DISSERTATIONES
TECHNOLOGIAE
UNIVERSITATIS
TARTUENSIS
23

MARIT ORAV

Study of the initial amplification of the human papillomavirus genome

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
23

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
23

MARIT ORAV

Study of the initial amplification of the human papillomavirus genome

UNIVERSITY OF TARTU
PRESS
1632

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on December 10th, 2015 by the Council of Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

Supervisor: Ene Ustav, PhD,
Senior Researcher,
Institute of Technology, University of Tartu, Estonia

Reviewers: Juhan Sedman, PhD,
Professor of Biochemistry,
Institute of Molecular and Cell Biology, University of Tartu, Estonia

Andres Merits, PhD,
Professor of Applied Virology,
Institute of Technology, University of Tartu, Estonia

Opponent: Laimonis A. Laimins, PhD,
Professor of Microbiology-Immunology,
Department of Microbiology-Immunology,
Feinberg School of Medicine, Northwestern University, Chicago

Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 09.15 on January 21st, 2016

Publication of this thesis is granted by the Institute of Technology, University of Tartu.

ISSN 2228-0855
ISBN 978-9949-77-014-4 (print)
ISBN 978-9949-77-015-1 (pdf)

University of Tartu Press
www.tyk.ee

“To try and understand”
– Arundhati Roy, “The cost of living”

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 9
LIST OF ABBREVIATIONS 10
1. INTRODUCTION 11
2. LITERATURE ANALYSIS 12
2.1 Introduction to human papillomaviruses 12
2.1.1 Medical impact of HPV infections 12
2.1.2 HPV genome organization and a brief introduction to the viral proteins 13
2.1.3 Overview of HPV infection in stratified epithelia 15
2.2 HPV replication cycle 16
2.2.1 Initial transient amplification replication 16
2.2.2 Stable maintenance replication 19
2.2.2.1 Maintenance of HPV genomes in host cells 21
2.2.3 Vegetative amplification replication 21
2.3 Overview of the cellular DNA damage response pathways 24
2.3.1 ATM signaling pathway 25
2.3.2 ATR signaling pathway 26
2.3.3 Recombination-dependent DNA replication 27
2.4 Association of HPV replication and cellular DDR 29
2.4.1 Activation of cellular DDR pathways during initial HPV amplification 29
2.4.2 Stable maintenance replication and cellular DDR 29
2.4.3 Engagement of cellular DDR for the efficient vegetative amplification of the HPV genome 29
2.4.4 NHEJ and HPV replication 30
2.4.5 HR and HPV integration 31
2.4.6 Possible implications of DDR activation during HPV replication 31
2.5 Brief overview of polyomavirus replication 32
3. OBJECTIVES OF STUDY 34
4. MATERIALS AND METHODS 35
5. RESULTS AND DISCUSSION 37
5.1 Generation of oligomeric molecules of the viral genome during the replication of HPV genomes in U2OS cells (I) 37
5.2 HPV genomic oligomers form via homologous recombination during the initial amplification of the HPV genome (II) 39
5.3 Evidence of the involvement of two different replication mechanisms in the initial amplification of the HPV genome (III) 43
5.3.1 Dual-mechanism model of papillomavirus replication 46
5.3.2 Perspectives on the dual-mechanism model of replication 47

6. CONCLUSIONS 49
SUMMARY IN ESTONIAN 50
ACKNOWLEDGMENTS 53
REFERENCES 54
PUBLICATIONS 71
CURRICULUM VITAE 129
ELULOOKIRJELDUS 130

LIST OF ORIGINAL PUBLICATIONS

I Jelizaveta Geimanen, Helen Isok-Paas, Regina Pipitch, Kristiina Salk, Triin Laos, **Marit Orav**, Tormi Reinson, Mart Ustav, Jr., Mart Ustav, and Ene Ustav (2011) Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses. *Journal of Virology*, 85 (7), 3315–3329

II **Marit Orav**, Liisi Henno, Helen Isok-Paas, Jelizaveta Geimanen, Mart Ustav, Ene Ustav (2013) Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. *Journal of Virology*, 87 (22), 12051–12068

III **Marit Orav**, Jelizaveta Geimanen, Eva-Maria Sepp, Liisi Henno, Ene Ustav & Mart Ustav (2015) Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. *Scientific Reports*, 5, 15952

My contributions to the publications are as follows:

I Analyzed the physical state of papillomavirus DNA during the initial transient, stable maintenance and vegetative amplification replication phase;

II Designed and performed the analysis of the prevalence of different forms of HPV18 genomes over time, the characterization of HPV18 oligomeric molecules as head-to-tail concatemeric episomes, the analysis of HPV18 mutant genomes, the analysis of HPV18 oligomers generated in different cell lines, and all the experiments with the HPV11 genome; helped design the experiments on co-transfecting the HPV18wt and HPV18E genome; participated in the analysis of patient samples; interpreted the results; and wrote the manuscript;

III Designed and performed the experiments related to the analysis of uncut HPV genomes, the time course analysis of HPV18wt genome, the 2D N/N analysis of the linearized HPV18 genomes, the analysis of subgenomic HPV18 fragments, the *in gelo* digestion analysis, and the analysis with the HaCaT cell line; interpreted the results; and wrote the manuscript.

Invention: Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication. Authors: Mart Ustav, Ene Ustav, Jelizaveta Geimanen, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, Mart Ustav, Triin Laos, **Marit Orav**, Anu Remm, Kristiina Salk, Andres Männik. PCT/EE2010/000010

LIST OF ABBREVIATIONS

1D – one-dimensional
2D – two-dimensional
AGE – agarose gel electrophoresis
ATM – Ataxia-telangiectasia mutated
ATR – ATM- and Rad3-related
ATRIP – ATR-interacting protein
BIR – break-induced replication
bp – base pair
BPV – bovine papillomavirus
Chk1 – checkpoint kinase 1
Chk2 – checkpoint kinase 2
C-terminal – carboxyl-terminal
DDR – DNA damage response
D-loop – displacement loop
DSB – DNA double-stranded break
dsDNA – double-stranded DNA
E. coli – *Escherichia coli*
E1BS – E1 protein binding site
E2BS – E2 protein binding site
HPV – human papillomavirus
HR – homologous recombination
kbp – kilo-base pair
LMW DNA – low-molecular weight DNA
MMBIR – microhomology-mediated break-induced replication
MMEJ – microhomology-mediated end-joining
MRN – Mre11-Rad50-Nbs1 complex
N/A – neutral/alkaline
N/N – neutral/neutral
N/N/A – neutral/neutral/alkaline
NHEJ – non-homologous end-joining
N-terminal – amino-terminal
Oligomeric (HPV) genome – viral DNA containing several copies of the HPV genome
ORF – open reading frame
RDR – recombination-dependent replication
RPA – replication protein A
S. cerevisiae – *Saccharomyces cerevisiae*
ssDNA – single-stranded DNA
TopBP1 – topoisomerase IIβ-binding protein 1
URR – upstream regulatory region

1. INTRODUCTION

The successful establishment of most viral infections requires productive proliferation of the viral genetic material. Papillomaviruses are similar to other small DNA viruses and utilize a small number of virus-encoded proteins in addition to numerous cellular factors to replicate their genome. The viral proteins are the clear minority among all of the factors involved in papillomavirus replication; however, the virus is extremely adept at manipulating normal cellular processes to achieve the efficient multiplication of its genome.

The viral and cellular factors involved in the replication of papillomavirus DNA (hereafter referred to as papillomavirus replication) have been the subject of thorough research. The recruitment of a host cell's replication proteins to facilitate papillomavirus replication by viral replication proteins is well characterized. However, recent research has established that the factors involved in cellular DNA damage signaling and repair pathways are also necessary for efficient papillomavirus replication. These findings raise an intriguing possibility that papillomaviruses may hijack the host cell's DNA repair machinery and use it as an additional mechanism for replicating the viral genome. The present thesis examines two aspects that are connected with the replication of human papillomaviruses (HPVs). First, we study the characteristics and generation of HPV DNA that contains several copies of the viral genome (referred to as oligomeric genomes for brevity), which appear during the early phases of HPV replication. We establish that the oligomeric genomes arise through homologous recombination and may represent the manifestation of the involvement of cellular DNA repair pathways in viral replication. Second, a detailed analysis of replication intermediates generated during the early phase of HPV replication indicates that two separate replication mechanisms are utilized for the initial replication of HPV genomes. We identify one of the mechanisms as bidirectional theta replication, which is frequently involved in the replication of small circular double-stranded DNA genomes such as the papillomavirus genome, and has already been implicated in the later stages of HPV replication. We suggest that the other mechanism is recombination-dependent replication. Collectively, we propose that the identification of replication intermediates generated via two separate mechanisms is indicative of a dual-mechanism model of papillomavirus replication.

The literature analysis provides information on the medical importance of HPV infections, an overview of the papillomavirus life cycle, a detailed description of the papillomavirus replication cycle, and an overview of cellular DNA damage response pathways and recombination-dependent replication.

2. LITERATURE ANALYSIS

2.1 Introduction to human papillomaviruses

Papillomaviruses are DNA viruses that infect the keratinocytes of cutaneous and mucosal epithelial tissue of vertebrates. To date, several hundred different papillomavirus variants, which are referred to as types, have been identified (1–3). Because of their clinical importance, scientific research has primarily concentrated on HPVs; however, bovine papillomavirus type 1 (BPV1) has served as a useful model for the study of papillomavirus infection in humans.

2.1.1 Medical impact of HPV infections

Papillomavirus infections in general are most commonly either asymptomatic or lead to the formation of benign lesions in the infected tissue, and HPV infections are no exception.

HPV is widely detected in samples collected from healthy skin and can be considered a component of typical skin microbiota (4, 5). HPV types that infect the cutaneous epithelium, such as HPV1 and HPV2, cause a variety of clinically insignificant benign epithelial growths, such as warts or papillomas. However, emerging evidence suggests a link between the infection of certain cutaneous HPV types (e.g., HPV5 and HPV8) and the development of non-melanoma skin cancer, especially in immunocompromised and immunosuppressed patients (reviewed in (6)).

HPV types that infect the mucosal epithelium have been established as the causative agents underlying several epithelial pathological states. Mucosal HPV types are considered to be either low risk or high risk based on the propensity of the resulting lesions to progress into malignant tumors. Low-risk HPV types (e.g., HPV6b and HPV11) mainly cause genital warts (7, 8); however, both HPV6b and HPV11 also cause recurrent respiratory papillomatosis (9), a rare yet potentially fatal disease. High-risk HPV types (e.g., HPV16, 18, 31 and 45) are implicated in the development of cervical (10, 11) and other anogenital cancers (12) as well as head and neck cancers (13, 14). The global prevalence of HPV infection in the cervix of asymptomatic women has been reported at approximately 11–12%, and there is considerable regional variation: the prevalence of HPV infection is significantly higher in developing countries than in the developed world (15). Less data are available on the prevalence of HPV infection among men; however, the prevalence of HPV infection among the women and men of the same population has been shown to correlate reasonably well (16). Approximately 5% of new cancers occurring worldwide are attributable to HPV (15, 17), and the incidence of HPV-related cancers is higher in less-developed regions than in more highly developed regions (15). The introduction of preventive HPV vaccines has led to a decrease in the prevalence of HPV infections (18, 19) and cervical lesions (20–23), especially in countries

where a national vaccination program has been implemented. However, the vaccines currently on the market are only targeted against nine of the most common HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52 and 58) (24) and have no reported therapeutic effects. Additionally, because of the high cost of the vaccines, they are largely unattainable in developing countries. Currently, there are no therapeutic procedures or drugs available to treat HPV infections by specifically targeting HPV replication.

2.1.2 HPV genome organization and a brief introduction to the viral proteins

HPVs have an approximately 8-kbp circular double-stranded DNA (dsDNA) genome that consists of three major regions: the non-coding region, which is also referred to as the upstream regulatory region (URR); the early coding region; and the late coding region (Fig. 1).

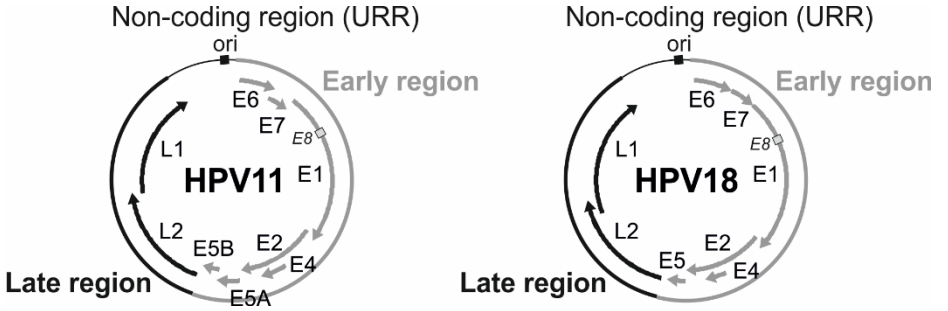

Figure 1. Schematic representation of HPV11 and HPV18 genomes. The open reading frames of HPV early proteins are marked with grey arrows, and the open reading frames of HPV late proteins are marked with black arrows. Ori denotes the approximate position of the origin of replication, and the short E8 open reading frame is marked with a grey square.

The non-coding region of HPV genomes contains binding sites for several cellular and viral regulatory proteins (25–27), transcription start sites (28–32) and the origin of replication (33–37).

The early coding region contains open reading frames (ORFs) for the HPV proteins E6, E7, E1, E2, E4 and E5.

The E1 and E2 proteins are the only viral proteins crucial for the initiation of HPV replication. The E1 protein is the main HPV replication protein with DNA helicase/ATPase activity (38). Functionally, the protein consists of three main domains: the N-terminal regulatory domain, the central DNA-binding domain, and the C-terminal helicase domain (reviewed in (39)). The E2 protein is the main viral transcriptional regulator, and it participates in the initiation of HPV genome replication and maintenance of viral genomes in the infected cells. HPV genomes encode a full-length E2 protein containing the N-terminal trans-

activation domain and the C-terminal DNA-binding/dimerization domain connected by a linker region; however, HPV genomes also encode several truncated versions of the E2 protein, which serve various regulatory functions. The most thoroughly studied truncated version of the E2 protein is the E8E2 protein. The E8E2 protein contains amino acids encoded by the short E8 open reading frame located within the E1 open reading frame and the C-terminal domain of the E2 protein, and it has been shown to be a strong repressor of HPV replication and transcription (30, 40–42). The E2 protein and its truncated versions bind to the HPV genome through specific 12-bp binding sites, which are mainly located in the non-coding regulatory region of the viral genome. The structure and numerous functions of the E2 protein have been reviewed in (43).

The E5, E6 and E7 reading frames encode viral oncogenes. There are significant differences in the characteristics of the oncoproteins encoded by the low-risk and high-risk types of HPV. The high-risk E6 proteins associate with and promote the degradation of cellular tumor suppressor p53 (44, 45) among other cellular targets (46). The high-risk E7 proteins modulate cell-cycle regulation of the host cell through several cellular proteins (47–49), most notably the retinoblastoma family proteins (50). For the high-risk HPV types, the E6 and E7 protein expression from the full-length viral genome is necessary and sufficient for the *in vitro* transformation of primary human keratinocytes (51). The E6 and E7 proteins encoded by low-risk and high-risk HPV types engage with similar major cellular targets and processes, although the low-risk E6 and E7 proteins bind their targets with lower affinity (45, 52, 53) and are not sufficient for host cell transformation (54, 55).

The E5 protein is likely a transmembrane protein, although its functions are poorly understood. However, BPV1 and HPV16 E5 proteins are known to affect the cell surface expression of the epidermal growth factor receptor and stimulate cell growth (56–60) and down-regulate the expression of cellular proteins involved in antigen presentation at the cell surface (61–64), thus contributing to host cell transformation and immune evasion. Sequence analyses have revealed that not all HPVs encode the E5 protein and certain HPVs encode two E5 proteins (e.g., HPV11); moreover, the HPV types that encode the E5 protein have increased carcinogenic potential (65, 66).

The E4 open reading frame is primarily expressed as part of the E1E4 protein, which is synthesized from a HPV transcript containing the beginning of the E1 open reading frame spliced with the E4 open reading frame (67). Despite its name, the E1E4 protein is most abundantly expressed during the late phase of HPV infection. The main function of the E1E4 protein is likely to disrupt the cellular keratin network (68), which may help to facilitate the release of viral particles from the host cell.

The late coding region contains open reading frames for the HPV capsid proteins L1 and L2. The L1 protein is the major capsid protein and assembles into capsid-like structures when expressed in cells (69), whereas the L2 minor capsid

protein contributes significantly to the efficiency of viral DNA encapsulation and the infectivity of viral particles (70).

2.1.3 Overview of HPV infection in stratified epithelia

HPVs infect the keratinocytes of stratified mucosal and cutaneous epithelia. Epithelial keratinocytes undergo a distinct differentiation program that also influences the life cycle of papillomaviruses.

Stratified mucosal and cutaneous epithelium is separated from underlying tissue by the basement membrane. The basal layer of keratinocytes located on the basement membrane contains epidermal stem cells and their actively proliferating progeny cells, which are referred to as transiently amplifying cells. When the daughter cells of transiently amplifying keratinocytes detach from the basement membrane, they stop dividing and begin terminal differentiation. As the cells move upward, the expression of keratins increases as programmed cell death occurs. This process results in the generation of keratin-filled cell husks that provide mechanical resistance to the stratified epithelia. The differentiation program of keratinocytes has been reviewed in (71, 72).

The establishment of productive HPV infection is generally expected to involve infecting the keratinocytes of the basal layer. Establishing an infection has been reported to require the host cell to enter mitosis (73), and in healthy epithelium, the only proliferation-competent cells are basal cells. After entry into the basal host cell, the HPV genome undergoes transient initial amplification followed by the stable maintenance of the viral genome copy number (reviewed in (74)). During infection with high-risk HPV types, the expression of oncoproteins E6 and E7 from the viral genome promotes active cell division, and the number of infected cells grows *in situ* (reviewed in (75)); however, the mechanisms underlying the initial proliferation of cells infected with low-risk HPV types are not well understood.

The detachment of HPV-infected cells from the basement membrane initiates the onset of the productive phase of the HPV life cycle. In normal epithelium, keratinocytes exit the cell cycle after leaving the basal layer. However, the expression of oncoproteins (both low risk and high risk) is sufficient to maintain a host cell environment capable of supporting viral replication (reviewed in (74, 76)). As the host cell enters its differentiation program, vegetative amplification of the viral genome is induced (reviewed in (74)). Following genome amplification, viral capsid proteins are synthesized and viral particles are assembled in the upper layers of the epithelium (77, 78). Finally, infectious HPV particles are released from the remains of the host cell (79, 80). An overview of the productive HPV infection in mucosal tissue is provided in Figure 2.

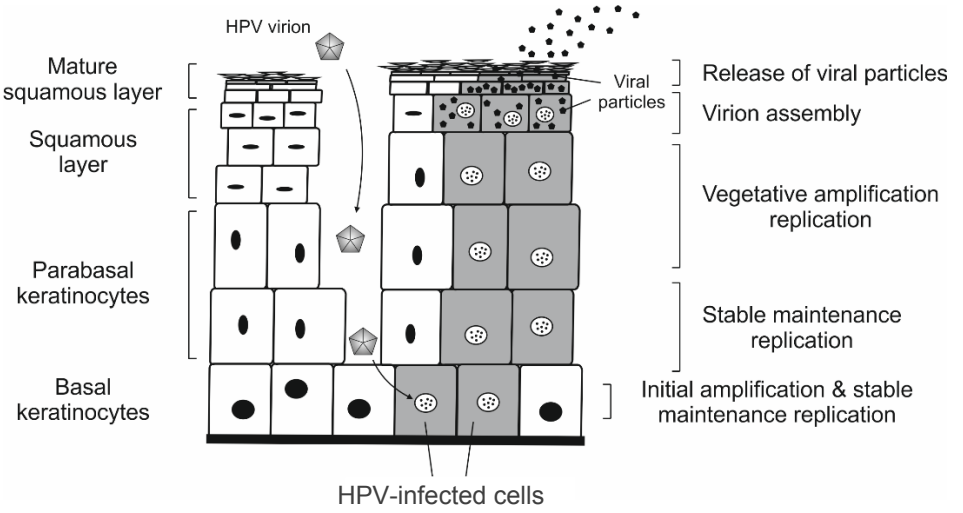

Figure 2. Simplified overview of productive HPV infection in stratified mucosal epithelium. Layers of mucosal epithelia are indicated on the left, and appropriate phases of the viral cycle are indicated on the right. See the text for details.

2.2 HPV replication cycle

The HPV genome replicates in three separate phases: initial transient amplification replication, stable maintenance replication, and vegetative amplification replication.

2.2.1 Initial transient amplification replication

The initial transient replication of the papillomavirus genome is generally believed to occur soon after viral entry into the host cell, although this has not yet been studied *in vivo*. The rapid amplification of viral genomes during the early stages of infection is assumed to occur because of the high copy number of viral genomes noted in HPV-positive cell lines established from the cervical samples of HPV-infected patients. For example, when the W12 cell line was established from a low-grade cervical lesion, it carried approximately one hundred copies of the episomal HPV16 genome per cell with the viral copy number remaining stable over multiple passages (81). Because notable changes in the viral copy number are not observed, the replication of HPV16 genomes during the cultivation of W12 cells under regular cell culture conditions likely represents the stable maintenance replication phase. Because it is highly unlikely that the precursor cell of the W12 cell line was infected by such a high number of HPV viral particles, it is reasonable to assume that the viral genome underwent a transient amplification phase prior to the stable maintenance replication phase.

Under laboratory conditions, the first stage of papillomavirus replication is mimicked by transfecting eukaryotic cells capable of supporting papillomavirus replication with the full viral genome. However, only a few cell lines are capable of supporting papillomavirus replication, and it usually occurs at a low efficiency; moreover, replication that occurs shortly after viral entry into the cells is the most difficult to analyze. Alternatively, cells can be co-transfected with DNA containing the origin of papillomavirus replication and expression vectors for the E1 and E2 proteins. Early research into papillomavirus replication used BPV1 as a model for determining the events underlying the initial transient amplification of papillomavirus genomes, whereas contemporary research has primarily focused on studying the different HPV types.

The only viral proteins that are sufficient and required for the initial replication of BPV1 and HPV genomes are the full-length E1 and E2 proteins (34, 37, 82–84). The initial transient replication of the HPV genome is initiated at the non-coding region of the viral genome, and the complete HPV origin of replication contains three E2 protein binding sites (E2BSs) flanking an A/T-rich segment containing the E1 binding site (E1BS) (34, 35, 85–89). The origin of replication location in the non-coding region (hereafter referred to as the URR origin) is marked in Figure 1 as "ori". The minimal requirements for the initiation of HPV replication are either the presence of the A/T-rich segment adjoining a single E2BS or the presence of two consecutive E2BSs (33, 35). The efficiency of replication initiation is determined by the number of E2 binding sites (35, 85, 86). Interestingly, the E1 and E2 proteins can support the initiation of replication from heterologous papillomavirus origin sequences (37, 82); however, the URR origin is the only active origin of replication thus far identified in the HPV genome.

The initial amplification replication is initiated when the full-length E1 and E2 proteins bind to the origin of replication. The E1 protein binds to the origin of papillomavirus replication as a dimer, and the process is facilitated by the formation of the E1-E2 protein complex (90–93). The E2 protein is required for efficient origin recognition (90, 91, 94, 95); however, the initiation of replication is absolutely dependent on the presence of the E1 protein (96). Experiments with purified BPV1 E1 and E2 proteins demonstrated that after the E1-E2 complex binds to the origin of replication, E2 is displaced and the E1-origin complex remains (95). Following the displacement of the E2 protein, the E1 protein forms a double-trimer complex at the origin of replication (97, 98). The E1 double-trimer melts the dsDNA at the origin of replication (97, 99, 100), after which a double-hexamer E1 complex is formed (97, 100), with each E1 hexamer encircling a single DNA strand (101). The initiation of papillomavirus replication in the presence of the minimal origin sequence (one E1BS and an adjoining E2BS) is depicted in Figure 3.

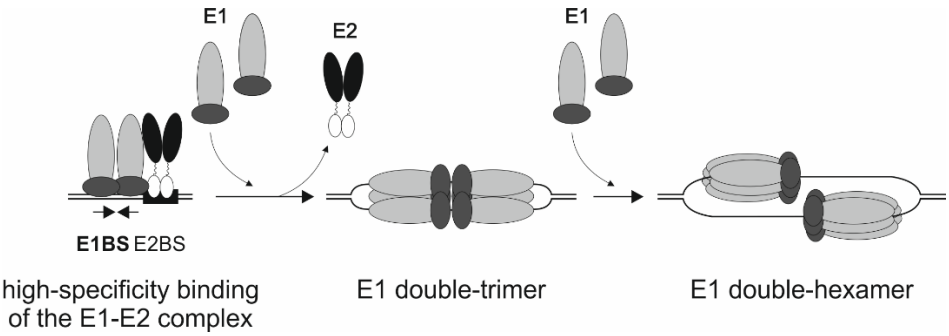

Figure 3. Schematic overview of the E1/E2-dependent initiation of papillomavirus replication from a minimal origin of replication sequence consisting of the E1 binding site (E1BS) and a single adjoining E2 binding site (E2BS) (adapted from (102)). See the text for details.

The E1 hexamer is a DNA helicase/ATPase (103, 104). The E1 protein also interacts with cellular DNA polymerase α-primase (91, 105, 106), replication protein A (RPA) (107, 108), topoisomerase I (109), and numerous other cellular proteins associated with DNA replication (reviewed in (39)). DNA polymerase α-primase initiates eukaryotic DNA synthesis and is required for the repeated re-initiation of replication during the synthesis of the lagging DNA strand; RPA is the most prevalent eukaryotic single-stranded DNA (ssDNA) binding protein; and topoisomerase I is a eukaryotic enzyme responsible for relaxing supercoiled DNA. In short, the E1 protein recruits cellular replication proteins to the papillomavirus origin of replication, thus initiating the replication of the viral genome. Cellular interaction partners of the E2 protein are also required to establish efficient HPV replication. Notably, E2 protein must be able to bind topoisomerase IIβ binding protein (TopBP1) to establish viral episomes (110). TopBP1 is required for the replication of cellular DNA and the rescue of stalled replication forks (111–113).

The replication initiated by the viral E1 and E2 proteins from the URR origin is generally presumed to proceed via bidirectional theta replication; however, experimental data are not available to either confirm or disprove the hypothesis. Determining the mechanism that underlies the initial amplification replication should involve an analysis of replication intermediates that arise during the initial phase of HPV genomic replication. Cervical samples for HPV-associated research are usually collected for diagnostic purposes and do not contain enough viral genetic material to allow for an analysis of replication intermediates, even if the sample is collected from recently infected tissue. Alternatively, samples can be collected from HPV-associated cervical lesions, which represent a much later phase of viral infection. Low replication efficiency has also hindered the analysis of replication intermediates arising during the initial amplification of full-length HPV genomes after transfection into eukaryotic cell lines.

Theta replication is a common replication mechanism for small circular dsDNA molecules, such as bacterial plasmids and certain viral genomes (e.g., the polyomavirus genome). During bidirectional theta replication, two replication forks assemble at the origin of replication and progress in the opposite direction until eventually converging upon the complete replication of the molecule. The converging of replication forks is followed by the separation of daughter molecules. A notable characteristic of theta replication is that it represents a so-called circle-to-circle type of replication in which the replication of a circular molecule generates circular daughter molecules. A schematic overview of bidirectional theta replication is depicted in Figure 4.

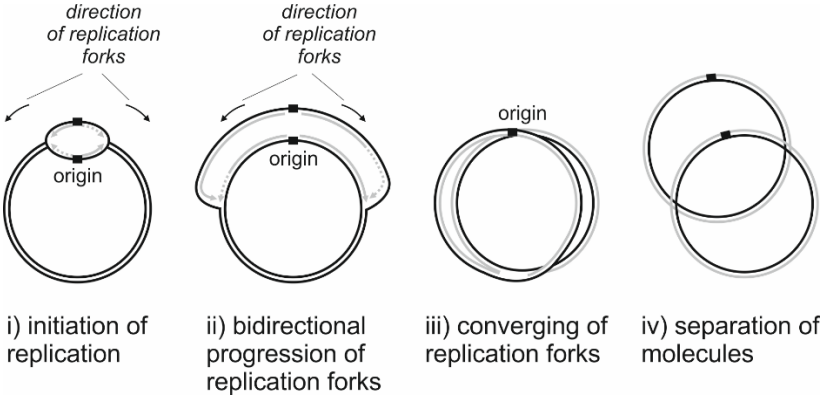

Figure 4. Simplified overview of bidirectional theta replication. Replication is initiated at the origin of replication, where two replication forks are assembled. The assembled replication forks then progress in opposite directions until they converge upon the nearly complete replication. Following the convergence of the replication forks, the daughter molecules are separated and the replication process is finalized.

Bidirectional theta replication is associated with the initial amplification of the HPV genome mainly because the mechanism has already been implicated in the later phases of papillomavirus replication (114–117). Replication of polyomavirus SV40, which is considered to be similar to papillomaviruses in many aspects, also occurs via bidirectional theta replication (118).

2.2.2 Stable maintenance replication

The initial amplification of the papillomavirus genomes in the host cell is soon replaced by a replication phase during which the viral genome copy number is maintained at a near constant level. Several cell lines established from low-grade cervical lesions, such as HPV16-positive W12 cells and HPV31b-positive CIN612 cells (81, 119), represent the stable maintenance replication phase when cultivated under regular cell culture conditions. HPV-positive cell lines suitable for the analysis of stable maintenance replication can also be created

through full-length HPV genome transfection of cell lines capable of supporting HPV replication. BPV1 stable maintenance replication has been studied using the ID13 cell line created by transforming the mouse fibroblast C127 cell line with BPV1 (120).

Because suitable model systems are available to study stable maintenance replication, several aspects of this particular replication phase have been intensively studied.

Although the initial transient replication of the papillomavirus genome is dependent on the availability of the viral replication proteins E1 and E2, the stable maintenance replication phase has been shown to proceed in the absence of the E1 protein (84, 121–123). However, an analysis of the replication intermediates generated during the stable maintenance replication phase suggests that during this phase the origin of replication is the same as that used during the initial transient replication phase (the URR origin) (114, 115, 117). The initiation of HPV genomic replication from the URR origin is a strictly E1/E2-dependent process during initial transient replication, thus reducing the likelihood that the initiation of replication from the URR origin during stable maintenance replication occurs via an E1-independent mechanism. Collectively, these data suggest the involvement of two different replication mechanisms in the stable maintenance replication of the HPV genome: an E1/E2-dependent mechanism initiated at the URR origin and a separate E1-independent mechanism utilizing different initiation sequences.

The E1/E2-dependent stable maintenance replication initiated at the URR origin proceeds via bidirectional theta replication (114, 115, 117); however, the mechanism underlying the E1-independent replication of HPV genomes has not been identified.

Whether papillomavirus DNA replicates in a strictly controlled once-per-S-phase mode or a random mode during the maintenance phase has also been the subject of thorough research. Determining the mode of replication offers insights into the factors controlling replication initiation and, ultimately, the mechanism involved in stable maintenance replication. It has been shown that HPV genomes are, in principle, inherently capable of both once-per-S-phase and random modes of replication during the stable maintenance phase, with the choice depending on the level of viral replication proteins in the host cell (124). In the presence of high viral replication factor levels, HPV replicates via a random-choice mode, whereas in the presence of low viral replication factor levels, the once-per-cell-cycle mode is utilized (124). These data suggest a certain degree of flexibility in the mode deployed for HPV replication and may reflect the previously discussed utilization of two separate replication mechanisms for the stable maintenance of the HPV genomes.

2.2.2.1 *Maintenance of HPV genomes in host cells*

To complete a productive viral life cycle, papillomavirus genomes must be maintained in host cells as extrachromosomal molecules. During stable maintenance replication, BPV1 and HPV DNA persist in eukaryotic cell lines as a mix of molecules containing a single copy of the viral genome and molecules containing several copies of the viral genome (oligomeric genomes) (114, 115, 125, 126). The presence of oligomeric HPV genomes has also been detected in HPV-associated cervical lesions (127–133). However, the mechanism underlying the generation of oligomeric HPV genomes and the biological relevance of the viral genomic oligomers have not been investigated.

High-risk HPV DNA can also be maintained in host cells in an integrated form. The integration of HPV DNA into the host genome is generally viewed as a hallmark of aberrant infection and frequently linked with the potential malignant progression of HPV-associated lesions (134). The E1 and E2-driven replication of integrated HPV sequences has been proposed as the mechanism underlying the genomic instability that potentially facilitates the malignant transformation of the host cell (135, 136).

Because the stable maintenance phase of replication occurs in the actively dividing keratinocytes of the basal layer, efficient segregation of extrachromosomal papillomavirus genomes between daughter cells is paramount for the maintenance of infection. The efficient segregation of papillomavirus genomes is achieved by tethering the viral genome to host mitotic chromosomes using the E2 protein and E2 binding sites (137–139).

2.2.3 Vegetative amplification replication

When the host cell detaches from the basement membrane and enters its differentiation program, a vegetative amplification of the viral genome is induced. The vegetative amplification replication of HPV genomes can be mimicked *in vitro* by cultivating HPV-positive keratinocyte cell lines in high-calcium medium, suspended in methylcellulose, or cultivating them as organotypic raft cultures. Vegetative amplification has also been analyzed using samples collected from HPV-infected patients. However, determining the exact phase of replication in patient samples can be difficult without the opportunity to observe possible HPV genome copy number variations over time; therefore, in patient samples, the vegetative amplification replication phase may be confused with the stable maintenance replication phase.

The vegetative amplification of HPV genomes depends on the presence of the E1 protein (84). Upon entering the vegetative amplification replication phase, HPV activates members of the cellular endoprotease protein family caspases, which in turn cleave the viral E1 protein from a conserved site located in the N-terminal regulatory domain (140). Cleavage of the E1 protein is required for the vegetative amplification of the HPV genome (140), although

the molecular mechanism underlying this phenomenon remains unknown. During host cell differentiation, HPV genomes also activate cellular DNA damage response pathways and recruit cellular proteins involved in homologous recombination to nuclear viral replication foci, which is a prerequisite for the vegetative amplification of HPV genomes (141–143).

It is generally believed that during the vegetative amplification phase, HPV genomes replicate via a different mechanism than during the two previous replication phases. In W12 and CIN612 cells, the vegetative replication of HPV16 and HPV31b genomes has been found to proceed via a unidirectional replication mechanism without specific origin or termination sequences (117). Initially, the mechanism was suggested to be rolling-circle replication, which is a common replication mechanism for several bacterial plasmids and viral ssDNA genomes.

Rolling-circle replication of bacterial plasmids is initiated by the site-specific generation of a nick at the dsDNA origin of replication, which is mediated by specific initiator proteins. The 3' DNA end generated during the nicking process is then extended by a DNA polymerase during leading strand synthesis, whereas the cleaved non-template DNA strand is simultaneously displaced from the replicating molecule. During the replication of bacterial plasmids, the leading strand synthesis usually proceeds until the nicking site is reached; subsequently, replication of the leading strand is terminated and the displaced strand is released from the replicated molecule. The displaced ssDNA then serves as the template for the synthesis of the lagging strand, with replication initiated from a special ssDNA origin sequence. Rolling-circle replication of bacterial plasmids has recently been reviewed in (144).

Alternatively, rolling-circle replication has been suggested to result in the generation of circular molecules with concatemeric linear "tails", such as the intermediates of bacteriophage λ late replication (145, 146). This type of rolling-circle replication is also referred to as sigma replication. Bacteriophage λ late replication intermediates contain more than two copies of the phage genome (146–148), indicating that replication of the leading strand is not terminated upon reaching the initiation site but proceeds for several rounds. The generation of dsDNA linear tails also implies that the synthesis of the lagging strand is initiated well before the termination of the leading strand synthesis; otherwise, replication intermediates with ssDNA tails would have been observed. The sigma replication mode is associated with recombination-dependent replication (149). A schematic overview of rolling-circle replication is presented in Figure 5.

Rolling-circle replication is a unidirectional mechanism that proceeds via a single replication fork similarly to the mechanism involved in the vegetative amplification of the HPV16 and HPV31b genomes (117); however, there are certain inconsistencies between the intermediates of rolling-circle replication and the intermediates observed during the vegetative amplification of the

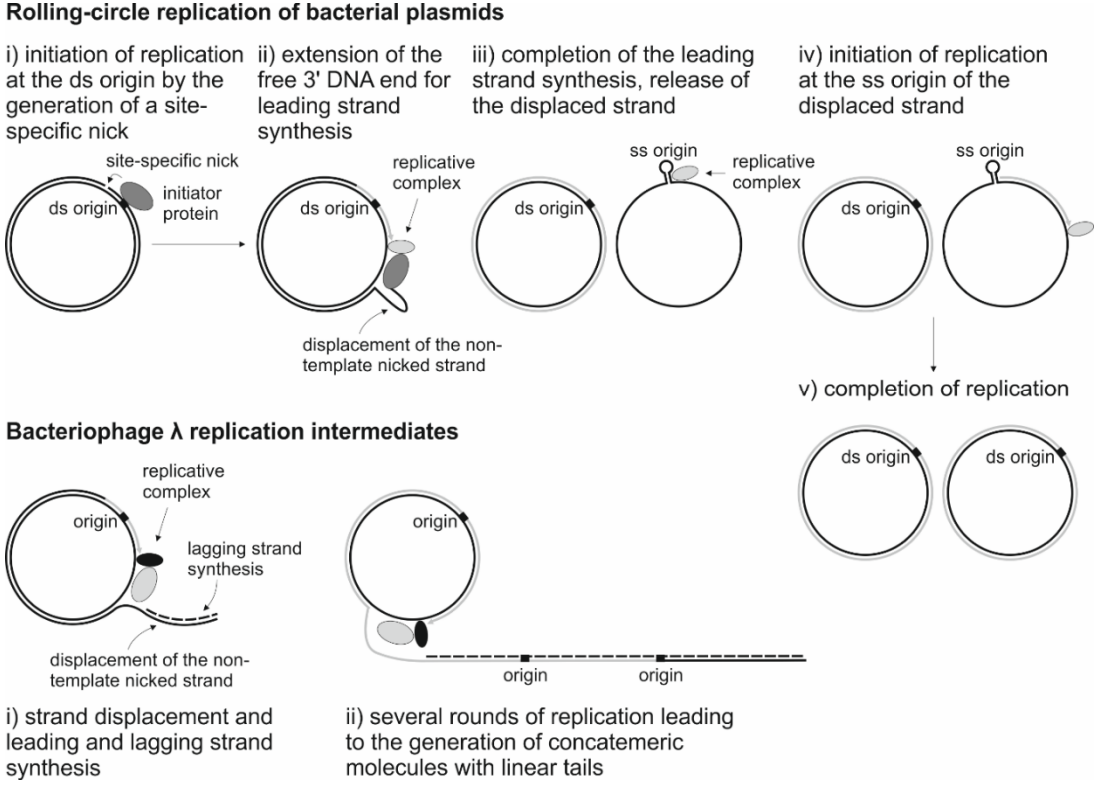

Figure 5. Overview of the rolling-circle replication of bacterial plasmids and a brief characterization of bacteriophage λ late replication intermediates generated through the sigma type of rolling-circle replication. See the text for details.

HPV16 and HPV31b genomes. Rolling-circle replication of bacterial plasmids initiates from a clearly defined origin sequence and requires another specific ssDNA origin sequence for the efficient synthesis of the lagging strand, whereas HPV vegetative amplification intermediates do not display a preference for a specific initiation sequence (117). Furthermore, rolling-circle replication of bacterial plasmids proceeds via a ssDNA replication intermediate, which has not been reported during analyses of replication intermediates generated during the vegetative amplification of the HPV16 and HPV31b genomes (117). The sigma type of rolling-circle replication generates highly specific σ-shaped replication intermediates, which can be easily identified during the analysis of uncut replication intermediates; however, uncut replication intermediates have not been analyzed along with HPV vegetative replication intermediates (117). Therefore, rolling-circle replication may not be the mechanism underlying the vegetative amplification of the HPV genome; moreover, because cellular homologous recombination proteins are recruited to HPV replication centers, recombination-dependent replication has recently been proposed as the alternative mechanism (142, 143, 150).

An analysis of the low-risk HPV type 11 replication intermediates isolated from vocal cord papillomas suggests that bidirectional theta replication can also be involved in the vegetative amplification replication of HPV genomes (116). However, it must be noted that the vocal cord papilloma tissue was presumed to represent vegetative amplification replication because of its high viral genome copy number, which was comparable with copy number of the HPV16 genome in the W12-E cell line used as a model for the stable maintenance replication (116, 117).

2.3 Overview of the cellular DNA damage response pathways

Maintaining a fully functional genome is of the utmost importance for all cells. In eukaryotic cells, several mechanisms have developed for the detection and repair of DNA lesions, and they collectively form the cellular DNA damage response (DDR) mechanism. The DDR utilizes two major signaling pathways whose key components are the ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-related) protein kinases. The most notable downstream target of ATM is checkpoint kinase 2 (Chk2) (151), which induces cell cycle arrest in response to DNA damage (152, 153). The effector of the ATR pathway is checkpoint kinase 1 (Chk1), which is similar to Chk2 in that it mediates cell cycle arrest in the presence of DNA lesions (154–157). However, the processes associated with the activation of the ATM and ATR signaling pathways and implementation of the DDR machinery are not completely understood.

2.3.1 ATM signaling pathway

ATM is primarily implicated in the response to DNA double-stranded breaks (DSBs). DSBs can be repaired via two principal mechanisms: homologous recombination (HR) and non-homologous end-joining (NHEJ).

HR repairs DNA lesions using a homologous sequence as a repair template and regenerates an intact (previously disrupted) sequence. The HR pathway is restricted to the S and G2 phases of the cell cycle when homologous molecules are present and available as templates, and it is initiated by the detection of DSBs by the Mre11-Rad50-Nbs1 (MRN) complex. Mre11 is a nuclease with both endonuclease and exonuclease activity (158), and it is crucial to HR licensing because Mre11 endonuclease activity is involved in resectioning the DSB DNA ends, which is required to initiate the HR pathway (159, 160). Rad50 provides a protein scaffold that bridges the DSB ends and maintains the proximity of the disjointed DNA ends to each other (161, 162), and Nbs1 is required for the activation of ATM kinase (163). The free 3' ssDNA strands generated via Mre11 resectioning invade a homologous molecule in a reaction mediated by a distinct set of proteins (e.g., Rad51), thus creating the displacement loop (D-loop) structure. Subsequent to the D-loop creation the synthesis of the missing sequences is initiated. HR has been reviewed in (164), and a simplified overview of HR is presented in Figure 6.

NHEJ is an error-prone mechanism that repairs DSBs by simply joining two DNA ends together, which leads to the generation of deletions in the repaired DNA sequence and potential rearrangements of genomic material. NHEJ can occur throughout the cell cycle and is initiated when DSBs are detected by the Ku70/Ku80 heterodimer (reviewed in (165)). A simplified overview of NHEJ is presented in Figure 6.

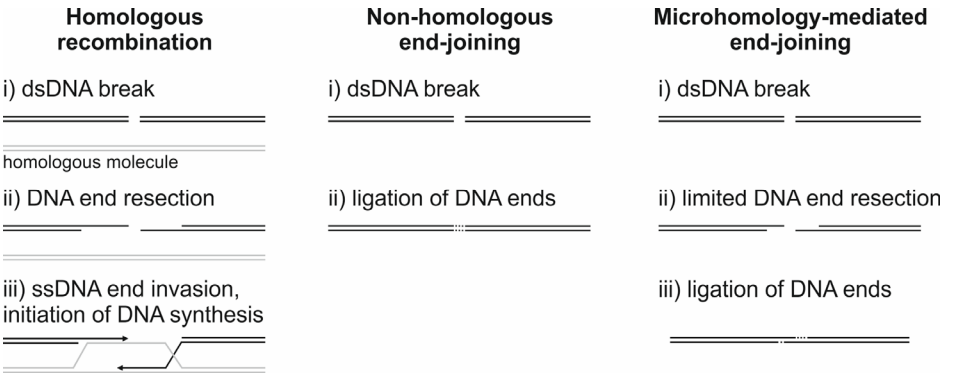

Figure 6. Simplified overview of the different pathways utilized to repair dsDNA breaks (DSBs). See the text for details.

It has been suggested that during the late S/G2 cell cycle phase when DSB repair can proceed via both the NHEJ and HR pathways, the Ku70/Ku80 heterodimer initially binds to DSBs and then NHEJ makes the first attempt at repair; if the NHEJ pathway cannot proceed rapidly, then the HR repair pathway is utilized (166). The distinct nuclease activities of Mre11 are important for determining the DSB repair pathway, and Mre11-mediated resectioning of DNA ends is crucial for HR pathway licensing and NHEJ repression (159, 160). The choice between the HR and NHEJ pathways also appears to be regulated by factors involved in more general cellular processes. For example, phosphorylation of Nbs1 by the cell cycle progression regulator cyclin-dependent kinase during the S, G2 and M phases of the cell cycle facilitates the MRN-dependent resectioning of DSB DNA ends to promote HR and suppress NHEJ (167).

The HR pathway is directly connected to ATM activation through Nbs1 (163, 168). However, it must be noted that the MRN complex is also involved in DNA damage repair mediated by the ATR signaling pathway (169), and the Nsb1 protein is both an upstream regulator and a downstream substrate of ATR kinase (170). Furthermore, the essential function of the MRN complex has been suggested to be the resolution of replication intermediates rather than the repair of DNA lesions (171).

The connections between the NHEJ pathway and ATM activation are not well understood, although several components of the NHEJ pathway have been identified as downstream components of ATM signaling (160, 172, 173).

DSBs can also be repaired via alternative pathways that share similarities with both the HR and NHEJ pathways, such as the microhomology-mediated end-joining (MMEJ) pathway. The MMEJ pathway relies on short sequence homologies (5–25 bp) for the ligation of DNA ends generated via DSBs and can, in principle, lead to the accurate repair of DNA molecules in the presence of fully complementary ssDNA ends. However, complementary ssDNA ends must frequently be generated prior to annealing in a manner similar to DSB end processing that leads to the initiation of HR (174). After suitable ssDNA ends are generated, the two DNA molecules are ligated, and small deletions usually occur in the repaired molecule. A simplified overview of MMEJ is presented in Figure 6, and MMEJ has been reviewed in (175).

2.3.2 ATR signaling pathway

The ATR signaling pathway responds to DNA breaks, adducts and crosslinks and replicative polymerase inhibition or stalling (176). ATR is activated by the presence of structures containing ssDNA and ssDNA-dsDNA junctions (177–179). ssDNA is bound by RPA, which is required for the recruitment of ATR via an association with ATR-interacting protein (ATRIP) (180). The ATRIP-ATR complex is then activated by the Rad checkpoint proteins (e.g., Rad9-Rad1-Hus1 complex) and topoisomerase IIβ-binding protein 1 (TopBP1) (113,

181). Alternatively, ssDNA/dsDNA junctions can be directly recognized by the Rad checkpoint proteins (182), which then activate ATR signaling via TopBP1 (183, 184).

The MRN complex is central to the ATM-mediated repair of DSBs via HR; however, it is also involved in the ATR signaling pathway. The MRN complex is likely required for ATR activation in response to stalled or collapsed replication forks and facilitates the ATR-mediated repair of replication-associated DSBs (169, 185).

2.3.3 Recombination-dependent DNA replication

Recombination-dependent DNA replication (RDR) was first demonstrated as essential for the late replication of bacteriophage T4 (186, 187), where RDR provides a mechanism for replicating the linear ends of the viral genome. RDR has also been studied in the bacterium *E. coli* and budding yeast *S. cerevisiae*, where it is usually referred to as break-induced replication (BIR). The primary role of RDR in *E. coli* has been suggested to be the rescue of collapsed replication forks (reviewed in (188)). In eukaryotic cells, RDR has been associated with an alternative maintenance pathway for chromosome ends (189), and it has also been suggested to be essential for the rescue of collapsed replication forks (190).

The initiation of RDR is similar to the initiation of DSB repair via HR, and there is considerable overlap among the factors involved in the two processes. RDR can be initiated in the presence of both DSBs and ssDNA regions, although the crucial prerequisite for the initiation of RDR is the generation of free 3' ssDNA ends, which is mediated by Mre11 nuclease in eukaryotic cells (159, 160, 167). The resectioned ssDNA ends subsequently invade a homologous dsDNA molecule in a reaction mediated by a distinct set of proteins, including the Rad51 protein, to create the D-loop structure. The D-loop serves as a substrate for recruiting the replicative complex. Although the repair of DSBs via HR only leads to the synthesis of the minimum DNA required to replace missing sequences, the initiation of RDR leads to the replication of extensive DNA sequences well beyond the site of the lesion. BIR has been reviewed in (191).

In bacteriophage T4 and *E. coli*, RDR has been shown to lead to the assembly of a fully functional replication fork and the establishment of semiconservative replication (192–195). In *S. cerevisiae*, BIR requires most of the replication factors associated with regular origin-dependent replication (196), although Pol32, an otherwise non-essential subunit of DNA polymerase delta, is the exception (189); however, in budding yeast, BIR leads to error-prone conservative replication that proceeds along with the migrating bubble (197, 198). A simplified schematic overview of RDR is provided in Figure 7.

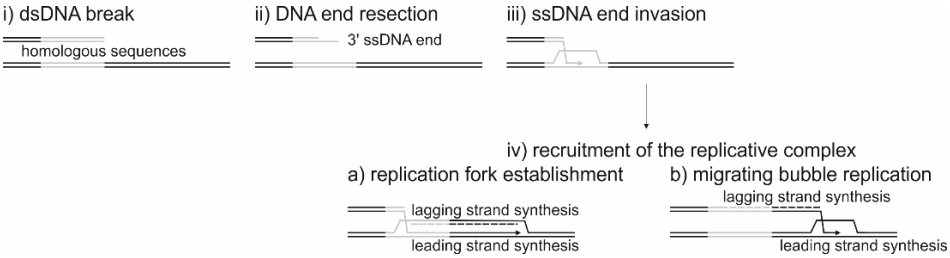

Figure 7. Simplified overview of recombination-dependent replication (RDR). See the text for details.

A distinctive error-prone BIR mechanism, microhomology-mediated break-induced replication (MMBIR), may be able to initiate via sequence microhomologies. MMBIR is likely similar to microhomology-mediated end-joining (MMEJ) (199), which is initiated by a limited resectioning of DNA ends by Mre11 (174) but is independent of Rad51 (200). The Rad51 protein is otherwise essential for eukaryotic RDR (201). MMEJ frequency has been shown to increase when cells enter the S phase, and it has been suggested that MMEJ is used to repair collapsed replication forks (174). Similarly, the collapse of replication forks in stressed cells has been suggested to lead to the initiation of MMBIR (199). MMBIR may be the mechanism underlying the creation of copy number variations in the human genome (199).

The involvement of RDR in the late replication of the linear genome of the bacteriophage T4 is well known; however, RDR is also involved in the replication of the bacteriophage SPP1 genome (reviewed in (202)). The SPP1 replication cycle is characterized by a distinctive switching between circular and linear genomic forms. At the onset of infection, the linear dsDNA SPP1 genome converts into a circular molecule upon entering the host cell. The circular viral genome initially undergoes several rounds of circle-to-circle theta replication, although the mode of replication quickly switches to the sigma type of replication via recombination-dependent restart of collapsed theta replication forks. The recombination-mediated sigma replication leads to the generation of linear concatemeric SPP1 genomes, which are recognized by the viral DNA packaging machinery and processed into mature linear viral genomes packaged into progeny viral particles. Although bacteriophage SPP1 is considered the primary example of this particular mode of replication, the same mechanism may be involved in the replication of bacteriophage λ and herpes simplex virus type 1 genomes (149), both of which also utilize the distinct linear-circular-linear genome conversion and generate linear concatemeric viral genomes during infection. The rolling-circle mechanism of bacteriophage λ late replication was previously discussed in chapter 2.3.3.

2.4 Association of HPV replication and cellular DDR

The links between HPV replication and cellular DDR components have been established for all three phases of papillomavirus replication.

2.4.1 Activation of cellular DDR pathways during initial HPV amplification

The E1 protein, which is required for both the initial and vegetative HPV amplification replication phases (84), has the intrinsic ability to activate the ATM-Chk2 signaling pathway and induce cell cycle arrest (203–205). Both the ATPase and DNA melting activities of the E1 protein are necessary for DDR activation, which is likely the result of E1-generated DSBs (204, 205). E1/E2-dependent HPV replication is insensitive to DSB-generated replication arrest mediated by DDR signaling (206); however, the presence of the E2 protein and the viral origin of replication decrease E1-dependent DDR activation during the initial amplification phase to the extent that large-scale damage to cellular DNA cannot be observed in their presence (203, 204). Furthermore, because inhibition of the ATM pathway does not eliminate the transient replication of the viral origin in the presence of the E1 and E2 proteins (203) or the initial amplification of the HPV genome (204), activation of the ATM-Chk2 pathway is likely unnecessary for an efficient initial amplification replication phase, although this does not necessarily indicate a general lack of involvement of DDR activation in HPV initial amplification. Notably, members of the ATR signaling pathway, the ATRIP and TopBP1 proteins, co-localize with HPV replication foci during the initial amplification of the viral genome, indicating a role for ATR activation in HPV replication (204). Moreover, TopBP1 is a known interaction partner of E2, and E2-TopBP1 binding is necessary for the establishment of HPV episomes (110).

2.4.2 Stable maintenance replication and cellular DDR

Several DDR and HR proteins (e.g., activated ATM and Chk2 proteins, RPA, Rad51, Mre11 and Rad50) co-localize with HPV replication centers during the stable maintenance replication phase; however, this co-localization only occurs in a fraction (approximately 50%) of HPV-positive cells (142, 143). Furthermore, the inhibition of DDR does not affect the efficiency of stable maintenance replication (141, 143).

2.4.3 Engagement of cellular DDR for the efficient vegetative amplification of the HPV genome

During the vegetative amplification replication phase, the co-localization of HPV replication centers and cellular DDR and HR proteins (e.g., RPA, Mre11,

Rad50 and Rad51) is significantly increased, and productive vegetative replication requires the activation of cellular DDR pathways (141–143). Notably, efficient vegetative amplification replication requires Mre11 protein nuclease activity and Nbs1-dependent localization of the MRN complex and Rad51 to the HPV genome (143). Mre11 protein nuclease activity, the MRN complex and the Rad51 protein are the essential requirements of both HR and RDR, and these data indicate a role for HR in the vegetative amplification of the HPV genome. However, it is important to note that inhibiting the components of HR does not eliminate the vegetative amplification replication altogether, suggesting that while HR may be important for HPV DNA replication, it is not indispensable.

Inhibiting the ATM-Chk2 pathway has been shown to disrupt the formation of HPV replication foci during vegetative amplification replication and decrease the efficiency of viral replication (141). However, fluctuations in the levels of activated ATM and Chk2 have not been shown to significantly affect the efficiency of vegetative amplification replication (143). Similarly, although the efficiency of the vegetative amplification replication phase depends on Nbs1-mediated recruitment of the MRN complex and Rad51 to the viral genome, Nbs1 depletion does not significantly affect ATM activation (143). The role of the ATM-Chk2 pathway in vegetative amplification replication is therefore controversial, and ATM activation may serve functions unrelated to the activation of HR machinery. For example, Chk2 activation during host cell differentiation leads to the activation of caspases required for the cleavage of the viral E1 protein involved in vegetative amplification (140, 141). Therefore, it is likely that HPV deploys different components of the cellular DDR pathways for separate albeit partially overlapping functions. Additionally, the uncoupling of ATM activation and the recruitment of the MRN complex to viral replication foci may indicate that the ATR pathway is engaged during the vegetative amplification phase of HPV replication, which would mirror the potential involvement of the ATR pathway during the initial amplification of the viral genome (204).

2.4.4 NHEJ and HPV replication

Components of the NHEJ DSB repair pathway have not been shown to localize to HPV replication foci (142), which indicates that the components of the NHEJ pathway are not involved in or required for HPV replication. Excluding the NHEJ pathway components from the HPV replication foci may simply be a result of the timing of viral replication with HPV replication shown to occur during the S and G2 phases of the cell cycle (207, 208); however, the HR is the pathway of choice for the repair of any detected DSBs during these phases of the cell cycle (167). Because the involvement of the NHEJ pathway during DSB repair has the potential to generate rearranged genomic sequences, excluding the NHEJ components from the viral replication centers is most likely beneficial for the replication of HPV genomes.

2.4.5 HR and HPV integration

In premalignant and malignant cervical lesions, high-risk HPV genomic material is often integrated into cellular DNA. In the case of HPV18, this integration appears to be a crucial step in the progression of malignant lesions (134); however, in the case of HPV16, the viral genome is often present in episomal or mixed episomal/integrated forms in tumor biopsies (134, 209). The genomes of low-risk HPV types are not generally believed to integrate into cellular DNA.

Based on the analysis of HPV16, the integration breakpoints in the viral genome occur in any part of the viral genome (210, 211), and a preference for the often-cited E2 open reading frame is not observed (211). However, data suggest that cellular genomic elements related to genomic instability (such as copy number variations, genomic rearrangements and common fragile sites) are enriched for integrated HPV sequences (210–212). In addition, a noticeable enrichment of microhomologies was observed between the cellular DNA and HPV genomes at or close to the integration loci (211). Several cellular genomic elements related to genomic instability have been associated with recombination events (reviewed in (213, 214)), and these results suggest a recombination-associated model of HPV integration (211). Similarly, integrated HPV sequences have been shown to recruit several cellular factors associated with HR and DDR (136).

2.4.6 Possible implications of DDR activation during HPV replication

The viral functions or factors responsible for activating DDR in HPV-positive cells are as yet unknown. Viral oncoprotein E7 expression from expression vectors has been shown to increase the activation of Chk2 (141, 143) as well as the level of the Mre11, Rad50 and Nbs1 proteins (143). However, full-length HPV18 genomes defective for E6 or E7 protein expression were still capable of inducing DDR in U2OS cells, suggesting that viral replication alone is sufficient for DDR activation (204).

Although the collective data clearly indicate that cellular DDR activation is required during HPV replication, the exact function of this activation remains unknown. The localization of DDR components to HPV replication foci has been suggested as indicative of the involvement of RDR in HPV (vegetative) amplification replication (142, 143, 150); however, definitive proof has not been established to support this hypothesis, particularly considering that DDR activation during DNA replication is expected. Because the ATM-mediated activation pathway appears to be dispensable for activating DDR during the early and late amplification phases of viral replication (143, 203, 204), ATR-mediated activation can reasonably be expected to be involved in the process, especially because components of the ATR pathway have been shown to localize to HPV replication centers during the initial viral amplification (204).

The recruitment of several factors required for the initiation of both HR and RDR to the viral replication centers is an essential pre-requirement for efficient vegetative amplification of the HPV genome (143). ATR activation and HR functions overlap during the rescue of collapsed replication forks (169, 185); therefore, it is plausible that DDR activation during HPV replication merely facilitates the repair of replication-associated DNA lesions. Alternatively, because the essential role of the MRN complex may be the resolution of replication intermediates (171), the activation of DDR may be required for the separation of newly replicated viral genomes.

Indications of the involvement of recombination events in the integration of HPV into cellular DNA (211) provide additional evidence of a possible link between HPV replication and cellular HR machinery. These data indicate that HPV replication generates intermediates that are actively recognized and processed by the cellular repair machinery. Microhomologies are enriched between cellular DNA and the HPV genome close to the viral integration loci (211), and these data more specifically implicate the engagement of microhomology-mediated repair mechanisms during HPV replication.

2.5 Brief overview of polyomavirus replication

The complexity of the papillomavirus life cycle has hindered the study of many features of HPV infection, including the exact mechanism of viral replication. Research into HPV replication would greatly benefit from the availability of a comparable well-studied replication system, and polyomavirus replication has been suggested to provide such an analogous model.

Polyomaviruses are small tumorigenic viruses that have an approximately 5.2-kbp dsDNA genome and typically establish asymptomatic persistent infections. In immunosuppressed patients, however, polyomavirus infection may lead to the development of severe pathologies (including tumors) (reviewed in (215)). Therefore, polyomavirus infections share considerable similarities with papillomavirus infections.

The overall genome organization of polyomaviruses and papillomaviruses is also similar, with both viral genomes containing a non-coding regulatory region, an early region, and a late region coding the capsid proteins. However, notable sequence similarities are not observed between the two groups of viruses. The only exception is an approximately 200 amino acid region located in the C-terminus of the papillomavirus E1 protein and the polyomavirus large T antigen (216). Similar to papillomavirus E1 protein, the large T antigen is the primary viral replication protein, and the C-terminal domain of both the E1 protein and the large T antigen is the helicase domain.

Polyomavirus genome replication shares several similarities with papillomavirus genome replication. Polyomaviruses require a number of host factors to replicate their genome, although the initiation of viral replication relies on the

assembly of the hexameric large T antigen helicase at the viral origin of replication. The assembly of hexameric helicases leads to the formation of two replication forks that replicate the viral genome via bidirectional theta replication. The initiation of polyomavirus replication and the functions of the large T antigen are reviewed in (217, 218). The bidirectional progression of polyomavirus replication forks is halted when replication is approximately 91% complete and occurs in preparation for the separation of daughter molecules (219, 220). The separation of daughter molecules appears to be a slow, rate-limiting process, with the nearly fully replicated molecules accumulating in polyomavirus-infected cells (219–221).

Polyomaviruses actively engage components of the ATM and ATR signaling pathways to viral replication foci (222–224) and inhibition of the ATM and ATR pathways decreases viral replication efficiency (223–228). An analysis of polyomavirus replication intermediates suggests that ATM and ATR are activated by replication stress and contribute to the maintenance of replication fork integrity during replication (222, 224). Additionally, the ATR pathway may participate in the separation of daughter molecules following the replication of the viral genome (224), while ATM activation represses NHEJ to prevent the creation of aberrant viral genomes during replication (229).

Interestingly, compared with HPV E1/E2-dependent replication, polyomavirus SV40 large-T-dependent DNA replication is arrested after the ATR-mediated activation of cellular checkpoint response to DSBs (206).

3. OBJECTIVES OF STUDY

Persistent papillomavirus infection is a considerable health risk that could potentially lead to the development of severe malignancies. However, the development of efficient and specific drugs against papillomavirus infections has been hampered by a lack of understanding of viral genome replication and maintenance in host cells. Our research group has long concentrated on identifying the mechanisms underlying these viral functions that are essential for the establishment of a productive and persistent papillomavirus infection.

The general objectives of the research detailed in the present thesis involve developing a cellular assay capable of supporting all three phases of papillomavirus replication and utilizing the developed cellular assay to investigate the initial amplification replication of the HPV genome.

The newly developed U2OS cell line-based model system was implemented to study the early events of HPV replication, and we specifically concentrated on the following:

- Examining and characterizing HPV DNA that contains several copies of the viral genome (referred to as oligomeric HPV genomes for brevity) generated during the initial amplification of HPV type 18 and type 11 genomes;
- Identifying the mechanisms underlying the generation of oligomeric HPV18 and HPV11 genomes;
- Analyzing the replication intermediates generated during the initial amplification replication of the HPV18 genome to identify the mechanism of replication utilized for HPV amplification.

4. MATERIALS AND METHODS

In the present thesis, we used the U2OS cell line-based assay system to study the characteristics and generation of oligomeric HPV genomes and to analyze replication intermediates generated during the initial amplification of HPV genomes. The development of the U2OS-based assay has been thoroughly described in the Materials and Methods section of paper I.

Most of the experiments discussed in the present thesis study events that occur during the initial amplification of HPV genomes, which was mimicked by transfecting the U2OS cells with plasmids containing HPV genomes. Initially, input HPV genomes were produced in bacteria as plasmids containing the full-length viral genome cloned into a bacterial vector. Prior to transfection, HPV genomes were cleaved out from bacterial vectors and recircularized at low DNA concentrations. The experimental procedures used to generate recircularized HPV genomes have been detailed in the Materials and Methods section of paper I. Prior to studying the characteristics and generation of oligomeric genomes, the production of input HPV genomes for the U2OS cell line-based assay system was modified. HPV11 and HPV18 full-length genomes that are essentially free from bacterial vector sequences were purified from *E. coli* strain ZYCY10P3S2T as covalently closed circular plasmids generated through minicircle production technology (230). The modified U2OS-based assay system was also used in paper III. The production of minicircle HPV genomes has been detailed in the Materials and Methods sections of papers II and III.

During the transient replication assay mimicking initial amplification replication, low-molecular weight (LMW) DNA containing viral DNA was extracted from HPV-transfected cells, and the intermediates and end products of viral replication were analyzed using agarose gel electrophoresis (AGE) followed by Southern blotting. HPV replication end products were analyzed using one-dimensional (1D) AGE, HPV replication intermediates were analyzed using two-dimensional (2D) neutral/neutral (N/N), 2D neutral/alkaline (N/A) and three-dimensional (3D) neutral/neutral/alkaline (N/N/A) AGE analysis. 2D and 3D AGE analyses are based on running consecutive perpendicular gel electrophoresis analyses under different electrophoresis conditions. 2D N/N AGE enables the separation of branched replication intermediates and linear molecules (231, 232), 2D N/A AGE facilitates the analysis of replication intermediates based on their nascent strand composition (233), and 3D N/N/A AGE combines the 2D N/N and N/A analyses (234, 235). The experimental procedures used during DNA extraction and analysis have been described in the Materials and Methods sections of papers I, II and III.

U2OS cells are not natural papillomavirus host cells. The U2OS (originally 2T) cell line was derived from a human mesenchymal tumor, specifically a moderately differentiated osteogenic sarcoma of the tibia from a 15-year-old patient (236). The cells are characterized by the preservation of mitotic activity (even in crowded cultures), an epithelial-like growth pattern, and extensive

chromosomal rearrangements (236). The U2OS cell line-based assay system cannot model the entire HPV life cycle culminating with the production of the viral particles; however, our data suggest the system is suitable to study HPV replication. The initiation of HPV replication in U2OS cells requires the expression of the full-length viral replication proteins E1 and E2, and later studies by our research group have demonstrated that the transcription patterns of the genomes of HPV types 18, 11 and 5 in U2OS cells are similar to those in keratinocytes (30, 31, 237). Additionally, the generation of oligomeric HPV genomes upon initial HPV replication was also observed in the SiHa, HeLa and C-33 A cells, and the HPV replication intermediates generated in U2OS cells were similar to the HPV replication intermediates observed during the initial amplification of HPV genomes in the HaCaT cells. The SiHa, HeLa and C-33 A cell lines were derived from cervical carcinomas (238–240), HaCaT cells were established from spontaneously transformed epithelial cells (241).

AGE analysis enables the characterization of replication intermediates and end products based on the molecular weight and shape of the analyzed molecules, and is not always sufficient to identify the analyzed molecules. For the characterization of oligomeric HPV genomes, restriction analyses and specific enzyme treatments were used prior to 1D AGE to alleviate the limitations of AGE analysis. During the characterization of HPV replication intermediates, several different AGE techniques were used in combination to avoid possible misinterpretations.

5. RESULTS AND DISCUSSION

5.1 Generation of oligomeric molecules of the viral genome during the replication of HPV genomes in U2OS cells (I)

To study the replication of HPV genomes, our research group developed a U2OS cell line-based assay system that is capable of supporting the replication of high- and low-risk mucosal and cutaneous HPVs (I). The initial transient amplification of HPV was mimicked by transfecting the U2OS cells with plasmids containing HPV genomes (I, Fig. 1); the stable maintenance replication of HPV was mimicked by generating stable HPV-positive U2OS subclone cell lines (I, Fig. 2); and the vegetative amplification replication of HPV was achieved by cultivating stable HPV-positive cell lines under dense cell culture conditions (I, Fig. 3). The U2OS cell line supported the initial transient, stable maintenance and vegetative amplification replication phases of HPV low-risk mucosal types 6b and 11, high-risk mucosal types 16 and 18, and cutaneous types 5 and 8 (I, Fig. 1, Fig. 2, Fig. 3). The initiation of HPV replication in U2OS cells is dependent on the expression of the viral replication proteins E1 and E2, which can be derived either from the viral genome or exogenous expression vectors (I, Fig. 1e).

We noticed that HPV genomic replication in U2OS cells not only yielded viral molecules containing a single copy of the HPV genome (hereafter referred to as monomeric HPV genomes) but also generated HPV genomes in a higher molecular weight form (I, Fig. 5a and b). An analysis of the HPV18 DNA extracted from transfected U2OS cells mimicking the initial transient replication phase of the viral genome revealed that the HPV DNA in the host cells was in a predominantly monomeric genomic form up to 4 days post-transfection (I, Fig. 5a, lanes 1–2 and 4–5), although higher molecular weight forms were also detected. At three weeks post-transfection, only the higher molecular weight forms were observed (I, Fig. 5a, lane 7), indicating a selective advantage for the maintenance of higher molecular weight forms during the stable maintenance replication phase.

The analysis of HPV18-positive U2OS subclone cell line #1.13 confirmed that the majority of HPV genomic material persists in U2OS cells in the higher molecular weight forms (I, Fig. 5b, lane 1). Under conditions mimicking the onset of vegetative amplification of the viral genome, robust amplification of both the monomeric and higher molecular weight HPV18 genomes occurred (I, Fig. 5b, lanes 2–6), demonstrating that both forms are replication competent. Restriction and 2D N/N AGE analyses revealed that the higher molecular weight forms of HPV18 DNA were episomal molecules containing several copies of the viral genome (hereafter referred to as oligomeric HPV genomes for brevity) (I, Fig. 5c, 5d, 5f, 5g and 5h).

In addition to the U2OS cell line-based assay system, oligomeric papillomavirus genomes have also been detected in numerous tissue samples collected

from HPV-infected patients (127–129, 131–133), HPV-transformed human keratinocyte cell lines established from cervical lesions (125, 126), and BPV1-positive mouse fibroblast cell line ID13 (115). Because an increased prevalence of oligomeric genomes was observed over time compared with the prevalence of monomeric genomes, the oligomeric genomes likely have a selective advantage over single-copy HPV genomes during prolonged maintenance in dividing cells. Thus, oligomerization is likely especially important for the stable maintenance phase of viral replication. The long-term maintenance of viral genomes depends on the replication and segregation functions of episomal molecules, and oligomeric HPV genomes can be expected to be efficient in both functions. During the replication of an oligomeric genome, a single initiation event would trigger the synthesis of several copies the HPV genome and ensure a high copy number of viral genomes in the host cell, even under circumstances when the expression of viral replication proteins is at a low level. Similarly, during the segregation of viral genomes between daughter cells, oligomeric molecules would have advantages over a large number of monomeric genomes.

Several mechanisms could potentially lead to the generation of oligomeric HPV genomes. Additionally, oligomerization may be a separate process or it could be linked to other viral functions, with genomic replication an especially likely candidate. Moreover, although oligomeric HPV genomes have been detected both *in vitro* and *in vivo* and hold advantages over monomeric genomes during long-term maintenance in host cells, they may represent an aberrant form of the HPV genome because only monomeric viral genomes are ultimately packaged into viral particles. Therefore, if oligomeric HPV genomes are part of the productive viral life cycle, they must eventually be rearranged back into monomeric genomes.

The generation of linear concatemeric molecules containing several copies of the viral genome has been described for bacteriophage SPP1 replication (chapter 2.3.3 Recombination-dependent DNA replication). The SPP1 dsDNA genome takes a circular form upon entering the host cell but is in linear form when packaged into viral particles. The linear concatemeric molecules generated during SPP1 replication through a switch from circle-to-circle theta replication to recombination-dependent rolling-circle replication serve as necessary substrates for the packaging machinery. The SPP1 rolling-circle mediated model for the generation of concatemeric viral genomes suggests that rolling-circle replication may also be involved in the generation of HPV viral oligomers. The involvement of rolling-circle replication in HPV vegetative amplification replication has been previously proposed (117), lending further plausibility to this particular hypothesis. However, there are inconsistencies between the oligomeric molecules generated during HPV initial amplification and SPP1 replication. Oligomeric HPV genomes are circular molecules and not linear; however, HPV oligomers may become circular after these molecules have been generated as linear concatemers and this inconsistency does not exclude the possibility of similarities among the mechanisms used by these viruses to

generate oligomeric molecules. A more significant inconsistency between the HPV oligomerization model and the mechanisms utilized by SPP1 is related to the purpose of the generated oligomers because while SPP1 linear concatemers are required for viral packaging, the biological relevance of HPV oligomers remains unknown.

5.2 HPV genomic oligomers form via homologous recombination during the initial amplification of the HPV genome (II)

To investigate the generation of oligomeric HPV genomes, we focused on the initial transient amplification phase of HPV replication when the viral genomic oligomers first appear (II). As previously indicated, the initial transient replication was mimicked by transfecting U2OS cells with plasmids containing HPV genomes. To further evaluate the importance of oligomerization in the HPV replication cycle, two HPV types were used during the analysis: high-risk type 18 and low-risk type 11.

First, we confirmed the results obtained in reference I Figure 5a and b (II, Fig. 1c) and again observed that when HPV18-transfected U2OS cells were cultivated for 2 weeks under subconfluent cell culture conditions with regular passaging, the initial prevalence of monomeric viral genomes at early time points (II, Fig. 1c, lanes 1–5) was replaced by the prevalence of oligomeric viral genomes at later time points (II, Fig. 1c, lanes 7–8). When the HPV18wt-transfected cells were cultivated under dense cell culture conditions to induce the vegetative amplification of viral genomes, the oligomeric genomes became the prevalent form of HPV DNA, although the monomeric genomes also underwent amplification (II, Fig. 1c, lanes 11–12). These data confirmed that oligomeric HPV genomes are generated during the initial amplification phase of viral replication, and they appear to have a selective advantage over monomeric genomes during the stable maintenance phase and are replication competent during the vegetative amplification phase of replication.

To further characterize the oligomeric molecules, LMW DNA from HPV18-transfected U2OS cells was subjected to a restriction enzyme analysis (II, Fig. 2a and b), topoisomerase I treatment (II, Fig. 2c) and 2D N/N AGE (II, Fig. 2e and 2f). These analyses confirmed that the HPV18 oligomers are episomal concatemers containing the viral genome in a head-to-tail orientation.

After determining the characteristics of the oligomeric genomes, we evaluated the role of viral proteins in the induction of oligomerization (II, Fig. 3b). Because oligomeric HPV genomes are generated during the initial amplification replication, it is highly unlikely that either of the late viral proteins that are expressed during the late stages of viral replication is involved in the process. Indeed, the subgenomic HPV18 construct that lacks the late open reading frames encoding viral capsid proteins L1 and L2 (the HPV18E construct)

demonstrated efficient replication and oligomerization (II, Fig. 5b, lanes 1–4), confirming that the L1 and L2 proteins are not necessary for oligomerization. To analyze the role of the HPV early proteins, a series of full-length HPV18 mutant genomes were generated, and they each lacked the expression of a single early viral protein. The HPV18 E6$^-$ (II, Fig. 3b, lanes 4–6), E7$^-$ (II, Fig. 3b, lanes 7–9), E8$^-$ (II, Fig. 3b, lanes 13–15), E4$^-$ (II, Fig. 3b, lanes 19–21), E1E4$^-$ (II, Fig. 3b, lanes 22–24) and E5$^-$ (II, Fig. 3b, lanes 25–27) mutant genomes were all replication capable and produced oligomeric viral genomes, indicating that the expression of these viral proteins is not necessary for oligomerization.

The HPV18 E1$^-$ (II, Fig. 3b, lanes 10–12) and E2$^-$ (II, Fig. 3b, lanes 16–18) mutant genomes were replication deficient as expected. To investigate the role of E1 and E2 proteins in the initiation of oligomerization, we co-transfected U2OS cells with a minicircle plasmid containing the non-coding region of the HPV18 genome (the HPV18 URR construct) and the E1 and E2 expression vectors (II, Fig. 4). The HPV18 URR construct contains the origin of HPV18 replication and viral regulatory regions but does not express any viral proteins. Nonetheless, the HPV18 URR construct was capable of oligomerization as well as replication in the presence of the viral replication proteins E1 and E2 (II, Fig. 4), raising the possibility that oligomerization is induced by replication. Whether the E1 and E2 protein have an additional function in the oligomerization process separate from their functions as replication factors could not be determined during these experiments. Collectively, these data suggest that oligomerization is linked to or the direct result of HPV genomic replication, and it requires no other viral factors than those associated with viral replication.

The replication of circular molecules, such as the HPV genome, could lead to the generation of catenated molecules; however, the generation of circular concatemeric molecules cannot be directly linked to any of the replication mechanisms associated with the replication of circular molecules. Circular genomes can replicate either via theta or rolling-circle replication, and neither of these mechanisms is capable of generating the observed oligomeric HPV genomes. However, rolling circle replication can generate linear concatemeric molecules, which may then be circularized by the host cell. Alternatively, the generation of head-to-tail concatemeric molecules may be associated with recombination. Both rolling-circle replication and HR have previously been associated with papillomavirus replication (117, 141, 150).

To elucidate the mechanism underlying the generation of HPV oligomeric molecules U2OS cells were co-transfected with two distinguishable HPV18 (II, Fig. 5) or HPV11 (II, Fig. 6) genomes. The HPV18wt genome was co-transfected with the truncated HPV18E construct and produced HPV18 genomic oligomers containing both the HPV18wt and HPV18E molecules (II, Fig. 5b, lanes 5–8). The co-transfection of HPV11 E1$^-$ and E2$^-$ mutant genomes, which are incapable of replication independently, produced HPV11 genomic oligomers containing both the E1$^-$ and E2$^-$ mutant genomes (II, Fig. 6, lanes 13–16). Rolling-circle replication uses a single molecule as a template for

generating multimers; however, HR occurs between two separate molecules. The generation of oligomeric molecules containing two different HPV genomes can only be achieved through HR, and these results indicate that HPV genomic replication is associated with cellular HR. However, these results do not rule out the possible involvement of rolling-circle replication in the initial amplification of the HPV genome. The concatemeric genomes of bacteriophage SPP1 are generated via recombination-dependent sigma type of rolling-circle replication. The first rounds of SPP1 replication proceed via unidirectional theta replication; however, it has been suggested that the progression of theta replication forks is quickly blocked, which leads to the collapse of these replication forks and generates DSBs (242, 243). The recombination-dependent re-initiation of replication subsequently triggers the onset of sigma replication and leads to the generation of concatemeric viral DNA (243). Thus, a mechanism that combines HR and rolling-circle replication may be involved in the generation of oligomeric HPV genomes.

The presence of oligomeric HPV genomes in tissue samples collected from HPV-infected patients has been previously noted (127–129, 131–133); however, we also determined that several eukaryotic cell lines in addition to the U2OS cell line were capable of supporting the oligomerization of HPV18E8$^-$ mutant and HPV11wt genomes during the initial amplification phase of viral replication (II, Fig. 7). The oligomerization of HPV genomes was analyzed in the HPV16-positive SiHa (II, Fig. 7a and 7c, lanes 1–9), HPV18-positive HeLa (II, Fig. 7b and 7c, lanes 10–18) and HPV-negative C-33 A (II, Fig. 7d and e) cell lines, which were all derived from cervical carcinomas (238–240). The SiHa, HeLa and C-33 A cell lines all supported HPV genomic oligomerization as well as replication, although not as efficiently as the U2OS cell line, indicating that HPV oligomeric genomes are generated in all cell lines capable of supporting HPV replication. We also initiated a collaboration with the Women's Clinic of the University of Tartu Hospital and analyzed cervical brush or colposcopy samples collected from HPV-infected women with persistent HPV18 or HPV16 infections. We published the results from the analysis of three patient samples, and they all contained oligomeric HPV molecules (II, Fig. 8). Our data together with the data from previously published research papers demonstrate that oligomeric HPV genomes can be found *in vivo*, suggesting that the oligomerization of HPV genomes is a naturally occurring phenomenon.

The efficient oligomerization of the HPV18 URR plasmid in the presence of E1 and E2 proteins suggests that oligomerization is replication-dependent. The co-transfection experiments with two distinguishable HPV18 or HPV11 genomes clearly demonstrates the presence of HPV genomic oligomers generated through HR. Collectively, these data indicate the involvement of cellular HR in HPV replication.

HR is involved in both ATM-mediated DSB repair and ATR-mediated repair of collapsed replication forks. The initial amplification replication of HPV

genomes is insensitive to the inhibition of the ATM-Chk2 pathway (203, 204); however, members of the ATR pathway (ATRIP and TopBP1) co-localize with HPV replication foci (204). TopBP1 is necessary for the establishment of HPV episomes in infected cells (110), and implicated in the ATR-mediated rescue of stalled replication forks in eukaryotic cells (111, 113). In eukaryotic cells, HR is facilitated by the MRN complex. The localization of MRN complex components with regard to viral replication centers has not been analyzed during the initial amplification phase of HPV replication; however, members of the MRN complex are recruited to HPV replication centers during stable maintenance and vegetative amplification replication (142, 143). Importantly, efficient vegetative amplification relies on the recruitment of the MRN complex and the Rad51 protein to HPV replication centers as well as the nuclease activity of Mre11 (143). Collectively, these data demonstrate the necessary engagement of the homologous repair machinery during HPV replication and indicate that this engagement may be mediated by the ATR signaling pathway. Our results, however, demonstrate that HR end products are generated during HPV replication.

The functions of cellular DDR and HR machinery activation during HPV replication remain unknown. The essential functions of the MRN complex in eukaryotic and prokaryotic cells may be the resolution of replication intermediates and the rescue of stalled replication forks (171, 188), and the MRN-mediated activation of the ATR signaling pathway has similarly been proposed as primarily facilitating the repair of stalled and collapsed replication forks (169, 185). During polyomavirus replication, the ATM and ATR signaling pathways are both activated by replication stress (222, 224), and cellular DDR activation is required for the maintenance of viral replication fork integrity (224, 229). Thus, the involvement of HR in HPV genomic replication may be limited to similar functions.

During bacteriophage SPP1 replication, the induced collapse of replication forks facilitates a shift to a recombination-dependent mode of replication. The involvement of a second replication mechanism has long been suspected in papillomavirus replication, especially during the later phases of viral replication (117), and the engagement of HR during viral replication may indicate the presence of a recombination-dependent mode of HPV replication (150). During bacteriophage SPP1 replication, the initiation of recombination-dependent mode of replication is facilitated by a viral recombination protein (243); however, with the possible exception of the HPV replication proteins E1 and E2, viral proteins are dispensable for the recombination-dependent generation of HPV oligomers (II, Fig. 3b and 4) and recombination-associated functions have not been indicated for any HPV proteins. The HPV genome is also significantly smaller than the SPP1 genome and heavily reliant on host factors for the replication of its genome. Therefore, while HPV may, similarly to SPP1, switch to a recombination-dependent mode of replication, the mechanisms these viruses use to facilitate the switch are likely different.

5.3 Evidence of the involvement of two different replication mechanisms in the initial amplification of the HPV genome (III)

The initial amplification of the HPV genome is dependent on the presence of the viral E1 and E2 proteins ((83); I, Fig. 1e; II, Fig. 3), and it is believed to proceed via bidirectional theta replication; however, initial amplification also produces oligomeric HPV genomes generated via HR (II), and the components of the ATR signaling pathway are recruited to HPV replication foci during this phase (204). To determine the mechanism underlying the initial amplification of the HPV genome, replication intermediates generated in HPV18-transfected U2OS cells were analyzed using 2D N/N, 2D N/A and 3D N/N/A AGE analysis.

An analysis of the replication intermediates generated during the initial amplification of the HPV18 genome in U2OS cells indicated that the initial rounds of HPV replication proceed via bidirectional theta replication initiated from the origin of replication located in the non-coding region of the HPV18 genome (the URR origin) (III, Fig. 2). Theta replication intermediates retained their strong presence throughout the analyzed time points up to 5 days post-transfection (III, Fig. 2). We noticed a considerable accumulation of late theta replication intermediates, which indicated difficulties with the completion of theta replication (III, Fig. 2, Fig. 3, and Fig. 4).

We also noted the presence of replication intermediates that do not resemble the intermediates of bidirectional theta replication (III, Fig. 2, Fig. 3, Fig. 4, Fig. 5, and Fig. 6). These novel or secondary replication intermediates were not initiated from a specific origin sequence (III, Fig. 3a and Fig. 5) and contained a unidirectional replication fork (III, Fig. 4 and Fig. 5). The unidirectional novel replication forks did not have a fixed polarity because these forks can run through the HPV genome in either direction (III, Fig. 6). Because the characteristics of the novel replication intermediates indicated essential differences with the intermediates of bidirectional theta replication, we concluded that a second mechanism was involved in the initial amplification of the HPV18 genome. Notably, we were unable to detect molecules representing the very early intermediates of the second replication mechanism because the observed intermediates of the second replication mechanism never emanated from the signal representing 1n linear non-replicating molecules (III, Fig. 2, Fig. 3, and Fig. 5). This result may indicate that the very early intermediates of the second replication mechanism are especially labile molecules. Alternatively, the structures generated following the initiation of the second replication mechanism may simply be larger and more structurally complex than the 1n linear molecules.

Both bidirectional theta and second replication intermediates were observed during the initial amplification of HPV18 genomes in the HaCaT cells (III, Fig. 7). The HaCaT cell line was established from spontaneously transformed epithelial cells and retains the ability to differentiate distinctive to normal keratino-

cytes (241). The generation of similar replication intermediates in both U2OS and HaCaT cells indicates that the replication intermediates observed during the HPV18 genome replication in U2OS cells represent authentic HPV replication intermediates.

Importantly, the analysis of uncut HPV18 replication intermediates was used to investigate the involvement of rolling-circle replication in the initial amplification of the HPV genome (III, Fig. 1c). Rolling-circle replication produces distinct σ-shaped replication intermediates, and these intermediates were not observed during the analysis of uncut HPV18 replication intermediates (III, Fig. 1c), indicating that rolling-circle replication is not involved in HPV replication.

Bidirectional theta replication has long been considered to be the mechanism underlying the stable maintenance replication of papillomavirus genomes (114, 115, 117), and it has also been implicated in the vegetative amplification of the HPV genome (116). Our data confirmed the involvement of bidirectional theta replication in the initial amplification replication of HPV genomes. Because the bidirectional theta replication intermediates appeared before the second replication intermediates and were likely initiated from the HPV18 URR origin (35), bidirectional theta replication is likely the mechanism underlying the E1/E2-dependent viral replication.

An analysis of replication intermediates alone is not sufficient to identify the mechanism underlying the generation of the second non-theta group of replication intermediates. However, because of the strong evidence for the involvement of HR in HPV genomic replication observed by our group and other research groups, RDR is a likely candidate for the mechanism underlying the generation of the second replication intermediates.

The characteristics of the intermediates of the second replication mechanism noted here are consistent with RDR. RDR is initiated by the invasion of a ssDNA 3’ end into a homologous sequence, and then either a fully functional replication fork is assembled and semiconservative replication is established or conservative migrating bubble replication is established (192–196). RDR does not require a specific viral sequence for initiation because the site of replication initiation is determined by the sequence of the invading ssDNA end. The initiation structures of RDR are larger than the 8 kbp HPV genome, and based on analogy with the branch migration of Holliday junctions, the strand invasion intermediates would also be quite labile molecules (244), thus accounting for our inability to detect the initiation structures of the second replication mechanism.

The origin of the free 3’ ssDNA ends and the trigger of the second replication mechanism are unknown. During the recombination-dependent rolling-circle replication of bacteriophage SPP1 genomes, RDR is likely initiated from a collapsed theta replication fork (243). An analysis of uncut HPV18 replication intermediates indicates that σ-shaped intermediates characteristic of rolling-circle replication are not present during the initial amplification of the HPV genome, thus ruling out the presence of a replication mechanism similar to that

of SPP1. However, the initiation of the papillomavirus second replication mechanism may result from a similar event.

The E1 protein is the sole component of the HPV hexameric helicase and has the intrinsic ability to generate DSBs in the viral genome (204, 205). However, the E1-mediated generation of DSBs occurs during the overexpression of the E1 protein, and large-scale DNA damage has not been observed during E1/E2-dependent viral replication (203, 204). However, there are still indications that the E1/E2-dependent bidirectional theta replication of HPV genomes may generate replication-associated DSBs leading to the initiation of a recombination-dependent mode of replication. For example, the E2-mediated recruitment of TopBP1 is required for productive HPV replication (110) and TopBP1 co-localizes with HPV replication foci during the initial amplification phase of replication (204). TopBP1 is associated with the ATR-mediated rescue of stalled replication forks (111, 113). The TopBP1-mediated rescue of stalled replication forks relies on the presence of the MRN complex and the nuclease activity of Mre11 (169), both of which are also necessary for the efficient replication of HPV genomes during the vegetative amplification phase (143).

It remains unclear whether triggering the second replication mechanism is a one-off event, meaning that once it is triggered, the second replication mechanism can continuously re-initiate without the need for further triggering events, or whether the second replication mechanism must be continuously induced. During SPP1 replication, once recombination-dependent rolling-circle replication is initiated, the process takes over the replication of the viral genome. During HPV replication, however, bidirectional theta replication intermediates occur at later time points simultaneously with the intermediates of the second replication mechanism, indicating a certain degree of flexibility in the choice of mechanism for HPV genome replication.

The accumulation of nearly fully replicated late theta intermediates may also be involved in inducing the second replication mechanism. This phenomenon is not novel because the accumulation of nearly full replicated late theta intermediates is also noted during polyomavirus replication. In the case of the polyomavirus SV40, replication forks initiated at the origin of replication and proceeding bidirectionally halted when replication was approximately 91% complete (219–221); the halting of replication forks likely occurred in preparation for the separation of daughter molecules. The separation of nearly fully replicated intermediates generates molecules containing either regions of ssDNA or even DSBs and potentially leads to the activation of RDR.

Based on the analysis of replication intermediates that are generated during the initial replication of the HPV18 genome in U2OS cells, we propose a dual-mechanism model of HPV replication.

5.3.1 Dual-mechanism model of papillomavirus replication

The initiation of HPV replication is dependent on the presence of E1 and E2 proteins and the origin of replication located in the non-coding region of the viral genome (the URR origin) (I, Fig. 1e; II, Fig. 3; (34, 37, 82–86)), and the initial HPV replication proceeds via bidirectional theta structures (III, Fig. 2). After the onset of E1/E2-dependent bidirectional theta replication, the second replication mechanism is initiated (III, Fig. 2) as a direct result of the ongoing E1/E2-dependent replication. The second replication mechanism is likely RDR (II, Fig. 5 and 6; (142, 143, 150, 204)). A possible trigger for the initiation of the second replication mechanism may be the stalling and eventual collapse of the theta replication forks, which results in the recruitment of cellular DDR factors to viral replication foci and the re-initiation of replication via a recombination-dependent mechanism. Alternatively, the recruitment of cellular DDR factors to viral replication centers may be facilitated by viral proteins involved in HPV replication similarly to the recruitment of cellular replication machinery to the viral origin of replication, and the process may proceed without requiring a specific triggering event. Throughout papillomavirus infection, however, the viral genome retains the availability of both E1/E2-mediated and recombination-dependent initiation of replication.

There are several indications that HPV genomes are inherently capable of replicating via two different mechanisms during the stable maintenance and vegetative amplification phases of replication.

The analysis of stable maintenance replication in HPV-positive cell lines derived from naturally infected cervical tissues demonstrated that HPV genomes replicate via two different modes, once-per-S-phase (similar to cellular DNA) or randomly, and the selected mode is determined by the availability of viral replication proteins (124). The presence of the E1 protein, however, is not required for the stable maintenance replication phase (84). However, during stable maintenance replication, bidirectional theta replication intermediates initiated from the E1/E2-dependent URR origin of replication are the prevalent replication intermediates (117), indicating that E1/E2-dependent bidirectional theta replication is involved during this phase of HPV replication. Similarly, proteins involved in cellular DDR and HR co-localize with HPV replication foci in approximately 50% of HPV-positive cells during the stable maintenance phase of replication (142, 143), although the inhibition of cellular DDR has no discernible effect on stable maintenance replication (141, 143).

The vegetative amplification of the HPV genome is dependent on the presence of the E1 protein (84). An analysis of the replication intermediates generated during the vegetative amplification of HPV types 16 and 31b, however, indicated that the most prevalent replication intermediates were generated via a unidirectional replication mechanism without specific initiation or termination sequences rather than bidirectional theta replication initiated from the E1/E2-dependent URR origin of replication (117). Cellular DDR activation contributes to vegetative amplification by activating caspases involved in the

cleavage of the E1 protein, which is required for efficient vegetative amplification of the HPV genomes (140, 141). However, whether E1 protein cleavage contributes to generating the unidirectional replication mechanism remains unknown. Additionally, the co-localization of HPV replication foci and proteins associated with cellular DDR and HR increases after the onset of the vegetative amplification of the HPV genome (141–143), and HPV vegetative amplification replication is notably decreased but not eliminated by disrupting the nuclease activity of Mre11 and the localization of the MRN complex and Rad51 protein to the HPV replication foci (143).

These data indicate that both E1/E2-dependent replication and a second, likely recombination-dependent, replication mechanism are simultaneously utilized for the replication of HPV genomes during the stable maintenance and vegetative amplification phases of viral replication. The analysis of the replication intermediates generated during the initial amplification phase of the HPV18 genome also indicated the involvement of two separate mechanisms during that phase of HPV replication. Collectively, these data suggest that papillomaviruses are capable of using two different replication mechanisms to multiply the viral genome throughout all three phases of HPV replication.

5.3.2 Perspectives on the dual-mechanism model of replication

The results presented in this thesis highlight the unique characteristics of the mechanism underlying HPV genomic replication. The utilization of several different mechanisms during the viral replication cycle is not uncommon, and the engagement of factors involved in cellular recombination and repair functions for the replication of the viral genome is not unusual. However, HPV replication obviously does not follow any of the previously described models for other DNA viruses (e.g., polyomavirus SV40 or bacteriophage SPP1 replication). The uniqueness of the HPV replication mechanism likely mirrors the uniqueness and complexity of the papillomavirus life cycle.

Additional work must be performed to identify with a degree of certainty the second mechanism involved in the initial amplification replication of the HPV genome. Because we propose that the second replication mechanism is recombination-dependent, different cellular repair and recombination factors should be knocked out and the associated effects on the intermediates of the second replication mechanism should be investigated. Additionally, the structure of the replication intermediates generated via the second replication mechanism should be further characterized using direct visualization via electron microscopy or atomic force microscopy. After identifying the replication mechanism, a detailed investigation of the events occurring during the initiation, elongation and termination phases of the second replication mechanism should be conducted.

Utilizing two different replication mechanisms would enable papillomaviruses to maintain HPV genomic replication under changing host cell environments, and such a construct would contribute to highly efficient replication

during the amplification phases. The exact mechanism underlying the second mode of replication remains unknown, although evidence suggests that the initiation of the second mechanism is not licensed by the (exact) same factors involved in the initiation of the bidirectional theta replication of HPV genomes. The presence of two replication mechanisms with different initiation requirements certainly offers a degree of flexibility for host cell conditions that are conducive to HPV replication. If the second replication mechanism is indeed largely driven by cellular repair and recombination machinery, it would provide a mechanism of replicating the viral genome that requires minimal viral replication factors (with the onset of the second replication mechanism being a likely exception). Such a mechanism would allow viral replication to occur even under host cell conditions that do not permit the large-scale expression of viral proteins, which would make the second replication mechanism especially important during the stable maintenance phase of replication when the expression of proteins from the HPV genome is low. Because of the apparent ability of the second replication mechanism to function simultaneously with bidirectional theta replication without generating aberrant replication intermediates, this mechanism would also be conducive to HPV replication during the initial and vegetative amplification phases. Therefore, a dual-mechanism replication model would be highly beneficial for the virus.

Demonstrating the presence of two different replication mechanisms during papillomavirus replication could have significant implications for the treatment of HPV-associated lesions. Specific inhibitors of HPV replication are not currently used for the treatment of HPV-associated afflictions; however, the involvement of two different mechanisms in the replication of the HPV genome indicates that any potential drug should be able to target both of these mechanisms to eradicate HPV infection. It is highly unlikely that a single inhibitor could be efficient against two mechanisms that display such considerable differences while maintaining the specificity required to only affect viral DNA replication. Thus, a combination of inhibitors should most likely be used, with one inhibitor that targets E1/E2-dependent replication via bidirectional theta structures and one inhibitor that targets the second replication mechanism. The dual-mechanism model of replication would also provide a reason to re-examine any potential drugs that were designed to inhibit conventional E1/E2-dependent papillomavirus replication but failed to eliminate viral replication. These drugs may have been functional against their intended targets, although the presence of the second replication mechanism could have resulted in the overall continuation of viral replication. Additionally, if the second replication mechanism is indeed RDR, drugs originally intended for the treatment of other diseases (e.g., anti-cancer drugs) might be repurposed for the treatment of papillomavirus infections in combination with inhibitors of E1/E2-dependent replication via theta structures.

6. CONCLUSIONS

1) The U2OS cell line-based assay system developed by our research group is suitable for the analysis of genomic replication of both high-risk and low-risk HPV types.

2) HPV initial amplification replication generates monomeric and oligomeric viral genomes. Although the monomeric genomes are initially the prevalent form of viral DNA, oligomeric forms appear to have a selective advantage during the long-term maintenance of HPV genomes in host cells.

3) Oligomeric HPV genomes are generated via HR, and the process is likely replication dependent. The generation of oligomeric viral genomes is a direct evidence of the actual engagement of cellular HR machinery during the replication of HPV genomes.

4) The replication intermediates generated during the initial amplification of the HPV18 genome were analyzed, and the results demonstrate that two separate mechanisms are involved in papillomavirus replication. One of the mechanisms is bidirectional theta replication, and we propose that the other mechanism is RDR.

5) We propose a dual-mechanism replication model for the replication of papillomavirus genomes throughout all three phases of papillomavirus replication.

SUMMARY IN ESTONIAN

Inimese papilloomiviiruse genoomi paljundamise varajaste etappide uurimine

Inimese papilloomiviirused (HPVd) nakatavad inimorganismis teadaolevalt ainult ühte tüüpi rakke, naha ja limaskestade epiteelkoes paiknevaid keratinotsüüte. Tavaliselt ei tekita HPV nakkus epiteelkoes märgatavaid haigustunnuseid, kuid viiruse osadele variantidele ehk tüüpidele on omane võime kutsuda esile peremeesrakkude ebatavaliselt kiiret paljunemist, mis võib viia nakatunud koes healoomuliste kasvajate arenguni. Pikaajaline HPV nakkus võib aga põhjustada viiruse tekitatud healoomulise kasvaja muutumise halvaloomuliseks. Püsivat HPV nakkust peetakse emakakaelavähi tekke kõige olulisemaks riskifaktoriks, kuid HPVd on seotud ka teiste genitaal- ja anaalpiirkonna ning pea- ja kaelapiirkonna kasvajate arenguga.

Iga eduka viirusnakkuse käigus toimub nakatunud rakkudes lõpuks viiruse päriliku materjali (ehk viiruse genoomi) paljundamine ning uute viirusosakeste tootmine. HPV pärilikkusmaterjaliks on kaheahelaline DNA, mis võimaldab viirusel kasutada oma genoomi paljundamiseks peamiselt peremeesraku DNA paljundamise (ehk replikatsiooni) masinavärki. HPV päriliku materjali paljundamise algatamisel ning peremeesraku replikatsiooni masinavärgi kaasamisel viiruse genoomi paljundamisesse mängivad aga võtmerolli kaks viirusliku päritoluga valku.

HPV peremeesrakke keratinotsüüte iseloomustab keeruline, ettemääratud rakusurmaga lõppev elutsükkel, mille käigus rakud kaotavad paljunemisvõime ning sünteesivad endale ümber tugeva sarvkesta. Eduka HPV nakkuse korral siseneb viirus keratinotsüüti peremeesraku elutsükli varajases etapis, kuid uute viirusosakeste tootmine toimub alles elutsükli viimaseid etappe läbivates peremeesraku jäänukites. Seega peab HPV nakkuse käigus suutma end säilitada ning oma pärilikku materjali paljundada pidevalt muutuvas keskkonnas. Ilmselt tulenevalt vajadusest kohaneda peremeesraku keerulise elutsükliga toimub HPV genoomi paljundamine peremeesrakus kolmes selgelt eristuvas etapis: varajane kiire viiruse genoomi koopiaarvu tõus, genoomi koopiaarvu stabiilne säilitamine ja hiline kiire viiruse genoomi koopiaarvu tõus.

Laboritingimustes on HPV peremeesrakkude kasvatamine ning nende elutsükli matkimine küllaltki keeruline ning äärmiselt kulukas. Lisaks ei võimaldanud kasutusel olnud metoodikad uurida HPV genoomi replikatsiooni varajasi etappe. Seetõttu ei ole HPV genoomi paljundamise täpne mehhanism ning protsessis osalevad rakulised faktorid veel täielikult teada, kuigi need teadmised on väga olulised HPV-vastaste ravimite väljatöötamiseks. HPV-ga nakatumise vältimiseks on välja töötatud mitmeid efektiivseid, kuid ainult väikese hulga HPV tüüpide vastu kaitset pakkuvaid vaktsiine, samuti on HPV tekitatud kasvajaid võimalik eemaldada erinevate meditsiiniliste protseduuridega. Siiski

ei ole praegu kasutusel mitte ühtegi ravimit, mis suudaks takistada viiruse paljunemist nakatunud koes.

Käeolevas doktoritöös kajastatud uurimisprojekti eesmärk oli luua mudelsüsteem, mida saaks rakendada HPV päriliku materjali paljundamise uurimiseks, ning rakendada loodud mudelsüsteemi viiruse replikatsiooni varajaste etappide uurimiseks.

Välja töötatud mudelsüsteem põhineb HPV päriliku materjali sisestamisel inimese sääreluu kasvajast algatatud U2OS rakkudesse. Kuigi U2OS rakud ei ole HPV looduslikud peremeesrakud, suudavad nad toetada HPV genoomide kõiki kolme replikatsiooni etappi. U2OS rakkudel põhinevat mudelsüsteemi rakendades uurisime HPV päriliku materjali paljundamise varajases etapis tekkivaid viiruse DNA molekule, mis sisaldavad mitut viiruse genoomi koopiat. Selliste eripäraste HPV genoomide (edaspidi nimetatud viiruse või HPV genoomi oligomeerideks) teket on varasemalt täheldatud nii püsivat HPV nakkust kandvatelt patsientidelt eraldatud koeproovides kui ka patsientidelt võetud koeproovidest algatatud HPV genoomi kandvate rakuliinide analüüsil. Me tegime kindlaks, et HPV genoomi oligomeeride teke on seotud viiruse genoomi paljundamisega ning toimub homoloogilise rekombinatsiooni kaudu. Homoloogiline rekombinatsioon osaleb inimese rakkudes mitmetes protsessides, kuid tavapäraselt rakendatakse seda rakus tekkinud DNA kahjustuste parandamisel. Päriliku materjali terviklikkuse säilitamine ja selle kahjustada saamise vältimine on rakkudele väga oluline, mistõttu inimese rakkudes on arenenud mitmed keerukad mehhanismid DNA kahjustuste avastamiseks ning parandamiseks. Juba varem on HPV uurimise käigus täheldatud, et viiruse nakkuse käigus aktiveeruvad peremeesrakus signaalid, mis algupäraselt peaksid tähistama rakulise DNA kahjustuste olemasolu. Nende signaalide olemasolu on teadaolevalt vajalik viiruse päriliku materjali efektiivseks paljundamiseks, samuti on kirjeldatud DNA kahjustuste parandamiseks vajalike rakuliste valkude värbamine kompleksidesse, mis paljundavad HPV genoomi. Kuid meie rühmal õnnestus esmakordselt näidata, et rakulised DNA parandamise mehhanismid on reaalselt kaasatud HPV päriliku materjali paljundamisesse.

Lisaks analüüsisime HPV molekule, mis tekivad viiruse päriliku materjali paljundamise varajaste etappide käigus HPV replikatsiooni vahesaadustena. Uurides molekule, mis tekivad paljunemise protsessi käigus, on võimalik saada andmeid HPV genoomi replikatsiooni taga oleva mehhanism kohta. Meie tulemused näitasid, et viirus kasutab oma päriliku materjali paljundamiseks kahte eraldiseisvat mehhanismi. Üks neist, väga iseloomulike replikatsiooni vahesaadustega kahesuunalise theta replikatsiooni nime kandev mehhanism, on varasemalt seostatud HPV genoomi paljundamise hiliste etappidega. Kuid meie töö käigus ilmnesid ka uudsed, varem kirjeldamata replikatsiooni vahesaadused, mille tekkimise mehhanismi ei olnud võimalik kasutatud analüüsi käigus lõplikult selgitada. Tuginedes meie uurimisrühma ja teiste uurimisrühmade poolt avaldatud andmetele, pakume teise replikatsiooni mehhanismina välja rakuliste DNA parandamise mehhanismidega seotud rekombinatsioonist sõltuva replikatsiooni.

Me tõstatame hüpoteesi, et HPV on võimeline kõigi kolme replikatsiooni etapi jooksul kasutama kahte erinevat mehhanismi oma genoomi paljundamiseks. Viirusele oleks kahe eraldiseisva mehhanismi rakendamine oma päriliku materjali paljundamiseks äärmiselt kasulik, kuna kahe erineva mehhanismi kasutamine võimaldab viiruse genoomi paljundada erinevate peremeesraku tingimuste juures, mis on keratinotsüütide elutsükli iseärasustest tingituna HPV jaoks väga oluline. Samas võimaldab kahe mehhanismi üheaegne kasutamine paljundada viiruse pärilikku materjali lühikese aja jooksul äärmiselt efektiivselt. Samas raskendab kahe mehhanismi kasutamine viiruse DNA paljundamiseks oluliselt HPV genoomi replikatsiooni takistavate ravimite väljatöötamist, kuna tõhus ravim peab toimima kahe erineva mehhanismi vastu.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my family for their lifelong support and instilling within me trust for my own abilities and judgement.

I am very grateful to my supervisors Ene and Mart for all of the knowledge they passed on to me and for all of the help they provided during my BSc, MSc and PhD studies. I would also like to thank all of the past and present members of our research group with whom I have had the pleasure to work, especially Liisi Henno, Eva-Maria, Liza, Helen, Regina, Eve, Liisi Võsa, Matu, Tormi, Mart Toots, Andrei and Mihkel. An additional thank you is extended to the co-authors of the publications with which I was involved. It must also be noted that Liisi and Eva-Maria were absolutely wonderful supervisees, and a special thank you is extended to Helen for first teaching me how to perform experiments.

I thank Andres Merits and Juhan Sedman for their helpful comments on the thesis, and Reet Kurg for providing the template for Figure 3.

I would also like to thank my past and present colleagues from other research groups located on the 3rd and 4th floor of the Institute of Technology, especially Kadri, Sandra, Age, Sirle, Margit, Ervin, Tom, and Rait. I also appreciated all of the help Merike and Inge provided me on various occasions.

I would also like to thank my dear friends Marja, Teele and Erik. An extra special thank you is reserved for Margit Nõukas.

My PhD studies and the work published in papers I, II and III were supported by the Estonian Science Foundation (research grants 9385 and 9467), the Estonian Research Council (SF0180175A, SF0180175B, IUT 20-27), the Center of Excellence in Chemical Biology (3.2.0101.08-0017) through the European Regional Development Fund, Enterprise Estonia (EAS) Project EU42266, and the Graduate School of Biomedicine and Biotechnology created under auspices of European Social Fund.

REFERENCES

1. **Bernard HU, Burk RD, Chen Z, van Doorslaer K, Hausen H, de Villiers EM.** 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology **401:**70–79.
2. **de Villiers EM.** 2013. Cross-roads in the classification of papillomaviruses. Virology **445:**2–10.
3. **Bzhalava D, Eklund C, Dillner J.** 2015. International standardization and classification of human papillomavirus types. Virology **476:**341–344.
4. **Antonsson A, Erfurt C, Hazard K, Holmgren V, Simon M, Kataoka A, Hossain S, Hakangard C, Hansson BG.** 2003. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. J Gen Virol **84:**1881–1886.
5. **Foulongne V, Sauvage V, Hebert C, Dereure O, Cheval J, Gouilh MA, Pariente K, Segondy M, Burguiere A, Manuguerra JC, Caro V, Eloit M.** 2012. Human skin microbiota: high diversity of DNA viruses identified on the human skin by high throughput sequencing. Plos One **7:**e38499.
6. **Accardi R, Gheit T.** 2014. Cutaneous HPV and skin cancer. Presse Medicale **43:**E435–E443.
7. **Gissmann L, Devilliers EM, Hausen HZ.** 1982. Analysis of Human Genital Warts (Condylomata-Acuminata) and Other Genital Tumors for Human Papillomavirus Type-6 DNA. International Journal of Cancer **29:**143–146.
8. **Guimera N, Lloveras B, Lindeman J, Alemany L, van de Sandt M, Alejo M, Hernandez-Suarez G, Bravo IG, Molijn A, Jenkins D, Cubilla A, Munoz N, de Sanjose S, Bosch FX, Quint W, Grp RHTHVS.** 2013. The Occasional Role of Low-risk Human Papillomaviruses 6, 11, 42, 44, and 70 in Anogenital Carcinoma Defined by Laser Capture Microdissection/PCR Methodology Results From a Global Study. American Journal of Surgical Pathology **37:**1299–1310.
9. **Smith EM, Pignatari SSN, Gray SD, Haugen TH, Turek LP.** 1993. Human Papillomavirus Infection in Papillomas and Nondiseased Respiratory Sites of Patients with Recurrent Respiratory Papillomatosis Using the Polymerase Chain-Reaction. Archives of Otolaryngology-Head & Neck Surgery **119:**554–557.
10. **Durst M, Gissmann L, Ikenberg H, zur Hausen H.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A **80:**3812–3815.
11. **Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJF, Peto J, Meijer CJLM, Munoz N.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. Journal of Pathology **189:**12–19.
12. **De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S.** 2009. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. Int J Cancer **124:**1626–1636.
13. **Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D.** 2000. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst **92:**709–720.

14. **Gillison ML, Lowy DR.** 2004. A causal role for human papillomavirus in head and neck cancer. Lancet **363:**1488–1489.
15. **Forman D, de Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L, Vignat J, Ferlay J, Bray F, Plummer M, Franceschi S.** 2012. Global Burden of Human Papillomavirus and Related Diseases. Vaccine **30:**F12–F23.
16. **Franceschi S, Castellsague X, Dal Maso L, Smith JS, Plummer M, Ngelangel C, Chichareon S, Eluf-Neto J, Shah KV, Snijders PJF, Meijer CJLM, Bosch FX, Munoz N.** 2002. Prevalence and determinants of human papillomavirus genital infection in men. Br J Cancer **86:**705–711.
17. **Parkin DM, Bray F.** 2006. The burden of HPV-related cancers. Vaccine **24:**11–25.
18. **Tabrizi SN, Brotherton JM, Kaldor JM, Skinner SR, Cummins E, Liu B, Bateson D, McNamee K, Garefalakis M, Garland SM.** 2012. Fall in human papillomavirus prevalence following a national vaccination program. Journal of Infectious Diseases **206:**1645–1651.
19. **Markowitz LE, Hariri S, Lin C, Dunne EF, Steinau M, McQuillan G, Unger ER.** 2013. Reduction in human papillomavirus (HPV) prevalence among young women following HPV vaccine introduction in the United States, National Health and Nutrition Examination Surveys, 2003–2010. Journal of Infectious Diseases **208:**385–393.
20. **Baldur-Felskov B, Dehlendorff C, Munk C, Kjaer SK.** 2014. Early impact of human papillomavirus vaccination on cervical neoplasia – nationwide follow-up of young Danish women. J Natl Cancer Inst **106:**djt460.
21. **Powell SE, Hariri S, Steinau M, Bauer HM, Bennett NM, Bloch KC, Niccolai LM, Schafer S, Unger ER, Markowitz LE.** 2012. Impact of human papillomavirus (HPV) vaccination on HPV 16/18-related prevalence in precancerous cervical lesions. Vaccine **31:**109–113.
22. **Ogilvie GS, Naus M, Money DM, Dobson SR, Miller D, Krajden M, van Niekerk DJ, Coldman AJ.** 2015. Reduction in cervical dysplasia in young women in British Columbia after introduction of the HPV vaccine: An ecological analysis. Int J Cancer doi:10.1002/ijc.29508.
23. **Hariri S, Bennett NM, Niccolai LM, Schafer S, Park IU, Bloch KC, Unger ER, Whitney E, Julian P, Scahill MW, Abdullah N, Levine D, Johnson ML, Steinau M, Markowitz LE.** 2015. Reduction in HPV 16/18-associated high grade cervical lesions following HPV vaccine introduction in the United States – 2008–2012. Vaccine **33:**1608–1613.
24. **Pils S, Joura EA.** 2015. From the monovalent to the nine-valent HPV vaccine. Clin Microbiol Infect **21:**827–833.
25. **Sibbet GJ, Campo MS.** 1990. Multiple interactions between cellular factors and the non-coding region of human papillomavirus type 16. J Gen Virol **71 (Pt 11):**2699–2707.
26. **Garcia-Carranca A, Thierry F, Yaniv M.** 1988. Interplay of viral and cellular proteins along the long control region of human papillomavirus type 18. J Virol **62:**4321–4330.
27. **Sichero L, Sobrinho JS, Villa LL.** 2012. Identification of novel cellular transcription factors that regulate early promoters of human papillomavirus types 18 and 16. Journal of Infectious Diseases **206:**867–874.
28. **Stubenrauch F, Malejczyk J, Fuchs PG, Pfister H.** 1992. Late Promoter of Human Papillomavirus Type-8 and Its Regulation. J Virol **66:**3485–3493.

29. **Wang XH, Meyers C, Wang HK, Chow LT, Zheng ZM.** 2011. Construction of a Full Transcription Map of Human Papillomavirus Type 18 during Productive Viral Infection. J Virol **85:**8080–8092.
30. **Sankovski E, Mannik A, Geimanen J, Ustav E, Ustav M.** 2014. Mapping of betapapillomavirus human papillomavirus 5 transcription and characterization of viral-genome replication function. J Virol **88:**961–973.
31. **Toots M, Mannik A, Kivi G, Ustav M, Ustav E, Ustav M.** 2014. The Transcription Map of Human Papillomavirus Type 18 during Genome Replication in U2OS Cells. Plos One **9**.
32. **Hummel M, Hudson JB, Laimins LA.** 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J Virol **66:**6070–6080.
33. **Lu JZJ, Sun YN, Rose RC, Bonnez W, Mccance DJ.** 1993. 2 E2 Binding-Sites (E2bs) Alone or One E2bs Plus an a/T-Rich Region Are Minimal Requirements for the Replication of the Human Papillomavirus Type-11 Origin. J Virol **67:**7131–7139.
34. **Sverdrup F, Khan SA.** 1994. Replication of Human Papillomavirus (Hpv) Dnas Supported by the Hpv Type-18 E1 and E2 Proteins. J Virol **68:**505–509.
35. **Sverdrup F, Khan SA.** 1995. 2 E2 Binding-Sites Alone Are Sufficient to Function as the Minimal Origin of Replication of Human Papillomavirus Type-18 DNA. J Virol **69:**1319–1323.
36. **Frattini MG, Laimins LA.** 1994. The Role of the E1 and E2 Proteins in the Replication of Human Papillomavirus Type 31b. Virology **204:**799–804.
37. **Del Vecchio AM, Romanczuk H, Howley PM, Baker CC.** 1992. Transient replication of human papillomavirus DNAs. J Virol **66:**5949–5958.
38. **Hughes FJ, Romanos MA.** 1993. E1 Protein of Human Papillomavirus Is a DNA Helicase/Atpase. Nucleic Acids Res **21:**5817–5823.
39. **Bergvall M, Melendy T, Archambault J.** 2013. The El proteins. Virology **445:**35–56.
40. **Stubenrauch F, Hummel M, Iftner T, Laimins LA.** 2000. The E8E2C protein, a negative regulator of viral transcription and replication, is required for extra-chromosomal maintenance of human papillomavirus type 31 in keratinocytes. J Virol **74:**1178–1186.
41. **Straub E, Dreer M, Fertey J, Iftner T, Stubenrauch F.** 2014. The viral E8^E2C repressor limits productive replication of human papillomavirus 16. J Virol **88:**937–947.
42. **Zobel T, Iftner T, Stubenrauch F.** 2003. The papillomavirus E8-E2C protein represses DNA replication from extrachromosomal origins. Mol Cell Biol **23:**8352–8362.
43. **McBride AA.** 2013. The papillomavirus E2 proteins. Virology **445:**57–79.
44. **Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM.** 1990. The E6 Oncoprotein Encoded by Human Papillomavirus Type-16 and Type-18 Promotes the Degradation of P53. Cell **63:**1129–1136.
45. **Werness BA, Levine AJ, Howley PM.** 1990. Association of Human Papillomavirus Type-16 and Type-18 E6 Proteins with P53. Science **248:**76–79.
46. **White EA, Kramer RE, Tan MJA, Hayes SD, Harper JW, Howley PM.** 2012. Comprehensive Analysis of Host Cellular Interactions with Human Papillomavirus E6 Proteins Identifies New E6 Binding Partners and Reflects Viral Diversity. J Virol **86:**13174–13186.

47. **White EA, Sowa ME, Tan MJA, Jeudy S, Hayes SD, Santha S, Munger K, Harper JW, Howley PM.** 2012. Systematic identification of interactions between host cell proteins and E7 oncoproteins from diverse human papillomaviruses. Proceedings of the National Academy of Sciences of the United States of America **109:**E260–E267.
48. **Jones DL, Alani RM, Munger K.** 1997. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21(Cip1)-mediated inhibition of cdk2. Genes & Development **11:**2101–2111.
49. **Katich SC, Zerfass-Thome K, Hoffmann I.** 2001. Regulation of the Cdc25A gene by the human papillomavirus Type 16 E7 oncogene. Oncogene **20:**543–550.
50. **Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J **8:**4099–4105.
51. **Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol **63:**4417–4421.
52. **Heck DV, Yee CL, Howley PM, Munger K.** 1992. Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. Proc Natl Acad Sci U S A **89:**4442–4446.
53. **Crook T, Tidy JA, Vousden KH.** 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and transactivation. Cell **67:**547–556.
54. **Halbert CL, Demers GW, Galloway DA.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J Virol **65:**473–478.
55. **Barbosa MS, Vass WC, Lowy DR, Schiller JT.** 1991. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. J Virol **65:**292–298.
56. **Pim D, Collins M, Banks L.** 1992. Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene **7:**27–32.
57. **Leechanachai P, Banks L, Moreau F, Matlashewski G.** 1992. The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. Oncogene **7:**19–25.
58. **Straight SW, Hinkle PM, Jewers RJ, McCance DJ.** 1993. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. J Virol **67:**4521–4532.
59. **Petti L, Nilson LA, DiMaio D.** 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J **10:**845–855.
60. **Drummond-Barbosa DA, Vaillancourt RR, Kazlauskas A, DiMaio D.** 1995. Ligand-independent activation of the platelet-derived growth factor beta receptor: requirements for bovine papillomavirus E5-induced mitogenic signaling. Mol Cell Biol **15:**2570–2581.

61. **Ashrafi GH, Tsirimonaki E, Marchetti B, O'Brien PM, Sibbet GJ, Andrew L, Campo MS.** 2002. Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins. Oncogene **21:**248–259.
62. **Campo MS, Graham SV, Cortese MS, Ashrafi GH, Araibi EH, Dornan ES, Miners K, Nunes C, Man S.** 2010. HPV-16 E5 down-regulates expression of surface HLA class I and reduces recognition by CD8 T cells. Virology **407:**137–142.
63. **Ashrafi GH, Haghshenas M, Marchetti B, Campo MS.** 2006. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. Int J Cancer **119:**2105–2112.
64. **Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS.** 2005. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. Int J Cancer **113:**276–283.
65. **Bravo IG, Alonso A.** 2004. Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. J Virol **78:**13613–13626.
66. **Schiffman M, Herrero R, DeSalle R, Hildesheim A, Wacholder S, Rodriguez AC, Bratti MC, Sherman ME, Morales J, Guillen D, Alfaro M, Hutchinson M, Wright TC, Solomon D, Chen ZG, Schussler J, Castle PE, Burk RD.** 2005. The carcinogenicity of human papillornavirus types reflects viral evolution. Virology **337:**76–84.
67. **Nasseri M, Hirochika R, Broker TR, Chow LT.** 1987. A human papilloma virus type 11 transcript encoding an E1–E4 protein. Virology **159:**433–439.
68. **Doorbar J, Ely S, Sterling J, Mclean C, Crawford L.** 1991. Specific Interaction between Hpv-16 E1–E4 and Cytokeratins Results in Collapse of the Epithelial-Cell Intermediate Filament Network. Nature **352:**824–827.
69. **Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC.** 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol Cell **5:**557–567.
70. **Holmgren SC, Patterson NA, Ozbun MA, Lambert PF.** 2005. The minor capsid protein L2 contributes to two steps in the human papillomavirus type 31 life cycle. J Virol **79:**3938–3948.
71. **Watt FM.** 1989. Terminal differentiation of epidermal keratinocytes. Curr Opin Cell Biol **1:**1107–1115.
72. **Eckhart L, Lippens S, Tschachler E, Declercq W.** 2013. Cell death by cornification. Biochim Biophys Acta **1833:**3471–3480.
73. **Pyeon D, Pearce SM, Lank SM, Ahlquist P, Lambert PF.** 2009. Establishment of Human Papillomavirus Infection Requires Cell Cycle Progression. PLoS Pathog **5**.
74. **Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, Stanley MA.** 2012. The biology and life-cycle of human papillomaviruses. Vaccine **30 Suppl 5:**F55–70.
75. **Doorbar J.** 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) **110:**525–541.
76. **Klingelhutz AJ, Roman A.** 2012. Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. Virology **424:**77–98.
77. **Finnen RL, Erickson KD, Chen XS, Garcea RL.** 2003. Interactions between papillomavirus L1 and L2 capsid proteins. J Virol **77:**4818–4826.

78. **Buck CB, Thompson CD, Pang YY, Lowy DR, Schiller JT.** 2005. Maturation of papillomavirus capsids. J Virol **79:**2839–2846.
79. **Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P, Omary MB, Doorbar J.** 2004. Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. J Virol **78:**821–833.
80. **Brown DR, Kitchin D, Qadadri B, Neptune N, Batteiger T, Ermel A.** 2006. The human papillomavirus type 11 E1–E4 protein is a transglutaminase 3 substrate and induces abnormalities of the cornified cell envelope. Virology **345:**290–298.
81. **Stanley MA, Browne HM, Appleby M, Minson AC.** 1989. Properties of a non-tumorigenic human cervical keratinocyte cell line. Int J Cancer **43:**672–676.
82. **Chiang CM, Ustav M, Stenlund A, Ho TF, Broker TR, Chow LT.** 1992. Viral-E1 and Viral-E2 Proteins Support Replication of Homologous and Heterologous Papillomaviral Origins. Proceedings of the National Academy of Sciences of the United States of America **89:**5799–5803.
83. **Ustav M, Stenlund A.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J **10:**449–457.
84. **Egawa N, Nakahara T, Ohno S, Narisawa-Saito M, Yugawa T, Fujita M, Yamato K, Natori Y, Kiyono T.** 2012. The E1 Protein of Human Papillomavirus Type 16 Is Dispensable for Maintenance Replication of the Viral Genome. J Virol **86:**3276–3283.
85. **Chiang CM, Dong G, Broker TR, Chow LT.** 1992. Control of Human Papillomavirus Type-11 Origin of Replication by the E2 Family of Transcription Regulatory Proteins. J Virol **66:**5224–5231.
86. **Remm M, Brain R, Jenkins JR.** 1992. The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18. Nucleic Acids Res **20:**6015–6021.
87. **Sun YN, Lu JZ, McCance DJ.** 1996. Mapping of HPV-11 E1 binding site and determination of other important cis elements for replication of the origin. Virology **216:**219–222.
88. **Ustav M, Ustav E, Szymanski P, Stenlund A.** 1991. Identification of the Origin of Replication of Bovine Papillomavirus and Characterization of the Viral Origin Recognition Factor-E1. Embo Journal **10:**4321–4329.
89. **Ustav E, Ustav M, Szymanski P, Stenlund A.** 1993. The Bovine Papillomavirus Origin of Replication Requires a Binding-Site for the E2 Transcriptional Activator. Proceedings of the National Academy of Sciences of the United States of America **90:**898–902.
90. **Frattini MG, Laimins LA.** 1994. Binding of the Human Papillomavirus E1 Origin-Recognition Protein Is Regulated through Complex-Formation with the E2 Enhancer-Binding Protein. Proceedings of the National Academy of Sciences of the United States of America **91:**12398–12402.
91. **Masterson PJ, Stanley MA, Lewis AP, Romanos MA.** 1998. A C-terminal helicase domain of the human papillomavirus el protein binds E2 and the DNA polymerase alpha-primase p68 subunit. J Virol **72:**7407–7419.
92. **Mohr IJ, Clark R, Sun S, Androphy EJ, Macpherson P, Botchan MR.** 1990. Targeting the E1 Replication Protein to the Papillomavirus Origin of Replication by Complex-Formation with the E2 Transactivator. Science **250:**1694–1699.

93. **Sedman J, Stenlund A.** 1995. Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro. Embo Journal **14:**6218–6228.
94. **Kuo SR, Liu JS, Broker TR, Chow LT.** 1994. Cell-Free Replication of the Human Papillomavirus DNA with Homologous Viral E1 and E2 Proteins and Human Cell-Extracts. Journal of Biological Chemistry **269:**24058–24065.
95. **Sanders CM, Stenlund A.** 1998. Recruitment and loading of the E1 initiator protein: An ATP-dependent process catalysed by a transcription factor. Embo Journal **17:**7044–7055.
96. **Sakai H, Yasugi T, Benson JD, Dowhanick JJ, Howley PM.** 1996. Targeted mutagenesis of the human papillomavirus type 16 E2 transactivation domain reveals separable transcriptional activation and DNA replication functions. J Virol **70:**1602–1611.
97. **Schuck S, Stenlund A.** 2005. Assembly of a double hexameric helicase. Mol Cell **20:**377–389.
98. **Sedman J, Stenlund A.** 1996. The initiator protein E1 binds to the bovine papillomavirus origin of replication as a trimeric ring-like structure. Embo Journal **15:**5085–5092.
99. **Liu XF, Schuck S, Stenlund A.** 2007. Adjacent residues in the E1 initiator beta-hairpin define different roles of the beta-hairpin in ori melting, helicase loading, and helicase activity. Mol Cell **25:**825–837.
100. **Schuck S, Stenlund A.** 2011. Mechanistic Analysis of Local On Melting and Helicase Assembly by the Papillomavirus E1 Protein. Mol Cell **43:**776–787.
101. **Enemark EJ, Joshua-Tor L.** 2006. Mechanism of DNA translocation in a replicative hexameric helicase. Nature **442:**270–275.
102. **Kurg R.** 2011. The Role of E2 proteins in Papillomavirus DNA replication. *In* Seligmann H (ed), DNA Replication – Current Advances. InTech.
103. **Sedman J, Stenlund A.** 1998. The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities. J Virol **72:**6893–6897.
104. **Fouts ET, Yu X, Egelman EH, Botchan MR.** 1999. Biochemical and electron microscopic image analysis of the hexameric E1 helicase. Journal of Biological Chemistry **274:**4447–4458.
105. **Conger KL, Liu JS, Kuo SR, Chow LT, Wang TSF.** 1999. Human papillomavirus DNA replication – Interactions between the viral E1 protein and two subunits of human DNA polymerase alpha/primase. Journal of Biological Chemistry **274:**2696–2705.
106. **Park P, Copeland W, Yang L, Wang T, Botchan MR, Mohr IJ.** 1994. The Cellular DNA-Polymerase Alpha-Primase Is Required for Papillomavirus DNA-Replication and Associates with the Viral E1 Helicase. Proceedings of the National Academy of Sciences of the United States of America **91:**8700–8704.
107. **Han YF, Loo YM, Militello KT, Melendy T.** 1999. Interactions of the papovavirus DNA replication initiator proteins, bovine papillomavirus type 1 E1 and simian virus 40 large T antigen, with human replication protein A. J Virol **73:**4899–4907.
108. **Loo YM, Melendy T.** 2004. Recruitment of replication protein A by the papillomavirus E1 protein and modulation by single-stranded DNA. J Virol **78:**1605–1615.

109. **Clower RV, Fisk JC, Melendy T.** 2006. Papillomavirus E1 protein binds to and stimulates human topoisomerase I. J Virol **80:**1584–1587.
110. **Donaldson MM, Mackintosh LJ, Bodily JM, Dornan ES, Laimins LA, Morgan IM.** 2012. An interaction between human papillomavirus 16 E2 and TopBP1 is required for optimum viral DNA replication and episomal genome establishment. J Virol **86:**12806–12815.
111. **Makiniemi M, Hillukkala T, Tuusa J, Reini K, Vaara M, Huang D, Pospiech H, Majuri I, Westerling T, Makela TP, Syvaoja JE.** 2001. BRCT domain-containing protein TopBP1 functions in DNA replication and damage response. J Biol Chem **276:**30399–30406.
112. **Yamane K, Chen J, Kinsella TJ.** 2003. Both DNA topoisomerase II-binding protein 1 and BRCA1 regulate the G2-M cell cycle checkpoint. Cancer Res **63:**3049–3053.
113. **Kumagai A, Lee J, Yoo HY, Dunphy WG.** 2006. TopBP1 activates the ATR-ATRIP complex. Cell **124:**943–955.
114. **Yang L, Botchan M.** 1990. Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. J Virol **64:**5903–5911.
115. **Schvartzman JB, Adolph S, Martinparras L, Schildkraut CL.** 1990. Evidence That Replication Initiates at Only Some of the Potential Origins in Each Oligomeric Form of Bovine Papillomavirus Type-1 DNA. Mol Cell Biol **10:**3078–3086.
116. **Auborn KJ, Little RD, Platt TH, Vaccariello MA, Schildkraut CL.** 1994. Replicative intermediates of human papillomavirus type 11 in laryngeal papillomas: site of replication initiation and direction of replication. Proc Natl Acad Sci U S A **91:**7340–7344.
117. **Flores ER, Lambert PF.** 1997. Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. J Virol **71:**7167–7179.
118. **Fareed GC, Garon GF, Salzman NP.** 1972. Origin and direction of simian virus 40 deoxyribonucleic acid replication. J Virol **10:**484–491.
119. **De Geest K, Turyk ME, Hosken MI, Hudson JB, Laimins LA, Wilbanks GD.** 1993. Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines. Gynecol Oncol **49:**303–310.
120. **Law MF, Lowy DR, Dvoretzky I, Howley PM.** 1981. Mouse Cells Transformed by Bovine Papillomavirus Contain Only Extrachromosomal Viral-DNA Sequences. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **78:**2727–2731.
121. **Angeletti PC, Kim K, Fernandes FJ, Lambert PF.** 2002. Stable replication of papillomavirus genomes in Saccharomyces cerevisiae. J Virol **76:**3350–3358.
122. **Kim K, Angeletti PC, Hassebroek EC, Lambert PF.** 2005. Identification of cis-acting elements that mediate the replication and maintenance of human papillomavirus type 16 Genomes in Saccharomyces cerevisiae. J Virol **79:**5933–5942.
123. **Kim K, Lambert PF.** 2002. E1 protein of bovine papillomavirus 1 is not required for the maintenance of viral plasmid DNA replication. Virology **293:**10–14.
124. **Hoffmann R, Hirt B, Bechtold V, Beard P, Raj K.** 2006. Different modes of human papillomavirus DNA replication during maintenance. J Virol **80:**4431–4439.

125. **Alazawi W, Pett M, Arch B, Scott L, Freeman T, Stanley MA, Coleman N.** 2002. Changes in cervical keratinocyte gene expression associated with integration of human papillomavirus 16. Cancer Res **62:**6959–6965.
126. **Hong S, Laimins LA.** 2013. The JAK-STAT transcriptional regulator, STAT-5, activates the ATM DNA damage pathway to induce HPV 31 genome amplification upon epithelial differentiation. PLoS Pathog **9:**e1003295.
127. **Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J **3:**1151–1157.
128. **Durst M, Kleinheinz A, Hotz M, Gissmann L.** 1985. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. J Gen Virol **66 (Pt 7):**1515–1522.
129. **Choo KB, Pan CC, Liu MS, Ng HT, Chen CP, Lee YN, Chao CF, Meng CL, Yeh MY, Han SH.** 1987. Presence of Episomal and Integrated Human Papillomavirus DNA-Sequences in Cervical-Carcinoma. Journal of Medical Virology **21:**101–107.
130. **Choo KB, Cheung WF, Liew LN, Lee HH, Han SH.** 1989. Presence of catenated human papillomavirus type 16 episomes in a cervical carcinoma cell line. J Virol **63:**782–789.
131. **Cullen AP, Reid R, Campion M, Lorincz AT.** 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J Virol **65:**606–612.
132. **Kristiansen E, Jenkins A, Holm R.** 1994. Coexistence of Episomal and Integrated Hpv16 DNA in Squamous-Cell Carcinoma of the Cervix. Journal of Clinical Pathology **47:**253–256.
133. **Adachi A, Kiyono T, Hayashi Y, Ohashi M, Ishibashi M.** 1996. Detection of human papillomavirus (HPV) type 47 DNA in malignant lesions from epidermodysplasia verruciformis by protocols for precise typing of related HPV DNAs. J Clin Microbiol **34:**369–375.
134. **Hudelist G, Manavi M, Pischinger KID, Watkins-Riedel T, Singer CF, Kubista E, Czerwenk KF.** 2004. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. Gynecol Oncol **92:**873–880.
135. **Kadaja M, Sumerina A, Verst T, Ojarand M, Ustav E, Ustav M.** 2007. Genomic instability of the host cell induced by the human papillomavirus replication machinery. Embo Journal **26:**2180–2191.
136. **Kadaja M, Isok-Paas H, Laos T, Ustav E, Ustav M.** 2009. Mechanism of genomic instability in cells infected with the high-risk human papillomaviruses. PLoS Pathog **5:**e1000397.
137. **Piirsoo M, Ustav E, Mandel T, Stenlund A, Ustav M.** 1996. Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator. EMBO J **15:**1–11.
138. **Ilves I, Kivi S, Ustav M.** 1999. Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which Is mediated by the viral E2 protein and its binding sites. J Virol **73:**4404–4412.
139. **Ustav M, Jr., Castaneda FR, Reinson T, Mannik A, Ustav M.** 2015. Human Papillomavirus Type 18 cis-Elements Crucial for Segregation and Latency. Plos One **10:**e0135770.

140. **Moody CA, Fradet-Turcotte A, Archambault J, Laimins LA.** 2007. Human papillomaviruses activate caspases upon epithelial differentiation to induce viral genome amplification. Proceedings of the National Academy of Sciences of the United States of America **104:**19541–19546.
141. **Moody CA, Laimins LA.** 2009. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. PLoS Pathog **5:**e1000605.
142. **Gillespie KA, Mehta KP, Laimins LA, Moody CA.** 2012. Human papillomaviruses recruit cellular DNA repair and homologous recombination factors to viral replication centers. J Virol **86:**9520–9526.
143. **Anacker DC, Gautam D, Gillespie KA, Chappell WH, Moody CA.** 2014. Productive replication of human papillomavirus 31 requires DNA repair factor Nbs1. J Virol **88:**8528–8544.
144. **Ruiz-Maso JA, Macho NC, Bordanaba-Ruiseco L, Espinosa M, Coll M, Del Solar G.** 2015. Plasmid Rolling-Circle Replication. Microbiol Spectr **3:**PLAS-0035-2014.
145. **Skalka A, Poonian M, Bartl P.** 1972. Concatemers in DNA replication: electron microscopic studies of partially denatured intracellular lambda DNA. J Mol Biol **64:**541–550.
146. **Bastia D, Sueoka N.** 1975. Studies on the late replication of phage lambda: rolling-circle replication of the wild type and a partially suppressed strain, Oam29 Pam80. J Mol Biol **98:**305–320.
147. **Enquist LW, Skalka A.** 1973. Replication of bacteriophage lambda DNA dependent on the function of host and viral genes. I. Interaction of red, gam and rec. J Mol Biol **75:**185–212.
148. **Smith MG, Skalka A.** 1966. Some properties of DNA from phage-infected bacteria. J Gen Physiol **49:**127–142.
149. **Weller SK, Sawitzke JA.** 2014. Recombination promoted by DNA viruses: phage lambda to herpes simplex virus. Annu Rev Microbiol **68:**237–258.
150. **Sakakibara N, Chen D, McBride AA.** 2013. Papillomaviruses Use Recombination-Dependent Replication to Vegetatively Amplify Their Genomes in Differentiated Cells. PLoS Pathog **9**.
151. **Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ.** 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proceedings of the National Academy of Sciences of the United States of America **97:**10389–10394.
152. **Matsuoka S, Huang MX, Elledge SJ.** 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science **282:**1893–1897.
153. **Ilves I, Tamberg N, Botchan MR.** 2012. Checkpoint kinase 2 (Chk2) inhibits the activity of the Cdc45/MCM2-7/GINS (CMG) replicative helicase complex. Proceedings of the National Academy of Sciences of the United States of America **109:**13163–13170.
154. **Furnari B, Blasina A, Boddy MN, McGowan CH, Russell P.** 1999. Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. Molecular Biology of the Cell **10:**833–845.
155. **Furnari B, Rhind N, Russell P.** 1997. Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. Science **277:**1495–1497.
156. **Walworth NC, Bernards R.** 1996. rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint. Science **271:**353–356.

157. **Liu QH, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo GB, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ.** 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes & Development **14:**1448–1459.
158. **Paull TT, Gellert M.** 1998. The 3' to 5' exonuclease activity of Mre11 facilitates repair of DNA double-strand breaks. Mol Cell **1:**969–979.
159. **Shibata A, Moiani D, Arvai AS, Perry J, Harding SM, Genois MM, Maity R, van Rossum-Fikkert S, Kertokalio A, Romoli F, Ismail A, Ismalaj E, Petricci E, Neale MJ, Bristow RG, Masson JY, Wyman C, Jeggo PA, Tainer JA.** 2014. DNA Double-Strand Break Repair Pathway Choice Is Directed by Distinct MRE11 Nuclease Activities (vol 53, pg 7, 2014). Mol Cell **53:**361–361.
160. **Dimitrova N, de Lange T.** 2009. Cell Cycle-Dependent Role of MRN at Dysfunctional Telomeres: ATM Signaling-Dependent Induction of Nonhomologous End Joining (NHEJ) in G(1) and Resection-Mediated Inhibition of NHEJ in G(2). Mol Cell Biol **29:**5552–5563.
161. **Hopfner KP, Karcher A, Shin DS, Craig L, Arthur LM, Carney JP, Tainer JA.** 2000. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell **101:**789–800.
162. **Williams RS, Tainer JA.** 2005. A nanomachine for making ends meet: MRN is a flexing scaffold for the repair of DNA double-strand breaks. Mol Cell **19:**724–726.
163. **Difilippantonio S, Celeste A, Fernandez-Capetillo O, Chen HT, San Martin BR, Van Laethem F, Yang YP, Petukhova GV, Eckhaus M, Feigenbaum L, Manova K, Kruhlak M, Camerini-Otero RD, Sharan S, Nussenzweig M, Nussenzweig A.** 2005. Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. Nature Cell Biology **7:**675–U656.
164. **Krejci L, Altmannova V, Spirek M, Zhao X.** 2012. Homologous recombination and its regulation. Nucleic Acids Res **40:**5795–5818.
165. **Ciccia A, Elledge SJ.** 2010. The DNA Damage Response: Making It Safe to Play with Knives. Mol Cell **40:**179–204.
166. **Shibata A, Conrad S, Birraux J, Geuting V, Barton O, Ismail A, Kakarougkas A, Meek K, Taucher-Scholz G, Lobrich M, Jeggo PA.** 2011. Factors determining DNA double-strand break repair pathway choice in G2 phase. Embo Journal **30:**1079–1092.
167. **Falck J, Forment JV, Coates J, Mistrik M, Lukas J, Bartek J, Jackson SP.** 2012. CDK targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination. Embo Reports **13:**561–568.
168. **Wu J, Zhang X, Zhang L, Wu CY, Rezaeian AH, Chan CH, Li JM, Wang J, Gao Y, Han F, Jeong YS, Yuan X, Khanna KK, Jin J, Zeng YX, Lin HK.** 2012. Skp2 E3 ligase integrates ATM activation and homologous recombination repair by ubiquitinating NBS1. Mol Cell **46:**351–361.
169. **Lee J, Dunphy WG.** 2013. The Mre11-Rad50-Nbs1 (MRN) complex has a specific role in the activation of Chk1 in response to stalled replication forks. Molecular Biology of the Cell **24:**1343–1353.
170. **Olson E, Nievera CJ, Lee AY, Chen L, Wu X.** 2007. The Mre11-Rad50-Nbs1 complex acts both upstream and downstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to regulate the S-phase checkpoint following UV treatment. J Biol Chem **282:**22939–22952.

171. **Bruhn C, Zhou ZW, Ai H, Wang ZQ.** 2014. The Essential Function of the MRN Complex in the Resolution of Endogenous Replication Intermediates. Cell Reports **6:**182–195.
172. **Riballo E, Kuhne M, Rief N, Doherty A, Smith GCM, Recio MJ, Reis C, Dahm K, Fricke A, Krempler A, Parker AR, Jackson SP, Gennery A, Jeggo PA, Lobrich M.** 2004. A pathway of double-strand break rejoining dependent upon ATM, artemis, and proteins locating to gamma-H2AX foci. Mol Cell **16:**715–724.
173. **Chen BPC, Uematsu N, Kobayashi J, Lerenthal Y, Krempler A, Yajima H, Lobrich M, Shiloh Y, Chen DJ.** 2007. Ataxia telangiectasia mutated (ATM) is essential for DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. Journal of Biological Chemistry **282:**6582–6587.
174. **Truong LN, Li YJ, Shi LDZ, Hwang PYH, He J, Wang HL, Razavian N, Berns MW, Wu XH.** 2013. Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America **110:**7720–7725.
175. **McVey M, Lee SE.** 2008. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends in Genetics **24:**529–538.
176. **Flynn RL, Zou L.** 2011. ATR: a master conductor of cellular responses to DNA replication stress. Trends in Biochemical Sciences **36:**133–140.
177. **Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA.** 2005. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes & Development **19:**1040–1052.
178. **Michael WM, Ott R, Fanning E, Newport J.** 2000. Activation of the DNA replication checkpoint through RNA synthesis by primase. Science **289:**2133–2137.
179. **MacDougall CA, Byun TS, Van C, Yee MC, Cimprich KA.** 2007. The structural determinants of checkpoint activation. Genes & Development **21:**898–903.
180. **Zou L, Elledge SJ.** 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300:**1542–1548.
181. **Navadgi-Patil VM, Burgers PM.** 2009. The unstructured C-terminal tail of the 9-1-1 clamp subunit Ddc1 activates Mec1/ATR via two distinct mechanisms. Mol Cell **36:**743–753.
182. **Zou L, Cortez D, Elledge SJ.** 2002. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes & Development **16:**198–208.
183. **Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, Karnitz LM.** 2007. The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. Genes & Development **21:**1472–1477.
184. **Lee J, Kumagai A, Dunphy WG.** 2007. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. J Biol Chem **282:**28036–28044.
185. **Shiotani B, Nguyen HD, Hakansson P, Marechal A, Tse A, Tahara H, Zou L.** 2013. Two distinct modes of ATR activation orchestrated by Rad17 and Nbs1. Cell Reports **3:**1651–1662.
186. **Formosa T, Alberts BM.** 1986. DNA synthesis dependent on genetic recombination: characterization of a reaction catalyzed by purified bacteriophage T4 proteins. Cell **47:**793–806.

187. **Kreuzer KN, Alberts BM.** 1985. A defective phage system reveals bacteriophage T4 replication origins that coincide with recombination hot spots. Proc Natl Acad Sci U S A **82:**3345–3349.
188. **Kuzminov A.** 1995. Collapse and Repair of Replication Forks in Escherichia-Coli. Molecular Microbiology **16:**373–384.
189. **Lydeard JR, Jain S, Yamaguchi M, Haber JE.** 2007. Break-induced replication and telomerase-independent telomere maintenance require Pol32. Nature **448:**820–U810.
190. **Llorente B, Smith CE, Symington LS.** 2008. Break-induced replication: what is it and what is it for? Cell Cycle **7:**859–864.
191. **Malkova A, Ira G.** 2013. Break-induced replication: functions and molecular mechanism. Current Opinion in Genetics & Development **23:**271–279.
192. **Liu J, Xu L, Sandler SJ, Marians KJ.** 1999. Replication fork assembly at recombination intermediates is required for bacterial growth. Proc Natl Acad Sci U S A **96:**3552–3555.
193. **Barry J, Alberts B.** 1994. Purification and Characterization of Bacteriophage-T4 Gene-59 Protein – a DNA Helicase Assembly Protein Involved in DNA-Replication. Journal of Biological Chemistry **269:**33049–33062.
194. **Xu LW, Marians KJ.** 2003. PriA mediates DNA replication pathway choice at recombination intermediates. Mol Cell **11:**817–826.
195. **Dudas KC, Kreuzer KN.** 2005. Bacteriophage T4 helicase loader protein gp59 functions as gatekeeper in origin-dependent replication in vivo. Journal of Biological Chemistry **280:**21561–21569.
196. **Lydeard JR, Lipkin-Moore Z, Sheu YJ, Stillman B, Burgers PM, Haber JE.** 2010. Break-induced replication requires all essential DNA replication factors except those specific for pre-RC assembly. Genes & Development **24:**1133–1144.
197. **Saini N, Ramakrishnan S, Elango R, Ayyar S, Zhang Y, Deem A, Ira G, Haber JE, Lobachev KS, Malkova A.** 2013. Migrating bubble during break-induced replication drives conservative DNA synthesis. Nature **502:**389–+.
198. **Donnianni RA, Symington LS.** 2013. Break-induced replication occurs by conservative DNA synthesis. Proceedings of the National Academy of Sciences of the United States of America **110:**13475–13480.
199. **Hastings PJ, Ira G, Lupski JR.** 2009. A Microhomology-Mediated Break-Induced Replication Model for the Origin of Human Copy Number Variation. Plos Genetics **5**.
200. **Bennardo N, Cheng A, Huang N, Stark JM.** 2008. Alternative-NHEJ Is a Mechanistically Distinct Pathway of Mammalian Chromosome Break Repair. Plos Genetics **4**.
201. **Davis AP, Symington LS.** 2004. RAD51-dependent break-induced replication in yeast. Mol Cell Biol **24:**2344–2351.
202. **Lo Piano A, Martinez-Jimenez MI, Zecchi L, Ayora S.** 2011. Recombination-dependent concatemeric viral DNA replication. Virus Research **160:**1–14.
203. **Fradet-Turcotte A, Bergeron-Labrecque F, Moody CA, Lehoux M, Laimins LA, Archambault J.** 2011. Nuclear accumulation of the papillomavirus E1 helicase blocks S-phase progression and triggers an ATM-dependent DNA damage response. J Virol **85:**8996–9012.
204. **Reinson T, Toots M, Kadaja M, Pipitch R, Allik M, Ustav E, Ustav M.** 2013. Engagement of the ATR-Dependent DNA Damage Response at the Human

Papillomavirus 18 Replication Centers during the Initial Amplification. J Virol **87:**951–964.
205. **Sakakibara N, Mitra R, McBride AA.** 2011. The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. J Virol **85:**8981–8995.
206. **King LE, Fisk JC, Dornan ES, Donaldson MM, Melendy T, Morgan IM.** 2010. Human papillomavirus E1 and E2 mediated DNA replication is not arrested by DNA damage signalling. Virology **406:**95–102.
207. **Wang HK, Duffy AA, Broker TR, Chow LT.** 2009. Robust production and passaging of infectious HPV in squamous epithelium of primary human keratinocytes. Genes Dev **23:**181–194.
208. **Reinson T, Henno L, Toots M, Ustav M, Jr., Ustav M.** 2015. The Cell Cycle Timing of Human Papillomavirus DNA Replication. Plos One **10:**e0131675.
209. **Arias-Pulido H, Peyton CL, Joste NE, Vargas H, Wheeler CM.** 2006. Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. J Clin Microbiol **44:**1755–1762.
210. **Akagi K, Li JF, Broutian TR, Padilla-Nash H, Xiao WH, Jiang B, Rocco JW, Teknos TN, Kumar B, Wangsa D, He DD, Ried T, Symer DE, Gillison ML.** 2014. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Research **24:**185–199.
211. **Hu Z, Zhu D, Wang W, Li WY, Jia WL, Zeng X, Ding WC, Yu L, Wang XL, Wang LM, Shen H, Zhang CL, Liu HJ, Liu X, Zhao Y, Fang XD, Li SC, Chen W, Tang T, Fu AS, Wang Z, Chen G, Gao QL, Li S, Xi L, Wang CY, Liao SJ, Ma XY, Wu P, Li K, Wang SX, Zhou JF, Wang J, Xu X, Wang H, Ma D.** 2015. Genome-wide profiling of HPV integration in cervical cancer identifies clustered genomic hot spots and a potential microhomology-mediated integration mechanism. Nature Genetics **47:**158–163.
212. **Schmitz M, Driesch C, Jansen L, Runnebaum IB, Durst M.** 2012. Non-Random Integration of the HPV Genome in Cervical Cancer. Plos One **7**.
213. **Liu PF, Carvalho CMB, Hastings PJ, Lupski JR.** 2012. Mechanisms for recurrent and complex human genomic rearrangements. Current Opinion in Genetics & Development **22:**211–220.
214. **Minocherhomji S, Hickson ID.** 2014. Structure-specific endonucleases: guardians of fragile site stability. Trends in Cell Biology **24:**321–327.
215. **DeCaprio JA, Garcea RL.** 2013. A cornucopia of human polyomaviruses. Nat Rev Microbiol **11:**264–276.
216. **Clertant P, Seif I.** 1984. A common function for polyoma virus large-T and papillomavirus E1 proteins? Nature **311:**276–279.
217. **An P, Saenz Robles MT, Pipas JM.** 2012. Large T antigens of polyomaviruses: amazing molecular machines. Annu Rev Microbiol **66:**213–236.
218. **Sowd GA, Fanning E.** 2012. A wolf in sheep's clothing: SV40 co-opts host genome maintenance proteins to replicate viral DNA. PLoS Pathog **8:**e1002994.
219. **Tapper DP, DePamphilis ML.** 1980. Preferred DNA sites are involved in the arrest and initiation of DNA synthesis during replication of SV40 DNA. Cell **22:**97–108.
220. **Seidman MM, Salzman NP.** 1979. Late replicative intermediates are accumulated during simian virus 40 DNA replication in vivo and in vitro. J Virol **30:**600–609.

221. **Tapper DP, DePamphilis ML.** 1978. Discontinuous DNA replication: accumulation of Simian virus 40 DNA at specific stages in its replication. J Mol Biol **120:**401–422.
222. **Verhalen B, Justice JL, Imperiale MJ, Jiang MX.** 2015. Viral DNA Replication-Dependent DNA Damage Response Activation during BK Polyomavirus Infection. J Virol **89:**5032–5039.
223. **Tsang SH, Wang X, Li J, Buck CB, You JX.** 2014. Host DNA Damage Response Factors Localize to Merkel Cell Polyomavirus DNA Replication Sites To Support Efficient Viral DNA Replication. J Virol **88:**3285–3297.
224. **Sowd GA, Li NY, Fanning E.** 2013. ATM and ATR Activities Maintain Replication Fork Integrity during SV40 Chromatin Replication. PLoS Pathog **9**.
225. **Jiang M, Zhao L, Gamez M, Imperiale MJ.** 2012. Roles of ATM and ATR-mediated DNA damage responses during lytic BK polyomavirus infection. PLoS Pathog **8:**e1002898.
226. **Dahl J, You J, Benjamin TL.** 2005. Induction and utilization of an ATM signaling pathway by polyomavirus. J Virol **79:**13007–13017.
227. **Shi YL, Dodson GE, Rundell K, Tibbetts RS.** 2005. Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. Journal of Biological Chemistry **280:**40195–40200.
228. **Boichuk S, Hu LA, Hein J, Gjoerup OV.** 2010. Multiple DNA Damage Signaling and Repair Pathways Deregulated by Simian Virus 40 Large T Antigen. J Virol **84:**8007–8020.
229. **Sowd GA, Mody D, Eggold J, Cortez D, Friedman KL, Fanning E.** 2014. SV40 Utilizes ATM Kinase Activity to Prevent Non-homologous End Joining of Broken Viral DNA Replication Products. PLoS Pathog **10**.
230. **Chen ZY, He CY, Kay MA.** 2005. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. Hum Gene Ther **16:**126–131.
231. **Bell L, Byers B.** 1983. Separation of branched from linear DNA by two-dimensional gel electrophoresis. Anal Biochem **130:**527–535.
232. **Brewer BJ, Fangman WL.** 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell **51:**463–471.
233. **Nawotka KA, Huberman JA.** 1988. Two-Dimensional Gel-Electrophoretic Method for Mapping DNA Replicons. Mol Cell Biol **8:**1408–1413.
234. **Lucas I, Hyrien O.** 2000. Hemicatenanes form upon inhibition of DNA replication. Nucleic Acids Res **28:**2187–2193.
235. **Garcia-Luis J, Machin F.** 2014. Mus81-Mms4 and Yen1 resolve a novel anaphase bridge formed by noncanonical Holliday junctions. Nature Communications **5**.
236. **Ponten J, Saksela E.** 1967. Two established in vitro cell lines from human mesenchymal tumours. Int J Cancer **2:**434–447.
237. **Isok-Paas H, Mannik A, Ustav E, Ustav M.** 2015. The transcription map of HPV11 in U2OS cells adequately reflects the initial and stable replication phases of the viral genome. Virology Journal **12**.
238. **Scherer WF, Syverton JT, Gey GO.** 1953. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J Exp Med **97:**695–710.

239. **Friedl F, Kimura I, Osato T, Ito Y.** 1970. Studies on a new human cell line (SiHa) derived from carcinoma of uterus. I. Its establishment and morphology. Proc Soc Exp Biol Med **135:**543–545.
240. **Auersperg N.** 1964. Long-Term Cultivation of Hypodiploid Human Tumor Cells. J Natl Cancer Inst **32:**135–163.
241. **Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE.** 1988. Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell-Line. Journal of Cell Biology **106:**761–771.
242. **Ayora S, Missich R, Mesa P, Lurz R, Yang S, Egelman EH, Alonso JC.** 2002. Homologous-pairing activity of the Bacillus subtilis bacteriophage SPP1 replication protein G35P. J Biol Chem **277:**35969–35979.
243. **Martinez-Jimenez MI, Alonso JC, Ayora S.** 2005. Bacillus subtilis bacteriophage SPP1-encoded gene 34.1 product is a recombination-dependent DNA replication protein. J Mol Biol **351:**1007–1019.
244. **Panyutin IG, Biswas I, Hsieh P.** 1995. A pivotal role for the structure of the Holliday junction in DNA branch migration. EMBO J **14:**1819–1826.

PUBLICATIONS

CURRICULUM VITAE

Nimi: Marit Orav
Date of birth: 28th of February 1986
Nationality: Estonian
Contact information: Institute of Technology, Nooruse 1, Tartu, 50411, Estonia
E-mail: marit.orav@gmail.com

Education and work experience

University of Tartu	Specialist	2012–
University of Tartu	PhD studies in Engineering and Technology, specializing in Biomedical technology	2010–
University of Tartu	MSc in Gene Technology, *cum laude*	2008–2010
National University of Ireland, Galway	UREKA Infectious Disease Programme	2007
University of Tartu	BSc in Gene Technology	2005–2008
Tallinn English College	primary and secondary education	1994–2005

Publications:

Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, Liisi Henno, Ene Ustav, Mart Ustav (2015) Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Scientific Reports, 5, 15952

Marit Orav, Liisi Henno, Helen Isok-Paas, Jelizaveta Geimanen, Mart Ustav, Ene Ustav (2013) Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. *Journal of Virology*, 87 (22), 12051–12068

Jelizaveta Geimanen, Helen Isok-Paas, Regina Pipitch, Kristiina Salk, Triin Laos, **Marit Orav**, Tormi Reinson, Mart Ustav Jr, Mart Ustav, Ene Ustav (2011) Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses. *Journal of Virology*, 85 (7), 3315–3329

Invention: Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication. Authors: Mart Ustav, Ene Ustav, Jelizaveta Geimanen, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, Mart Ustav, Triin Laos, **Marit Orav**, Anu Remm, Kristiina Salk, Andres Männik. PCT/EE2010/000010

Additional information

Language skills: Estonian, English, German

ELULOOKIRJELDUS

Nimi: Marit Orav
Sünniaeg: 28. veebruar 1986
Rahvus: eestlane
Kontaktandmed: Tehnoloogiainstituut, Nooruse 1, Tartu, 50411, Eesti
E-post: marit.orav@gmail.com

Haridustee ja teenistuskäik

Tartu ülikool	spetsialist	2012–
Tartu ülikool	doktoriõpingud Tehnika ja tehnoloogia õppekaval, Biomeditsiini tehnoloogia erialal	2010–
Tartu ülikool	MSc Geenitehnoloogias, *cum laude*	2008–2010
National University of Ireland, Galway	UREKA Infectious Disease Programme	2007
Tartu ülikool	BSc Geenitehnoloogias	2005–2008
Tallinna Inglise Kolledž	põhi- ja keskkooli haridus	1994–2005

Teadusartiklid:

Marit Orav, Jelizaveta Geimanen, Eva-Maria Sepp, Liisi Henno, Ene Ustav, Mart Ustav (2015) Initial amplification of the HPV18 genome proceeds via two distinct replication mechanisms. Scientific Reports, 5, 15952

Marit Orav, Liisi Henno, Helen Isok-Paas, Jelizaveta Geimanen, Mart Ustav, Ene Ustav (2013) Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. *Journal of Virology*, 87 (22), 12051–12068

Jelizaveta Geimanen, Helen Isok-Paas, Regina Pipitch, Kristiina Salk, Triin Laos, **Marit Orav**, Tormi Reinson, Mart Ustav Jr, Mart Ustav, Ene Ustav (2011) Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses. *Journal of Virology*, 85 (7), 3315–3329

Leiutis: Method and a kit for identifying compounds capable of inhibiting human papilloma virus replication. Authors: Mart Ustav, Ene Ustav, Jelizaveta Geimanen, Regina Pipitš, Helen Isok-Paas, Tormi Reinson, Mart Ustav, Triin Laos, **Marit Orav**, Anu Remm, Kristiina Salk, Andres Männik. PCT/EE2010/000010

Lisaks
Keeleoskus: eesti keel, inglise keel, saksa keel

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto.** Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.
3. **Hannes Luidalepp**. Studies on the antibiotic susceptibility of *Escherichia coli*. Tartu 2012, 111 p.
4. **Vahur Zadin**. Modelling the 3D-microbattery. Tartu 2012, 149 p.
5. **Janno Torop**. Carbide-derived carbon-based electromechanical actuators. Tartu 2012, 113 p.
6. **Julia Suhorutšenko.** Cell-penetrating peptides: cytotoxicity, immunogenicity and application for tumor targeting. Tartu 2012, 139 p.
7. **Viktoryia Shyp.** G nucleotide regulation of translational GTPases and the stringent response factor RelA. Tartu 2012, 105 p.
8. **Mardo Kõivomägi.** Studies on the substrate specificity and multisite phosphorylation mechanisms of cyclin-dependent kinase Cdk1 in *Saccharomyces cerevisiae*. Tartu, 2013, 157 p.
9. **Liis Karo-Astover.** Studies on the Semliki Forest virus replicase protein nsP1. Tartu, 2013, 113 p.
10. **Piret Arukuusk**. NickFects–novel cell-penetrating peptides. Design and uptake mechanism. Tartu, 2013, 124 p.
11. **Piret Villo.** Synthesis of acetogenin analogues. Asymmetric transfer hydrogenation coupled with dynamic kinetic resolution of α-amido-β-keto esters. Tartu, 2013, 151 p.
12. **Villu Kasari.** Bacterial toxin-antitoxin systems: transcriptional cross-activation and characterization of a novel *mqsRA* system. Tartu, 2013, 108 p.
13. **Margus Varjak.** Functional analysis of viral and host components of alphavirus replicase complexes. Tartu, 2013, 151 p.
14. **Liane Viru.** Development and analysis of novel alphavirus-based multifunctional gene therapy and expression systems. Tartu, 2013, 113 p.
15. **Kent Langel.** Cell-penetrating peptide mechanism studies: from peptides to cargo delivery. Tartu, 2014, 115 p.
16. **Rauno Temmer**. Electrochemistry and novel applications of chemically synthesized conductive polymer electrodes. Tartu, 2014, 206 p.
17. **Indrek Must.** Ionic and capacitive electroactive laminates with carbonaceous electrodes as sensors and energy harvesters. Tartu, 2014, 133 p.
18. **Veiko Voolaid.** Aquatic environment: primary reservoir, link, or sink of antibiotic resistance? Tartu, 2014, 79 p.
19. **Kristiina Laanemets.** The role of SLAC1 anion channel and its upstream regulators in stomatal opening and closure of *Arabidopsis thaliana.* Tartu, 2015, 115 p.

20. **Kalle Pärn**. Studies on inducible alphavirus-based antitumour strategy mediated by site-specific delivery with activatable cell-penetrating peptides. Tartu, 2015, 139 p.
21. **Anastasia Selyutina.** When biologist meets chemist: a search for HIV-1 inhibitors. Tartu, 2015, 172p.
22. **Sirle Saul**. Towards understanding the neurovirulence of Semliki Forest virus. Tartu, 2015, 136 p.