure of populations and the evolutionary forces shaping them. It combines Darwinian evolution with Mendelian genetics and gives a mathematical framework to study past, present and future genetic diversity (Gillespie 2004).

Genetic Diversity

Genetic variation comes in different flavors, like single nucleotide polymorphisms (SNPs) in protein coding genes, which are either synonymous or non-synonymous depending on whether they change the encoded amino acid or not (Li, et al. 1985). Alterations also include different sizes of insertions or deletions at particular positions within the genome, so called loci (singular locus) (Mills, et al. 2006). Different variants of genes are called alleles, which can differ by multiple means: by origin in a physical sense, by state if they encode different DNA sequences or by decent if the alleles do not share a common ancestor (Gillespie 2004). A locus with two alleles different by state (A_1 and A_2) with relative frequencies p and $q = 1 - p$, would yield relative diploid genotype frequencies for A_1A_1 with p^2, A_1A_2 with $2pq$ and A_2A_2 with q^2 under non-selective conditions (Hardy 1908; Verein für vaterländische Naturkunde in, et al. 1908). This ratio, known as the Hardy-Weinberg law, is the cornerstone of modern population genetics (Mayo 2008). The overall homozygosity of the population, the frequency of individuals with two copies of the same allele at the locus, can be calculated in general by:

$$G = \sum_{i=1}^{k} p_i^2 \tag{1.1}$$

with G being the homozygosity and p_i the allele frequency of the respective allele. Consequently, the heterozygosity, the frequency of individuals with two different alleles, can be calculated as

$$H = 1 - G = 1 - \sum_{i=1}^{k} p_i^2 \tag{1.2}$$

Since the heterozygosity (H) is calculated only from individual allele frequencies, the measurement can also be used for haploid, asexual populations that do not meet the Hardy-Weinberg assumptions to describe genetic diversity at any locus (Gillespie 2004; Boca, et al. 2019).

Genetic Drift, Selection and Mutation

Three major evolutionary forces form the genetic structure of a population (Figure 1.4). These are genetic drift, selection and mutation (Stephens 2010). Genetic diversity is created by mutation and lost due to genetic drift. Selection, however, requires genetic diversity for adaptation (Gillespie 1994; Fisher and Bennett 1999). The decay of heterozygosity mediated by genetic drift is given as

$$\Delta_N H = -\frac{1}{nN} H \tag{1.3}$$

with N being the population size and n the ploidy of the population. Therefore, the overall change of heterozygosity can be described as the sum of what is created from mutation and what is lost by genetic drift:

$$\Delta H = \Delta_N H + \Delta_u H \approx -\frac{1}{nN} H + 2u(1 - H) \tag{1.4}$$

with u as the mutation frequency per locus per generation. Changes in allele frequencies due to selection can be stated as:

$$\Delta_s p = \frac{pqs(ph + q(1 - h))}{1 - 2pqhs - q^2 s} \approx qhs \tag{1.5}$$

with s defining the selection coefficient and h the heterozygous effect. The approximation is valid for $q \approx 0$. The denominator $1 - 2pqhs - q^2 s = \bar{w}$ is also known as the mean fitness of the population considering that specific locus (Gillespie 2004). Asexual haploid populations do not feature diploid stages, although upon coinfection and especially when mutations arise within infected cells, heterozygotic stages can model the situation well enough (Wilke and Novella 2003; Bushman and Antia 2019). Considering the two respective homozygous and the heterozygous genotypes, three kinds of selection can occur: directional selection when one allele is clearly favored over the other, balancing selection when heterozygotes and therefore intermediate allele frequencies are favored as well as disruptive selection when either homozygote outcompetes the heterozygote (Bürger and Gimelfarb 1999; Rieseberg, et al. 2002; Rueffler, et al. 2006; Sellis, et al. 2011; Cicconardi, et al. 2017). Allele frequencies also depend on mutations:

$$\Delta_u p = -up \approx -u \tag{1.6}$$

Approximations can be used for very small frequencies of $q \approx 0$ and $p = 1 - q \approx 1$ (Gillespie 2004). The overall change in allele frequencies can be stated as the sum of both selective and mutational changes, which at equilibrium yields:

$$\hat{q} \approx \frac{u}{hs} \tag{1.7}$$

Mutations impact population fitness by accumulating deleterious variants, measured as genetic load, the relative difference between the maximal fit genotype and the mean fitness of the locus.

$$L = \frac{w_{max} - \bar{w}}{w_{max}} \approx 2u \tag{1.8}$$

This allows for an easy demonstration that genetic load and therefore fitness loss is independent of selection itself (Haldane 1937; Bertorelle, et al. 2022).

1.2.2 Population and Genomic Structures

"If you think being descended from apes is bad for your self esteem, then get used to the idea that you are also descended from viruses." Matt Ridley, Genome: The Autobiography of a Species, 1999

Viruses occur in several different configurations. Their genomes vary in size, chemistry and structure (Baltimore 1971; Flint, et al. 2015a). Similarly, their population dynamics and lifestyles are highly diverse. All of that influences how genetic diversity is produced, maintained and changed over time.

Sequence Space

Sequence space is depicted as a multidimensional hypercube that arranges genomes according to their genomic distances (Gates 1986). Genomes that differ only at single positions are subsequently closer to each other than those that feature larger numbers of mutations, insertions or deletions (Figure 1.5A). Sequence space can be depicted in genomic or in proteomic terms. This distinction is crucial as coding for the same protein with different genomic sequences highly influences viral fitness, virulence, transmission and evolution (Moratorio, et al. 2017) (Figure 1.5B). For example, codon deoptimization (CD) or codon pair deoptimization (CPD) is a known mechanism to attenuate viruses and is used to engineer live attenuated vaccines (Kunec and Osterrieder 2016; Eschke, et al. 2018; Groenke, et al. 2020; Trimpert, et al. 2021). The reason for that is supposedly multifactorial. Firstly, deoptimizing a coding sequence influences transcript stability and secondary structure (Groenke, et al. 2020). It also yields lower levels of protein biosynthesis, probably by tRNA availability and protein folding. Furthermore, recoding may increase the amount of CpG dinucleotides, which are detected by Toll-like receptors (Atkinson, et al. 2014). Evolutionarily more interesting though, is the distance in genomic sequence space. New mutations, that occur regularly during genomic replication, can have very different impacts according to their genomic context (Figure 1.5B) (Moratorio, et al. 2017). Low fidelity genome replication requires genetic sequences to evolve a certain level of mutational robustness (McBride, et al. 2008; Ogbunugafor, et al. 2009; Gabzi, et al. 2022). Redirecting viruses in genomic sequence space, which initially might not show a fitness disadvantage, can progressively devastate future prospects of the growing population.

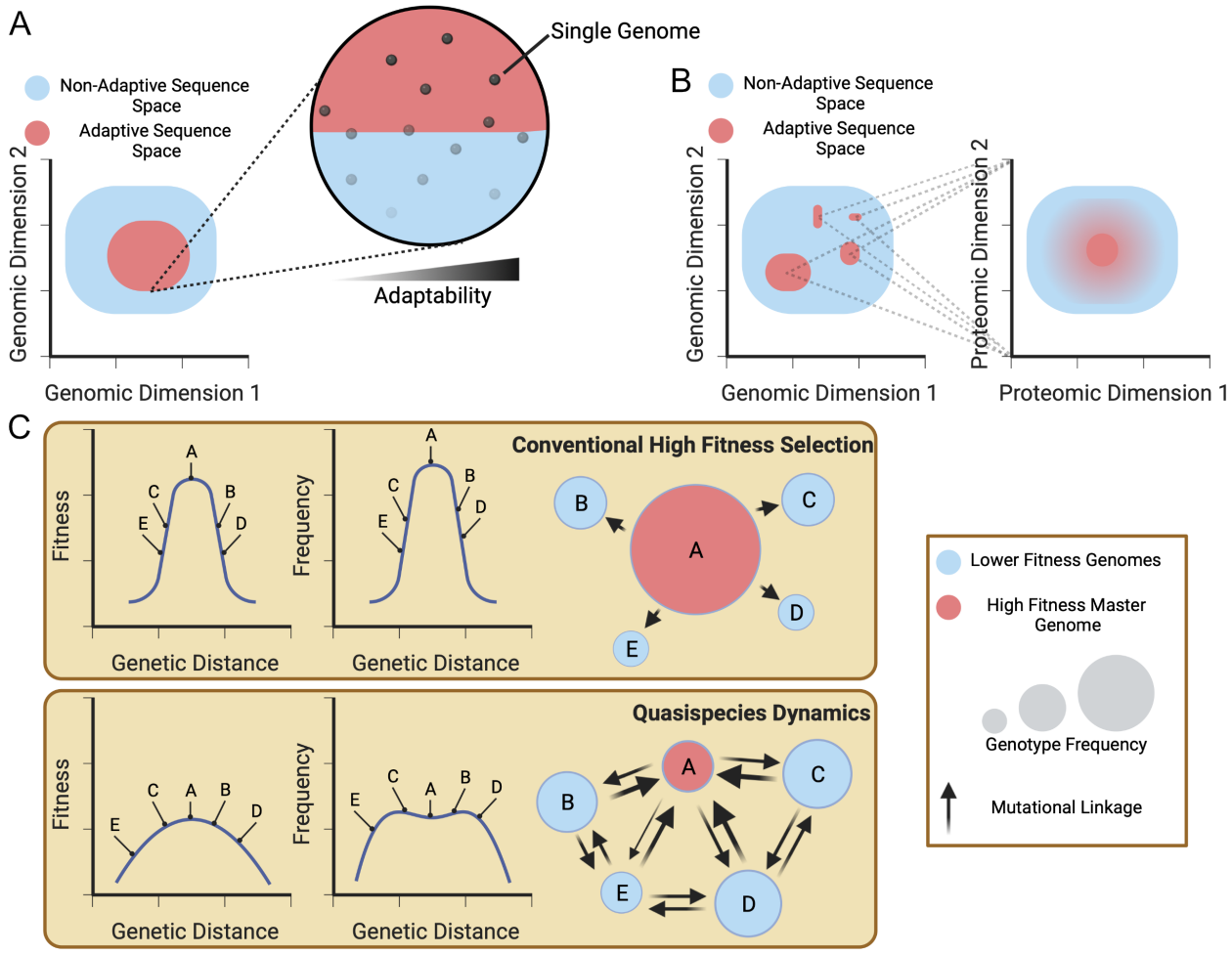

Figure 1.5: Quasispecies dynamics as visualized in sequence space. A) Sequence space describes every possible combination of a nucleotide sequence within multidimensional space. Dimension reduction is usually applied to visualize it in two dimensions. Single genomes are assigned fitness values that describe how adaptable each position is. A genotype surrounded by high fitness sequences is more likely to be adaptable than one close to non-adaptable genomes. B) Multiple areas in genomic sequence space can translate into similar areas in proteomic sequence space, based on the degenerated nature of the genetic code. However, recoding on a genomic level might lead to other consequences than simple changing amino acids in protein coding genes. Mutations have different impacts and some areas of the protein are more likely to change amino acids than others. This is represented by different sizes of adaptable areas in genomic sequence space which all code for the exact same protein. C) Fitness and genotype frequencies under conventional high fitness selection (upper) and quasispecies dynamics (lower panel). Most important distinction is the relation between individual fitness and the mutation rate. In quasispecies dynamics mutational linkage between higher and lower fitness genotypes allows for selection of later, simply by proximity in sequence space.

Quasispecies Dynamics

Barely any concept is as controversially debated as quasispecies dynamics in viral populations (Biebricher and Eigen 2006; Domingo and Perales 2019). First introduced in the 1970s by Manfred Eigen and Peter Schuster, it described the behavior of primordial genetic replicators (Eigen 1971; Eigen and Schuster 1978). The original theory assumed a fully occupied sequence space (infinite population sizes, no genetic drift), that displayed mutational linkage between individual genotypes. This results in a mutant spectrum that does not necessarily feature high fitness genotypes at highest frequencies, but maintains many closely related genotypes with increased frequencies, as selection acts on individual and mutationally linked genomes alike (Eigen 1996) (Figure 1.5C).

Mathematically, there are two formulas that define quasispecies dynamics:

$$\frac{dx_i}{dt} = (A_i Q_i - D_i)x_i + \sum_{k=1,k\neq i}^{n} W_{ik}x_k - \Phi_i \tag{1.9}$$

$$v < v_{max} = \frac{\ln \sigma_0}{1-\bar{q}} = \frac{\ln \sigma_0}{\bar{p}} \text{ and } \bar{p} < \bar{p}_{max} = \frac{\ln \sigma_0}{v} \tag{1.10}$$

with x_i, x_k being the frequencies of the i^{th} and k^{th} genotype, A_i, Q_i and D_i the growth rate, accuracy and degradation of i, W_{ik} the rate of synthesis of i from k, Φ_i the flux of i from the system, v the sequence length, σ_0 the superiority of the master sequence and $\bar{q}$ as well as $\bar{p}$ the fidelity and error rate respectively (Eigen and Schuster 1978; Domingo and Perales 2019). In biological terms this describes that the frequency of any genotype is dependent on its own replication, the amount of creation from other genotypes and the destruction by mutation. Furthermore, the amount of information and the error rate are co-dependent on the superiority of the master sequence. Consequently, the genetic diversity increases while the individual fitness of different genotypes decreases, resulting in the flattening of the fitness curve ("survival of the flattest"; Figure 1.5C) (Codoñer, et al. 2006). Because fitness depends on the genetic neighborhood, genomes are expected to evolve mutational robustness to decrease the impact of deleterious mutations (Manrubia, et al. 2005). Santiago Elena recently demonstrated that quasispecies dynamics can reproduce Hamilton's rule of kin selection (Hamilton 1964), arguing for social interactions that drive the evolution of quasispecies (Elena 2023; Leeks, Bono, et al. 2023). Importantly, quasispecies theory is not at odds with mutation-selection-balance based population genetics (Wilke 2005). All equations used for conventual mutation-selection models can be generated from quasispecies dynamics, the major difference is only the mutation rate, which enables mutational linkage and restructures individual genotype frequencies.

Mutation and recombination rates interact differently in quasispecies dynamics. In general, recombination is necessary to overcome genetic load on single genomes, which accumulates due to genetic drift (known as muller's ratchet) (Muller 1964; Jaramillo, et al. 2013). To overcome the ratchet, co-infection and recombination is necessary, upon which natural selection prefers the fitter genotype. However, quasispecies do not tolerate high recombination rates, as deleterious mutations accumulate faster (Boerlijst, et al. 1996). Therefore, RNA viruses show reduced rates of recombination. The highest observed rates of recombination occur in retroviruses, which consequently also display lower mutation rates, arguing furthermore for a trade-off between the two attributes (Jetzt, et al. 2000).

Additionally, quasispecies maintain viral genomes from earlier selection pressures, commonly referred to as quasispecies "memory" (Ruiz-Jarabo, et al. 2000). It describes the above average frequency of genotypes that conferred fitness advantages in the past and is dependent on the initial advantage as well as the passing time (Ruíz-Jarabo, et al. 2002). This results in a gradual decrease of memory genomes, which is accelerated by small population bottlenecks.

1.2.3 Ecology

"Life finds a way" Steven Spielberg, Kathleen Kennedy & Gerald R. Molen, Jurassic Park, 1993

Populations do not live in a vacuum. They depend on and compete with other living and non-living things. Ecology studies the interwoven relationships of species in different environments (Begon and Townsend 2021).

Conditions and Resources

Biological life is depending on abiotic conditions as well as available resources (abiotic or biotic) (Hutchinson 1957). Organisms have specific ranges of temperature, pH, salinity and some tolerance for catastrophe and pollution (Brock 1985; Yeo 1998; Gillooly, et al. 2001; Dhakar and Pandey 2016; Reid, et al. 2016). Resources like oxygen, water, minerals and food together with conditions form ecological niches that allow organisms to live in certain habitats (Hutchinson 1957; Leibold 1995). While conditions can be altered, they cannot be consumed like resources, yet both influence one another (Begon and Townsend 2021). For example, the solubility of oxygen in water depends on temperature and salinity (Tromans 1998). Individual metabolism is influencing available resources and altering conditions, which in turn changes environments and therefore puts pressure on other organisms living in the habitat.

Competition

The struggle for life, as Darwin called it, is a direct consequence of a finite amount of resources available for any sustaining population (Darwin 1859). This is best described in terms of competition. Members of the same species consume the same resources, which in turn depletes those und leads to finite populations sizes (Begon M. 1996) (Figure 1.6A). This indirect way of competition by depletion is called exploitation (Dulvy, et al. 2004; Ward, et al. 2006). Another form of competition, called interference, occurs whenever direct interaction leads to consummation or defense of resources (Case and Gilpin 1974; Delong and Vasseur 2013). Animals with territorial behavior or harem structures are examples for interference.

Intraspecific competition is density dependent, as crowding leads to more conflict (Begon M. 1996; Begon and Townsend 2021). This culminates in slower growth rates, increased deaths and a decline in birth rates. Smaller or weaker individuals are usually the ones suffering the most and therefore contribute less to following generations. An interesting consequence of intraspecific competition is adaptive radiation, the rapid evolution of closely related species that rely on different resources to minimize competition (Gottlieb 1998; Gavrilets and Vose 2005; Gavrilets and Losos 2009).

In contrast, interspecific competition is more nuanced, as it usually drives one of the species away from the respective habitat (Volterra 1928; Lotka 1932; Weber 1999). Whenever compet-

ing species coexist spatially, they are finely subdivided (Jaeger 1974; León and Tumpson 1975; Price 1978) (Figure 1.6B). The existence of one species and absence of the other may not be adaptive, but an inability to inhabit the area while the one still occupies the niche (Case and Gilpin 1974). Therefore, ecological niches are usually subdivided into fundamental and realized niches (Malanson, et al. 1992; Soberón and Arroyo-Peña 2017). Fundamental niches include all the conditions and resources a species requires to inhabit a space whereas realized niches additionally also include other species that are potentially harmful but still allow the species to coexist in a given habitat (Begon and Townsend 2021). The competitive exclusion principle postulates that competing species only coexist in the same habitat if niche differentiation occurred (Jaeger 1974; Weber 1999). Niche differentiation can be as subtle as a single resource or how certain resources are utilized. In nature two species are assumed to compete if removing one allows the other to flourish, however, not every natural setting does tolerate such experiments (Begon and Townsend 2021).

1.2.4 Genetic Conflict

"Now, here, you see, it takes all the running you can do, to stay in the same place" Lewis Carrell, Through the Looking-Glas, 1871

Competition can occur even on genetic levels (Burt and Trivers 2006). Biological interactions, especially antagonistic relationships, drive evolution as no other mechanism can (Magurran, et al. 1998; Werren 2011; Rice 2013). Populations are moving targets, which is why adapting to one's competitors usually requires more genetic innovations.

Red Queen Conflicts (Arms Races)

The Red Queen Hypothesis (RQH), as introduced by van Valen in 1973, describes the impact of biotic interaction between prey and predator or host and pathogen (van Valen 1973). The concept is named after the Red Queen in Lewis Carroll's "Through the Looking Glass" based on the above-mentioned quote.

The RQH is theorized to, among others, play important roles for speciation and extinction events, for the evolution of sex and for high evolutionary rates in immune genes (Murlas Cosmides and Tooby 1981; Hurst, et al. 1992; Magurran, et al. 1998; Burt and Trivers 2006; Werren 2011; Kölliker, et al. 2015). Considering viral evolution, Red Queen Conflicts (RQCs) or arms races to overcome host immune defenses are the most noteworthy (Elde, et al. 2012; Rheinemann, et al. 2021; Fixsen, et al. 2022). Viruses require at least one mechanism to evade immunity to establish reliably spreading chains of infection in their host population (Vossen, et al. 2002; Schönrich, et al. 2017; Zhu and Zheng 2020). As host immune defenses are immensely diverse on multiple levels (Murphy and Weaver 2017), immune pressure is among the biggest hurdles' parasites face (Vossen, et al. 2002; Harris and Dudley 2015). Consequently, high survival pressure applied by

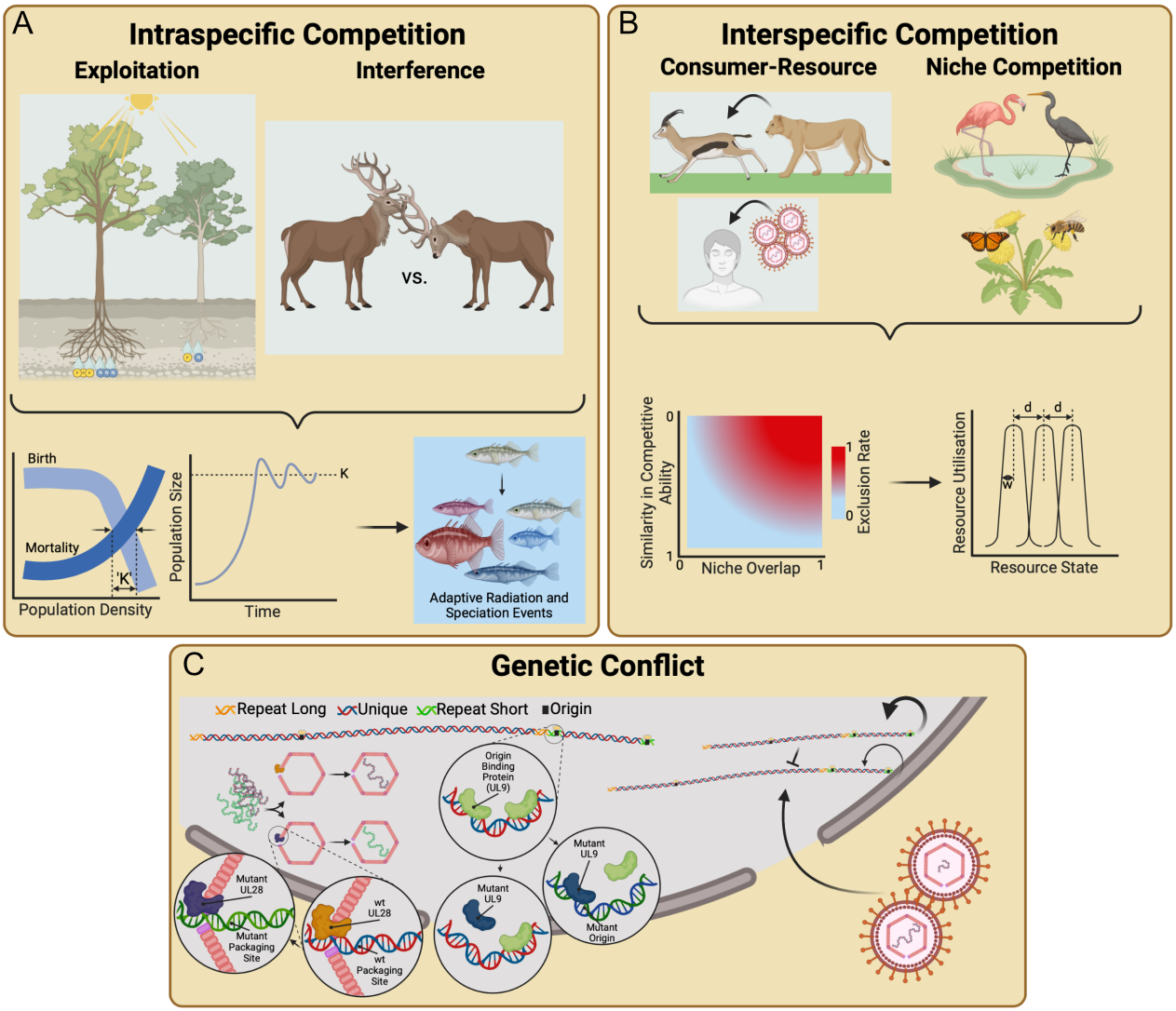

Figure 1.6: Competition and genetic conflict. A) Conflict within species occurs indirect via exploitation or direct by interference. Exploitation describes the difference in resource availability for different individuals of the same species, whereas direct fights for resources or occupation of nutricious habitats is described by interference. Regardless whether direct or indirect, intraspecific conflict is regulating population sizes and, overtime, might lead to adaptive radiation and new speciation events. B) Conflict between different species also comes in two flavours. Consumer-resource competition includes preditor-prey as well as host-parasite interactions. Niche competition however, is closer to intraspecific conflict as similar lifestyles and resource requirements lead to conflict. Exclusion of a species depends on how similar they are in their competitive ability as well as how similar their resource requirements are. This usually results, overtime, in niche differentiation. C) Genetic conflict occurs whenever genes from different individuals or within the same genome are opposing each other. Examples are differences in recognition and binding of origins of replication or packaging of genomes with varying packaging sites. However, selfish genomes can also interfere with productive genome replication and viral reproduction.

constant exposure to potentially lethal pathogens shapes host immune functions (Regoes, et al. 2014; Zhu and Zheng 2020; Tenthorey, et al. 2022). This explains the high evolutionary rate as well as their high diversity, as many different pathogens require different variants and defense systems (Hughes, et al. 2005).

Intraspecies competition can drive divergence and eventually speciation events (Begon M. 1996). However, for speciation to occur reproductive isolation is necessary (Dieckmann and Doebeli 1999). Intergenomic RQCs can be the source of that (Magurran, et al. 1998). To be genetically compatible, multiple DNA-protein as well as protein-protein interactions are required. For instance, during meiosis spindle fibers attach to special histones localized at the centromere (Ma-

lik and Henikoff 2001; Amor, et al. 2004). If those histone-DNA or spindle-histone interactions do not take place, no fertile offspring can emerge and the organism is doomed to extinction (Akera, et al. 2019). Hybrids usually face those fates. Again, viruses face similar problems. Interaction between replication origins and origin binding proteins or between packaging sites and packaging machinery do influence which genomes are replicated or packaged and therefore determine transmission efficiency (Figure 1.6C) (Elias, et al. 1990; Diffley and Cocker 1992; Nadal, et al. 2010).

Selfish Genomes

Replication leads inevitably to mutations (Sanjuan, et al. 2010; Peck and Lauring 2018). Some of those are genomic rearrangements which left to their own devices, yield no to little infectious offspring (Zeltzer, et al. 2022; Shitrit, et al. 2023). However, coinfection with functional helper viruses rescues those genomes while sacrificing overall reproduction (Rezelj, et al. 2018). Most studies define them as defective interfering particles or genomes (DIPs or DIGs), as devoting cellular and viral resources to the replication of DIGs interferes with productive genome replication (Figure 1.6C).

Selfish genomes can occur in form of DIGs but also as satellite viruses (A F Murant and Mayo 1982). Satellite viruses or virophages as they are known for giant viruses (Zhou, et al. 2013), are similar to DIGs in so far as they also cannot establish any productive viral replication by themselves, however, they usually do not originate from the helper virus population but are already established cheats (Leeks, Bono, et al. 2023). Therefore, both satellite viruses and *de novo* cheats drive viral genome evolution (Frígols, et al. 2015). Among others, superinfection exclusion (SIE) is one mechanism to protect viruses from cheats by rendering a cell unsuitable for superinfecting viruses (Hunter and Fusco 2022). In a time depend manner, SIE can act on different stages of the infectious cycle. For example, SIE in human papillomaviruses occurs during entry and is dependent on the minor capsid protein L2 (Biryukov and Meyers 2018), while alphaherpesviruses mediate SIE also early but require some gene expression (Criddle, et al. 2016; Cwick, et al. 2022). In contrast, SIE in RNA viruses depends often on cross-reactive RNA interference mechanisms (Zou, et al. 2009; Perdoncini Carvalho, et al. 2022; Sims, et al. 2023).

Cheats in virus populations are also theorized to drive the evolution of multipartite viruses (Leeks, Young, et al. 2023), viruses that split their genome in multiple segments that get packaged into individual capsids. The model suggests that, under rather realistic circumstances, the co-occurrence of different cheats that complement each other could outcompete the monopartite progenitor. Other studies proposed a beneficial aspect of DIGs by increasing effective mutation rates and reintroducing them into functional genomes via recombination (Dimmock and Easton 2014; Vignuzzi and López 2019; Rezelj, et al. 2021; Leeks, Bono, et al. 2023).

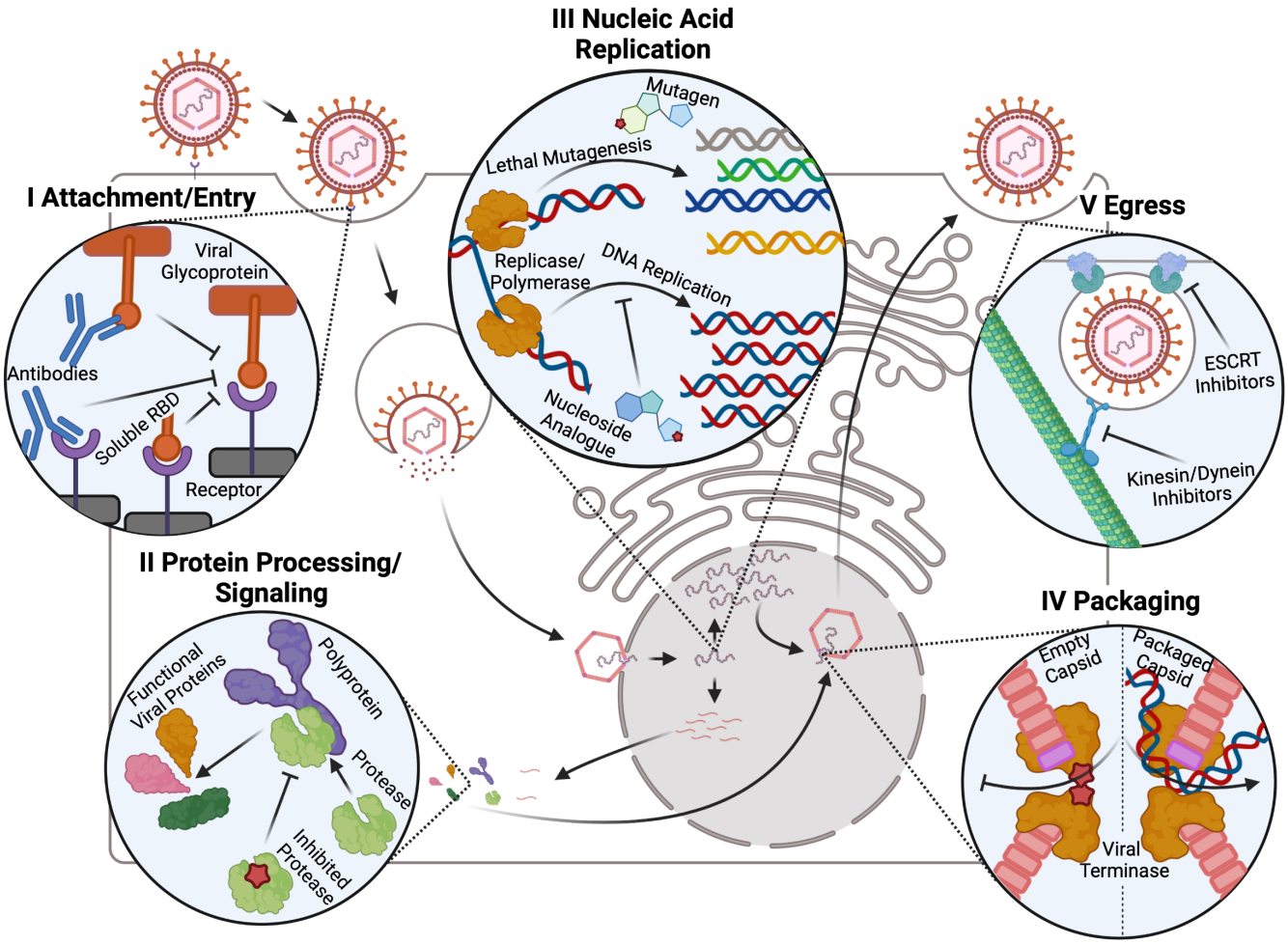

Figure 1.7: Targets for antiviral therapy. Viral reproduction can be inhibited at numerous stages of the infectious cycle. (I) Attachment and entry inhibitors like monoclonal antibodies against viral glycoproteins or cellular receptors as well as soluble forms of receptor binding domains (RBDs) or receptors can be utilized. (II) Interfering with protein processing or signaling by inhibiting proteases or kinases has been employed as antiviral strategies. (III) Nucleic acid replication can be inhibited by means of lethal mutagenesis or by chain determination. (IV) Disturbing packaging of viral genomes into infectious particles renders infections manageable. (V) Hindering transport and egress of viral particles successfully inhibits their release and lowers infectious burdens.

1.3 Antiviral Treatment

"Suggested remedy for the common cold: A good gulp of whiskey at bedtime-it's not very scientific, but it helps." Sir Alexander Fleming

Infectious diseases are treated by the administration of antimicrobials (Leekha, et al. 2011). Because viruses are very dependent on host cells to replicate, the best way to inactivate viruses is to destroy the host cell. In most cases this approach is not well suited for medical practice. Better suitable are highly specific drugs, acting against unique enzymes of closely related viruses (De Clercq 2002).

1.3.1 Modes of Action

"Not knowing how venom kills does not take away the usefulness of knowing that it does." Mokokoma Mokhonoana

Finding targets for antiviral drug design is challenging, especially since there are no striking dif-

ferences in molecular functions between viruses and their hosts (Lou, et al. 2014). To circumvent that, antivirals usually inhibit specific viral enzymes or facilitate population collapse. The aim often is not to inactivate viruses but to stall them till the immune system takes over.

Reproductive Inhibitors

Maybe the most obvious way to treat viral infection is to prevent them from reproducing. Therefore, most antivirals available on the market are directed against different steps of the infectious cycle (Andrei, et al. 2009; De Clercq and Li 2016) (Figure 1.7). Both viral and host factors are targeted to reduce or block viral reproduction in order to overcome the infection.

Attachment/Entry. Many antivirals aim to already stop attachment and entry of virions into susceptible host cells (Moore and Doms 2003; Teissier, et al. 2011) (Figure 1.7, part I). Most important among those, are antibodies, which will be discussed later. However, other entry inhibitors often utilize a soluble form of the cell receptor to compete for binding (Byrn, et al. 1989; Ohlin, et al. 1994). Additionally, the reverse is also possible, to engineer a soluble form of the receptor binding domain (RBD) (Wong, et al. 2004; Zahradník, et al. 2021).

Protein Processing/Signaling. Viruses rely on proteome maturation (Xue, et al. 2008; Mangel and San Martín 2014; Mattei, et al. 2016) (Figure 1.7, part II). Consequently, inhibiting protein processing interferes with viral reproduction. Protease inhibitors are often part of combinational treatment of hepatitis C (HCV) or human immunodeficiency virus (HIV) infections (Gortmaker, et al. 2001; Lamarre, et al. 2003; Drenth 2013). Recently, a protease inhibitor against SARS-CoV-2 was developed and licensed for human use (Najjar-Debbiny, et al. 2022). Furthermore, cellular signaling pathways and especially those manipulated by viral enzymes, also occur to be fantastic antiviral targets (Raghuvanshi and Bharate 2022). In the case of herpesviruses, a highly conserved protein kinase (UL13 in HSV-1) phosphorylates multiple proteins (Li, et al. 2011). The nucleoside analogue maribavir is used to inhibit the HCMV homologue UL97 (Trofe, et al. 2008). Additionally, inhibiting cellular kinases also shows promising antiviral effects (Schang, et al. 2000).

Nucleic Acid Replication. Probably the most common antiviral target is nucleic acid replication (De Clercq 2002; De Clercq and Li 2016) (Figure 1.7, part III). Differential selectivity of viral compared to cellular polymerases as well as prodrugs activated by viral enzymes are utilized to block viral replication (Leinbach, et al. 1976; Faulds and Heel 1990; Wagstaff, et al. 1994; Rezende and Prasad 2004; Hayden and Shindo 2019). However, even if nucleotide selectivity does not exclude incorporation into host DNA, limited levels of replication in terminally differentiated cells also prohibits major host damage while still detrimental to viral reproduction (Nouspikel and Hanawalt 2002; Estefanía, et al. 2012). Polymerase inhibitors usually come in two flavors, nucleoside analogues and non-nucleoside inhibitors (Tian, et al. 2021). A frequently used mechanism is chain termination, either mediated by incorporated nucleotides that cannot be elongated (Wagstaff, et al. 1994; Chen, et al. 2014) or because inhibitors result in polymerase collisions and stall the replicase (Kokic, et al. 2021). Other nucleoside analogues can lead to lethal mutagen-

esis which is discussed later. Apart from polymerase inhibitors, helicase and primase inhibitors also effectively limit viral replication. Those targets are studied for multiple viruses including HCV, CoVs, enteroviruses and HSV (Crute, et al. 2002; Tanner, et al. 2003; Li, et al. 2012; Fang, et al. 2021).

Packaging/Egress. If neither entry into nor replication within cells can be inhibited, inhibiting packaging and egress from infected cells might still be a valid target to break viral transmission chains (Andrei, et al. 2009; Piret and Boivin 2021; Pachota, et al. 2023). Interfering with packaging of viral DNA (Figure 1.7, part IV) turned out to effectively decrease HCMV reproduction (Kim 2018; Ligat, et al. 2018). Furthermore, herpesviruses were recently found to package cellular kinesin molecules to motorize their capsids and allow for retrograde axonal transport and subsequent neuro invasion (Pegg, et al. 2021). Targeting capture and packaging of cellular factors into viral particles might result in new effective antiviral therapies. Other examples include anti-influenza neuraminidase inhibitors, which interfere with budding from infected cells (Kim, et al. 1999) and broad-spectrum inhibition of ESCRT pathways (Kharkwal, et al. 2014; Robinson, et al. 2018; Rheinemann, et al. 2021; Dai, et al. 2024) (Figure 1.7, part V).

Monoclonal Antibodies and Convalescent Plasma

Administering antibodies against a virus is a very effective and low risk treatment (Both, et al. 2013). They are well tolerated and highly specific. Both, convalescent plasma and monoclonal antibodies are used clinically. Convalescent plasma is a mixture of all the antibodies a donor features in their blood (Hung, et al. 2011; Rajendran, et al. 2020). Therefore, it must be checked for cross reactive and self-antibodies as well as the antibody of interest (Carter, et al. 2021; Gilchuk, et al. 2022). These requirements make clinical trials, to evaluate efficacy, more difficult to design.

In contrast, monoclonal antibodies are produced *in vitro* and were shown to have protective abilities against the respective pathogen (Both, et al. 2013). They are usually isolated from people that respond well against natural infections by molecular cloning and can be produced on high scales (Boyd, et al. 1984). As the formulation of the monoclonal is precisely defined, proper placebo controlled double blinded clinical trials can be utilized to evaluate efficacy.

Regardless if convalescent or monoclonal, antibodies can act against viral pathogens in multiple ways. Neutralizing antibodies inhibit cell entry by binding at the RBD, by inhibiting binding protein processing like furin cleavage or locking proteins in prefusion conformations (Figure 1.7, part I) (Hook, et al. 2008; Corti and Lanzavecchia 2013; Lee, et al. 2013). Recognized epitopes can be linear or conformational, that is, are specific for single stretches of amino acids or are highly dependent on three-dimensional structure and potential protein-protein interactions (Ito, et al. 2003).

Even though more difficult to demonstrate, non-neutralizing antibodies can be just as effective (Schmaljohn, et al. 1982; Chandler, et al. 2023). Most mechanisms involve the antibody Fc

region and subsequent receptors on effector cells. Opsonized viral particles or infected cells are recognized by immune cells like natural killer cells or macrophages and are killed by apoptosis or phagocytosis (Pollara, et al. 2011; Reichert 2014; Tay, et al. 2019; Eberhard, et al. 2021). Furthermore, Fc regions can activate complement effectors and stimulate formation of the membrane attacking complex (Vogt, et al. 2011). Additionally, non-neutralizing antibodies might also expose neutralizing epitopes (Howell, et al. 2017). Binding to viral epitopes intracellularly blocks egress or triggers degradation and MHC presentation (Feng, et al. 2002; Caddy, et al. 2020).

Lethal Mutagenesis

Another popular method to treat viral infections is the application of specific mutagens. This particular form of therapy, lethal mutagenesis, aims for within-host population extinction (Perales, et al. 2011). Most extinction processes require massive reductions of population sizes to accumulate deleterious mutations (Raup 1986; Wiens and Slaton 2012). In contrast, lethal mutagenesis works even in large populations by reducing the amount of fit and viable offspring (Bull, et al. 2007). Here it is important to make some necessary distinctions between error threshold, error catastrophe and extinction threshold (Holmes 2009). The error threshold describes the mutation rate at which an evolutionary shift from fitness to mutational robustness selection occurs. Beyond the error threshold, error catastrophe leads to a successive loss of genetic information due to high mutation rates. Extinction, however, might occur at lower or higher mutation rates, depending on the initial relationship between fitness and the genotype replacement rate:

$$w(1-\mu) < 1 \tag{1.11}$$

with w being the fitness and μ the mutation rate (Bull, et al. 2005).

Importantly, lethal mutagenesis requires both evolutionary and demographic parameters to be effective. Increases in mutation rates lower the mean fitness of the population, yet if the particular virus has a high reproduction rate, that might not be enough to cause extinction, especially in the case of error catastrophe (Bull, et al. 2007). Consequently, lethal mutagenesis works best in combination with other antiviral drugs or if the mutagen itself also inhibits viral growth (Graci and Cameron 2006). Lethal mutagenesis could already be successfully employed for multiple RNA virus infections like arenaviruses, poliovirus, food-mouth-disease virus, HIV and SARS-CoV-2 (Perales, et al. 2011; Hadj Hassine, et al. 2022; Swanstrom and Schinazi 2022). Approved mutagenic drugs include ribavirin, favipiravir and molnupiravir (Hadj Hassine, et al. 2022). Treatment with molnupiravir in SARS-CoV-2 infections is less effective than replicative inhibitors like paxlovid and remdesivir (Wen, et al. 2022; Gudima, et al. 2023). Interestingly, SARS-CoV-2 features lower intrinsic mutation rates than other RNA viruses because of the 3'-5' exonuclease which during replication acts as a proofreading enzyme (Zhao, et al. 2004; Eckerle, et al. 2007; Graepel, et al. 2017; Domingo, et al. 2021). Therefore, the virus is thought to maintain mutation rates far below

the error threshold, which in turn would render SARS-CoV-2 less susceptible to lethal mutagenesis. Along those lines, so far, no mutagen against DNA virus infections is approved for clinical usage, probably because DNA viruses, especially large dsDNA viruses, display far lower mutation rates while maintaining similar fertility (Sanjuan, et al. 2010; Sanjuan and Domingo-Calap 2016).

1.4 Aims of This Study

"Perfection is impossible, but we don't stop aiming for it" Saquon Barkley

1.4.1 Exploring the Limits of Hypermutation in Herpes Simplex Virus Type 1

Early work on HSV-1 polymerases concluded that proofreading deficient viruses are not viable (Hall, et al. 1995; Kuhn and Knopf 1996; Baker and Hall 1998; Lawler and Coen 2018). However, we could recently show that exonuclease mutants with impaired and also absent proofreading could be recovered for MDV, a related alphaherpesvirus of chickens (Trimpert, et al. 2019; Xing, et al. 2022). We therefore concluded that HSV-1 exonuclease deficient viruses are probably viable, but will accumulate deleterious mutations at such an enormous speed, that they do not survive for long *in vitro*. So, the first aim of this thesis was to explore the limits of hypermutation in HSV-1 and to determine the cause of population extinction in exonuclease deficient virus.

1.4.2 Establishing Stably Replicating Viral Populations with Increased Mutation Rates

A further aim of this study was to establish stably replicating viral populations with hypermutator properties. Since experimental evolution is a labor and time-consuming endeavor (Ogbunugafor, et al. 2009; Kawecki, et al. 2012; Sanjuán, et al. 2021), working with hypermutators that increase genetic diversity but still maintain wt-like replication dynamics and evolutionary patterns is greatly advancing the field (Xing, et al. 2022). This would not only accelerate experimental research on viral evolution and adaptation, it would also allow to select for more complex phenotypes which, under wt conditions, would take years and a complex series of selection pressures.

1.4.3 Investigating Antiviral Resistance in Hypermutator Populations

The third and last aim of this thesis was to investigate the evolution of antiviral resistance in hypermutator HSV-1 populations. Antiviral resistance is one of the most challenging phenotypes in clinical virology (Bestman-Smith and Boivin 2003; Chen, et al. 2014; Piret and Boivin 2016; Morrison and Zembower 2020). Developing antiviral drugs is difficult as well as expensive and extensive usage of antivirals leads to resistance. All of these reasons make drug development very unattractive for pharmaceutical companies. However, if an easy and fast to do screen for antiviral resistance mutations would be available, developers could determine how fast resistance

would occur and how to respond. Evolution of co-resistance and trade-offs between drugs could be evaluated and used to guide clinical recommendations.

Chapter 2

Suicidal Phenotype of Proofreading-Deficient Herpes Simplex Virus 1 Polymerase Mutants

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2.1 Abstract

Herpes simplex virus type 1 (HSV-1) encodes a family B DNA polymerase (Pol) capable of exonucleolytic proofreading whose functions were extensively studied in the past. Early studies on the *in vitro* activity of purified Pol protein found that the enzymatic functions of the holoenzyme are largely separated. Consequently, exonuclease activity can be reduced or abolished by certain point mutations within catalytically important regions, with no or only minor effects on polymerase activity. Despite unimpaired polymerase activity, recovery of HSV-1 mutants with a catalytically inactive exonuclease was so far unsuccessful. Hence, mutations such as D368A, which abolish exonuclease activity, are believed to be lethal. Here we show that HSV-1 can be recovered in absence of Pol intrinsic exonuclease activity and demonstrate that a lack of proofreading causes rapid accumulation of likely detrimental mutations. Although mutations that abolish exonuclease activity do not appear to be lethal, the lack of proofreading yields viruses with a suicidal phenotype that cease to replicate within few passages following reconstitution. Hence, we conclude that high replication fidelity conferred by proofreading is essential to maintain HSV-1 genome integrity and that a lack of exonuclease activity produces an initially viable but rapidly suicidal phenotype. However, stably replicating viruses with reduced exonuclease activity and therefore elevated mutation rates can be generated by mutating a catalytically less important site located within a conserved exonuclease domain.

2.2 Importance

Recovery of fully exonuclease-deficient herpes simplex virus 1 (HSV-1) DNA polymerase mutants has been so far unsuccessful. However, exonuclease activity is not known to be directly essential for virus replication, and the lethal phenotype of certain HSV-1 polymerase mutants is thus attributed to factors other than exonuclease activity. Here, we showed that the recovery of a variety of exonuclease-deficient HSV-1 polymerase mutants is possible and that these mutants are initially replication competent. We, however, observed a progressive loss of mutant viability upon cell culture passaging, which coincided with the rapid accumulation of mutations in exonuclease-deficient viruses. We thus concluded that a lack of DNA proofreading in exonuclease-deficient viruses causes an initially viable but rapidly suicidal hypermutator phenotype and, consequently, the extinction of mutant viruses within few generations following recovery. This would make the absence of exonuclease activity the primary reason for the long-reported difficulties in culturing exonuclease-deficient HSV-1 mutants.

2.3 Introduction

Herpes simplex virus type 1 (HSV-1) is a human alphaherpesvirus and member of the large *Herpesviridae* family (1). To achieve high replication fidelity, all members of the order *Herpesvirales* encode a viral replication machinery, including a family B DNA polymerase (Pol). In HSV-1, this enzyme is encoded by the open reading frame (ORF) unique long 30 (*UL30*). Family B polymerases coordinate two opposite enzymatic functions in one holoenzyme and carry a 5'-3' polymerase domain for DNA replication activity and a 3'-5' exonuclease (Exo) domain capable of exonucleolytic proofreading (2). Structurally, herpesvirus family B polymerases share seven conserved polymerase domains, named I to VII (according to the degree of conservation), the δ-C region and three exonuclease domains, named Exo I to III (3, 4). The first conserved exonuclease domain (Exo I) does not overlap with any other part of the enzyme with known catalytic function, while Exo II and Exo III overlap the conserved Pol domain IV and the δ-C region respectively (Figure 2.1A). Several studies investigating the role of Exo in HSV-1 Pol have been carried out in the past (5-10). A hallmark study on the *in vitro* activity of HSV-1 Pol identified regions in the Pol gene where Exo activity can be manipulated with no or only minor effect on Pol activity. However, a strong interdependence of both functions is apparent in other regions of the enzyme. Specifically, mutations within Exo II and Exo III domains typically affect both enzymatic functions simultaneously (9). Mutants that show strong reduction of polymerase activity are not able to replicate DNA efficiently and thus yield nonviable viruses (5). On the other hand, specific mutations targeting the exonuclease catalytic core within Exo I abrogate Exo activity entirely by preventing the coordination of the Mg^{2+} required for catalytic activity (9, 11) but without compromising the efficiency of DNA replication. Previously, several studies on HSV-1 Pol D368A mutant, an Exo I mutant in which exonuclease activity is eliminated without any negative effect on DNA synthesis,

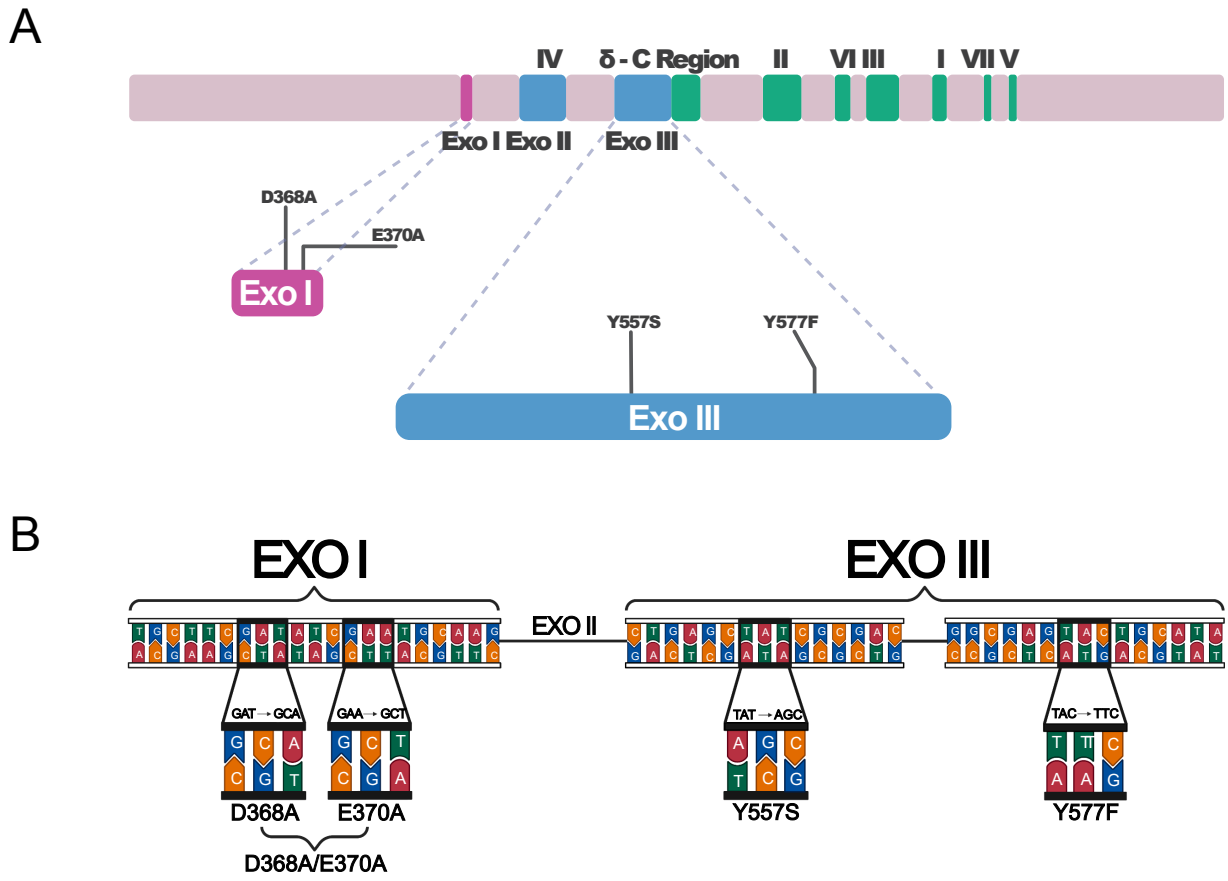

Figure 2.1: Mutations introduced into the HSV-1 DNA-polymerase gene. (A) Schematic representation of HSV-1 *UL30*, the polymerase gene with highlighted functional domains. (B) To generate Exo deficient Pol mutants used in this study, catalytically important residues within conserved Exo domains I and III were mutated based on a previously published mutational analysis of HSV-1 Pol (9).

found this mutation to be lethal, as reconstitution of mutant viruses in non-complementing cell lines failed (9, 10, 12, 13). It has been proposed that the reason for the observed lethal phenotype is not the lack of Exo activity but rather a not yet fully characterized effect of the mutation on expression of the holoenzyme (13). Recently, we have constructed a series of Exo-deficient Pol mutants in Marek's Disease Virus (MDV), an oncogenic alphaherpesvirus of chicken classified as gallid alphaherpesvirus 2 (GaAHV-2) (14). We found catalytically important residues to be highly conserved between MDV and HSV-1 (15). Moreover, we determined that the *in vitro* enzymatic activity of purified MDV Exo I or Exo III mutant Pol is in good agreement with published reports "on homologoues HSV-1 Pol mutants". However, in contrast to literature published on HSV-1 Pol mutants, we found both MDV Exo I and Exo III Pol mutants to be viable upon reconstitution in non-complementing cells. Importantly, this does include D358A, the homologue of D368A, which could not be reconstituted in case of HSV-1. However, we found that Exo deficient mutants such as D358A displayed a suicidal phenotype and typically ceased from forming viable virus progeny within few passages following reconstitution. This suicidal phenotype could be linked to rapid accumulation of mutations in viral genomes; we thus surmise that the observed aggregation of mutations, facilitated by a lack of exonucleolytic proofreading in Exo-deficient mutants, is causal

for the observed loss of replication capacity. In this case, the excessively error prone genome replication would cause viral genomes to accumulate deleterious mutations faster than they can be cleared, resulting in steep declines in replicative fitness, thus presenting the primary reason for the successive loss of viral viability observed by us. A situation in which a viral population continues to hypermutate to a degree which renders the vast majority of its progeny unfit is described as lethal mutagenesis. Lethal mutagenesis requires that, by means of deleterious mutation, an infectious virus particle creates on average fewer than one infectious offspring (16). This scenario is well described for RNA viruses and exploited as antiviral therapy concept (17, 18). Based on our previous findings for MDV and accounting for the highly conserved nature of alphaherpesvirus replicative DNA polymerases, we hypothesized a lethal mutagenesis-like scenario for proofreading deficient HSV-1 Exo mutants. To elucidate the phenotype of Exo deficient HSV-1 Pol mutants, we constructed three Exo I mutants, D368A, E370A and D368A/E370A, which are expected to abolish exonuclease activity; and two Exo III mutants, Y557S and Y577F, which are expected to retain some amount of residual exonuclease activity (9). In this study, we investigate the phenotype of these mutants; our results suggest that all Exo mutants presented here can be reconstituted in noncomplementing cells while most of them exhibit a suicidal phenotype similar to what we observed for MDV mutant homologues.

2.4 Results

2.4.1 Exonuclease Deficient HSV-1 Pol Mutants are Initially Viable in Cell Culture

Based on previous results obtained for MDV (GaAHV-2) polymerase mutants (14), we hypothesized that point mutations within the 3'-5'-exonuclease domain of the viral DNA polymerase are not lethal but increase mutation rates, in some cases to an extent that yields viruses with suicidal phenotypes. These phenotypes would be caused by lethal mutagenesis due to the accumulation of deleterious mutations and increased genetic load. To test this hypothesis for HSV-1, we generated mutants harboring mutations in Exo I and Exo III (Figure 2.1B) using a reverse genetic system (19). Exo I mutants D368A (GAT->GCA), E370A (GAA->GCT) and D368A/E370A (GAT->GCA/GAA->GCT) have been described as catalytically inactive (9) due to their inability to coordinate the catalytically required Mg^{2+} ion. On the other hand, Exo III mutants Y557S (TAT->AGC) and Y577F (TAC->TTC) are expected to retain a certain amount of exonuclease activity (9). We were able to reconstitute all HSV-1 Pol mutants that were subjects of this study by transfecting Vero cells with the respective virus DNA. In all cases, a visible cytopathic effect (CPE) became apparent within 4 h posttransfection, but the focus sizes differed considerably between mutants (Figure 2.2A). Upon serial passage, differences between mutants became even more obvious, with only Y557S retaining a typical HSV-1 plaque phenotype, while in all other mutants, infection foci did not extend to more than a few cells by passage II (pII; see "Infection of cells and propagation of virus" for an explanation of passage numbers) (Figure 2.2B; see Figure 2S1 in

supplemental material).

2.4.2 Viral Reproduction is Greatly Impaired in Most Pol Mutants

In our attempts to characterize the phenotype of Pol mutant viruses, we also observed a decrease in the number of infection foci for all mutants except Y557S over passages. To investigate if the reduction in focus size as well as the decrease in focus numbers is due to a global decrease in viral reproduction, we quantified viral genome copies in supernatants collected from infected cell monolayers at p0 to V via quantitative PCR (qPCR) (Figure 2.2B). Viral genome copy numbers of all mutants except Y557S were several orders of magnitude lower than that of the parental wild type (WT) in all passages following reconstitution, with a steep decrease for most mutants from pI to pII. Of note, all Exo I mutants and Exo III mutant Y577F behaved similarly, while Y557S was indistinguishable from the WT.

Accounting for the weak growth of all Pol mutants except Y557S, we were unable to passage these mutants using an inoculum similar to that for the WT and the Y557S mutant. We thus aimed to provide a more standardized assessment of virus growth using multistep growth kinetics of viruses at pII, in which we measured viral genome copies in the supernatant resulting from infection with a standardized inoculum of 200 focus forming units (FFU) per virus (Figure 2.2C). Results confirmed decreased viral reproduction in Exo I and Y577F mutants compared to that of the WT and Y557S, which again showed very similar growth. Additionally, Exo I and Y577F hypermutators drastically increased genome-to-FFU ratios compared to WT and Y557S, indicating that genomes are less likely to be associated with infectious virions (Figure 2.2D). The horizontal spread of infection foci is highly dependent on Pol activity, as treatment with foscarnet (FOS, herpesvirus polymerase inhibitor) abolishes plaque formation similar to disrupting *UL30* itself (Figure 2.2E and F). Overall, our results reflected the notion that the Y557S mutant replicates similarly to parental WT virus while all other Pol mutants are significantly impaired in growth.

2.4.3 Mutational Load is Drastically Increased in Most Pol Mutants

Along with progressive growth deficits, we observed massively increased mutation frequencies in Exo mutants compared to those of the WT (Figure 2.3A; Table S2). At p0, the overall numbers of mutations in all tested viruses were similar, although major genetic variants (single nucleotide polymorphisms [SNPs] with allele frequencies over 50%) were slightly less abundant in the WT and Y557S. After three passages, this difference became distinct when most Exo mutants accumulated double the number of minor genetic variants (SNPs with allele frequencies below 50%) compared to that of the WT and Y557S. Importantly, major variants again diverged more drastically, with the WT and Y557S, displaying 10 times fewer SNPs than the most conservative hypermutator (D368A/E370A).

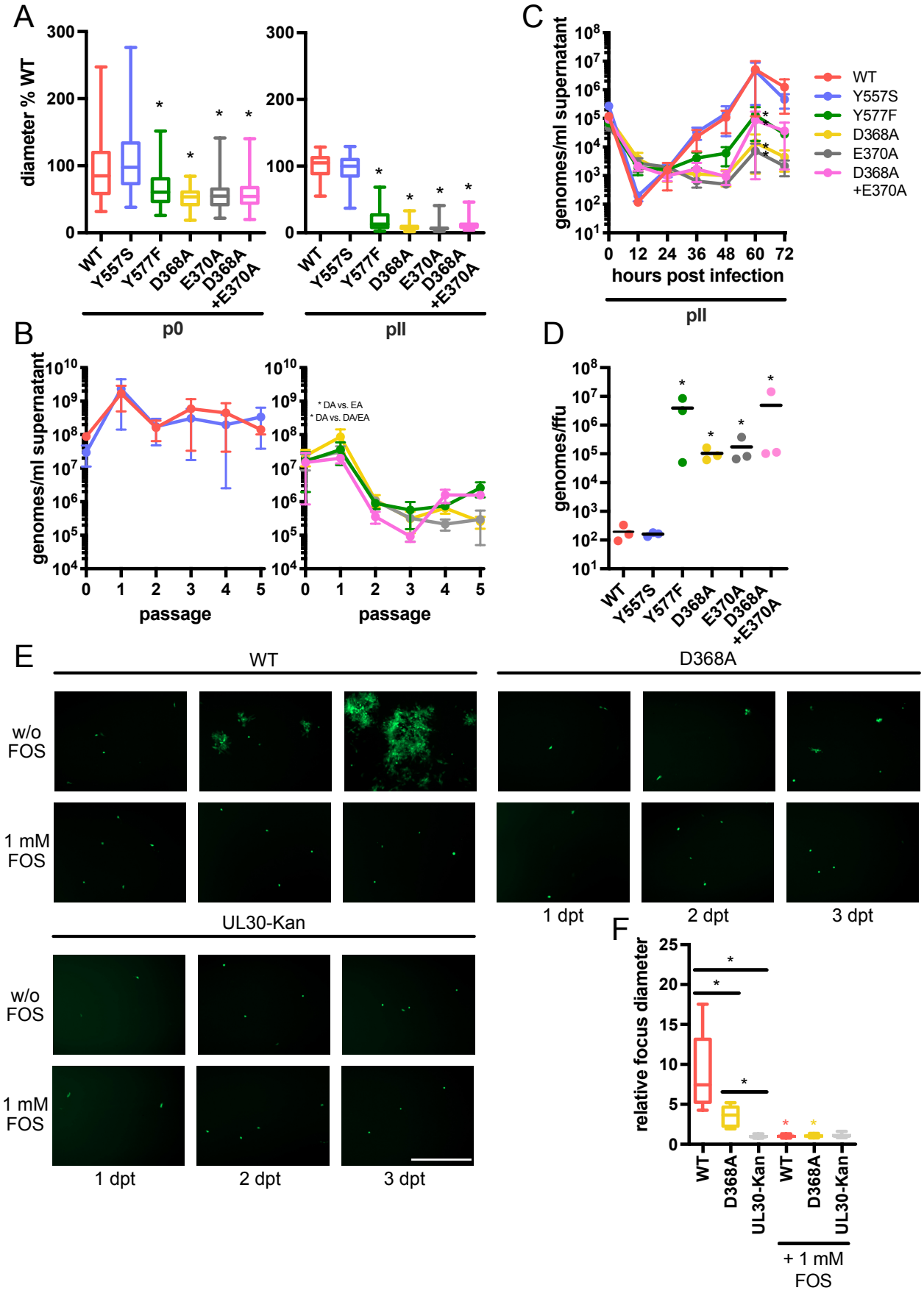

Figure 2.2: Exo I and Exo III Y577F mutants display severe growth deficits. (continued)

(continued) (A) Focus sizes in relation to the respective WT control are shown for p0 (left) and pII (right). (B) Endpoint titers for DNA copy numbers of passaged viruses measured by qPCR. WT and Y557S (left) were, from pI onwards, passaged by infecting fresh cells with 25 µl of supernatant, whereas all other viruses (right) were passaged by splitting infected cells 1:3 for all passages. (C) Multi-step growth kinetics of viruses measured at indicated time points via qPCR. (D) Genome/FFU ratios for p0 stocks measured by qPCR and focus assay. (E) Pictures of growing foci taken with a Zeiss Axio Vert.A1 inverted fluorescence microscope 1, 2 and 3 days posttransfection. Scale bar, 500 µm. (F) Relative focus diameters 3 days posttransfection with or without foscarnet (FOS; 1 mM) treatment. Focus areas were measured with ImageJ and transformed into diameters. Diameters were normalized to WT foci treated with FOS. Analysis of variance (ANOVA) was performed for all datasets. Focus size assays and genome/FFU measurement were analyzed by one-way ANOVA followed by Dunnett's multiple-comparisons test; end point titer and multi-step growth curves were analyzed by two-way ANOVA followed by Tukey's multiple-comparisons test. An asterisk indicates significant ($P < 0.05$) differences as indicated or against WT if not further specified. Colored asterisks in panel F indicate significant ($P < 0.05$) differences in results in comparison to those of the respective untreated viruses.

Next, we investigated whether the lack of exonucleolytic proofreading influences the type of mutations observed by us. Generally, nucleotide changes can be of two types: transitions if pyrimidine or purine bases are replaced by other pyrimidine or purine bases, respectively, and transversions if pyrimidine changes to purine or vice versa (20). All tested viruses show a higher level of transversions at p0, in minor and major variants alike (Figure 2.3B). Upon passaging, the spectrum of mutations observed in most hypermutators shifts towards a higher level of transitions. The WT and Y557S display the same distribution of mutations at both passages, with higher levels of transversions and overall higher genetic stability compared to all other Pol mutants.

Since HSV-1 features a large yet densely packed genome with about 80 to 200 ORFs (21), many of the mutations observed by us are located within coding regions. To test if the rapid accumulation of mutations in most Exo mutants could be responsible for the progressive growth deficit observed by us, we examined changes in protein coding genes more closely. At p0, silent and nonsilent mutations were fairly balanced in the minor variants, whereas all major variants were silent (Figure 2.4A). In contrast, results from pIII again clearly set our strong hypermutators apart from the WT and Y557S. Not only were mutations in coding regions in Exo I and Y577F mutants more abundant, they also were enriched for amino acid changes in minor and major variants alike, as similar levels of silent and nonsilent mutations could be detected. On the other hand, not only did the WT and Y557S show fewer mutations in coding regions in general, but minor and major variants also featured lower nonsilent-to-silent ratios. Of note, no amino acid-changing mutation became dominant by pIII in WT and Y557S viruses. Further analysis of amino acid-changing mutations by SIFT prediction (22) suggests that many of them have deleterious consequences, especially in major variants (Figure 2.4B).

Deleterious mutations in Exo I and Y577F mutant pIII populations occur in different proteins important for viral replication (Figure 2.4C). These include proteins involved in DNA metabolism (DNA polymerase UL30/42, 5'-3' exonuclease UL12, single strand binding protein UL29, etc.) (23, 24), structural proteins (glycoproteins UL10/27/US8, capsid proteins UL18/19/26/26.5) (25, 26) and others. Only two deleterious mutations occurred in Y557S minor variants by pIII, which very distinctly affect tegument proteins (UL36/47) (27). No deleterious mutations could be detected for WT or Y557S major variants.

In line with our hypothesis that random hypermutation caused by lack of HSV-1 Pol proofreading causes the elevated mutation frequencies observed for the mutants described here, we found

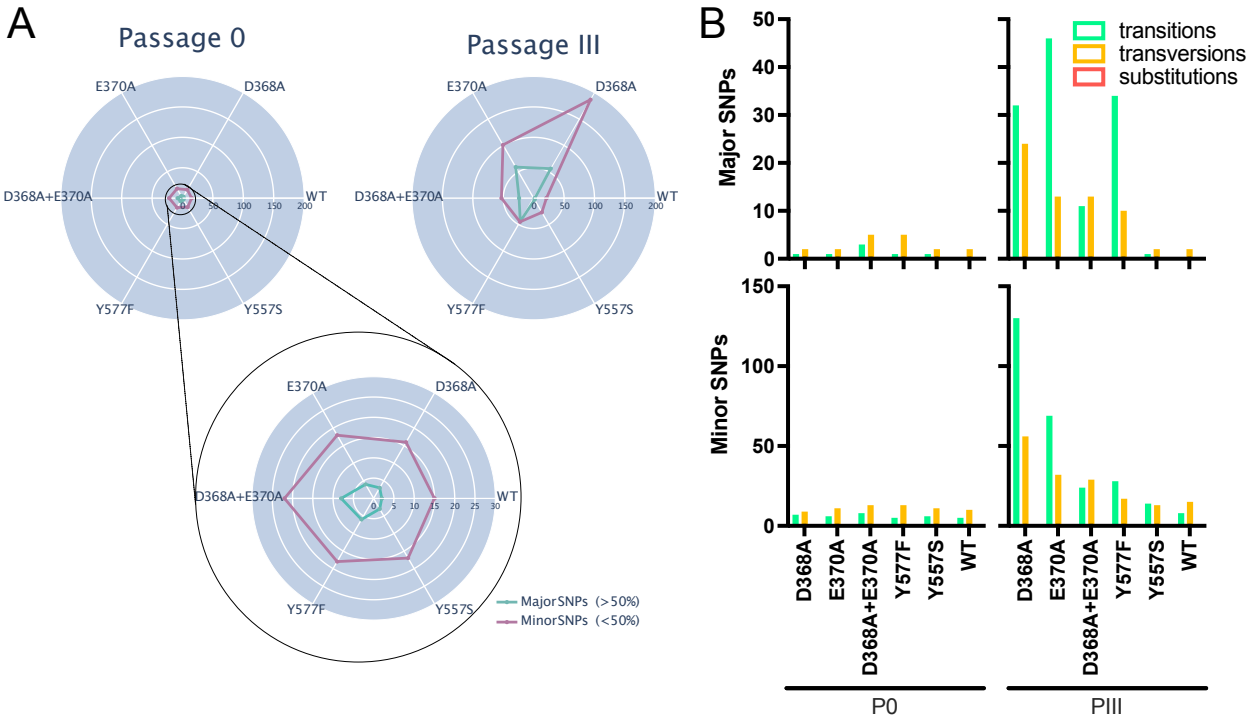

Figure 2.3: Exo I and Exo III Y577F mutants drastically increase mutations frequencies. (A) SNP counts broken down to major (allele frequencies above 50%) and minor (allele frequencies below 50%) for all viruses at p0 and pIII. SNPs were included if allele frequency was greater than 5%. (B) Major and minor SNPs of p0 and pIII viruses displayed according to the type of nucleotide exchange.

no apparent mutational hot spots in genomes analyzed up to pIII.

2.4.4 Exo III Mutant Y557S Maintains Stable Growth at Elevated Mutation Frequencies

Although we tried to maintain virus replication by passaging large amounts of infected cells and supernatants, Y577F and all Exo I mutants ceased to cause visible CPE beyond pIII in three replicate trials. On the other hand, Y557S showed stable growth but accumulated only slightly more mutations than the WT. We thus decided to further passage those two viable viruses to determine whether Y557S would accumulate a higher mutational load while still maintaining WT-like growth (Figure 2.5A). Indeed, Y557S grew just like the WT at passage X (pX) as determined by multistep growth kinetics (Figure 2.5B). On the other hand, plaques measured for pX at 2 days postinfection (dpi) showed significantly larger diameters produced by Y557S replicate 3 than by WT replicate 1, although all viruses produced increased plaque sizes over passaging time (Figure 2.5C). Apart from plaque sizes, plaque phenotypes also changed predominantly to syncytia in Y557S and partially in WT replicate 2 (Figure 2.5D).

Overall Y557S accumulated slightly more mutations than the WT (Figure 2.6A). Nucleotide change type distribution in the WT and the Y557S mutant were similar at pX, with transversions dominating over transitions in minor variants and transitions dominating transversions in major variants (Figure 2.6B). The WT and Y557S featured more mutations in protein coding regions at pX than at pIII, with elevated nonsilent-to-silent mutation ratios in major variants and more

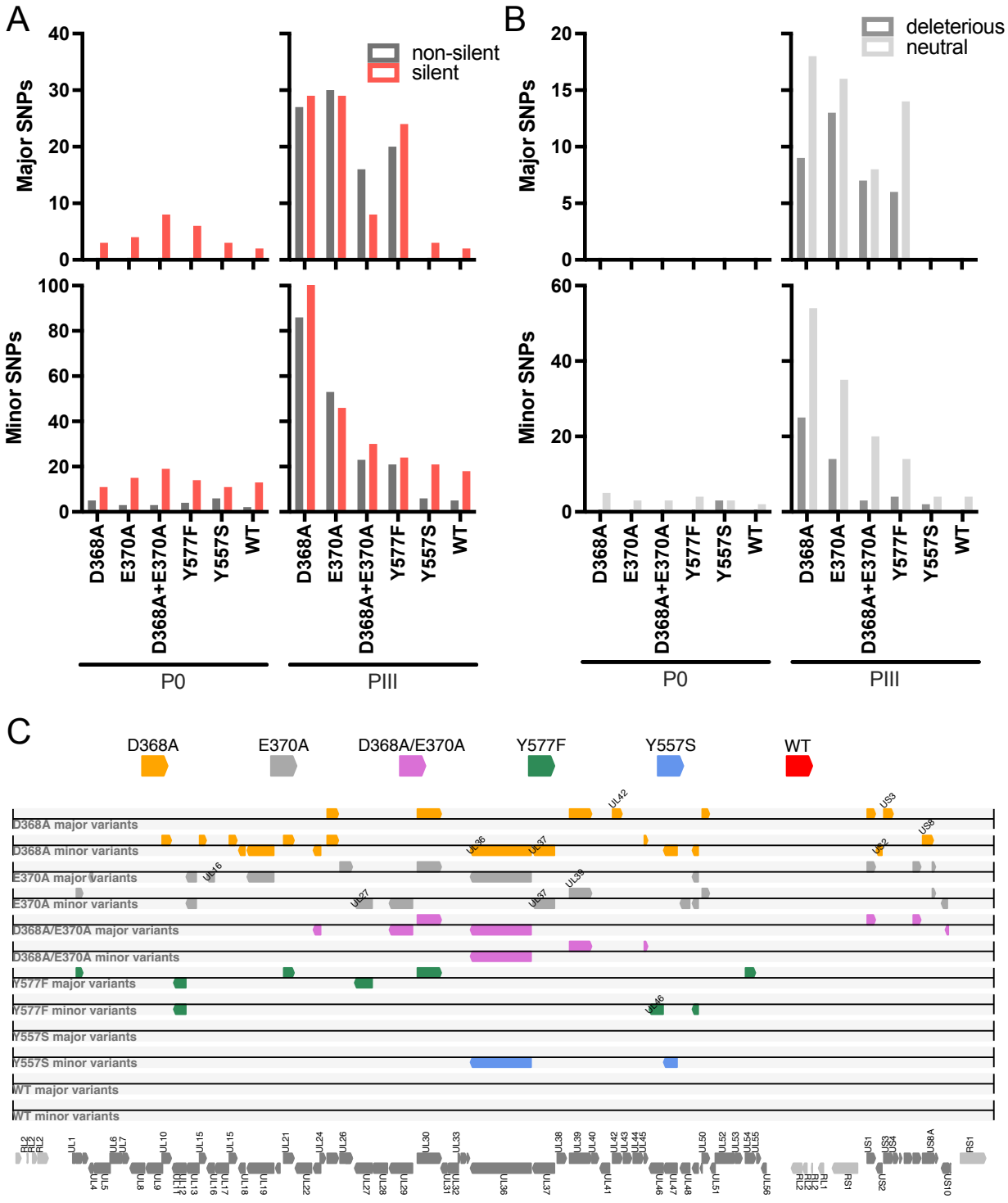

Figure 2.4: Hypermutators accumulate nonsilent, deleterious mutations in essential gene sets. (A) Major and minor SNPs in protein coding regions of p0 and pIII viruses grouped as silent or nonsilent according to their effects on an amino acid level. (B) Nonsilent mutations from A into deleterious and neutral mutations according to SIFT predictions. (C) Genes affected by deleterious major or minor mutations at pIII, respectively, are depicted. Genes labeled with their respective names feature more than one deleterious mutation.

silent changes in minor variants (Figure 2.6C). Most of those mutations were neutral (Figure 2.6D). Deleterious mutations observed at pX affected again very distinct groups of proteins like tegument (UL36/46/48), structural proteins (UL27/53 and US6/7/8) as well as proteins involved in DNA metabolism (UL12/23/30) (Figure 2.6E).

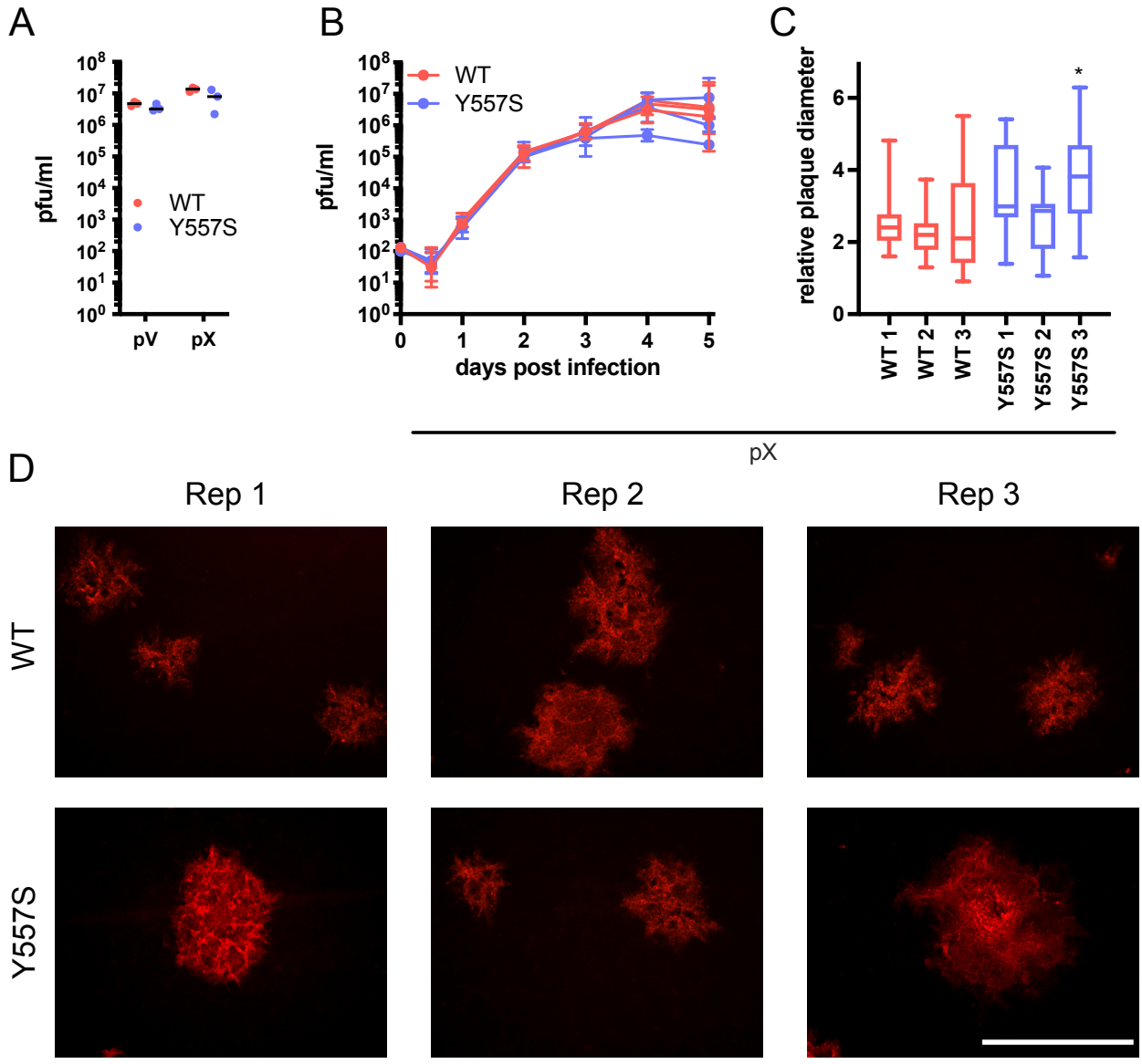

Figure 2.5: Y557S mutant replicates stably at later passages. (A) Viral endpoint titers for passaged WT and Y557S viruses. (B and C) Viral growth kinetics measured as multi-step growth curves (B) and plaque size (C). Significances were calculated by two-way ANOVA followed by Šidák's multiple-comparisons test for multistep growth curves and one-way ANOVA followed by Dunnett's multiple-comparison test for plaque size assays, respectively. An asterisk indicates significant ($P < 0.05$) differences in comparison to the WT for multistep growth curves and against WT replicate 1 for plaque size assays. (D) Plaque images taken at x100 magnification of indicated viruses and passages. Scale bar, 500 µm.

2.5 Discussion

Mutation rates are delicate to balance: while mutations are required for adaptation and prerequisite to evolution, they can interfere with molecular functionality and may cause unfavorable effects potentially culminating in lethal dysfunction. Similar to many other forms of cellular life, large DNA-viruses such as HSV-1 encode replicative DNA polymerases with 3'-5' exonuclease activity to increase replication fidelity and thus enable maintenance of large DNA genomes. While abrogation of exonuclease function was reported to be lethal for HSV-1 in the past (5), we report successful reconstitution of all Pol mutants generated for the purpose of this study. However, we did observe a severe, progressive growth deficit in all Exo I mutants as well as in Exo III

Y577F mutant. This growth defect was explicit in single-focus measures as well as in multistep growth kinetics and titers that could be achieved over passaging. Genome/FFU ratios were shifted considerably towards more genomes per infectious unit for all Pol mutants except Y557S. This indicates that, although DNA is efficiently replicated early after reconstitution, most of the progeny created by hypermutators are unfit to form infectious particles. Strikingly, Exo I and Y577F mutants could not maintain growth when usual transmission bottlenecks were applied. Passaging of these viruses required transfer of substantial amounts of infected cells in each passage, whereas WT and Y557S passed without difficulty through quite narrow bottlenecks. Despite our efforts, long-term survival of most Pol mutants was not achievable and pIII was typically the last point at which infectious foci could be observed. We suppose that the great speed at which mutations in Exo I and Y577F accumulate is the reason for the clearly suicidal phenotype we observe. Muller's ratchet is a concept that describes the accumulation of mutations in single genomes and the consequences that arise from an inability of clearing deleterious mutations (28, 29). Even when recombination between different co-infecting viruses is limited, recombination can act on progeny genomes produced from the original infecting viruses, presenting the prime mechanism that unlinks beneficial from deleterious mutations. If the mutation rates, however, increase to the extent that genomes produced in cells infected with a single virus are highly likely to feature at least one deleterious mutation, recombination between those genomes is less effective in purging these mutations and accumulation of deleterious mutations cannot be avoided. The most probable cause for the suicidal phenotype observed in Exo I and Y577F Pol mutants, is the number and location of deleterious mutations which, in case of these hypermutators, are highly enriched in viral populations by pIII. The gene sets featuring deleterious mutations are essential for productive viral replication and reproduction. No penetrant mutation in any of these important gene sets is found in WT or Y557S at pIII. Although some mutations within essential genes can be found in WT and Y557S at later passages, they remain far fewer compared to Exo I and Y577F mutants at pIII and they also are mainly featured in minor variants. Thus, this rapid and detrimental accumulation of mutations over few replication cycles could explain the suicidal phenotype of most Pol mutants described here. Based on their inability to perform proofreading, all our Exo I mutants should be uniformly depleted in their proofreading capability. Although all of these mutants present with similar phenotypes, the accumulation of mutations is not equal among them. Since some of the mutations we discover in Exo I mutants map to UL30, the gene encoding Pol, it is not unlikely that strong selection for higher replication fidelity yields viruses that repair their replication fidelity in Pol domains other than Exo. Such a possibility has been previously described and could affect the accumulation of mutations we observe by pIII (30). This could explain the milder hypermutation observed for the D368A/E370A mutant. By pIII, this particular mutant had gained 5 additional mutations within UL30 that could affect replication fidelity.

DNA mismatch repair proteins such as MSH2 and MLH1 are known to play important roles for efficient viral growth, MSH2 in general and MLH1 especially in primary humane foreskin fibroblasts (24, 31). ICP8 interacts with MSH6 and UL12 and MSH2 colocalizes with HSV-1 replication sites,

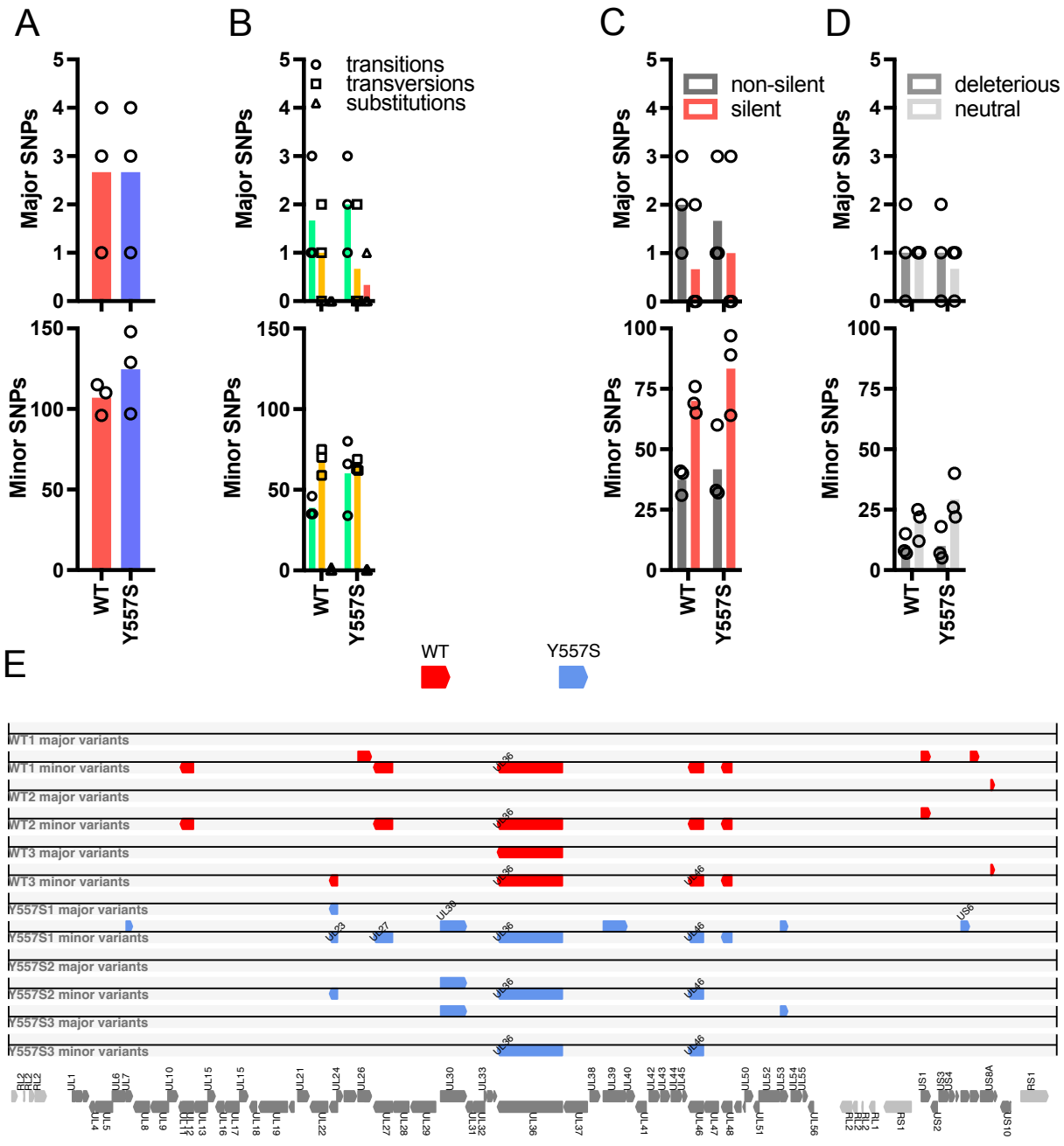

Figure 2.6: Stably replicating Y557S mutant accumulates more mutations than the WT. (A) SNP counts broken down to major and minor variants for WT and Y557S viruses at pX are shown. SNPs were included if allele frequency was greater than 5%. (B) Major and minor SNPs of pX WT and Y557S viruses displayed according to the type of nucleotide exchange. (C) Major and minor SNPs in protein coding regions of pX WT and Y557S viruses grouped according to whether they lead to amino acid changes (nonsilent) or not (silent). (D) Nonsilent mutations from C into deleterious and neutral mutations according to SIFT predictions. (E) Genes affected by deleterious major or minor mutations, respectively, are depicted. Genes labeled with their respective name contain more than one deleterious mutation.

while MLH1 seems to be important for immediate early gene expression (31, 32). However, the cellular DNA mismatch repair machinery has so far not been shown to impact the mutation profile of HSV-1. Evidently, DNA repair mechanisms are not sufficient to rescue proofreading-deficient mutants.

Many molecular evolution studies observe a bias in transition/transversion substitution rates towards transitions (20, 33). It has been controversially discussed whether this bias is due to

mutational bias (34-36) or selection bias (37-40). A mutational bias would suggest a bias toward transitions, because purine-to-pyrimidine changes and vice versa are sterically unfavorable and thus less likely to occur. A selection bias would presume that amino acid-changing mutations via transitions are more conservative than radical changes in the biochemical properties of replaced amino acids via transversions. Our study is not sufficiently powered to confirm or clearly reject any of these hypotheses. Despite that, it is tempting to speculate that the transition bias observed in our pIII sequencing dataset, especially if compared to p0, is due to a mutation bias. Even if selection acts to preserve the biochemical integrity of viral proteins, the large bottleneck passaging performed here would limit the effect of genetic drift and therefore most mutations are carried to the subsequent passages. Furthermore, for our pX WT and Y557S populations, transversions are more common in minor variants, suggesting that selection is favoring radical amino acid changes. Clearly, more work needs to be done to untangle transition/transversion biases in HSV-1 Pol mutants.

The WT and Y557S are not just stable in their growth kinetics, there is also some evidence for higher adaptability in the Y557S moderate hypermutator. This is indicated by larger plaque sizes and increased frequencies of syncytial plaque phenotypes, which have been shown to be selected for in Vero cell culture (41). Unfortunately, one Y557S replicate was harvested at a smaller population size which might explain why this replicate featured fewer mutations than the other two. Overall, 10 passages provide insuffcient evolutionary time to address differences in adaptability, so more research on this subject is necessary. We recently showed higher adaptability of MDV Pol Y547S mutant, the homolog of HSV-1 Y557S, which suggests that since Pol is highly conserved among alphaherpesviruses, the Y557S mutation can also confer evolutionary advantages (42).

Our results stand in contrast to previous reports on the lethal phenotype of Exo I mutants, specifically D358A (13). Although we cannot exclude that effects other than increased mutation rates are contributing to the observed unfit phenotype of most Pol mutants described here, the initial ability of these mutants to replicate their genome and form infectious progeny, in combination with the extremely fast accumulation of mutations observed by us, strongly suggest that hypermutation caused by a lack of proofreading activity is the reason for the observed disability of Pol mutant viruses to sustain replication. While caution is mandated when directly comparing results between our study and previously published literature, there is a number of reasons that could explain these differences. The rapidly suicidal phenotype of Exo I mutants renders detection of virus recovery difficult. In our case, the green fluorescent protein (GFP) reporter gene carried by our viruses facilitated early identification of infectious foci. Although visible CPE developed for all viruses within 48-72 hours following transfection, CPE typically disappeared as early as pI if not enough virus was transferred. In these cases, identification of infectious foci became impossible without fluorescence-based detection. Previously, the rescue of Pol mutants on Pol complementing cells was reported to be possible; however, transfer to non-complementing cells remained unsuccessful (13, 43). Although no description on the longer-term growth and stability of Pol mutants propagated on complementing cells is available, we assume that in such a case,

both the WT polymerase expressed by the complementing cell and the mutant polymerase expressed by the virus would replicate virus genomes. This would likely stabilize virus replication as abundant cellular expression of WT polymerase would enable high fidelity replication of viral genomes. At the same time, DNA replication by the mutant polymerase would cause hypermutation but likely with less drastic effects because alternative high-fidelity replication is available. Upon transfer of Pol mutant viruses propagated on complementing cells to non-complementing cells, these populations might already be weakened by previous hypermutation that interferes with their ability to replicate and cause CPE. Consequently, detection of these viruses might again be very difficult. Of course, many other factors, such as different virus strains, different methods employed in virus reconstitution, and different ways of assaying virus replication, could contribute to the difference in phenotypes observed for Pol mutants between our and previous studies. Of note, literature suggests that in case of human cytomegalovirus (HCMV), exonuclease-deficient mutants are selected by their ganciclovir-resistant phenotype (44). Although HCMV is a betaherpesvirus and thus not directly related to HSV-1, the existence of viable HCMV variants that carry mutations within highly conserved exonuclease domains of viral Pol does support the notion that such exonuclease-deficient herpesvirus mutants are viable in principle.

In conclusion, we show that HSV-1 Exo mutants are viable but exhibit suicidal phenotypes early after reconstitution. Furthermore, we provide evidence that this phenotype is caused by greatly increased accumulation of deleterious mutations in essential gene sets. Thus, the primary reason of the observed phenotype is drastically increased mutation rates due to a lack of exonucleolytic proofreading. Although the integrity of the exonuclease domains contained in Pol would in this case not be directly essential for the ability of HSV-1 to infect cells and form virus progeny, proofreading performed by the exonuclease is crucial to maintain genome integrity and thus is essential for maintaining virus replication in the long run. This would also make the exonuclease domain of the HSV-1 polymerase a possible target for future antiviral drug development. Lethal mutagenesis is an established concept in antiviral therapy (18) but is typically applied to RNA viruses that exist, in comparison to large DNA viruses, closer to a lethal error threshold (45-47). Since large DNA viruses employ their proofreading capabilities to replicate their genome with high fidelity (48, 49), lethal mutagenesis is difficult to achieve in the presence of proofreading. Therefore, inactivation of exonuclease domains might be key to antiviral treatments based on lethal mutagenesis for DNA viruses. In a recent paper, HSV-1 Pol and Exo are both described as potential targets for antiviral therapy, the rapid decline of Exo deficient populations observed by us would generally support such strategy (50). Despite the strongly suicidal phenotype of most Pol mutants described here, we could identify Exo III Y557S mutant as a stably replicating virus that replicated with slightly elevated mutation rates and potentially features higher adaptability than that of the WT. This stands in agreement with the substantial remaining exonuclease activity reported for this mutant (9) and suggests that Y557S is a prime candidate for experimental studies on major aspects of viral evolution, such as antiviral resistance, immune evasion, spillover, and others. Higher mutation rates of moderate hypermutators could allow accelerated evolution (42)

and would make studies on experimental evolution, a very time and labor-intensive field, much more approachable in the future.

2.6 Materials and Methods

2.6.1 Cells and Viruses

Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified eagle medium (DMEM; Pan Biotech) supplemented with 5 to 10% fetal calf serum (FCS), 100 IU/mL penicillin G (Carl Roth) and 100 µg/mL streptomycin (Carl Roth). All viruses used in this study are derived from the HSV-1 F strain pYEbac102 kindly provided by Y. Kawaguchi, University of Tokyo, Japan (51). For easier detection of cellular virus infection, an immediately early CMV promotor driven GFP was inserted into the Mini-F cassette of the virus construct.

2.6.2 Transfection

Vero cells were transfected using a standard polyethylenimine (PEI; Polysciences) transfection procedure (52). For transfection, 10 µL PEI (1 mg ml^{-1}) were diluted in 50 µL Opti-MEM (Thermo Fisher Scientific) and mixed with bacterial artificial chromosome (BAC) DNA (5 µg of BAC DNA in 50 µL of Opti-MEM). Following 25 min of incubation at room temperature, the DNA-PEI complex was added to a 90% confluent Vero cell monolayer, and one reaction mixture as described above was used per well of a 6-well plate. For transfection of 10-cm plates, an upscaled reaction (8-fold) was used. Cell culture medium was changed 4 h posttransfection.

2.6.3 Infection of Cells and Propagation of Virus

Virus was propagated by transferring infected cell lysate (obtained by freezing and thawing of infected cells) to a confluent monolayer of uninfected Vero cells. When the CPE became apparent 2 to 3 days postinfection, virus was transferred to fresh cells as described above. To maintain growth of mutants D368A, E370A, D368A/E370A and Y577F, in each passage, one third of the cells in an infected dish had to be transferred to a new dish without addition of fresh cells. Cells were suspended using trypsin/EDTA (0.25% trypsin [Sigma-Aldrich], 0.5 mM EDTA [AppliChem] in phosphate bufferd saline [PBS]). In case of Y557S and the WT, transfer of a small amount of supernatant (0.25% of the total volume) obtained from infected cell cultures was sufficient to maintain virus growth.

To monitor virus growth over passaging, HSV-1 was transfected in 6-well plates and supernatants were collected before passaging at 72 h posttransfection for passage 0 (p0) and at 48 h postinfection for subsequent passages I to V (pI to pV). From p0 to pI, all viruses were passaged by transferring one-third of the infected cells to a new dish without addition of fresh cells. From pI

to pV, viruses were passaged on 10-cm dishes as described above. Prior to passaging, 1 mL of supernatant was collected for each virus and frozen at -80℃ for at least 24 h. DNA was isolated from these cell-free virus stocks and used for qPCR as described below. The experiment was replicated three times, starting from independent transfections each time.

2.6.4 Virus Titration and Focus/Plaque Size Assays

Since most Pol mutants fail to cause readily visible CPE by the early passages after reconstitution in Vero cell culture, we determined infections virus particles as FFU either identified by the GFP carried in the BAC construct or by immunofluorescent staining (GFP is lost after 5-10 passages). Virus stocks were titrated by plating 10-fold serial dilutions on subconfluent Vero cells in 6-well dishes. Infectious foci were determined by counting fluorescent foci using a Zeiss Axio Vert.A1 inverted fluorescent microscope. Cells were transfected as described above or infected with 200 FFU per well using a 6-well plate. For p0, pictures were taken 72 h posttransfection, and for subsequent passages, pictures were taken 48 h postinfection using a Zeiss Axio Vert.A1 inverted fluorescent microscope. For the WT and Y557S mutant, cell culture medium was replaced by a semisolid overlay of methylcellulose (0.75% methylcellulose in 1x DMEM supplemented with 5% FCS, 100 IU/ml penicillin G, 100 µg streptomycin and 0.075% $NaHCO_3$) 1 h postinfection. For all other mutants, settling of transferred cells was allowed to occur before replacement of the medium 24 h postinfection. Focus size areas were measured with the NIH Image J 1.52n software and converted to focus diameters relative to the WT of the respective passage (for early p0 and pIII viral populations) or to the WT p0 (for later pX). As the GFP marker was readily lost after pX, plaques were stained using a cross-reactive simplex virus gB monoclonal antibody (C2D8) (53).

2.6.5 Cryopreservation of Viruses

Cell-free virus was collected by directly freezing infected cell cultures at -80℃. To maximize virus titers, infected cell lysates were generated by freezing plates at -80℃ followed by thawing to release virus particles. Then, both supernatant and cells were used to create viral stocks, which were transferred immediately to -80℃ and stored until further use.

2.6.6 DNA Extractions for qPCR and Transfection

Viral DNA was extracted, using the RTP DNA/RNA virus minikit (Stratec Molecular), from supernatant or from infected cell lysates (after freezing and thawing). Supernatants were cleared by centrifugation (1,000x g, 5 min), and cell lysates were used entirely. DNA was extracted according to the manufacturer's instructions, with 400 µL of sample and the addition of 5 µL proteinase K (20 mg/mL) into the extraction tube. For the purpose of transfection (virus reconstitution), viral

BAC DNA from Escherichia coli cultures was isolated using Qiagen's column-based midiprep kit according to the manufacturer's instructions.

2.6.7 Isolation of Viral DNA for Next Generation Sequencing (NGS)

Foci were picked by positioning a cell culture plate under a Zeiss Axio Vert.A1 inverted fluorescence microscope at x50 magnification. A p200 pipet was used to pick infected cells and some supernatant in a volume of 50 µL. A total of 30 foci for each virus were collected. Viral DNA was extracted using the RTP DNA/RNA virus minikit (Stratec Molecular).

For pX of WT and Y557S, viral DNA was isolated by micrococcal nuclease DNA extraction (54). In brief, completely infected confluent 10-cm plates were harvested. Cell pellets were permeabilized twice in 5 mL permeabilization buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5], 1% Triton X-100) by resuspension and centrifugation at 1,300x g and 4℃. Pelleted nuclei were resuspended in 50 µL nuclei buffer (10 mM Tris-HCl [pH 7.5], 2 mM $MgCl_2$, 10% sucrose) and mixed with equal amounts of 2x nuclease buffer (40 mM PIPES [piperazine-N,N"-bis(2-ethanesulfonic acid)], 7% sucrose, 20 mM NaCl, 2% $CaCl_2$, 10 mM 2-mercaptoethanol, 200 µM phenylmethylsulfonyl fluoride [PMSF]) supplemented with 1 µL of RNAse A (10 mg/mL) and 1.5 µl micrococcal nuclease (2,000 gel units/µL, NEB). After incubation at 37℃ for 90 min, the reaction was stopped by adding 2.4 µL of 0.5 M EDTA. Protein digestion was performed by adding 400 µL of digestion buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 25 mM EDTA, 0.5% SDS) supplemented with 2.5 µL of proteinase K (20 mg/mL) at 50℃ for 18 h. After three rounds of phenol-chloroform extraction, DNA was precipitated by adding 800 µL ethanol and 200 µl 7.5 M ammonium acetate to 400 µL of sample. After pelleting at 20,000x g and 4℃ for 20 min pellets were washed with 70% ethanol and pelleted as described before. Air-dried pellets were dissolved in 70 µL of 10 mM Tris-HCl (pH 7.5).

2.6.8 Viral Growth Kinetics

For growth kinetics of pII viruses, approximately $1x10^6$ Vero cells were infected with 200 FFU virus in triplicate using 6-well plates. For each time point, three replicate wells were harvested by freezing at -80℃ every 12 h for 3 days. Viral DNA was isolated from 400 µL sample (including supernatant and cells) and used for qPCR in duplicate reactions. For growth kinetics of pX viruses, Vero cells were infected at a multiplicity of infection (MOI) of 0.001 in 6 well plates in triplicates. Each time point and replicate consisted of one separate well. The respective wells were frozen to stop the infection at the indicated time points and thawed to create stocks, which were titrated by 10-fold serial dilutions. The medium was changed to a methylcellulose overlay 1 h postinfection and incubated at 37℃ and 5% CO_2 until plaques formed. The plates were washed twice with PBS, fixed with 0.4% formaldehyde for 20 min and stained with 0.75% crystal violet. Titers were calculated per milliliter of stock.

2.6.9 BAC Mutagenesis

HSV-1 mutants were generated by en passant mutagenesis, a two-step Red-mediated recombination protocol (19). PCR products containing recombination-specific sites, the desired point mutation, an I-SceI recognition site and a kanamycin resistance gene (*aphAI*) as a selection marker were generated and used to transform electrocompetent and recombination-competent *E. coli* carrying the HSV-1 F strain BAC. Arabinose-inducted I-SceI expression initiated a double-strand break in the DNA, followed by homologous recombination to remove *aphAI*. Kanamycin-susceptible clones were selected and confirmed by restriction fragment length polymorphism analysis and Sanger sequencing of the relevant loci (19). (See Table S1 in the supplemental material for a list of all primers used for BAC mutagenesis). The UL30-Kan mutant used in Figure 2.2E and F is the UL30 D368A *aphAI*-containing intermediate prior to resolution of *aphAI* cointegration

2.6.10 PCR

PCRs were performed for Sanger sequencing, mutagenesis (high fidelity DNA polymerases such as S7 Fusion [Mpbidiag] or PrimeStar® [TaKaRa Bio]), and to determine presence and orientation of specific sequences (using DreamTaq [Thermo Scientific] polymerase). PCRs were carried out in a total volume of 50 µL. All polymerases and reaction buffers were used according to the manufacturer's instructions. The NEB Tm Calculator (http://tmcalculator.neb.com) was used to calculate an annealing temperature that was suitable for both the polymerase and the respective primers (Table S1).

qPCR reactions were performed to quantify the amount of viral DNA in virus stocks using a TaqMan protocol. For the detection of viral DNA, primers and probe were designed to match a conserved region in *UL30*, the gene encoding the viral DNA polymerase. Standard curves were generated using HSV-1 BAC DNA of known concentration. PCRs were carried out in 20 µL with a 100 nM concentration of each primer and probe and 2 µL of sample DNA. Duplicate reactions were performed on an ABI 7500 fast real-time PCR machine (Thermo Fischer) using the SensiFast master mix (Bioline, Luckenwalde, Germany) with the following conditions: initial denaturation at 95℃ for 180 s and then 35 cycles of denaturation for 15 s at 95 ℃, followed by primer annealing for 15 s at 55℃ and elongation for 30 s at 68℃. Normalized gene copy numbers per mililiter of supernatant were calculated and provided for analysis.

2.6.11 NGS and Target Enrichment

Samples from every mutant at p0 and pIII (as well as pX for the Y557S mutant and the WT) underwent whole-genome DNA sequencing using the Illumina MiSeq platform. To this end, sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep kit (NEB). DNA fragmenta-

tion was performed by using a Covaris M220 focused-ultrasonicator on 1 to 5 µg of extracted DNA that was diluted up to 130 µL 0.1x Tris-EDTA (TE) buffer. The size selection step of the NEBNext Ultra II DNA library prep protocol was performed for selection of fragments between 500 and 700 bp. Sequencing libraries for p0 and pIII samples were then enriched for HSV-1 genomic DNA using the myBaits hybridization capture for targeted NGS kit (Arbor Biosciences) with a custom designed set of RNA probes to target the whole HSV-1 F-Strain genome (Genbank accession no GU734771.1). The hybridization reaction was maintained for 20 h; enriched libraries were then recovered "off-bead" per the manufacturer's instructions, and the final PCR cleanup was performed using SPRI beads (AMPure XP Beads; Beckman Coulter) at a ratio of 0.9. Final enriched libraries were pooled and loaded into the MiSeq instrument as per Illumina's instructions.

The resultant Illumina sequencing data were processed with Trimmomatic v.0.39 (55) and mapped against an HSV-1 strain F-based reference (GU734771.1) using the Burrows-Wheeler aligner v.0.7.17 (56). Since the sequence of the BAC-derived HSV-1 F strain virus differs slightly from the RefSeq entry (GU734771.1), an updated version of the reference was manually generated and deposited at Github (https://github.com/mmnascimento/HSV1polmutants). Mapping statistics were generated using Samtools v1.10 (57) and alignments were visualized using IGV v2.9.4 for Linux (58). For detection of single-nucleotide polymorphisms (SNPs), Freebayes, a Bayesian genetic variant detector (5), was used. All SNPs with a minimum mapping quality of 5, minimum count of 3 and minimum fraction of 0.01 were considered. Consensus sequences for each sample were obtained using BCFtools (57). All SNP-containing ORF sequences were extracted from these consensus genomes and translated using a custom-made script also deposited in the Github repository for this work (https://github.com/mmnascimento/HSV1polmutants).

2.6.12 Data Availability

Generated raw sequencing data were deposited with the Sequencing Read Archive and can be found under BioProject accession number PRJNA864599.

2.7 Acknowledgments

We thank Nikolaus Osterrieder and Dusan Kunec for providing materials and reagents as well as productive discussions. We further thank Annett Neubert, Ann Reum and Michaela Zeitlow for expert technical assistance as well as Kerstin Borchers for anti-herpes simplex virus antibody.

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2.8 Supplemental Material

Supplemental tables can be found at https://github.com/hoeflet/ThesisSupplemental.

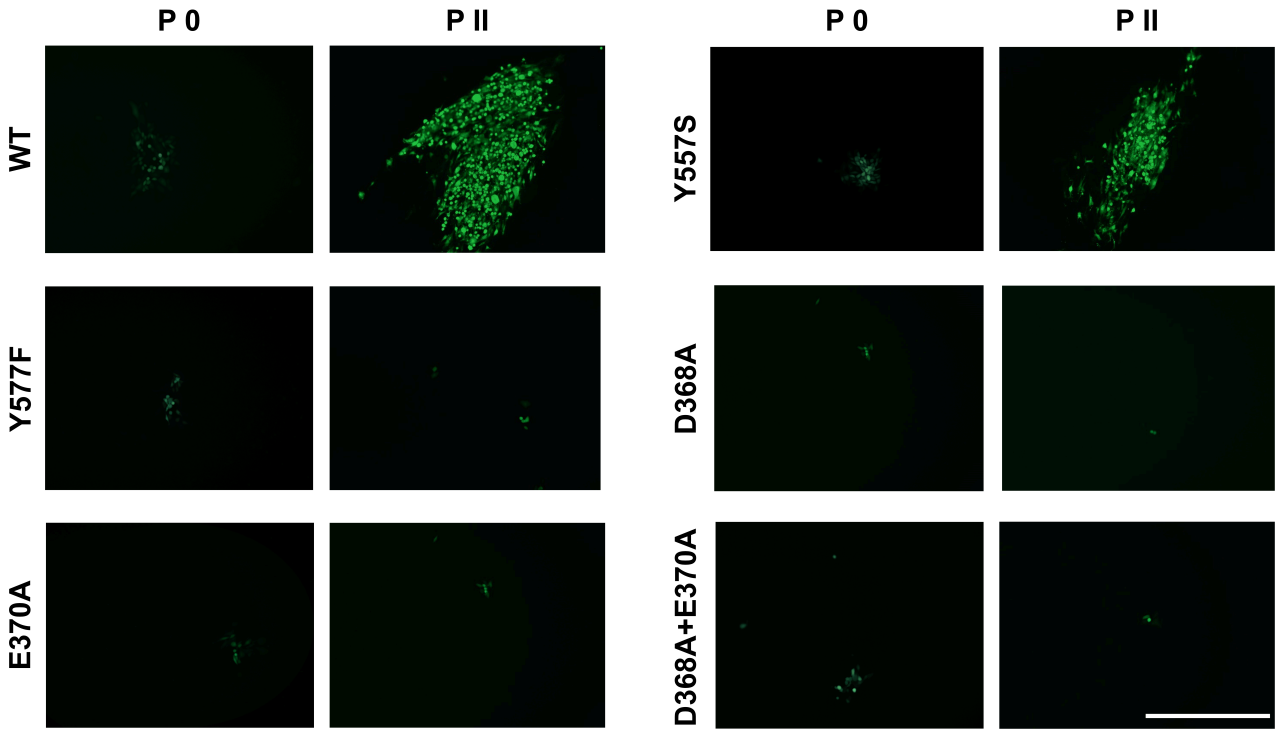

Figure 2S1: Focus pictures of Exo mutants at p0 and II. Pictures taken at a Zeiss Axio Vert.A1 inverted fluorescent microscope 48 h post infection. Scale bar marks 500 µm.

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Chapter 3

Evolutionary Dynamics of Accelerated Antiviral Resistance Development in Hypermutator Herpesvirus

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3.1 Graphical Abstract

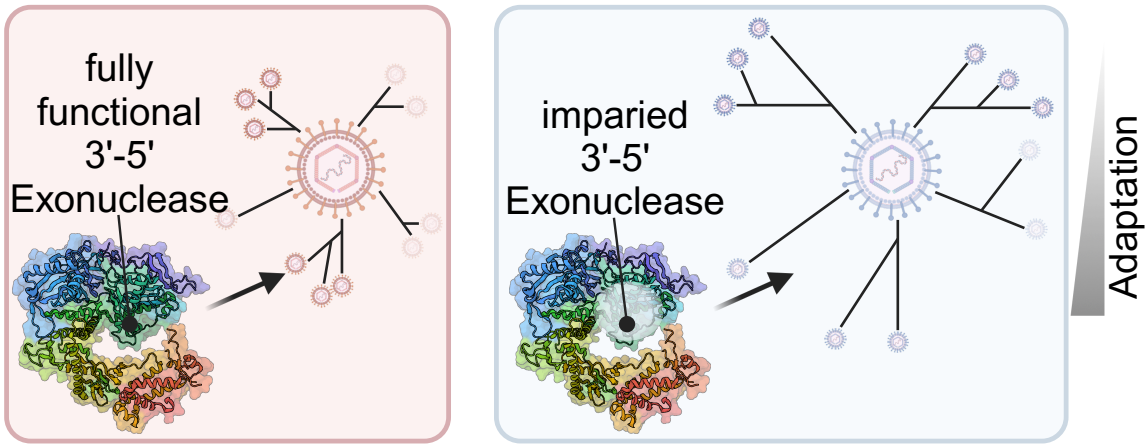

Figure G.A.

3.2 Abstract

Antiviral therapy is constantly challenged by the emergence of resistant pathogens. At the same time, experimental approaches to understand and predict resistance are limited by long periods required for evolutionary processes. Here, we present a herpes simplex virus 1 (HSV-1) mutant with impaired proofreading capacity and consequently elevated mutation rates. Comparing this hypermutator to parental wild type virus, we study the evolution of antiviral drug resistance *in vitro*. We model resistance development and elucidate underlying genetic changes against three antiviral substances. Our analyses reveal no principle difference in the evolutionary behavior of both viruses, adaptive processes are overall similar, however significantly accelerated for the hypermutator. We conclude that hypermutator viruses are useful for modelling adaptation to antiviral therapy. They offer the benefit of expedited adaptation without introducing apparent bias and can therefore serve as an accelerator to predict natural evolution.

3.3 Introduction

Development and clinical use of antimicrobial agents are among the greatest achievements in medical history. However, the evolution of many pathogens is unbowed and antimicrobial resistance is developed just as fast as new antimicrobials are (Christaki, et al. 2020; Larsson and Flach 2022). Large population sizes combined with relatively high mutation rates and intense genetic exchange enable microorganisms to adapt to environmental conditions including selective pressure presented by antimicrobial therapy and vaccination (Arber 1991). Understanding the evolution of, and the mechanisms underlying resistance is therefore critical for sustainable and effective usage of existing, as well as development of novel antimicrobials.

Infections with the human pathogen herpes simplex virus 1 (HSV-1), member of the *Herpesviridae* family of large double stranded DNA viruses (Gatherer, et al. 2021), are ubiquitous (WHO 2023). Although infection is often asymptomatic or causes only mild to moderate symptoms, severe disease can result from HSV-1 infection and antiviral therapy is frequently applied (Kukhanova, et al. 2014; Whitley and Baines 2018). All clinically used antivirals are polymerase inhibitors, most of them nucleoside analogs such as aciclovir (ACV) and its derivatives (Gnann, et al. 1983; Wagstaff, et al. 1994; Klysik, et al. 2020). Other anti-herpetic drugs include ganciclovir (GCV) which is a nucleoside analog frequently used to treat human cytomegalovirus (HCMV) infections (Andrei, et al. 2009) and the second line drug foscarnet (FOS), a pyrophosphate mimetic (Faulds and Heel 1990; Chrisp and Clissold 1991). While ACV and GCV are prodrugs and need to be activated through phosphorylation by viral and cellular kinases to inhibit viral nucleic acid synthesis (Littler, et al. 1992; Andrei, et al. 2009), FOS is administered in its active form (Zeichner 1998). Resistance to antiviral therapy is frequently described in the context of HSV infections and ACV as well as GCV resistance is often mediated by changes in the enzymes essential for prodrug activation (thymidine kinase encoded by *UL23* in HSV-1 and the protein kinase encoded

by *UL97* in HCMV) or in the DNA-polymerase (*UL30* and *UL54* in HSV-1 and HCMV respectively) (Ruiz-Carrascoso, et al. 2013; Piret and Boivin 2016). As FOS does not depend on viral genes for activation, resistance is mostly conferred by polymerase mutations (Piret and Boivin 2016).

Experimental work has elucidated many aspects of microbial evolution (Conrad, et al. 2011; Kawecki, et al. 2012; Dragosits, et al. 2013; Shapiro and Turner 2018; Maeda, et al. 2020; Fixsen, et al. 2022). However, modelling virus evolution remains time and labor intensive. As mutations are required for populations to diversify and for selection to act upon, increasing mutation rates can lead to experimental systems for expedited evolution (Jayaraman 2011). Several factors are known to influence viral mutation rates, among them antiviral host factors, DNA-damage responses, cellular translesion polymerases, tertiary ssRNA structure, “genomic accordions” or replication mode (Sardanyes and Elena 2011; Elde, et al. 2012; Luftig 2014; Geller, et al. 2015; Harris and Dudley 2015; Sanjuan and Domingo-Calap 2016; Zeltzer, et al. 2022). However, high fidelity genome replication conferred by replicative viral DNA polymerases (Pol) is likely of greatest importance. Large dsDNA viruses such as HSV-1 encode a family B DNA-Pol with intrinsic 3’-5’ exonuclease (Exo) activity to allow proofreading of newly synthesized DNA and thereby increase replication fidelity (Trimpert and Osterrieder 2019).

Our work with Exo depleted Pol mutants in Marek’s disease virus (MDV, GaAHV-2) shows that these are initially viable but are prone to extinction within few cell culture passages. These mutants could only be cultured by establishing large and highly diverse populations in which replication fidelity was presumably restored partially or deleterious gene variants complemented in trans (Trimpert, et al. 2019). We however also identified a mutation within Exo domain III (Y547S), which had only mild effects on Exo activity (Trimpert, et al. 2019). This mutant did not only replicate stably over the course of many *in vitro* passages but showed elevated mutation frequencies enabling better adaptation to non-permissive cell lines, which also resulted in stronger attenuation *in vivo* (Xing, Höfler, et al. 2022). Recently, we observed a similar behavior for HSV-1 Pol mutants, with strongly suicidal phenotypes in all tested Exo mutants, except Y557S (YS, homologous to MDV Exo III mutant Y547S) which replicated with wt-like kinetics, while showing a tendency for increased mutation rates (Brunialti, et al. 2023). Here, we used this mutant to investigate evolution of resistance to three anti-herpetic drugs under conditions of mild hypermutation. We observed faster and stronger adaptation in YS compared to wt populations despite similar evolutionary patterns, suggesting on overall similar but accelerated evolution of the hypermutator compared to parental wt. This demonstrates the power of using hypermutator viruses to study virus evolution.

3.4 Results

3.4.1 Baseline Values of Growth and Resistance for wt and YS Genotypes

We first established baseline values for viral growth and drug resistance to identify any constitutive differences between our wt and Pol^{Y557S} (YS) HSV-1 viruses, in BAC derived clonal populations

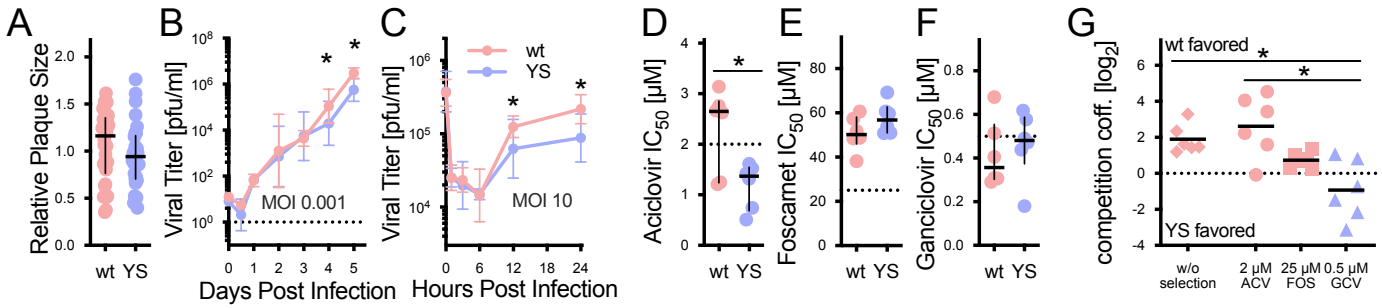

Figure 3.1: Differences in growth and resistance between wt and YS. A) Plaque size assay for wt and YS on Vero cells. To wt normalized plaque diameters of 30 plaques as well as median and interquartile range are displayed. No significant differences are observed (unpaired t-test, $p>0.05$). Growth kinetics for wt and YS with a starting MOI of 0.001 (multi-step, B) and MOI of 10 (single-step, C) on Vero cells. Curves present geometric mean and 95% confidence interval of 3 replicates per viral stock. * indicates significant difference ($p<0.05$) between wt and YS at the given timepoint determined by 2-way ANOVA followed by Šidák's multiple comparisons test. IC_{50} values calculated from plaque reduction assays for D) aciclovir, E) foscarnet and F) ganciclovir. Individual datapoints from 6 independent biological replicates as well as median and interquartile range are displayed, dotted lines indicate chosen selection levels. * indicates significant difference ($p<0.05$) between wt and YS determined by unpaired t-test. G) Competition assays between mCherry labled wt and GFP labled YS viruses under non-selective and selective conditions. Log_2 transformed competition coefficients (number of wt plaques/number of YS plaques) are shown for 6 independent competitions. * indicates significant differences ($p<0.05$) determined by 1-way ANOVA followed by Tukey's multiple comparisons test.

(Brunialti, et al. 2023). Plaque sizes 2 days post infection (dpi) for wt and YS are similar on Vero cells (Figure 3.1A) and growth only differs at late time points in multi- and single-step growth curves (Figure 3.1BC). Resistance against aciclovir (ACV) is about twice as high in wt compared to YS (Figure 3.1D), whereas no significant differences were observed for foscarnet (FOS; Figure 3.1E) or ganciclovir (GCV; Figure 3.1F). Importantly, those IC_{50} values are below common molarity thresholds for resistance (3 and 330 µM for ACV and FOS respectively), indicating that our initial stocks can be considered drug sensitive (Schmidt, et al. 2015). Based on our baseline resistance measurement, we decided to select at concentrations of 2 µM, 25 µM and 0.5 µM for ACV, FOS and GCV respectively.

Under non-selective conditions, wt was favored over YS by about 2 log_2 units in competition assays with differentially labeled viruses (Figure 3.1G). ACV and FOS selection at above-mentioned concentrations did not interfere with this observation. In contrast, GCV selection favored YS over wt by about 1 log_2 unit. Since we did not use mixed populations for *in vitro* evolution and wt as well as YS GCV resistance was similar for both viruses, the higher selection value for YS over wt under GCV conditions was not expected to be problematic.

Following the observation of only minor differences between our two viruses, we went on to passage wt and YS separately under ACV, FOS, GCV and non-selective (H_2O) conditions in triplicate populations for 30 passages in Vero cell culture. Relatively narrow bottlenecks (about 10^{-4} of the endpoint population of each passage, see material/methods section for more details) were applied, attempting to mimic natural transmission cycles of HSV-1 (Rathbun and Szpara 2021; Rathbun, et al. 2022). This passaging strategy applies tighter bottlenecks than similar *in vitro* evolution studies in HSV-1 and 2 do, which increases the amount of genetic drift and might influence evolutionary rates (Kuny, et al. 2020; López-Muñoz, et al. 2021). Viral stocks were collected, titrated, sequenced and tested for antiviral resistance every 5 passages (Figure 3.2A). No significant differences in end-point titers were observed (Figure 3.2B), indicating that

comparable population sizes were maintained and the selective force was similar for all replicates.

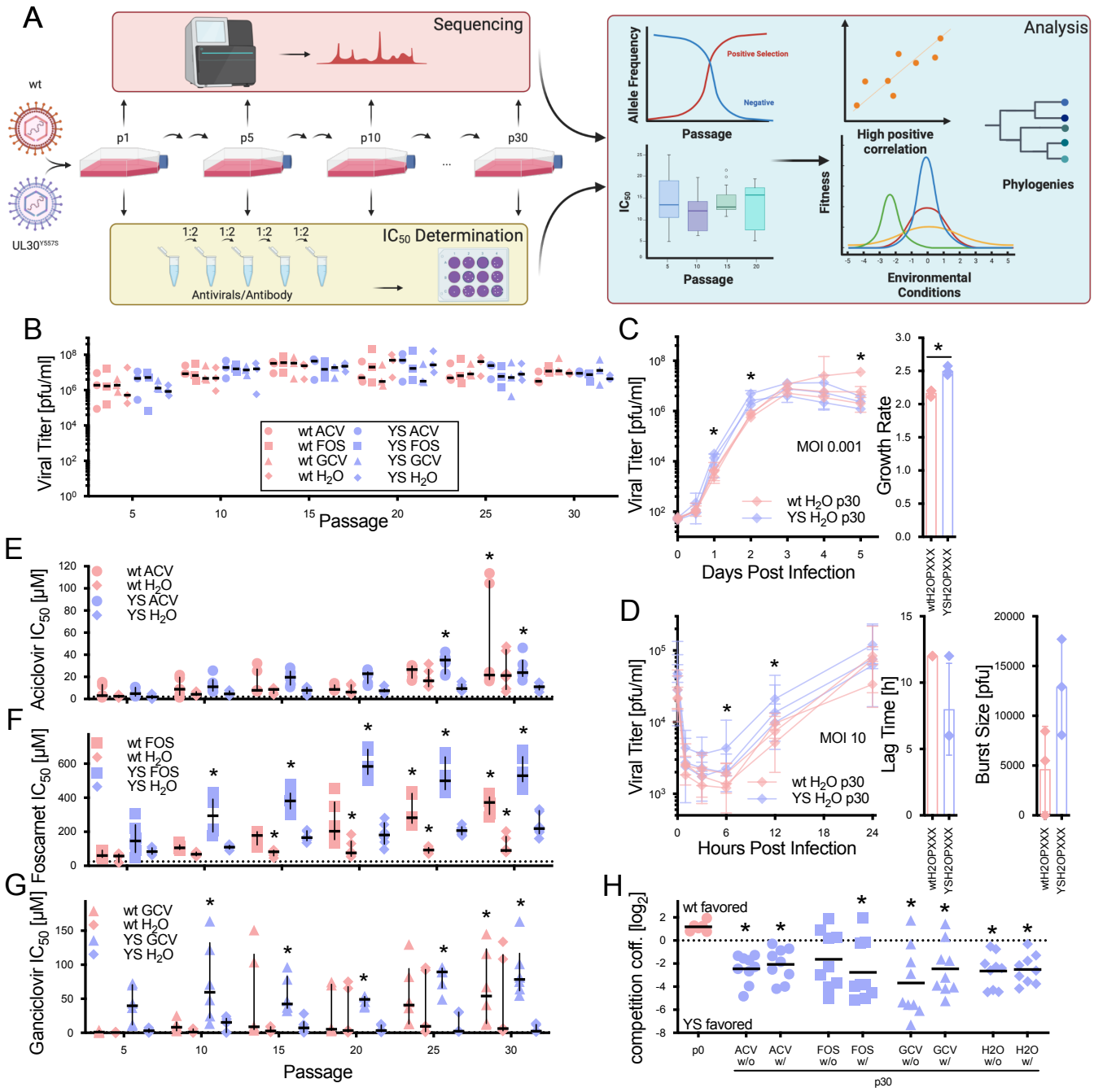

Figure 3.2: Faster adaptation to cell culture and antiviral conditions in YS populations. A) Experimental setup for *in vitro* evolution. Viral stocks were collected every 5 passages and subjected to genotypic and phenotypic evaluation. B) Endpoint titers for viral stocks measured by plaque assay. No significant differences could be detected using 1-way ANOVA followed by Tukey's multiple comparisons test. Growth curves for wt and YS H_2O p30 populations infected C) at MOI 0.001 (multi-step) and D) at MOI 10 (single-step). Curves present geometric mean and 95% confidence interval of 3 technical replicates per replicate virus population. * indicates significant difference ($p<0.05$) between wt and YS at the given timepoint determined by 2-way ANOVA followed by Šidák's multiple comparisons test. Differences in the growth rate (multi-step; C) as well as lag time and burst size (single-step; D) are shown on the right. * indicates significant difference ($p<0.05$) between wt and YS measured by unpaired t-test. Resistance measured by plaque reduction assays against E) aciclovir, F) foscarnet and G) ganciclovir over the course of passaging. Single IC_{50} values of 2 independent measurements per replicate as well as median and interquartile range are shown. Dotted lines show respective levels of drug selection * indicates significant differences ($p<0.05$) against YS H_2O at the given passage measured by 2-way ANOVA followed by Dunnett's multiple comparisons test. H) Competition assays between indicated wt and YS populations under selective and non-selective conditions on Vero cells. Log_2 transformed competition coefficient (number of wt genomes/number of YS genomes) is shown for the 9 respective competitions measured in duplicates via qPCR. * indicates significant differences ($p<0.05$) against p0 competition as determined by 1-way ANOVA followed by Dunnett's multiple comparisons test.

3.4.2 Increased Adaptability of YS Populations

Replicative fitness is among the most important traits of viral populations (Domingo and Holland 1997; Wargo and Kurath 2012). Therefore, viruses are rapidly selected for efficient growth, as individual genomes compete with each other for available resources. To monitor growth in Vero culture, we performed growth curves for our H_2O passage 30 (p30) samples. In multi- and single-step growth kinetics, YS H_2O outgrew wt H_2O populations early on (1 and 2 dpi as well as 6 and 12 hpi) but plateaued earlier when compared to wt (Figure 3.2C and D respectively). This observation is supported by increased growth rates in multi-step kinetics and higher burst size as well as shorter lag time in single-step growth curves. Since the main focus of this study was to observe antiviral resistance evolution under mild hypermutation conditions, we monitored IC_{50} values against all antivirals used for selection. Resistance against the respective antiviral treatment (defined by significant increases compared to H_2O controls) developed faster for all YS populations. Although YS was initially more susceptible to ACV, resistance developed faster by 5 passages (p25 for YS ACV compared to p30 for wt ACV; Figure 3.2E). Interestingly, after 30 passages, one out of three wt populations not specifically selected for (H_2O control) developed some resistance against ACV. For this reason, differences were assessed against YS H_2O in this experiment (Figure 3.2EFG). Remarkably, evolution of resistance against FOS and GCV was even more accelerated, with respective YS populations becoming resistant within 10 passages (Figure 3.2F and G respectively), whereas wt populations reached FOS and GCV resistance by p25 and p30 respectively. Notably, YS H_2O also increased FOS resistance to levels above wt H_2O.

We also observed substantial development of cross-resistance (Figure 3S1). Especially wt populations selected on FOS displayed high ACV resistance, but also wt GCV, wt ACVGCV (double selection at lower concentrations) and YS ACVGCV populations established resistance similar to ACV populations by p30. Double selections of ACVFOS and ACVGCV also increased FOS resistance at different timepoints in YS populations. However, GCV treatment (especially in wt populations) increased susceptibility to FOS. Cross resistance against GCV occurred exclusively in YS populations selected on ACV or ACVGCV.

Since both a growth advantage and antiviral resistance developed faster in YS populations, we wanted to directly compare the fitness of wt and YS populations evolved under the same conditions using competition assays. Initial advantages for wt populations completely disappeared after 30 passages of *in vitro* evolution. YS was now favored over wt under all conditions (Figure 3.2H), with the exception of FOS in which YS was only favored upon FOS selection.

3.4.3 Higher Mutation Frequencies and More Rapid Protein Evolution in YS Populations

To understand the reasons behind the observed faster adaptation of YS to cell culture and antiviral treatment, we sequenced DNA obtained from our viral populations. Over passaging time, YS accumulated more single nucleotide polymorphisms (SNPs) than wt populations (Figure 3.3A).

To demonstrate differences in mutation load on a population level, we pooled SNP counts from all selections applied. Indeed, YS in general accumulated about 50% more SNPs at p5 and even 2-times more at p30 (Figure 3.3B). Similarly, about 2-times more substitutions were counted in plaque purified clonal YS compared to clonal wt viruses at p30 (Figure 3.3C). In addition to assessing the overall mutational load, we calculated non-synonymous to synonymous (dN/dS) substitution rates, an often-used metric to quantify rapid protein evolution (Yang and Bielawski 2000). Doing this for p5 and 30 samples, for all HSV-1 genes with subsequent 2-dimensional principal component analysis revealed that YS populations occupy more evolutionary space than wt populations (Figure 3.3D). Furthermore, *de novo* genome assembly for passage 30 populations supports those findings. Reads map similarly well to genomes assembled *de novo* and to the reference genome (compare Figure 3S2 and 3S3A). Variant calling from those alignments (Figure 3S3B), as well as phylogenetic analysis of consensus genomes (Figure 3S3C), shows higher genetic diversity within and between YS populations compared to wt populations.

SNPs are distributed across the whole genome with only *RL1* and *RL2* being fully conserved following selection in Vero cells (Figure 3.3E), however, as repeat regions are particularly difficult to sequence the observed absence of mutations in these regions might be due to a lack of sufficient coverage (Figure 3S2). Furthermore, we find many genes with convergently increased dN/dS ratios (Figure 3.3F). As expected, *UL23* and *UL30* (coding for the thymidine kinase [TK] and the DNA-polymerase [Pol] respectively) displayed high SNP frequencies (Figure 3.3E) and elevated dN/dS ratios (Figure 3.3G), which prompted us to analyze these genes more closely.

3.4.4 Genetic Variation in *UL23* and *UL30*

SNPs in *UL23* were most common after ACV and GCV selection, especially for wt populations (Figure 3.4A). Only three mutations within *UL23* reached fixation in their respective populations: those encode of amino acid changes R222C in wt ACV as well as L188P and A93V in wt GCV (Figure 3.4B). Interestingly, no *UL23* mutation reached fixation in YS populations. Analysis of recombination junctions as well as monitoring of coverage data for *UL23* in p30 samples both suggest genomic rearrangements in this gene, specifically after ACV and GCV selection (Figure 3.4C). However, indel calling usually underestimates the real frequency and number of genomic rearrangements (Hasan, et al. 2015).

Within *UL30*, we detected SNPs in all applied virus/selection combinations, even though not in all respective replicates (Figure 3.4D). Non-synonymous mutations occurred almost exclusively downstream of the conserved Exo II region. Most SNPs were located in conserved polymerase regions II, VI and III. The introduced mutation in the Exo III domain (Y557S) was stably maintained in all YS populations (Figure 3.4E). Three changes in wt ACV populations (R700G, M784T and S513N), one in wt FOS (R700G), one in YS H_2O (S720G) as well as one each in YS ACV and FOS (A719V) reached fixation by p30. Notably, GCV selection yielded few to no SNPs in *UL30* and none reached fixation.

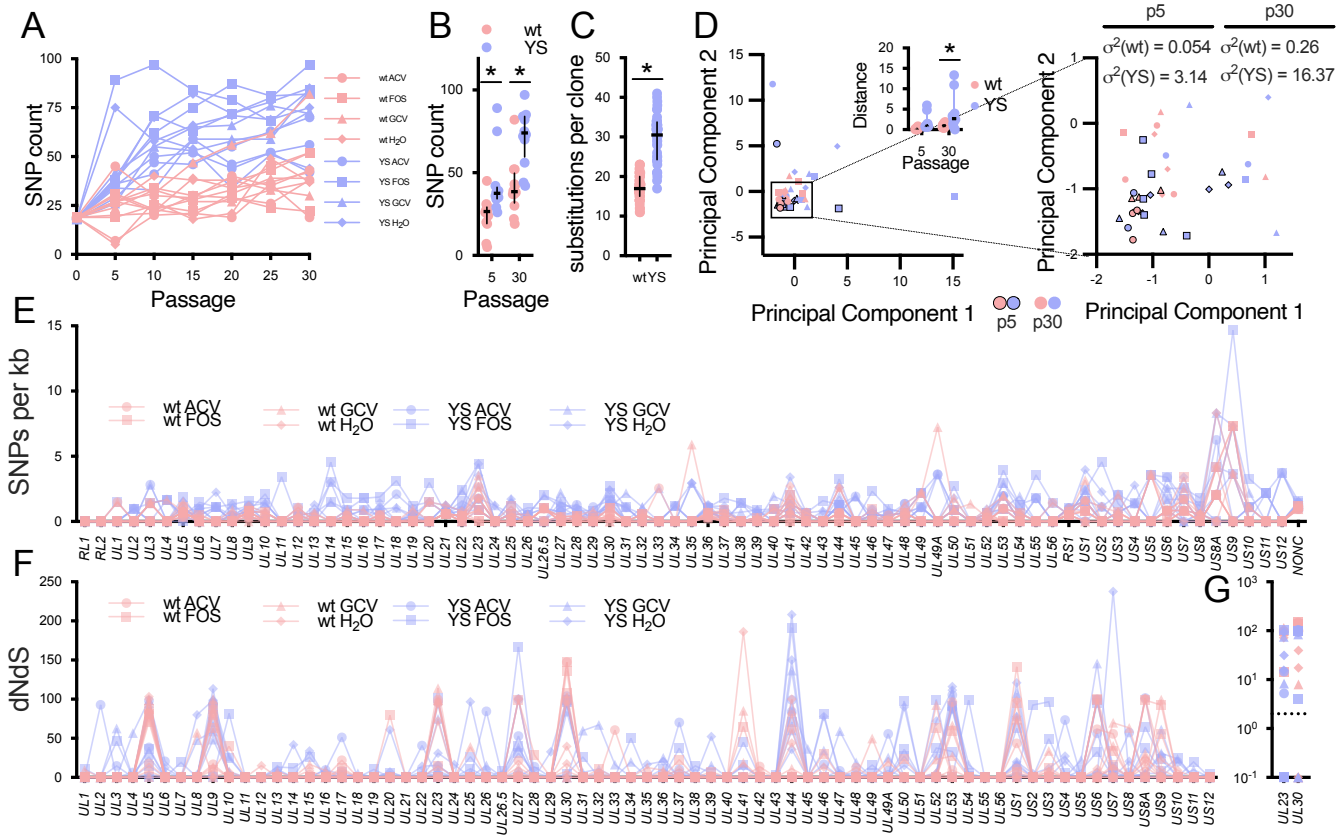

Figure 3.3: Higher mutation frequency of YS populations facilitates faster movement in genetic space. A) Population SNP count including allele frequencies of at least 0.05 for individual lineages over time. B) Pooled SNP counts from A at p5 and p30 for wt and YS populations respectively. * indicates significant differences ($p<0.05$) at given passage as determined by 2-way ANOVA followed by Šidák's multiple comparisons test. C) Base substitutions (allele frequency>0.5) per clonal genome from plaque purified clones of two p30 populations each. * indicates significant difference ($p<0.05$) determined by unpaired t-test. D) 2-dimensional principal component analysis of non-synonymous to synonymous per nucleotide site substitution rates (dN/dS) for p5 and p30 samples. The full figure (D, left) was zoomed into (D, right) to highlight less diverse populations. The inset on the left panel shows differences in their respective distance from the origin. * indicates significant differences ($p<0.05$) at given passage determined by 2-way ANOVA followed by Šidák's multiple comparisons test. Diversity in distance (σ^2) for p5 and p30 populations are given on the right. E) All SNPs that occur at least once throughout the passaging experiment (at allele frequency>0.05), depicted according to their loci and normalized by gene length. NONC stands for non-coding regions. F) dN/dS ratios for all genes and G) for *UL23* and *UL30* specifically. If at least 2 out of the 3 replicate populations displayed dN/dS ratios above 2, the gene was considered under positive selection.

Almost all detected variants in *UL23* and *UL30* occur solitarily within called haplotypes, which agrees with our initial variant call (Figure 3.4F). The only exception is co-occurrence in low frequency haplotypes in YS H_2O Rep3 and YS GCV Rep1. An overview of all occurring variants with their respective allele frequencies at all sampled passages can be found in Sup. Table 2.

3.4.5 Thymidine Kinase and DNA-Polymerase Amino Acid Changes Facilitate Antiviral Resistance *in vitro*

To score the impact of the amino acid changes we detected in our evolved populations, we performed SIFT (Figure 3.5A) and alphafold2 predictions (Figure 3S4AB). As expected, most TK amino acid changes have functional impacts, indicated by low probabilities of tolerance (Figure 3.5A and Figure 3S4A). This suggests that a loss of function leads to antiviral resistance. In contrast, the detected Pol amino acid changes are tolerated much better (Figure 3.5A and Figure

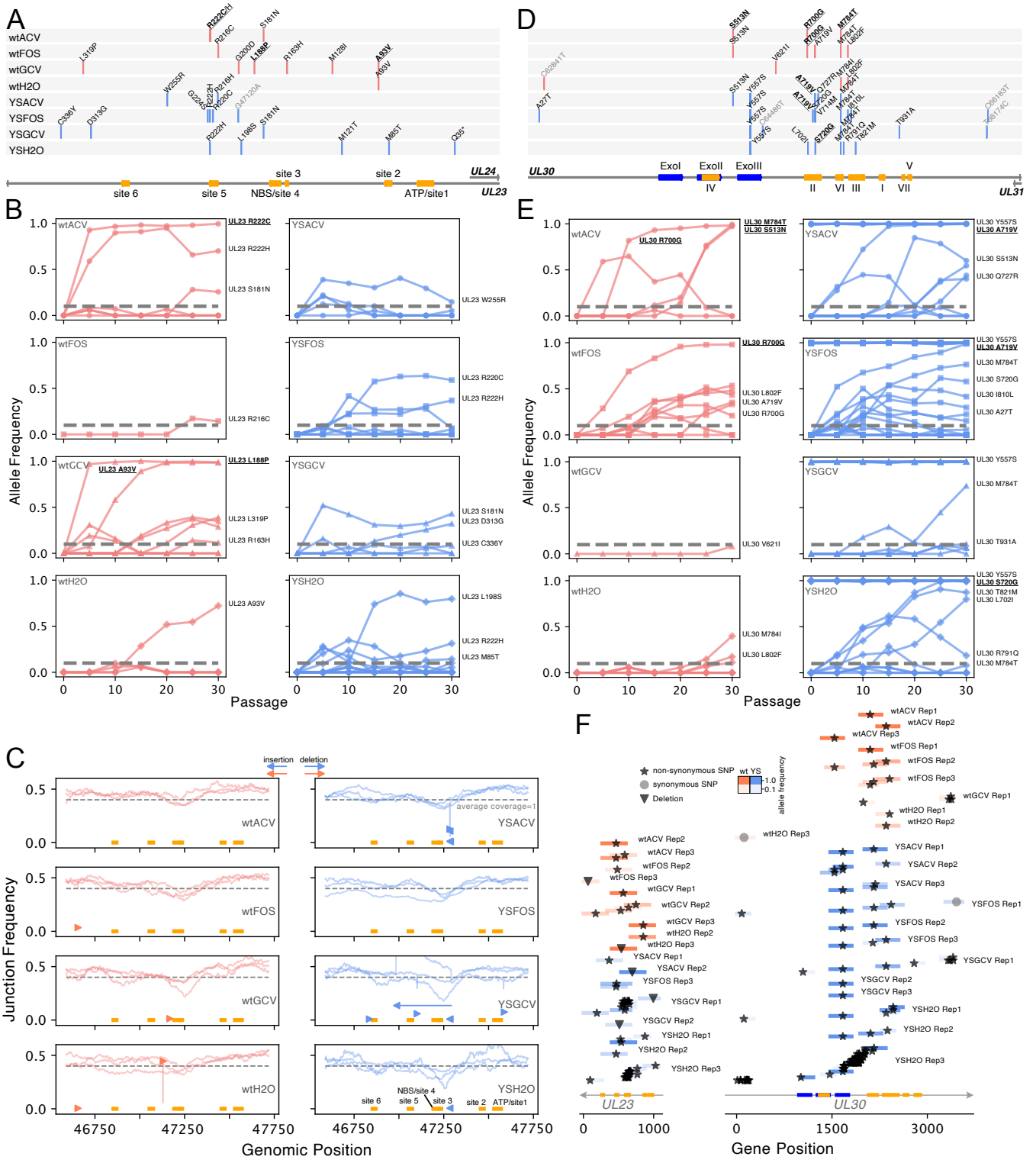

Figure 3.4: *UL23* and *UL30* mutations detected in evolved populations. All mutations in A) *UL23* (thymidine kinase) and D) *UL30* (DNA-polymerase) that could be detected (allele frequency>0.05) at p30 plotted according to their location within the respective gene. SNP allele frequencies for B) *UL23* and E) *UL30* plotted over time for individual populations, dotted lines show limit of detection (allele frequency of 0.05). SNPs that reach fixation are depicted as bold and underlined (ABDE). C) Genomic position and orientation of recombination junctions in *UL23* determined by ViReMa. Occurrence of recombination junctions was overlayed with coverage data. Orange boxes mark functional regions of the gene. F) Haplotypes (allele frequency>0.1) called for *UL23* (left) and *UL30* (right) in p30 populations by VILOCA and ShoRAH.

3S4B).

Mutations in *UL23* and *UL30* are known to be involved in conferring resistance to herpesvirus

polymerase inhibitors (Piret and Boivin 2016), to confirm this in the context of our study, we set out to reverse-engineer the observed mutations. This allows us to compare the effect of mutations introduced by wt or YS DNA polymerases. As *UL23* is a non-essential gene (Dogrammatzis, et al. 2021) and structural analysis suggests a loss of function, we disrupted this ORF by creating a partial knock out, maintaining only the first 393 bp of the gene to avoid any interference with *UL24* expression. As expected, deletion of *UL23* led to increased resistance to both ACV (Figure 3.5B) and GCV (Figure 3.5D). Interestingly, for wt, susceptibility to FOS increased following disruption of *UL23*, whereas YS only trends towards higher susceptibility (Figure 3.5C). Additionally, we also assessed competitive fitness of reverse engineered mutants against their respective parental viruses. Knock outs in both virus backgrounds provided competitive advantages, even under non-selective conditions on Vero, HFF and MRC-5 cells alike (Figure 3.5E). Applying antiviral pressure (same amount as in the passaging experiment) increased these advantages. High concentrations of ACV and GCV even lead to outcomes with undetectable levels of parental virus (black bordered points).

To investigate *UL30*'s role in antiviral resistance, we used the parental wt and YS backbones to reverse-engineer all *UL30* mutations ($>$0.05 allele frequency) observed at p30. In contrast to *UL23* mutations, and consistent with the enzymes essential function, Pol amino acid changes are generally better tolerated (Figure 3S4B), even though substitutions likely affect protein function (Figure 3.5A). Most of them, especially from amino acid positions 700 to 810, facilitated increased resistance against ACV and FOS in both backgrounds (Figure 3.5FG). However, no amino acid change in Pol observed by us led to increased resistance against GCV (Figure 3.5H). More so, most Pol mutations even increased susceptibility to GCV. Notably, some changes (Q727R – I810L) were better tolerated in YS background. We also checked for competitive advantages of Pol amino acid changes under non-selective conditions. Contrary to what we expected, no amino acid change came at a fitness cost, not even in human cells (Figure 3.5I). Many substitutions even conferred some fitness advantage over wt on Vero cells. Only 4 changes (detected at allele frequencies above 0.05, two of which are known to confer resistance to ACV and FOS) were shared between wt and YS populations, and only one variant was found for all different selections applied (Figure 3.5J).

3.4.6 Standing Genetic Variation Accelerates Phenotypic Development

To determine the influence of existing genetic variation on phenotypic plasticity in populations under strong selective pressure, we passaged early (p15) and late (p30) samples separately on increased antiviral concentrations (10 µM ACV, 100 µM FOS and 5 µM GCV respectively) as well as without antivirals (Figure 3.6A, end point titers shown in Figure 3S5AB). No apparent difference in speed or magnitude of phenotypic change was observed in early populations (Figure 3.6B), whereas late YS populations increased their respective resistance faster and for FOS and GCV even to higher levels than wt, which might be due to better initial adaptation to Vero cell

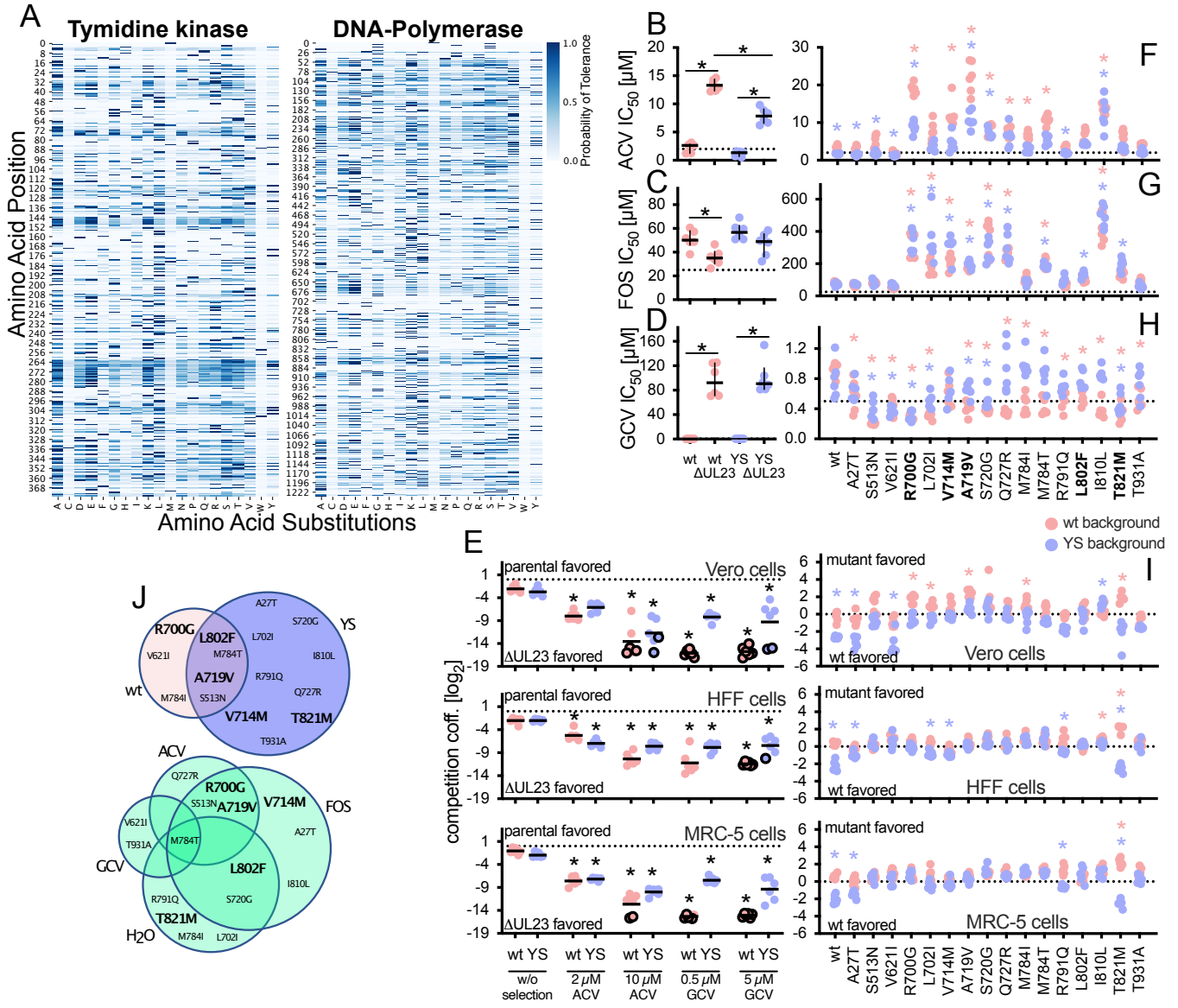

Figure 3.5: Tymidine kinase and DNA-polymerase alterations lead to antiviral resistance without fitness costs *in vitro*. A) SIFT predictions for all possible amino acid changes in the thymidine kinase (TK, left) and DNA-polymerase (Pol, right). Scores below 0.05 (white) are considered to impact molecular function. Resistance measured by plaque reduction assays and displayed as IC_{50} values for 6 independent replicates (median + interquartile range) against B) aciclovir, C) foscarnet and D) ganciclovir for *UL23* deletion mutants as well as against F) aciclovir, G) foscarnet and H) ganciclovir for Pol amino acid substitutions. * (red: wt, blue: YS) indicates significant differences ($p<0.05$) determined by 1-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test for TK and Pol changes respectively. Competition assays between E) *UL23* deletion mutants and their respective parental virus as well as between I) Pol mutants and wt virus on Vero, HFF and MRC-5 cells. Log_2 transformed competition coefficients (number of mutant plaques/number of control plaques) are shown for 6 independent replicates. Circled values identify competitions at which no parental virus could be detected. * indicates significant differences ($p<0.05$) against the respective non-selective condition as determined by 1-way ANOVA followed by Tukey's multiple comparisons test for *UL23* deletion. For Pol mutants, * indicates significant differences observed for wt (red) and YS (blue) mutants, measured against wt/wt competition as determined by 1-way ANOVA and Dunnett's multiple comparisons test. I) Venn diagram showing mutations observed in either wt or YS background (upper panel) and following specific selection (lower panel). Amino acid changes in bold font indicate changes already associated with increased resistance.

culture and increased antiviral pressure (Figure 3.6C). Notably, this increase at elevated antiviral concentrations occurs despite an observed flattening of the resistance curve in the original antiviral selection, especially in YS FOS populations (compare to Figure 3.2EFG and Figure 3S1). As expected, we found more SNPs in YS populations, however, the overall distribution of *de novo* mutations (not observed in parental populations) and already present SNPs does not differ significantly between selection pairs (Figure 3.6D). Interestingly, many SNPs increase in allele

frequency under relaxed selection or do not change between selective and non-selective conditions. SNPs from the originating populations are shown in Figure 3.6E according to whether they increase after further antiviral pressure or not.

Similarly, we also passaged our early and late H_2O populations on different antivirals (2 µM ACV, 25 µM FOS and 0.5 µM GCV respectively; Figure 3.6F, end point titers shown in Figure 3S5CD). No major differences between early and late as well as wt and YS populations could be observed here (Figure 3.6G). However, we found that pre-passaged H_2O populations in general diversified more rapidly than clonal starting populations (compare to Figure 3.2EFG). Again, H_2O controls showed about the same amount of *de novo* mutations in early and late populations (Figure 3.6H). In wt populations selection of SNPs already present sums up to about half of the *de novo* mutations, especially in non-synonymous variants. In YS populations specifically, selection of already established non-synonymous mutations reaches or surpasses *de novo* mutation levels.

3.4.7 YS Populations Display Higher Genetic and Phenotypic Variation at Single Clone Resolution

We next decided to investigate genetic and phenotype variation on a clonal level. To this end, we plaque purified 20 clones from 4 different p30 populations (wt as well as YS, GCV and H_2O) and characterized both their phenotype and genotype. Plaque sizes differed substantially, especially in YS compared to wt populations (Figure 3.7A; Figure 3S6). Furthermore, we also characterized individual antiviral resistance and found significant correlation between ACV and FOS, as well as ACV and GCV resistance, for both wt and YS GCV populations (Figure 3.7B). Even GCV and FOS resistance correlated in YS GCV populations. By contrast, no linear relationship or correlation could be observed in H_2O populations, except for wt H_2O between ACV and GCV resistance (Figure 3.7C).

Phylogenetically, YS is populated by genetically more diverse individuals compared to wt, however, both wt and YS populations are very similar in their structure, exemplified by lineage selection under GCV selection (Figure 3.7D). Overall, pairwise sequence identity is higher in wt, although all examined populations display high genetic homogeneity with identity scores around 0.99 (Figure 3.7E). In line, heterozygosity, a population genetics measure to describe variability at particular loci (Gillespie 2004), is higher for nearly every gene in YS populations compared to wt (Figure 3.7F).

In summary, our analyses on both populations and clones show higher variability and adaptability of YS, they however do not suggest any principal difference in the evolution of wt and YS viruses.

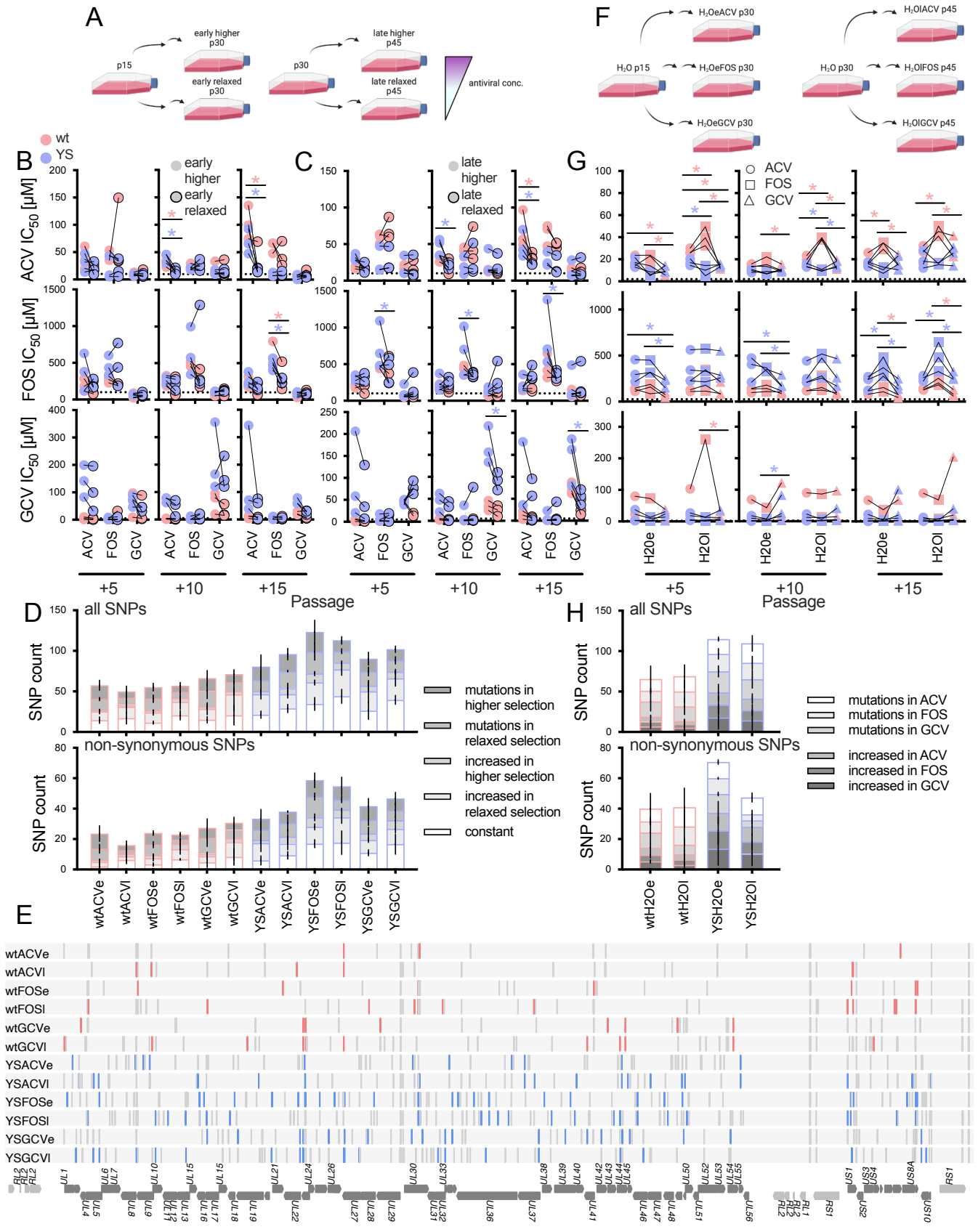

Figure 3.6: Pheno- and genotypic responses to selection changes in already diversified populations.(continued)

(continued) Passaging schemes for A) populations already selected on antivirals and F) H_2O control populations. Antiviral resistance for B) early (originated from passage 15), C) late (originated from passage 30) on antivirals pre-selected populations as well as G) early and late H_2O control populations selected on antivirals. Bordered dots in B and C represent populations under non-selective conditions (relaxed selection). Red and blue * indicate significant differences ($p<0.05$) as determined for wt and YS populations respectively, calculated by 2-way ANOVA followed by Šidák's multiple comparisons test for B and C and Tukey's multiple comparisons test for G. Mutations detected in early and late populations 15 passages after the respective change in selection was applied for D) pre-selected antiviral and H) H_2O control populations. SNPs were categorized according to whether they were first observed after selection change (*de novo* mutations in higher, relaxed, ACV, FOS or GCV selection) or when already present in originating populations, if they increase (>1.2 times) in allele frequency relatively to the paired populations (for higher and relaxed selections) or to the originating population (for H_2O controls). Variants in D labeled as constant did not differ between higher and relaxed selection pairs. E) Genomic locations of standing genetic diversity in preselected populations. Colored lines indicate SNPs with higher frequency after increased antiviral pressure, grey lines show SNPs selected under relaxed conditions.

3.5 Discussion

Antimicrobial resistance is ubiquitous and among the greatest medical challenges to date (Christaki, et al. 2020; Morrison and Zembower 2020). Experimental evolution offers an attractive way to study mechanisms of acquired resistance. However, adaptation can be time-consuming, especially when considering the period and scale available to natural evolution. Increasing viral adaptability in experimental setups is thus of interest and can be achieved through increased mutation rates and consequently faster exploration of sequence space (Domingo and Holland 1997; Jayaraman 2011; Xing, Höfler, et al. 2022). Here we used a proofreading impaired HSV-1 mutant and demonstrate higher adaptability while maintaining wt-like evolutionary processes.

Throughout this study, we have observed similar phenotypes for both wt and YS (Figure 3.2, 3.5, 3.6 and 3.7). However, wt is initially more ACV resistant (Figure 3.1D), indicating a potential role of Pol 3'-5' exonuclease activity in the excision of DNA incorporated ACV. All antivirals used in this study are DNA-polymerase inhibitors (Kukhanova, et al. 2014), ACV as well as GCV require phosphorylation to exert their pharmacological action (Andrei, et al. 2009; Klysik, et al. 2020), while FOS acts directly within the active center of Pol (Piret and Boivin 2016). Consequently, mutations conferring resistance to either treatment are expected to reside in *UL30* and *UL23* (Piret and Boivin 2016).

Many of the TK and Pol amino acid changes detected by us (Figure 3.4) are also found in clinical isolates and are confirmed to cause resistance. Those include A93V, R163H, S181N, G200D, R216C/H, R220C, R222C/H and C336Y in TK as well as R700G, V714M, A719V, L802F and T821M in Pol (Sauerbrei, et al. 2010; Sauerbrei, et al. 2015; Schmidt, et al. 2015; Piret and Boivin 2016). This indicates the usefulness of our hypermutator model system to predict and evaluate antiviral resistance. Furthermore, some of those amino acid changes are known to influence protein function, like T821M and R700G in Pol as well as R163H, R216C/H and C336Y in TK (Sauerbrei, et al. 2015).

Indeed, our analyses confirm that Pol and TK are both under positive selection for most antiviral treatments (Figure 3.3G). As expected, TK changes are prominent in ACV and GCV selections, but not selected for by FOS treatment (Figure 3.4BC). Interestingly, we find that GCV treatment has a conservative effect on Pol (Figure 3.4DE), a phenomenon so far not explicitly reported, but potentially relevant for combinatorial treatments. Specifically, the combination of FOS and GCV may suppress resistance development in complicated cases of HSV infection.

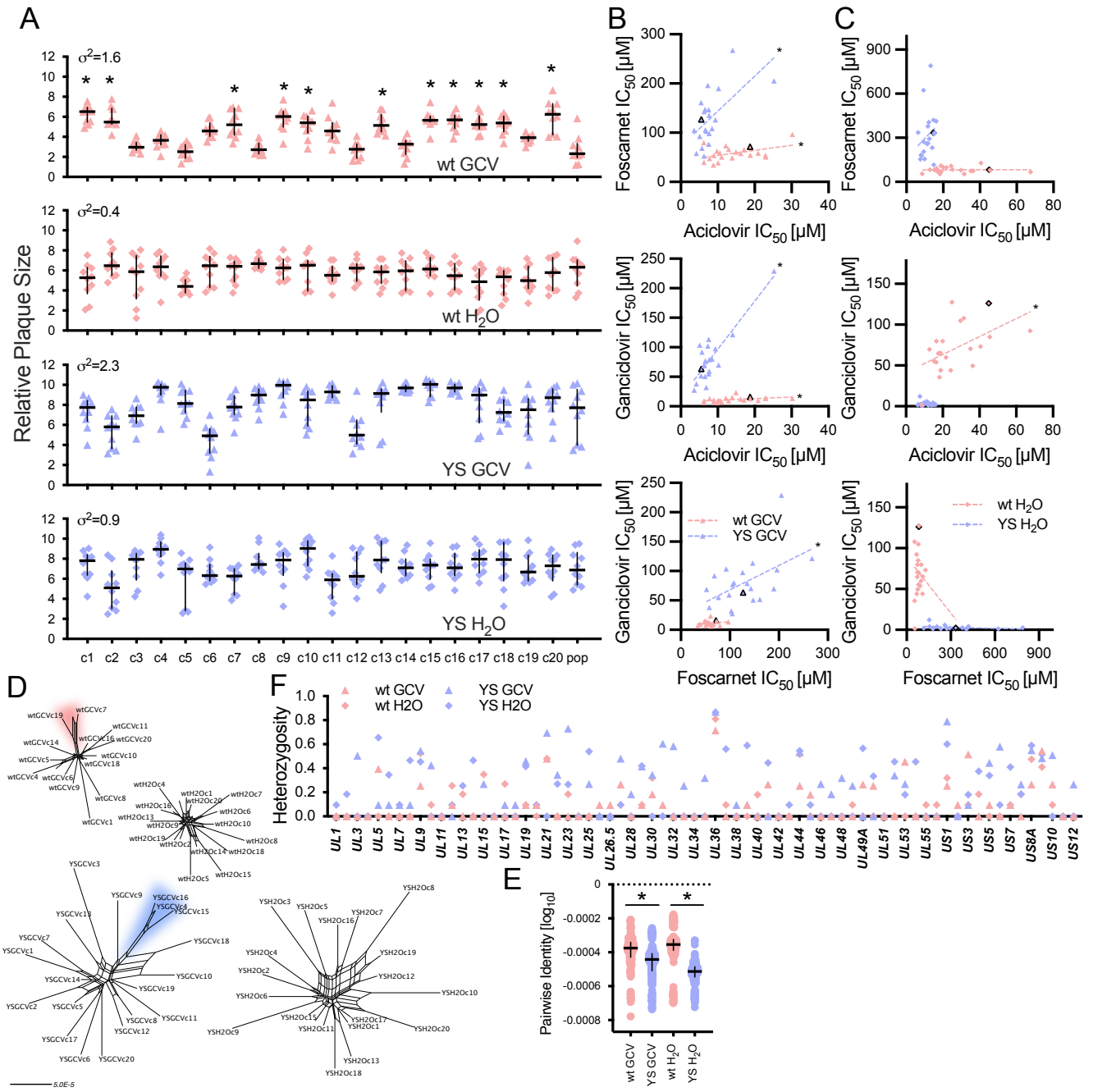

Figure 3.7: Single clone analysis elucidates phenotypic and genotypic diversity upon *in vitro* evolution. A) Plaque size assay for 20 single clones per population as well as the population itself. Median and interquartile range are provided as well as individual sizes of 10 plaques per clone/population. Variance (σ^2) was calculated using the mean plaque sizes of all 20 clones per population. * indicates significant differences ($p<0.05$) against population value as determined by 1-way ANOVA followed by Dunnett's multiple comparisons test. Antiviral resistance measured via plaque reduction assays and plotted against one another for B) wt and YS GCV clones as well as C) wt and YS H_2O clones. Bordered values indicate resistance measurements for populations. * indicates significant correlation and linear regression ($p<0.05$) determined by Pearson's correlation test and simple linear regression. D) Phylogenetic trees of individual populations calculated and visualized with SplitsTree. Lineage selection is indicated by colored branches. E) Pairwise sequence identity scores for genomes from D. Dotted line indicates 100% identity. F) Heterozygosity, a measure to describe genetic variability within populations, for all genes in unique regions was calculated using a custom python script.

Our observations are confirmed by reverse engineering of *UL23* knock out and *UL30* mutants. Most of the observed Pol amino acid changes increased ACV and FOS resistance (Figure 3.5FG), surprisingly without apparent fitness costs (Figure 3.5I), whereas not a single Pol amino acid change observed in this study conferred resistance to GCV (Figure 3.5H). Considering that

both ACV and GCV are guanosine analogs which diverge only in their sugar backbone, opposite effects of Pol mutations on drug susceptibility may be due to differentiation in the sugar-phosphate backbone. While ACV only contains the 5'-OH and thus terminates chain elongation, GCV possess the 3'-OH permitting further nucleotide incorporation into nascent DNA strands. Using purified HCMV DNA-polymerases, Chen et.al showed that GCV inhibits DNA synthesis only after initial extension of GCV containing DNA strands (Chen, et al. 2014). It seems possible that exclusion of nucleotides lacking 3'-OH increases affinity for 3'-OH containing nucleotides. By this mechanism, ACV resistance could lead to GCV susceptibility, and higher affinity for canonical nucleotides might explain fitness advantages in Vero cells (Figure 3.5I). This seems to be the case for I810L amino acid substitutions. This change is located in the Pol finger domain (Figure 3S4B), which is known to interact with incoming nucleotides selectively (Hwang, et al. 1999). I810L vastly increases resistance against ACV and FOS but renders the polymerase more susceptible to GCV. Contrarily, V714M, the only amino acid change that does not cause significantly increased GCV susceptibility, is located in the palm domain.

Chen et.al further showed that Exo deficient HCMV mutants are GCV resistant (Chen, et al. 2014). In agreement, we observed Pol alterations that increased GCV susceptibility in wt but not in YS (Figure 3.5H). Among those amino acid changes are I810L, Q727R, M784T and A719V, all of them reside either in the finger domain or in the hinge between finger and palm (Figure 3S4B). Despite impairment, YS retains substantial 3'-5' exonuclease activity (Kuhn and Knopf 1996), which may explain why baseline differences in GCV susceptibility are only observed in direct competition between wt and YS (Figure 3.1G).

Experimental evolution studies often use either plaque purified or clonal DNA derived viruses (McBride, et al. 2008; Ogbunugafor, et al. 2009) which disregards the role of standing genetic variation in virus evolution. However, adaptation to Vero cell culture is accelerated in diverse populations compared to plaque purified virus (Kuny, et al. 2020). We found that when resistance-conferring mutations are not yet fixed, standing variation can compensate for lower mutation rates (Figure 3.6B). Contrarily, YS diversified faster than wt after 30 passages of pre-selection (late populations, Figure 3.6C). As resistance development is flattening in those viruses, further increase or decrease is most likely dependent on *de novo* rise of mutations rather than selection from standing variation. Adaptation to antiviral selection in H_2O control populations further suggests that standing genetic variation can, to some degree, compensate for lower mutation rates (Figure 3.6G).

Single clone analysis elucidated within population diversity in wt and YS populations. Plaque sizes were more variable in YS and GCV populations, compared to wt and H_2O (Figure 3.7A). Selection is also apparent in antiviral profiles and correlations. Individuals obtained from GCV populations show significant correlations of ACV/FOS and ACV/GCV resistance (for YS GCV also FOS/GCV, Figure 3.7B), whereas in H_2O populations only wt H_2O correlates ACV and GCV resistance (Figure 3.7C). As FOS resistance increases GCV susceptibility and vice versa, correlation in YS GCV might occur because clones with Pol changes also contain mutated TK. Pol medi-

ated FOS resistance occurs in absence of TK dependent GCV activation. Similarly, correlation of ACV/GCV resistance in wt H_2O could be facilitated by TK alterations. Apart from phenotypic evidence, genetic analysis also indicates selection in GCV populations. Phylogenetically, clones from H_2O populations are more related to each other with no evidence for selection, while GCV clones show beginning lineage selection (Figure 3.7D). Despite similar phylogenetic structure, YS populations are more diverse, demonstrated by higher heterozygosities in nearly all genes (Figure 3.7F). Thus, also on an individual level, we observe similar evolutionary patterns but higher diversity in YS hypermutators.

A limitation of our study is the sole focus on cell culture models, which generally lack the complexity of host organisms. Invariant host cells and lack of an immune system are among the most important shortcomings. Using cell culture methods, we also did not consider the impact of latency on virus evolution, which overall is an understudied subject. However, our experiments with MDV suggest that the YS mutant presented here can be employed in *in vivo* studies where it shows accelerated adaptation similar to what we describe here (Xing, Höfler, et al. 2022). Many mutations observed *in vitro* are deleterious or lethal *in vivo*, therefore, evolutionary constrains presented by animal models would yield physiologically more relevant information about how viruses evolve. Additionally, important phenotypes like virulence or neuroinvasion are difficult or even impossible to study in cell culture systems.

With this study, we established the Exo impaired hypermutator virus YS as a tool to understand viral evolution on shorter timescales as well as to elucidate mechanisms and correlations of antiviral resistance in HSV-1. To illustrate the benefit offered by our system, we calculated how much time one can save by using YS. Early on, passages take 3-4 days before endpoint criteria are reached, however, since both viruses adapt to cell culture conditions and YS even more so, we calculated 3 days for wt and 2.5 days for YS passages. Considering that phenotypes are developing 5-10 passages faster for YS than for wt, one would save 15-30 days of experimental work. For phenotypes that require 30 passages in wt, this would add up to 25-40 days, saving nearly half the time and about a third of the cell culture material. Our results thus advocate the use of hypermutator viruses when experimentally addressing virus evolution. Although this study focuses on HSV-1, the high degree of conservation found in viral DNA polymerases (Takemura, et al. 2015; Xing, Höfler, et al. 2022) suggests that our approach is feasible for a variety of other large DNA viruses.

3.6 Material and Methods

3.6.1 Cells and Viruses

Vero cells (ATCC CCL-81), HFF cells (ATCC SCRC-1041) and MRC-5 cells (ATCC CCL-171) were cultured in Dulbecco's modified eagle medium (DMEM, Pan Biotech) supplemented with 10% fetal calf serum (FCS, Pan Biotech), 100 IU/ml penicillin G (Carl Roth) and 100 µg/ml strep-

tomycin (Carl Roth) at 37 °C and 5% CO_2. All HSV-1 viruses presented in this study derived from F strain pYEbac102 kindly provided by Dr. Y. Kawaguchi (Tanaka, et al. 2003), University of Tokyo, Japan. A cytomegalovirus immediate early promoter driven GFP marker within the bacterial artificial chromosome (BAC) mini-F backbone was used for easy detection upon reconstitution.

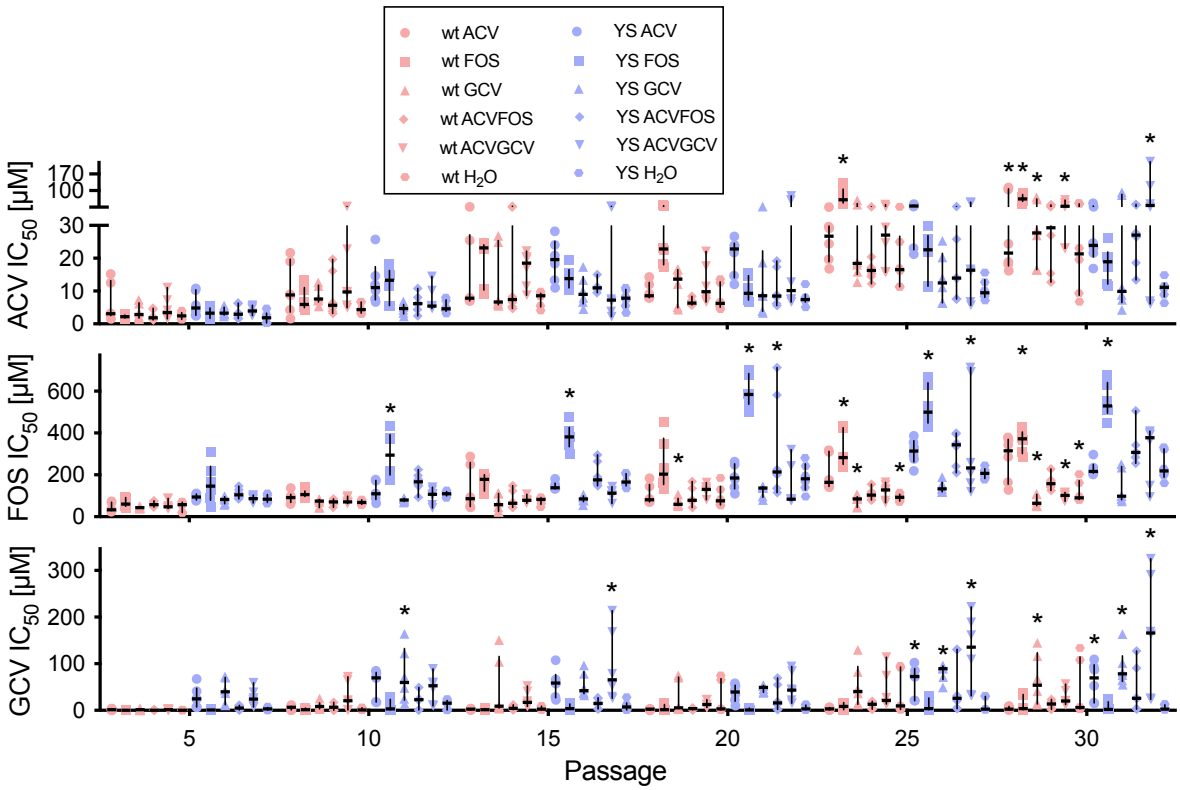

Figure 3S1: Cross resistance to antivirals. Antiviral resistance measurements via plaque reduction assays for all populations against all antivirals used in this study. Individual measurements from two technical replicates obtained from three biological replicate populations as well as median and interquartile range are provided. * indicates significant differences ($p<0.05$) against YS H_2O populations, calculated by 2-way ANOVA followed by Dunnett's multiple comparisons test.

3.6.2 Viral Reconstitution

Viral BAC mutants were reconstituted by a standard polyethylenimine (pei) protocol. In brief, 2-3 µg of DNA were diluted in 100 µl Opti-mem (Thermo Fisher Scientific) and supplemented with 12 µl of pei (1 mg/ml polyethylenimine, linear (mw 25,000); Polysciences). After 30 min of room temperature (RT) incubation, reactions were mixed with 1 ml cell culture medium and put on a sub-confluent 6-well plate well. Medium was changed 4 h post transfection. Plates were kept in culture till 70-100% of cells showed cytopathic effects (CPE).

3.6.3 Viral Stock Preparation

Virus stocks were prepared by freezing 90-100% infected cell culture plates. After thawing at RT,

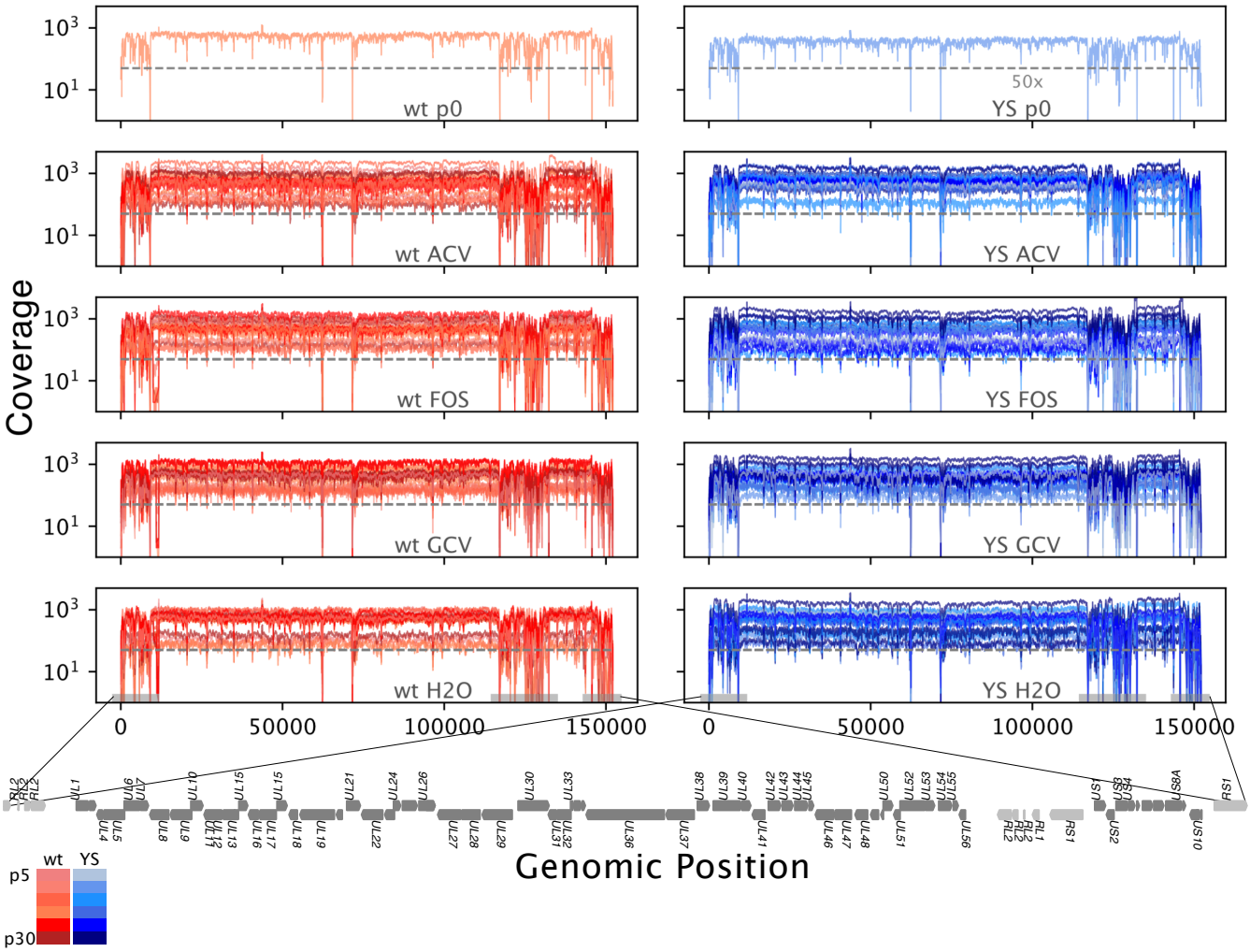

Figure 3S2: Sequencing coverage for evolved viral populations. Per nucleotide sequencing depth is presented for all analyzed samples. Dotted lines indicate minimal average depth for inclusion of the sample in the NGS dataset (50x). Grey areas in the lower plots indicate areas of repetitive sequence (terminal and internal repeats). Below the coverage plots, the genomic organization of HSV-1 strain F is provided for orientation.

lysed cells were resuspended in supernatant and 1.8 ml were put into cryotubes and stored at -80 °C.

3.6.4 Propagating of Cells and Virus

Viruses were passaged on Vero cells. Confluent 10 cm plates were infected with 500 plaque forming units (pfu) for p1 and with 100 µl of a 10^{-2} dilution for all subsequent passages (corresponding to a 10^{-4} dilution of endpoint populations and to a MOI of 0.001). Passages took place till 90-100% of cells showed CPE (2-4 days) and were immediately frozen afterwards (-80 °C). Thawed stocks, prepared and stored as described above, were created every 5 passages for viral populations. For antiviral selection cell culture medium was supplemented with 10 µl of the respective antiviral stock solution (2 mM aciclovir, Ratiopharm; 25 mM foscarnet, Alfa Aesar; 0.5 mM ganciclovir, Acros Organics) or with DEPC H_2O (Carl Roth).

3.6.5 Viral Titer Determination

Viral titers were measured by plaque assays in 24-well plates. Stocks were subjected to 10-fold serial dilutions and 100 µl of each dilution were incubated for 1 h on separate wells. Afterwards, inoculate was removed and cells were overlayed with a semisolid colloid cellulose cell culture

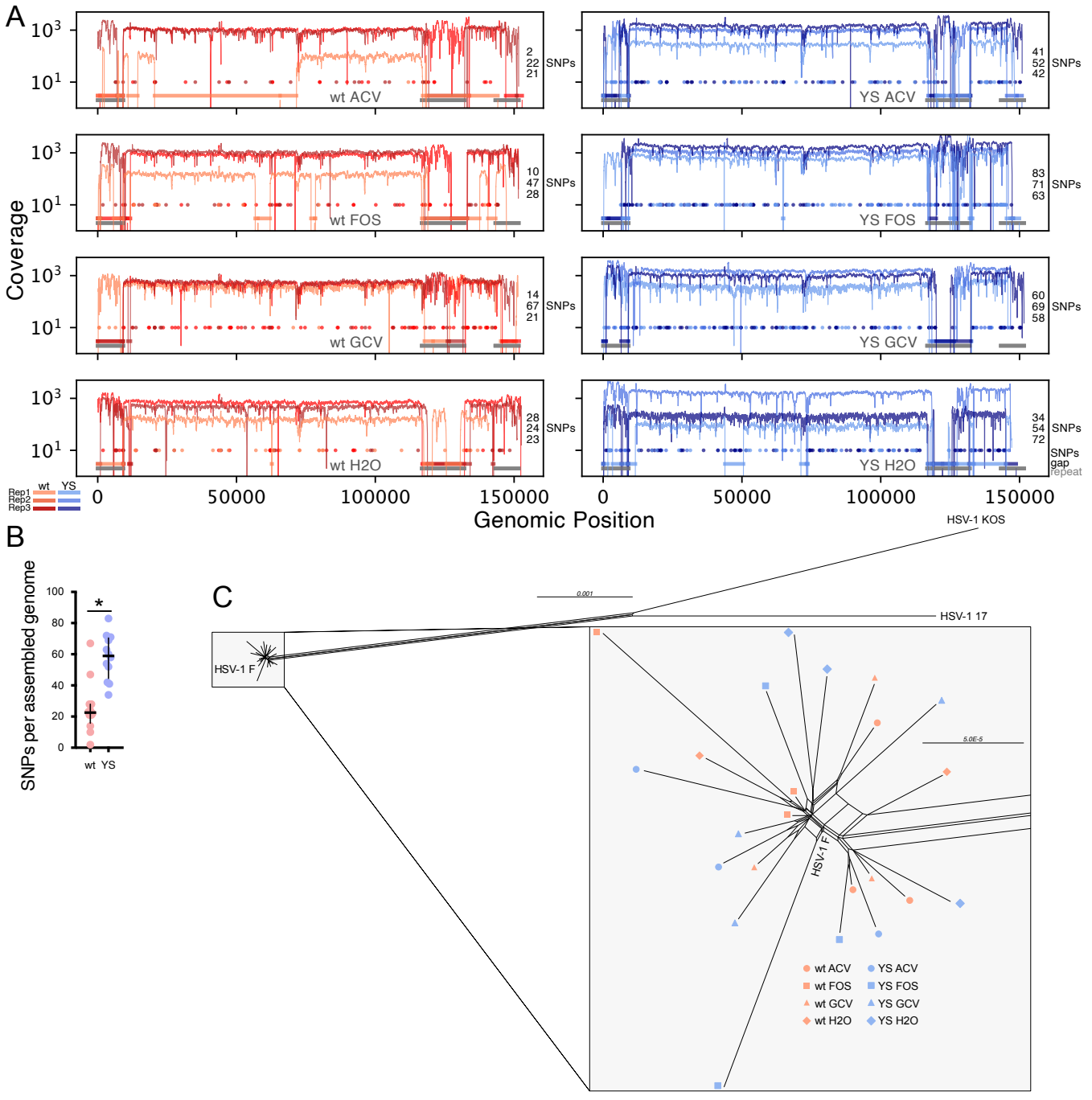

Figure 3S3: *de novo* genome assemblies for passage 30 viruses. A) Per nucleotide sequencing depth for reads back mapped to the *de novo* assembled consensus genome is presented for all passage 30 samples. Dots indicate single nucleotide polymorphisms detected at the indicated position. Grey lines represent areas of repetitive sequence (terminal and internal repeats), colored lines above signify gaps in the *de novo* assembly. B) SNP counts in wt and YS populations in relation to the respective *de novo* assembled genomes. SNPs were counted as such if they occurred with allele frequencies above 0.05. * indicates significant difference demonstrated by two-sided unpaired t-test. C) SplitsTree representation of *de novo* assembled consensus genomes. HSV-1 strains 17 (genbank accession number: NC_001806) and KOS (genbank accession number: KT899744) were included as outgroups.

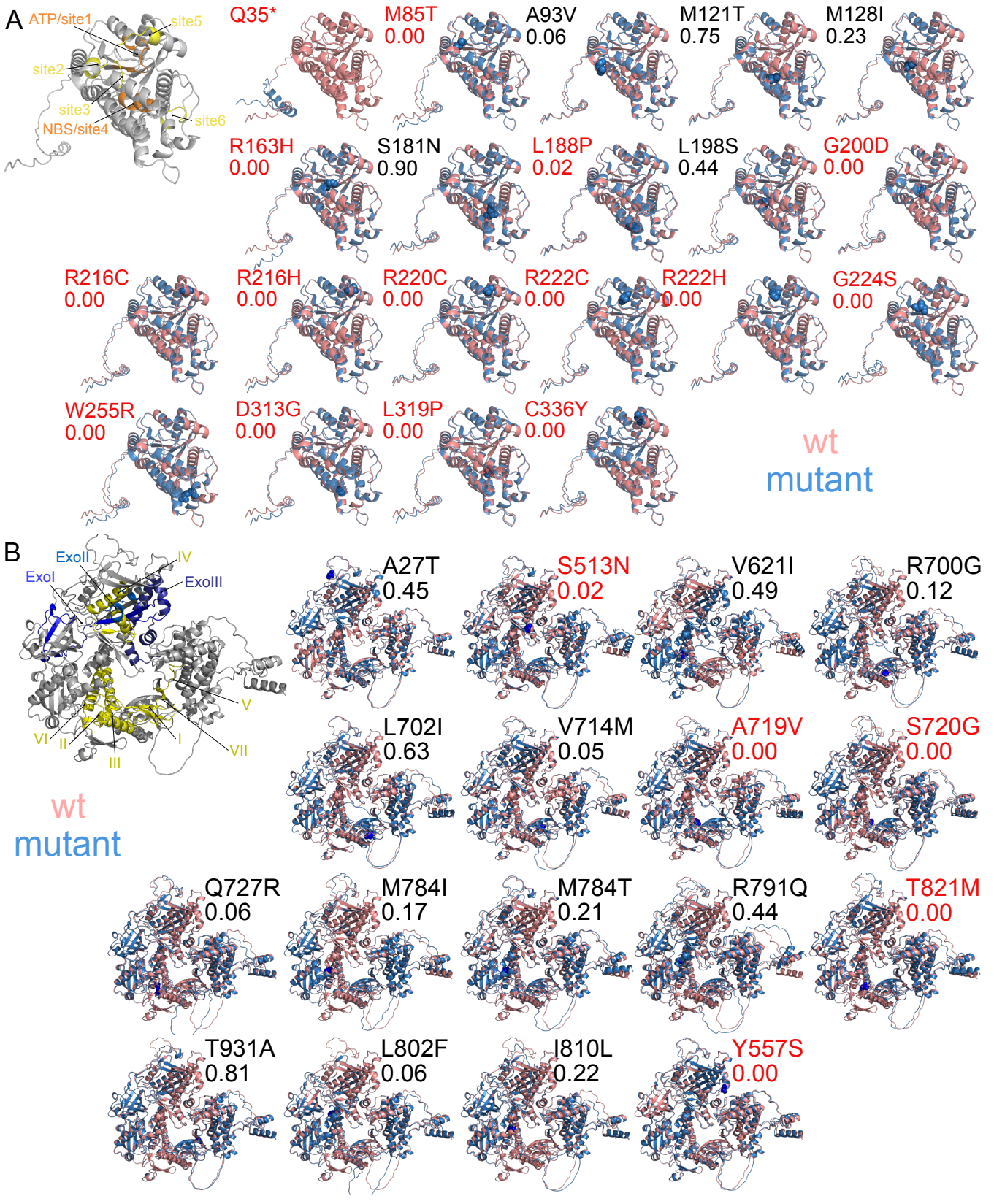

Figure 3S4: Structural impacts of amino acid changes on thymidine kinase and DNA-polymerase. Alphafold2 structure predictions for A) observed TK and B) Pol mutations at passage 30. Predictions of wt structures with labeled functional domains are shown on the left. Numbers indicate predicted SIFT probabilities of tolerance, probabilities smaller than 0.05 (marked by red text) are likely to affect molecular function. Mutant predictions are shown in blue (amino acid changes as spheres) and wt in red.

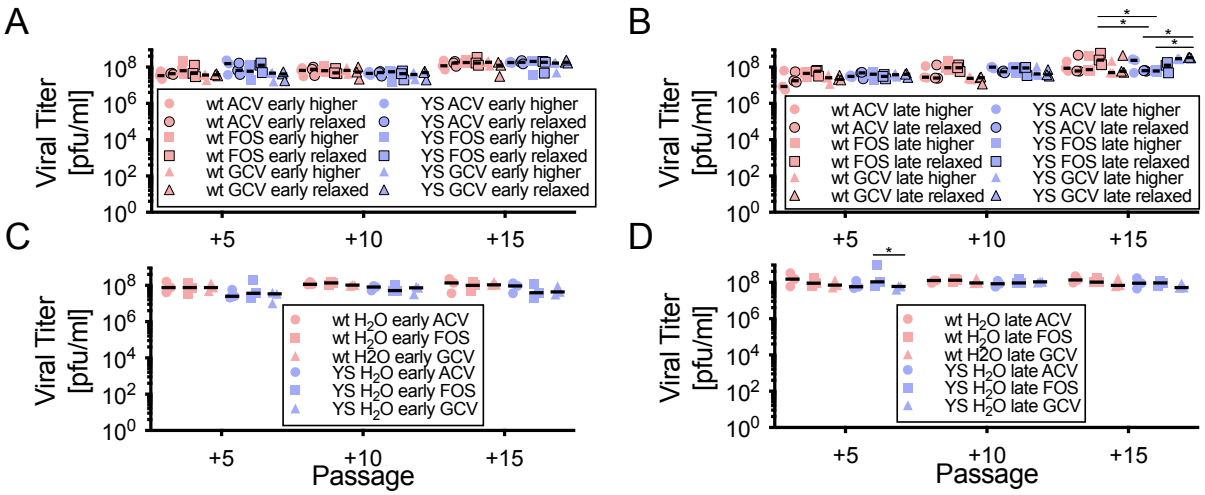

Figure 3S5: Endpoint titers for viral stocks upon selection changes. A) Endpoint titers for viral stocks of early selection changes from passage 15 on, B) for late selection changes from passage 30 on, C) for early H_2O controls upon antiviral selection from passage 15 on and D) for late H_2O controls upon antiviral selection from passage 30 on. * indicates significant differences ($p<0.05$) as shown, calculated by 2-way ANOVA followed by Tukey's multiple comparisons test.

medium (2.5% colloid microcrystalline cellulose, Aldrich; in 1x DMEM, Biochrom; supplemented with 10% FCS, 100 IU/ml penicillin G, 100 µg/ml streptomycin and 0.15% sodium bicarbonate, Sigma Life Science). Plates incubated till macroscopically visible plaques formed (2-4 days) and were washed twice with PBS. After fixing for 20 min with 4% formaldehyde, plates were stained with 0.75% crystal violet. Wells featuring at least 10 plaques were counted and used to calculate infectious titers.

3.6.6 Plaque Size Assays

Single wells of 6-well plates were infected with ca. 100 pfu of virus. After 1 h of incubation, inoculate was removed and cells were overlayed with semisolid colloid cellulose cell culture medium. Plates were incubated for 2 days, afterwards washed twice in PBS and fixed with 4% formaldehyde for 20 min. After permeabilization with PBS supplemented with 0.1% triton X-100 for 10 min, wells were blocked with PBS containing 1% FCS for 2 h. First antibody (C2D8, 1:100 dilution in PBS + 1% FCS) (Borchers and Ludwig 1991) was incubated overnight at 4℃ and second antibody (goat-anti-mouse alexa fluor 568 conjugated, 1:3000 dilution in PBS + 1% FCS, Thermo Fisher Scientific) was incubated for 2 h at RT. In between and after antibody incubations, wells were thoroughly washed with PBS. Plaque pictures were taken at a Zeiss Axio Vert.A1 inverted fluorescence microscope with 100x magnification. Plaque areas were measured using NIH ImageJ 1.52n (Schneider, et al. 2012) and normalized to wt p0 areas. Normalized areas were converted to diameters and used for further analysis.

3.6.7 Viral Growth Kinetics

Cells were infected in triplicates at multiplicity of infection (MOI) 0.001 in 6-well plates or at MOI 10

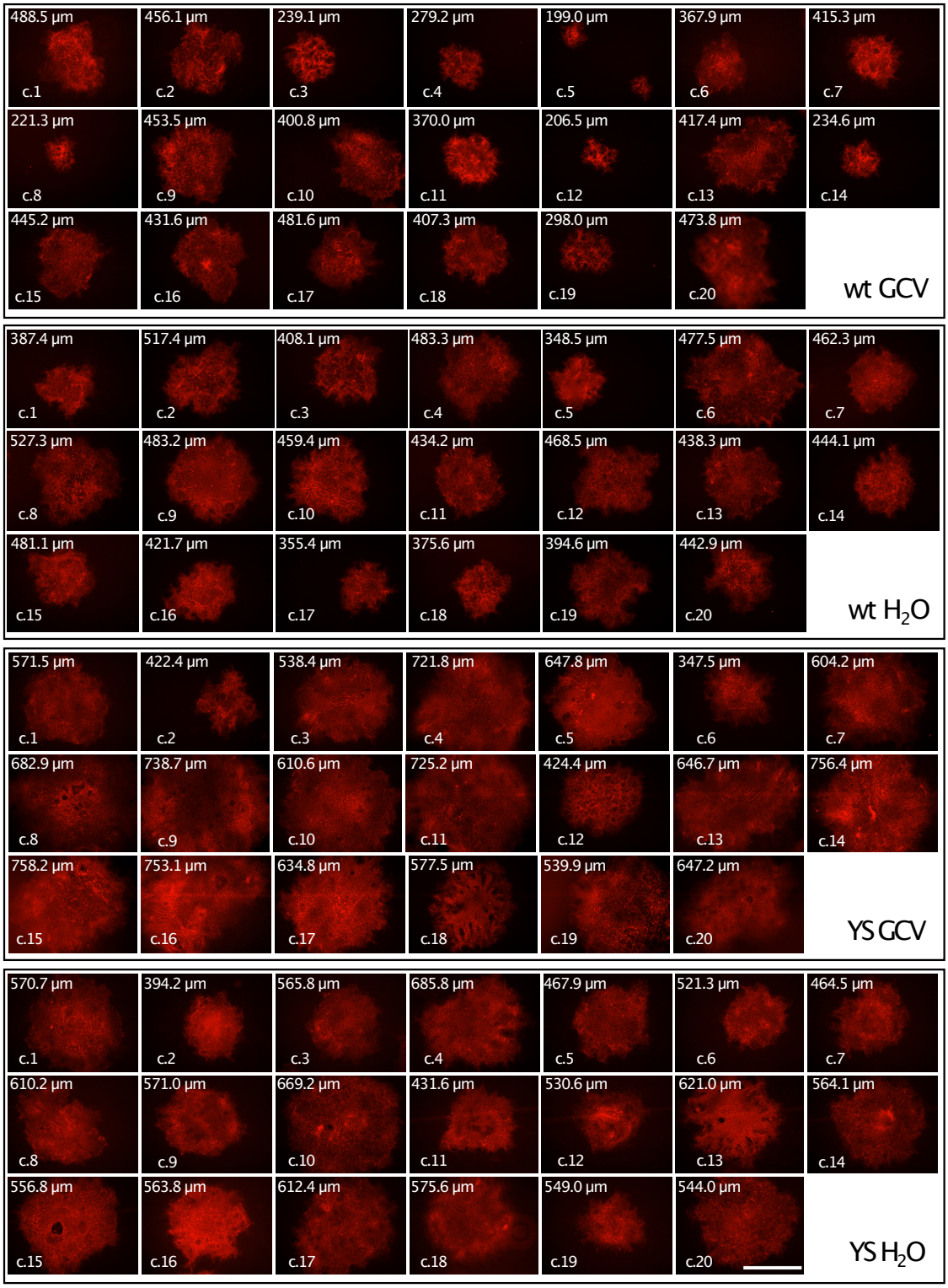

Figure 3S6: Within population diversity of plaque sizes and phenotypes. Plaque pictures for single clones originating from passage 30 populations, taken with an inverted Zeiss Axio Vert.A1 fluorescence microscope at 100x magnification (scalebar marks 500 µm). Infections were stained with cross-reactive anti-simplexvirus gB antibody and detected after secondary goat-anti-mouse AF568 incubation. Upper left corner shows mean plaque diameters for respective clone.

in 24-well plates for multi- and single-step growth curves respectively. For single-step, inoculum was removed after 1 h, washed and overlayed with fresh medium. Each timepoint and replicate consisted of 1 well. Virus was harvested after 12 h and afterwards every day till 5 days post infection (dpi) for multi-step and after 1, 3, 6, 12 and 24 h for single-step. Stocks were prepared and titrated as described above.

3.6.8 Plaque Reduction Assays

Antiviral resistance was measured by plaque reduction assays in 24-well plates. Confluent wells were infected with 50 pfu of virus. After 1 h incubation, inoculate was replaced by a semisolid colloid cellulose cell culture medium supplemented with 2-fold dilutions of antivirals (2 wells were left without antivirals as growth control). After plaques formed (2-4 days post infection), overlay was removed and wells were washed, fixed and crystal violet stained as described above. Plaques in all wells were counted if macroscopically observable and 50% inhibitory concentrations (IC_{50}) were calculated following a non-linear model:

$$\frac{f_a}{f_u} = \left(\frac{IC_{50}}{d}\right)^m \tag{3.1}$$

With f_a and f_u being the affected (untreated pfu-treated pfu, normalized to untreated pfu; the plaques that vanish) and unaffected (treated pfu/untreated pfu; the plaques that survive) fraction respectively, d being the antiviral concentration and m the magnitude.

3.6.9 Competition Assays

Viral fitness was measured by competition assays. Cells were infected with MOI 0.001 for both viruses respectively and kept in culture till 90-100% of cells showed CPE. For p0 competitions, GFP and mCherry marked viruses were used. Stocks were prepared and titrated as described above. Fixed and unstained plates were used to count green and red plaques with a Zeiss Axio Vert.A1 inverted fluorescence microscope with 100x magnification. Competition coefficients were calculated by dividing green and red plaque counts and $\log_2$ transformation afterwards. To exclude an influence of different marker genes (GFP/mCherry) on assay outcome, assays were tested reciprocally for wt/wt, YS/YS as well as wt/YS competitions. For p30 competitions wt and YS genomes were discriminated by allele specific qPCR in duplicates. DNA was isolated from infected cell pellets via sarcosine lysis as described below. For qPCR 10 ng sample DNA was mixed with 0.9 µl of each primer (10 µM, Sup.Table 1), 0.2 µl probe (10 µM, Sup.Table 1), 10 µl sensiFast master mix (2x; Bioline, Luckenwalde) and 1 µl DMSO. Reaction volume was filled up to 20 µl with DEPC H_2O. After 5 min of initial denaturation (95℃), 30 cycles of denaturation (95℃ for 10 s) and annealing/extension (64℃ for 30 s) were performed on a qTower^3/G (Analytik Jena). Cycle

threshold (Ct) values were calculated if a threshold of 5 was reached. Competition coefficients were calculated by subtracting mean Ct values for YS and wt of the respective sample.

3.6.10 DNA Isolation

DNA for sequencing was isolated by a micrococcal nuclease extraction protocol (Volkening and Spatz 2009), as described previously (Brunialti, et al. 2023). In order to use it for Illumina sequencing, DNA was size selected to exclude fragmented chromosomal DNA (<3000 bp) (Clarke, et al. 2014). SPRI Beads (AMPure XP Beads, Beckman Coulter) were washed twice in TET buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.05% Tween 20) and resuspended in PEG buffer (8% polyethylene glycol, 1 M NaCl, 10 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.05% Tween 20). DNA samples were mixed with 1.8x PEG/beads and incubated for 5 min. After 2 rounds of washing with 150 µl of 70% ethanol, air dried beads were resuspended in 10 mM Tris/HCl pH 7.5 for elution. Supernatant was collected after another 5 min of incubation and used for subsequent sequencing. DNA for qPCR was isolated by sarcosine lysis. Cell pellets harvested from single wells of 6-well plates were resuspended in 100 µl of TEN buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 100 mM NaCl). After addition of 50 µl of sarcosine lysis buffer (75 mM Tris/HCl pH 8.0, 25 mM EDTA, 3% N-lauryl-sarcosine) and incubation for 15 min at 65°C, RNA was removed by adding 1 µl of RNase A (10 mg/ml) and incubation for 20 min at 37°C. Protein was digested using 1 µl of proteinase K (20 mg/ml) for 16 h at 55°C. After 3 rounds of phenol/chloroform extraction as well as ethanol precipitation and washing, pellets were solved in 30 µl of 10 mM Tris/HCl pH 7.5. BAC DNA was isolated from 200-500 ml of *E. coli* liquid cultures using Qiagen's MidiPrep kit according to the manufacturer's instructions.

3.6.11 Next Generation Sequencing and Bioinformatics

Sampled populations were sequenced on the Illumina MiSeq platform. Sequencing libraries were prepared using the NEBNext Ultra II DNA Library kit. A starting 100 ng of DNA was fragmented either by enzymatic fragmentation or by using a Covaris M220 ultrasonicator. DNA was size selected for 500-700 bp fragments. Equimolar pooled libraries were diluted to 4 nM, denatured, further diluted in hybridization buffer (Illumina) and loaded onto MiSeq flow cells according to the manufacturer's instructions. Paired-end Illumina sequencing was performed for 600 cycles, yielding single reads with 300 bp. Sequencing data was required to yield an average coverage depth of at least 50x (see Sup. Figure 2). Obtained FASTQ files (available at NCBI SRA, BioProject accession number PRJNA927130) were processed with Trimmomatic v0.39 (Bolger, et al. 2014), mapped back to the paternal reference genome (https://github.com/hoeflet/antiviral-resistance-evolution.git) (Brunialti, et al. 2023) with Burrows-Wheeler aligner v0.7.17 (Li and Durbin 2009) and variants were called using Samtools v1.10 (Danecek, et al. 2021), BCFtools v1.11 and LoFreq v2.1.3.1 (Wilm, et al. 2012). Haplotypes were called using the VILOCA viral haplotype

caller (https://github.com/cbg-ethz/VILOCA), applying the ShoRAH tool (Zagordi, et al. 2011). Recombination junctions were mapped using ViReMa (Routh and Johnson 2014; Sotcheff, et al. 2023). The VirGA pipeline was employed for *de novo* genome assemblies (Parsons, et al. 2015). The NGS analysis pipeline was run on the CURTA HPC cluster of ZEDAT, Freie Universität Berlin (Bennett, et al. 2020). Single nucleotide polymorphisms (SNPs) were processed by a custom python script (https://github.com/hoeflet/antiviral-resistance-evolution.git). This included genetic loci, amino acid changes, allele frequencies, depth and non-synonymous to synonymous substitution rates (dN/dS). dN/dS was calculated by normalizing nucleotide replacements to per site rates (dN and dS), multiplied by allele frequency and summarized per gene:

$$\frac{dN}{dS}_{gene} = \frac{\sum_{i=1}^{n} \frac{AF_{N,i}}{c_{N,i}}}{\sum_{i=1}^{m} \frac{AF_{S,i}}{c_{S,i}}} \tag{3.2}$$

With AF_N and AF_S being the allele frequency of a non-synonymous and synonymous mutations respectively as well as c_N and c_S being how often a mutation at that nucleotide site leads to an amino acid change or not ($1 \leq c_N, c_S \geq 3$). Phylogenetics for p30 clones and populations were performed by aligning consensus genomes, shortened of terminal repeat regions, with MAFFT v7.475 (Katoh and Standley 2013). Trees were calculated and visualized with SplitsTree v4.19.1 (Huson and Bryant 2005). Heterozygosity was calculated per gene as follows:

$$H_{gene} = 1 - \sum_{i=1}^{n} p_i^2 \tag{3.3}$$

With H_{gene} being the heterozygosity per gene and n the number of different alleles of that gene with their respective allele frequency p_i. Alphafold2 predictions were performed by using CollabFold (Mirdita, et al. 2022) and visualized with PyMOL (Retrieved from http://www.pymol.org/pymol). Thymidine kinase and DNA-polymerase mutations were scored as tolerated or affecting protein function by SIFT predictions (Sim, et al. 2012). Principal component analysis was performed with a custom python script (https://github.com/hoeflet/antiviral-resistance-evolution.git). In brief, dN/dS ratios were standardized per open reading frame using the sklearn StandardScaler. Python's sklearn PCA package was utilized for two-dimensional principal component analysis and results were stored in pandas data frames for further use.

3.6.12 BAC Mutagenesis and Reverse Genetics

Viral mutants were reverse engineered using en passant mutagenesis in *E. coli* (Tischer, et al. 2010). In brief, transformation and recombination competent *E. coli* GS1783 [pHSV-1] or [pHSV-1Y557S] were transformed with a PCR product featuring a kanamycin resistance gene cassette flaked by homologous arms carrying the respective mutation and on one side a I-SceI restriction site (primers can be found in Sup. Table 1). Kanamycin resistant (KanR) clones were selected

and screened for insertion by colony PCR (cPCR, DreamTaq-polymerase, Thermo Scientific) and restriction fragment length polymorphism (RFLP) electrophoresis. After successful insertion at the particular loci, KanR clones were resolved by arabinose induced expression of I-SceI and temperature (42℃) induced expression of the recombination machinery. Kanamycin sensitive clones were again screened by cPCR and RFLP. Positive clones were further confirmed by high fidelity PCR (Q5, NEB) and Sanger sequencing (LGC Genomics). To generate differentially labelled viruses for competition assays the EGFP contained in the MiniF Region of our BAC construct was replaced with mCherry, by using a mCherry construct with the removable Kan-I-SceI cassette already inserted, as described earlier (Xing, Wang, et al. 2022).

3.6.13 Statistical Analysis

All statistics given in this study were performed in GraphPad Prism v.9.4.0. For further information regarding specific tests, please see the respective figure legends.

3.7 Code and Data Availability

Code and sequencing data are available via github (https://github.com/hoeflet/antiviral-resistance-evolution.git) and the NCBI SRA under BioProject accession number PRJNA927130, respectively.

3.8 Author Contribution

T.H. and J.T. conceived and designed the study. T.H., M.M.N. and M.Z. and J.Y.K. collected data. T.H. analyzed the data. T.H. and J.T. wrote the paper, all authors read, edited and agreed to the finished manuscript. J.T. supervised the study and provided funding.

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3.11 Conflict of Interest

The authors declare no conflict of interest.

3.12 Supplemental Material

Supplemental tables can be found at https://github.com/hoeflet/ThesisSupplemental.

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Chapter 4
Discussion

4.1 General Discussion

"A philosopher who is not taking part in discussions is like a boxer who never goes into the ring." Ludwig Wittgenstein, 1930

Herpes simplex virus (HSV) is among the most prevalent human pathogens on the planet (WHO 2023). Nearly every adult person is infected and harbors virus in a latent fashion. Although mostly considered harmless, severe complications including encephalitis, keratitis or neonatal infections do occur occasionally (Reichman 1984; Cherpes, et al. 2012). Even though no effective vaccine has been developed as of the writing of this thesis, antivirals against HSV and other related herpesviruses are available and effective (Lou, et al. 2014; Whitley and Baines 2018; Piret and Boivin 2021). Most of those are polymerase inhibitors and resistance against any one of them is already described (Kariya, et al. 2000; Sauerbrei, et al. 2015; Piret and Boivin 2016). Some newer developments include kinase inhibitors, terminase inhibitors as well as helicase/primase inhibitors (Crute, et al. 2002; Trofe, et al. 2008; Andrei, et al. 2009; Baines 2011; Li, et al. 2011; Kukhanova, et al. 2014; Ligat, et al. 2018; Pachota, et al. 2023). Recent studies also introduced the use of gene drive cassettes as a potential way to combat viral infections (Walter and Verdin 2020).

The aim of this thesis was to study the evolution of HSV-1. Here we established boundaries to the HSV-1 mutation rate and elucidated how those mutation rates influence the adaptability of those viruses. Highly proofreading deficient viruses are initially viable, but display suicidal behavior shortly after reconstitution. High levels of genetic load in concert with overall small population sizes due to inefficient transfection destines Exo I hypermutators to extinction (Bull, et al. 2007). However, we employed a stably replicating hypermutator to study antiviral resistance development and observed similar evolutionary behavior as in wild type (wt) populations, though accelerated. Accelerated evolution was observed phenotypically, genetically, as well as in within population dynamics. Overall, we found that HSV-1 can establish populations with increased mutation rates. However, high genomic loads need to be overcome by complementation and masking of deleterious and lethal variants, or by recombination and natural selection thereafter (Jaramillo, et al. 2013). Moderately increased mutation rates are tolerable and can be employed to accelerate evolution by providing more genetic diversity from which to select variants with higher

fitness (Xing, et al. 2022).

4.2 Viral Mutation Rates

"Evolutionary plasticity can be purchased only at the ruthlessly dear price of continuously sacrificing some individuals to death from unfavourable mutations. Bemoaning this imperfection of nature has, however, no place in a scientific treatment of this subject." Theodosius Dobzhansky

Per nucleotide mutation rates in viruses span multiple orders of magnitude (Sanjuan, et al. 2010). They are inversely correlated to their respective genome sizes, with smaller genomes featuring higher mutation rates and vice versa. Apart from genome size, genome nature and secondary structures play also important roles (Sanjuan and Domingo-Calap 2016). Therefore, even without considering the multitude of possible enzymatic interactions (Luftig 2014; Harris and Dudley 2015), the genomic starting conditions already determine where on the mutation rate scale the respective virus can be found (Sanjuan, et al. 2010; Geller, et al. 2015). However, replication strategies and modes also highly influence mutation rates (Sardanyes and Elena 2011).

4.2.1 How Do Viruses Mutate?

"Mutants. Since the discovery of their existence they have been regarded with fear, suspicion, often hatred. Across the planet, debate rages. Are mutants the next link in the evolutionary chain or simply a new species of humanity fighting for their share of the world? Either way it is a historical fact: Sharing the world has never been humanity's defining attribute." Bryan Singer, Lauren Shuler Donner & Ralph Winter, X2: X-Men United, 2003

Generally, RNA viruses feature higher mutation rates than DNA viruses (Sanjuan, et al. 2010). There are multiple reasons for that, for one RNA viruses usually feature smaller genomes. As soon as they reach genome sizes comparable to that of DNA viruses, the observed mutation rate also lowers, as exemplified by coronaviruses (Zhao, et al. 2004; Eckerle, et al. 2007; Graepel, et al. 2017; Domingo, et al. 2021). This is because small RNA viruses do not encode 3'-5' exonucleases. The transition from RNA to DNA as the major genetic material included the involvement of proofreading to increase genetic stability and replication fidelity (Forterre 2002). At what point viruses evolved is still up to debate, however, a popular theory postulates that RNA viruses first established themselves before DNA was the predominant genetic material (Forterre 2006; Koonin, et al. 2006).

Furthermore, since RNA and DNA metabolism are different from each other, and host cells are more versatile in working with DNA, repair and recombination systems are more readily available for DNA genomes (Anantharaman, et al. 2002; Luftig 2014). Therefore, DNA is more easily repaired than RNA.

Regardless of initial differences, as soon as error catastrophe is established and genome evolution favors mutational robustness over individual fitness, high mutation rates are difficult to abandon (Codoñer, et al. 2006; Lauring, et al. 2013; Fares 2015). Fitness selection, in that

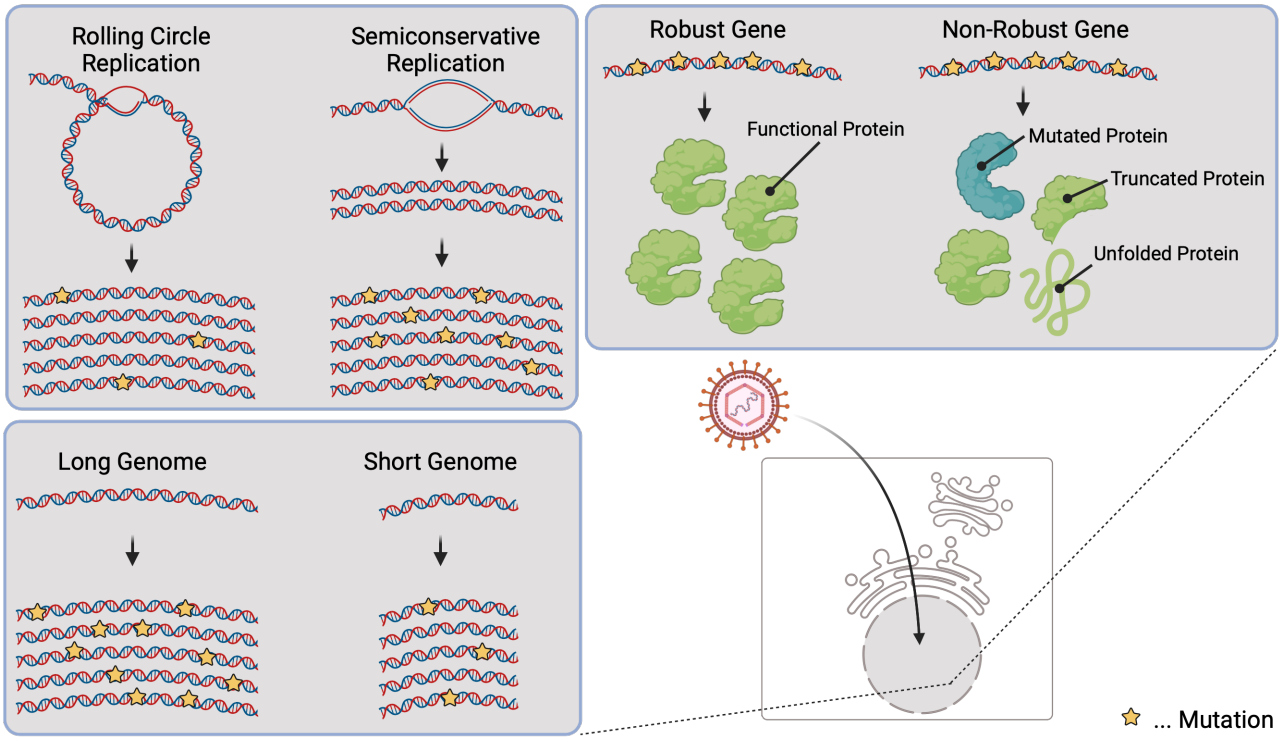

Figure 4.1: Requirements for hypermutation in HSV. Genomic structures influence mutation rates. Hypermutation is more likely if there is a stamp machine like mode of replication (like rolling circle replication). Additionally, smaller genomes allow for higher per nucleotide mutation rates. Finally, if essential genes develop a certain amount of mutational robustness, high mutation rates are less likely to cause detrimental outcomes.

scenario, mainly occurs via mutational linkage between closely related genotypes (Manrubia, et al. 2005; Domingo and Perales 2019). Individual genomes are not only selected because they are more fit than the average, but also because their surroundings in sequence space are favorable, resulting in a population structure that not necessarily features the most fit genotype at the highest frequency (Eigen 1971; Eigen and Schuster 1978). This flat landscape does not agree with lower mutation rates, because genotypes with substantial higher fitness are far removed in sequence space (Codoñer, et al. 2006). The population is trapped.

Therefore, in order for hypermutation in HSV-1 populations to arise, some necessary adaptations need to happen (Figure 4.1). Preferably, the genome would get significantly shortened. Since HSV-1 undergoes genomic rearrangements which can lead to large deletion events (Shitrit, et al. 2023), some self-replicating genomes might be able to survive. Additionally, essential genes should evolve some levels of mutational robustness to ensure functionality (Lauring, et al. 2013). Replication mode in HSV-1 is not fully understood, but rolling circle DNA replication is better tolerated for high mutation rates, as within cells only a few genomes act as templates (Boezen, et al. 2022). To conclude, hypermutation could arise spontaneously in HSV-1 if certain requirements are met, however, the specifications of those adaptations are as of now still unclear.

Earlier work with Marek's disease virus (MDV) could already establish an Exo III mutant (Pol^{Y567F}) that replicated and evolved with quasispecies-like dynamics (Trimpert, et al. 2019). MDV's lifestyle is somewhat different from HSV-1 (Osterrieder, et al. 2006). Firstly, MDV is highly cell associated, meaning that no cell free viremia is established in infected birds. Furthermore,

MDV is also oncogenic, causing the most prevalent cancer in the animal kingdom (Bertzbach, et al. 2020). Both of those reasons allow for a more nuanced intracellular interaction between co- and superinfecting genomes, masking deleterious variants and complementing one another (Xue, et al. 2016). Additionally, slower replication combined with larger initial population sizes might help MDV hypermutators to overcome the high genomic load (Osterrieder, et al. 2006; Trimpert, et al. 2019; Vychodil, et al. 2021; Xing, et al. 2022). Along those lines, preliminary data on human cytomegalovirus (HCMV) Exo I mutants Pol^{E303A} and $Pol^{D301A/E303A}$ shows that hypermutators go extinct within 5 passages in small, but maintain replication in larger populations. HCMV is the most diverse human herpesvirus, despite its relatively slow replication rates (Emery, et al. 1999; Renzette, et al. 2015; Cagliani, et al. 2020). Since HCMV Exo mutants can confer resistance to ganciclovir (Chen, et al. 2014), occurrences of such variants in patients might lead to the evolution of diverse HCMV genomes that feature mutational robustness. Overall, herpesviruses can establish hypermutating populations that are sustainable.

4.2.2 Drift-Barrier Hypothesis

"The two go hand in hand like a dance: chance flirts with necessity, randomness with determinism. To be sure, it is from this interchange that novelty and creativity arise in Nature, thereby yielding unique forms and novel structures." Eric Chaisson, Epic of Evolution: Seven Ages of the Cosmos, 2005

Genetic drift plays a substantial role for evolutionary change, even though it is very difficult to predict (Honnay 2013). Many evolutionary biologists formulate mathematical frameworks to describe genetic drift and incorporate it into their models, despite its random nature (Nagylaki 1978; Gillespie 2000; Chen, et al. 2017). With the introduction of the neutral theory of molecular evolution in 1968, Motoo Kimura first argued for a more substantial role of neutral mutations in evolution, and that these are mostly subject to genetic drift (Kimura 1968, 1983; Kimura 1991). Furthermore, genetic drift also decreases the force of selection in smaller populations, rendering evolution in those scenarios factually neutral (Saccheri and Hanski 2006; Bell and Collins 2008). Additionally, the difference between mutation rate and evolutionary rate determines whether evolution precedes in a neutral or deleterious fashion (Kimura 1989).

Genetic drift also plays an unappreciated role for mutation rate evolution (Lynch 2008). Mutations are difficult to balance, as most are deleterious or yield no viable offspring, however, some are beneficial and fuel adaptive evolution (Drake, et al. 1998). To decrease the amount of random deleterious mutations, mutation rates usually evolve towards higher replication fidelity, but there is a lower limit for mutation rates which is defined by genetic drift (Lynch 2011). This boundary is known as the drift-barrier and the concept itself consequently as the drift-barrier hypothesis (DBH). It postulates that any molecular trait can only improve in functionality until the effect of beneficial mutations and subsequent selection is outperformed by genetic drift (Lynch, et al. 2016). This is universally applicable for any trait such a growth rate, body mass, genome structure as well as organismal complexity, mutation rates and speciation events (Lynch and Conery 2003; Lynch

2007; Lynch and Walsh 2007; Lynch, et al. 2022; Bergeron, et al. 2023; Kalirad, et al. 2024). There have been experimental studies that explored mutation rate evolution in terms of the DBH. A study in which yeast was evolved under clonal bottleneck conditions and without mismatch repair (MMR) showed that, after restoration of MMR, mutation rates were comparable to that of the progenitor and other natural yeast isolates (Liu and Zhang 2021). The authors there failed to measure mutation rates in the MMR^- progenitor and in the MMR^- evolved strains, therefore it is difficult to evaluate whether the observed effects are in line with the DBH. However, another study in *E. coli* observed evolutionary behavior according to the DBH (Wei, et al. 2022). Mutation rates increased in smaller populations as well as due to environmental factors such as resource depletion in wt populations. However, MMR^- populations generally evolved towards lower mutations rates, regardless of population bottlenecks. This indicates that higher initial mutations rates, far removed from the drift-barrier, do not respond to genetic drift as wt rates would.

In terms of mutation rate evolution in herpesviruses this might explain why genome replication occurs with such high fidelity. Genomic studies of transmission pairs observe survival of numerous low frequency SNPs (Rathbun, et al. 2022). This points towards large infectious inoculums and subsequently less genetic drift. Furthermore, lifelong latency of herpesviruses also increases the overall population size (Everett 2014; Rathbun and Szpara 2021). These increases in overall population size, by life style and broad transmission bottlenecks, might explain relatively low mutation rates in herpesviruses.

4.3 Practical Implications

"Be practical as well as generous in your ideals. Keep your eyes on the stars, but remember to keep your feet on the ground." Theodore Roosevelt

The findings of this thesis have some important practical implications, that should not be overlooked. First among them may be that viral evolution is often neglected when considering clinical problems (Iwasa, et al. 2005; Piret and Boivin 2016; Christaki, et al. 2020; Rathbun and Szpara 2021). Applying antiviral therapy should go hand in hand with thinking about potential resistances and clinical outcome. In order to consider those potential risks, more information about what facilitates resistance and how it can be avoided is necessary (Regoes and Bonhoeffer 2006; Andrei, et al. 2009; Whitley and Baines 2018). Furthermore, our works shows that lethal mutagenesis in herpesviruses is possible and could technically be combined with other antivirals to achieve better results (Bull, et al. 2007; Swanstrom and Schinazi 2022). Implications and applications of lethal mutagenesis and accelerated experimental evolution are discussed below.

4.3.1 Lethal Mutagenesis in Herpesviruses

"We are all mutants with ticking time bombs hidden inside." Lone Frank, My Beautiful Genome: Exposing Our Genetic Future, One Quirk at a Time, 2010

Here we report that highly proof-reading deficient HSV-1 yields suicidal virus populations. Generally, lethal mutagenesis has evolutionary and demographic components, meaning that lowering the population mean fitness by means of hypermutation does not necessarily mean population extinction (Bull, et al. 2007). Antiviral therapy in herpesviruses is widely distributed and applied, however, resistance against all used antivirals is already described (Kariya, et al. 2000; Bestman-Smith and Boivin 2003; Kukhanova, et al. 2014; Piret and Boivin 2016). Using specific exonuclease inhibitors in combination with approved anti-herpetics probably would yield better results and might increase the barrier for resistance development (DiScipio, et al. 2022). As of now, no resistance against exonuclease inhibitors has been described, as necessary experimental evolution studies have not yet been done. Lethal mutagenesis itself is a very robust antiviral mechanism, yet, resistance against incorporation of the mutagen into the polymerase or the DNA strand could rapidly develop (Beaucourt and Vignuzzi 2014). Resistances associated with antiviral mutagens often increase replication fidelity. Our earlier work with MDV showed that highly proofreading deficient Exo I mutants select for amino acid changes in the Pol finger and palm domains (Trimpert, et al. 2019). Those very likely increase fidelity, as homologous changes in HSV-1 do (preliminary data, not shown). Furthermore, suboptimal concentrations of the mutagen can lead to only marginal increases in mutation rates which in turn would enable viral populations to benefit from accelerated evolution by increasing genetic variability (Sanjuán 2012; Xing, et al. 2022).

Lethal mutagenesis in HSV-1 works probably as well as we observed, because the genome did not yet evolve the necessary mutational robustness to cope with hypermutation (Lauring, et al. 2013; Fares 2015). However suboptimal mutagen concentrations again could provide the circumstances for the virus to establish enough error catastrophe to evolve towards higher genomic stability (Holmes 2009). Although lethal mutagenesis seems a valuable and promising new therapy for herpesvirus infections, potential development of resistance and therapeutic ranges for mutagens and exonuclease inhibitors need to be established in advance.

4.3.2 Accelerated Experimental Evolution

"There's no short-cut in life but with right knowledge, you can fast-track things to come to pass." Ifeanyi Enoch Onuoha

HSV-1 virus populations that display higher adaptability and evolvability are potentially very valuable for future experimental evolution studies (Xing, et al. 2022). Especially phenotypes that generally are very complex might be evolvable if the initial mutational input and genetic variability is high enough. Some of those phenotypes include, but are not limited to host spillovers and within-host speciation, immune evasion and virulence evolution, social interaction within viral population as well as overall genome evolution of herpesviruses (Hamilton 1964; Witter 1997; Vossen, et al. 2002; Gavrilets and Vose 2005; Moratorio, et al. 2017; Trimpert, et al. 2017; Brito, et al. 2021; Leeks, Bono, et al. 2023).

Especially the concept of social evolution in viruses is getting more and more attention from researchers and evolutionary theorists alike (Díaz-Muñoz, et al. 2017; Sanjuán 2021; Leeks, Bono, et al. 2023). As social interaction can be mutual beneficial or exploitative, some very interesting evolutionary dynamics could be involved (Sanjuán 2021). For example, HSV-1 was recently shown to produce certain noncanonical genomic rearrangements during infectious cycles in different cells (Shitrit, et al. 2023). Those generally are known as defective interfering genomes (DIG), selfish genomes or cheats (Rezelj, et al. 2018; Vignuzzi and López 2019; Leeks, et al. 2021; Leeks, Young, et al. 2023). Many studies describe similar genetic occurrences in RNA virus populations, there are even studies that use DIGs as antivirals to lessen infectious burdens (Dimmock and Easton 2014; Rezelj, et al. 2021). But if cheats are necessarily interfering with productive replication, why do viral populations protect themselves so poorly? A potential defense mechanism would be superinfection exclusion as well as privatization of viral gene products (Zou, et al. 2009; Criddle, et al. 2016; Perdoncini Carvalho, et al. 2022; Sims, et al. 2023). Are cheats mostly produced as a byproduct of fast genome replication without substantial quality control or do they fulfill some evolutionary benefits? Some studies speculate that DIGs are produced and packaged to act as a decoy for humoral immune factors (Handke, et al. 2009). Others theorize that the overactivation of innate immune responses could be employed to allow functional virus to explore more distant tissues (Vignuzzi and López 2019). Furthermore, beneficial aspects of increasing effective mutation rates by accumulating mutations on DIGs and reintroducing them into functional genome via recombination were brought up. Social interactions between individual viral genomes are highly relevant for clinical outcomes and can be very complex depending on how many different parties are involved (Díaz-Muñoz, et al. 2017). Accelerated experimental evolution might be one of the few methods to study those occurrences in reasonable short timeframes.

Other phenotypes which would probably be too complex to study under wt-mutation rate conditions include extended phenotypes, like manipulation of host behavior or restructuring of transmission sites (Martinez, et al. 2012; Dawkins 2016). Cold sores for example are transmission sites for HSV-1. Variants of the virus that manage to produce more virions per cold sore would feature higher transmission rates and therefore are evolutionarily more fit. Along those lines, viruses that could somehow influence host hormones or neurotransmitters could manipulate host behavior in a way to maximize their transmission. As of now, no such phenotypes have been studied in herpesviruses. Hypermutator based accelerated experimental evolution however, would allow for circumstances under which such phenotypes could develop more easily.

4.4 Final Remarks and Outlook

"Then there they were... the last words." A.D. Aliwat, In Limbo, 2021

All replicating stretches of nucleic acid are subject to evolution. However, how evolution influences the genetic diversity in different populations is very much dependent on genetic and demographic

structures and parameters (Gillespie 2004). Viruses are very diverse, coming in different flavors, sizes and styles, which makes it very difficult to introduce a general framework to study their evolution (Baltimore 1971; Elena and Sanjuán 2007; Peck and Lauring 2018; Koonin, et al. 2021). This thesis provides valuable insight into the limits and advantages of increased mutation rates for *in vitro* replication of HSV-1 populations (Figure 4.2).

New evolutionary inventions are very difficult to obtain by employing single nucleotide mutations only. Therefore, genomic rearrangements or multiple nucleotide changes that happen simultaneously are needed to evolve complicated phenotypes (Arber 1991; Jetzt, et al. 2000; Schumacher, et al. 2012; Luftig 2014; Sanjuan and Domingo-Calap 2016). Consequently, high fidelity genome replication is not able to produce the necessary genetic diversity to substantially change phenotypic behavior. However, over the course of thousands of years, viruses and all other organisms could establish a remarkable array of diverging phenotypes. One way to accelerate those innovations is by increasing mutations rates and increase overall genetic diversity, which in turn allows for multi-locus selection and co-evolution of different gene variants on single genomes (Holmes and Rambaut 2004; Elena and Sanjuán 2007; Sardanyes and Elena 2011; Lyons and Lauring 2018). However, another way to gather the genetic requirement for innovation is recombination and also the capture of host genes (Loncoman, et al. 2017; Schönrich, et al. 2017; Fixsen, et al. 2022). This allows for easy incorporation of foreign genetic material and facilitates *de novo* gene birth. Herpesviruses are known to occasionally capture host genes and employ them for immunomodulatory functions (Schönrich, et al. 2017). Furthermore, some herpesviruses do integrate into host chromosomes (Morissette and Flamand 2010; Kheimar, et al. 2017; Aimola, et al. 2020; You, et al. 2021; Wight, et al. 2022), which upon excision might allow for stealing more genetic material. As herpesviruses that integrate into host DNA usually also feature arrays of telomeric repeats, those themselves might have been ancient gene capture events that nowadays allow for easy genome integration and latent persistence of viral DNA.

This study established different hypermutator HSV-1 viruses that can be used for accelerated experimental evolution. Amino acid exchange Y557S in the Pol Exo III domain increases mutation rates to an extent that simple phenotypes conferred by little genetic change can arise substantially faster than in the wt (Figure 4.2). In contrast, hypermutation conferred by Exo I amino acid changes is so substantial that, under the conditions tested, no population could sustain itself to overcome the created genomic load. We recently found compensatory mutations in the polymerase fingers and palm domains that were readily selected in Exo I MDV populations (Trimpert, et al. 2019). Introducing the homologous mutations into HSV-1 Exo I mutants allowed for more substantial increases in mutation rates, while still maintaining an overall wt-like growth phenotype. The compensatory mutations (F918L and Q807H respectively) by themselves increase replication fidelity, barely producing mismatches at all (preliminary data, not shown). Since higher fidelity genome replication can be achieved without proofreading, some fidelity/speed trade-offs seem to be causal (Regoes, et al. 2013; Fitzsimmons, et al. 2018). The F918L change is located within the palm domain, the catalytic center of the polymerase reaction (Knopf and Weisshart

1988; Trimpert and Osterrieder 2019). It seems very likely that this alteration allows for slower but more accurate incorporation of correctly pairing nucleotides. The Q807H change is located in the finger domain. While not being in the very center of the reaction, the finger domain interacts with incoming nucleotides (Hwang, et al. 1992; Trimpert and Osterrieder 2019). Therefore, high fidelity might be achievable by discriminating more selectively between the nucleotide substrates.

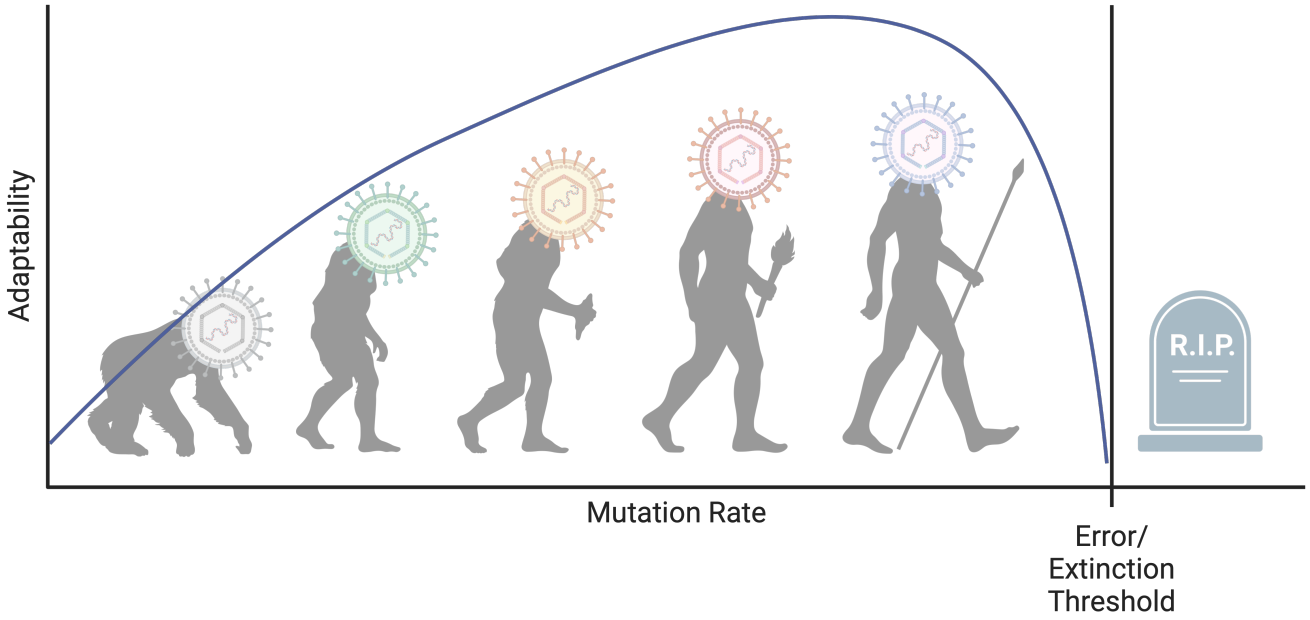

Figure 4.2: Mutation rates influence adaptability and survival of HSV-1 populations. Increases in the HSV-1 mutation rate initially also increase adaptability. However, as soon as the error/extinction threshold is crossed lethal mutagenesis takes place and rapidly abolishes the number viable offspring.

The fact that Exo I mutants are initially viable and only go extinct after accumulating huge genomic loads, as well as the survival of proofreading deficient hypermutators with compensatory mutations argues for a less essential role of the 3'-5' exonuclease in other areas than replication fidelity itself (Marcy, et al. 1990; Lawler and Coen 2018). This suggests that it might be possible to engineer an HSV-1 hypermutator that behaves according to quasispecies dynamics (Figure 4.1). Ways to accomplish that might require streamlining of the genome by deleting non-essential parts. Furthermore, selecting for mutational robustness of streamlined genomes would overall increase the genetic and phenotypic stability of the population. Stepwise engineering of HSV-1 viruses with quasispecies behavior would enlighten the requirements for such in the first place. Additionally, if such a virus could be produced, reintroducing the high-fidelity polymerase would allow for a nice demonstration of how important low fidelity genome replication is for mutationally robust quasispecies, without compromising the replication speed, as high-fidelity mutants in RNA viruses are known to do (Regoes, et al. 2013; Fitzsimmons, et al. 2018).

Overall, the mutants described in this study can be used to further study the relationship between replication speed and fidelity, to speed up evolutionary processes and to study more demanding and complicated phenotypes. Evolution is a process mostly dependent on chance.

Something occurs randomly and selection acts upon whatever is present and works in the specific environment and context (Gillespie 2004). This study acted as the producing force to creating new tools for researchers across the world to employ. From here on, the scientific community acts as the selective force that decides how to use the new material.

Abstracts

On Fidelity, Adaptation and Reproduction: A Study of Hypermutation in Herpes Simplex Virus 1

Balancing mutation rates always comes with trade-offs. Often deleterious or even lethal, mutations also produce the necessary genetic diversity to fuel evolution by natural selection. Many factors influence viral mutation rates; however, replicative polymerases play an essential role. Large dsDNA viruses, like herpesviruses, encode a DNA-polymerase with intrinsic 3'-5' exonuclease (Exo) activity, increasing replication fidelity by orders of magnitude. Abolishing this proofreading consequently yields viral populations with increased mutation rates.

Here we described the first successful reconstitution of proofreading deficient herpes simplex type 1 viruses. Although those viruses are initially viable, we found that after a few passages they accumulate huge mutational loads that interfere with productive viral replication. Especially deleterious mutations in major variants map specifically to essential gene sets important for nucleic acid metabolism, structural proteins and others. Notably, mild effects on proofreading conferred by Exo III mutant Y557S (YS) did increase mutation rates but yielded populations with wild type (wt) like replication kinetics.

To follow up on the adaptability of mild hypermutators we passaged both wt and YS under antiviral pressure to screen for resistance mutations. We observed faster resistance development in YS by about 5-10 passages. This increase in adaptability is due to 2-3 times more mutations occurring in YS populations compared to wt. Importantly, we could detect no general differences in the underlying evolutionary processes. Furthermore, changing selection pressures shed light on the importance of standing genetic variation, especially in populations that already plateaued in their phenotypes. Finally, within population diversity on a phenotypic and genetic level is vastly increased in YS populations.

Taken together we managed to explore the limits of hypermutation in herpes simplex type 1. Marginal increases in their mutation rates lead to greater adaptability which in turn can be employed to speed up evolutionary processes for experimental applications without compromising the underlying mechanism. However, increases beyond that, mediated by abolishing proofreading completely, lead to lethal mutagenesis and suicidal phenotypes. So far lethal mutagenesis is a process exclusively described for RNA virus populations. Defining the circumstances for lethal mutagenesis in herpes simplex virus type 1 might be interesting for future antiviral developments.

Especially combination of replicative in concert with Exo inhibitors might yield promising antiviral therapies.

Über Treue, Anpassung und Reproduktion: Eine Studie über Hypermutation in Herpes Simplex Virus 1

Jede Mutation gleicht einer Runde am Roulette Tisch. Obwohl eine geringe Chance besteht den großen Gewinn abzuräumen, verliert man meist doch. Genauso verhält es sich mit Mutationen, der überwiegende Teil ist schädlich oder sogar letal, dennoch besteht die Möglichkeit mit hilfreichen Änderungen einen Vorteil zu erlangen und den Rest der Population abzuhängen.

Mutationsraten werden durch eine Vielzahl von Faktoren beeinflusst, jedoch spielen replikative Polymerasen eine entscheidende Rolle. Doppelstrang DNA Viren mit relativ großen Genomen, wie Herpesviren, codieren eine eigene DNA-Polymerase mit eingebauter 3‘-5‘ Exonuklease (Exo) welche jede eingebaute Base korrekturliest um Mutationsraten um Größenordnungen zu verringern. Ein Verlust der Korrekturlesefunktion erhöht folglich die Mutationsrate.

In dieser Arbeit beschreiben wir erstmals die erfolgreiche Herstellung von Korrekturlese defizienten Herpes Simplex Typ 1 Viren. Obwohl anfangs lebensfähig, konnten wir zeigen, dass wenige Zellkultur Passagen zur Ansammlung von enorm vielen Mutationen in diesen Populationen führte. Diese wiederum, verhindern eine produktive Virusreplikation. Auffällig waren dabei vor allem schädliche Mutationen in essentiellen Gengruppen, die in der Population überhandnahmen. Diese Gene sind wichtig für die Struktur des viralen Partikels, für den Nukleinsäure Stoffwechsel als auch für weitere Prozesse. Eine mildere Reduktion der Exo Funktion war hingegen gut verträglich und resultierte in einen Hypermutator der Wildtyp (wt) ähnliche Replikationskinetik aufwies.

Dieser milde Hypermutator (YS) wurde verwendet um die Evolution von Virostatika Resistenz zu untersuchen. YS wies schnellere Resistenzentwicklung auf, was auf 2-3-mal mehr Mutationen in diesen Populationen zurückgeführt werden konnte. Beeindruckender Weise konnten wir keine generellen Unterschiede in den zu Grunde liegenden evolutionären Prozessen sehen, außer die zuvor erwähnte höhere Mutationsrate. Des Weiteren, konnten wir diese Ergebnisse auf klonaler Ebene bestätigen und auch die Bedeutung von genetischer Variabilität hervorheben, besonders wenn Änderungen in den Selektionsdrücken stattfinden.

Zusammenfassend behandelt diese Arbeit die Vor- und Nachteile von Mutationen in Herpes Simplex Virus Typ 1. Erhöhungen in der Mutationsrate sind möglich, in gewissem Umfang und unter bestimmten Bedingungen sogar hilfreich, sobald allerdings ein kritischer Punkt erreicht wird, wird aus erhöhter Anpassungsfähigkeit letale Mutagenese, welche wiederum zum Aussterben der Population führt. Bisher wurde letale Mutagenese nur in RNA Virus Populationen beschrieben, allerdings zeigt diese Arbeit, dass letale Mutagenese auch in Herpes Simplex Typ 1 stattfinden kann. Dies könnte unter anderem zu neuen antiviralen Therapien führen, welche beispielsweise

replikative und Exo Inhibitoren kombinieren um Viren an zwei Fronten zu bekämpfen.

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Höfler T., Nascimento M., Trimpert J. **Talk** (22th of March 2022): **Adaptability of Proofreading Impaired Viruses: Why Making Mistakes is Not Too Bad!** 1st "The Social Lives of Viruses" Meeting, Oxford, UK

Höfler T., Brunialti M., Xing N., Nascimento M., Trimpert J. **Talk** (31st of August 2022): **Risks and Benefits of Hypermutation in Herpesviruses.** ZIBI Retreat, Berlin, Germany

Höfler T., Xing N., Brunialti M., Nascimento M., Zeitlow M., Trimpert J. **Poster** (29th of March 2023): **Hypermutation in Herpesviruses - From Lethal Mutagenesis to Increased Adaptability.** 32nd Annual Meeting of the Society for Virology, Ulm, Germany

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Appendix

About Nomenclature

The scientific community subscripted to certain rules to name and write things. Whenever a certain level of taxonomical classification is mentioned in this text the name of said taxa is written in italic font (e.g. *Herpesviridae*). Furthermore, genes and transcripts are also written in italic, whereas protein names are written in roman font (compare gene *UL9* to protein UL9). For many gene/protein combinations I tried to use different names to avoid confusion (e.g. *UL30* and Pol). Lastly, Latin descriptions were also given in italic font. Those include phrases like *in vitro, in vivo, de novo, in sito, in silico* and *ex vivo*. Note that not all of those phrases are used in this thesis.

Glossary

Adaptive Radiation

Rapid speciation event, in which one species subdivides into multiple which utilize different ecological niches. See introduction section 1.2.3 and figure 1.6.

Alphafold

Alphafold (versions 1-3 available as of the writing of this thesis) is a deep learning based protein structure prediction tool developed by Google Deepmind.

Antivirals

See introduction section 1.3.

Antibodies

See introduction section 1.3.1.

Apoptosis

Programmed cell death. Different from necrotic or pyroptotic cell deaths.

Baltimore Scheme

See introduction section 1.1.1.

Bivalves

Bivalves are a class of animals (*Bivalvia*) that is part of the phylum *Mollusca*. They include marine and freshwater molluscs like mussels, clams and oysters.

Bottleneck

A dilution process at which a large population is reduced to a substantially smaller one. Infectious doses or founding populations are examples of bottlenecks. Consequently, bottlenecks are contributing massively to the amount of genetic drift between transmission events.

Capsid

A capsid is the protein shell that surrounds the viral genome. Virologist differentiate between helical, icosahedral or complex structure.

Centromere

The central part of eukaryotic chromosomes. This area is tightly packed and acts as the connection points for sister chromatids after DNA-replication.

Codon (Pair) Deoptimization

Organisms tend to have certain biases as to which codons they prefer. This is influenced by tRNA availability, protein folding, GC content, tolerance for CpG dinucleotides and more. Similarly, also codon pairs - meaning which codon follows which - are biased. This allows researchers and vaccine manufactures to deoptimize coding regions in order to attenuate pathogens. Codon deoptimization and codon pair deoptimization is abbreviated as CD and CPD, respectively.

Complement System

Pathway that complements antibodies in the humoral immune system. It consists of a proteolytic cascade that can get activated by multiple ways, which results in marking pathogens or infected cells for destruction.

Concatemeric DNA

DNA is considered concatemeric if it is made up of multiple repetitions of the same genomic sequence in a linear fashion.

Cytopathic Effect

Observable difference in cell morphology between infected and uninfected cells. Abbreviated as CPE.

***de novo* Gene Birth**

The spontaneous acquirement of molecular function by any stretch of genomic material is known as *de novo* gene birth. For protein coding genes this requires transcription and translation initiating sites as well as the absence of multiple in-frame stop codons.

Defective Interfering Particles/Genomes

Abbreviated as DIP or DIG. See introduction section 1.2.4.

DNA-Ligase

DNA ligases covalently join DNA strands in an 5'-3' manner. See introduction section 1.1.3.

DNA Mismatch Repair

DNA repair mechanism that fixes mismatches independently from replication. Important for mismatch repair are MSH and MLH proteins. Abbreviated as MMR.

DNA-Terminase

The terminase complex consists of multiple proteins which recognize packaging sites on viral DNA, package genomes into forming capsids and cut DNA afterwards. See introduction section 1.1.3.

DNA-Polymerase

See introduction section 1.1.3 and figure 1.3A.

Drift-Barrier Hypothesis

Abbreviated as DBH. See discussion section 4.2.2.

E3 Ubiquitin Ligase

Protein involved in the post-translational modification of ubiquitination. E3 ubiquitin ligases are specific for the protein substrates they ubiquitinate. Mono-ubiquitination can be modulatory, poly-ubiquitination marks proteins for degradation by the proteosome.

Ecological Niche

The combination of resources and conditions required for a particular species to exist in a given habitat. The "job" of the species. See introduction section 1.2.3.

Endosomal Sorting Complex Required for Transport

Protein complex involved in membrane restructuring, vesicle transport, viral entry and budding, protein turnover and more. Abbreviated as ESCRT.

Epitope

Antigenic structure recognized by adaptive immune effectors such as antibodies and T-cell receptors.

Error Threshold/Catastrophe and Extinction Threshold

Distinctly different phase shifts in lethal mutagenesis and quasispecies dynamics. Error threshold describes the transition point at which mutational robustness is favoured over individual high fitness. Error catastrophe indicates a successive loss of genetic information by accumulating high levels of mutations, it occurs at mutation rates beyond the error threshold. The extinction threshold defines the mutation rate at which the genotype replacement rate gets smaller than one. See introduction section 1.3.1.

Exon

Functional subunit of a gene. Exons are assembled by splicing introns from the pre-mature RNA. See also glossary entry for introns.

Exploitation

Exploitation describes an indirect form of competition that occurs by depletion of resources. See introduction section 1.2.3 and figure 1.6.

Extended Phenotypes

Phenotypic manifestations beyond the confines of the individual body are generally considered to be extended phenotypes. Those are most often divided into zoological architecture, action at a distance and parasite manipulation of host behavior. See discussion section 4.3.2.

Extinction

Ecological process that describes the loss or death of a species. Extinction is ecologically opposing speciation and contributes to the species turnover in the biosphere.

Fitness

Fitness describes the survival and eventual contribution to the next generation (that is offspring) of an individual relative to others in the same habitat. See introduction section 1.2.1.

Genetic Drift

See introduction section 1.2.1 and figure 1.4.

Genetic Load

Genetic load describes the relative difference between the mean and maximum fitness in the population. It corresponds to the amount of deleterious mutations at the particular locus. When talking about the whole genome the term genomic load is used. See introduction section 1.2.1.

Github

Online repository for software and version control.

Habitat

The physical space a species exists in. The "address" of the species.

Haplotype

Haplotypes define which genomic variants are co-occurring on the same molecule. For animal species it usually describes the haploid chromosome set.

Hardy-Weinberg Equilibrium

See introduction section 1.2.1.

Heterozygosity

Heterozygosity is a population genetics based measurement for genetic diversity at a particular locus. It is based on individual allele frequencies and describes the likelihood of two alleles, randomly drawn from the population's gene pool, to be different from each other by state. See introduction section 1.2.1.

Holoenzyme

An enzyme that includes multiple molecular functions within one polypeptide. For example, the HSV-1 DNA-polymerase (encoded by *UL30*) features polymerase as well as 3'-5' exonuclease (see glossary entry for proofreading) functions within one holoenzyme.

Homologous

Homologous means that something is comparable or analogue. In evolutionary biology it defines genes or structures that are related and come from a common ancestor.

Host Spillover

Host Spillover describes the instance when a pathogen gains the ability to infect a new host species, in which it did not circulate initially.

Hybrid

Offspring resulting from mating of members from different species. Hybrids are often sterile. See introduction section 1.2.4.

I-SceI

I-SceI is a mitochondrial homing endonuclease isolated from *Saccharomyces cerevisiae*.

Interference

Interference describes a direct form of competition that occurs by physically restricting an individuals access to resources. See introduction section 1.2.3 and figure 1.6.

Intron

Introns are DNA sequences interspersed in functional genes. They get excised from pre-RNAs by splicing.

Kinase

Enzyme that phosphorylates substrates (e.g. proteins or other ligands). Reaction is ATP dependent.

Kin Selection

Kin selection is one of the cornerstones of social evolutionary theory. It describes social interaction based on how related the individuals are, which in turn influences selection and evolution as a

whole. Important is Hamilton's rule for altruistic behavior:

$$rb > c \tag{A1}$$

where r is the relatedness between actor and recipient, b is the benefit for the recipient and c the cost for the actor. This in turn leads to the observation that individuals act to maximize their inclusive fitness, which is the direct fitness of the individual void of all the influences of the social environment + the indirect fitness the individual gains from beneficial or harmful behavoir towards members of the social environment.

Latency

Dormant state of a virus, often benign without any clinical symptoms. See introduction section 1.1.3.

Lethal Mutagenesis

See introduction section 1.3.1.

microRNA

Small non-coding RNA that have multiple functions. Most prominently they influence gene expression and epigenetic modification.

Muller's Ratchet

Accumulation of numerous deleterious mutations due to a lack of recombination, reassortment and/or sexual reproduction.

Multipartite Virus

A virus with segmented genome which is packaged into physically different particles. Successful viral replication requires all particles carrying individual genome segments to co-infect the same cell.

Multiplicity of Infection

Statistical ratio that defines how much virus infects how many cells. Calculated as:

$$MOI = \frac{n_{pfu}}{n_{cells}} \tag{A2}$$

with MOI being the multiplicity of infection, n_{pfu} the number of plaque forming units (or any other measurement for infectious virus) and n_{cells} the number of cells. A poisson distribution describes which part of the cell population harbours how many viruses:

$$P(n) = \frac{MOI^n}{n!} e^{-MOI} \tag{A3}$$

Where MOI is the mean as well as the variance of the distribution, n is the number of viral particles infecting a single cell, and $P(n)$ the proportion of cells infected with n viral particles.

Mutation

See introduction section 1.2.1 and figure 1.4.

Mutational Robustness

Genes that can endure many mutations without much impact on the selective advantage of the mutated allele are considered mutational robust.

Natural Selection

See introduction section 1.2.1 and figure 1.4.

Neuraminidase

Enzyme that cleaves sialic acid from glycans.

Neutral Theory of Molecular Evolution

Introduced by Motoo Kimura in 1968, the neutral theory of molecular evolution states that most allelic variation is in fact without selective impact. This allows for the creation of genetic diversity without the interference of natural selection. Only once beneficial or deleterious mutation occur can selection itself act upon them. See discussion section 4.2.2.

Non-Synonymous to Synonymous Substitution Rates

Value used in population genetics to measure selection in protein coding genes. Ratios above 2 usually describe proteins under positive selection whereas ratios below 1 define purifying selection. See chapter 3 methods section. Abbreviated as dN/dS.

Nuclear Egress Complex

Protein complex which mediates the transport of viral capsids out of the nucleus and into the cytoplasm. See introduction section 1.1.3.

Nucleoside Analogue

Biomolecule that mimics the structure of canonical nucleosides (base + sugar backbone).

Open Reading Frame

Protein coding nucleic acid sequence. Features a start and stop codon with mulitple amino acid coding codons in between. Frames define sequences of nucleotide triplets, which consequently results in three potential frames for any nucleotide sequence. Abbreviated as ORF.

Opsonization

The marking of molecular structures for phagocytosis.

Phagocytosis

Engulfment of extracellular structures by specialized cells (phagocytes or macrophages).

Phylogenetics

Phylogenetics studies the evolutionary relationships between species, individuals, genomic or protein sequences. The most common representation is the phylogenetic tree.

Plaque/Focus

Plaques are holes in cell culture monolayers, that occur after a virus kills cells in a concentric manner. Infectious foci (singular focus) however, are clusters of cells that show cytopathic effects (see glossary entry on cytopathic effects) but do not come off the monolayer.

Ploidy

Ploidy defines the number of chromosome sets. Haploid organisms feature only one copy of each chromosome whereas diploids and triploids carry two and three respectively.

Polymerase Chain Reaction

Laboratory method to amplify stretches of DNA. Requires primers, dNTPs, heat-stable DNA-polymerases, Mg^{2+}, template DNA and a specific temperature programme. Abbreviated as PCR.

Portal

An opening in one of the 12 icosahedral vertices of the herpesvirus particle. It allows entrance and exit for DNA in order to get packaged and released. See introduction section 1.1.3

Prevalence

Prevalence indicates how common certain things are. For infectious diseases prevalence is used for endemic pathogens to describe which percentage of the population carries said pathogen.

Principal Component Analysis

Mathematical break-down of multi-dimensional data into underlying components which describe the observed variability. Abbreviated as PCA.

Privatization

Defense mechanism against co- and superinfection. It makes sure that viral gene products are only utilized for the replication and transmission of the genome they originated from.

Proofreading

Molecular process that increases genome replication fidelity. Mediated by 3'-5' exonucleases that degrade wrongly incorporated nucleotides and allows for error correction. See introduction section 1.1.3, figure 1.3A as well as throughout chapters 2 and 3.

Protease

Enzyme that degrades proteins.

Quasispecies

See introduction section 1.2.2 and figure 1.5C.

Recombination

A molecular process that rearranges genetic material. There are different mechanisms: homologous recombination, single-strand annealing recombination, non-homologous end-joining, site-specific recombination and more. See introduction section 1.1.3 and figure 1.3B.

Reconstitution

Reconstitution is the process of recovering virus from DNA.

Red Queen Hypothesis

The Red Queen Hypothesis (abbreviated as RQH) describes the intimate adaptive relationship between coexisting species, especially antagonistic interactions like predator-prey or host-pathogen. See introduction section 1.2.4.

Replication

See introduction section 1.1.3 and figure 1.3A.

Rolling Circle DNA Replication

DNA-replication mode for circular DNA. One strand is cut and elongated while the old strand gets replaced. This yields long concatemeric DNA that is only copied from a single template.

Satellite Virus

A virus that cannot establish an infection without a helper virus. See also glossary entry on defective interfering particles/genomes and introduction section 1.2.4.

Sequence Space

See introduction section 1.2.2.

Speciation

The evolutionary process of forming new taxonomic species is called speciation. What is considered a species however is less strictly defined. For animals individuals are considered part of the same species if they can mate and produce fertile offspring. For non-sexual individuals it usually is defined on a sequence level. There are different forms of speciation, depending on the spacial organisation of the originating and new populations.

Standing Genetic Variation

Standing genetic variation, contrarily to *de novo* mutations, describes already present allelic variation within a population.

Superinfection Exclusion

Abbreviated as SIE. See introduction section 1.2.4.

Tegument

See introduction section 1.1.3 and figure 1.2B.

Telomere

The outer part of eukaryotic chromosomes. This area consists of repetitive sequences that shorten after DNA-replication.

Topoisomerase

Topoisomerases are enzymes that change the topology of DNA. More precisely, they change the super coiling of DNA by cutting and ligating DNA strands.

Transition

Nucleic acid mutation which replaces a pyrimidine with a pyrimidine or a purine with a purine.

Transversion

Nucleic acid mutation which replaces a pyrimidine with a purine or vice versa.

Translesion Polymerase

DNA-polymerases that are required for DNA-replication, recombination and repair. They fill in gaps by overriding unconventional nucleotide conformations (e.g. thymidine dimers) and generally feature low replication fidelity due to a lack of proofreading.

Triangulation Number

The triangulation number of an icosahedral capsid defines how many viral capsid proteins are necessary to form the structure as well as how they are arranged. See also introduction section 1.1.3 and figure 1.2B.

Tropism

See introduction section 1.1.3.

Virema

Infectious phase in which virus can be detected in the host's blood.

Virions

The word virion describes an infectious viral particle. As not all particles are capable of initiating an infectious cycle, virologist differentiate between virions and viral particles (which include virions and non-infectious particles).

Virulence

Virulence is the ability of a pathogen to cause disease.

Virus

See introduction section 1.1.1.

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English

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Conflict of Interests

I declare no conflict of interest.

Declaration of Independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated.

Berlin, 22.11.2024 Thomas Höfler