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Effects of the pseudorabies virus US3 protein kinase on actin and actin-controlling proteins

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About the cover

The front cover shows LifeAct (red) transduced swine testicle cells that were transfected with a plasmid encoding PRV US3. US3 protein was stained with primary mouse anti-US3 antibody and secondary FITC-labeled (green) anti-mouse antibody. Nuclei were counterstained with Hoechst 33342 (cyan). This image was taken using a Leica TCS SPE confocal microscope (XY length: 174.6 x 174.6 µm; Z = 6,8 µm, objective: ACS APO 63.0 x 1.30 (oil)).

“What is written without effort is, in general, read without pleasure.”

- Samuel Johnson

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Abbreviation list

ABP	actin binding protein
ADF	actin depolymerizing factor
cAMP	cyclic AMP
CF	corneal fibroblasts
CHO	Chinese hamster ovary
CHX	cycloheximide
CIN	chronophin
CRIB	cdc42/Rac-interactive binding protein
CTL	cytotoxic T-lymphocyte
CytD	cytochalasin D
DAD	diaphanous-autoregulatory domain
DIP	diaphanous-interacting protein
Drf	diaphanous-related formins
dTDP	deoxythymidine diphosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuracyl triphosphate
ED	equine dermal
EHV-1	equine herpesvirus 1
F-actin	filamentous actin
FH	formin homology domain
G-actin	globular actin
GAP	GTPase activating protein
GBD	GTPase binding domain
GDI	guanine nucleotide dissociation inhibitor

GDP	guanoside diphosphate
GTP	guanoside triphosphate
HDAC	histone deacetylase
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
ILTV	infectious laryngotracheitis virus
IRS	internal repeat sequence
ISG	interferon stimulating gene
JMY	junction-mediating and regulatory protein
KO	knock out
LIMK	LIM (Lin-11, Isl1 and Mec-3) kinase
MDCK	Madin-Darby canine kidney
mDia	mammalian homolog of diaphanous
MDV	Marek's disease virus
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MLC	myosin light chain
Nap1	Nck-associated protein 1
NEC	herpesviral nuclear egress complex
NPF	nucleation promoting factor
ORF	open reading frame
PAK	p21-activated kinase
PAM	pulmonary alveolar macrophage
PAMP	pathogen-associated molecular pattern

PBD	p21-binding domain
PDCD4	programmed cell death protein 4
PI(4,5)P2	phosphatidylinositol-4,5-bis-phosphate
PK-15	porcine kidney 15
PKA	protein kinase A
PRR	pattern-recognition receptor
PRV	pseudorabies virus
RK13	rabbit kidney 13
ROCK	rho associated protein kinase
ST	swine testicle
SuHV1	suid herpesvirus 1
TCR	T-cell receptor
TESK	testicular kinase
TRS	terminal repeat sequence
VCA	verprolin homology domain, cofilin homology domain and acidic region
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
WASH	WASP and SCAR homolog
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin-homologous protein
WH2	WASP homology 2 domain or verprolin homology domain
WHAMM	WASP homolog associated with actin, membranes, and microtubules
WIP	WASP interacting protein
WISH	WASP-interacting SH3-protein
WT	wild type

Chapter 1

Introduction

Chapter 1: Introduction

1. Pseudorabies virus (PRV)

1.1 Introduction

Pseudorabies virus (PRV), also known by its taxonomic name suid herpesvirus 1 (SuHV1) or its original name Aujeszky's disease virus belongs to the family of the *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* and is the causative agent of Aujeszky's disease in pigs.

Based on their biological properties, genome content and organization, most herpesviruses can be categorized into one of three major subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. These subfamilies vary in host range, the cell type where virus latency is established, and duration of the viral replication cycle (Pellet & Roizman, 2007). The broadest host range is displayed by the alphaherpesviruses, which are characterized by a fast replication cycle in host cells, producing viral particles in a matter of hours. For most alphaherpesviruses, latency is established in sensory ganglia. Betaherpesviruses have the most restricted host range and slowest rate of replication. Latency is induced in a wide range of tissues and cells, including kidneys, secretory glands and lymphoreticular cells. Gammaherpesviruses predominantly infect T or B lymphocytes and establish latency in lymphoid tissue (Pomeranz *et al.*, 2005).

Human alphaherpesviruses include herpes simplex viruses (HSV-1 and HSV-2) and varicella zoster virus (VZV), causing cold sores (HSV-1), genital lesions (HSV-2), chickenpox and shingles (VZV). In certain circumstances, often associated with a suppressed immunity, these viruses can cause severe symptoms, such as keratitis, blindness and encephalitis (HSV-1). Several important animal herpesviruses belong to the alphaherpesviruses, including PRV, bovine herpesvirus 1 and 5 (BHV-1, BHV-5), equine herpesvirus 1 and 4 (EHV-1, EHV-4), avian Marek's disease virus (MDV) and infectious laryngotracheitis virus (ILTV). *Table 1* gives an overview of currently characterized alphaherpesviruses.

Table 1: Alphaherpesvirus taxonomy based on the International Committee on Taxonomy of Viruses (ICTV) (from: http://ictvonline.org/)

	Genus	Species
Alphaherpesvirinae	Iltovirus	Gallid herpesvirus 1
		Psittacid herpesvirus 1
	Mardivirus	Anatid herpesvirus 1
		Columbid herpesvirus 1
		Gallid herpesvirus 2
		Gallid herpesvirus 3
		Meleagrid herpesvirus 1
	Scutavirus	Chelonid herpesvirus 5
	Simplexvirus	Ateline herpesvirus 1
		Bovine herpesvirus 2
		Cercopithecine herpesvirus 2
		Human herpesvirus 1
		Human herpesvirus 2
		Leporid herpesvirus 4
		Macacine herpesvirus 1
		Macropodid herpesvirus 1
		Macropodid herpesvirus 2
		Papiine herpesvirus 2
		Saimiriine herpesvirus 1
	Unassigned	Chelonid herpesvirus 6
	Varicellovirus	Bovine herpesvirus 1
		Bovine herpesvirus 5
		Bubaline herpesvirus 1
		Canid herpesvirus 1
		Caprine herpesvirus 1
		Cercopithecine herpesvirus 9
		Cervid herpesvirus 1
		Cervid herpesvirus 2
		Equid herpesvirus 1
		Equid herpesvirus 3
		Equid herpesvirus 4
		Equid herpesvirus 8
		Equid herpesvirus 9
		Felid herpesvirus 1
		Human herpesvirus 3
		Phocid herpesvirus 1
		Suid herpesvirus 1

1.2 Structure

The herpesvirus family is characterized by virions containing a double-stranded DNA genome, packaged in virions ranging from 200 to 250 nm. The virion consists of three structural components: a capsid, tegument and envelope (Pellet & Roizman, 2007). *Figure 1* shows a schematic representation and an electron microscope image of a PRV virion.

1.2.1 Genome

The full PRV genome sequence and gene arrangement have been characterized (Klupp *et al.*, 2004). Each PRV virion contains a double stranded DNA genome of approximately 143 kbp (Ben-Porat & Kaplan, 1985; Klupp *et al.*, 2004). The highly GC-rich genome (about 73%) consists of a unique long (U_L) and a unique short (U_S) region, the latter being flanked by the internal repeat sequences (IRS) and terminal repeat sequences (TRS) (Ben-Porat & Kaplan, 1985). The genome contains 72 open reading frames (ORFs), coding for different proteins which all have orthologs in other herpesviruses (Klupp *et al.*, 2004; Pomeranz *et al.*, 2005). As the PRV gene arrangement is largely collinear with the HSV-1 genome, which was established earlier, the nomenclature used for HSV-1 was also adopted for PRV (Roizman & Pellet, 2001).

1.2.2 Capsid

Most information regarding the PRV capsid is extrapolated from studies on HSV-1 (Newcomb *et al.*, 1993). The capsid consists of the proteins VP19C (UL38), VP26 (UL35), VP5 (UL19), VP23 (UL18) and the products of UL25 and UL6 genes (Zhou *et al.*, 1998). Its icosahedral shape (12 vertices, 20 faces and 30 sides) of about 125 nm originates from the specific arrangement of 162 capsomers (150 hexons and 12 pentons), mainly composed of major capsid protein VP5, which is highly conserved in all herpesviruses (Newcomb *et al.*, 1993). The hexons, composed of six VP5 monomers, are complemented by six VP26 molecules, forming the outer edges and faces of the capsid, while the pentons, containing five VP5 monomers, form the vertices. Hexons and pentons are linked by binding a triplex formed by VP19C/VP23/VP23 (Newcomb & Brown, 1991; Okoye *et al.*, 2006). Eleven of the pentons are VP5 pentamers, while the twelfth is a cylindrical portal, composed of 12 UL6 molecules (Newcomb *et al.*, 2001). This portal functions as a channel through which the viral genome enters the capsid (Homa & Brown, 1997; Moore & Prevelige, 2002). The genome surrounded by the capsid is referred to as the nucleocapsid.

1.2.3 Tegument

The space between the capsid and the envelope membrane is called the tegument, occupying two thirds of the virion volume (Grunewald *et al.*, 2003). The tegument consists of at least two distinct structures: an inner, tightly associated layer, associated with capsid proteins, and an outer, heterogeneous layer interacting with the cytoplasmic domain of viral membrane proteins (Pomeranz *et al.*, 2005). Cellular actin and at least fourteen viral tegument proteins are integrated in the tegument layer. These tegument proteins are involved in the process of taking over the host-cell, as they are released in the cell together with the nucleocapsid upon entry. While most of the tegument proteins

share little sequence homology between different herpesviruses, tegument protein US3 is conserved throughout the alphaherpesvirus subfamily (McGeoch & Davison, 1986) and will be discussed thoroughly below (see **2. US3**).

1.2.4 Envelope

The outer layer of the virion is called the envelope and is derived from intracellular membranes of vesicles from the trans-Golgi apparatus (Granzow *et al.*, 1997; Whealy *et al.*, 1991). Viral proteins are embedded in the envelope and are often glycosylated, referred to as glycoproteins. Eleven glycoproteins have been described in PRV: gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN, based on the unified nomenclature for herpesvirus glycoproteins (Mettenleiter, 2000). Some glycoproteins bind in pairs, to form homodimers, such as the gB/gB complex, or heterodimers, such as gE/gI, gH/gL, and gM/gN. Except for gG, which is secreted by infected cells, all of these glycoproteins are present in the viral envelope (Mettenleiter, 2000). Additionally, UL20, UL43, US9 and possibly also UL24 are non-glycosylated viral proteins that reside in the viral envelope. UL34 has been found in the primary virion envelope (see **1.3.4 Egress**), but not in mature virions (Fuchs *et al.*, 2002b; Klupp *et al.*, 2000). Envelope proteins are involved in a variety of processes including viral entry, egress, cell-to-cell spread, neuro-invasion, regulation of immune responses, and syncytia formation (Mettenleiter, 2000; Nauwynck *et al.*, 2007; Pomeranz *et al.*, 2005).

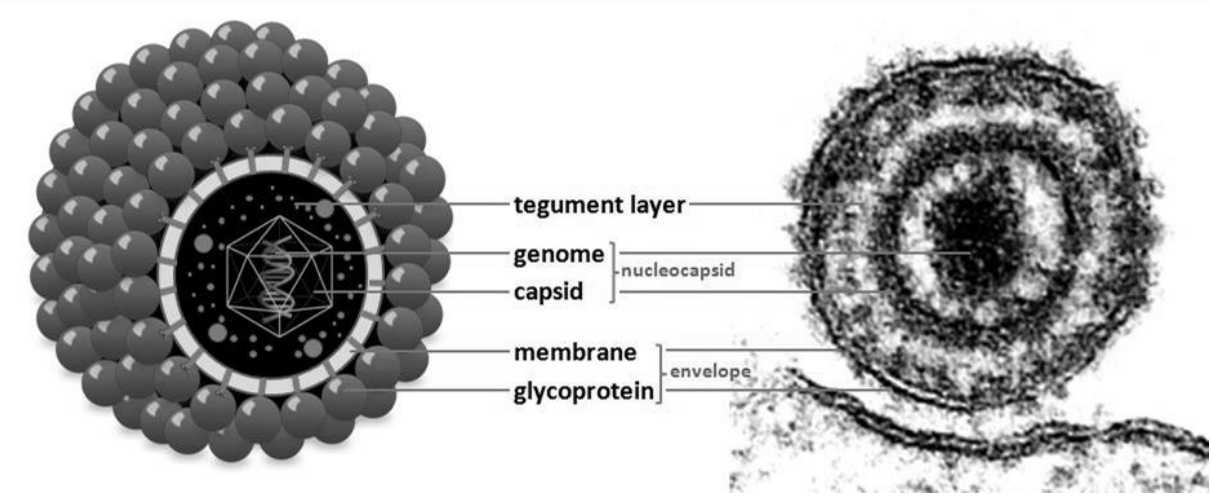

Figure 1: Schematic representation of PRV-virion (adapted from (Granzow *et al.*, 1997)).

1.3 Replication cycle

The herpesviral replication cycle is divided into several steps: entry, transport of nucleocapsids to the nucleus, transcription and DNA replication, virion assembly and finally egress. Many of the details for PRV replication are based on findings obtained on HSV-1 and are summarized in *Figure 2*.

1.3.1 Entry

In order to establish a successful infection, a PRV virion first needs to gain access to the cell. This is a multistep process and is initiated by the attachment of PRV gB and gC to host cell heparan sulphate proteoglycans. Although this labile, electrostatic binding is non-essential for infection, it increases viral entry efficiency (Mettenleiter *et al.*, 1990; Spear *et al.*, 2006). Attachment is further stabilized by PRV gD binding to its cellular receptor (Karger & Mettenleiter, 1993). Currently, three cellular PRV gD entry receptors, also known as herpesvirus entry mediators, have been identified: HveB (PRR2, nectin2), HveC (PRR1, nectin1) and HveD (PVR, CD55) (Spear *et al.*, 2000). Two additional gD receptors (HveA (TNFRSF14) and 3-O-sulfated heparan sulphate) have been described in HSV-1 (Mettenleiter, 2000; Spear, 2001; Spear *et al.*, 2000). In contrast to the labile gB/gC binding to heparan sulphate, this interaction between gD and one of its receptors is required for infection (Campadelli-Fiume *et al.*, 2000; Mettenleiter, 2002; Spear *et al.*, 2000).

Subsequently, the viral envelope and the cellular membrane fuse (Mettenleiter, 1994), allowing the viral capsid and tegument to reach the cytoplasm. During this process, viral glycoproteins gB, gH/gL and gD play essential roles, mediating fusion between the viral envelope and the cellular plasma membrane and releasing the nucleocapsid with its tegument in the cytoplasm (Klupp *et al.*, 1997; Mettenleiter, 2000; Pomeranz *et al.*, 2005; Schroter *et al.*, 2014). Next to cellular gD receptors, cellular gB receptors have also been described. The paired immunoglobulin-like type 2 receptor PILRα plays an important role as a gB associating coreceptor in both HSV-1 and PRV infection (Arii *et al.*, 2009; Satoh *et al.*, 2008). While gB, gH and gL are conserved within herpesviruses, gD is not (Mettenleiter, 2004; Spear *et al.*, 2000).

This process of direct entry through fusion at the plasma membrane is considered as the main route of entry for herpesviruses. However, viral hijacking of host cell endocytosis pathways, such as macropinocytosis and phagocytosis-like endocytosis have also been described. This is the case for HSV-1, EHV-1 and KSHV entry in particular cell types (Akula *et al.*, 2003; Clement *et al.*, 2006; Devadas *et al.*, 2014; Frampton *et al.*, 2007; Nicola *et al.*, 2003; Nicola & Straus, 2004; Raghu *et al.*, 2009).

Following entry, the viral capsids are transported via cellular dynein, a microtubule-associated motor protein that moves the capsid along microtubules from the cell periphery to the nucleus (Granzow *et al.*, 1997; Sodeik *et al.*, 1997). The nuclear pore complex serves as a docking station where PRV genomic DNA is released into the nucleus (Dohner *et al.*, 2002; Granzow *et al.*, 2005).

1.3.2 Transcription/Replication

Once the linear viral genomic DNA has entered the nucleus, it circularizes and serves as a template for transcription. The transcription of herpesviruses is strictly regulated, occurring in a cascade-like manner (Ben-Porat & Kaplan, 1985; Roizman & Pellet, 2001). First, regulatory proteins encoded by immediate-early (IE) genes are expressed. Promoters of the IE genes are recognized by cellular transcription factors and RNA polymerase II, and expression of these genes does not require new viral DNA synthesis. The mechanism of IE gene expression is a conserved process in alphaherpesviruses and is partly regulated by viral tegument protein UL48 (VP16) (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Fuchs *et al.*, 2002a; Misra *et al.*, 1994; Moriuchi *et al.*, 1993; Pomeranz *et al.*, 2005; Stern *et al.*, 1989). PRV, unlike several other herpesviruses, encodes only one immediate-early (IE) gene, IE180. The resulting protein is homologous to the HSV-1 IE protein ICP4 (Cheung, 1989; Taharaguchi *et al.*, 1994). HSV-1 contains four other IE genes, including ICP0, ICP22 and ICP27 and ICP47, which, except the latter, all have orthologs in PRV (EP0, RSp40 and UL54 respectively), but these are regulated in PRV as early (E) genes (Baumeister *et al.*, 1995; Cheung, 1991; Huang & Wu, 2004; Mettenleiter, 2000; Pomeranz *et al.*, 2005).

PRV IE180 encodes for a viral transactivator of early (E) genes and is essential for viral spread and replication (Wu *et al.*, 2014). Gene products of E genes include proteins required for DNA replication and other enzymatic factors. In order to start DNA replication, and begin synthesis of the leading and lagging DNA strands, the site for initiation of DNA synthesis must be recognized, the supercoiled DNA unwound and the DNA strands separated, which is mostly regulated by viral proteins, but likely also by host proteins (Boehmer & Lehman, 1997; Chang *et al.*, 2004; Ou *et al.*, 2002; Taharaguchi *et al.*, 1994). Certain host proteins, such as DNA polymerase, α-primase, DNA ligase I, and topoisomerase II have also been reported to play a role (Boehmer & Lehman, 1997). The PRV EP0 gene, an E protein, serves as a viral transactivator and in general stimulates viral gene expression (Ho *et al.*, 1999; Ono *et al.*, 1998; Watanabe *et al.*, 1995). However, EP0 has a suppressive effect on the vhs (Chang *et al.*, 2004) and gE (Chang *et al.*, 2002) promotors. US3 is also an early protein and will be discussed in more detail later, seen its importance in this work (see **2. US3**). Other notable early viral proteins are thymidine kinase (UL23), dUTPase (UL50) (Jons *et al.*, 1997) and ribonucleotide reductase (UL39/UL40) (de Wind *et al.*, 1993). The viral encoded dUTPase is responsible for the cleavage of deoxyuracyl-triphosphate

(dUTP) into deoxyuracyl-monophosphate (dUMP) and pyrophosphate. Further enzymatic activity leads to the conversion of dUMP to deoxythymidine-monophosphate (dTMP), which is phosphorylated by cellular thymidine kinase to deoxythymidine-diphosphate (dTDP). Viral thymidine kinase is required for the phosphorylation of dTDP to deoxythymidine-triphosphate (dTTP), one of the four crucial DNA building blocks (Chen *et al.*, 2002; Kit *et al.*, 1987; McGregor *et al.*, 1985). Ribonucleotide reductase is required for the reduction of ribonucleotides into deoxyribonucleotides (Kaliman *et al.*, 1994). While these enzymes are also encoded by host cells, viral encoded enzymes ensure viral replication in non-dividing or terminally differentiated host cells (i.e., neurons) (Boldogkoi & Nogradi, 2003; Chen *et al.*, 2002). Furthermore, as opposed to cellular ribonucleotide reductase, the PRV-encoded enzyme is resistant to dTTP induced feedback inhibition (Ben-Porat & Kaplan, 1985) and PRV lacking this enzyme as well as lacking thymidine kinase is described to be highly attenuated in several animal models (Kit *et al.*, 1985).

Viral DNA synthesis occurs via a rolling-circle mechanism, resulting in linear, concatomeric (containing multiple copies of the same DNA sequences) genomes (Newcomb *et al.*, 1999).

Finally, late genes are transcribed, sometimes subdivided into early-late and true-late genes, encoding capsid, tegument and envelope proteins (Mettenleiter, 2000; Pomeranz *et al.*, 2005). While the expression of immediate-early and early genes occurs entirely before viral genome replication, the expression of early-late genes already starts before replication but reaches a maximum level of transcripts during/after replication. The expression of true-late genes only occurs after replication.

1.3.3 Virion assembly

Assembly of new capsids occurs in the nucleus (Homa & Brown, 1997; Newcomb *et al.*, 1999; Steven & Spear, 1997). Every capsid building block (UL38, UL35, UL25, UL19, UL18 and UL6) as well as two scaffold proteins UL26 and UL26.5 (VP24 and VP22a) need to be present (Pomeranz *et al.*, 2005; Zhou *et al.*, 1998). The latter two are only necessary for capsid formation, and are not present in the mature virion. Finally, the replicated, concatomeric PRV DNA is cleaved into smaller single-genome units, before it is packed into the capsid. PRV UL28 and UL17 are both known to be required for DNA cleavage and encapsidation (Klupp *et al.*, 2005; Kwong & Frenkel, 1989; Ladin *et al.*, 1980; Mettenleiter *et al.*, 1993; Salmon & Baines, 1998).

1.3.4 Egress

To get to the cytoplasm, the nucleocapsid has to cross the nuclear membrane, which is composed of an inner nuclear membrane, lined on the nuclear side by the nuclear lamina, and an outer nuclear

membrane. The capsids acquire their primary envelope and primary tegument by budding through the inner nuclear membrane into the perinuclear space, a process dependent on the herpesviral nuclear egress complex (NEC) consisting of UL31 and UL34 (Klupp *et al.*, 2000; 2001; Passvogel *et al.*, 2015). The primary enveloped virion then loses its envelope through fusion with the outer nuclear membrane, resulting in the release of naked capsids into the cytoplasm, a process that appears to be fundamentally different from fusion during entry (Fuchs *et al.*, 2002b; Klupp *et al.*, 2000). Fusion of the primary enveloped virion involves the activity of the viral US3 protein and will be further discussed below (see **2.2.2 Nuclear Egress**) (Klupp *et al.*, 2001; Schaap *et al.*, 2005; Wagenaar *et al.*, 1995). For HSV-1, gB and gH are also involved in this de-envelopment of primary enveloped virions, while this is not the case for PRV (Farnsworth *et al.*, 2007; Klupp *et al.*, 2008). Herpesviruses, including PRV, can also leave the nucleus by inducing nuclear envelope breakdown (NEBD) (Grimm *et al.*, 2012; Klupp *et al.*, 2011), which has recently been demonstrated to be UL46 dependent (Schulz *et al.*, 2014).

The cytoplasmic capsids contain only very little tegument (Granzow *et al.*, 1997) and the addition of tegument proteins to the capsid is thought to be regulated by VP5 and UL37 (Fuchs *et al.*, 2004; Klupp *et al.*, 2002; Zhou *et al.*, 1999). The secondary envelopment step then occurs at the trans-Golgi network (TGN), where the capsid buds into its lamellae and vesicles, generating a complete virus within a vesicle (Granzow *et al.*, 1997; Whealy *et al.*, 1991). Based on data from both PRV and HSV-1, secondary envelopment is instructed by UL11 and gM, directing tegument proteins, capsids and envelope glycoproteins to the TGN budding site. These proteins are conserved in all herpesviruses, probably indicative for their pivotal role in this process (Kopp *et al.*, 2003; Mettenleiter, 2004; Mettenleiter *et al.*, 2009). Recently, tegument proteins UL36 (VP1/2) and UL37 have been reported to be essential for secondary envelopment for HSV-1 viral egress (Kelly *et al.*, 2014).

Finally, the secondary enveloped, mature virion is transported to the cell surface, where exocytosis releases it in the extracellular space (Granzow *et al.*, 2001; Granzow *et al.*, 1997). Cellular proteins involved in the constitutive secretory pathway such as Rab6a, Rab8a and Rab11a were recently found to play a role in this process. They were found to be increased at the site of exocytosis just before and rapidly diffused after exocytosis (Hogue *et al.*, 2014).

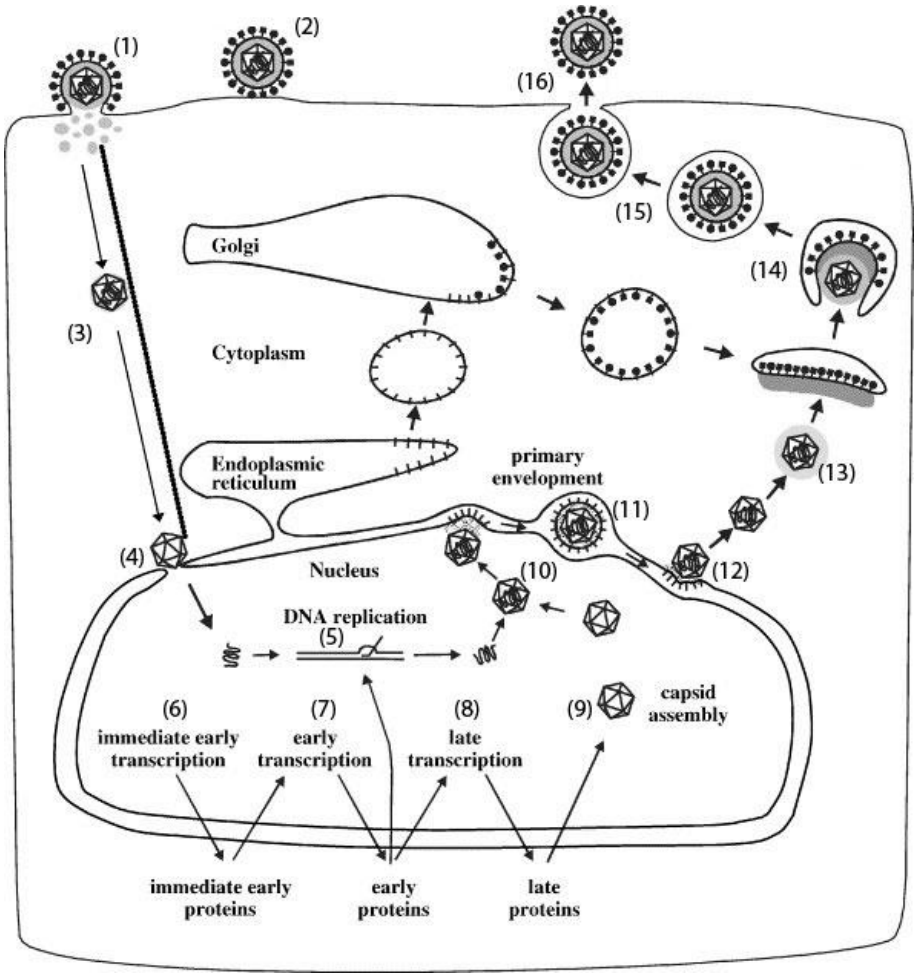

Figure 2: PRV replication cycle. Following attachment (1), virions fuse with the plasma membrane (2). Upon fusion, the nucleocapsid and tegument proteins are released in the cell and nucleocapsids are transported along microtubules towards the nuclear pore complex (3), where the viral DNA is released into the nucleus (4), and is replicated according to a rolling circle mechanism (5). VP16 transactivates cellular RNA polymerase II transcription of PRV's only immediate early gene IE180 (6), inducing IE proteins, leading to transcription and expression of E genes (7), in turn regulating replication and transcription and expression of L proteins (8). Next, capsids assemble (9), encapsulate the genome (10) and bud into the perinuclear space, resulting in primary enveloped virions with a primitive tegument (11). The nucleocapsid loses its primary envelope through fusion with the outer nuclear membrane (12) and gains its final tegument and envelope by associating with tegument and envelope proteins (13) and budding in vesicles derived from the trans-Golgi network (14). A sorting vesicle finally delivers the mature virus to the cell surface (15), which is released in the extracellular space through exocytosis (16) (Adapted from (Mettenleiter, 2004)).

1.4 PRV cell-associated spread

Exocytosis of virus particles exposes them to extracellular detrimental molecules, such as antibodies, complement factors and enzymes, and allows their elimination by phagocytes. It therefore comes as no surprise that herpesviruses have developed several ways of cell-associated spread that protect them from the extracellular milieu and are mechanistically different depending on the cell type and the distance between cells (Nauwynck *et al.*, 2007). An infected cell can either fuse with a neighboring

non-infected cell, leading to syncytium (multinucleated cell) formation. This process is coordinated by envelope glycoproteins gB, gH/gL and gK, which are essential for this process, while gE/gI and gM have a modulating function (Mettenleiter, 2000). Distant cells can also be connected by US3-mediated protrusions, formed by PRV-infected cells, allowing virus to travel to non-infected cells. This will be discussed in more detail below (see **2.2.5 Actin rearrangements**). Furthermore, cellular adhesion molecules may also control the attachment of cells followed by virus spread from one to the other cell. This has been described for PRV-infected monocytes and endothelial cells (Van de Walle *et al.*, 2003).

1.5 Aujeszky's disease

Aujeszky's disease was first described as "mad itch" in cattle, a disease characterized by heavy itching (Hanson, 1954). The first isolation of PRV was reported by the Hungarian veterinary surgeon, Aládar Aujeszky (Aujeszky, 1902). He inoculated rabbits with PRV-infected material, leading to symptoms that were reminiscent, yet distinguishable from rabies virus, hence the name pseudorabies virus. Soon after its discovery, the viral nature of PRV (Schmiedhofer, 1910) as well as it being the causative agent of mad itch (Shope, 1931) were confirmed.

Although eradication programs have been successful in major parts of the USA and Europe (Pomeranz *et al.*, 2005), PRV still remains an interesting tool to study alphaherpesvirus biology in general, which will be discussed further (see **1.6 PRV as a model organism to study alphaherpesvirus biology**).

1.5.1 Pathogenesis

The natural host of PRV is the pig, but the virus infects a broad range of vertebrates, including sheep, cattle, dogs, cats, goats, chicken, raccoons, possums, skunks, rodents, rabbits, guinea pigs and rarely also horses, and is usually lethal in non-natural hosts (Field & Hill, 1974; Gustafson, 1986; Kimman *et al.*, 1991; McCracken *et al.*, 1973; Pensaert & Kluge, 1989; Pomeranz *et al.*, 2005; Wittmann *et al.*, 1980). Symptoms before dying include ataxia, itching, scratching, and paralysis (Pomeranz *et al.*, 2005). Higher order primates, including man, are not affected by the virus (Enquist, 1999; Kluge *et al.*, 1999).

Primary replication in pigs occurs in the upper respiratory tract, more specifically the nasal and oropharyngeal mucosa (Masic *et al.*, 1965). This allows the virus to access the sensory nerve endings innervating the site of infection, leading viral particles to the trigeminal, glossopharyngeal and olfactory nerves through retrograde transport (Maes *et al.*, 1997). The virus spreads through lymph and blood vessels to internal organs, with reproductive organs being an important target. As replication occurs in the respiratory tract, central nervous system and reproductive organs, it is not

surprising that the main pathological symptoms are respiratory, nervous and reproductive disorders (Pomeranz *et al.*, 2005).

In the trigeminal and sacral ganglia, PRV is described to enter a state of quiescence, called latency, concealing the presence of the virus infection. These sites are also the predominant sites of latency for human herpesviruses HSV-1 and HSV-2 (Whitley, 2001). During latency, the virus is present as an episome (segment of DNA that exists autonomously) in infected cells, without detectable production of viral proteins and virions. Reactivation and shedding of the virus in latently infected individuals is often triggered by stress factors. For PRV, these might be concomitant disease conditions, vehicular transport, poor animal care, farrowing and treatment with immunosuppressive agents (Davies & Beran, 1980; Rziha *et al.*, 1986; Rziha *et al.*, 1989; Thawley *et al.*, 1984; van Oirschot & Gielkens, 1984; Wittmann *et al.*, 1983).

PRV infection in suckling piglets usually leads to their death, as a result of viral encephalitis. Initial symptoms include listlessness and disinterest in nursing, which later change to neurologic symptoms as trembling, excessive salivation, uncoordinated movements, ataxia and seizures within 24 h of the initial symptoms (Kluge *et al.*, 1999). In weaned pigs from 3-6 weeks old, the symptoms are comparable, except that the mortality rate drops to ± 50%. Infected animals develop respiratory signs (including sneezing, nasal discharge, coughing and breathing difficulties, leading to significant weight reduction) a direct economic loss for farmers (see **1.5.2 Economic impact**). Careful nursing may reduce mortality rates to 10% (Kluge *et al.*, 1999). Next to respiratory symptoms, adult swine may exhibit reproductive disorders (including stillbirths, abortions or weakened piglets), again having economic consequences (see **1.5.2 Economic impact**). Additionally, sporadic neurological symptoms have been described, varying from mild muscle tremors to severe convulsions. Since late 2011, however, a highly virulent strain characterized by neurologic symptoms and high mortality even in adult pigs has been detected on several farms in China, the PRV TJ strain (or HeN1/JS-2012 strain) (An *et al.*, 2013; Luo *et al.*, 2014; Wang *et al.*, 2014a; Wu *et al.*, 2013; Yu *et al.*, 2014). Sequence analysis of 39 PRV isolates demonstrated that the PRV gE gene of PRV TJ showed 2 aspartic acid insertions (An *et al.*, 2013). In other PRV strains, mortality in infected adult animals is usually relatively low, and rarely exceeds 1-2%.

1.5.2 Economic impact

Severe outbreaks of pseudorabies occurred during 1960-1970 in the US and Europe, correlating with an increase in pork production (Kluge *et al.*, 1999; Nauwynck *et al.*, 2007). These outbreaks resulted in devastating disease with great economic impact worldwide. Economic losses are caused by the development of pneumonia in infected animals, leading to significant weight loss (Kluge *et al.*, 1999) or result from stillbirths, abortions or weakened piglets born from an infected sow, as PRV might cross

the placenta and infect animals in utero (Kluge *et al.*, 1999). To limit these losses, expensive eradication programs were introduced, including slaughter of infected animals, vaccination, limitations in animal transport, decontamination procedures and improvements in herd management. Eventually, these measures led to the eradication of PRV in large parts of the US and Europe, including Belgium (Pomeranz *et al.*, 2005).

However, full eradication has been compromised. Infection is re-emerging from the increasing reservoir of infected feral swine in the USA as well as in Japan (Hahn *et al.*, 2010; Mahmoud *et al.*, 2011; Yamane *et al.*, 2015). While PRV isolates from feral swine are more attenuated than isolates from domestic swine, this could still pose an important economic risk (Hahn *et al.*, 2010). Additionally, the Bartha-K61 vaccine, which provided 100% protection against lethal challenge with PRV (An *et al.*, 2013; Gu *et al.*, 2015), only provided 50% protection against the recent and more virulent Chinese PRV TJ strain (or JS-2012/HeN1 strains) (An *et al.*, 2013; Luo *et al.*, 2014; Wang *et al.*, 2014a). Hence, to allow early detection of recent strains, novel and rapid PRV detection assays were required (Luo *et al.*, 2015; Wernike *et al.*, 2014; Zeng *et al.*, 2014). Recent research shows that, following immunization with a gE deleted TJ strain, piglets did not exhibit clinical signs and were protected from lethal challenge with PRV TJ, pointing towards the potential of this gE deleted strain to control the ongoing epidemic in China (Wang *et al.*, 2014a).

1.6 PRV as a model organism to study alphaherpesvirus biology

Despite its eradication in several Western countries, PRV remains an important research target because of its homology with other alphaherpesviruses, providing considerable insights in general aspects including virology, neurobiology, cellular biology and immunology (Enquist, 1999; Enquist & Card, 2003; Mettenleiter, 2000; Pomeranz *et al.*, 2005). These are summarized in *Figure 3*.

Due to its broad host range in vertebrates, excluding higher primates, a lot of animal models are available for experiments, without posing a threat towards the researcher. Infections can be studied in (cells of) the natural host, which is especially relevant since no natural alphaherpesviruses of small laboratory animals have been described thus far. Additionally, infection of cell cultures leads to high viral titers, which is a useful technical advantage (Enquist, 1999; Pomeranz *et al.*, 2005). The full genome sequence of PRV is known, DNA can be purified and is still infectious, bacterial artificial chromosomes (BACs) containing the full genome have been made (Kopp *et al.*, 2003; Smith & Enquist, 1999) and different mutations have already been successfully introduced (Enquist, 1999). This allows the functional analysis of every PRV gene in tissue culture or animal models, providing an excellent tool to increase knowledge of not only PRV, but also other herpesviruses.

Additionally, because of its neurotropic properties and broad host range, PRV can be used as a neuronal tracer to identify the structure of synaptic pathways. The Bartha strain of PRV is often used to identify neuronal networks, as this strain only displays retrograde (towards to neuronal cell body through its axon) and not the opposite anterograde spread (Aston-Jones & Card, 2000; Enquist, 1999; Enquist & Card, 2003; Enquist *et al.*, 1998).

General knowledge of alphaherpesvirus biology obtained through PRV research can lead to the development of new vaccines, diagnostical applications and antiviral therapies for man and animal, but also to new insights into host cell biology and the immune system of the host (Enquist, 1999; Pomeranz *et al.*, 2005).

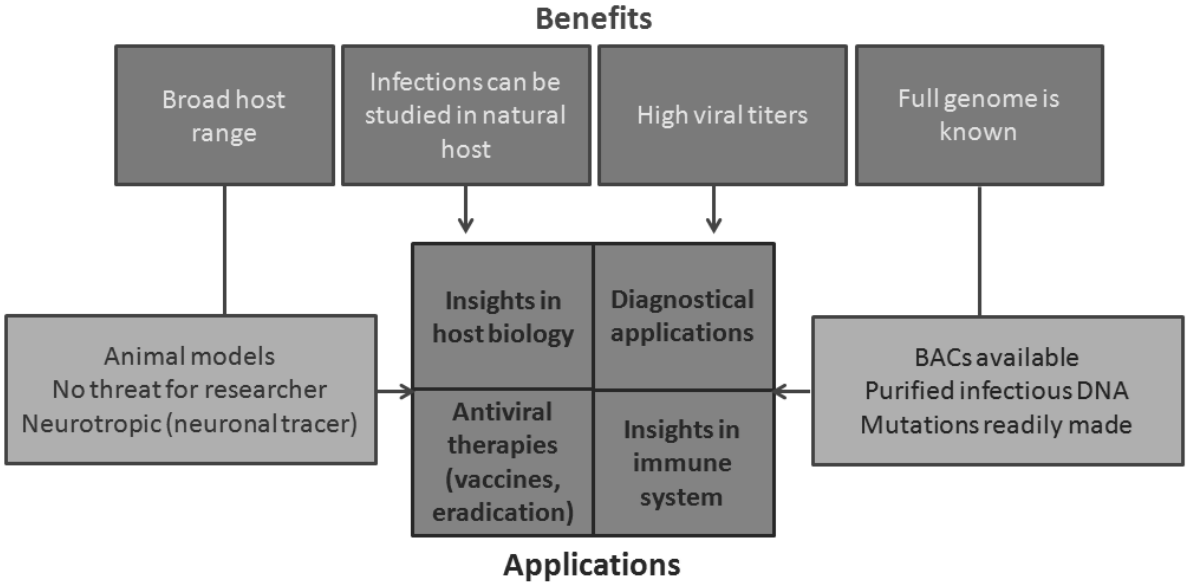

Figure 3: PRV as a model system for alphaherpesviruses. This figure summarizes the most important benefits and applications/advantages using PRV as an alphaherpesvirus model organism.

2. US3

2.1 Introduction

The US3 protein is encoded by every alphaherpesvirus that has currently been identified (Frame *et al.*, 1987; Hanks *et al.*, 1988; Heineman *et al.*, 1996; McGeoch & Davison, 1986; Purves *et al.*, 1987). Soon following the discovery that US3 of HSV-1 and HSV-2 showed homology with cellular protein kinases (McGeoch & Davison, 1986), US3 was identified in HSV-1 as a viral serine/threonine protein kinase by using kinase assays and antisera (Frame *et al.*, 1987).

The minimal consensus phosphorylation sequence of PRV US3 is RnX(S/T)ZZ, where n is larger or equal to 2; X can be Arg, Ala, Val, Pro, Ser or absent, and Z can be any non-acidic amino acid except proline (Leader *et al.*, 1991; Purves *et al.*, 1986). In these consensus sequences, arginine (R) residues may be substituted by lysine (K) residues, without loss of recognition and phosphorylation (Pike *et al.*, 2008). The optimal consensus phosphorylation sequence is similar, except that $n \geq 3$ and X is not absent (Leader, 1993; Leader *et al.*, 1991; Purves *et al.*, 1986). Interestingly, the PRV US3 consensus site is largely comparable to that of HSV-1, HSV-2 and VZV US3 (ORF66) (Daikoku *et al.*, 1993; Eisfeld *et al.*, 2006; Purves *et al.*, 1986) and to that of the cellular protein kinase A (PKA) which is RRX(S/T)Φ where Φ tends to be a hydrophobic residue (Benetti & Roizman, 2004; Kato *et al.*, 2009). US3 function and localization both seem to be affected by autophosphorylation on serine 147 in HSV-1 and HSV-2 (Kato *et al.*, 2008; Morimoto *et al.*, 2009).

Every alphaherpesvirus US3 ortholog has a kinase domain of around 280-300 amino acids that includes the ATP-binding domain and the catalytic active site (van Zijl *et al.*, 1990). PRV US3 shares 39% amino acid identity with HSV-1 and HSV-2 orthologs, while HSV-1 and HSV-2 US3 share 75% identity (McGeoch & Davison, 1986). However, the sequence similarity of the kinase domains between different alphaherpesviruses is significantly higher. Two amino acids are absolutely conserved in the US3 kinase domain of alphaherpesviruses (Deruelle *et al.*, 2010). In PRV, these are found at lysine 136, which is a critical residue for ATP binding and at aspartic acid 223, which is a crucial residue for catalytic activity. These residues were targeted by mutagenesis, resulting in recombinant viruses and expression vectors encoding kinase-inactive US3 (Coller & Smith, 2008; Deruelle *et al.*, 2007; Finnen & Banfield, 2010; Kinchington *et al.*, 2000; Ryckman & Roller, 2004). Recombinant PRV or HSV-1 viruses either lacking US3 or expressing kinase-deficient US3 show only slightly reduced growth characteristics in most cell cultures whereas their virulence is strongly impaired in pigs (PRV) and mice (HSV) (Coller & Smith, 2008; Inagaki-Ohara *et al.*, 2001; Kimman *et al.*, 1994; Meignier *et al.*, 1988; Nishiyama *et al.*, 1992; Purves *et al.*, 1987; Reynolds *et al.*, 2002; Ryckman & Roller, 2004; Sagou *et al.*, 2009; Van den

Broeke *et al.*, 2009a). This is comparable to findings in VZV, where kinase-deficient ORF66 displayed severe growth and replication defects in primary human corneal stromal fibroblasts, while an insignificantly reduced growth was observed in a fibroblast cell line (Erazo *et al.*, 2008).

A particular feature of the PRV and HSV-1 US3 genes, compared to those of other alphaherpesviruses, is that both contain two transcriptional start sites and encode two US3 isoforms: a minor long isoform (390 AA, 53 kDa) and a major short isoform (336 AA, 41 kDa) (Poon *et al.*, 2006a; Poon & Roizman, 2005; van Zijl *et al.*, 1990). In PRV, the short truncated protein for which translation starts at the second start codon represents more than 95% of the total US3 protein in infected cells (van Zijl *et al.*, 1990). While both isoforms are expressed in infected cells, only the short one is incorporated in the PRV virion (Klupp *et al.*, 2001; Lyman *et al.*, 2003; Zhang *et al.*, 1990). The isoforms have different cellular localizations, as the long isoform contains an N-terminal mitochondrial localization signal (Calton *et al.*, 2004; Van Minnebruggen *et al.*, 2003), causing the long isoform to predominantly reside in mitochondria, while the short isoform is mainly located in the nucleus. US3 has also been detected in the cytoplasm and at cell membranes (Brzozowska *et al.*, 2010; Calton *et al.*, 2004). These different localizations may be reflected in the different US3 functions.

2.2 Functions

Many viruses have evolved multifunctional proteins, probably because of their restricted genome size. Due to their ability to affect different substrates, viral kinases almost invariably have several functions. The US3 protein is associated with a plethora of different functions, including anti-apoptotic activity, nuclear egress, gene expression, immune evasion and actin rearrangements. It is likely that not all phenotypes that are caused by US3 are equally important for the virus, and an important challenge is to discriminate the relevant biological interactions from the less important interactions or artifacts. An overview of the functions attributed to US3 orthologs from different alphaherpesviruses is given in *Figure 4*.

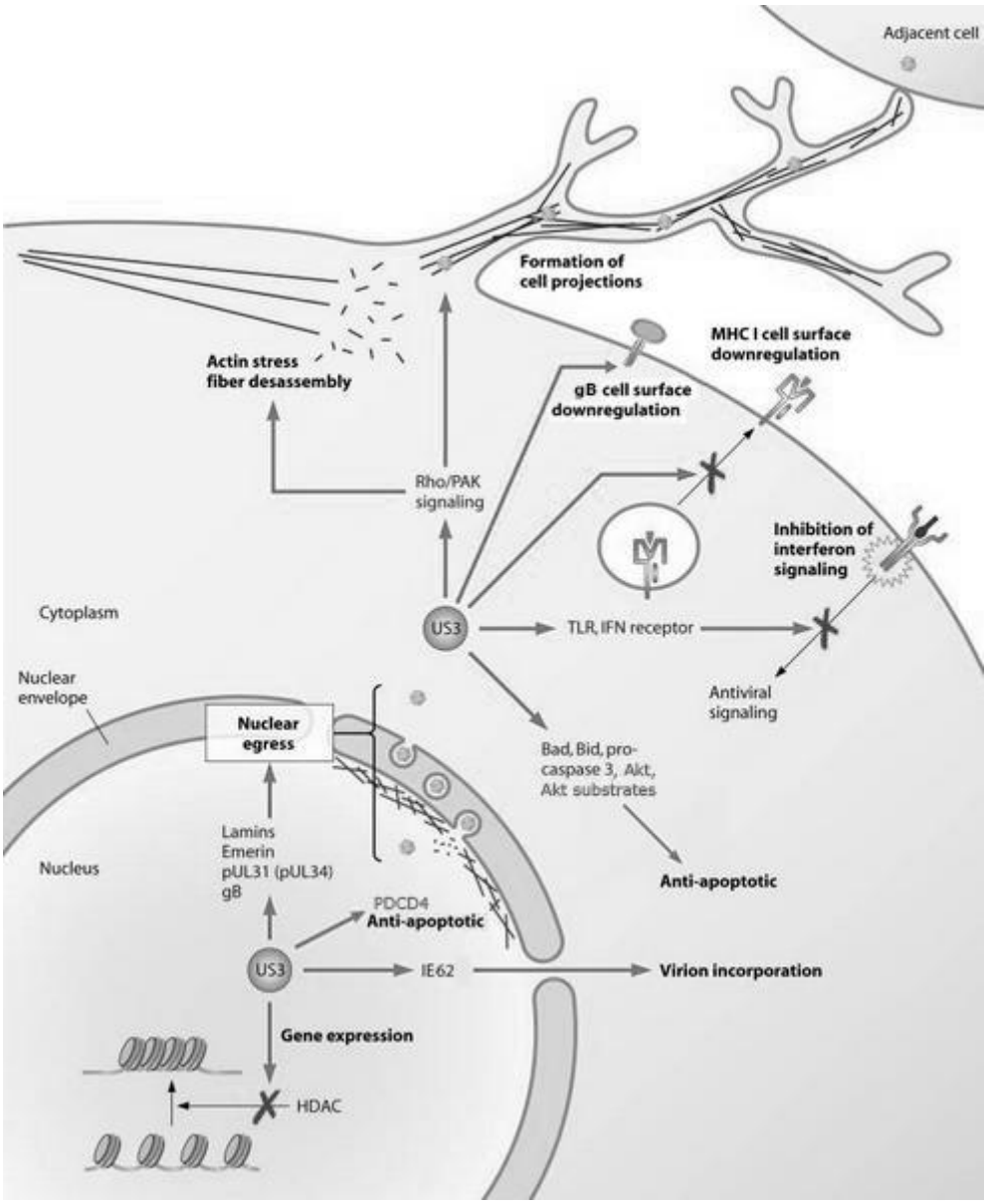

Figure 4: Functions associated with US3 orthologs of different alphaherpesviruses. (Adapted from (Jacob *et al.*, 2011)).

2.2.1 Anti-apoptotic activity

Apoptosis of infected cells may result from either the direct cellular response to viral infection or may be triggered by immune cells, particularly cytotoxic T-lymphocytes (CTLs) or natural killer (NK) cells. Apoptosis of virus-infected cells early in the viral replication represents an intrinsic antiviral response, a form of innate immunity. Cellular proteins eliciting and/or regulating these responses are often constitutively present in uninfected cells, so they can act as soon as infection occurs, or they may be upregulated by viral infection (Koyama *et al.*, 2000; Yan & Chen, 2012). In order to prolong intracellular

replication and promote spread, several viruses, including herpesviruses, have developed strategies to prevent apoptosis.

The US3 protein of several herpesviruses, including HSV-1, HSV-2, PRV, BHV-5, and MDV, displays anti-apoptotic activity, but BHV-1 US3 has no anti-apoptotic effect (Asano *et al.*, 1999; Geenen *et al.*, 2005; Ladelfa *et al.*, 2011; Leopardi *et al.*, 1997; Murata *et al.*, 2002b; Schumacher *et al.*, 2008; Takashima *et al.*, 1999). The kinase activity of HSV-1, PRV, BHV-5 and MDV US3 has been reported to be required for this function (Cartier *et al.*, 2003b; Deruelle *et al.*, 2007; Ladelfa *et al.*, 2011). For PRV, the long isoform of US3 appears to mediate the strongest anti-apoptotic effect, although this is the less abundant isoform in infected cells (Chang *et al.*, 2013; Geenen *et al.*, 2005). This can likely be explained by its mitochondrial localization, where some of its potential targets in the apoptotic pathway are located. The short isoform of US3, as mentioned higher, is predominantly located in the nucleus.

Several phosphorylation targets involved in the HSV-1 US3-mediated suppression of apoptosis have been identified, including Bad, Bid, and procaspase-3, pointing at the involvement of US3 in different anti-apoptotic pathways (Benetti & Roizman, 2007; Cartier *et al.*, 2003b). PRV US3 has also been reported to lead to phosphorylation (and thereby inactivation) of the pro-apoptotic cellular protein Bad (Deruelle *et al.*, 2007). Protein kinase A (PKA) inhibits apoptosis by phosphorylation and inactivation of Bad (Harada *et al.*, 1999). US3 of HSV-1 activates PKA and at the same time functionally overlaps PKA by targeting the same phosphorylation substrates, thereby interfering with apoptosis (Benetti & Roizman, 2004). Recently, anti-apoptotic signaling proteins PI3K/Akt and NF-κB were also shown to be targeted by HSV-1 and PRV US3 (Chang *et al.*, 2013; Wang *et al.*, 2014b) (see **2.2.4 Immune evasion**). However, whereas HSV-1 US3 inactivates NF-κB activity, PRV US3 activates NF-κB activity (Chang *et al.*, 2013), demonstrating the requirement for more studies to elucidate the precise role of these processes. HSV-1 US3, but also VZV, HSV-1 and PRV US3 phosphorylate and activate Akt substrates (Chuluunbaatar *et al.*, 2010; Erazo *et al.*, 2011), while PRV US3 is also described to activate Akt itself and its upstream molecule PDK-1 (Chang *et al.*, 2013), thus having a similar outcome. Furthermore, HSV-1 US3 was described to interact with programmed cell death protein 4 (PDCD4), causing retention of PDCD4 in the nucleus and subsequently blocking apoptosis (Wang *et al.*, 2011). To some extent, PRV US3 also seems to interact with the cellular p21-activated kinase PAK1 to inhibit apoptosis (Van den Broeke *et al.*, 2011). Finally, HSV-1 gB and US3 were reported to collaborate in downregulating cellular antigen presenting molecule CD1d to inhibit recognition and killing by Natural killer T cells (NKT) (Rao *et al.*, 2011). HSV-1 US3 also reduces induction of cytotoxic T lymphocytes in mice by indirectly downregulating MHC-I (Rao *et al.*, 2011) (see **2.2.4 Immune evasion**).

This broad substrate spectrum might explain how US3 is able to inhibit apoptosis induced by very diverse apoptotic stimuli, including herpesvirus infection itself, granzyme B released by cytotoxic T lymphocytes, overexpression of Bcl-2 family members Bad and Bax, staurosporine, and sorbitol (Asano *et al.*, 1999; Cartier *et al.*, 2003a; Cartier *et al.*, 2003b; Chang *et al.*, 2013; Hata *et al.*, 1999; Jerome *et al.*, 1999; Leopardi *et al.*, 1997; Munger & Roizman, 2001; Murata *et al.*, 2002a; Ogg *et al.*, 2004; Van den Broeke *et al.*, 2011).

The anti-apoptotic activity of US3 during early stages of infection is often assumed to permit increased replication of the virus, which may result in an increased yield of progeny virus. This may explain the lower end-point titers of kinase-dead US3 or US3null variants of PRV, HSV or MDV in several cell types (Coller & Smith, 2008; Demmin *et al.*, 2001; Deruelle *et al.*, 2010; Kimman *et al.*, 1994; Reynolds *et al.*, 2002; Ryckman & Roller, 2004; Schumacher *et al.*, 2008; Van den Broeke *et al.*, 2009a; Van Minnebruggen *et al.*, 2003). However, in contrast to this view, inhibition of apoptosis using a caspase inhibitor did not increase virus progeny yield of US3null PRV, questioning the contribution of the anti-apoptotic effect of US3 to increased virus production and viral spread (Deruelle *et al.*, 2010).

2.2.2 Nuclear egress

In 1995, a first report was published illustrating the involvement of US3 in nuclear egress (Wagenaar *et al.*, 1995). Alphaherpesviruses and herpesviruses in general, use a unique system to transport progeny nucleocapsids out of the nucleus and into the cytoplasm. As described higher, nucleocapsids undergo primary envelopment by budding in the inner nuclear membrane, followed by fusion with the outer nuclear membrane, releasing the capsid into the cytoplasm (Mettenleiter *et al.*, 2009) (see **1.3.4 Egress**). In cells infected with US3null PRV, HSV-1, or MDV, infective virions aggregate within the perinuclear space in large invaginations (Klupp *et al.*, 2001; Reynolds *et al.*, 2002; Schumacher *et al.*, 2005; Wagenaar *et al.*, 1995; Wild *et al.*, 2015), suggesting a conserved role for the US3 kinase in the de-envelopment step during nuclear egress. However, this defect in capsid nuclear export is not absolute, since extracellular virus titers are only mildly reduced in the absence of US3 (Coller & Smith, 2008; Reynolds *et al.*, 2002; Ryckman & Roller, 2004; Van den Broeke *et al.*, 2009a; Van Minnebruggen *et al.*, 2003; Wild *et al.*, 2015). Since these initial reports, US3 has been described to be implicated in different steps of the nuclear egress pathway. Lamin A/C and emerin, key elements of the nuclear lamina network, are phosphorylated by HSV-1 US3 (Leach *et al.*, 2007; Morris *et al.*, 2007; Mou *et al.*, 2007; Mou *et al.*, 2008), which likely contributes to disruption of the nuclear lamina, a barrier for virions to reach the inner nuclear membrane.

Additionally, infection with US3null HSV-1 or PRV also results in an altered distribution of the viral UL34 and UL31 proteins, both crucial regulators of primary envelopment of nucleocapsids (**see 1.2.4**

Envelope and **1.3.4 Egress**), from a roughly continuous distribution to one in discrete aggregates (Klupp *et al.*, 2001; Reynolds *et al.*, 2001). For HSV-1, this relocalization of the envelopment machinery is regulated by phosphorylation of the N-terminus of UL31 by US3 (Mou *et al.*, 2009). HSV-1 US3 forms a complex not only with UL31 and UL34, but also with IE protein ICP22 and UL47 (VP13/VP14), both recently identified as regulators of nuclear egress (Liu *et al.*, 2014; Maruzuru *et al.*, 2014).

In HSV-1, gB, together with gH, has been suggested to be involved in de-envelopment of the primary enveloped virion with the outer nuclear membrane (Farnsworth *et al.*, 2007). HSV-1 US3 was described to phosphorylate the cytoplasmic domain of gB, which may contribute to the role of gB during this de-envelopment step (Imai *et al.*, 2011; Imai *et al.*, 2010; Wisner *et al.*, 2009). The latter may not be a conserved function of alphaherpesviruses since, for PRV, neither gB nor gH appears to be present at the nuclear membranes and neither protein functions in the nuclear egress of virions (Granzow *et al.*, 2001; Klupp *et al.*, 2008). Besides affecting nuclear egress, HSV-1 US3-mediated gB phosphorylation regulates endocytosis of gB and also affects HSV-1 pathogenesis in mouse models (Imai *et al.*, 2011; Imai *et al.*, 2010; Kato *et al.*, 2009; Wisner *et al.*, 2009).

2.2.3 Gene expression

Over the last couple of years, it has become clear that US3 is able to affect gene expression. US3 orthologs of HSV-1, HSV-2, PRV, and VZV were all described to phosphorylate histone deacetylases (HDAC)-1 and -2, affecting their enzymatic activity, localization and interaction with downstream substrates (Morimoto *et al.*, 2009; Poon *et al.*, 2006b; Walters *et al.*, 2009; Walters *et al.*, 2010). HDACs coordinate deacetylation of lysine residues of histone tails, inducing chromatin condensation and thereby repressing gene expression (Grozinger & Schreiber, 2002). Herpesviruses need to interfere with HDAC activity in order to enable efficient viral gene expression (Danaher *et al.*, 2005; Poon *et al.*, 2003).

HSV-1 and HSV-2 US3 phosphorylate HDAC-1 and -2 directly (Kato *et al.*, 2008; Morimoto *et al.*, 2009; Poon *et al.*, 2006b; Poon *et al.*, 2003; Poon & Roizman, 2005; 2007), although there are indications that not only VZV and PRV US3, but also HSV-1 US3 phosphorylate HDACs indirectly by activating a yet unknown cellular kinase pathway (Walters *et al.*, 2009; Walters *et al.*, 2010).

In contrast to its HSV-1 and VZV US3 orthologs, PRV US3 appears not essential for phosphorylation of HDACs, as phosphorylation still occurred in the absence of US3 (Walters *et al.*, 2010). Furthermore, HDAC inhibition induced a higher plaque number in PRV and VZV US3null virus infected cells, but not in HSV-1 US3null virus infected cells. Both findings point to virus-dependent differences in the

mechanism and importance of US3-mediated HDAC modification (Walters *et al.*, 2009; Walters *et al.*, 2010).

VZV US3 has also been described to regulate gene expression in another way. Both ORF66 and the second viral kinase, ORF47, phosphorylate IE62, a viral nuclear transcription regulating protein. Phosphorylated IE62 accumulates in the cytoplasm, where it is incorporated in newly formed virions. The nuclear import of IE62 is thereby reduced, obstructing gene expression (Eisfeld *et al.*, 2006; Erazo & Kinchington, 2010). The authors postulate that this interaction may come to play during VZV latency (Erazo & Kinchington, 2010).

2.2.4 Immune evasion

Herpesviruses have developed various mechanisms to interfere with major components of the host immune system (Costa *et al.*, 2012). US3 has been implicated in some of these virus immune evasion strategies, particularly in herpesvirus interference with the interferon system, with cytotoxic T lymphocytes and with virus-neutralizing antibodies.

The most immediate line of response to pathogens is generated by the innate immunity. The innate response is very rapid and potent but relatively unspecific, in contrast to the adaptive immune response, which is more specific, but needs more time to come to full effect. Several components of the innate immune system work through recognition of specific pathogen-associated molecular patterns (PAMPs) by host pattern-recognition receptors (PRRs), which initiates signal transduction pathways that result in elimination of virions and virus-infected cells and the production of interferon (IFN) and pro-inflammatory cytokines, such as IL-1 and TNF-α (Takeuchi & Akira, 2007). Interferon regulates the expression of interferon stimulated genes (ISGs), inducing an antiviral, antiproliferative and immunoregulatory state in the host cells (Haller *et al.*, 2007; Haller & Weber, 2007; Weber & Haller, 2007). All herpesviruses trigger the induction of type I IFN during a primary infection (Costa *et al.*, 2012).

US3 acts on different fronts to counteract **interferon**-induced antiviral effects. HSV-1 US3 has been reported to interfere with IFN-β synthesis (Peri *et al.*, 2008) and was also suggested to be involved in overcoming the antiviral state induced by IFN (Piroozmand *et al.*, 2004). HSV-1 US3 also downregulates type I interferon-inducible protein MxA expression, one of the IFN-induced antiviral proteins (Peri *et al.*, 2008; Piroozmand *et al.*, 2004). Furthermore, HSV-1 US3 phosphorylates the interferon gamma (IFN-γ) receptor, leading to disturbed IFN-γ dependent gene expression (Liang & Roizman, 2008). VZV US3 (ORF66) has also been shown to reduce IFN signaling, when adding IFN-γ to infected T-cells (Schaap *et al.*, 2005).

US3 may also interfere with signaling initiated by **TNF-α**, another pro-inflammatory cytokine. Indeed, HSV-1 US3 inhibits TNF-α-stimulated NF-κB signaling (see **2.2.1 Anti apoptotic activity**) via hyperphosphorylation of p65, thereby decreasing the expression of the inflammatory chemokine interleukin-8 (IL-8) (Wang *et al.*, 2014b).

US3 may also affect the adaptive immune response. **Cytotoxic T-lymphocytes** (CTLs) have been reported to be functionally altered following contact with HSV-infected cells, losing their ability to release cytotoxic granules and to synthesize cytokines when triggered through the T-cell receptor (TCR) (Sloan *et al.*, 2003; York & Johnson, 1993). In HSV-1 and -2, this CTL inactivation seems to depend on US3, ICP4 and UL54 (Sloan *et al.*, 2003). The effect on the T-cells and the mechanisms underlying inactivation are not completely understood, but it may give HSV an advantage in establishment of latency or during reactivation and could favor viral replication through the observed selective production of the anti-inflammatory cytokine interleukin-10 (IL-10) (Sloan & Jerome, 2007; Sloan *et al.*, 2003).

CTLs recognize infected cells because the latter express major histocompatibility complex I (MHC-I) molecules loaded with viral peptides on their surface, which can then be recognized by the T cell receptor (Hansen & Bouvier, 2009; Horst *et al.*, 2011). The MHC I antigen presentation pathway therefore represents an important threat for the virus. It may therefore come as no surprise that numerous reports show that several herpesvirus proteins interfere with MHC I antigen presentation, via a variety of mechanisms (Hansen & Bouvier, 2009; Horst *et al.*, 2011). Proteins like the immediate-early ICP47 in HSV (Ahn *et al.*, 1996; Cioni *et al.*, 2013; Fruh *et al.*, 1995; Jugovic *et al.*, 1998; Tomazin *et al.*, 1996; Tomazin *et al.*, 1998) and UL49.5 (gN) in BHV-1, PRV, EHV-1 and -4 as well as MDV (Koppers-Lalic *et al.*, 2005; Koppers-Lalic *et al.*, 2008) are major players in herpesvirus interference with MHC class I presentation by interfering with TAP (Transporter associated with antigen processing)-mediated transport of cytoplasmic viral peptides to the MHC I molecules in the ER.

In VZV, US3 (ORF66) has also been implicated in interference with MHC-I antigen presentation, by abrogating transport of MHC I molecules through the Golgi complex (Abendroth *et al.*, 2001; Eisfeld *et al.*, 2007). Interestingly, the kinase activity of US3/ORF66, although beneficial, is not required for MHC-I downregulation (Eisfeld *et al.*, 2007).

PRV US3 has been reported to be involved, but not sufficient, in the downregulation of MHC I cell surface expression. This involvement was highly cell-type dependent, as it was observed in porcine kidney (PK-15) cells but not in swine testicle (ST) or pulmonary alveolar macrophage (PAM) cells and the underlying mechanism is still unclear (Deruelle *et al.*, 2009). Also in HSV-1, US3-mediated downregulation of MHC I has been reported. Like for PRV US3, this effect appeared to rely on an

indirect mechanism, since US3 expression alone was not sufficient for MHC I downregulation, nor did US3 trigger phosphorylation of MHC I (Imai *et al.*, 2013). In addition, HSV-1 US3, together with gB, has been reported to downregulate cell surface MHC I like molecule CD1d, potently abrogating recognition of CD1d by **natural killer T** (NKT) cells (Rao *et al.*, 2011) (see **2.2.1 Anti apoptotic activity**).

US3 also evades host immune responses by reducing viral glycoprotein expression on the cell surface. HSV-1 US3 specifically phosphorylates gB on position T887 (cytoplasmic domain), thereby regulating nuclear egress (see **2.2.2 Nuclear egress**) (Imai *et al.*, 2010; Kato *et al.*, 2009; Wisner *et al.*, 2009). This phosphorylation also results in a downregulation of gB from the surface of infected cells, which may serve as an important immune evasion mechanism as surface expressed gB is a potent inducer of both B- and T-cell responses (Kato *et al.*, 2009) (see **2.2.1 Anti apoptotic activity**). Furthermore, this may also affect **NK cell**-mediated cell lysis, since lysis of HSV-infected cells by NK-cells is correlated with the level of surface expressed gB (Kato *et al.*, 2009). Downregulation of cell surface gB has also been observed in PRV-infected cells and was also suggested to serve as a viral immune evasion mechanism there, although it is unknown whether US3 is involved in this process (Favoreel *et al.*, 1999; Favoreel *et al.*, 2002; Van Minnebruggen *et al.*, 2004).

As mentioned above, during cell-free spread, virions in the extracellular environment are vulnerable for inactivation by several host factors, particularly **neutralizing antibodies**. Both in infected and transfected cells, PRV US3 induces the formation of cellular protrusions, consisting of both actin and microtubules, contacting distant uninfected cells (Favoreel *et al.*, 2005). Virus particles can migrate in these protrusions, providing a virus cell-cell spread route that is shielded from virus neutralizing antibodies (Favoreel *et al.*, 2000; Favoreel *et al.*, 2005). US3-induced cell projections have also been reported for HSV-2, BHV-1, and BHV-5 (Brzozowska *et al.*, 2010; Finnen *et al.*, 2010; Ladelfa *et al.*, 2011). This function of US3 will be described in more detail further (see **2.2.5 Actin rearrangements** and **4.2 Egress**).

2.2.5 Actin rearrangements

US3 of several alphaherpesviruses induces drastic cytoskeletal rearrangements in infected or transfected host cells, typically consisting of actin stress fiber breakdown and/or the formation of long protrusions (sometimes also referred to as filamentous processes), containing both actin and microtubules. The latter were reported for PRV, HSV-2, BHV-1 and BHV-5 (Brzozowska *et al.*, 2010; Calton *et al.*, 2004; Favoreel *et al.*, 2005; Finnen *et al.*, 2010; Ladelfa *et al.*, 2011; Van den Broeke *et al.*, 2009a; Van den Broeke *et al.*, 2009b). This will be discussed in more detail further on (see **4.2 Egress**).

3. The actin cytoskeleton and Rho GTPases

3.1 Introduction

The actin cytoskeleton is a dynamic, three-dimensional network composed of actin polymers and various associated actin binding proteins (ABP). Its main functions are providing cellular rigidity and structure, as well as regulating crucial cellular processes including phagocytosis, cellular movement, cytokinesis and intracellular trafficking and communication (Croise *et al.*, 2014; Deschamps *et al.*, 2013; Disanza *et al.*, 2005; Pantaloni *et al.*, 2001; Pollard & Borisy, 2003; Pollard & Cooper, 2009).

The actin cytoskeleton is anchored to regions of cell-cell contact termed adherens junctions. In continuous sheets of epithelial cells, these junctions keep cells together in a tissue by forming an interconnected lateral bridge that links the actin cytoskeleton of neighboring cells (Harris & Tepass, 2010; Meng & Takeichi, 2009). Cadherins form a complex with cytoplasmic proteins called catenins, serving as a site of attachment of actin bundles, thereby mediating intercellular contacts at adherens junctions (Cooper, 2000; Huveneers & de Rooij, 2013; Weber *et al.*, 2011). Focal adhesions or cell-matrix adhesions consist of integrin complexes, connecting the extra cellular matrix (ECM) to actin filaments, providing strength to lateral contacts between adjacent cells on planar substrates (Partridge & Marcantonio, 2006) (*Figure 5*).

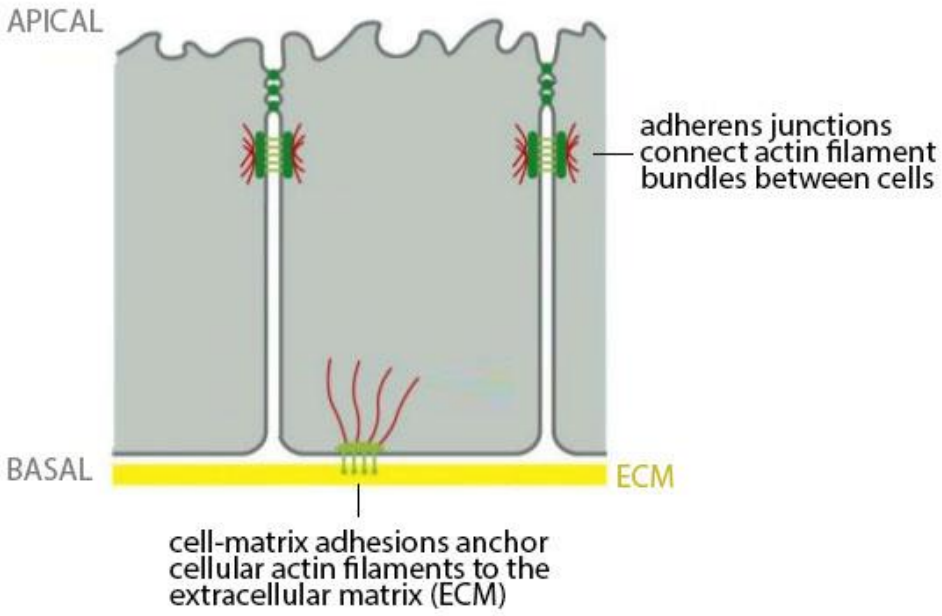

Figure 5: Adherens junctions and focal adhesions mediate adhesion in epithelial cells. Adherens junctions facilitate cell-cell adhesion by connecting actin filament bundles between adjacent cells, while focal adhesions or cell-matrix adhesions anchor cellular actin filaments to the extracellular matrix (ECM). Red lines: actin filaments.

3.2 Composition

Actin is a globular protein with a mass of 43 kDa and represents the most abundant protein in eukaryotic cells. Two basic forms of actin can be distinguished: monomeric globular actin (G-actin) and polymeric fibrous or filamentous actin (F-actin) (Disanza *et al.*, 2005). Actin filaments are formed by head to tail-association of G-actin. Actin filaments are consequently structurally and kinetically polarized, characterized by a fast-growing (barbed) end and a slow-growing (pointed) end and are subject to continuous ATP hydrolysis. Under physiological conditions, Mg^{2+}/ATP-bound actin molecules are incorporated at the barbed end. Slow hydrolysis then converts ATP-actin monomers into ADP-Pi-actin monomers. As only the barbed end grows, the relative position of an actin monomer in the filament moves to the pointed end, where it dissociates and recycles back into the actin monomer pool. Pi is not released immediately and remains noncovalently bound for some time to ADP-actin in the filament (Vavylonis *et al.*, 2005). The nucleotide of released ADP-actin is exchanged for ATP, a process catalyzed by the actin binding protein (ABP) profilin (Mockrin & Korn, 1980), allowing participation of ATP-actin in a new polymerization cycle. This continuous dynamic actin filament turnover or treadmilling is required for the formation of protrusive actin structures, cellular motility and movement of cytosolic components (Blanchoin *et al.*, 2014; Chang *et al.*, 2003; Disanza *et al.*, 2005; Van Troys *et al.*, 2008) (*Figure 6*). Essential herein is the orientation of the polarized actin filaments, e.g. with their fast growing barbed end towards the membrane in protrusive structures (see **3.3.3 Other actin structures**).

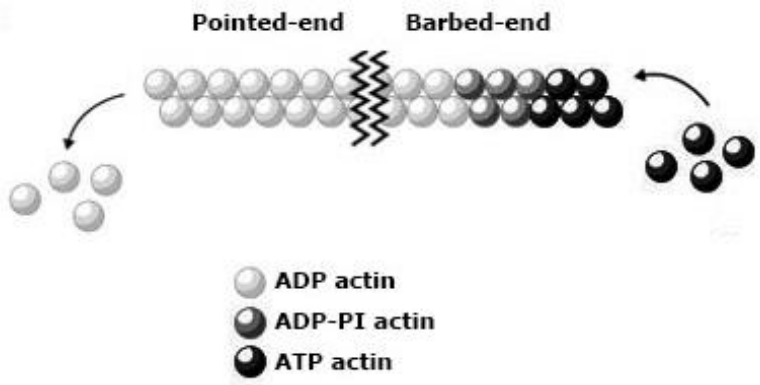

Figure 6: Actin treadmilling occurs through association of ATP-monomers at barbed ends and dissociation of ADP-monomers at pointed ends, resulting in monomer cycling through the filament. Light grey circles: ADP actin monomers; medium grey circles: ADP-Pi actin monomers (ADP with inorganic phosphate (Pi) bound noncovalently in the γ-phosphate position as a result of ATP hydrolysis); dark grey circles: ATP actin monomers (Adapted from (Littlefield & Fowler, 2002)).

3.3 Actin filament-based structures

The coordinated action of actin nucleation, polymerization and depolymerization ultimately leads to the formation of different filamentous actin structures. In cells this is driven by the regulated activity of a vast array of actin binding proteins. Actin meshworks or bundles can be formed, such as cortical actin or stress fibers (Taylor *et al.*, 2011). Furthermore, sheet-like extensions can be formed, such as lamellipodia, membrane ruffles, but also finger-like protrusions, such as microvilli and filopodia (Taylor *et al.*, 2011). Podosomes are dot-like actin structures (Taylor *et al.*, 2011). The most relevant cytoskeletal structures for this thesis will be discussed below and are schematically summarized in *Figure 7*.

3.3.1 Stress fibers

Stress fibers are composed of actin filaments, bundled together in packs of 10-30 (Cramer *et al.*, 1997). The actin crosslinking protein α-actinin keeps these bundles together, although other actin-crosslinking proteins, such as fascin, espin and filamin have also been described (Adams, 1995; Chen *et al.*, 1999; Lazarides & Burridge, 1975; Wang *et al.*, 1975). Stress fibers are attached to the plasma membrane at focal adhesions through interactions with integrins (Cooper, 2000; Weber *et al.*, 2011). Stress fibers show a strong similarity with muscle sarcomeres, as in both cases α-actinin shows a periodic staining across the bundle, alternating with myosin II (Lazarides & Burridge, 1975). However, the sarcomeric model does not describe the complexity of stress fibers very adequately: analysis of non-muscle cells has shown that actin filaments within stress fibers indeed show alternating polarity, as is the case in sarcomeres, but there is a range of possible orientations. While some stress fibers display a uniform filament polarity, others have a complete random orientation (Cramer *et al.*, 1997). In motile cells, however, the polarity of the majority of stress fibers is organized in the same way: barbed ends pointing outwards at the ends of the fiber (at the focal adhesion) and alternated polarization within the middle of the fiber (Cramer *et al.*, 1997).

Three types of actin stress fibers have been distinguished: (1) ventral stress fibers, which are many micrometers long and are anchored at each end of the cell by focal adhesions, (2) dorsal stress fibers (also referred to as radial stress fibers), which are usually shorter and are only anchored at one end of the cell to a focal adhesion or focal adhesion complex and (3) transverse arcs, which are contractile bundles of actin filaments in migrating or spreading cells, typically displaying a convex shape and move away from the leading edge of the cell (Heath & Holifield, 1993; Soranno & Bell, 1982). Because the latter are not directly anchored at adhesions, they were not originally classified as stress fibers, but they frequently are, as they give rise to ventral stress fibers, together with dorsal stress fibers (Burridge

& Wittchen, 2013; Hotulainen & Lappalainen, 2006). In endothelial cells, another type of stress fibers are characterized, which are essentially identical to ventral stress fibers, except that these insert into adherens junctions rather than into focal adhesions, thereby linking endothelial cells together (Millan *et al.*, 2010).

Stress fibers only develop when cells are cultured on rigid substrata. This leads to development of isometric tension in the cells, which is required for stress fiber formation (Grinnell, 1994; Halliday & Tomasek, 1995; Mochitate *et al.*, 1991; Tomasek *et al.*, 1992) and depends on RhoA activation (Paszek *et al.*, 2005; Wozniak *et al.*, 2003) (see **3.4.1 RhoA-pathway**). RhoA is also associated with myosin-driven stress fiber formation and contractility which enhances cellular permeability (Burridge & Wittchen, 2013).

3.3.2 Cortical actin

Cortical actin, also called the actin cortex, is situated immediately beneath the plasma membrane and consists of an extremely dense arrangement of actin filaments, formed by crosslinking by α-actinin (Charras *et al.*, 2006) and branching (Bovellan *et al.*, 2014; Charras *et al.*, 2006). Cortical actin also contains myosin I and II and has been described to have a contractile function, such as in amoeboid motility (Miyoshi *et al.*, 2013; Papadopulos *et al.*, 2013). The cortical actin provides the plasma membrane with properties that intracellular membranes lack, such as a high mechanical resistance and tension and allows it to undergo structural changes, including local extension and retraction of protrusions and the formation of invaginations, often involved in various types of endocytosis and exocytosis (de Curtis & Meldolesi, 2012).

This cortical actin meshwork is constantly being remodeled in response to diverse signaling pathways to support the cells' needs, playing an important role in the normal functioning of the cell, as it helps maintain the cell shape and provide the driving force for cell migration (Charras *et al.*, 2006). Remodeling of cortical actin is also essential for endocytosis and exocytosis (de Curtis & Meldolesi, 2012; Eitzen, 2003; Engqvist-Goldstein & Drubin, 2003; Malacombe *et al.*, 2006; Qualmann & Kessels, 2002). Remodeling of cortical actin is directly regulated by Rho GTPases and mediated by numerous actin-binding proteins, which are arranged in large multimolecular complexes that also include membrane-binding and signaling domains (de Curtis & Meldolesi, 2012) (see **3.4 Rho GTPase signaling**).

3.3.3 Other actin structures

During cell migration, the cytoplasm is continuously remodeled and induces membrane protrusions in the form of lamellipodia and filopodia (Small, 2010). The formation of these protrusions is regulated by WASP and WAVE proteins (see **3.5.1 WASP**). Actin treadmilling induced by WASP and WAVE proteins is of critical importance in generating protrusive forces in cells, and thereby potentiates cellular migration and motility (Chang *et al.*, 2003; Ladwein & Rottner, 2008; Takenawa & Suetsugu, 2007). **Filopodia** are one-dimensional, rod-like extensions of about 0.1-0.2 µm in diameter that protrude beyond the cell periphery. They function as important sensory organelles (such as in neuronal growth cones or during the fusion of sheets of epithelial tissues) and serve as a precursor in adhesion site of stress fiber formation (Faix *et al.*, 2009). Like stress fibers, they are composed of a bundle of actin filaments, crosslinked by α-actinin, fascin, fimbrin and proteins from the filamin family (Aratyn *et al.*, 2007; Bretscher & Weber, 1980; Nishita *et al.*, 2006; Ohta *et al.*, 1999; Sobue & Kanda, 1989). These bundles are oriented with their fast-growing ends toward the tip of the bundle (Goldman & Knipe, 1973; Lindberg *et al.*, 1981; Nemethova *et al.*, 2008; Small & Celis, 1978; Small *et al.*, 1978) and are reminiscent of those in microvilli, but are longer, thinner and more dynamic (Alberts *et al.*, 2002). In contrast to filopodia, **lamellipodia** are composed of a crisscross arrangement of highly dynamic actin filaments, forming a two-dimensional, sheet-like structure, with the fast-growing filament ends adjacent to the plasma membrane. Filaments are crosslinked by α-actinin, coronin and filamin (David *et al.*, 1998; de Hostos, 1999; Langanger *et al.*, 1984; Stossel *et al.*, 2001; van der Flier & Sonnenberg, 2001). The broad and sheet-like protrusions caused by lamellipodia are driven by actin filament polymerization, growing in front of the lamellum. The latter consists primarily of condensed linear actin bundles, leading to a more stable and less dynamic actin filament network) and contains myosin II, required to drive and guide cell locomotion (Ladwein & Rottner, 2008). **Podosomes**, often subsumed together with invadopodia as invadosomes, are complex dot-like F-actin accumulations (Linder, 2009). Podosomes are typically formed in monocytic cells, endothelial cells and smooth muscle cells, whereas invadopodia are mostly found in invasive cancer cells (Linder, 2007). These structures are crosslinked by AFAP-110 (Albiges-Rizo *et al.*, 2009) surrounded by several cytoskeletal proteins and matrix metalloproteases (Sato *et al.*, 1997) that, together with signaling molecules, remodel the cytoskeleton and extracellular matrix during tissue invasion (Saltel *et al.*, 2011).

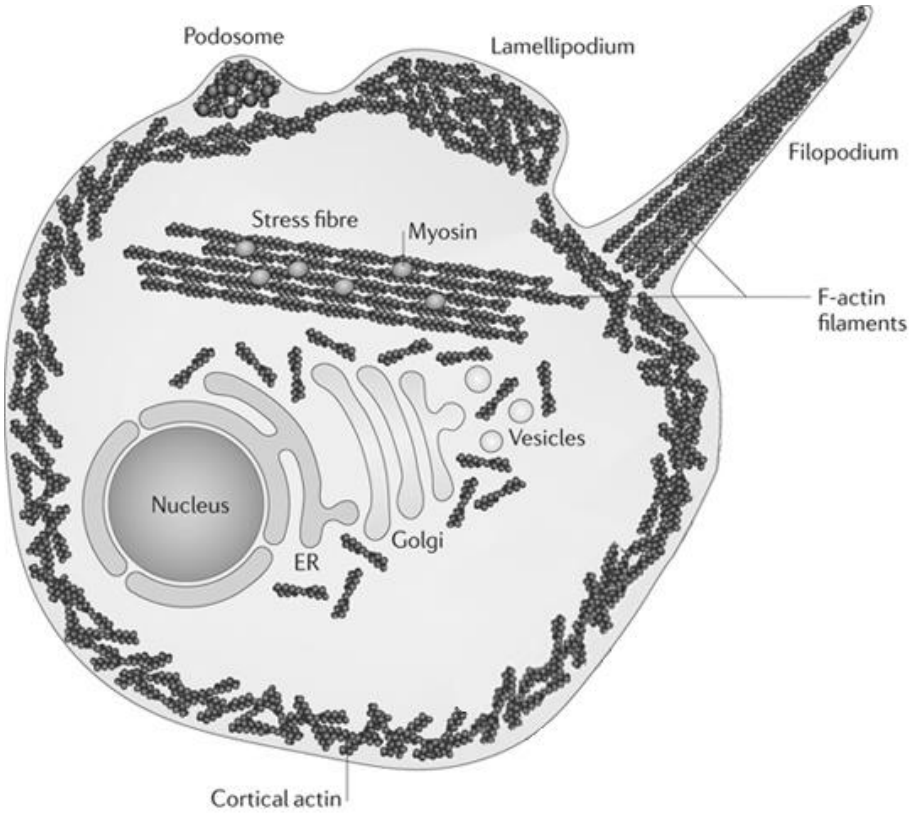

Figure 7: Different forms of actin filament based structures. Stress fibers are large bundles of actin filaments that can span the length of the cell, with myosin enabling their contractility. Just beneath the plasma membrane lies the cortical actin. Actin filaments can also be organized to produce a range of cellular extensions, including podosomes, lamellipodia, filopodia, microvilli and large membrane ruffles. Podosomes contain (next to several actin-binding proteins, signaling molecules) metalloproteases (black balls in podosome). (ER = endoplasmic reticulum) (Adapted from (Taylor *et al.*, 2011)).

3.4 Rho GTPase signaling

The actin cytoskeleton is mainly regulated by the Rho family of GTPases (Rho GTPases) (Hall, 1998). These monomeric, low-molecular weight proteins constitute a distinct family with 22 mammalian members within the superfamily of Ras-related small GTPases, and are subdivided in the Rac subfamily (Rac1, Rac2, Rac3, and RhoG), Cdc42 subfamily (Cdc42, TC10, TCL, Chip, and Wrch-1), RhoA subfamily (RhoA, RhoB, and RhoC) and other Rho GTPases (RhoE/Rnd3, RhoH/TTF, Rif, RhoBTB1, RhoBTB2, Miro-1, Miro-2, RhoD, Rnd1, and Rnd2). Besides regulating the actin cytoskeleton, Rho GTPases are also involved in regulation of the microtubule network and in linking membrane receptors to the actin cytoskeleton, which explains their fundamentality and omnipresence throughout eukaryotic cells (Aspenstrom *et al.*, 2004; Hall, 2009) and their requirement to be strictly controlled in a spatiotemporal manner. They can be viewed as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound form. This switch is tightly regulated by three sets of proteins: guanine nucleotide-

exchange factors (GEFs) that catalyze the activating exchange of GDP for GTP (Schmidt & Hall, 2002), GTPase activating proteins (GAPs) that stimulate the intrinsic GTPase activity to inactivate the switch (Bernards, 2003; Heasman & Ridley, 2008) and guanine nucleotide-dissociation inhibitors (GDIs), sequestering Rho GTPases in the cytoplasm, away from the plasma membrane and their regulators and targets (Garcia-Mata *et al.*, 2011; Olofsson, 1999) (*Figure 8*).

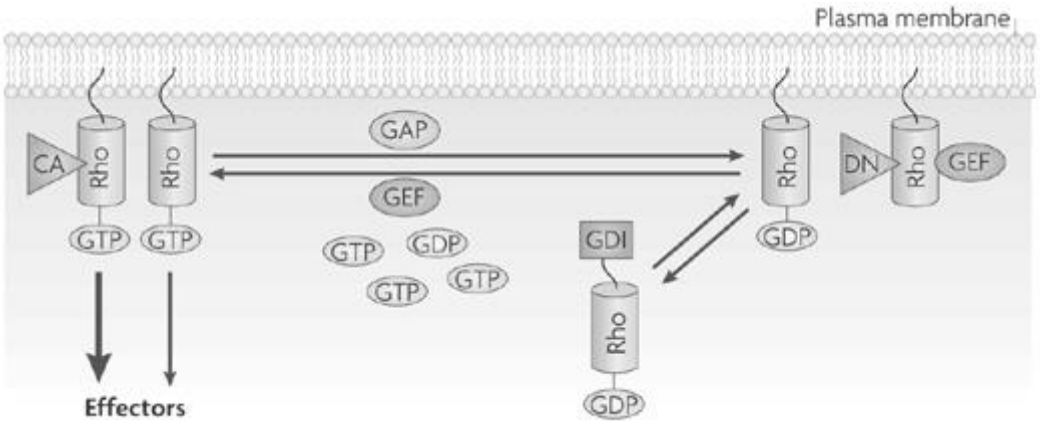

Figure 8: Regulation of Rho GTPases. Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the release of GDP and the binding of GTP. Dominant-negative (DN) Rho GTPases contain a substitution mutation of Thr for Asn at amino acid 17 (numbering according to Rac1 sequence), allowing binding to GEFs but inhibiting downstream interactions with effector proteins. GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing the intrinsic GTPase activity of Rho proteins. Constitutively active (CA) mutants cannot hydrolyze GTP and therefore signal constitutively to their effector proteins. Common constitutively active mutations are Gly to Val at amino acid 12 (numbering according to Rac1) or Gln to Leu at amino acid 61. Guanine nucleotide-dissociation inhibitors (GDIs) bind to C-terminal prenyl groups on some Rho proteins, sequestering them in the cytoplasm away from their regulators and targets (From (Heasman & Ridley, 2008))**.**

In their active GTP-bound state, Rho GTPases act through a conformation-specific interaction with their target effector proteins. The best characterized members of the Rho GTPases are RhoA, Rac1, and Cdc42, which show high degrees of conservation in higher vertebrates. Activation of RhoA leads to formation of actin stress fibers and focal adhesion assembly, Rac1 typically regulates the formation of lamellipodia or membrane ruffles, while Cdc42 induces the formation of protrusive filopodia. As Rho GTPases especially affect membrane dynamics, they need to be localized near the cellular membrane. This is mediated by prenylation (farnesylation or geranylgeranylation) and sometimes palmitoylation at their C-terminus, which enhances the interaction with membranes (Adamson *et al.*, 1992). Rho GDIs can also inhibit Rho GTPase activity by masking these prenyl groups, thereby preventing Rho GTPase interaction with membranes and downstream targets (DerMardirossian & Bokoch, 2005; Michaelson *et al.*, 2001).

Over the past decades, many of the molecular details of the signal transduction pathways that connect Rho GTPase activity to rearrangements of the actin cytoskeleton have been unraveled. Nevertheless, identification of downstream targets of Rho GTPases has proven to be challenging. A combination of affinity chromatography, protein purification and yeast-two hybrid screening revealed over 100 potential Rho GTPase targets (Bishop & Hall, 2000). Rho, Rac1, and Cdc42 alone are each capable of interacting with about 20–30 different proteins in a GTP-dependent manner, which is reflected by their broad signal transduction potential (Hall, 2012). Rac1 and Cdc42 signaling converges to group I serine/threonine p21 activating kinase (PAK) signaling, which is interconnected with downstream RhoA signaling (Xie *et al.*, 2008; Yamaguchi *et al.*, 2001). In general, RhoA pathway signaling counteracts the Rac1 and Cdc42 signaling axes and vice versa (Kozma *et al.*, 1997; Leeuwen *et al.*, 1997; Nimnual *et al.*, 2003; Parri & Chiarugi, 2010; Sander *et al.*, 1999). *Figure 9* represents a (non-exhaustive) schematic visualization summarizing these interwoven signaling pathways. Because of the importance of the RhoA pathway and the Cdc42/Rac1 effector PAK in the context of the current thesis, these two components of Rho GTPase signaling will be discussed in more detail below.

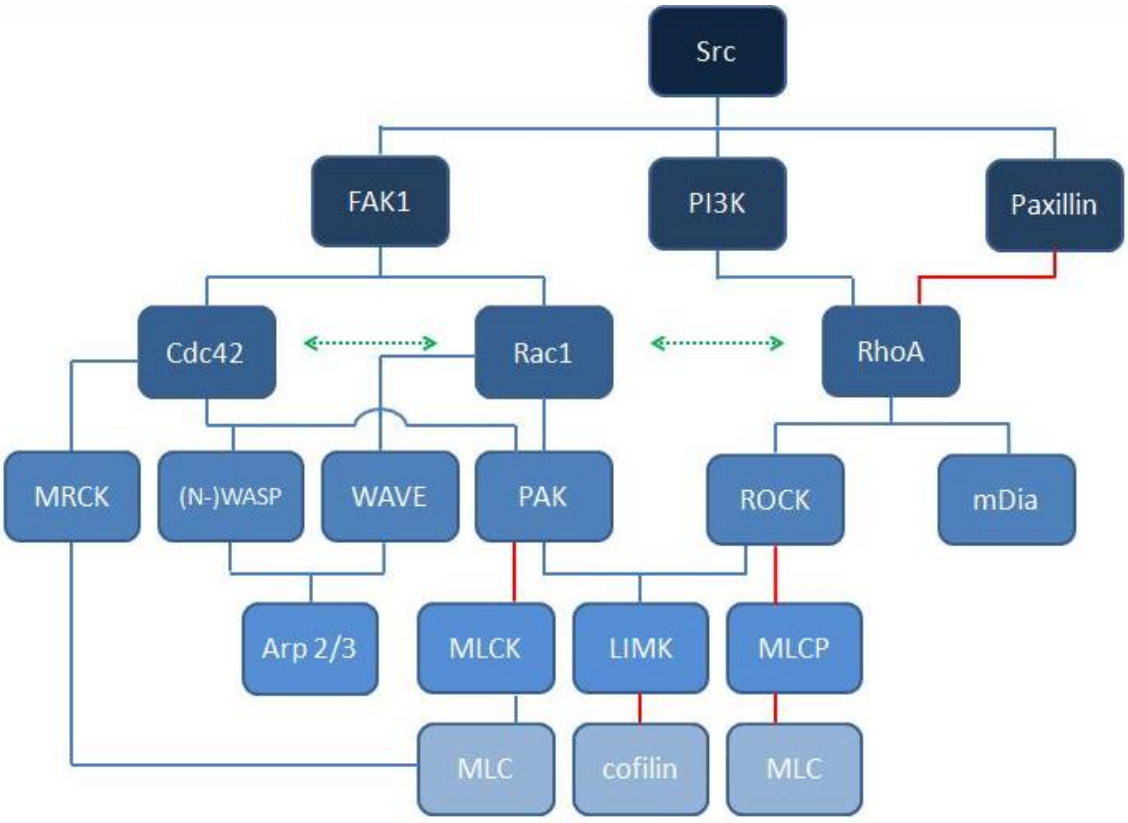

Figure 9: Schematic overview of selected Rho GTPase signaling. Cdc42 and Rac1 signaling leads to activation of the common effector protein p21-activated kinase (PAK) and scaffold proteins belonging to the WASP/WAVE family, which are key regulators of actin filament nucleation and polymerization (via binding the Arp (actin related protein)2,3 complex). Active PAK phosphorylates myosin light chain kinase (MLCK), thereby inactivating it and inhibiting myosin light chain (MLC) phosphorylation and actomyosin-based contractility. PAK also phosphorylates and activates LIMK which may lead to phosphorylation of cofilin, thereby inhibiting its actin-

function. Downstream targets of RhoA include the serine/threonine kinase ROCK which is involved in the formation of stress fibers and focal adhesions. ROCK phosphorylates downstream MLC, leading to actin–myosin contractility. At the same time, ROCK inhibits MLC dephosphorylation by inhibiting MLC phosphatase (MLCP). LIMK is also a downstream effector of ROCK. The mammalian homolog of the Drosophila diaphanous protein (mDia) is another important RhoA effector mediating actin filament nucleation. Red line = inhibitory effect; blue line = activating effect; dotted green arrows = counteracting effect (From (Van den Broeke *et al.*, 2014)).

3.4.1 RhoA-pathway

The best characterized effectors of RhoA are the mammalian homolog of the Drosophila Diaphanous protein (mDia), member of the formin family and Rho-associated coiled-coil forming kinase (ROCK) (Narumiya *et al.*, 1997; Satoh & Tominaga, 2001; Watanabe *et al.*, 1999; Watanabe *et al.*, 1997). Other RhoA effectors include members of the ezrin/radixin/moesin (ERM) proteins (Matsui *et al.*, 1999) and citron kinase (Yamashiro *et al.*, 2003).

mDia, as a member of the formin protein family, belongs to a subclass of the formins, the *Diaphanous*-related formins (Drfs), that can act as effectors for Rho GTPases. They consist of three formin homology (FH) domains, an N-terminal Rho GTPase binding domain (GBD) and the intramolecular autoregulatory partner of the GBD, the Diaphanous-autoregulatory domain (DAD) (Alberts, 2001; Shi *et al.*, 2009; Tominaga *et al.*, 2000; Wallar & Alberts, 2003) (*Figure 10*). Interaction with RhoA triggers mDia activation by disrupting auto inhibitory intramolecular constraints between the GBD and DAD of mDia (Alberts, 2001; Shi *et al.*, 2009). The FH2-domain is responsible for driving actin nucleation, moving progressively as the filament elongates. After nucleation, this domain remains bound at the barbed end, preventing the access of capping proteins to the filament (Chhabra & Higgs, 2007; Higashida *et al.*, 2004). All formins studied to date function in a homodimeric way, as their FH2 domains dimerize, allowing a dynamic association with the barbed end of growing filaments (Chhabra & Higgs, 2007). FH1, a proline rich sequence, is reported to aid actin nucleation and polymerization through binding with complexed actin-profilin (reviewed by (Wallar & Alberts, 2003)). Actin from this complex is then built in at the barbed end of the filament by the FH2 domain (Courtemanche & Pollard, 2012). Very little is known about the FH3-domain function, which was described by Petersen and colleagues (Petersen *et al.*, 1998).

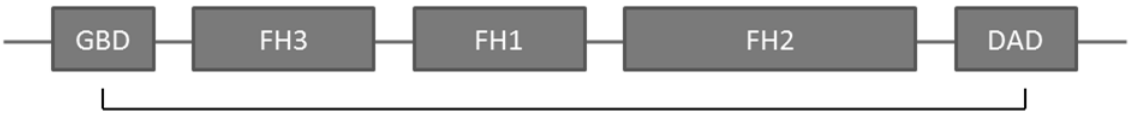

Figure 10: mDia1-3 domain organization. GBD: Rho GTPase binding domain; FH3: formin homology 3 domain; FH1: formin homology 1 domain; FH2: formin homology 2 domain; DAD: Diaphanous-autoregulatory domain. The black line connecting the GBD and DAD domain represents the intramolecular auto inhibitory interaction (Adapted from (Goh *et al.*, 2012)).

Although mDia is a direct actin filament nucleator, binding of Src to mDia's FH1-region has been described, acting downstream of mDia to assist in stress fiber formation (Tominaga *et al.*, 2000; Young & Copeland, 2010). Diaphanous-interacting protein (DIP) was also reported to bind to the FH1-region of mDia and leads to activation of Src (Satoh & Tominaga, 2001). Later research showed that DIP is actually phosphorylated by Src downstream of mDia activation (Meng *et al.*, 2004). This phosphorylation was found to be required for DIP-dependent phosphorylation of p190RhoGAP (a RhoA GAP) and Vav2 (a Rac1 GEF), resulting in inhibition of RhoA and activation of Rac1 respectively (Meng *et al.*, 2004). The latter illustrates formin-induced negative feedback regulation of RhoA signaling (Meng *et al.*, 2004; Satoh & Tominaga, 2001). DIP has also been reported to directly bind and activate the Arp2/3 complex (see **3.5.1 WASP**) (Kim *et al.*, 2006), inducing actin filament nucleation. Rho GTPase Rif was also demonstrated to interact with mDia and is able to trigger the formation of both filopodia (see next paragraph) and stress fibers (Fan *et al.*, 2010; Goh *et al.*, 2011; Pellegrin & Mellor, 2005).

Three mDia isoforms are currently known in mammals, namely mDia1, mDia2 and mDia3. Several studies have investigated the function of each isoform. mDia1 demonstrated the broadest activity spectrum, ranging from promoting stress fiber formation in cultured cells (Nemethova *et al.*, 2008; Watanabe *et al.*, 1999), filopodia formation, cell polarization and migration of certain cell lines, acting as a sensor for mechanical forces (Higashida *et al.*, 2013; Jegou *et al.*, 2013; Riveline *et al.*, 2001) to axon elongation (Arakawa *et al.*, 2003). Different roles of mDia1 in serum response factor (SRF)-mediated transcription, macrophage phagocytosis, exocrine vesicle secretion and cell-cell adhesion of epithelial cells have also been reported (Colucci-Guyon *et al.*, 2005; Sahai & Marshall, 2002; Satoh & Tominaga, 2001). mDia2 seems especially involved in filopodia formation and cytokinesis (Beli *et al.*, 2008; Pellegrin & Mellor, 2005; Watanabe *et al.*, 2008; Yang *et al.*, 2007). Finally, the mDia3 isoform is involved in chromosome alignment through spindle positioning and in endocytosis (Gasman *et al.*,

2003; Yasuda *et al.*, 2004). It might seem confusing that mDia1 and mDia2 are both reported to induce filopodia, while we stated above that filopodia are typically the result of Cdc42 signaling, which counteracts RhoA signaling. This can be explained by the apparent involvement of mDia1 and -2 in Cdc42-mediated filopodia formation (Goh *et al.*, 2012; Pellegrin & Mellor, 2005; Peng *et al.*, 2003). While Rho GTPase Rif has been described to interact with all mDia isoforms, the biological relevance for mDia3 has yet to be investigated (Fan *et al.*, 2010; Goh *et al.*, 2011).

The ROCK serine/threonine kinases consist of three major domains: the kinase domain, the RhoA binding domain (RBD) located in a coiled-coil region and the cysteine-rich pleckstrin-homology domain that is thought to participate in protein localization (*Figure 11*).

Figure 11: ROCK domain organization. RBD: RhoA binding domain; PH: pleckstrin homology domain (Adapted from (Ueda *et al.*, 2003)).

There are two ROCK isoforms in mammals, namely ROCK-I and ROCK-II (Nakagawa *et al.*, 1996). Rho-mediated ROCK activation regulates phosphorylation of myosin light chain (MLC), either directly or through inactivation of MLC phosphatase, resulting in increased actomyosin contractility (Kimura *et al.*, 1996; Narumiya *et al.*, 2009). There is a lot of data concerning biological ROCK functions because of the discovery of Y-27632, a highly specific (albeit not isoform-specific) ROCK-inhibitor (Uehata *et al.*, 1997). Using this inhibitor, the involvement of ROCK in cell migration and cell-cell adhesion in multiple cells, transcription, apoptosis, axogenesis, cell polarization and migration of T-cells was reported (Bito *et al.*, 2000; Coleman *et al.*, 2001; Heasman *et al.*, 2010; Hirose *et al.*, 1998; Itoh *et al.*, 1999; Ohgushi & Sasai, 2011; Sahai *et al.*, 1999; Sahai & Marshall, 2002; Sebbagh *et al.*, 2001; Tsuji *et al.*, 2002). More recently, a role for ROCK in apoptosis was shown in dissociated human embryonic stem cells (Kurosawa, 2012; Rungsiwiwut *et al.*, 2013). LIMK is another downstream target of ROCK, which phosphorylates and inhibits the actin regulating protein cofilin (Yang *et al.*, 1998), which will be discussed below (**see 3.6.2 Cofilin**).

3.4.2 Group I p21-activated kinases (PAKs)

The p21-activated kinases (PAKs) are serine/threonine kinases downstream of Rho GTPases Cdc42 and Rac1. The PAK family consists of six members that can be divided into two subfamilies based on their structure and biochemical properties: group I PAKs (PAK1, -2 and -3) and group II PAKs (PAK4, -5 and -

6). While PAK2 and -4 are ubiquitously expressed, PAK1 is expressed in the brain, spleen, muscle and mammary gland and PAK3 and -5 are restricted to the brain. PAK6 is expressed in testis, prostate, placenta, and kidney, but especially high in the brain (Jaffer & Chernoff, 2002; Manser *et al.*, 1994; Pandey *et al.*, 2002) (reviewed in (Rane & Minden, 2014)).

Group I and II PAKs both contain a p21-binding domain (PBD or Cdc42/Rac1-interacting (CRIB) domain), found in the N-terminal region of the protein (Morreale *et al.*, 2000) and a C-terminal kinase domain (Eswaran *et al.*, 2008). Proteins of both groups contain an additional N-terminal auto inhibitory domain (AID), partly overlapping the PBD in group I PAKs (Baskaran *et al.*, 2012; Eswaran *et al.*, 2008; Ha *et al.*, 2012; Zhao & Manser, 2012). Only group I PAKs function as dimers (Eswaran *et al.*, 2008; Rane & Minden, 2014; Zhao & Manser, 2012). The AID interacts with the kinase domain, keeping the molecule in a dimeric (group I PAKs) or folded monomeric (group II PAKs), auto inhibitory conformation (Baskaran *et al.*, 2012; Rane & Minden, 2014) (*Figure 12*). Since group I PAKs in particular are associated with regulation of the actin cytoskeleton, the remainder of the text will mainly focus on these.

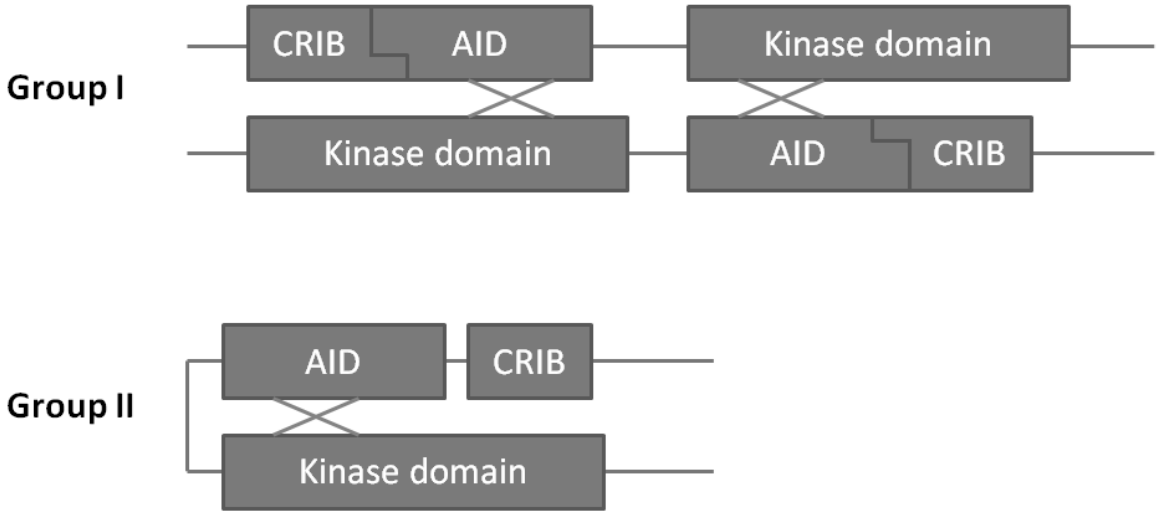

Figure 12: Group I and II PAK domain organization. CRIB: Cdc42/Rac1-interacting domain; AID: auto inhibitory domain. Proteins are represented in their inhibited conformations (diagonal crossed lines indicate inter- (group I PAK) and intramolecular (group II PAK) interactions) (Adapted from (Rane & Minden, 2014)).

PAKs can be activated in both Rho GTPase dependent and independent manners. Binding of group I PAKs to activated GTP-Rac1 of GTP-Cdc42 through its PBD/CRIB domain leads to a conformational change in PAK and causes it to become a monomer, which subsequently becomes autophosphorylated on Thr423 in PAK1 (or Thr402 in PAK2 or Thr421 in PAK3) in its activation loop (Jaffer & Chernoff, 2002; Zenke *et al.*, 1999). This phosphorylation prevents refolding, even in the absence of GTPases.

Phosphorylation of this residue by specific cellular kinases such as Akt (Tang *et al.*, 2000) or PDK1 (King *et al.*, 2000) can also directly cause full catalytic activity of PAK towards its substrates (Gatti *et al.*, 1999). Furthermore, PAK cleavage by caspase 3, recruitment to the plasma membrane through Nck and Grb2 to activated tyrosine kinase receptors or direct stimulation by sphingosine and sphingosine-derived lipids have also been described as group I PAK activation mechanisms (Bokoch, 2003; Lu & Mayer, 1999).

Activation of group I PAKs, and PAK4 (Dan *et al.*, 2001), leads to loss of stress fibers and focal adhesion complexes. Depending on the cell type, polarized lamellipodia are formed, cell-cell adhesion is weakened and cell motility increased (Manser *et al.*, 1994; Sells *et al.*, 1998; Wang *et al.*, 1999). In addition to their role in regulating cytoskeletal dynamics, PAKs are implicated in cell motility, neurogenesis, angiogenesis and cancer metastasis (reviewed in (Bokoch, 2003)). Group I PAKs are also involved in macropinocytosis, a cellular endocytosis process which is also hijacked by several viruses to gain entry to the cell (Dharmawardhane *et al.*, 2000).

3.5 Downstream effectors of Rho GTPase signaling that regulate actin polymerization

Rho GTPases directly or indirectly (e.g. via PAKs and other downstream effectors) activate molecules that regulate actin polymerization. Two important types of such molecules are WASP-family proteins and the ADF/cofilin family proteins. ADF/cofilin family proteins are actin binding proteins (ABP), while WASP-family proteins can recruit proteins that bind to actin. Cofilin is of particular importance in the context of the current thesis, and will be elaborately discussed.

3.5.1 WASP

WASP (Wiskott-Aldrich syndrome protein)-superfamily proteins are major actin polymerizing factors of the actin cytoskeleton, also termed nucleation promoting factors (NPFs) (Chhabra & Higgs, 2007; Kurisu & Takenawa, 2009; Miki & Takenawa, 2003; Stradal *et al.*, 2004). At least one WASP superfamily homolog has been characterized in all eukaryotic organisms that are examined thus far. In humans, eight superfamily members have been described: WASP, expressed exclusively in hematopoietic lineages; its ubiquitous homolog neural WASP (N-WASP); brain enriched WAVE-1 (WASP family verprolin-homologous protein 1) and WAVE-3; and ubiquitously expressed WAVE-2 (Kurisu & Takenawa, 2009; 2010). The latest characterized members are WASP and SCAR homolog (WASH), WASP homolog associated with actin, membranes, and microtubules (WHAMM) and junction-mediating and regulatory protein (JMY), but little information is available about their biological roles (Kurisu & Takenawa, 2009; 2010). A variety of cellular processes associated with actin dynamics

depends on members of the WASP family, such as vesicular trafficking and the formation of membrane protrusions (Yamaguchi *et al.*, 2005).

The WASP and WAVE family proteins have a conserved C-terminal VCA domain (consisting of the verprolin homology (or WASP homology 2 (WH2)) domain, central/cofilin homology domain (C) and acidic region (A)), through which they bind to and activate the Arp2/3 complex. The WH2-domain binds actin, while the C and A domains bind the Arp2/3 complex (Machesky & Insall, 1999). Adjacent to their VCA domain, a proline rich segment characterizes both WASP en WAVE family proteins (Lane *et al.*, 2014). The N-terminal sequence of WASP subfamily proteins is different from that of WAVE proteins. WASP subfamily proteins possess an N-terminal WASP homology 1 (WH1 or Ena-VASP homology 1 (EVH1)) domain, responsible for binding to WASP-interacting protein (WIP) family proteins (Anton *et al.*, 2007) and a GTPase-binding domain (GBD or Cdc42/Rac-interactive binding (CRIB) domain), which is critical for the activity of WASPs. This CRIB domain binds to the WASP C-terminal VCA-region, forming an inhibitory intramolecular interaction (Kim *et al.*, 2000). WAVE subfamily proteins on the other hand lack the WH1 and CRIB domains and are characterized by an N-terminal WAVE homology domain/SCAR homology (WHD/SHD) domain, followed by a stretch of basic residues (Kurisu & Takenawa, 2009) (*Figure 13*).

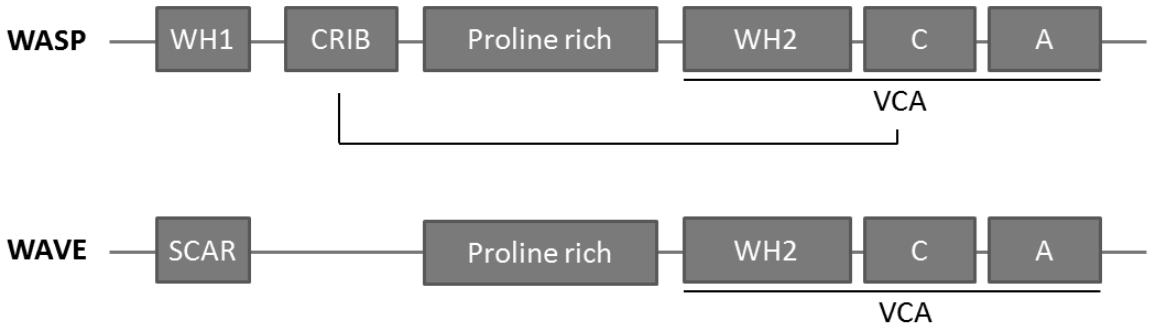

Figure 13: WASP and WAVE family proteins domain organization. WH1: WASP homology 1 (WH1 or Ena-VASP homology 1 (EVH1)) domain; CRIB: Cdc42/Rac-interactive binding or GTPase-binding domain (GBD); WH2: WASP homology 2 domain; C: central/cofilin homology domain; A: acidic region; VCA: domain comprising WH2, C and A; SCAR: WAVE homology domain (WHD or SCAR homology domain (SHD)). The black line connecting the CRIB and VCA domain represents the intramolecular auto inhibitory interaction of WASP (Adapted from (Lane *et al.*, 2014)).

In order to activate WASP proteins, Cdc42 and Rac1 can cooperate with phosphatidylinositol-4,5-bis-phosphate (PI(4,5)P2) to bind the CRIB domain, folding open the protein and thereby overcoming auto inhibition (Miki *et al.*, 1998; Symons *et al.*, 1996). Several other proteins, such as Nck, Grb and WISH (WASP-interacting SH3-protein) (Yamaguchi *et al.*, 2005) have been described to bind and activate N-

WASP, by binding its proline rich region and thereby exposing the VCA domain. Additionally, WIP (WASP-interacting protein) has been reported to bind to the WH1 domain in N-WASP, stabilizing its closed, inactive conformation (Fried *et al.*, 2014; Moreau *et al.*, 2000). Another potential binding partner of WASP is cortactin, which stabilizes actin branches, by both binding with the Arp2/3 complex and polymerized actin (Pant *et al.*, 2006). Furthermore, WASP family proteins are described to be involved in the formation of podosomes (Kaverina *et al.*, 2003; Linder *et al.*, 1999; Mizutani *et al.*, 2002; Pollard & Cooper, 2009).

Unlike WASP proteins, which exist independently in cells, each WAVE protein is associated through its SCAR-domain with additional proteins including Abi (Abelson-interacting protein), Nap1/Hem-2, PIR121/Src1/Cyfip1 and HSPC300/Brick1 , which have also been shown to copurify along with WAVE-2 and WAVE-3 (Gautreau *et al.*, 2004; Innocenti *et al.*, 2004; Kurisu & Takenawa, 2009; Stovold *et al.*, 2005). These proteins form the WAVE-regulatory complex (WRC), keeping WAVE in an intrinsically inactive state (Derivery *et al.*, 2009). Rac1 has been described to activate WAVE by recruitment to the plasma membrane through interaction with the WASP WRC (Koronakis *et al.*, 2011; Steffen *et al.*, 2013; Steffen *et al.*, 2004). The mechanism is still unclear, but Rac1 binding to the WRC potentially leads to dissociation of WAVE from the inhibitory WRC (Eden *et al.*, 2002).

In their activated state, WASP and WAVE family proteins induce activation of ABP Arp2/3 (actin-related protein 2 and 3), leading to its binding to the side of an existing (mother) filament and assembling a new (daughter) filament at a 70° angle, producing a branched structure (Millard *et al.*, 2004; Welch & Mullins, 2002). These branched actin filaments grow in successive generations, like the twigs of a bush, leading to the generation of a meshwork, as observed in lamellipodia (Pollard & Borisy, 2003). WASP and WAVE couple the cell membrane to Arp2/3-dependent actin polymerization, to achieve coordinated membrane cytoskeleton dynamics (Kurisu & Takenawa, 2009). More recently, the Arp2/3 complex has been shown to serve as a key upstream factor for the recruitment of modulators of lamellipodia formation, such as capping proteins and cofilin, which might be crucial for lamellipodia-based cellular migration (Koestler *et al.*, 2013) (see **3.5.2 Cofilin**). The binding and activation of the Arp2/3 complex through WASP-superfamily members and the formation of branched actin networks is shown in *Figure 14*.

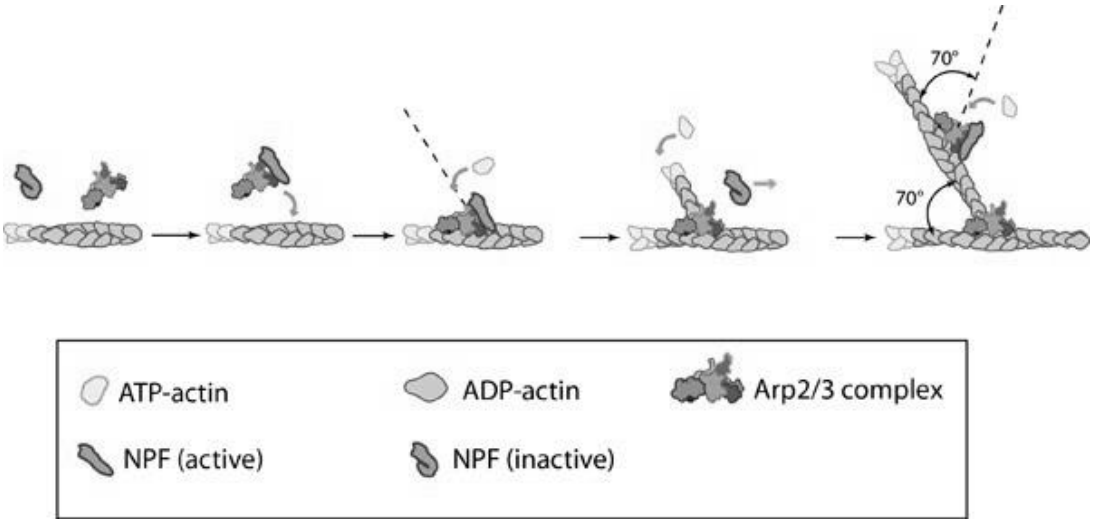

Figure 14: The binding and activation of the Arp2/3 complex through WASP-superfamily members and the formation of branched actin networks. Activated nucleation promoting factors **(**NPFs) (WASP and WAVE) bring together the Arp2/3 complex and actin monomers to nucleate actin filaments that form new branches from the side of preexisting filaments. The Arp2/3 complex remains at the minus end of the filament (From (MBInfo, 2014)).

3.5.2 ADF/Cofilin

The ADF/cofilin family in mammals consists of three paralogs: Cofilin-1, Cofilin-2 and actin depolymerizing factor (ADF) or destrin (Van Troys *et al.*, 2008). The three cofilin isoforms each have their own tissue specificity, as was determined in mice (Vartiainen *et al.*, 2002). Cofilin-1 is the dominant isoform, omnipresent in most cell types, while cofilin-2 is expressed only in muscle cells. ADF expression is reported to be restricted to epithelia and endothelia, but is likely more widespread as its presence was recently reported in B-cells (Ono *et al.*, 1994; Rochelle *et al.*, 2013; Vartiainen *et al.*, 2002). Cofilin family members are critical regulators of actin dynamics in cells.

Cofilins have been termed actin-dynamizing proteins based on their capacity to enhance the turnover of actin filaments *in vitro* (Carlier *et al.*, 1999). Important mechanical insight was provided by Andrianantoandro and Pollard (Andrianantoandro & Pollard, 2006). Using real-time microscopy, they could distinguish cofilin-decorated actin filaments. When only a few cofilin molecules were bound to an F-actin filament, there was maximal straining between (cofilin decorated) twisted and non-twisted regions, leading to frequent breaking points, hence F-filament severing (Bobkov *et al.*, 2006). In contrast, severing was no longer observed at higher cofilin concentrations, as the high degree of cofilin binding now limited straining (Andrianantoandro & Pollard, 2006; Bobkov *et al.*, 2006). These findings led to the new paradigm stating that cofilin activity is fine-tuned based on its concentration in the cell, with F-actin severing activity being predominant at optimal low cofilin concentrations, which was in

line with several previous studies (Ichetovkin *et al.*, 2002; Orlova *et al.*, 2004; Pavlov *et al.*, 2007). At this cofilin concentration, capping of the severed barbed ends leads to actin monomer dissociation from pointed ends, resulting in actin disassembly (Andrianantoandro & Pollard, 2006). Higher cofilin concentrations favor the release of Pi from ADP-Pi filaments (Blanchoin & Pollard, 1999), which is normally a slow process (see **3. The actin cytoskeleton and Rho GTPases** and **3.2 Composition**). This Pi-release results in debranching of actin filaments as 1) ADP-actin is more prone to depolymerization and 2) Arp2/3 has a higher affinity for ATP-actin than ADP-actin (Andrianantoandro & Pollard, 2006; Blanchoin *et al.*, 2000). Finally, at very high concentrations of cofilin versus actin monomers, monomer binding and actin nucleation seem enhanced as cofilin stabilizes actin monomers and actin assembly is stimulated (Andrianantoandro & Pollard, 2006; Yeoh *et al.*, 2002) (*Figure 15*).

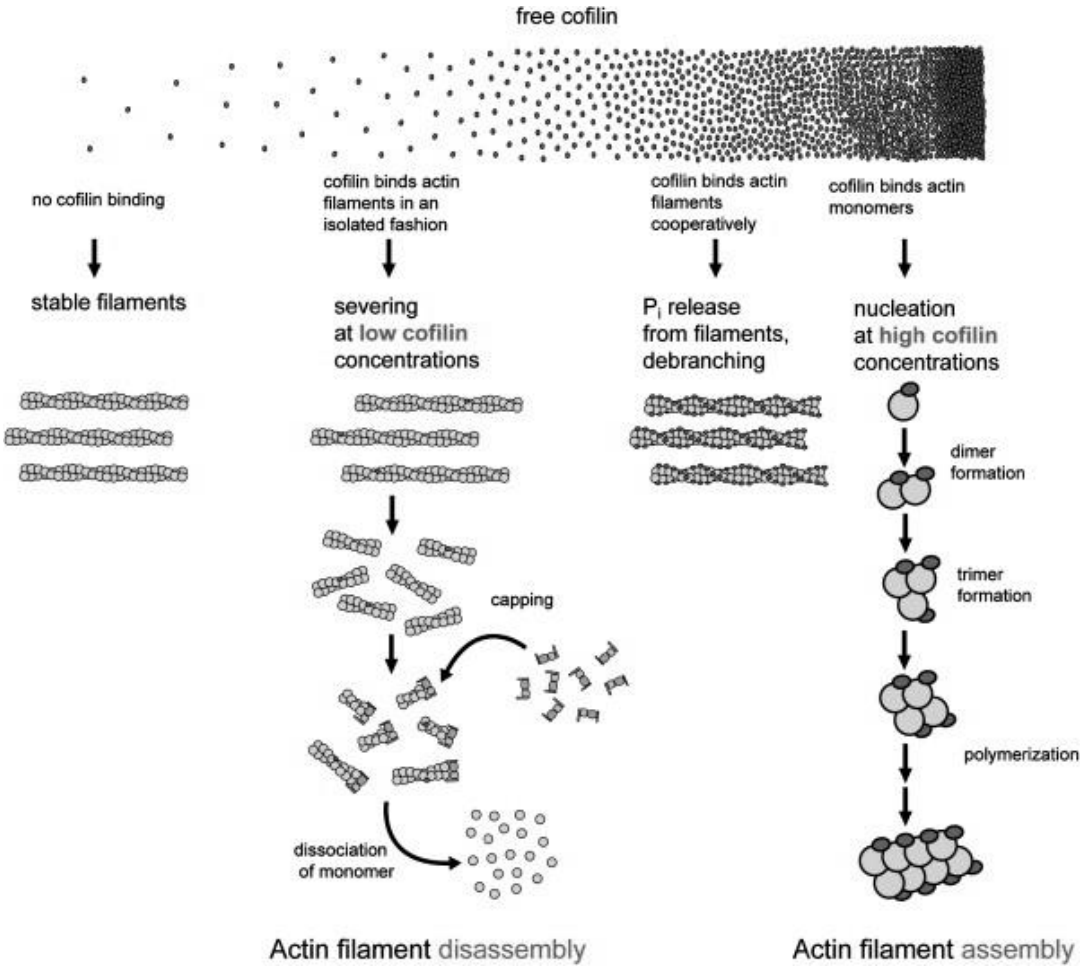

Figure 15: The activity of cofilin depends on its local concentration in the cell. Very low concentrations of cofilin (blue ovals) do not bind actin filaments (green strands). At an optimal low concentration of cofilin, single cofilin molecules bind and sever actin filaments. Capping of severed filament barbed ends promotes dissociation of actin monomers (green circles) from pointed ends, leading to filament disassembly. Higher concentrations of cofilin bind cooperatively to actin filaments and promote the release of inorganic phosphate (Pi) but do not sever these filaments. Very high concentrations of cofilin bind actin monomers and stimulate nucleation, leading to actin filament assembly (From (Andrianantoandro & Pollard, 2006)).

Next to concentration dependency, cofilin activity also varies between the different isoforms. Cofilin-2 promotes actin filament assembly rather than disassembly, compared to Cofilin-1 and ADF. ADF, with its weaker nucleating activity and promotion of pH-dependent actin disassembly, is the most potent of the three isoforms with regard to its effect on actin turnover (Chen *et al.*, 2004; Nakashima *et al.*, 2005; Vartiainen *et al.*, 2002; Yeoh *et al.*, 2002).

Multiple cofilin regulation mechanisms have been identified, including phosphorylation at Ser3, interaction with polyphosphoinositides, pH-dependent effects and synergistic or competitive interactions of cofilin with other actin binding proteins. Ser3 phosphorylation of cofilin, a hallmark of cofilin inactivation, is regulated by both kinases and phosphatases. Two families of ubiquitous kinases with related catalytic domains have been reported to phosphorylate cofilin at Ser3: the LIM (Lin-11, Isl1 and Mec-3) kinases (LIMK) and testicular kinases (TESK) (Scott & Olson, 2007b; Toshima *et al.*, 2001). Dephosphorylation and consecutive activation of cofilin can be achieved via phosphatases of the Slingshot (SSH) family, the haloacid dehalogenase phosphatases chronophin (CIN) and protein phosphatase 1 and 2A (PP1 and PP2A) (Huang *et al.*, 2006; Takuma *et al.*, 1996). The relative contributions of these phosphatases and kinases on cofilin activity depend on their expression levels, tissue distribution, subcellular localization, activation pathways, and activity levels. In addition, different isoforms of both types of enzymes have been described, and isoform-specific differences in expression levels have been observed (Acevedo *et al.*, 2006; Foletta *et al.*, 2004). LIMK has two isoforms, namely LIMK-1 and -2, SSH has three isoforms, SSH-1, -2 and -3, TESK has two isoforms, TESK-1 and -2 and CIN only has one isoform (Van Troys *et al.*, 2008). Many of the data on kinase/phosphatase functionality that is currently available, however, has been derived from studies using only one isoform, and contradictory results have sometimes been obtained (Huang *et al.*, 2006; Ono, 2007; Scott & Olson, 2007b; Van Troys *et al.*, 2008; Wang *et al.*, 2007).

Downstream Rho GTPase signaling converges to these cofilin phosphatases and kinases to regulate control of cofilin activity. PAKs play a central role in this respect. PAK activity has been associated with cofilin phosphorylation and therefore inactivation, mainly because LIMK isoforms are important downstream substrates of PAK that can directly phosphorylate cofilin (Kobayashi *et al.*, 2006; Li *et al.*, 2006; Scott & Olson, 2007a; Zoudilova *et al.*, 2007). PAK1 and PAK4 can activate LIMK-1, but not LIMK-2, leading to cofilin Ser3 phosphorylation and therefore inactivation. Cofilin inactivation via LIMK through Rac1 via PAK1 or PAK2 signaling has been described (Zhou *et al.*, 2013), as well as cofilin inactivation via LIMK through RhoA/ROCK or through Cdc42/MRCKα signaling (Misra *et al.*, 2005). Currently, SSH1L (long isoform) is the only phosphatase known to dephosphorylate and inactivate LIMKs (Soosairajah *et al.*, 2005). As SSH1L also directly dephosphorylates cofilin, it is probably involved in a positive feedback loop regulating cofilin activation. Interestingly, next to causing cofilin

inactivation through LIMK1, PAK4 also negatively regulates SSH1L activity in different cell types, further contributing to cofilin inactivation (Soosairajah *et al.*, 2005). Contrastingly, other data in other cell types suggest a positive effect of Rac1 signaling on SSH activation (Kligys *et al.*, 2007).

More recently, increasing evidence indicates that PAK activity may not only lead to cofilin inactivation (phosphorylation), but may also signal to several of the cofilin phosphatases like PP2A, CIN (chronophin) and/or the SSH (slingshot) family and thereby activate cofilin (Ke *et al.*, 2004; Oleinik *et al.*, 2010). Most likely, cell type-specific or environmental factors may influence the outcome of PAK activation on cofilin activity (Davidson & Haslam, 1994; Okada *et al.*, 1996; Samstag *et al.*, 1996; Samstag *et al.*, 1994; Takuma *et al.*, 1996). These different cofilin regulation mechanisms are summarized in *Figure 16* (from (Van Troys *et al.*, 2008).

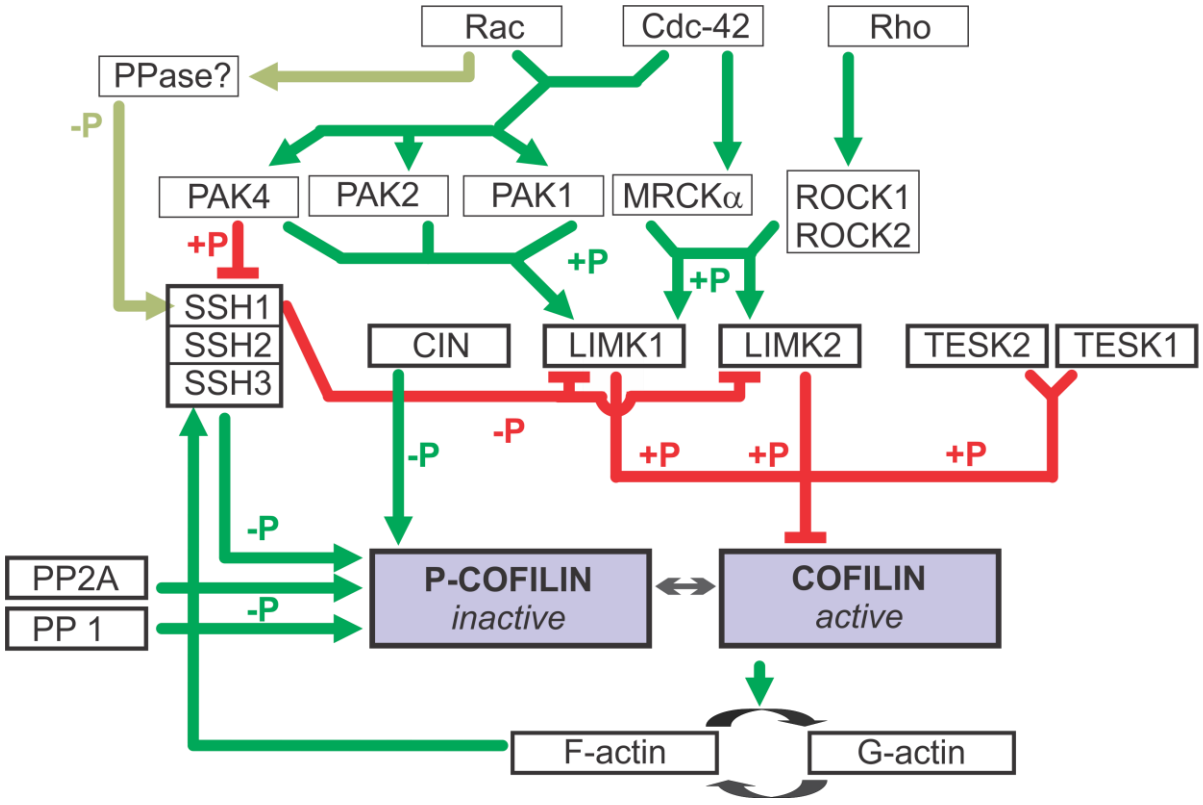

Figure 16: The cofilin regulatory system: signals controlling cofilin phosphorylation and dephosphorylation. Phosphorylated ADF/cofilin (P-cofilin) is inactive and no longer binds or severs F-actin. Rho GTPases have a prominent role in controlling kinases and phosphatases that affect cofiline activity. Red line = inhibitory effect; dark green line = activating effect; light green line = activation with no/unknown effect; +P = phosphorylation; –P = dephosphorylation (Adapted from (Van Troys *et al.*, 2008)).

4. Alphaherpesvirus interactions with the actin cytoskeleton and Rho GTPases during virus entry and egress

Because Rho GTPase signaling and actin are involved in a plethora of cellular processes, it is perhaps not surprising that several viral gene products have evolved to interfere with and modulate these cellular factors during several steps of their replication cycle. During entry and egress, viral interactions with actin and Rho GTPases may allow or facilitate viral particle passage across the cortical actin barrier and may trigger rearrangements of actin to conformations that promote virus infection and spread. This section will focus on interactions between the alphaherpesvirus subfamily members and Rho GTPase signaling/actin during virus entry and egress.

4.1 Entry/Transport to the nucleus

The first report suggesting the involvement of Rho GTPases in alphaherpesvirus entry showed that HSV-1 entry in Madin-Darby canine kidney II (MDCKII) cells was associated with activation of Cdc42 and Rac1, and that overexpression of genetically engineered Rho GTPases with altered activity influenced HSV-1 infectivity (Hoppe *et al.*, 2006). This is in line with findings that PRV induces Cdc42-dependent signaling upon infection in sensory neurons (De Regge *et al.*, 2006).

Interestingly, herpesviruses and several other viruses, including retroviruses, papillomaviruses, poxviruses, dengue virus and vesicular stomatitis virus (VSV), engage Rho GTPase signaling even before entering the cell. In order to reach the cell body, virions attach to filopodia-like structures, leading to unidirectional movements toward the cell, followed by viral entry. This process, called viral surfing, was first described by Lehmann and colleagues for murine leukemia virus (MLV), avian leucosis virus, human immunodeficiency virus (HIV) and VSV in HEK-293-T cells and relies on activation of myosin II and actin rearrangements (Lehmann *et al.*, 2005; Mercer & Helenius, 2008; Schelhaas *et al.*, 2008). HSV-1 and dengue virus not only travel along filopodia during entry, but may also actively induce filopodia formation at this stage through activation of Cdc42 and Rac1 signaling (Oh *et al.*, 2010; Zamudio-Meza *et al.*, 2009).

Depending on the cell type and virus, alphaherpesviruses may directly fuse their envelope with the plasma membrane (Barocchi *et al.*, 2005). During fusion, the cortical actin layer forms a barrier that the virus needs to overcome to access the cytoplasm. Local actin rearrangements might be required to allow passage of the capsids through the cortical actin meshwork underneath the plasma membrane. Although direct fusion with the plasma membrane has been shown to lead to a productive infection, it is unclear whether capsid access to the cytoplasm requires (local) disassembly of cortical

actin filaments. Perhaps pointing in this direction, early F-actin depolymerization was seen in HSV-1, coinciding with increased viral penetration (Zheng *et al.*, 2014). Similarly, electron microscopy experiments indicated local cortical F-actin disassembly at the sites of HSV-1 entry (Maurer *et al.*, 2008). HSV-1-induced F-actin depolymerization was found to rely on activation of cofilin, a central mediator in actin dynamics (Zheng *et al.*, 2014). Other viruses that enter host cells via direct fusion at the plasma membrane, like HIV, have also been reported to (locally) disrupt the cortical F-actin layer to allow access of incoming virus particles to the cytoplasm through activation of cofilin (Campbell et al., 2004; Yoder et al., 2008).

In particular cell types, alphaherpesviruses entry can rely on cellular endocytic processes for transport across the cell membrane and the cortical actin layer. Endocytic virus uptake routes often involve Rho GTPase signaling, seen their involvement in several different endocytic uptake processes (Marsh & Helenius, 2006; Mercer *et al.*, 2010; Schelhaas, 2010; Sieczkarski & Whittaker, 2002). HSV-1 follows a phagocytosis-like uptake route in corneal fibroblasts (CF) and nectin1-overexpressing Chinese hamster ovary (CHO) cells, during which brief Cdc42 activation is followed by sustained RhoA activation (Clement *et al.*, 2006). In line with the HSV-1 results, EHV-1 entry into CHO-K1 cells occurs via an endocytic or phagocytic mechanism that depends on ROCK activation (Frampton *et al.*, 2007).

Manipulation of Rho GTPase signaling, inducing actin rearrangements, thus seems a common aspect during viral entry, although the specific Rho GTPase signaling axes involved are highly variable, depending on the entry route used by the virus. Evidence is accumulating that entry routes not only differ between different viruses but also between different target cells. The specific impact of Rho GTPase signaling and actin modulations and their sometimes conflicting roles in entry processes merit further attention in the future. More insights into the different entry routes and the role of particular actin-controlling signaling pathways in different cell types may lead to the development of antiviral drugs that block viral entry in pathogenically important target cells.

4.2 Egress

During egress, newly formed virus particles again have to overcome the cortical actin layer and the plasma membrane in order to exit the cell (Delorme-Axford & Coyne, 2011; Taylor *et al.*, 2011). Depletion of the F-actin cortex has been described for HSV-1 egress in Vero cells (Mingo *et al.*, 2012) and EHV-1 egress in primary murine neurons (Slonska *et al.*, 2014). The viral factor that is responsible for this depletion has not been investigated yet, but could rely on US3 (see **Chapter 5**).

During egress, viruses manipulate the actin cytoskeleton to assist in viral spread to neighboring cells. As indicated higher (see **2.2.5 Actin rearrangements**), alphaherpesviruses induce actin

rearrangements including disassembly of actin stress fibers and/or the formation of cellular projections, associated with increased viral spread in cell cultures. These actin rearrangements are induced by the conserved US3 protein kinase for PRV, HSV-2, BHV1- and -5, and MDV (Calton *et al.*, 2004; Favoreel *et al.*, 2005; Murata *et al.*, 2000; Schumacher *et al.*, 2005; Van Minnebruggen *et al.*, 2003). Newly formed progeny viral particles have been reported to migrate inside these protrusions to neighboring cells, allowing intercellular virus transmission, even in the presence of virus-neutralizing antibodies (Favoreel *et al.*, 2005). Treatment with the actin-stabilizing drug jasplakinolide as well as treatment with actin depolymerizing drugs cytochalasin D or microtubule polymerization inhibitor nocodazole abrogates the formation of these protrusions, and negatively influences viral spread (Favoreel *et al.*, 2005; Schumacher *et al.*, 2005). Interestingly, and in line with the requirement for intact microtubules, HSV-1 US3 has recently been reported to stabilize microtubules, which also contributes to virus spread (Naghavi *et al.*, 2013). US3 also induces stress fiber breakdown in several alphaherpesviruses, such as HSV-2, MDV, PRV, BHV-1 and BHV-5 (Brzozowska *et al.*, 2010; Favoreel *et al.*, 2005; Ladelfa *et al.*, 2011; Murata *et al.*, 2000; Schumacher *et al.*, 2008; Schumacher *et al.*, 2005; Van den Broeke *et al.*, 2009a; Van den Broeke *et al.*, 2009b; Van Minnebruggen *et al.*, 2003).

A first suggestion that US3 may interfere with Rho GTPase signaling came from Murata and coworkers for HSV-2 (Murata *et al.*, 2000), and has been unraveled in more detail for PRV US3. For PRV US3, group I p21-activated kinases (PAKs), downstream effectors of Cdc42 and Rac1 Rho GTPases (see **3.5 PAK**) were identified as central downstream phosphorylation targets. PRV US3 was found to phosphorylate and activate both PAK1 and PAK2 (Van den Broeke *et al.*, 2009b). Experiments using PAK1 and PAK2 knockout (KO) mouse embryonic fibroblast (MEF) cells demonstrated that US3-induced stress fiber disassembly depends on PAK2 and US3-induced cell projection formation on PAK1 (Van den Broeke *et al.*, 2009b). In line with the importance of cytoskeletal rearrangements for viral spread, infection of these PAK KO cells was associated with a reduced viral spread (Van den Broeke *et al.*, 2009b). In addition, treatment of US3null PRV-infected cells with Y27632, an inhibitor of the RhoA downstream kinase ROCK (see **3.4.1 RhoA pathway**), induced US3-like cytoskeletal rearrangements and partly compensated for the decreased intercellular spread of US3null PRV (Favoreel *et al.*, 2005). This suggests that, besides activation of the Cdc42/Rac1/PAK signaling axis, US3 may also suppress the RhoA/ROCK pathway, although such putative effect of US3 on RhoA/ROCK signaling had not yet been investigated (see **Chapter 3**).

Actin rearrangements by the US3 protein kinase typically depend on its kinase activity in HSV-2, PRV and BHV-1 US3, while it is redundant for MDV US3 and only required for induction of cell projections in BHV-5 (Brzozowska *et al.*, 2010; Finnen *et al.*, 2010; Ladelfa *et al.*, 2011; Murata *et al.*, 2000; Schumacher *et al.*, 2008; Van den Broeke *et al.*, 2009a). Intact and kinase-inactive US3 of MDV display

equal abilities to induce actin rearrangements, although these are less dramatic compared with those of most other alphaherpesviruses, as MDV US3 induces actin stress fiber disassembly but has not been reported to induce cell projections (Schumacher *et al.*, 2008). Also, unlike for other alphaherpesviruses, MDV US3-induced stress fiber disassembly is temporary, and stress fibers reassemble 24-48 hours after disruption (Schumacher *et al.*, 2008; Schumacher *et al.*, 2005). This points towards a more robust effect of US3 on the actin cytoskeleton when induced by catalytic activity, underscoring the importance of downstream phosphorylation targets (Van den Broeke *et al.*, 2009a). Similarly, interference of MDV with Rho GTPase signaling is different compared with other alphaherpesviruses as, unlike for PRV, the Rac1/PAK signaling axis appears inhibitory to MDV spread, whereas the RhoA/ROCK signaling axis promotes spread (Richerioux *et al.*, 2012).

Interestingly, actin-based projection formation enabling the virus to reach and infect neighboring cells is documented for more viruses than alphaherpesviruses. Vaccinia virus induces US3-like actin rearrangements through its viral protein F11, contributing to viral spread (Morales *et al.*, 2008; Valderrama *et al.*, 2006). Similar to PRV US3 (see **Chapter 3**), F11 induces these actin rearrangements through interference with RhoA signaling (Handa *et al.*, 2013; Valderrama *et al.*, 2006). Furthermore, HIV induces tunneling nanotubes (TNTs), also referred as viral filopodia (VF), allowing viral spread (Aggarwal *et al.*, 2012; Kimura *et al.*, 2013; Sowinski *et al.*, 2008). HIV Nef was found to be responsible for the formation of these TNTs in infected T lymphocytes and dendritic cells (Aggarwal *et al.*, 2012; Eugenin *et al.*, 2009; Kimura *et al.*, 2013; Nikolic *et al.*, 2011; Nobile *et al.*, 2010; Xu *et al.*, 2009). Nef shows other functional similarities with US3, such as activation of PAKs (Kouwenhoven *et al.*, 2013; Stolp *et al.*, 2010), interference with RhoA activity (Lu *et al.*, 2008; Tan *et al.*, 2013) (see **Chapter 3**) and F-actin disassembly facilitating viral entry (see **Chapter 5**) through cofilin activation (see **Chapter 4**) (Campbell *et al.*, 2004; Yoder *et al.*, 2008). So, although Nef and F11 are no protein kinases like US3 and do not share amino acid homology, they appear to show considerable functional homology. These proteins will be further discussed later (see **Chapter 6**).

Eventually, Rho GTPase signaling often leads to the activation of common downstream proteins. It will therefore be important to obtain clear insights into the downstream targets of Rho signaling, as they may be valuable to identify putative common signaling nodes. Similar to the development of viral entry inhibitors, leading from a better knowledge of different viral entry routes, identifying common downstream targets of viral interference with Rho signaling could allow the development of antiviral drugs that might block viral hijacking of several cellular signaling cascades.

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Chapter 2

Aims

Chapter 2: Aims

Several viruses have developed sophisticated strategies to interfere with the actin cytoskeleton of their host cell during particular steps of their replication cycle in order to improve their replication and spread. For alphaherpesviruses, particularly pseudorabies virus (PRV), the conserved viral US3 serine/threonine protein kinase causes dramatic changes in the actin cytoskeleton, consisting of actin stress fiber disassembly and the formation of actin-based cell projections, that are associated with enhanced viral cell-to-cell spread (Favoreel et al., 2005). Other proteins of other virus families, such as HIV Nef (Haller & Fackler, 2008; Rudnicka & Schwartz, 2009) or vaccinia virus F11 (Morales et al., 2008) induce similar actin rearrangements.

The general aim of the current thesis was to obtain novel insights in the mechanisms and biological consequences of PRV US3-mediated actin rearrangements, via three specific studies.

It has been previously reported by our research group that US3 phosphorylates and thereby activates p21 activated kinases (PAKs), downstream effectors of Rac1/Cdc42 signaling, and that this plays a central role in causing PRV US3-induced actin rearrangements (Van den Broeke *et al.*, 2009). Rac1 and Cdc42 signaling axes typically counteract RhoA signaling and vice versa (Van den Broeke *et al.*, 2014). Other viruses, like vaccinia virus, have been reported to inhibit RhoA signaling to trigger actin rearrangements very similar to the ones observed with PRV US3 (Valderrama *et al.*, 2006). Hence, in **Chapter 3**, we investigated whether PRV US3 also affects the RhoA signaling axis to mediate its actin rearrangements.

Little is known on the effect that PRV US3 generates on proteins that directly affect actin polymerization and how this may contribute to the US3-induced actin rearrangements. Cofilin is a central regulator of actin polymerization. As a second specific aim, we investigated whether PRV (US3) affects cofilin activation and whether this contributes to the US3-mediated effects on the actin cytoskeleton (**Chapter 4**).

Although it is well documented that the actin rearrangements induced by US3 are associated with enhanced viral spread from one cell to another in cell culture, it has not yet been investigated whether these may also be involved in virus entry in a host cell. During entry, other viruses like HIV have been reported to induce disassembly of F-actin to dissolve the cortical actin barrier to gain access to the cytoplasm (Delorme-Axford & Coyne, 2011). US3-induced rearrangements of the actin cytoskeleton are known not to affect total actin protein levels (Van Minnebruggen *et al.*, 2003). We aimed at investigating whether US3 results in an overall disassembly of F-actin and whether US3 may contribute to certain aspects of virus entry in host cells (**Chapter 5**).

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Chapter 3

Pseudorabies virus US3 triggers RhoA phosphorylation to reorganize the actin cytoskeleton

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Chapter 3: Pseudorabies virus US3 triggers RhoA phosphorylation to reorganize the actin cytoskeleton

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Abstract

The conserved alphaherpesviral serine/threonine kinase US3 causes dramatic changes in the actin cytoskeleton, consisting of actin stress fiber breakdown and protrusion formation, associated with increased viral spread. In this report, we show that US3 expression leads to RhoA phosphorylation at serine 188 (S188), one of the hallmarks of suppressed RhoA signaling, and that expression of a non-phosphorylatable RhoA variant interferes with the ability of US3 to induce actin rearrangements. Furthermore, inhibition of cellular protein kinase A (PKA) abrogates the ability of US3 to induce S188 RhoA phosphorylation, pointing to a role for PKA in US3-induced RhoA phosphorylation. Hence, the US3 kinase leads to PKA-dependent S188 RhoA phosphorylation, which contributes to US3-mediated actin rearrangements. Our data suggest that US3 efficiently usurps the antagonistic RhoA and Cdc42/Rac1/PAK signaling branches to rearrange the actin cytoskeleton.

Introduction

Alphaherpesviruses represent the largest subfamily of the herpesvirus family, comprising closely related pathogens of man and animal, including herpes simplex virus (HSV) and varicella zoster virus (VZV) in man.

The pseudorabies virus (PRV) is a porcine alphaherpesvirus and is often used to study general aspects of alphaherpesvirus biology (Pomeranz *et al.*, 2005). Infection with PRV can lead to dramatic changes in the actin cytoskeleton of the host cell, consisting of actin stress fiber breakdown and protrusion formation (Favoreel *et al.*, 2005). These actin rearrangements are associated with increased viral cell-to-cell spread and depend on the kinase activity of the viral serine/threonine kinase US3 (Favoreel *et al.*, 2005; Van den Broeke *et al.*, 2009b). US3 is conserved within the alphaherpesvirus subfamily, and comparable US3-induced cytoskeletal changes have been described for several other alphaherpesviruses, including HSV-2 and bovine herpesvirus-1 and -5 (BHV-1/-5) (Brzozowska *et al.*, 2010; Finnen *et al.*, 2010; Ladelfa *et al.*, 2011).

Rearrangements of the actin cytoskeleton are generally regulated by Rho GTPase signaling pathways (Hall, 1998). RhoA, Rac1 and Cdc42 are the best characterized Rho GTPases and regulate many actin-driven processes. Activation of RhoA generally leads to actin stress fiber formation, while Rac1 and Cdc42 are typically associated with the formation of different actin-based protrusions, most notably filopodia (Cdc42) and lamellipodia (Rac1) (Hall, 1998). Rac1/Cdc42 signaling typically counteracts RhoA signaling and vice versa (Van den Broeke *et al.*, 2014).

We have demonstrated previously that PRV US3 influences the Rac1/Cdc42 signaling branch, via activation of p21 activated kinases (PAKs), downstream effectors of Rac1 and Cdc42. In particular, US3 phosphorylates and thereby activates both PAK1 and PAK2, contributing to stress fiber breakdown and protrusion formation (Van den Broeke *et al.*, 2009b).

It is currently not know whether US3 may also affect the opposing RhoA signaling branch. RhoA GTPase activity is regulated via several mechanisms, including phosphorylation at serine residue 188 (S188). S188 phosphorylation of RhoA suppresses RhoA signaling via RhoGDI–mediated relocalization of (GTP-bound) RhoA from the cellular membrane to the cytoplasm, thereby keeping RhoA away from its site of activity and preventing downstream signaling (Lang *et al.*, 1996; Rolli-Derkinderen *et al.*, 2005). Cyclic AMP (cAMP)-dependent protein kinase A (PKA) has been reported to phosphorylate RhoA at position S188 in several cell types (Dong *et al.*, 1998; Ellerbroek *et al.*, 2003; Jones & Palmer, 2012; Lang *et al.*, 1996; Lapetina *et al.*, 1989; Quilliam *et al.*, 1991; Tkachenko *et al.*, 2011). This is of particular interest since the US3 protein kinase homologs of alphaherpesviruses like HSV-1 and VZV have been

shown (i) to functionally overlap with cellular PKA with regard to cellular substrates and/or (ii) trigger activation of cellular PKA during infection (Benetti *et al.*, 2003; Benetti & Roizman, 2004; Erazo & Kinchington, 2010; Munger & Roizman, 2001; Ogg *et al.*, 2004).

In the current report, we investigated whether expression of the US3 protein of the alphaherpesvirus PRV affects RhoA phosphorylation at S188 and, if so, whether this contributes to the US3-mediated effects on the actin cytoskeleton. We report that expression of US3 leads to increased RhoA S188 phosphorylation, that overexpression of a non-phosphorylatable S188A RhoA mutant interferes with the US3-mediated actin rearrangements, and that inhibition of PKA interferes with the ability of US3 to trigger RhoA phosphorylation.

Materials and methods

- Cell cultures and viruses

ST (Swine testicle) cells were cultured according to published literature (Geenen *et al.*, 2005). WT NIA3 PRV and isogenic US3null NIA3 PRV viruses were described before (Baskerville, 1973; de Wind *et al.*, 1990).

- Plasmids

HA-tagged WT and S188A RhoA plasmids were described earlier and were kindly provided by Gervaise Loirand (Inserm, Nantes, France) (Rolli-Derkinderen *et al.*, 2005). The plasmids encoding PRV US3 (pKG1) and the kinase-inactive US3 mutant (pHF61) (point mutation in ATP-binding site, K136G) were described before (Deruelle *et al.*, 2007; Geenen *et al.*, 2005). The empty control plasmid pcDNA 3.1 was bought from Invitrogen.

- Reagents

Primary antibodies included mouse anti RhoA (Santa Cruz, sc-418, diluted 1/250 for Western blots), rabbit anti P-RhoA (Abcam, ab-41435, diluted 1/1000 for Western blot), mouse anti-US3 (kindly provided by LeighAnne Olsen and Lynn Enquist (Princeton University), diluted 1/50 for immunofluorescence and 1/100 for Western blots), monoclonal mouse anti-PRV gE 13D12 (described earlier by Nauwynck and Pensaert (Nauwynck & Pensaert, 1995), diluted 1/100 for Western blot), rabbit anti-HA tag ((Sigma-Aldrich, diluted 1/100 for immunofluorescence and 1/200 for Western blot), anti-phospho PKA substrates (Cell Signaling, 6921, diluted 1/1000 for Western blots) and rabbit anti-tubulin (Abcam, ab-18251, diluted 1/500 for Western blot). Secondary antibodies included goat anti-rabbit Alexa Fluor 647 (Invitrogen, diluted 1/200 for immunofluorescence) and goat anti-mouse TexasRed (Invitrogen, diluted 1/100 for immunofluorescence) and we used the fluorescent probe phalloidin-FITC for actin staining (diluted 1/200). Horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1/3000) and goat anti-mouse antibody (1/2000) for Western blot detection were purchased from Dako Cytomation. As a nuclear counterstaining in immunofluorescence assays, Hoechst 33342 (Invitrogen, diluted 1/200) was used. For immunofluorescence, antibodies were always diluted in PBS, while for Western blot assays antibodies were diluted in PBS with 5% Tween 20 (Sigma Aldrich) and 5% non-fat, dry milk (Nestlé). Protein Kinase A inhibitor PKI (fragment 14-22, myristoylated trifluoroacetate salt) was bought from Sigma-Aldrich.

- Transfection and infection assays

Transfection was performed using the JetPrime transfection kit (PolyPlus, Westburg) according to the manufacturer's instructions. One day prior to transfection, ST cells were seeded at a density of 75,000 cells/ml. For infection assays, ST cells were seeded at 150,000 cells/ml and two days later inoculated at a MOI (multiplicity of infection) of 10 or 30 in 1 ml medium (MEM complemented with 10% FCS, 1% glutamin, 1% P/S, 1% NaPy, 0.5% gentamycin).

- Western blotting

6h post-inoculation (hpi) or 24 h post-transfection (p.t.), cells were washed in PBS and collected in lysis buffer consisting of 50 mM Tris-HCL, 5 mM EDTA, 150 mM NaCl, 1% NP40, 1 tablet of protease inhibitors (Roche, EDTA-free, complete mini) per 7 ml of lysis buffer and phosphatase inhibitors (2 mM NaVO3, 5 mM NaF). After 1 h incubation, the lysate was centrifuged for 5 min at 13,000 x *g* and the supernatant was collected. After SDS-PAGE, Western blotting and detection of the bands using ECL Western Blotting substrate (Pierce) using the ChemiDoc (Biorad), band intensities were quantified using the "Analyse Gel" option in ImageJ.

- Immunofluorescence

For immunofluorescence experiments, cells were seeded on glass coverslips. Either 6 hpi or 24 h p.t., cells were washed in PBS, fixed for 10 min in a 3% paraformaldehyde/PBS solution, washed, permeabilized for 2 min with 0.2% Triton X-100, washed again, incubated with primary antibody for 1 h at 37°C, washed two times and incubated at 37°C for 1 h with fluorescently labeled secondary antibodies. Finally, the cells were incubated for 10 min with a 1/200 Hoechst 33342 (Invitrogen) dilution in PBS and washed two more times, before mounting the coverslips in glycerin-DABCO (1,4-diazabicyclo[2.2.2]octane). Samples were imaged using a Leica TCS SPE confocal microscope (Leica DM RBE, Leica Microsystems, GmbH, Heidelberg, Germany).

- In vitro kinase assay

This protocol was performed according to previous published literature (Lang *et al.*, 1996; Van den Broeke *et al.*, 2009b). In short, 1 µg recombinant RhoA (Sigma-Aldrich) was added to 1 µg recombinant GST-tagged US3 or 2500 units of the cAMP-dependent Protein Kinase (PKA) catalytic subunit (New England BioLabs), in kinase buffer (50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 20 µM ATP), each reaction containing a total volume of 20 µl. After 30 min incubation at 30°C, samples were subsequently used for SDS-PAGE/Western blotting.

Results and discussion

- PRV infection leads to US3-dependent RhoA S188 phosphorylation

To investigate whether PRV infection leads to RhoA S188 phosphorylation, and, if so, whether this depends on US3 expression, porcine ST cells were mock-inoculated or inoculated with wild type (WT) PRV or isogenic US3null PRV NIA3 (containing a translational stop codon in US3) (Baskerville, 1973; de Wind *et al.*, 1990). At 6 hpi, cells were lysed and subjected to SDS-PAGE and Western blotting to detect levels of phospho (P)-S188-RhoA, total RhoA, viral proteins US3 and gE (13D12 (Nauwynck & Pensaert, 1995)) and loading control tubulin (*Figure 1A*). P-S188-RhoA and RhoA band intensities were measured with the "Analyze gels" option in ImageJ, and P-S188-RhoA ratios were normalized to mock levels (*Figure 1B*). Figure 1A&B show that WT PRV infection triggers a substantial increase in RhoA phosphorylation, compared to mock-infected or US3null PRV infected cells. A time course assay showed that RhoA phosphorylation can be detected from early in infection, gradually increasing up to 6 hpi and reaching a plateau from then onwards (*Supplementary Figure 1*). Hence, PRV infection induces US3-mediated RhoA S188 phosphorylation. In addition, transfection of a WT US3-encoding eukaryotic expression plasmid (Geenen *et al.*, 2005) in ST cells was sufficient to trigger S188 RhoA phosphorylation, while transfection of kinase-inactive US3 (K136G mutation in the ATP-binding site) (Deruelle *et al.*, 2007; Van den Broeke *et al.*, 2009a) did not (*Figure 1C&D*). Concluding, expression of US3 leads to S188 RhoA phosphorylation in infected and transfected ST cells.

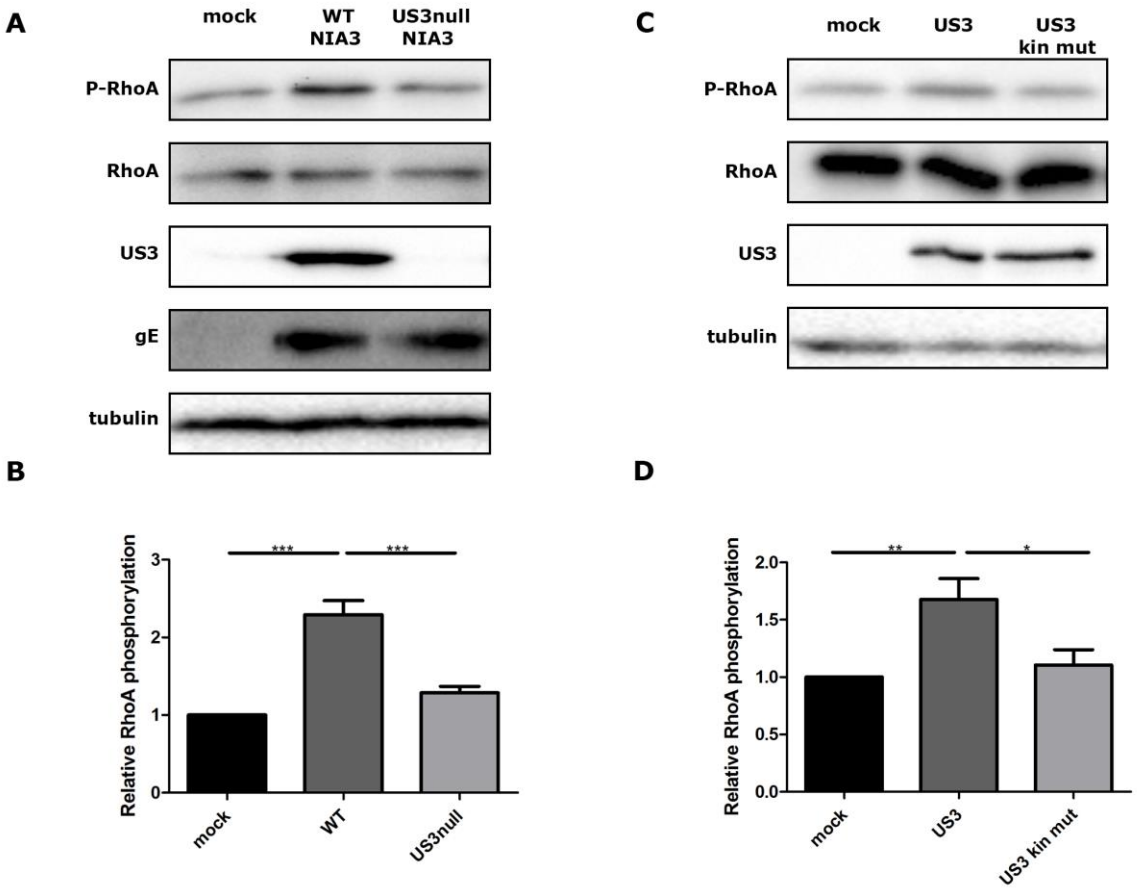
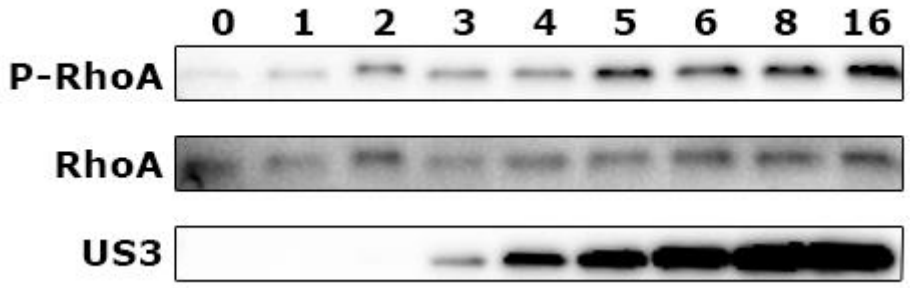


Fig. 1: Expression of US3 during infection or transfection triggers RhoA-S188 phosphorylation. (A-B) ST cells were mock-inoculated or inoculated with WT PRV (strain NIA3) or isogenic US3null PRV. At 6 hpi, cell lysates were subjected to Western blotting to detect P-S188-RhoA, total RhoA, US3, gE or tubulin (A). (B) shows relative RhoA phosphorylation levels (P-S188-RhoA/RhoA ratio with mock infection set to 1) from three independent repeats. Data represent means + standard errors of the means, with *** = $P < 0.001$ (determined by one-way ANOVA and Tukey post test). (C-D) ST cells were mock-transfected or transfected with a eukaryotic expression vector encoding PRV US3 or kinase-inactive PRV US3 (comprising a point mutation in the predicted ATP-binding site, K136G). At 24 h post-transfection, cell lysates were subjected to Western blotting to detect total P-S188-RhoA, total RhoA, US3, and tubulin expression levels (C). (D) shows relative RhoA phosphorylation levels (P-S188-RhoA/RhoA ratio with mock transfection set to 1) from three independent repeats. Data represent means + standard errors of the means, with * = $P < 0.05$ and ** = $P < 0.01$ (determined by one-way ANOVA and Tukey post test). Antibody panels in A and C were obtained from separate gels, but derive from the same sample. All gels have all been run with the same amount of sample, blotted, incubated and detected together in parallel.



Suppl. Fig. 1: Kinetic time course of RhoA phosphorylation during PRV infection. ST cells were mock-inoculated (0 hpi) or inoculated with WT PRV (strain NIA3). At 1, 2, 3, 4, 5, 6, 8 and 16 hpi, cell lysates were collected and subjected to Western blotting to detect levels of P-S188-RhoA, total RhoA, and US3. Every antibody panel was obtained from separate gels, but derives from the same sample. All gels have all been run with the same amount of sample, blotted, incubated and detected together in parallel.

- Expression of a non-phosphorylatable S188A-RhoA variant interferes with US3-mediated actin rearrangements

If RhoA S188 phosphorylation is important for US3-induced actin rearrangements, one would expect that overexpression of wild type RhoA may interfere with US3-induced actin rearrangements, since accumulated levels of RhoA may exceed the ability of US3 to inactivate it. Overexpression of a non-phosphorylatable S188A-RhoA mutant, which cannot be inactivated through phosphorylation, would then be expected to interfere to an even larger extent with US3-induced actin rearrangements. To test this, ST cells were transfected with empty vector pcDNA3.1 alone or cotransfected with US3 together with pcDNA3.1 or either HA-tagged WT RhoA or S188A-mutated RhoA (as described in (Rolli-Derkinderen *et al.*, 2005)). At 24 h post-transfection, cells were fixed using 3% paraformaldehyde, permeabilized with Triton X-100 and fluorescently labeled with antibodies against US3, the HA-tag and a probe against actin (phalloidin-FITC). Finally, cells were counterstained for nuclei using Hoechst 33342 (*Figure 2A&B*).

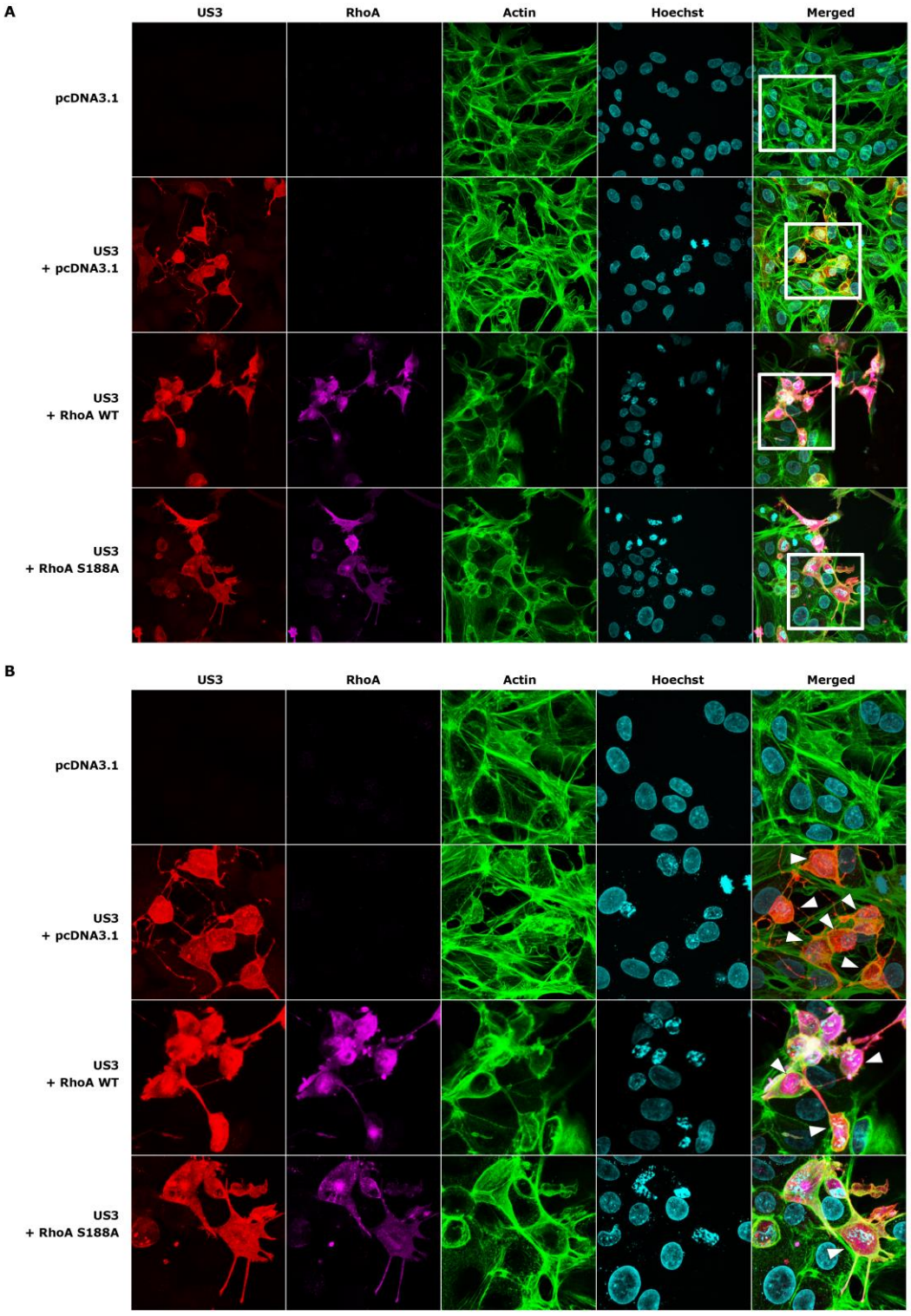


Fig. 2: Overexpression of non-phosphorylatable S188A-RhoA and, to a lesser extent, WT RhoA interferes with the ability of US3 to cause actin rearrangements. (A) ST cells were transfected with empty vector pcDNA3.1 alone or cotransfected with US3 together with pcDNA3.1 or either HA-tagged WT RhoA or S188A RhoA. At 24 h post-transfection, cells were fixed and stained for US3 (Texas Red, red), HA-RhoA (Alexa Fluor 647, purple), actin (phalloidin-FITC, green) and nuclei (Hoechst 33342, cyan). White squares in 2A indicate zoomed areas in 2B. (B) Cells marked by white arrowheads demonstrate cells with the US3-phenotype (cell rounding and stress fiber breakdown) that were scored positive in Figure 3A.

Figure 3A summarizes results from three independent experiments where each time 200 cotransfected cells (both high US3 and RhoA expressing cells) per condition were scored for cell rounding (actin stress fiber disassembly) and cell projection formation (white arrows on the merged views of *Figure 2B* indicate cells that were scored positive)**.** Western blot assays of total cell lysates 24 hours post-transfection detecting US3, RhoA-HA and tubulin expression indicate that US3 expression is similar in all samples (*Figure 3B*). Hence, our data shows that overexpression of RhoA interferes with the ability of US3 to trigger actin rearrangements. Importantly, co-expression of S188A RhoA with US3 caused a significantly stronger suppression of US3-mediated actin rearrangements compared to co-expression of WT RhoA with US3. Hence, non-phosphorylatable S188A RhoA more potently suppresses the ability of US3 to induce actin rearrangements, supporting a role for US3-triggered RhoA S188 phosphorylation in these actin rearrangements.

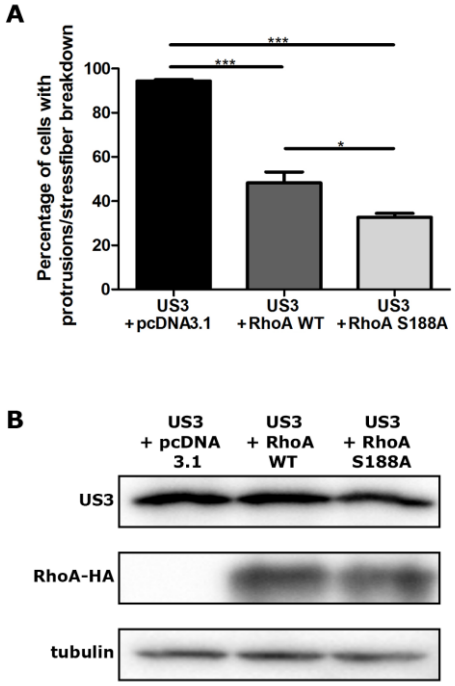


Fig. 3: Overexpression of non-phosphorylatable S188A-RhoA and, to a lesser extent, WT RhoA interferes with the ability of US3 to cause actin rearrangements. ST cells were transfected with US3 together with either empty vector pcDNA3.1, HA-tagged WT RhoA or HA-tagged S188A RhoA. At 24 h post-transfection, cells were fixed and stained for US3 and HA-RhoA. For each experiment, 200 cells expressing high and comparable levels of US3 and HA-tagged RhoA were scored for US3-mediated actin rearrangements. (A) shows the percentage of transfected cells displaying actin rearrangements representing means + standard errors of the means from three independent repeats, with * = $P < 0.05$ and *** = $P < 0.001$ (determined by one-way ANOVA and Tukey post test). (B) shows total cell lysates at 24 h post-transfection subjected to Western blotting to detect US3, HA-tagged RhoA constructs and endogenous tubulin expression levels. Antibody panels in B were obtained from separate gels, but derive from the same sample. All gels have all been run with the same amount of sample, blotted, incubated and detected together in parallel.

- US3-triggered RhoA phosphorylation occurs via PKA

To investigate whether US3 phosphorylates RhoA directly, a kinase assay was performed according to published literature (Lang *et al.*, 1996; Van den Broeke *et al.*, 2009b). In short, 1 µg recombinant RhoA (Sigma-Aldrich) was added to 1 µg recombinant GST-tagged US3 or 2500 units of the positive control PKA catalytic subunit, in kinase buffer. The used lot of GST-US3 exhibited kinase activity on the p21-activated kinase PAK1 (data not shown), in line with what we published earlier (Van den Broeke *et al.*, 2009b). After 30 min incubation at 30°C, samples were boiled in loading buffer and used for SDS-PAGE/Western blotting. While this assay confirmed that active PKA directly phosphorylates RhoA, US3 did not induce detectable RhoA phosphorylation, suggesting that US3 may not directly phosphorylate RhoA and that a cellular kinase may be involved (*Figure 4A*).

US3 of HSV-1 has been reported before to trigger PKA activation (Benetti *et al.*, 2003; Benetti & Roizman, 2004; Munger & Roizman, 2001). To investigate whether this is also the case in PRV, ST-wells were either mock-inoculated or inoculated with WT PRV or US3null PRV. At 6 hpi, cells were lysed and subjected to Western blotting to detect levels of phospho-PKA substrates, viral proteins US3 and gE and loading control tubulin (*Figure 4B*). WT PRV infection resulted in substantially increased phospho-PKA substrate signal, while this was considerably less prominent for US3null PRV infection.

Next, we examined whether PRV-mediated RhoA phosphorylation depends on PKA activity. To this end, ST cells were mock-inoculated or inoculated with WT PRV in the presence or absence of 50 µM of the cell-permeable PKA inhibitor PKI (14-22 myristoylated), added from 30 min before viral inoculation. At 6 hpi, cells were lysed and subjected to SDS-PAGE and Western blotting to detect levels of phospho (P)-S188-RhoA, total RhoA, phospho-PKA substrates, viral proteins US3 and gE and loading control tubulin (*Figure 4C&D*). Phospho-PKA substrate signal confirmed increased PKA activity during PRV infection. PKI largely suppressed this PRV-induced phospho-PKA substrate signal, confirming that this signal specifically correlates with increased PKA activity (*Figure 4C*). Interestingly, PKI abrogated PRV-induced RhoA phosphorylation, indicating that PRV-triggered RhoA phosphorylation depends on PKA activity (*Figure 4C&D*). Hence, our data indicate that US3 indirectly triggers RhoA phosphorylation, via the cellular PKA kinase.

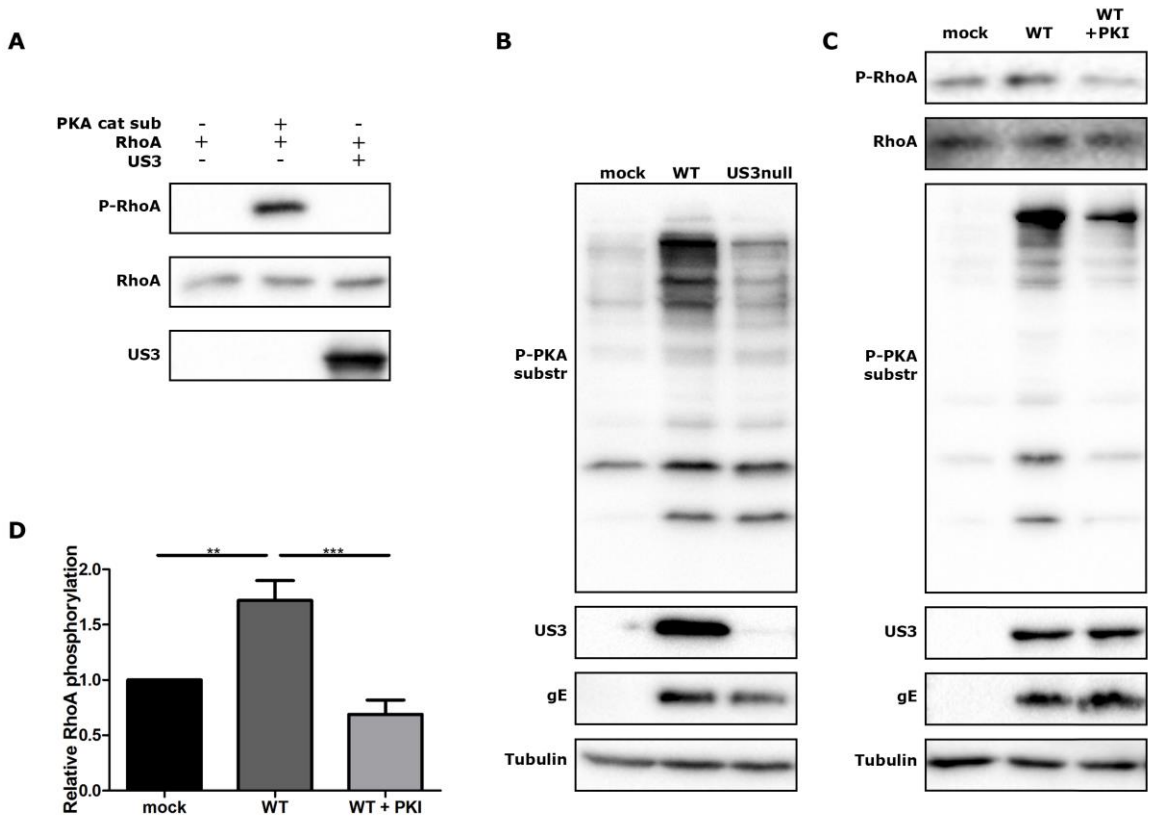


Fig. 4: US3-induced RhoA phosphorylation is PKA-dependent. (A) In vitro kinase assay: recombinant RhoA was added to either recombinant US3-GST or cAMP-dependent Protein Kinase (PKA) catalytic subunit in kinase buffer. After incubation, samples were subjected to SDS-PAGE/Western blotting to detect levels of P-S188-RhoA, RhoA and US3. (B) ST cells were mock-inoculated or inoculated with WT PRV or US3null PRV. At 6 hpi, cell lysates were subjected to Western blotting to detect levels of phospho-PKA substrate signal, US3, gE, and tubulin. (C) ST cells were mock-inoculated or inoculated with WT PRV in the presence or absence of PKI, a cell-permeable PKA inhibitor. At 6 hpi, cell lysates were subjected to Western blotting to detect levels of P-S188-RhoA, RhoA, phospho-PKA substrate signal, US3, gE and tubulin. (D) shows relative RhoA phosphorylation levels (P-S188-RhoA/RhoA ratio with mock infection set to 1) from three independent repeats. Data represent means + standard errors of the means, with ** = $P < 0.01$ and *** = $P < 0.001$ (determined by one-way ANOVA and Tukey post test). Antibody panels in A, B and C were obtained from separate gels, but derive from the same sample. All gels have all been run with the same amount of sample, blotted, incubated and detected together in parallel.

In line with this, it has been reported before that PKA-induced RhoA S188 phosphorylation leads to changes in cell morphology that correspond well with US3-induced actin rearrangements. For example, in epithelial SH-EP cells, addition of forskolin, which increases PKA activity via increased intracellular cAMP levels, resulted in PKA-mediated RhoA S188 phosphorylation, and consequent cell retraction and formation of long, branched cell projections that resemble US3-induced cell projections (Dong *et al.*, 1998).

Phosphorylation and activation of PAK, another effect of US3 signaling (Van den Broeke *et al.*, 2009b), has also been associated with US3-like actin rearrangements, stress fiber disassembly and cell projection formation (Zhao *et al.*, 1998). Hence, US3 both suppresses RhoA signaling via RhoA phosphorylation and triggers Cdc42/Rac1 signaling via PAK phosphorylation, and both appear to contribute to the US3-induced actin rearrangements. Why does US3 interfere with both pathways? In this context, it is interesting that there is negative feedback between RhoA signaling on the one hand and Cdc42/Rac1/PAK signaling on the other hand, and reciprocal control of both branches of signaling has been reported in different cellular settings (Kozma *et al.*, 1997; Leeuwen *et al.*, 1997; Sander *et al.*, 1999; Xie *et al.*, 2008; Yamaguchi *et al.*, 2001). For example, mesenchymal and amoeboid migration rely on antagonistic reciprocal control of RhoA and Rac signaling (Parri & Chiarugi, 2010). Relevant to this work, RhoA activation promotes formation of stress fibers whereas PAK activation destabilizes stress fibers (Etienne-Manneville & Hall, 2002; Van Aelst & D'Souza-Schorey, 1997). By simultaneously triggering both PAK activating signaling and RhoA inactivating signaling, US3 seems to very efficiently usurp this feedback system.

Interestingly, a US3-like phenotype of actin rearrangements has also been described for the F11 protein of vaccinia virus, a poxvirus (Arakawa *et al.*, 2007). F11 exerts its effect also via suppression of the RhoA signaling axis, albeit via a different mechanism than US3. F11 interferes with the Rho GTPase-activating protein (GAP) Myosin-9A (Handa *et al.*, 2013), thereby blocking the interaction of RhoA with its downstream effectors ROCK and mDia (Valderrama *et al.*, 2006). Several other viruses have also been reported to mediate RhoA inactivation at some point in their replication cycle to lead to successful infection or spread, such as human cytomegalovirus (HCMV) (Frampton *et al.*, 2007), simian virus 40 (SV40) (Stergiou *et al.*, 2013), hepatitis C virus (HCV) (Brazzoli *et al.*, 2008), Epstein Barr virus (EBV) (Loesing *et al.*, 2009), and human immunodeficiency virus (HIV-1) (del Real *et al.*, 2004). To our knowledge, the current study is the first report describing virus-triggered phosphorylation of RhoA.

To conclude, US3 expression leads to PKA-dependent RhoA S188 phosphorylation, which contributes to the US3-induced actin rearrangements. The combination of activation of PAK signaling and suppression of RhoA signaling may provide the ideal signaling conditions to generate the robust US3-induced cytoskeletal rearrangements.

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Chapter 4

Alphaherpesviral US3 kinase induces cofilin dephosphorylation to reorganize the actin cytoskeleton

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Chapter 4: Alphaherpesviral US3 kinase induces cofilin dephosphorylation to reorganize the actin cytoskeleton

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Abstract

The conserved alphaherpesviral serine/threonine kinase US3 causes dramatic actin rearrangements, associated with increased viral spread. Here, we show that US3 of pseudorabies virus (PRV) leads to activation (dephosphorylation) of the central actin regulator cofilin. A mutation that impairs US3 kinase activity and the group I p21-activated kinase inhibitor IPA-3 inhibited US3-mediated cofilin activation. Additionally, expression of phosphomimetic S3D cofilin significantly suppressed the ability of US3 to cause cell projections and cell rounding. In conclusion, the US3 kinase of PRV leads to activation (dephosphorylation) of cofilin, and cofilin contributes to US3-mediated actin rearrangements.

Introduction

The US3 kinase is conserved among *Alphaherpesvirinae*. We and others have shown that this kinase induces dramatic rearrangements of the actin cytoskeleton, including disassembly of actin stress fibers and formation of cellular projections, which are associated with increased viral spread in cell culture (Calton *et al.*, 2004; Favoreel *et al.*, 2005; Finnen *et al.*, 2010; Ladelfa *et al.*, 2011; Murata *et al.*, 2000; Schumacher *et al.*, 2005; Van Minnebruggen *et al.*, 2003). For the alphaherpesvirus pseudorabies virus (PRV), we previously reported that the US3-induced changes in the actin cytoskeleton are mediated through p21-activated kinases (PAKs), central regulators in Rho GTPase signaling (Van den Broeke *et al.*, 2009b). Apart from the involvement of PAKs, relatively little is known about the factors contributing to US3-mediated actin rearrangements.

Cofilin, a member of the actin depolymerizing factor (ADF)/cofilin family, is a central player in actin dynamics known to be activated through dephosphorylation on serine residue 3 (S3) (Moriyama *et al.*, 1996). Phosphorylation and dephosphorylation of cofilin at S3 is complexly regulated by multiple kinases and phosphatases (Van Troys *et al.*, 2008). Increasing evidence indicates that cofilin constitutes an important cellular target affected by both bacterial and viral infections (Berkova *et al.*, 2007; Han *et al.*, 2011; Moffatt *et al.*, 2012; Stolp *et al.*, 2009; Xiang *et al.*, 2012). With regard to alphaherpesviruses, herpes simplex virus 1 (HSV-1) has been reported to induce a cell-type dependent upregulation of cofilin levels and modulation of cofilin activity (Pei *et al.*, 2011; Vorster *et al.*, 2011a). This may affect viral replication, although the underlying mechanism is unclear (Pei *et al.*, 2011; Xiang *et al.*, 2012). The best characterized viral modulation of cofilin activity has been documented for HIV, which triggers cofilin S3 phosphorylation and thus inactivation through gp120-mediated activation of the Rac-PAK-LIMK pathway, which is involved in initiation of infection of CD4 T cells (Vorster *et al.*, 2011b). HIV Nef also leads to cofilin inactivation through the activity of PAK2, thereby restricting migration of infected T lymphocytes (Stolp *et al.*, 2009). On the other hand, HIV-mediated activation of cofilin has also been described to affect initiation of infection (Jimenez-Baranda *et al.*, 2007; Vorster *et al.*, 2011b; Yoder *et al.*, 2008).

In the current report, we investigated whether the US3 protein of the alphaherpesvirus PRV affects cofilin phosphorylation, and, if so, whether this contributes to the US3-mediated effects on the actin cytoskeleton.

Materials and methods

- Cell cultures and viruses

ST (Swine testicle) cells were cultured as described previously (Geenen *et al.*, 2005). Wild-type NIA3 PRV, isogenic US3null NIA3 PRV (constructed by insertion of a palindromic oligonucleotide containing translational stop codons in all reading frames, in the 5' part of the open reading frame) (de Wind *et al.*, 1990) and revertant NIA3 PRV viruses have been described before (Baskerville, 1973; de Wind *et al.*, 1990), as well as VP26-mRFP expressing Becker PRV variants including wild-type PRV-GS847, PRV-GS976 encoding a kinase-inactive variant of US3 and the revertant PRV-GS3000 Becker virus (Coller & Smith, 2008; Van den Broeke *et al.*, 2009a)

- Plasmids and reagents

Human cofilin-1 (indicated as cofilin) coding constructs were constructed as described (Leyman S et al. 2009, Mol Biol Cell). Briefly, WT cofilin was cloned into pEGFP.N1 (Clontech), producing a cofilin-GFP fusion protein. S3A and S3D mutations were introduced using the QuikChange mutagenesis kit (Stratagene). The plasmid encoding PRV US3 was described earlier (Geenen *et al.*, 2005). The plasmid encoding DsRed was kindly provided by R. Y. Tsien (UCSD, La Jolla, CA). Group I PAK inhibitor IPA-3 (a kind gift from Professor J. Chernoff, Fox Chase Cancer Center) was used at 33 µM, as decribed before (Deacon *et al.*, 2008). Antibodies used included rabbit anti-cofilin (Santa Cruz, sc-42824) (1/600 for Western blot), rabbit anti-phospho-S3 cofilin (Santa Cruz, sc-12912) (1/500 for immunofluorescence and 1/1000 for Western blot), mouse anti-US3 (kindly provided by LeighAnne Olsen and Lynn Enquist, Princeton University) (1/50 for immunofluorescence and 1/100 for Western blot), monoclonal mouse anti-PRV gE antibody 13D12 (Nauwynck & Pensaert, 1995) (1/100 for Western blot). Secondary antibodies for immunofluorescence included goat anti-rabbit FITC (Invitrogen) (1/200) and goat anti-mouse TR (Invitrogen) (1/100). Secondary Western blotting antibodies included horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1/3000) and goat anti-mouse antibody (1/2000) (Dako Cytomation). To stain nuclei in immunofluorescence experiments, Hoechst 33342 (Invitrogen) (1/200) was used. For immunofluorescence, antibodies were diluted in PBS, for Western blot assays antibodies were diluted in PBS with 5% Tween 20 (Sigma Aldrich) and 5% non-fat, dry milk (Nestlé).

- Transfection and infection assays

One day prior to transfection or infection assays, ST cells were seeded at a density of 100,000 cells/ml or 150,000 cells/ml, respectively. JetPrime (Polyplus, Westburg) was used for transfection according to manufacturer's guidelines. For infection assays, cells were inoculated with WT virus at MOI

(multiplicity of infection) 10 or 30 in 1 ml medium (MEM complemented with 10% FCS, 1% glutamin, 1% P/S, 1% NaPy, 0.5% gentamycin).

- Western blotting

At 6 h post-inoculation (hpi) or 24 h post-transfection, cells were washed twice in PBS and incubated in lysis buffer (50mM Tris-HCL, 5mM EDTA, 150mM NaCl, 1% NP40, 1 tablet of protease inhibitors (Roche, EDTA-free, complete mini) per 7 ml of lysis buffer, phosphatase inhibitors (2mM $NaVO_3$, 5mM NaF) at 4°C for 1 h. The lysate was centrifuged at 12,000 x *g* and the supernatant was collected. After SDS-PAGE, Western blotting and detection of protein bands, band intensities were quantified using the 'Analyse Gel' tool in ImageJ.

- Immunofluorescence

For immunofluorescence, cells were grown on coverslips. At 6 hpi or 24 h post-transfection, cells were washed in PBS, fixed in 3% paraformaldehyde for 10min, permeabilized for 2 min in 0.2% Triton X-100, washed in PBS and subjected to immunofluorescence staining consisting of a 1 h incubation step at 37°C with primary antibodies, two washing steps in PBS, 1 h incubation at 37°C with fluorescently tagged secondary antibodies and again two washing steps in PBS. After 10 min incubation with a 1/200 dilution of Hoechst 33342 (Invitrogen), coverslips were mounted in glycerin-DABCO (1,4-diazabicyclo[2.2.2]octane) and imaged using a Leica TCS SPE confocal microscope (Leica DM RBE, Leica Microsystems, GmbH, Heidelberg, Germany). Quantification of fluorescence in the images was performed through the ImageJ 'Analyze Particles' procedure, where the amount of cells was detected automatically using the 'Treshold'-function.

Results and discussion

- US3 is required for PRV-mediated suppression of cofilin phosphorylation

We determined whether US3 modulates the activity of cofilin through altered phosphorylation at the critical S3 residue in cofilin. ST cells were inoculated with isogenic NIA3 strains wild-type (WT) PRV, or US3null PRV (containing a translational stop codon in US3) or a revertant virus of the latter (8). At 6 h postinoculation (hpi), cells were subjected to Western blotting (WB). Antibodies used were directed against phospho-S3 cofilin, total cofilin, US3, and the viral membrane protein gE (18E8). Band intensity was measured with the "Analyze gels" option in ImageJ and phospho-S3 cofilin levels were normalized to mock levels. *Figure 1* shows that, compared to mock-infected ST cells, WT and US3rescue PRV infection led to a strong decrease in S3 cofilin phosphorylation, in contrast to US3null PRV (*Figure 1A&B*). Phospho-S3 cofilin levels in US3null PRV-infected cells were even increased, albeit not significantly, when compared to mock-infected cells. In line with the early kinetics of US3 expression, the decrease in phospho-S3 cofilin could be observed already early in infection (from 4 hpi onward) (*Figure 1C*). The ability of US3 to modulate cofilin activity levels is underscored by the fact that transfection of a WT US3-encoding construct in ST cells was sufficient to suppress phospho-S3 cofilin levels, as shown in *Figure 1D&E*. Transfection with a control plasmid encoding red fluorescent protein DsRed plasmid was used as a control (Campbell *et al.*, 2002). Hence, US3 leads to suppressed phospho-S3 cofilin levels in infected and transfected ST cells.

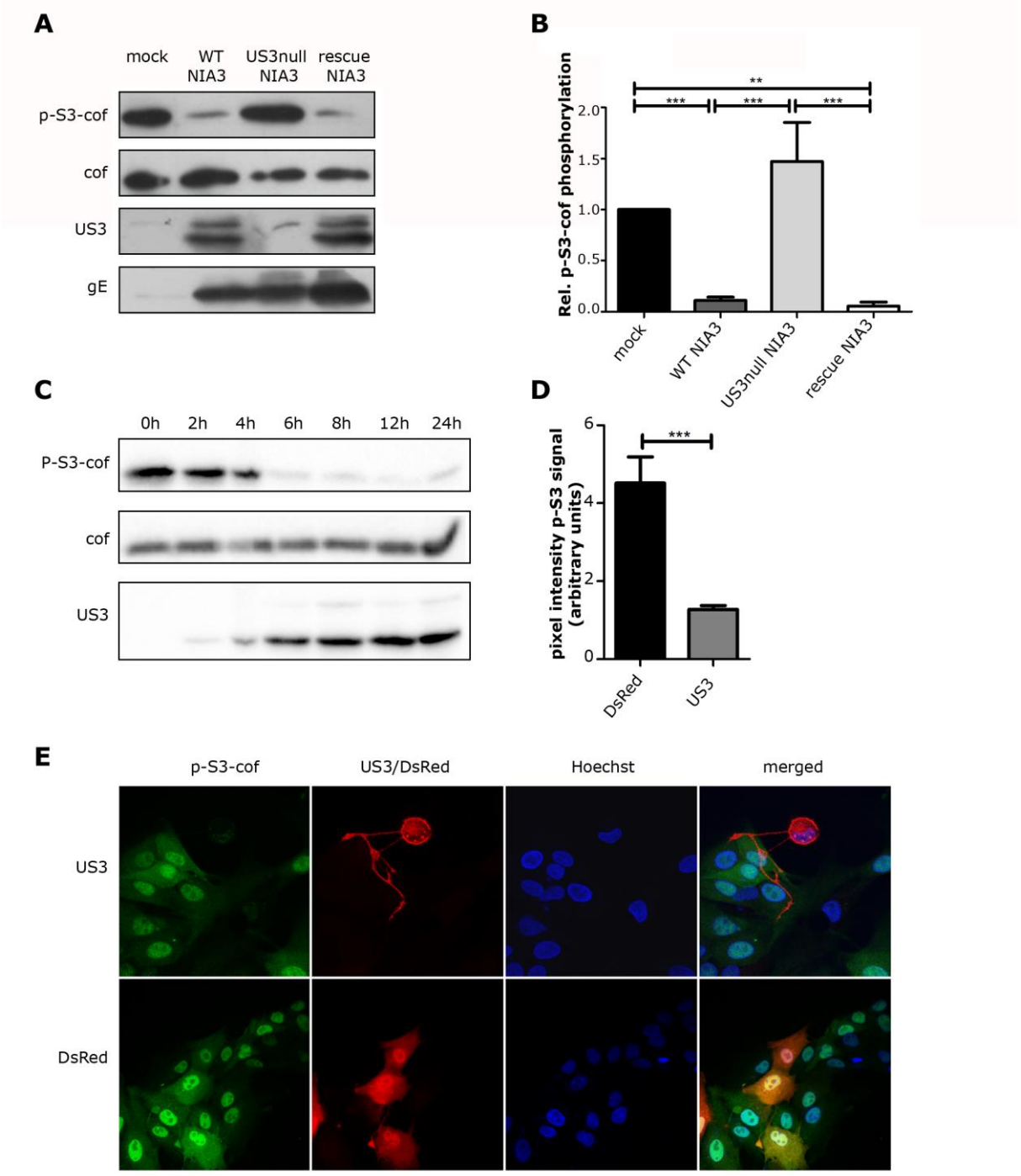

Fig. 1. PRV infection leads to a US3-dependent suppression in S3 cofilin phosphorylation. (A) ST cells were mock-inoculated or inoculated (MOI of 10) with WT PRV, US3null PRV, or US3rescue PRV. At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, US3, and gE. (B) Relative cofilin phosphorylation levels based on the phospho-S3 cofilin/cofilin ratio (with mock infection set to 1) are represented as means + standard errors of the means of data from three independent experiments, ** indicating P values of <0.01 and *** indicating P values of <0.001. (C) ST cells were inoculated with WT PRV (MOI of 10), and lysed at 0 h, 2 h, 4 h, 6 h, 8 h, 12 h or 24 h post-infection. Total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, and US3. (D and E) ST cells were transfected with US3 or with a control plasmid encoding DsRed (3) and stained for US3 and phospho-S3 cofilin. Panel D shows quantification of fluorescein isothiocyanate (FITC) (phospho-S3 cofilin) pixel intensities of 8 randomly chosen US3- or control plasmid-transfected cells, which were determined using ImageJ. Data shown represent means + standard errors of the means, with *indicating P values of <0.05.

- The kinase activity of US3 is required to suppress phosphorylation of cofilin

To assess the involvement of the kinase activity of US3 in suppressing cofilin phosphorylation in ST cells, cells were inoculated with a previously described PRV strain expressing a kinase-inactive US3 protein, containing a point mutation (D223A) in the catalytic base required for phosphotransfer (PRV-GS976) (Coller & Smith, 2008; Van den Broeke *et al.*, 2009a). At 6 hpi, phospho-S3 cofilin, total cofilin, US3, and gE levels were evaluated by WB (*Figures 2A&B*). The PRV strain Becker expressing a kinase-inactive US3, unlike isogenic wild-type PRV (PRV-GS847), did not suppress phospho-S3 levels of cofilin. A rescue strain in which the D223A mutation in US3 was restored (PRV-GS3000) acted like the WT virus and induced a strong suppression in cofilin phosphorylation. As observed for US3null PRV (*Figure 1*), infection with PRV encoding kinase-inactive US3 resulted in increased phosphorylation of cofilin compared to that of mock-infected cells. Hence, the ability of US3 to suppress S3 phosphorylation of cofilin in ST cells relies on its kinase activity.

Interestingly, infection with US3null PRV or D223A US3 PRV resulted in increased phospho-S3 cofilin levels compared to those of mock-infected cells (*Figure 1&2*). One hypothetical way to explain this may be that infection leads to cofilin inactivation (S3 phosphorylation) and that US3 activity counteracts this and even reduces phospho-S3 cofilin levels below normal levels. Why would infection lead to increased phospho-S3 cofilin levels? Viral infection is known to lead to a stress response in cells (Clemens, 2005; Jindal & Malkovsky, 1994; Santoro, 1996), which may perhaps be involved in increased phosphorylation of cofilin. Indeed, other cellular stress stimuli have been reported to lead to increased S3 cofilin phosphorylation, including heat shock (Simard *et al.*, 2011), fluid shear stress (Fu *et al.*, 2008; Liu *et al.*, 2010) and scavenging of reactive oxygen species (Popova *et al.*, 2010). It will be interesting to investigate the potential biological consequences of increased levels of phospho-S3 cofilin during US3null PRV infection for both virus and cell.

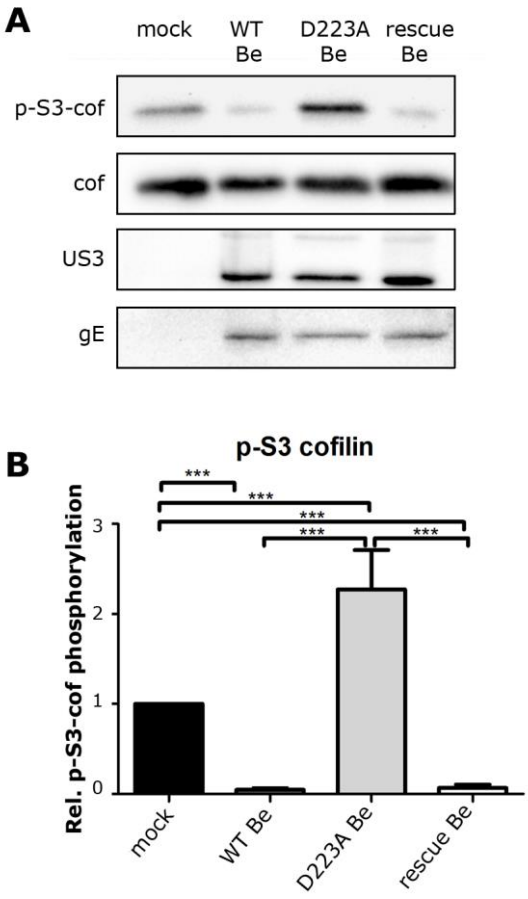

Fig. 2. The kinase activity of US3 is required to suppress phosphorylation of cofilin (A) ST cells were mock-inoculated or inoculated (MOI of 10) with WT PRV, kinase-inactive D223A US3 PRV, or D223Arescue PRV. At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, US3, and gE. (B) Means + standard errors of the means relative cofilin phosphorylation levels from three independent experiments, with *** indicating P values of <0.001.

- A constitutively inactive, S3D phosphomimetic cofilin variant interferes with US3-mediated cell rounding and cell projections

The experiments described above indicate that US3 leads to substantial S3 cofilin dephosphorylation, a hallmark of cofilin activation (Moriyama *et al.*, 1996). If this cofilin activation is important for PRV US3-induced actin rearrangements, one would expect that overexpression of a constitutively inactive (phosphomimetic) S3D cofilin mutant will interfere with US3-mediated actin rearrangements, whereas overexpression of wild-type cofilin or a constitutively active S3A cofilin mutant should not. Likewise, overexpression of S3D (but not S3A) cofilin has been reported to suppress the formation of long actin-containing dendritic cell protrusions in hippocampal neurons (Pontrello *et al.*, 2012).

To assess this, ST cells were cotransfected with US3 and constructs expressing previously described green fluorescent protein (GFP) fusions of wild-type cofilin, S3D cofilin or S3A cofilin (Leyman *et al.*, 2009). At 24 h post-transfection, cells were stained with anti-US3 antibody and scored for US3-mediated effects on the actin cytoskeleton. In brief, 200 randomly chosen transfected cells per condition were scored for cell rounding (actin stress fiber disassembly) and cell projection formation. Phosphomimetic S3D cofilin, but not wild type or S3A cofilin, significantly suppressed the ability of US3 to induce actin rearrangements in ST cells (*Figure 3A,B&C*). Overexpression of either WT or S3D cofilin on itself did not cause apparent changes in cell morphology. Overexpression of S3A cofilin on itself did not lead to obvious cell rounding but did induce cell projections that were shorter and less branched than observed upon transfection of US3 (data not shown). Notwithstanding the apparent colocalization of cofilin with US3 in some of the immunofluorescence images, immunoprecipitation experiments were not indicative for a direct interaction between US3 and cofilin (data not shown). In conclusion, expression of phosphomimetic S3D cofilin in ST cells interferes with the ability of US3 to induce actin rearrangements.

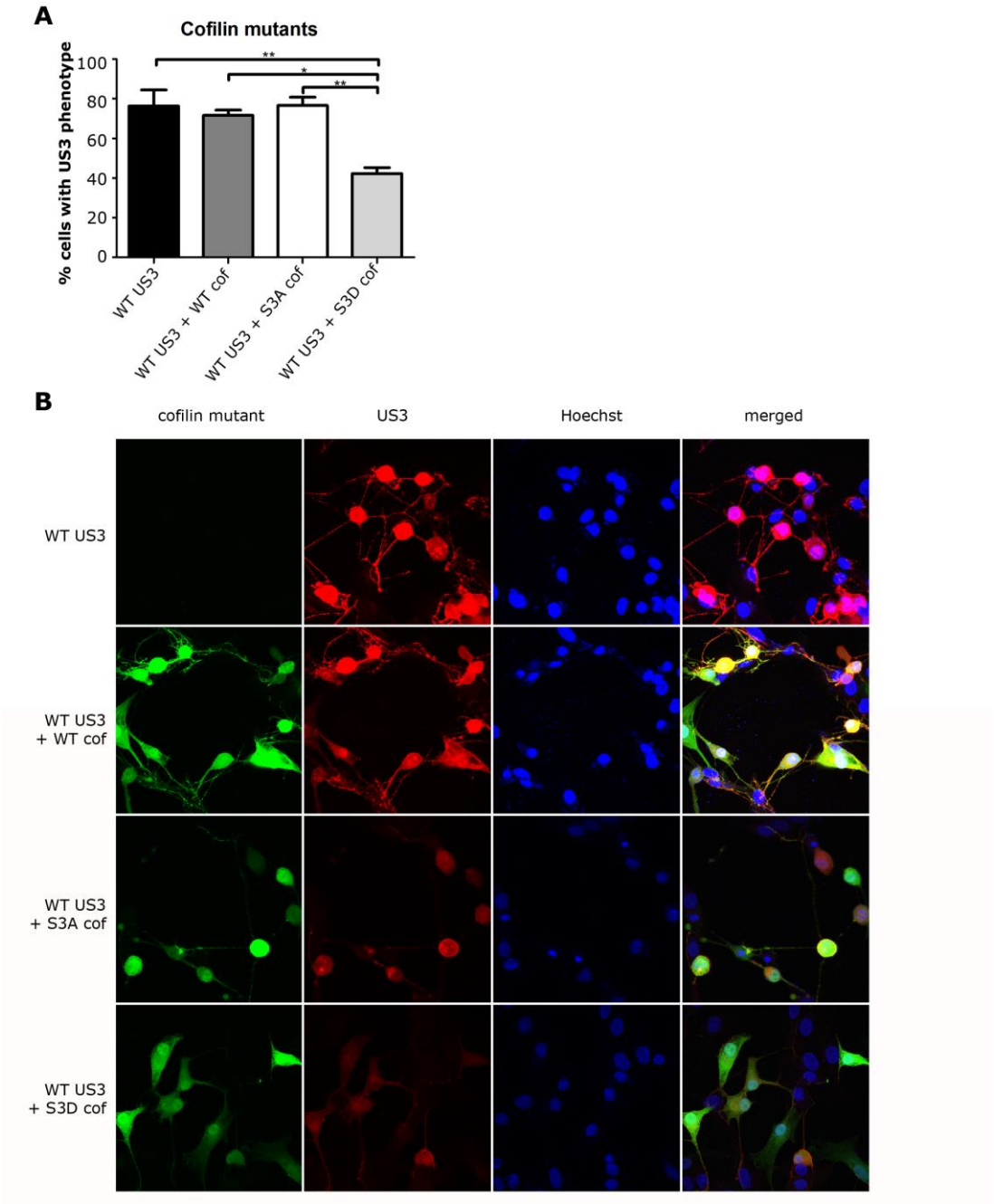

Fig. 3. Overexpression of S3D phosphomimetic cofilin interferes with the ability of US3 to cause cell rounding and cell projections. (A and B) ST cells were transfected with US3 encoding plasmids or cotransfected with plasmids encoding US3 and GFP-tagged WT cofilin, S3A cofilin, or S3D cofilin. At 24 h post-transfection, cells were fixed and stained for US3 and nuclei and analyzed for expression of cofilin (GFP; green) and US3 (red). Panel A shows the percentage of transfected cells displaying actin rearrangements, as assessed by cell rounding and the formation of cell projections (means + standard errors of the means; data from three independent experiments), with * indicating P values of <0.05 and ** indicating P values of <0.01. Small blue dots in panel B represent leftover plasmid DNA-containing transfection reagent in cells and on the cover glass.

- Group I PAKs are involved in the US3-mediated dephosphorylation of cofilin

The ability of PRV US3 to induce actin rearrangements has been shown to depend on the ability of US3 to phosphorylate and thereby activate group I PAKs (Van den Broeke *et al.*, 2009b). As a consequence, the group I PAK inhibitor IPA-3 is able to inhibit US3-mediated actin rearrangements in ST cells (Takuma *et al.*, 1996; Van den Broeke *et al.*, 2009a). We investigated whether IPA-3 is also capable of reverting the observed US3-mediated suppression of S3 cofilin phosphorylation. To this end, ST cells were either mock-inoculated or inoculated with WT PRV in the absence or presence of 33 µM IPA-3, used as decribed before (Deacon *et al.*, 2008). At 6 hpi, cells were lysed and phospho-S3 cofilin, total cofilin, and US3 levels were evaluated. The addition of IPA-3 restored the phospho-S3 cofilin signal in PRV-infected cells (*Figure 4B*), while it did not influence phospho-S3 cofilin levels in mock-infected cells (*Figure 4A*). Hence, the use of an inhibitor of group I PAK activity in ST cells interferes with the US3-mediated suppression of S3 cofilin phosphorylation.

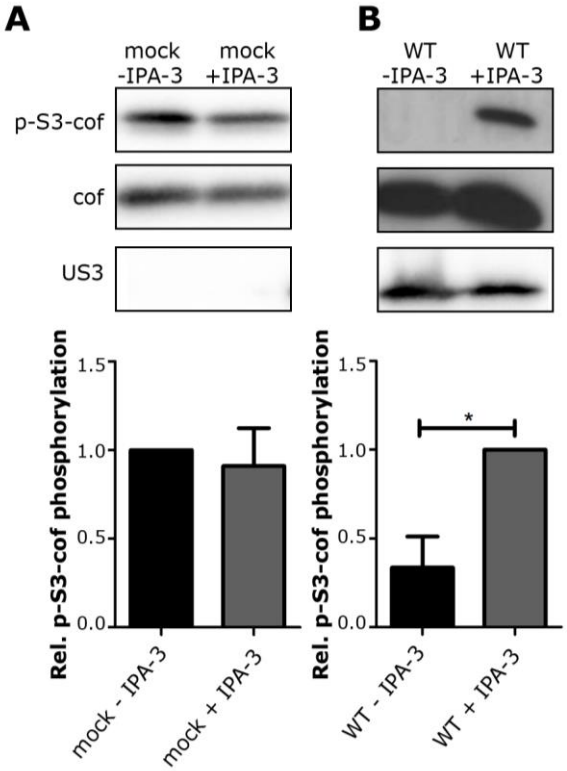

Fig. 4. Group I PAKs are involved in US3-mediated suppression of S3 cofilin phosphorylation. (A and B) ST cells treated with or without 33 µM of the group I PAK inhibitor IPA-3 were either mock-inoculated (A) or inoculated with WT PRV (B). At 6 hpi, total cell lysates were subjected to Western blotting to detect phospho-S3 cofilin, total cofilin, and US3. Values were normalized to mock (A) or to PRV and IPA-3 (B). The graphs represent the means + standard errors of the means from three independent experiments, with * indicating P values of <0.05.

This is in apparent contradiction with studies in HIV, where virus-induced activation of PAK2 (a group I PAK member) leads to S3 cofilin phosphorylation and thus inactivation in Jurkat-cells (Stolp *et al.*,

2010; Stolp *et al.*, 2009; Vorster *et al.*, 2011b). Nevertheless, ambiguity exists in the literature as to whether PAK activation leads to cofilin phosphorylation or dephosphorylation at S3. On the one hand, PAK activity has been associated with cofilin phosphorylation, mainly because LIM kinase isoforms are important downstream substrates of PAK that can lead to phosphorylated cofilin (Kobayashi *et al.*, 2006; Li *et al.*, 2006; Scott & Olson, 2007; Zoudilova *et al.*, 2007). On the other hand, more recently, increasing evidence indicates that group I PAK activity may also signal to several of the phosphatases like PP2A, chronophin (CIN) and/or the slingshot (SSH) family, that are known to dephosphorylate and activate cofilin (Coniglio *et al.*, 2008; Ke *et al.*, 2004; Nagata-Ohashi *et al.*, 2004; Oleinik *et al.*, 2010). Most likely, cell-type specific or environmental factors may influence the outcome of PAK activation on cofilin activity (Davidson & Haslam, 1994; Okada *et al.*, 1996; Samstag *et al.*, 1996; Samstag *et al.*, 1994; Takuma *et al.*, 1996). Future research aimed at further dissecting the mechanistic details of US3-PAK-mediated cofilin dephosphorylation will further clarify the other molecular players in this pathway and may therefore generate important cell biological insights on PAK-mediated cofilin regulation.

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Chapter 5

PRV US3 leads to F-actin disassembly and contributes to viral genome delivery to the nucleus

Adapted from: Jacob, T., Van den Broeke, C., Grauwet, K., Baert, K., Claessen, C., De Pelsmaeker, S., Van Waesberghe, C., Favoreel, H. W. (2015). Pseudorabies virus US3 leads to filamentous actin disassembly and contributes to viral genome delivery to the nucleus. Vet Microbiol. 177 (3-4): 379-8

Chapter 5: Pseudorabies virus US3 leads to filamentous actin disassembly and contributes to viral genome delivery to the nucleus

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Abstract

The conserved alphaherpesvirus US3 tegument protein induces rearrangements of the actin cytoskeleton, consisting of protrusion formation and stress fiber breakdown. Although US3 does not affect levels of total actin protein, it remains unclear whether US3 modulates the total levels of filamentous (F) actin. In this report, we show that the pseudorabies virus (PRV) US3 protein, via its kinase activity, leads to disassembly of F-actin in porcine ST cells. F-actin disassembly has been reported before to contribute to host cell entry of HIV. In line with this, in the current study, we report that US3 has a previously uncharacterized role in viral genome delivery to the nucleus, since quantitative polymerase chain reaction (qPCR) assays on nuclear fractions demonstrated a reduced nuclear delivery of of US3null PRV compared to wild type PRV genomes. Treatment of cells with the actin depolymerizing drug cytochalasin D enhanced virus genome delivery to the nucleus, particularly of US3null PRV, supporting a role for F-actin disassembly during certain aspects of viral entry. In conclusion, the US3 kinase of PRV leads to F-actin depolymerization, and US3 and F-actin disassembly contribute to viral genome delivery to the nucleus.

Introduction

Different viruses, including several herpesviruses, HIV, rabies virus and influenza virus have been reported to disassemble the filamentous (F) actin cytoskeleton of host cells (Arcangeletti *et al.*, 2000; Jones & Kilpatrick, 1988; Murata *et al.*, 2000; Nayak *et al.*, 2009; Schumacher *et al.*, 2005; Slonska *et al.*, 2014; Song *et al.*, 2013; Van Minnebruggen *et al.*, 2003; Vorster *et al.*, 2011; Wagenaar *et al.*, 1995). The cortical F-actin meshwork underneath the plasma membrane represents an important barrier that viruses need to modify or bypass during host cell entry to access the intracellular compartments, either by hijacking cellular endocytosis pathways or by virus-triggered F-actin disassembly upon virus fusion as has been described for HIV (Delorme-Axford & Coyne, 2011; Spear *et al.*, 2013; Yoder *et al.*, 2008).

Alphaherpesviruses represent the largest subfamily of the herpesvirus family, comprising closely related pathogens of humans and animals, including herpes simplex virus (HSV) and varicella zoster virus (VZV) in humans. We and others have reported before that the conserved and multifunctional alphaherpesvirus US3 serine/threonine protein kinase induces rearrangements of the actin cytoskeleton, consisting of actin stress fiber breakdown and formation of actin-based protrusions, which are associated with increased intercellular viral spread (Deruelle & Favoreel, 2011; Favoreel *et al.*, 2005; Van den Broeke *et al.*, 2009; Van Minnebruggen *et al.*, 2003). Although these US3-induced rearrangements of the actin cytoskeleton do not affect total actin protein levels (Van Minnebruggen *et al.*, 2003) it has not been investigated whether US3 results in an overall disassembly of F-actin and whether US3 may contribute to certain aspects of virus entry in host cells.

In the current study, we investigated whether the alphaherpesvirus pseudorabies virus (PRV) leads to disassembly of F-actin in infected cells and, if this is the case, whether the US3 protein kinase is involved. We also investigated whether US3 plays a hitherto unrecognized role during particular aspects of virus entry by focusing on nuclear delivery of viral genomes. To this end, we generated LifeAct mCherry transduced swine testicle (ST) cells, combined with flow cytometric analysis. LifeAct specifically labels F-actin and does not affect actin polymerization or stability, permitting quantification of changes in F-actin in living cells (Bovellan *et al.*, 2014; Riedl, 2010; Riedl *et al.*, 2008).

We report that PRV infection of ST cells results in a US3-dependent progressive reduction in F-actin, requiring US3 kinase activity. Using quantitative polymerase chain reaction (qPCR), we demonstrate that the wild-type (WT) PRV genome is delivered more efficiently at the nucleus compared to the genome of virions lacking the US3 protein, indicating a role for the US3 tegument protein during this aspect of virus entry. In line with this, the actin depolymerizing drug cytochalasin D (cytD) significantly enhanced delivery of the US3null viral genome to the nucleus, supporting a role for F-actin disassembly during particular stages of virus entry.

Materials and Methods

- LifeAct mCherry ST cells

Semi-confluent swine testicle (ST) cells were transduced using a lentiviral vector expressing the LifeAct gene coupled to an mCherry fluorescent marker, kindly supplied by Dr. Isabelle Maridonneau Parini (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France). Virus was diluted in 0.6 ml of medium (MEM complemented with 10% FCS, 1% glutamin, 1% P/S, 1% NaPy, 0.5% gentamycin), and washed away after 24 h. After expansion to a 75-cm² bottle, LifeAct expressing cells were separated from non-transduced cells using a BD FACSAria III Cell sorter (BD Biosciences), using the 488 nm laser, exciting the mCherry reporter. During sorting, cells were kept on ice in MEM containing 40% fetal calf serum (FCS) for 2-3 h, until the medium was replaced with regular medium before seeding in a 75-cm² bottle. After sorting, the population was at least 96% pure, stably expressing LifeAct for at least 12 passages whereafter, when required, the cells were sorted again.

- Infections and treatments

WT PRV NIA3 strain, isogenic US3null PRV NIA3 strain (constructed by insertion of a palindromic oligonucleotide containing translational stop codons in all reading frames, in the 5' part of the open reading frame) and revertant PRV NIA3 strain have been described in earlier literature (Baskerville, 1973; de Wind *et al.*, 1990). PRV Becker PRV-GS847, its isogenic mutant PRV-GS976 encoding a kinase-inactive US3 protein containing a mutation in the catalytic base required for phosphotransfer (D223A) and its revertant strain PRV-GS3000 have also been described before (Coller & Smith, 2008; Van den Broeke *et al.*, 2009). For flow cytometric experiments, LifeAct ST cells were seeded at 150,000 cells/ml in 6-well plates. Two days later, the medium was replaced by 1 ml of virus-containing medium at the indicated MOI (multiplicity of infection). At the indicated time points, cells were washed and collected. For qPCR experiments, new viral protein expression was blocked using 10 µg/ml cycloheximide (CHX, Sigma-Aldrich) from 30 min before infection until cell collection. The actin depolymerizing drug cytD (Sigma-Aldrich) was added at 1 µM, together with CHX.

- Antibodies and probes

Rabbit anti-tubulin was bought from Abcam (ab18251, 1/500 for Western blotting). Rabbit antibody against the inner nuclear membrane protein SUN2 (Sad1 and UNC84 domain containing 2) was kindly provided by Didier Hodzic (Washington University, Saint Louis) (1/1000 for Western blotting) (Crisp *et al.*, 2006). Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1/3000) for Western blotting detection was purchased from Dako Cytomation. Antibodies were diluted in PBS with 5% Tween 20 (Sigma-Aldrich) and 5% non-fat, dry milk (Nestlé).

- Flow cytometry

ST cells were washed in PBS and subsequently detached using 5 mM EDTA and 0.1 M trypsin, washed again and incubated with the viability marker Sytox Blue (Molecular Probes, Invitrogen). Cells were kept on ice until flow cytometric analysis using the BD Biosciences FacsCanto II Cell Analyzer. LifeAct mCherry was excited using the 488 nm laser and detected in the phycoerythrin (PE)-channel. Dead cells were excluded using the Sytox Blue staining, which was excited by the 405 nm laser. In every sample, 20,000 living, LifeAct positive cells were measured, and mean fluorescence intensity ratio (MFIR) was used for statistics.

- Western blotting

6 h post-inoculation (hpi), cells were washed in PBS and collected in lysis buffer consisting of 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% NP40, 1 tablet of protease inhibitors (Roche, EDTA-free, complete mini) per 7 ml of lysis buffer and phosphatase inhibitors (2 mM Na_3VO_4, 5 mM NaF). After incubation for 1 h on ice, the lysate was centrifuged for 5 min at 13,000 x *g* and the supernatant was collected and subjected to SDS-PAGE and Western blotting according to standard procedures. Blots were detected using home-made ECL-substrate (A: 1 M Tris, 250 mM luminol, 90 mM P-coumaric acid; B: 10% H_2O_2) and visualized using the ChemiDoc (Biorad) imaging system.

- Nuclear fractions and qPCR

Cells were collected at 4 hpi as described before (Veettil *et al.*, 2008). In brief, cells were washed once with PBS/EDTA (1 mM) at 37°C and subsequently incubated for 5 min in PBS/trypsin (0.25%) at 37°C. The cells were detached using gentle pipetting, and were collected after centrifugation. The "Rapid, Efficient And Practical" (REAP) fractionation protocol was used for cell fractionation, as described before (Suzuki *et al.*, 2010). For all samples, DNA was isolated through Qiagen's DNeasy Blood and Tissue-kit, according to the manufacturer's instructions. As a control for the qPCR assay, non-fractionated, total-cell samples were taken along. DNA concentration was analyzed by measuring the absorbance at 260 nm with NanoDrop 2000 (Thermo-Fisher). Purity of the DNA samples was tested by obtaining the 260/280 nm ratio, and was between 1.8 and 2.1.

Primers against NIA3 PRV gB (FW: 5'-ACAAGTTCAAGGCCCACATCTAC-3'; REV: 5'-GTCCGTGAAGCGGTTCGTGAT-3'; 60°C) from (Ma *et al.*, 2008) and GAPDH (FW: 5'-CTGCCGTCTGGAGAAACCTG-3'; REV: 5'-CCACCACCCTGTTGCTGTAG-3'; 60°C) from (Van Opdenbosch *et al.*, 2011) were blasted to check for endogenous off-target genes (Blast NCBI) and ordered through IDT (Integrated Device Technology, California, USA). All the assays were performed on the StepOnePlus Real-Time PCR-system (Applied Biosystems), using the fluorescent double-stranded DNA dye SYBR

Green (Applied Biosystems) as a detection method for PCR product. The suitability of the primer pairs was verified by means of non-template control samples (mock-infected cells) and melting curves of different samples. PCR-efficiency was determined through at least two independent qPCR-reactions on mixed sample, and was used for calculation of Rq-values. The efficiency-corrected calculation model was used, based on multiple samples and GAPDH as reference gene (Pfaffl, 2004). GAPDH expression was not affected by viral infection. All Rq-values were normalized to unfractionated Rq-values of total cell DNA samples and fold induction of gB was calculated in comparison to untreated infected samples.

- Statistics

For flow cytometry data, mean fluorescence intensities of LifeAct ST cells were normalized to mock-infected samples and statistically processed with GraphPad Prism followed by a one-way analysis of variance (ANOVA) test and Tukey post-testing. For qPCR data, normalized Rq-values (see above) were compared using a Student t-test. CytD gB qPCR Rq-values were analyzed using a non-parametric ANOVA test (Kruskall-Wallis), followed by Dunn's post-testing.

Results

- PRV US3 induces disassembly of filamentous actin

To investigate in living cells whether PRV infection leads to filamentous (F) actin disassembly, we first generated swine testicle (ST) cells stably expressing LifeAct mCherry. LifeAct is a small 17 amino acid peptide originating from *Sacharomyces cerevisiae* and absent in other organisms. As Live Act displays a non-disturbing, low-affinity, high specificity binding with F-actin, it is a very suitable marker to (quantitatively) determine changes in F-actin in living cells (Riedl *et al.*, 2008).

LifeAct transduced ST cells were inoculated for 1 h, 2 h, 3 h and 6 h at a multiplicity of infection (MOI) of 30 with WT NIA3 PRV. After collection of cells, F-actin levels were analyzed by flow cytometry. Already at 2 hpi, a trend of F-actin disassembly could be observed, that became statistically significant from 3 hpi onwards (*Figure 1A*). Importantly, US3 is required for PRV-induced F-actin disassembly, as inoculation with an isogenic US3null virus did not affect F-actin levels (*Figure 1B*). Increasing the inoculation dose of US3null PRV to an MOI of 100 still did not lead to F-actin disassembly (*Figure 1C*). Infection with virus where the US3 mutation had been restored (US3null rescue NIA3 PRV) led to a similar actin disassembly as observed for WT virus (*Figure 1D*). Hence, PRV infection results in a US3-dependent disassembly of F-actin.

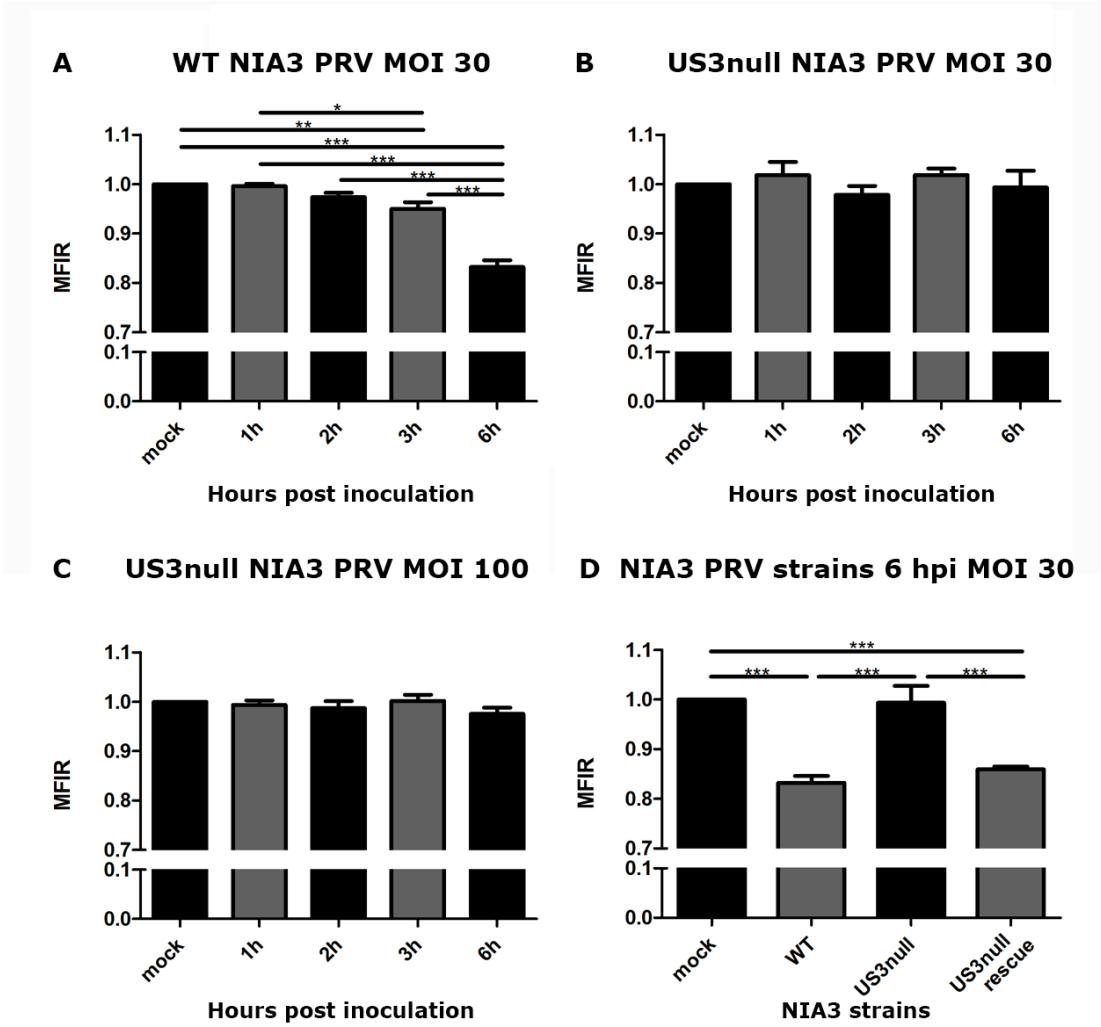

Figure 1: PRV triggers US3-dependent depolymerization of F-actin. LifeAct ST cells were inoculated with WT NIA3 PRV at an MOI of 30 (A) or isogenic US3null NIA3 PRV at an MOI of 30 (B) or an MOI of 100 (C). After collection, cells were analyzed by flow cytometry and mean fluorescence intensity ratios (MFIR) of the LifeAct signal were plotted (mock infection was normalized to 1). (D) shows data obtained at 6 hpi using WT NIA3 PRV, US3null NIA3 PRV, and US3null rescue NIA3 PRV, all at an MOI of 30. Data represent means + standard errors of the means from at least three independent experiments, with * = $P < 0.05$; ** = $P < 0.01$ and *** = $P < 0.001$.

To assess the involvement of the kinase activity of US3 in F-actin depolymerization, cells were inoculated for 1 h, 2 h, 3 h and 6 h at an MOI of 30 with a WT Becker PRV strain or an isogenic variant expressing a kinase-inactive US3 protein (D223A US3 Be PRV) (Coller & Smith, 2008; Van den Broeke *et al.*, 2009). Flow cytometric analysis confirmed that, unlike WT PRV, PRV expressing a kinase-inactive US3 did not cause F-actin disassembly (*Figure 2A and 2B*). Infection with virus where the US3 D223A mutation had been restored (US3 D223A rescue Becker PRV) led to a similar actin disassembly as observed for WT virus (*Figure 2C*). This confirms that the kinase activity of US3 is required for F-actin depolymerization.

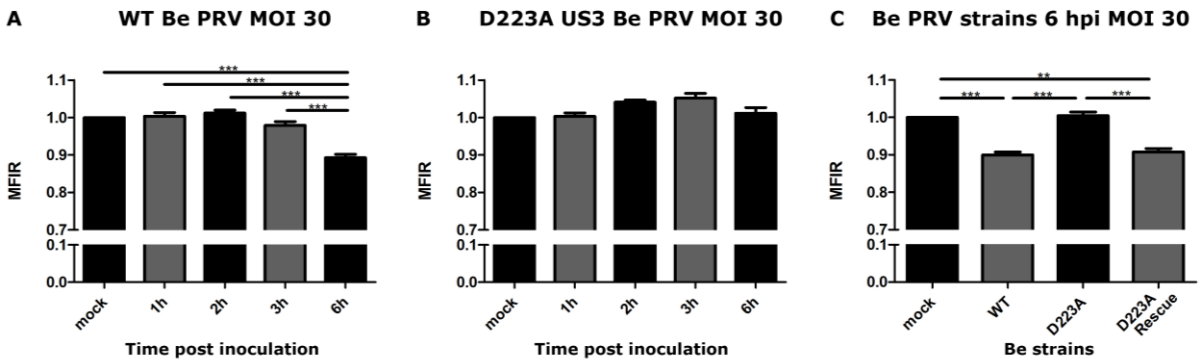

Figure 2: The kinase activity of US3 is required for PRV-induced F-actin depolymerization. LifeAct ST cells were inoculated at an MOI of 30 with WT Becker PRV (A) or isogenic US3 kinase inactive D223A Becker PRV (B). After collection, cells were analyzed by flow cytometry and mean fluorescence intensity ratios (MFIR) of the LifeAct signal were plotted (mock infection was normalized to 1). (C) shows data obtained at 6 hpi using WT Becker PRV, US3 kinase inactive D223A Becker PRV and US3 D223A rescue Becker PRV, all at an MOI of 30. Data represent means + standard errors of the means from at least three independent experiments, with *** = $P < 0.001$.

- US3 plays a role in viral genome delivery to the nucleus

To investigate whether US3 may play a role during virus genome delivery to the nucleus, a qPCR based assay was set up as described before (Fehr & Yu, 2011). ST cells were treated with 10 µg/ml cycloheximide (CHX) from 30 min prior to inoculation at an MOI of 30 with WT PRV or US3null PRV. At 4 hpi, cells were treated with trypsin to remove virus that was bound to but had not entered cells. Cells were collected and nuclear fractions were separated using the REAP fractionation protocol (Suzuki *et al.*, 2010). By isolating nuclear fractions, the amount of viral genome that had reached the cell nucleus could be assessed. Efficiency of nuclear fraction isolation was confirmed by Western blotting for the cytoplasmic protein tubulin and the nuclear protein SUN2 (*Figure 3C*). DNA was isolated and subsequently subjected to qPCR. To correct for subtle differences in initial viral administration, Rq-values were normalized to viral DNA in non-fractionated cells. This experiment showed that US3null virus DNA reached the nucleus significantly less efficiently compared to WT virus DNA (*Figure 3A*), pointing to a previously uncharacterized role for US3 during virus genome delivery to the nucleus.

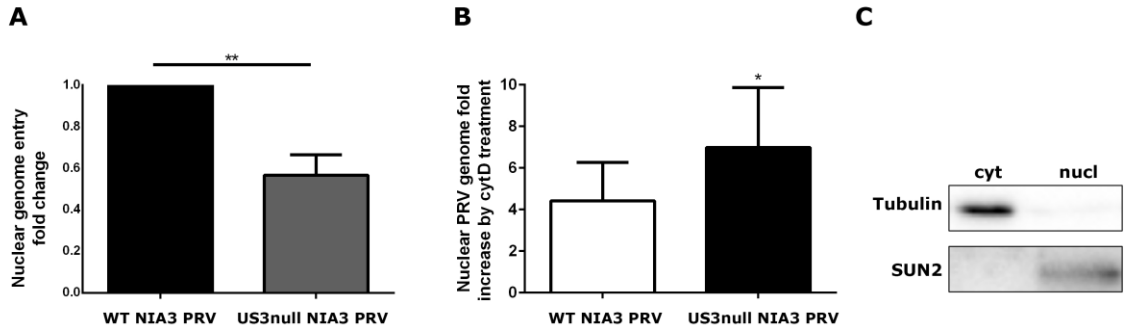

Figure 3: Viral genome delivery to the nucleus is impaired in virus lacking US3 and is enhanced by F-actin disassembly. (A) ST cells were treated with 10 µg/ml cycloheximide from 30 min before inoculation with WT NIA3 PRV or isogenic US3null NIA3 PRV at an MOI of 30. At 4 hpi, cells were collected, fractionated and DNA was isolated from nuclear fractions as well as from unfractionated cells. qPCR was performed and GAPDH- and total cell DNA-corrected Rq-values for PRV genome were normalized to WT. Data represent means + standard errors of the means from at least three independent experiments, where ** = $P < 0.01$. (B) 30 min before inoculation with WT NIA3 PRV or US3null NIA3 PRV at an MOI of 30, ST cells were treated with 10 µg/ml cycloheximide either or not supplemented with 1 µM cytochalasin D (cytD). 4 hpi, cells were collected, fractionated and DNA was isolated from nuclear fractions as well as from unfractionated cells. GAPDH- and total cell-corrected Rq-values for PRV genome were normalized to untreated WT PRV or untreated US3null PRV (each was normalized to 1). Data represent cytD-induced fold increase over respective normalized untreated sample (WT or US3null PRV) and show means + standard errors of the means from at least three independent experiments, where * = $P < 0.05$. (C) ST cells were subjected to different centrifugation steps and lysed in order to separate cytoplasmic (cyt) from nuclear (nucl) fractions. Successful fractionation was assessed through Western blotting and detection of nuclear protein SUN2 and the cytoplasmic protein tubulin.

- F-actin disassembly promotes viral genome delivery to the nucleus

To further corroborate our findings, we analyzed whether addition of the actin depolymerizing drug cytD increased nuclear delivery of WT and particularly US3null PRV genomes. Therefore, ST cells were treated with 10 µg/ml CHX supplemented with or without 1 µM cytD, 30 min prior to inoculation at an MOI of 30 with WT PRV or US3null PRV. This cytD concentration was optimized earlier in ST cells (Favoreel *et al.*, 2005). At 4 hpi, cells were treated with trypsin and cells were subsequently collected, fractionated to isolate nuclei and DNA was isolated as described above. qPCR analysis showed that actin depolymerization using cytD treatment significantly increased viral genome delivery of US3null PRV (*Figure 3B, right bar*). In addition, cytD treatment also, although not significantly, increased viral genome delivery efficiency of WT PRV (*Figure 3B, left bar*). Since the amount of viral genome delivery to the nucleus for US3null PRV was ± 50-60% of that of WT PRV (*Figure 3A*), and since addition of cytD led to a ± 7-fold increase in viral genome delivery of US3null PRV and a ± 4-fold increase for WT PRV(*Figure 3B*), addition of cytD in fact overcompensated for the lack of US3 and led to levels of viral

genome delivery that were similar for both WT and US3null PRV and reached roughly 4x the amount of viral genome delivery for WT PRV without cytD.

In conclusion, the actin depolymerizing drug cytD increases efficiency of PRV genome delivery to the nucleus, particularly in the absence of the viral F-actin depolymerizing US3 protein, suggesting a beneficial effect of F-actin breakdown during viral genome delivery to the nucleus.

Discussion

In this report, we show that the US3 protein kinase of PRV induces F-actin disassembly. Quantification of incoming nuclear viral genomes upon infection with WT and US3null PRV in the presence or absence of the actin depolymerizing drug cytD indicated that US3 and F-actin disassembly play a role during virus genome delivery to the nucleus.

Our findings are in line with studies on HSV-1, another alphaherpesvirus. Electron microscopy experiments indicated local cortical F-actin disassembly at the sites of HSV-1 entry (Maurer *et al.*, 2008) and drugs that affect actin dynamics were found to modulate HSV-1 entry (Zheng *et al.*, 2014). HSV-1-induced F-actin depolymerization was found to rely on activation of cofilin, a central mediator in actin dynamics (Zheng *et al.*, 2014). Interestingly, we recently showed that PRV US3 triggers activation of cofilin, further in line with our current findings (Jacob *et al.*, 2013).

We showed that PRV lacking US3 reaches the nucleus less efficiently than its WT counterpart. This suggests that the US3 tegument protein plays a previously unrecognized role during this aspect of virus entry. We hypothesize that this is due to US3-induced F-actin depolymerization since the F-actin depolymerizing drug cytD significantly increased delivery of the PRV genome at the nucleus. It has been reported for other viruses like HIV and SV40 that entry is associated with local F-actin depolymerization to overcome the cortical actin barrier, located just beneath the plasma membrane (Bukrinsky, 2008; Delorme-Axford & Coyne, 2011; Gordon-Alonso *et al.*, 2013; Pelkmans & Helenius, 2002; Pelkmans *et al.*, 2002; Taylor *et al.*, 2011; Yoder *et al.*, 2008). Although our current data may suggest that US3 has a similar function during PRV entry, our virus entry assay, which consisted of qPCR-based analysis of viral genome delivery at the nucleus, does not exclude that US3 may play a role in different aspects of viral entry preceding viral genome delivery to the nucleus, including capsid transport along microtubules.

Why is virus-induced F-actin depolymerization only significantly detectable from 3 hpi, while our results suggest that it may be triggered during viral entry? Probably, like for other viruses, F-actin disassembly occurs localized, at sites of virions entering the cells, which is also supported by electron microscopy studies on HSV-1 (Maurer *et al.*, 2008). Such localized F-actin disassembly is likely insufficient to be detectable by flow cytometry at early time points after infection.

Our findings on a viral structural protein that induces F-actin disassembly and that is involved in particular aspects of virus entry are in line with reports on HIV (Campbell *et al.*, 2004; Yoder *et al.*, 2008). For HIV, the viral causative factor for F-actin disassembly is the structural protein Nef, and, in line with our current data on US3, HIV variants lacking Nef show a virus entry defect (Campbell *et al.*,

2004; Dorfman *et al.*, 2002; Luo *et al.*, 1998). Further in line with our current data, disruption of the actin cytoskeleton by drugs that affect actin polymerization can complement the lack of Nef during entry (Campbell *et al.*, 2004). HIV Nef shows remarkable functional similarities with US3, including activation of p21-activated kinases (central molecules in Cdc42/Rac1 RhoGTPase signaling), disruption of actin stress fibers and formation of intercellular cell protrusions and, based on our current data, F-actin disassembly and contributing to virus entry (Lu *et al.*, 2008; Tan *et al.*, 2013; Xu *et al.*, 2009; Yoder *et al.*, 2008). So, although Nef is not a protein kinase and Nef and US3 do not show amino acid homology, both proteins appear to show considerable functional homology.

Despite our current data, ambiguities exist in literature on whether actin depolymerizing drugs are beneficial for viral infectivity, or rather inhibitory. This is also the case for alphaherpesviruses. Much of this confusion derives from the fact that actin polymerization and depolymerization play different roles during different steps of the viral replication cycle. There are strong indications, including our current report, that actin depolymerizing drugs can increase alphaherpesvirus entry and genome delivery to the nucleus (Mundy *et al.*, 2002; Zheng *et al.*, 2014). However, at later stages of infection, actin depolymerizing drugs can be inhibitory as alphaherpesviruses may rely on actin polymerization for viral egress and intercellular transport and spread (Dixit *et al.*, 2008; Elliott & O'Hare, 1997; Favoreel *et al.*, 2005; Xiang *et al.*, 2012). Very recently, it was shown that several F-actin depolymerizing drugs failed to affect intranuclear herpesvirus capsid motility (Bosse *et al.*, 2014), while this process was previously thought to be F-actin dependent (Feierbach *et al.*, 2006; Forest *et al.*, 2005). Hence, when analyzing the effect of actin destabilizing drugs on infection, timing is key to interpretation.

In conclusion, we report that PRV US3 triggers F-actin disassembly and that US3 and F-actin disassembly are involved in efficient delivery of the viral genome to the nucleus during virus entry.

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Chapter 6

General discussion

Chapter 6: General discussion

Herpesviruses, and viruses in general, have several reasons to interfere with the cellular actin cytoskeleton. When they enter cells through fusion, they need to transverse the cortical actin barrier, situated just below the cellular membrane. The same process may need to be repeated during egress. Furthermore, alphaherpesviruses have evolved a distinct way to facilitate viral spread. By inducing long, tubular protrusions that contain microtubules and actin, they can contact and spread to neighboring cells, thereby avoiding the harmful extracellular environment (Favoreel *et al.*, 2005). As Rho GTPases are central and conserved regulators of actin, it seems only natural that viruses evolved virulence factors targeting these GTPases. By deregulating Rho GTPase pathways, they adapt the actin cytoskeleton according to their needs.

US3 was identified in the second part of the '80s as a new viral serine/threonine protein kinase encoded by HSV-1 (Frame *et al.*, 1987), and soon after its discovery, its potential to function as a protein kinase was confirmed (McGeoch & Davison, 1986). US3 is conserved throughout the alphaherpesvirus subfamily, which is also reflected by a similar consensus phosphorylation sequence for US3 kinases from PRV, HSV-1, HSV-2 and VZV (Daikoku *et al.*, 1993; Eisfeld *et al.*, 2006; Leader, 1993; Leader *et al.*, 1991; Purves *et al.*, 1986). Among other effects, US3 of several alphaherpesviruses induces drastic cytoskeletal rearrangements in infected or transfected host cells, including actin stress fiber breakdown and/or the formation of the abovementioned protrusions (sometimes also referred to as filamentous processes) (Calton *et al.*, 2004; Favoreel *et al.*, 2005; Murata *et al.*, 2000; Schumacher *et al.*, 2005; Van Minnebruggen *et al.*, 2003).

Below, the results that were obtained in this thesis are discussed. First, our RhoA results are considered, and are linked to our earlier results showing US3 induced PAK activity. This leads to the hypothesis that PRV US3 inhibits RhoA, which has been reported for several other viruses. Finally, PKA-dependency of viral induced RhoA inhibition is discussed. Next, our cofilin results are reviewed, first focusing on the ambiguity existing in literature on whether activated PAK leads to cofilin activation or inactivation. We discuss possible phosphatases that could be involved in US3-mediated cofilin dephosphorylation and conclude this part by suggesting a potential RhoA involvement in cofilin activation. In the next part, our results concerning US3 induced disassembly of filamentous F-actin are discussed, which we link to viral entry, and is reported for other viruses. Cofilin activation is hypothesized as a possible factor in viral induced F-actin disassembly, and we elaborate on biphasic activation of cofilin during infection, which impedes interpretation of previous literature and underscores the importance of the stage of infection for assessing the cofilin activation status. Next, possible mechanisms for our results showing cofilin inactivation following infection with virus lacking

US3 will be discussed. Finally, the conclusions and importance of this research are highlighted, with emphasis on using our stable US3-induced phenotypes as a model system for the development of antiviral strategies, but also to study cell biological processes.

RhoA

Some years ago, our research team demonstrated that US3-mediated actin rearrangements occur predominantly through activation of group I p21 activated kinases (PAKs) (Van den Broeke *et al.*, 2009), downstream effectors of Rho GTPases Rac1 and Cdc42 (Hall, 2012). PAK-involvement was demonstrated a.o. by the use of the group I PAK inhibitor IPA-3 (Van den Broeke *et al.*, 2009), which almost completely abrogated US3-mediated actin rearrangements. These and other data led to the conclusion that US3 acts through the Cdc42/Rac1/PAK branch of Rho GTPase signaling (*Figure 1*, green '+' sign).

Interestingly, PAK and RhoA signaling are counteracting pathways that are interconnected through several feedback mechanisms, resulting in a network that is dynamically regulated in time and space (*Figure 1,* black double arrow)(Guilluy *et al.*, 2011). Negative feedback between both signaling branches has been reported in different cellular settings (Arthur *et al.*, 2002; Kozma *et al.*, 1997; Leeuwen *et al.*, 1997; Li *et al.*, 2002; Nimnual *et al.*, 2003; Parri & Chiarugi, 2010; Rosenfeldt *et al.*, 2006; Sander *et al.*, 1999; Sanz-Moreno *et al.*, 2008; Xie *et al.*, 2008; Yamaguchi *et al.*, 2001), although positive feedback mechanisms between RhoA and Rac1 signaling were also described (Liu *et al.*, 2009; Ridley *et al.*, 1992; Tsuji *et al.*, 2002).

A first question addressed in the current thesis was to investigate whether in addition to triggering PAK activity, US3 perhaps may also negatively affect RhoA signaling. This could provide for an optimal signaling environment for the dramatic actin rearrangements observed upon US3 expression (*Figure 1,* red '-' sign).

It was observed before that treatment of ST cells with an inhibitor against downstream RhoA effector ROCK (Y-27632) triggered actin rearrangements that were very similar to those induced by US3 (Favoreel *et al.*, 2005). A first real hint that US3 indeed may do more to Rho GTPase signaling than just activate PAK was the finding that cotransfection of dominant active mDia (downstream of RhoA) with PRV US3 led to a partial abrogation of US3-induced stress fiber breakdown and protrusion formation (unpublished results).

Using both PRV infection and US3-encoding plasmid transfection assays, we demonstrated a US3-induced RhoA phosphorylation at serine residue 188. This phosphorylation is linked with RhoA inhibition, as phosphorylated S188 can be recognized by RhoGDI which sequesters RhoA in the

cytoplasm. US3-induced RhoA inhibition seems to some extent in contrast to an earlier report, where co-expression of HSV-2 US3 with dominant active RhoA (RhoA V14) or dominant negative RhoA (RhoA N19) in HEp-2 cells did not affect US3-mediated actin rearrangements (Murata *et al.*, 2000). The latter findings imply that the activation state of RhoA is not important for US3-induced effects on the actin cytoskeleton. Importantly, S188 phosphorylated RhoA can be recognized by Rho GDI independent of its GTP-bound state (Lang *et al.*, 1996; Murthy *et al.*, 2003; Tamma *et al.*, 2003). So the contrasting results from Murata and colleagues could perhaps be explained by US3-induced altered RhoA localization, rather than activation status *per se*. However, while we reported an inhibitory effect of recombinant RhoA overexpression on US3-induced actin rearrangements, Murata and colleagues did not (Murata *et al.*, 2000). Potentially, their use of HEp-2 cells, which is a human laryngeal carcinoma cell line, is the cause for this difference. We think that RhoA overexpression may affect US3-induced actin rearrangements through negative feedback on PAK-activity. As PAK copy numbers are increased in several cancer types (Ong *et al.*, 2011), this could very well compensate for negative feedback effects of RhoA overexpression in HEp-2 cells.

We performed Rhotekin assays, which can be used to specifically pull down GTP-bound RhoA, and found no differences in active RhoA levels between mock, WT and US3null infected cell lysates (unpublished results). This was in line with the hypothesis that US3 does not affect the activation status of RhoA. Attempts to investigate potential effects of US3 on RhoA localization using immunofluorescence were too variable to draw meaningful conclusions. Further research could be performed through Western blot analysis of cytosolic and membrane fractions of infected cells obtained using ultracentrifugation, as reported in other RhoA localization studies (Lang *et al.*, 1996; Oishi *et al.*, 2012; Rolli-Derkinderen *et al.*, 2005).

In line with our data, several other viruses have been reported to interfere with RhoA signaling. For example, human cytomegalovirus (HCMV) (Frampton *et al.*, 2007), simian virus 40 (SV40) (Stergiou *et al.*, 2013), hepatitis C virus (HCV) (Brazzoli *et al.*, 2008), Epstein Barr virus (EBV) (Loesing *et al.*, 2009), human immunodeficiency virus (HIV-1) (del Real *et al.*, 2004) and vaccinia virus (VV) (Valderrama F, 2006) have all been reported to suppress RhoA signaling at some point in their replication cycle. VV and HIV both induce actin rearrangements that strongly resemble those observed with US3, including actin stress fiber disassembly and protrusion formation (Lu *et al.*, 2008; Valderrama *et al.*, 2006). The viral proteins that are responsible for this are VV F11 and HIV Nef. VV F11 binds RhoA and inactivates it through activation of Rho GAP Myosin 9A (Handa *et al.*, 2013), while HIV Nef interacts with diaphanous interacting protein (DIP), which activates Vav and p190RhoGAP, leading to Rac1 activation and RhoA inhibition (Lu *et al.*, 2008). Hence, it appears that evolutionary distinct viruses have evolved similar infection strategies to overcome some of the host barriers to infection.

We found that PRV US3-induced RhoA phosphorylation is PKA-dependent. This is in line with several other reports showing PKA-mediated S188 RhoA phosphorylation in several cell types (Dong *et al.*, 1998; Ellerbroek *et al.*, 2003; Jones & Palmer, 2012; Lang *et al.*, 1996; Lapetina *et al.*, 1989; Quilliam *et al.*, 1991; Tkachenko *et al.*, 2011). Interestingly, RhoA modulation through PKA can occur at several levels. Aside from phosphorylating RhoA at S188, leading to suppressed RhoA signaling through recognition by RhoGDI, PKA can also induce RhoGDI phosphorylation at S174, which increases the binding affinity between RhoA and RhoGDI (Oishi *et al.*, 2012). So US3-mediated PKA activation could regulate effective RhoA inactivation, by creating a recognition motive on RhoA for RhoGDI and making sure it stays bound during sequestration by phosphorylating RhoGDI itself.

Before our current findings, only human papillomavirus 16 protein E7 was reported to induce PKA-dependent RhoA inhibition (Cardone *et al.*, 2008). However, many viruses appear to induce PKA activity upon infection, such as HCV (Colpitts *et al.*, 2015), HIV (Oyeyemi *et al.*, 2015), dengue virus (Tsai *et al.*, 2014), adenovirus (Scherer *et al.*, 2014), astrovirus (Tange *et al.*, 2013), KSHV (Sharma-Walia *et al.*, 2010), HCMV (Yuan *et al.*, 2009), rotavirus (Rossen *et al.*, 2004), HBV (Tacke *et al.*, 2005), and also alphaherpesviruses like VZV (Desloges *et al.*, 2008; Erazo *et al.*, 2011) and HSV-1 (Benetti & Roizman, 2004). Interestingly, as we found for PRV, HSV-1- and VZV-induced PKA activation is US3-dependent (Benetti & Roizman, 2004; Daikoku *et al.*, 1993; Eisfeld *et al.*, 2006; Kato *et al.*, 2009). As we demonstrated for PRV, Western blot assays showed an upregulation of phosphorylated PKA substrates upon infection with VZV, HSV-1 or PRV, which was largely diminished upon infection with corresponding viruses lacking US3 (Erazo *et al.*, 2011).

The mechanism behind US3-mediated activation of PKA is still unclear, but for HSV-1, it has been suggested that US3 triggers phosphorylation at the α-position of regulatory subunit II of PKA (Poon *et al.*, 2006), where cAMP-binding can induce a conformational change which leads to PKA activation (Murray, 2008). Possibly, US3-phosphorylation at this cAMP-binding position induces the conformational change that is required to release the PKA catalytic subunits, hence leading to PKA activation.

Cofilin

Signaling from Rho GTPases converges to downstream effector proteins, ultimately eliciting an effect on actin. Important effector proteins of Rho GTPase signaling are actin binding proteins cofilin, Arp2/3 and WASP family proteins. US3 interference with these actin effector proteins likely would affect actin polymerization or depolymerization processes. Evidence for Arp2/3 mediated activation through WASP during viral infection is relatively limited but has been reported to play a role in HIV receptor clustering and fusion pore formation during entry (Harmon *et al.*, 2010) and VV-induced actin

polymerization (Dodding & Way, 2009; Moreau *et al.*, 2000). We did not investigate whether US3 affects WASP and/or Arp2/3, an item that certainly may merit attention in future research.

We did demonstrate that US3 induces dephosphorylation of cofilin at serine residue S3, a hallmark of cofilin activation. Cofilin, a member of the actin depolymerizing factor (ADF)/cofilin family, is a central player in actin dynamics and its activity is regulated through phosphorylation/dephosphorylation at S3 (Moriyama *et al.*, 1996). We found that cofilin dephosphorylation and activation is involved in US3-mediated actin rearrangements, since expression of a phosphomimetic cofilin mutant interfered with the ability of US3 to trigger stress fiber disassembly and cell projection formation. In line with our findings illustrating the role of active cofilin in US3-induced actin rearrangements, cofilin activation was also described for the growth regulation of filopodia and dendrites (Chen *et al.*, 2006; Gehler *et al.*, 2004).

Using the group I PAK inhibitor IPA-3, we found that US3-triggered cofilin activation relies on group I PAK activity. However, the most accepted 'default' pathway in literature is that activated PAK leads to LIMK activation and subsequent cofilin inactivation (Kobayashi *et al.*, 2006; Li *et al.*, 2006; Scott & Olson, 2007; Van Troys *et al.*, 2008; Zoudilova *et al.*, 2007). More recently however, other groups have reported cofilin activation following PAK activation (Cameron *et al.*, 2010; Coniglio *et al.*, 2008; Pandey *et al.*, 2009). As PAKs are kinases, we assume that PAK activates a phosphatase in order to regulate cofilin activation. Cofilin activation is regulated by phosphatases such as slingshots (SSH), chronophin (CIN) and phosphatase 1 and 2A (PP1/PP2A) (Gohla *et al.*, 2005; Huang *et al.*, 2006; Niwa *et al.*, 2002; Oleinik *et al.*, 2010; Westphal *et al.*, 1999) (*Figure 1*).

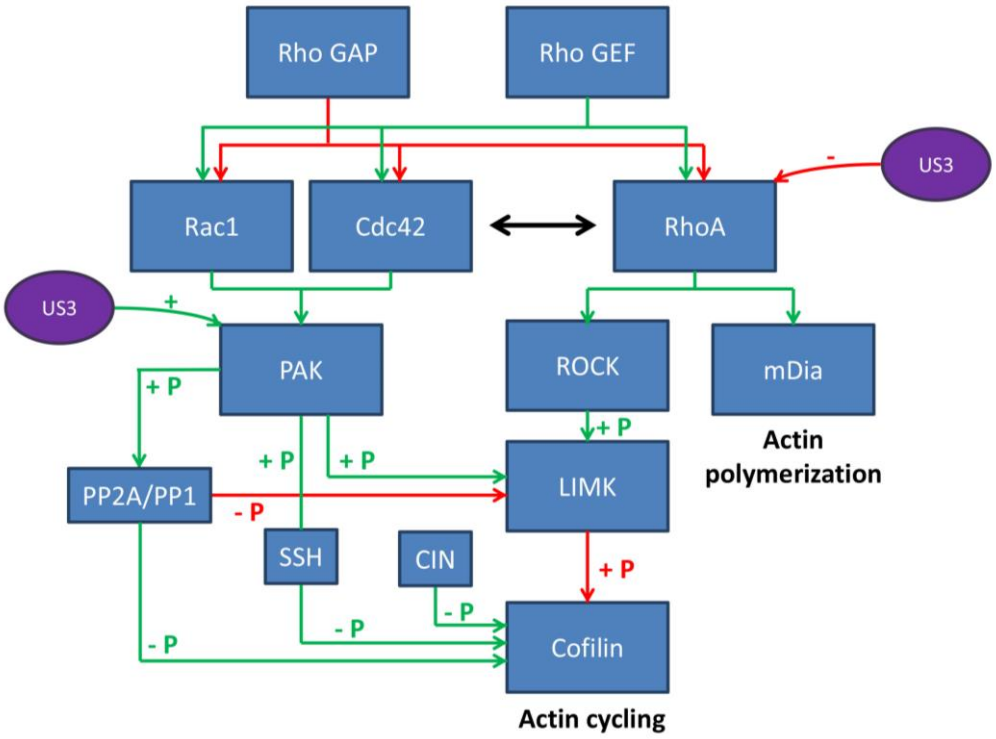

Figure 1: Downstream signaling from Rho GTPases towards cofilin regulation mechanisms. + P = phosphorylation; - P = dephosphorylation; green arrow = activating effect; red arrow = inhibitory effect. US3 interacts with and activates PAK (+) to induce actin rearrangements (Van den Broeke *et al.*, 2009) and, as demonstrated in the current thesis, also suppresses the RhoA signaling (-) to generate these actin rearrangements.

Unfortunately, we currently do not know which phosphatase is involved in PAK-mediated cofilin activation, but there are some indications pointing towards the involvement of slingshot phosphatases. Heregulin and thrombin treatment both have been described to induce PAK-dependent cofilin activation through activation of cofilin phosphatase SSH1L (Leonard *et al.*, 2013; Nagata-Ohashi *et al.*, 2004). In addition, and in line with the requirement of active cofilin for US3-mediated actin rearrangements, insulin treatment activates cofilin through SSH1L which is associated with the formation of cellular protrusions in 293T cells, MCF-7 and PC12 cells (Meberg *et al.*, 1998; Nishita *et al.*, 2004). Active cofilin seemed to accumulate in these insulin-induced membrane protrusions, indicating the importance of active cofilin in protrusion formation (Nishita *et al.*, 2004). Preliminary experiments using antibodies against both phosphorylated and total SSH isoforms did not lead to any conclusions regarding a possible involvement of slingshot phosphatases in US3-mediated cofilin activation thus far, as these antibodies did not react with SSH isoforms from our porcine ST cells (unpublished results).

However, other cofilin phosphatases may also be involved. For example, there are indications for the involvement of phosphatase PP2A in PAK-mediated cofilin activation. PAK1 is able to form a complex

with and activate PP2A (Ke *et al.*, 2004; Westphal *et al.*, 1999) and based on inhibitor studies, PP1 and PP2A can cause cofilin dephosphorylation (Oleinik *et al.*, 2010). We tried to investigate the involvement of PP1 and PP2A in US3-mediated cofilin activation through treatment of WT PRV and US3null PRV infected cells using okadaic acid, which inhibits both PP2A and PP1 and using cantharidin, which inhibits PP2A. Unfortunately, these inhibitors did not lead to conclusive data, as cantharidin treatment did not abrogate US3-mediated cofilin dephosphorylation and okadaic acid treatment generated variable results (unpublished results). The cantharidin experiments argue against a role for PP2A in US3-mediated cofilin activation. The variable results observed with okadaic acid could potentially be explained by the possible involvement of PP1 or PP2A phosphatases in dephosphorylation and inactivation of LIMKs (Vorster *et al.*, 2011). Hence, treatment with okadaic acid may affect cofilin phosphorylation status both directly and indirectly (via LIMKs), which may lead to variable outcomes. The combined use of several inhibitors and more specific approaches like siRNA will probably be needed to elucidate the role of PP1 and PP2A in US3/PAK-mediated cofilin dephosphorylation.

When addressing the US3-PAK-cofilin link in detail, our data pointing to US3-mediated interference with RhoA signaling should also be taken into account. Indeed, the effects of US3 on RhoA may also influence the cofilin phosphorylation status, as downstream RhoA effector ROCK can activate LIMK, and cause cofilin inactivation (Lin *et al.*, 2003). Indications for a possible RhoA involvement in cofilin dephosphorylation were deduced from our experiments where treatment of ST cells with the ROCK inhibitor Y-27632, which triggers actin rearrangements very similar to those observed with US3, induced cofilin dephosphorylation (unpublished results).

US3-induced disassembly of filamentous F-actin

Since alphaherpesviruses typically enter host cells via direct fusion of their envelope with the plasma membrane, although viral entry via endocytic pathways has also been described, this process results in delivery of capsid and tegument just beneath the plasma membrane. The cortical filamentous F-actin layer is located just below the plasma membrane and forms a physical barrier for virus particles (Radtke *et al.*, 2006; Taylor *et al.*, 2011). Other viruses that enter host cells via direct fusion at the plasma membrane, like HIV, have been reported to (locally) disrupt the cortical F-actin layer to allow access of incoming virus particles to the cytoplasm (Campbell *et al.*, 2004; Yoder *et al.*, 2008). In addition, for HSV-1, transmission electron microscopy tomography experiments have indicated that F-actin may be locally disrupted at sites where virions enter the host cell (Maurer *et al.*, 2008).

While US3 has been reported to trigger actin rearrangements, it does not affect total protein levels of actin (Van Minnebruggen *et al.*, 2003). However, it remained uninvestigated whether US3 modulates filamentous F-actin levels and if US3 contributes to viral entry in host cells. We report a US3-induced

net F-actin disassembly, which could already be detected early in infection. As we demonstrated a role for US3 in viral genome delivery to the nucleus, a process that we found to be also enhanced by addition of the actin depolymerizing drug cytochalasin D, our results suggest that US3 plays a previously unrecognized role in viral entry via depolymerization of the cortical F-actin barrier.

Likewise, other viruses have also been reported to depolymerize actin in order to facilitate their entry. Early F-actin depolymerization was reported for HIV and HSV-1 and coincided with and increased viral penetration (Campbell *et al.*, 2004; Maurer *et al.*, 2008; Yoder *et al.*, 2008; Zheng *et al.*, 2014). Interestingly, HSV-1 and EHV-1 both have been reported to induce a late decrease in F-actin (Mingo *et al.*, 2012; Slonska *et al.*, 2014), which may enhance viral egress, since it has been proposed that the cortical actin barrier needs to be breached again late in infection, to allow viral exocytosis (Sattentau, 2008). Hence, it may be interesting to investigate whether US3-triggered F-actin disassembly also contributes to viral egress.

Currently, we do not know which cellular factors are involved in US3-mediated F-actin disassembly. Considering the findings in this work, this could occur through US3-mediated PAK activation and/or RhoA inhibition, or through other currently unidentified US3 targets. Irrespective of the signaling pathway involved, US3-mediated activation of cofilin, as we reported here, may be involved in F-actin disassembly. In support of this, cofilin and cortical F-actin disassembly have been linked earlier (Fritzsche *et al.*, 2013; Tinevez *et al.*, 2009). Expression of constitutively active S3A cofilin leads to a reduction in cortical F-actin, while constitutively inactive cofilin S3E leads to an increase in F-actin (Fritzsche *et al.*, 2013). Local cortical F-actin disassembly at the sites of HSV-1 entry was also found to rely on activation of cofilin (Zheng *et al.*, 2014). Correspondingly, HIV entry studies were also able to link F-actin depolymerization to cofilin activation (Yoder *et al.*, 2008).

Nevertheless, there seem to be conflicting reports in literature on whether cofilin is activated or inactivated during (alphaherpes)virus infection. When assessing activation status of cofilin or any other actin-regulating protein, it is important to take into account the particular stage of infection, as viruses may rely on both actin polymerization and depolymerization during their infection cycle. Indeed, for alphaherpesviruses, actin depolymerizing drugs can increase alphaherpesvirus entry and genome delivery to the nucleus on the one hand (Mundy *et al.*, 2002; Zheng *et al.*, 2014), but can be inhibitory later in infection as alphaherpesviruses may rely on actin polymerization for their egress, intercellular transport and spread (Dixit *et al.*, 2008; Elliott & O'Hare, 1997; Favoreel *et al.*, 2005; Xiang *et al.*, 2012). In line with this, both in HSV-1 and HIV, cofilin seems to be regulated in a biphasic manner: cofilin is phosphorylated (inactivated) very early in infection (Vorster *et al.*, 2011; Zheng *et al.*, 2014), possibly playing a role in actin stabilization during receptor clustering (Jimenez-Baranda *et al.*, 2007; Stolp &

Fackler, 2011; Zheng *et al.*, 2014), and is then dephosphoryled (activated) at later time points, potentially allowing actin rearrangements to facilitate crossing of the cortical actin barrier (Cameron *et al.*, 2010; Stolp & Fackler, 2011; Xiang *et al.*, 2012; Yoder *et al.*, 2008; Zheng *et al.*, 2014). Furthermore, while F-actin depolymerization could be linked to a local weakening of the host barrier, the same could result from local F-actin polymerization, as this leads to a restructured barrier (Cameron *et al.*, 2010; Vorster *et al.*, 2011; Xiang *et al.*, 2012; Yoder *et al.*, 2008; Zheng *et al.*, 2014). This is the case for HIV fusion pore formation, which relies on actin polymerization and increases viral entry (Harmon *et al.*, 2010). In short, a dynamic cytoskeleton is often required in order to facilitate viral infection, and when analyzing the signaling pathways that are involved, one needs to keep in mind that timing is key to interpretation.

We could observe US3-induced cofilin dephosphorylation from the first time point that we analyzed, 2 hpi. Likewise, HSV-1 infection also induced cofilin dephosphorylation at 2 hpi (Xiang *et al.*, 2012), whereas the authors observed cofilin phosphorylation very early in infection, already from 10 minutes post inoculation (Zheng *et al.*, 2014). It would be interesting to investigate whether PRV US3-mediated cofilin dephosphorylation is also preceded by an early cofilin phosphorylation or inactivation, and if so, which viral proteins are involved and what the biological consequences are of early cofilin inactivation. Since, as indicated above, rapid cofilin inactivation and consequent decreased actin remodeling could contribute to receptor clustering and since subsequent cofilin activation and actin filament fragmentation could loosen the actin cortex and increase viral penetration, this mechanism could be usurped by more pathogens than currently known. Interestingly, and possibly in line with an ability of PRV to cause cofilin inactivation, we did observe PRV-induced cofilin inactivation upon infection with virus lacking US3 or containing kinase deficient US3. Hence, in the absence of the viral cofilin activating protein US3, PRV infection was associated with cofilin inactivation. It is unclear at this stage whether this cofilin inactivation process occurs before the onset of US3-mediated cofilin activation, and whether it is of biological relevance for the virus or host. Hypothetically, cofilin inactivation upon infection could be explained by a cellular stress response and subsequent phosphorylation of cofilin, which is counteracted by US3. Indeed, other cellular stress stimuli have been reported to lead to increased S3 cofilin phosphorylation, including heat shock (Simard *et al.*, 2011), fluid shear stress (Fu *et al.*, 2008; Liu *et al.*, 2010; Slee & Lowe-Krentz, 2013), hyperosmotic stress (Thirone *et al.*, 2009) and scavenging of reactive oxygen species (Popova *et al.*, 2010). All these stress stimuli were reported to increase LIMK1 activity, leading to cofilin inactivation. Hence, stress responses lead to a decreased actin severing activity of cofilin, which could help to preserve the cellular F-actin network and promote cell survival. In this way, cofilin phosphorylation and inactivation could be beneficial for the virus.

However, cofilin phosphorylation could also result from a cellular antiviral response. Protein kinase R (PKR) could play a role in this antiviral response, as it is activated by dsRNA (Lemaire *et al.*, 2008), and its activation induces a LIMK-mediated cofilin phosphorylation (Xu *et al.*, 2012), which leads to decreased cell migration. The latter might play a role in antiviral responses, as decreased cell migration could limit virus spread. Further in support of the antiviral response hypothesis, we noticed that phosphorylated cofilin was predominantly located in the cellular nucleus following infection (unpublished results). This is especially interesting, as cofilin has been reported to play a role in transcription (Obrdlik & Percipalle, 2011). Hence, cofilin inactivation might lead to suppressed transcription and potentially may play a role in obstructing virus replication. Hence, it would be interesting to further investigate the mechanism behind alphaherpesviral induced cofilin phosphorylation and whether or not this is detrimental for the virus.

The outcome of this thesis is summarized in *Figure 2*. It will be interesting to further identify US3 binding partners, to be able to fully understand the complete picture of US3 interference within the complex and interwoven Rho GTPase signaling pathways.

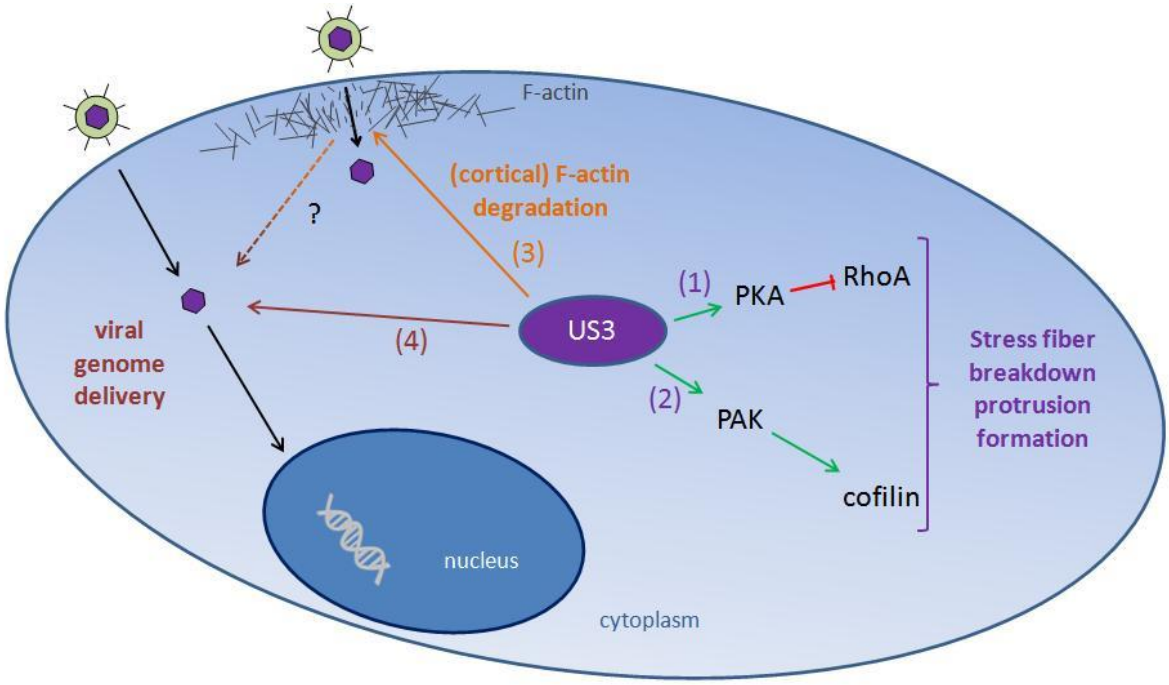

Figure 2: Summary of the three research lines of this thesis. (1) US3 phosphorylates and potentially inhibits RhoA through PKA. (2) US3 activates cofilin by a PAK-dependent mechanism. Both these processes contribute to US3-induced actin rearrangements. (3) US3 leads to F-actin degradation, likely contributing to (4) US3-mediated increased viral genome delivery.

Conclusions/importance

US3 is an early protein, that is expressed from very early time points post infection (detectable by Western blot from 2 hpi or earlier) and its expression levels increase gradually during the course of infection. In every one of our three studies, we were able to demonstrate a constant and stable US3-induced phenotype. US3 induces a RhoA phosphorylation from 2 hpi, (nearly) steadily reaching a plateau at 6 hpi, after which phosphorylated RhoA levels remain high and unchanged until the end of our time course study at 16 hpi. Likewise, cofilin phosphorylation levels decrease from 2 hpi and remain low until the last detection point at 24 hpi. And finally, US3-induced F-actin disassembly is induced from 2 hpi, gradually becoming more pronounced at later timepoints until the end of our observations at 6 hpi. These very stable and progressive phenotypes could not only be used as model systems to study viral signaling processes, but may also be useful in cell biology to unravel cellular signaling pathways associated with the actin cytoskeleton. Indeed, physiological signaling processes, including actin-regulating signaling, are typically temporal, making it much more difficult to e.g. identify signaling complexes and particular effects on the cytoskeleton and cellular behavior.

We and others have shown that RhoA, cofilin and PAK and their interconnecting signalization branches might represent promising targets for the development of antiviral strategies, as they are involved in viral entry and spread. For instance, small peptide derivates of RhoA are currently being tested as fusion inhibitors against RSV and HIV (Maselko *et al.*, 2011; Pastey *et al.*, 1999; Pastey *et al.*, 2000). However, this approach may be particularly interesting for alphaherpesviruses, as symptoms are mostly treated topically, limiting systemic overreactions to these drugs, or toxicity, which should be considered seen the conserved nature of Rho GTPases and their involvement in many cellular processes. Moreover, Rho GTPases and their effectors such as RhoA, Cdc42, Rac1, PAK, ROCK, LIMK and cofilin are involved in cancer, cardiovascular diseases, hyperaemia, metabolic disorders, asthma, Alzheimer disease and many more (Bamburg *et al.*, 2010; Chiba *et al.*, 2010; Dong *et al.*, 2010; Gong *et al.*, 2012; Grise *et al.*, 2009; Kume, 2008; Martin *et al.*, 2014; Mashiach-Farkash *et al.*, 2012; Montalvo-Ortiz *et al.*, 2012; Mu *et al.*, 2015; Murray *et al.*, 2010; Noda *et al.*, 2014; Ohashi *et al.*, 2014; Patel *et al.*, 2012; Patel *et al.*, 2014; Prudent *et al.*, 2012; Prudnikova *et al.*, 2015; Radu *et al.*, 2014; Rosenblatt *et al.*, 2011; Subramanian *et al.*, 2015; Van de Velde *et al.*, 2014; Yin *et al.*, 2015; Zins *et al.*, 2013a; Zins *et al.*, 2013b). Hence, Rho GTPase signaling pathways offer great potential for the development of inhibitors or activators. However, in order to limit toxicity and side effects of these products, it is important to fully understand the signaling pathways involved in particular disease complexes.

The process of elucidating the molecular details of Rho GTPases signaling pathways remains a challenge: a combination of affinity chromatography, protein purification and yeast-two hybrid

screening revealed over 100 potential Rho GTPase targets (Bishop & Hall, 2000). Crosslinking between different signaling pathways, leading to positive of negative feedback even increases the difficulty of mapping and understanding the different interactions and their effects. The stability of our US3-induced phenotypes may perhaps contribute to the unraveling of these signaling pathways, using inhibitors/activators and/or siRNA against several targets, and monitor the US3-effect as readout for the outcome.

For instance, the stable process of US3-mediated protrusion formation and our finding that this phenotype involves both RhoA inhibition and PAK activation may allow to better characterize the feedback systems between these two signaling pathways. We have indications that these pathways are interconnected and do not function independently during the US3-triggered actin rearrangements, as treatment with class I PAK inhibitor IPA-3 does not lead to partial inhibition of US3-induced actin rearrangements but to virtually complete abrogation (Van den Broeke *et al.*, 2009). This suggests that PAK is central in the US3-induced actin rearrangements, and hence that US3-mediated suppression of RhoA signaling probably also culminates in modulation of PAK activity. A hypothetical model could be set up to explain this. RhoA signaling is known to trigger mDia activation. Interestingly, phosphatase POPX2 binds with active mDia and plays a role in stress fiber maintenance (Xie *et al.*, 2008) and has been reported to suppress activity of PAK (Koh *et al.*, 2002), indicative for a RhoA-POPX2-PAK negative feedback signaling. Hence, hypothetically, US3-mediated inhibition of RhoA signaling could disrupt this negative feedback signaling and prevent suppression of PAK activity. Possibly in support of this, we found that triple transfection of cells with US3, dominant active mDia and phosphatase deficient POPX2, hence POPX2 that is not able to inactivate PAK, partially reversed the inhibitory effect of dominant active mDia on US3-mediated actin rearrangements (unpublished results). In any case, exploiting US3-induced actin rearrangements, RhoA phosphorylation and/or cofilin dephosphorylation in combination with dominant active/negative signaling proteins, signaling inhibitors and/or siRNA could help further elucidate Rho GTPase signaling pathways in general.

US3-induced actin rearrangements can also be used as a model system to identify the signaling pathways that are involved in cellular protrusion formation. US3-induced actin protrusions resemble to some extent lamellipodia and filopodia, but also show important differences, as the latter protrusions lack microtubules. Other protruding actin structures that do contain microtubules are invadopodia, nanotubes interconnecting cells, podocytes and neuronal dendrites (Kobayashi *et al.*, 2004; Onfelt *et al.*, 2006; Schoumacher *et al.*, 2010). US3-induced actin rearrangements could therefore be used to study conserved processes in the formation of protrusions, and this knowledge could possibly be extrapolated to the formation of neurites, invadopodia, podocytes and nanotubes. US3-expression could be used to induce protrusions in a series of different cell types, perhaps

accompanied by inhibitors/siRNA, in order to investigate whether PAK and/or cofilin, or other actin regulators play a conserved role in the formation of these cell projections.

Furthermore, as also mentioned higher, we noticed an increased nuclear localization of phosphorylated (inactive) cofilin following PRV infection (unpublished results). While the occurrence and biological role of filamentous actin in physiological processes is still a matter of debate, herpesviruses have been reported to induce nuclear actin filaments (Feierbach *et al.*, 2006), although the biological consequences as well as the mechanisms behind it are unknown. It would be interesting to determine whether nuclear cofilin plays a role in the generation of these nuclear actin filaments, which could help unravel their biological significance.

In conclusion, the results of this thesis have shed new light on the interaction between the conserved alphaherpesvirus US3 protein and Rho GTPase signaling, providing evidence for US3-mediated RhoA phosphorylation, cofilin activation, F-actin disassembly and enhanced viral entry. We have demonstrated reliable US3-induced phenotypes, which not only could help unravel virological processes and possibly identify new targets for antiviral therapy, but may also serve as an important tool to study cell biological processes.

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Summary

Summary

US3 is a serine/threonine kinase conserved throughout the alphaherpesvirus subfamily. This viral kinase is responsible for dramatic changes in the actin cytoskeleton of the host cell, consisting of stress fiber disassembly and the formation of actin-based protrusions. These protrusions are associated with enhanced viral cell-to-cell spread and are dependent on US3-mediated phosphorylation and activation of p21 activated kinases (PAKs). The general aim of this study was to obtain better insights in the mechanism and biological consequences of pseudorabies virus (PRV) US3-mediated actin rearrangements, which was investigated through three specific studies.

The **first chapter** gives an introduction on PRV, along with its viral structure and replication cycle. An overview of PRV-induced Aujeszky's disease and its economic impact is given, followed by reasons to use PRV as a model organism to study alphaherpesvirus biology. US3 is elaborately introduced, along with its functions, followed by a thorough section concerning the actin cytoskeleton, where the most important actin filament based structures are highlighted. Furthermore, Rho GTPase signaling, with special attention for the RhoA- and PAK-pathway and their downstream effectors, including the WASP superfamily and ADF/cofilin family is reviewed. Finally, the current knowledge on alphaherpesvirus interactions with the actin cytoskeleton and Rho GTPases during viral entry and egress is described.

The **second chapter** describes the aims of this study.

In the **third chapter**, we showed that the US3 kinase of PRV affects the RhoA signaling pathway to mediate stress fiber breakdown and protrusion formation. To induce these cytoskeletal rearrangements, US3 was described earlier to phosphorylate and activate p21 activated kinases (PAKs), which are able to counteract RhoA signaling and vice versa. RhoA phosphorylation on serine residue 188 (S188) is an important RhoA inactivation mechanism through its sequestration to the cytoplasm by RhoGDI. We demonstrated a US3-mediated RhoA S188 phosphorylation following PRV infection as well as transfection of US3 in ST cells. Furthermore, co-expression of US3 with non-phosphorylatable S188A RhoA in ST cells caused a suppression on US3-induced actin rearrangements compared to co-expression of wild type RhoA with US3, underscoring the importance of US3-triggered RhoA S188 phosphorylation for these rearrangements. Kinase assays indicated the US3 probably does not directly phosphorylate RhoA S188, but may activate a cellular kinase that phosphorylates RhoA instead. Cellular protein kinase A (PKA) has been reported earlier to phosphorylate RhoA at S188 and the HSV-1 and VZV US3 orthologs have been found earlier to trigger PKA activation. Here, we found indications that PRV US3 may also trigger PKA activation, since detection of phosphorylated PKA substrates on Western blot showed substantially increased protein bands following inoculation with

WT PRV compared to US3null PRV. Importantly, treatment with PKA inhibitor PKI during infection abrogated PRV-induced RhoA phosphorylation, indicating that PRV US3 indirectly triggers RhoA phosphorylation on S188 via PKA, a process that is involved in US3-mediated actin rearrangements.

In the **fourth chapter**, we investigated how PRV US3-mediated effects on actin-controling cell signaling translates to downstream actin regulators. A central player in actin dynamics is cofilin, which is activated through dephosphorylation on serine residue 3 (S3). ST cells infected with WT PRV showed decreased phospho-S3 cofilin levels, while this was not the case for US3null PRV and mock-infected cells. Further supporting US3-mediated cofilin dephosphorylation, transfection of ST cells with US3 and consecutive staining with phospho-S3 cofilin antibody demonstrated suppressed phospho-S3 cofilin levels in transfected cells. The kinase activity of US3 was required to suppress phosphorylation of cofilin, as infection with kinase-deficient D223A Be PRV was not able to suppress phospho-S3 cofilin levels, in contrast to WT Be PRV. Unexpectedly, we found that infection with both US3null and D223A PRV led to increased phospho-S3 cofilin levels compared to mock-infected cells, which could indicate currently unknown biological consequences of viral infection and might be interesting to further investigate. Overexpression of US3 together with constitutively inactive S3D or constitutively active S3A cofilin mutants allowed us to demonstrate the involvement of cofilin dephosphorylation in PRV US3-induced actin rearrangements, as phosphomimetic S3D cofilin suppressed US3-induced actin rearrangements in ST cells, while wild type or S3A cofilin did not. Interestingly, group I PAKs are involved in US3-induced cofilin dephosphorylation, as treatment with group I PAK inhibitor IPA-3 restored the phospho-S3 cofilin signal in PRV-infected cells, while leaving mock-infected cells unaffected, supporting recent evidence for a signaling axis that connects PAK activation to cofilin dephosphorylation. In conclusion, we report that PRV US3 leads to S3 dephosphorylation (activation) of cofilin, which contributes to US3-mediated actin rearrangements.

In the **fifth chapter**, we further investigated the biological consequences of US3-mediated actin rearrangements. LifeAct is a small peptide capable to bind filamentous (F)-actin in a non-disturbing, low-affinity, highly specific manner and thereby represents a suitable marker to (quantitatively) determine changes in F-actin in living cells. Using flow cytometric analysis of LifeAct transduced ST cells, we found that infection with WT PRV induces F-actin disassembly from 2 hours post inoculation (hpi), becoming more pronounced at later timepoints until the end of our observations at 6 hpi, while this was not the case for infection with US3null PRV. Furthermore, the kinase activity of US3 was required for F-actin disassembly, as infection with kinase-deficient D223A Be PRV could not induce decreased F-actin levels. As F-actin disassembly promotes viral entry in HIV, we wanted to investigate whether US3 also contributes to nuclear delivery of viral genomes. Therefore, qPCR assays were performed on nuclear fractions of infected cells, revealing that US3null virus DNA reached the nucleus

significantly less efficiently in comparison to WT virus DNA, indeed showing a role for US3 during viral genome delivery to the nucleus. Addition of cytochalasin D not only significantly increased viral genome delivery of US3null PRV, but also increased viral genome delivery of WT PRV, showing that actin depolymerization in fact overcompensates for the lack of US3, pointing towards a beneficial effect of F-actin breakdown during viral genome delivery to the nucleus. Taken together, the PRV US3 kinase induces F-actin disassembly and plays a role in viral genome delivery to the nucleus. As our findings show that actin depolymerization leads to increased viral genome delivery to the nucleus, our data suggest that US3-induced F-actin disassembly plays a role during virus entry in host cells.

The **sixth chapter** contains a general discussion on the results that were obtained in this thesis.

Conclusion

Previous work from our research group showed that PRV US3 phosphorylates and activates PAKs, downstream effectors of Rho GTPases Rac1/Cdc42, which plays a central role in US3-mediated actin stress fiber breakdown and protrusion formation. Here, we found that, in addition, US3 also regulates actin stress fiber breakdown and protrusion formation through phosphorylation of RhoA on serine 188, which leads to RhoA inactivation through RhoGDI. We also found that downstream US3 signalization leads, via a PAK-dependent mechanism, to cofilin dephosphorylation and activation, which is important for US3-induced actin rearrangements. In addition, we have shown that US3 induces a kinase dependent disassembly of filamentous (F)-actin and plays a previously uncharacterized role in nuclear delivery of viral genomes during entry. Treatment with actin depolymerizing drug cytochalasin D increased nuclear delivery of PRV, supporting a role for F-actin disassembly during viral entry.

Samenvatting

Samenvatting

US3 is een serine/threonine kinase dat geconserveerd is binnen de alfaherpesvirus subfamilie. Dit viraal kinase is verantwoordelijk voor veranderingen in het actine cytoskelet van de gastheercel, zoals stressvezel afbraak en vorming van celuitlopers. Deze celuitlopers dragen bij tot het cel-tot-cel spreiden van het virus en worden gevormd onder invloed van US3-gemedieerde fosforylatie en activatie van p21-geactiveerde kinasen (PAKs). De doelstelling van dit onderzoek was om een beter inzicht te verkrijgen in het mechanisme en de biologische consequenties van de actineveranderingen die veroorzaakt worden door US3 van het pseudorabies virus (PRV). Dit werd onderzocht door middel van drie specifieke studies.

In het **eerste hoofdstuk** wordt een introductie gegeven over PRV, meer specifiek over de virale structuur en replicatiecyclus van PRV. Er wordt een introductie gegeven rond de ziekte van Aujeszky, die veroorzaakt wordt door PRV, en de economische impact hiervan, waarna wordt overgegaan naar het gebruik van PRV als modelsysteem voor de studie van alfaherpesvirus biologie. Hierna wordt het viraal kinase US3 grondig toegelicht, samen met zijn verschillende functies, gevolgd door een uiteenzetting van het actineskelet en de belangrijkste structuren gevormd door actinefilamenten. Rho GTPases worden nader besproken, met speciale aandacht voor RhoA- en PAK-signalisatie en hun downstream effectoren zoals WASP en ADF/cofilin. Tenslotte wordt de huidige stand van zaken in verband met de interacties tussen alfaherpesvirussen en het actine cytoskelet en Rho GTPases tijdens virus binnenkomst in en uittrede uit gastheercellen besproken.

In het **tweede hoofdstuk** worden de doelstellingen van dit werk geschetst.

In het **derde hoofdstuk** wordt aangetoond dat het US3 kinase van PRV RhoA signalisatie beïnvloedt om zo stressvezel afbraak en uitlopervorming te bewerkstellingen. Eerder werd aangetoond dat US3 PAKs forforyleert en activeert om deze cytoskeletveranderingen te induceren. PAK-signalisatie is in staat om RhoA-signalisatie tegen te werken en vice versa. RhoA fosforylatie op serine 188 (S188) is een belangrijk mechanisme om RhoA te inactiveren, daar dit zorgt voor relocalisatie van RhoA naar het cytoplasma door RhoGDI. Door middel van infectie en transfectie experimenten werd aangetoond dat US3 fosforylatie induceert op positie S188 van RhoA. Verder werd aangetoond dat coëxpressie van US3 met niet-fosforyleerbaar S188A RhoA in ST cellen zorgt voor een onderdrukking van US3-gemedieerde actineveranderingen, terwijl dit niet het geval is bij cotransfectie met wild type RhoA, wat het belang van US3-geïnduceerde RhoA fosforylatie in dit proces benadrukt. Kinase experimenten suggereerden echter dat US3 niet rechtstreeks leidt tot RhoA fosforylatie, maar dat vermoedelijk een cellulair kinase betrokken is in dit proces. Vroeger werd reeds aangetoond dat het cellulaire kinase

proteïne kinase A (PKA) in staat is om RhoA te fosforyleren op positie S188. Eerder werd ook aangetoond dat US3 orthologen van HSV-1 en VZV leiden tot activatie van PKA. In de huidige studie werden indicaties gevonden dat ook PRV US3 leidt tot activatie van PKA, aangezien Western blot experimenten, waarbij gefosforyleerde PKA substraten werden gedetecteerd, aantoonden dat infectie met wild type (WT) PRV aanleiding gaf tot een verhoogde hoeveelheid eiwitbanden, in vergelijking met infectie met US3null PRV. Belangrijk hierbij is dat behandeling van cellen met de PKA-inhibitor PKI de US3-gemedieerde RhoA fosforylatie verhinderde, wat de betrokkenheid van PKA in de US3-gemedieerde RhoA fosforylatie ondersteunt. Als conclusie kan gesteld worden dat PRV US3 onrechtstreeks, via PKA, leidt tot fosforylatie van RhoA op positie S188, wat betrokken is bij de actineveranderingen die veroorzaakt worden door US3.

In het **vierde hoofdstuk** werd onderzocht hoe de manipulatie van actine-regulerende signalisatie door US3 zich vertaalt naar meer stroomafwaarts gelegen effector eiwitten. Een belangrijke regulator van actine dynamiek in cellen is cofiline, dat geactiveerd wordt door defosforylatie op positie serine 3 (S3). In dit hoofdstuk werd aangetoond dat infectie van ST cellen met WT PRV leidt tot verlaagde fosfo-S3 cofiline niveaus in vergelijking met cellen geïnfecteerd met US3null virus of mock-geïnfecteerde cellen. Transfectie van US3 in ST cellen, gevolgd door kleuring met een antistof tegen fosfo-S3 cofiline toonde eveneens aan dat de expressie van US3 leidde tot verlaagde fosfo-S3 cofiline niveaus. De kinase functie van US3 bleek cruciaal voor cofiline defosforylatie, aangezien deze cofiline defosforylatie niet waargenomen werd na infectie met kinase-deficiënt D223A PRV. Onverwacht werd waargenomen dat infectie met zowel US3null als D223A PRV leidde tot verhoogde niveaus van cofiline fosforylatie, wat een indicatie kan zijn voor nog ongeïdentificeerde biologische of virale factoren die cofiline fosforylatie moduleren tijdens infectie, wat een interessante verdere onderzoekspiste kan vormen. Overexpressie experimenten van US3 samen met een constitutief inactief S3D cofiline construct of een constitutief actief S3A cofiline construct suggereerden dat cofiline defosforylatie betrokken is bij de US3-geïnduceerde actineveranderingen, aangezien S3D cofiline deze veranderingen onderdrukte, terwijl S3A cofiline dit niet deed. Tenslotte werd aangetoond dat groep I PAKs betrokken zijn bij de US3-gemedieerde cofiline defosforylatie aangezien de groep I PAK inhibitor IPA-3 de PRV-geïnduceerde cofiline defosforylatie teniet deed. Dit ondersteunt het bestaan van een signalisatieweg die PAK activatie koppelt aan cofiline defosforylatie. Op basis van deze studie kan dus gesteld worden dat PRV US3 leidt tot S3 defosforylatie (activatie) van cofiline, wat bijdraagt tot US3-gemedieerde actineveranderingen.

In het **vijfde hoofdstuk** werd dieper ingegaan op de biologische consequenties van US3-gemedieerde modulatie van actine. LifeAct is een klein eiwit dat in staat is om filamenteus (F)-actine te binden met een lage affiniteit, doch met hoge specificiteit, zonder actine te verstoren, wat het een geschikte

merker maakt om F-actineveranderingen te bestuderen in levende cellen. Flow cytometrische analyse van LifeAct getransduceerde ST cellen toonde aan dat WT PRV infectie een F-actine afname veroorzaakt vanaf 2h na inoculatie, welke verder afneemt tot het einde van de observaties op 6 h na inoculatie, terwijl dit niet het geval was voor US3null PRV. Opnieuw bleek de kinase functie van US3 cruciaal voor dit proces, gezien infectie met kinase-deficiënt D223A PRV geen F-actine afname veroorzaakte. Eerder werd gerapporteerd dat F-actine afname een positieve invloed heeft op HIV binnenkomst in gastheercellen. Om na te gaan of PRV US3 ook bijdraagt tot virus binnenkomst, werden qPCR experimenten uitgevoerd op nucleaire fracties van geïnfecteerde cellen. US3null virus DNA bereikte de kern minder efficiënt dan WT virus DNA, wat inderdaad bevestigt dat US3 een rol speelt in aflevering van het virale genoom in de kern. Behandeling met actine depolymerizator cytochalasine D leidde er toe dat het DNA van US3null PRV efficiënter in de kern werd afgeleverd, wat wijst in de richting van een positief effect van F-actine afname tijdens aflevering van virale genomen in de kern. Samenvattend voor deze studie kan gesteld worden dat PRV US3 een afname veroorzaakt in F-actin en een rol speelt in de aflevering van virale genomen in de kern.

Het **zesde hoofdstuk** omvat een discussie waarin bovenstaande resultaten werden getoetst met de literatuur.

Conclusie

Eerder werk aan onze onderzoeksgroep toonde aan dat PRV US3 actine veranderingen induceert door fosforylatie en activatie van PAKs, downstream effectoren van Rho GTPases Rac1 en Cdc42. In de huidige thesis werd aangetoond dat US3 om deze actine veranderingen te bewerkstelligen ook RhoA manipuleert via fosforylatie op S188, wat kenmerkend is voor herkenning van RhoA door RhoGDI, leidend tot RhoA-inactivatie. Tevens werd aangetoond dat stroomafwaartse signalisatie van US3 leidt tot een PAK-afhankelijke activatie van de actine regulator cofiline, die tevens betrokken is bij de US3-gemedieerde actineveranderingen. Verder werd aangetoond dat US3 een kinase-afhankelijke F-actine afbraak bewerkstelligt en een voorheen ongekende rol speelt in aflevering van virale genomen in de kern. Behandeling met actine depolymerisator cytochalasine D verhoogde de nucleaire aflevering van PRV, wat de rol van F-actine afbraak tijdens virale binnenkomst in gastheercellen ondersteunt.

Curriculum Vitae

Curriculum Vitae

Personalia

Thary Jacob werd geboren op 3 juni 1986 te Aalst. Zij beëindigde in 2004 haar secundaire studies Wetenschappen-Wiskunde aan het Koninklijk Atheneum te Aalst. Datzelfde jaar startte zij haar universitaire studies Biomedische Wetenschappen aan de Faculteit Gezondheidswetenschappen aan de Universiteit Gent en koos in haar tweejarige masteropleiding voor de major Immunologie. In 2009 onderzocht ze "MAPPIT-analyse van Toll-Like receptor signaalwegen" aan het labo van Jan Tavernier (Rommelaere Instituut, Gent) voor haar masterthesis en behaalde zij haar diploma met onderscheiding. Van januari 2010 tot het einde van haar onderzoek beschikte zij over een persoonlijke doctoraatsbeurs van het agentschap voor Innovatie door Wetenschap en Technologie (IWT). Het onderzoek werd uitgevoerd aan de faculteit Diergeneeskunde van de Universiteit Gent aan de vakgroep Virologie, Parasitologie en Immunologie. De doelstelling van dit onderzoek was om een beter inzicht te verkrijgen in het mechanisme en de biologische consequenties van de actineveranderingen die veroorzaakt worden door US3 van het pseudorabies virus (PRV), wat resulteerde in drie A1 publicaties. Thary is tevens eerste auteur van twee review A1 artikels. Zij gaf voordrachten op verscheidene nationale en internationale congressen en begeleidde een studente bij haar bachelorproef.

Publicaties in peer-reviewed international wetenschappelijke tijdschriften

Jacob T, Van den Broeke C, Grauwet K, Baert K, Claessen C, De Pelsmaeker S, Van Waesberghe C, Favoreel H. 2015. Pseudorabies virus US3 leads to filamentous actin disassembly and contributes to viral genome delivery to the nucleus. Vet Microbiol. 177 (3-4): 379-85.

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Beurzen

Jan 2011	Persoonlijke IWT onderzoeksbeurs (4 jaar)
Mei 2013	Accommodatie Beurs Internationaal herpesviruscongres 2013 (Grand Rapids)
Mei 2013	FCWO reisbeurs

Bijgewoonde conferenties met posterpresentaties (p) en mondelinge presentaties (pr)

11 dec, 2009	Brussel	Belgian Society for Microbiology (BSM) 2009	
16 nov, 2011	Brussel	Belgian Society for Microbiology (BSM) 2011	
30 nov, 2012	Brussel	Belgian Society for Microbiology (BSM) 2012	p + pr
17-18 jan, 2013	Zürich	Europ. Society for Veterinary Virology (ESVV) 2013	p + pr
20-24 jul, 2013	Grand Rapids	International Herpesvirus Workshop (IHW) 2013	p + pr

Titel poster/presentatie: The US3 kinase of pseudorabies virus leads to activation of the actin regulator cofilin to induce actin cytoskeleton changes

Doctoral schools diploma

Verdiepende studies

Biogazelle Q-PCR cursus

Basis van photonics en electronica voor life scientists

Drug Discovery Anno 2014: from target identification to personalized medicine

Vaardigheidstraining

Statistische analyse met behulp van SPSS voor beginners

Statistische analyse met behulp van SPSS voor gevorderden

VIBes in Biosciences 2012

Advanced Academical English: Conference Skills - Academic Posters

6th From PhD to Job Market

7th from PhD to Job Market

Dankwoord

Dankwoord

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Thary