Determining the role of histone modification during Vaccinia virus infection

Noelia Garcia Calavia

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

University College London
MRC Laboratory for Molecular Cell Biology

Supervised by
Dr Jason Mercer and Dr Ricardo Henriques

October 2018

Declaration

I, Noelia Garcia Calavia, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed

Date

Abstract

Vaccinia is a Poxvirus widely known as the vaccine used to eradicate Smallpox in 1980. Today, it is extensively used in research as an easy to work with biological tool. Unlike most viruses, it replicates in the cell cytoplasm and for decades it had been postulated that the nucleus is not necessary for viral infection. As a consequence, interactions between Vaccinia and the cell nucleus have been overlooked. Recently, some studies have shown that the virus recruits host cell nuclear proteins to replication factories in order to facilitate transcription. However, little is known about the extent and nature of poxvirus-nucleus interactions. We know that pathogens modulate cell chromatin, by controlling histone marks, in order to dampen the cell's immune response. This project sheds light on the mechanisms used by Vaccinia to modulate host cell chromatin. We have special interest in how the virus may be preventing the expression of immune response genes. Using Immunofluorecence and Cell fractionation I have seen one phosphatase (H1) enter the cell nucleus during infection. A survey of histone modifications during the course of infection showed a drastic decrease in the phosphorylation of Histone 3 at Threonine 3, Serine 10, and Serine 28. As H3 S10 has been linked to cytokine gene repression I followed up this mark. I show, using in vitro dephosphorylation assays and cell transfections, that the Vaccinia phosphatase H1 is partially responsible for H3 S10 dephosphorylation. I further link this phenotype to dampening of the cellular immune response through reduction of cytokine gene expression. Finally, though modulation of H3 S10 phosphorylation I demonstrate that this modification is vital for productive Vaccinia infection.

Impact statement

Despite Smallpox eradication in 1980, it is still considered a potential bioterrorism threat. Additionally, other closely related Poxviruses, such as Molluscum contagiosum, affect today's population, impacting children and immunocompromised individuals. The presence of animal reservoirs for other pox viruses, e.g. Monkeypox and Vaccinia, makes the fear of a zoonotic spread imminent– confirmed by the recent reports of three people infected with Monkeypox in the UK. As a consequence, investigations of how the virus dampens the immune response of the cell could have an impact on how we treat pox infections. This work addresses the importance of Vaccinia-nucleus interactions and how these can be key for immunomodulatory processes during viral infection. Currently, it is known that the NFκB pathway is targeted by at least 10 Vaccinia proteins. However, there are still some remaining. Here, we show that the viral phosphatase H1 is at least partially responsible for the repression of TNFα-mediated cytokine gene expression.

Acknowledgments

First of all, I would like to thank my supervisor, Dr. Jason Mercer, for being the coolest P.I. around, for the initial Thursday pub nights, for the great lab trips to the beach and the snow, for the amazing barbeques and for putting up with all my nonsense. Also, thanks to my secondary supervisor, Dr. Ricardo Henriques, who was always available for help. I will also like to thank every member of the Mercer lab, David Albrecht, Becky Bamford, Corina Beerli, Susanna Bidgood, Robert Gray, Moona Huttunen, Victoria Gould, Melanie Krause, Caroline Martin, Harriet Mock, Jerzy Samolej, Artur Yakimovich and Jennifer Wood – for they have helped me grow over these years. Special thanks to Susanna, Moona and Artur for their help this last year in the lab. Also, thanks to Corina, who was essential during my first years of PhD, to Moona for her music playlists that always cheered me up, to Jerzy for his atrocious comments and numerous 'mehs' and to Jen for her amazing cakes that made you forget anything existed. Thanks to Janos Kriston-Vizi, for his absolutely great help in imaging and his even better never-ending chats. Finally, I would also like to thank Maria Elena Ledesma Fernandez, for being always there, for the human connection and for keeping me engaged with everything that happened in her life.

Second, I would like to thank my friends Dr. Elina Jacobs, Margarita Maria Semsi, Gabriela Lopez Lopez and Martina Bucsaiova. Elina for being a great support, for the lunches under the sun and for the understanding. Margarita for having the most awesome and philosophical concept of the world and Gabriela and Martina for showing me that we all go through the same, either out loud or in silence, but that is all part of the iLive package.

Thirdly, I would like to thank Andres Lerner Waen and Yasmin Saleh Cavero, for being my part of my London family, for the days around the city, for the days at home, for the best bubble tea, for the laughs and for bringing a little one to this world that I can spoil. Thanks to Graciela Waen Herrendorf, for her craziness and open vision of life, Alberto Lerner Liebermann, for his driving and his art skills and to Daniela and Pablo Lerner Menendez, whose videos and photos always cheered me up.

Fourthly, many many many many many thanks to my partner Guido, because without him, I would not be here today. For his true support, for his unconditional love, for all the hugs, for all our good and bad times, for letting me grow next to him and helping me grow, for making the little things ten thousand times much more fun, for our trips, for our days at home and for all the amazing meals he cooked. Gui, te amo, eres lo que me permite salir adelante cada día a pesar de que mi familia esté lejos.

And finally, thanks to my family, because they were the rock that held me together. Thanks for being there day and night, through all the possible phone apps that exist, thanks for our times together, for our Christmases, for our trips and for being my guide. Thanks to my mom for her strength, to my sister for being an excellent role model and to my dad for his wisdom. I love you too much to be able to express it with words. Mami, papi, enana, os quiero mucho.

Table of contents

List of tables

List of figures

Abbreviations

βTrCP	Beta Transducin repeat Containing Protein
2aa	2-AminoAcetophenone
Gumigatus	Aspergillus fumigatus
Phagocytophilum	Anaplasma phagocytophilum
Ac	Acetylation
AC	Adenylate Cyclase)
AGK2	2-cyano-3-[5-(2,5-dichlorophenyl)-2-furanyl]-N-5-quinolinyl-2-propenamide
AP-1	Activator Protein 1
ASF	Alternative Splicing Factor
ATR	Ataxia Telangiectasia and Rad3 related protein
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked
BCI	BCI - NSC 150117
Bcl	B-cell CLL/lymphoma
BHC80	BRAF35/HDAC2 Complex (80 KDa)
BSA	Bovine Serum Albumin
CLR	C-type lectin Receptors
C	Core
C. albicans	Candida Albicans
C. neoformans	Cryptococcus neoformans
C. perfringens	Clostridium perfringens
C. trachomatis	Chlamydia trachomatis
CARD	Caspase Activation and Recruitment Domain
CBP	CREB Binding Protein

CCL	C-C Motif Chemokine Ligand
CDC	Cholesterol-Dependent Cytolysins
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
CHD	Chromodomain Helicase DNA-binding protein
cIAP	cellular Inhibitor of Apoptosis
CIITA	Class II Major Histocompatibility Complex TransActivator
CLR	C-type lectin receptors
CLS	Cytoplasmic Localization Signal
CP	Copenhagen
CPC	Chromosome Passenger Complex
CPXV	Cowpox virus
CREB	cAMP Response Element Binding
CXCL	C-X-C motif ligand
DAG	Diacylglycerol
DC	Dendritic Cells
DD	Death Domain
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DNApol	DNA polymerase
DNMT	DNA Methyl-Transferase
Dot	Disruptor of telomeric silencing
DPF	Double PHD Fingers
dsDNA	double stranded DNA
DTT	Dithiothreitol

DUSP	Dual Specificity Phosphatase
EBV	Epstein-barr virus
eGFP	enhanced Green Fluorescent Protein
EM	Electron Microscopy
EPX	Eosinophil Peroxidase
EV	Extracellular Mature Virions
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
GAS sequence	Interferon-Gamma Activated Sequence
GPCR	G protein Coupled Receptor
H. pylori	Helicobacter pylori
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HAT	Histone Acetyltransferase
HCMV	Human Cytomegalovirus
HDA	Histone DeAcetylase
HDAC	Histone Deacetylase
HEK293	Human Embryonic Kidney 293
HIV	Human immunodeficiency virus
HP	Heterochromatin Protein
HSP	Heat Shock Protein
IκB	Inhibitor of kappa B
ICAM	Intercellular Adhesion Molecule
ICE	IL-1 converting enzyme

iE-DAP	g-D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
IgSF	Immunoglobulin superfamily receptors
IKK	IκB Kinase
IL	Interleukin
IL1R	Interleukin Receptor 1
IMV	Intracellular Mature Virions
iNos	inducible Nitric oxide synthase
IP3	Inositol 3,4,5-triphosphate
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory transcription factor
ISRE	IFN Stimulated Response Element
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
IκB	Inhibitor of kappa B
K	Lysine
KSHV	Kaposi's-Sarcoma Associated Herpesvirus
L. monocytogenes	Listeria monocytogenes
L. pneumophila	Legionella pneumophila
LGP2	laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2
LLO	Listeriolysin O
lncRNA	long non-coding RNA
LSD	Lysine Specific Demethylase
M	Membrane

m	Monomethylation
MDA	Melanoma Differentiation Associated Factor
Malt	Mucosa-associated lymphoid tissue lymphoma translocation protein
MAPK	Mitogen-Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MDP	Muramyl DiPeptide
Me2a	Asymmetrical dimethylation
Me2s	Symmetrical dimethylation
Me3	Trimethylation
MeCP	Methyl-CpG-binding protein
MIEP	Major Immediate-Early Promoter
MKK	Mitogen-Activated Protein Kinase Kinase
MLL	Histone-Lysine N-Methyltransferase
MOCV	Molluscum contagiosum virus
MPXV	Monkeypox virus
MSK	Mitogen- and stress-activated protein kinase
mTNF	membrane bound TNF
MV	Mature Virions
MyD88	Myeloid differentiation primary response 88
NAP1	NAK associated protein 1
NCOR	Nuclear Receptor Co-repressor
NEMO	NFκB Essential Modulator
NFκB	Nuclear Factor kappa-B
NIC	Nicotinamide

NIK	NFκB inducing kinase
NLR	NOD-like receptor
NLRP	Nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein
NLS	Nuclear Localization Signal
nNos	neuronal Nitric oxide synthases
NUP	Nucleoporin
NuRD	Nucleosome Remodeling Deacetylase
P	Phosphorylation
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-associated factor
PcG	Polycomb
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PHD	Plant Homeodomain
PIK3R3	Phosphatidylinositol 3-kinase regulatory subunit gamma
PKA	Protein Kinase A
PKB or Akt	Protein Kinase B
PLAD	Pre-Ligand binding Assembly Domain
PLC	Phospholipase C
PRC	Polycomb Repressor group Complex
PREs	Polycomb Group Response Elements
PRMT	Protein Arginine Methyltransferases
PRRs	Pattern-Recognition Receptors

PTMs	Post-translational modifications
qPCR	quantitative PCR
R	Arginine
RD	Repressor Domain
RHD	Rel Homology Domain
RIG-1	Retinoic acid-inducible gene I
RIP	Receptor Interacting Protein
RLRs	RIG-1-like Receptors
RNA pol	RNA polymerase
RNA	Ribonucleic Acid
RPA	Replication Protein A
RTqPCR	Reverse Transcription quantitative Polymerase Chain Reaction
S	Serine
S. flexneri	Shigella flexneri
S. pneumoniae	Streptococcus pneumoniae
SALL	Spalt Like Transcription Factor
SAM	S-adenosylmethionine
SB	SB 747651 tetrahydrochloride
SCRF	Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SEC	Super Elongation Complex
SINTBAD	Similar to NAP1 TBK1 Adaptor
siRNA	small interfering RNA
snRNA	small nucleolar RNA

SP	Specificity Protein
SSLs	Staphylococcal Superantigen-like Proteins
STAT	Signal transducer and Activator of Transcription
sTNF	soluble TNF
Syk	Spleen Tyrosine Kinase
T	Threonine
T. gondi	Toxoplasma gondii
TACE	TNF converting enzyme
TAD	Trans-Activation Domain
TAK	Transforming growth Factor beta activated Kinase 1
TANK	TRAF Family Member Associated NFkB Activator
TBK	TANK binding kinase 1
TBST	Tris-buffered saline plus Tween
TFEC	Transcription Factor EC
TFIID	Transcription Factor II D
TICAM	TIR domain containing adaptor protein inducing IFNβ "TRIF"/TIR domain containing molecule
TIRAP/MAL	TIR associated protein/MyD88 adaptor like
TLRs	Toll-like Receptors
TNF	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor alpha
TPA	Tetradecanolphorbol acetate / 12-O-tertadecanoylphorbol-13-acetate

TRADD	Tumor necrosis factor receptor type 1-associated Death domain protein
TRAF	TNF Receptor Associated Factor
TRAM	TRIF Related Adaptor Molecule
TREs	Trithorax Group Response Elements
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	Tripartite Motif-containing
tRNA	transfer RNA
TrX	Trithorax
TSA	Trichostatin A
TSS	Transcription Start Sites
US	United States
VARV	Variola virus
VCAM	Vascular cell Adhesion Protein
VISA	Virus-Induced Signalling Adapter
VV	Vaccinia virus
WB	Western blot
WHO	World Health Organization
WR	Western Reserve
YY	Ying Yang

Chapter I. Poxviruses and epigenetics

Section 1.01 Poxviruses

Poxviruses belong to the Poxviridae family. These viruses are characterised by their brick-like shape, their complex structural symmetry – unlike most viruses, and their large size. Additionally, they have a large double stranded DNA genome, ranging between 130-360 kb long, which encodes approximately 200 genes. They replicate in the cell cytoplasm and carry their own DNA replication and transcription machinery.

The Poxviridae family is comprised by two subfamilies: the Entomopoxvirinae that infect insects, and Chordopoxvirinae that infect vertebrates. Due to their host tropism, the Entomopoxvirinae family has received little attention during the years. On the other hand, Chordopoxviruses have been extensively studied due to their relevance in medical and veterinary areas. Among them, we find Variola virus (VARV), the causative agent of Smallpox which was eradicated in 1980, Molluscum contagiosum virus (MOCV), Monkeypox virus (MPXV), Cowpox virus (CPXV) and Vaccinia virus (VV) (Hughes, Irausquin et al. 2010) (McFadden 2005) (Oliveira, Rodrigues et al. 2017).

(a) Smallpox and zoonotic poxviruses

Variola virus is the best-known member of the Pox family and is commonly known as Smallpox. The main clinical manifestation is the formation of white pustules, filled with virus, that spread all over the body of the infected individual – from which it gets its name (Figure 1.1).

Smallpox gave rise to a global epidemic that killed and disfigured hundreds of millions of people. Its origin remains a mystery with some speculating that Variola is possibly an evolution of Camelpox virus to a virulent form. It is believed to be an ancient disease since there are reports describing the symptoms dating from the 4th century A.D. in China. Additionally, it has been suggested that the Pharaoh Ramses V died of smallpox in 1157 B.C. (Hughes, Irausquin et al. 2010).

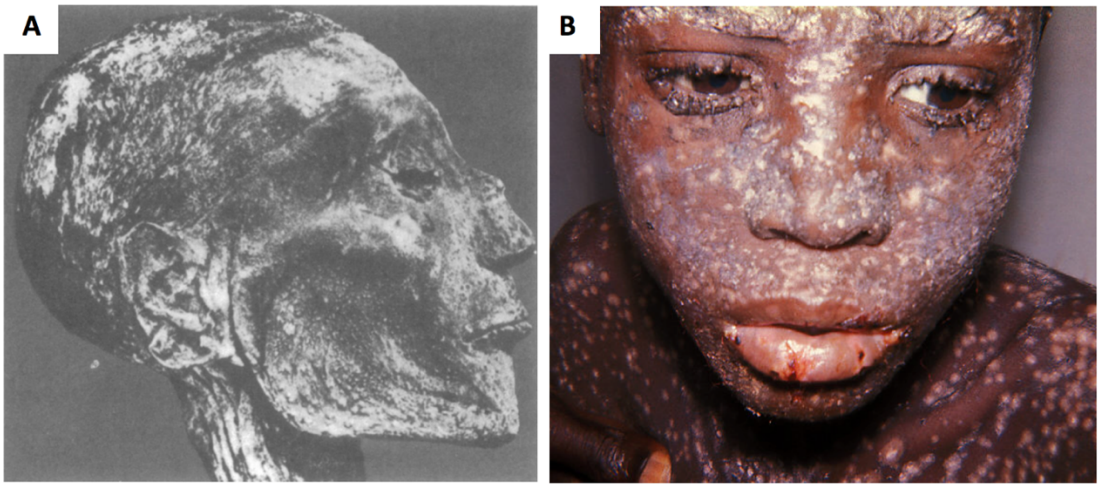


Figure 1.1. Smallpox infection in humans. A) Mummy of Pharaoh Ramses V, obtained from Behbehani, 1983. B) Ghanaian boy with Smallpox, obtained from Humanosphere.org.

In the 18th century, it caused the death of approximately 400,000 people per year and 1/3 of all the blindness in Europe by the end of the 18th century. Despite the practice of variolation, administration of material from lesions of people infected with uncomplicated smallpox to healthy individuals, the disease continued to spread. This practice was not very safe, and it was not until 1796 that vaccination was born, brought by the British physician Edward Jenner (1749-1823). He showed that people who had been exposed to milking cows presenting pustules similar to smallpox were resistant to variolation. This was thanks to infection with Cowpox virus,

which conferred immunity. However, it took years until vaccination campaigns were put in place. It wasn't until 1966 that the WHO (World Health Organization) led the smallpox eradication, with the last naturally occurring case in Somalia in 1977. Eradication of this atrocious virus was possible due of two factors: the vaccination campaign and the lack of a zoonotic Variola reserve (Behbehani 1983, McFadden 2005).

Apart from Variola, other Chordopoxviruses can also infect humans and give rise to viral outbreaks worldwide. While some of them have a selective tropism for humans, as it is the case for Molluscum contagiosum, some others have a different animal host but can affect humans, such as Monkeypox and Cowpox viruses. As a consequence, they are considered as emergent zoonotic diseases (McFadden 2005, Oliveira, Rodrigues et al. 2017).

Molluscum contagiosum virus is a mild virus that infects mainly 7% of children in the UK. It can give rise to an infectious dermatosis that usually resolves by itself within 9 months. Additionally, 18% of adults with HIV and some immunocompromised children are also affected by it. They belong to the risk group as they can be refractory to treatment (Forbat, Al-Niaimi et al. 2017).

Monkeypox virus is an endemic disease in Central and West Africa. Monkeypox presents with similar pustules as Smallpox and so its diagnosis needs to be done through specific tests. Although it does not pose serious danger and it is not highly transmissible among humans, there is a concern that the virus may become more virulent. Additionally, in 2003 there was an outbreak in the US and in 2018 there were three cases of Monkeypox in the United Kingdom (BBC news, 2018)(Weaver and Isaacs 2008).

Finally, Cowpox virus is carried by rodents and cattle, but it can also infect humans. Despite transmission among humans has not been reported, there have been increasing numbers of Cowpox infections in humans. Additionally, this virus generates self-limiting infections that cause only localised skin lesions but infection of immunocompromised patients can lead to fatal outcomes (Doellinger, Schaade et al. 2015).

(b) Vaccinia virus

Vaccinia is a pathogen that played its most important role in the eradication of Smallpox, a virus with which it shares 96% sequence identity (Massung et al. 1994). Since then, it has been used as the prototypic Poxvirus in laboratories for research purposes, as its genome can be easily manipulated, it is of large size and is able to infect most cell types. Due to these characteristics it was the first mammalian virus ever to be visualised by electron microscopy, being initially confused with bacteria.

The origin of this virus is unknown. It was believed to be the virus present in Edward Jenner's vaccine, therefore its name, however genetic analysis revealed that it was Cowpox instead (Schrick et al. 2017, Medaglia et al. 2015, Noyce, Lederman, and Evans 2018, Esparza et al. 2017). Vaccinia can also be found outside the lab. It has been reported to be carried by rodents and some farm animals in Brazil. Additionally, vaccination can also lead to Vaccinia infection in some people (Oliveira et al. 2017).

(i) Morphology and life cycle

Like all poxviruses, Vaccinia is a large dsDNA virus that encodes around 250 different genes. It is a brick-like shaped particle with the dimensions of 350nm x 300nm x 265nm (Figure 1.2). It is formed by a proteinaceous core, that comprises the factors necessary for early gene expression, and two lateral bodies – one on each side of the core, which carry proteins involved in immunomodulation. Finally, these three structures are wrapped by a membrane, giving rise to the mature virion (Bidgood and Mercer 2015).

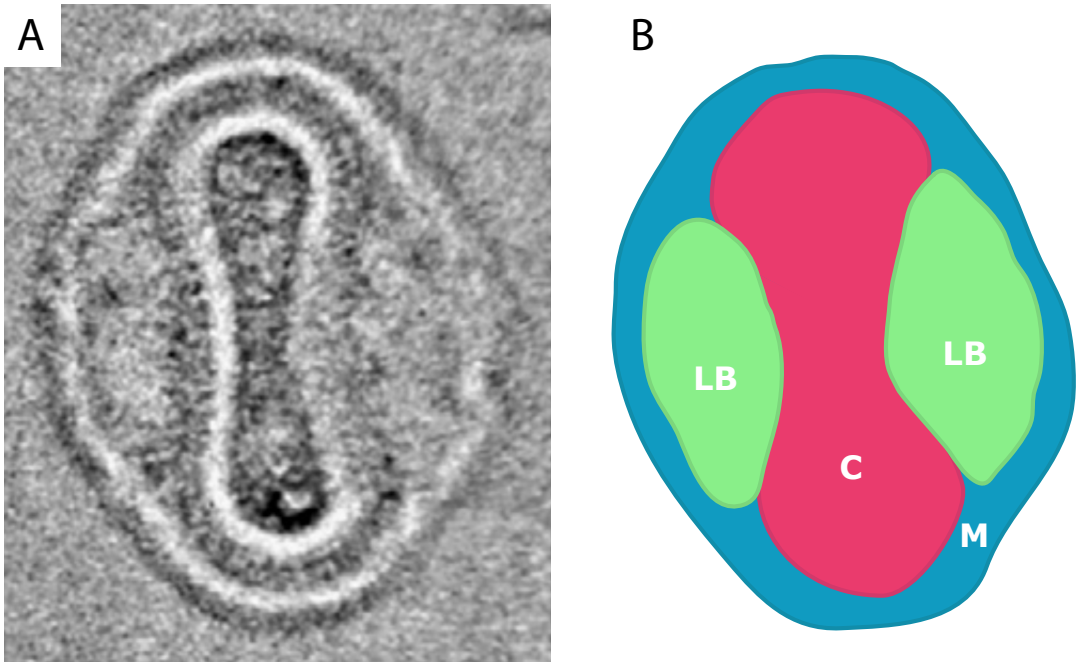


Figure 1.2. Mature virion of Vaccinia virus. A) Electron microscope image of Vaccinia (Condit et al. 2006). B) Graphical representation of Vaccinia, showing its core in red (C), lateral bodies in green (LB) and membrane in blue (M).

Vaccinia infection starts when the virus binds to the host cells and triggers macropinocytosis (Figure 1.3). This leads to the uptake and trafficking of the virus in macropinosomes. The low pH environment in the macropinosomes leads to membrane fusion and delivery of viral cores into

the cytoplasm (Laliberte, Weisberg et al. 2011)(Chang, Shih et al. 2012)(Mercer, J. and Helenius, A. 2009). Upon fusion, the lateral bodies dissociate from the core and release their contents inside the cell. These consist of proteins involved in the modulation of the cellular immune response.

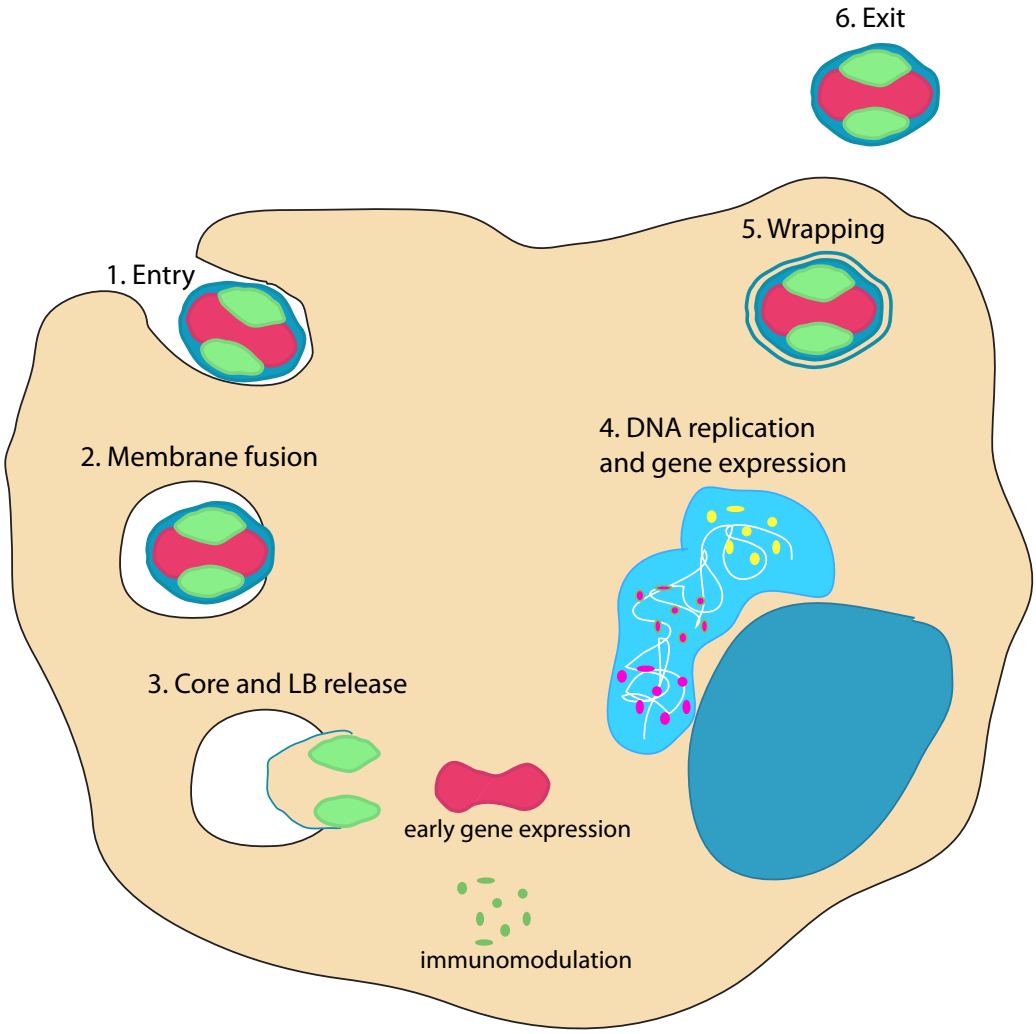


Figure 1.3. Vaccinia infection cycle. Vaccinia enters the cell by stimulating blebbing and membrane ruffling. A decrease in the pH of the vesicle in which it is internalised allows for membrane fusion. This is followed by core release and activation and lateral bodies disintegration, allowing for immunomodulatory proteins to suppress the cellular response. Then, viral DNA replication and early gene expression take place, which are followed by intermediate, late gene expression and viral morphogenesis. The virions are then wrap and exit the cell 8 hours post infection.

Vaccinia cores initiate early gene expression within 30 minutes of cytoplasmic delivery. The early genes include factors required for Vaccinia DNA replication which gives rise to the formation of viral replication factories within the cell cytoplasm. These can be recognized because they appear as electron dense masses by Electron Microscopy (Figure 1.4). Early gene expression is followed by intermediate and late gene expression, 4 hours and 6 hours post infection, respectively. This culminates with the complex morphogenesis of new Vaccinia virions. This begins with the formation of viral crescents which grow to encapsidate a portion of the viral factory. The immature virions formed include all the viral proteins needed for assembly. Once the viral DNA is packaged into these circular immature virions they undergo a series of morphological changes becoming mature virions. There are two types of mature viral particles: Intracellular mature virions or mature virions (IMVs or MVs) and the Extracellular mature virions (EVs). MVs which contain a single lipid bilayer are released at 72 hours post infection as a consequence of cells lysis and are involved in host-host transmission. EVs are formed when MVs are wrapped by a double lipid bilayer and released from host cells by exocytosis at 8 hours post infection. EVs are involved in spread of viral particles within and between tissues (Bidgood and Mercer 2015).

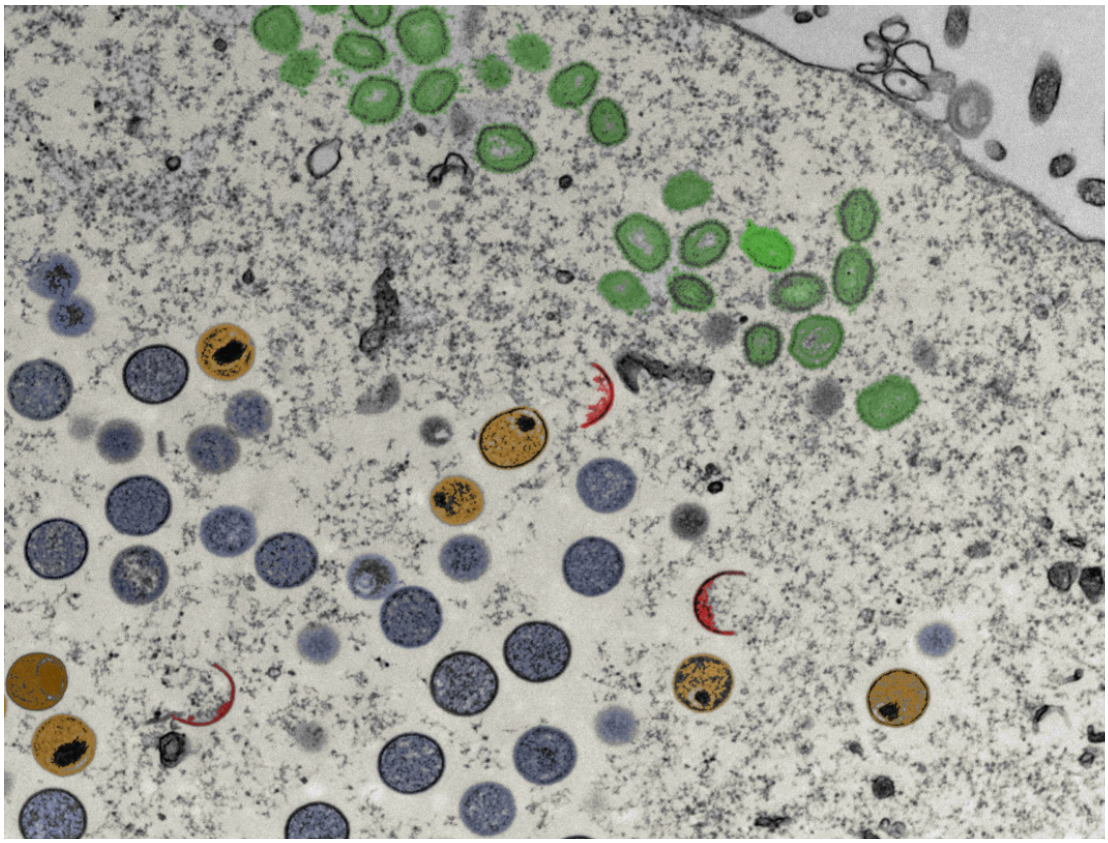


Figure 1.4. Viral replication factories seen by EM. Cell infected with Vaccinia virus shows the different intermediates of viral assembly until a mature virus is obtained within the replication factory. Red = crescents, Blue = Immature virions, Yellow = Immature virions with nucleoid, Green = Intracellular mature virions (Mercer, J. unpublished).

(ii) Vaccinia and the host cell nucleus

Vaccinia virus has always been considered an autonomous virus as it expresses its own DNA replication machinery and was thought to not require the cell nucleus for early viral expression and DNA replication. Experiments with enucleated cells demonstrated that the virus can enter cells and undergo uncoating and core activation without the nucleus being present. Additionally, early gene expression and viral DNA replication can take place in these cells, even if that happened at lower levels than in nucleated cells. As a consequence, it was postulated that Vaccinia must carry all proteins necessary for these two processes (D.M. Prescott 1971). Later on,

Pennington et al. discovered that Vaccinia infection in enucleated cells led to an 91.7% reduction in the production of viral particles and none were infectious, despite the presence of viral factories in the cells (Follet 1974).

Additionally, follow up of certain nuclear proteins during Vaccinia infection has shown their nuclear-cytoplasmic shuttling and their involvement in viral replication. Some nuclear transcription factors, YY1, SP1 and TATA binding protein, were shown to be recruited to viral factories where YY1 was seen to associate with Vaccinia replication complexes. Furthermore, some proteins involved in cellular transcription, RNA polymerase II, TAFIIp32 and HDAC8 (histone deacetylase 8), were seen to localise in the cell cytoplasm (Oh and Broyles 2005). Supporting the recruitment of nuclear proteins to viral factories, Postigo et al. provided evidence of the cellular proteins ATR (ataxia telangiectasia and Rad3 related protein), RPA (replication protein A) and PCNA (proliferating cell nuclear antigen) presence in viral transcription factories. Moreover, they showed that uncoating of Vaccinia's genome activated cytoplasmic ATR, which was essential for genome replication. On top of that, RPA and PCNA were shown to interact with the viral proteins H5, I3 and E9; where ATR and RPA play a role in viral replication and PCNA acts as the DNA sliding clamp. As a consequence, unlike previously thought, Vaccinia does recruit and rely on host nuclear proteins for genome replication (Postigo, Ramsden et al. 2017).

Recent studies have also shown that viral proteins can travel to the cell nucleus. Such is the case of Vaccinia C6, protein which has been shown by Smith et al. to travel to and act inside the cell nucleus, in addition to its cytoplasmic role in repressing interferon signalling through the IRF3 protein.

C6 associates with the transactivation domain of Stat2, necessary for the recruitment of chromatin remodelling factors, and as a consequence it may inhibit the expression of IFN responsive genes through prevention of chromatin modifications in genes containing ISRE (Interferon stimulated response elements) sequences (Stuart, Sumner et al. 2016).

Despite the fact that Vaccinia-nuclear interactions have been overlooked for decades due to its cytoplasmic replication, new research is trying to assess the importance that the nucleus may have for successful infection and how Vaccinia may be exploiting it for replication and repression of the cellular immune response.

Section 1.02 Epigenetics

The term epigenetics was coined by Conrad Waddington (1942) with regard to all those changes in phenotype that were not accompanied by a change in the genotype. Currently we know epigenetics consists of the dynamic modifications in gene expression that do not involve any changes in the DNA sequence. Epigenetics allows for the diversity of multicellular organisms: all cells carry the same genome, however the expression of only a subset of genes gives each cell specific properties. These reversible but stable marks can be classified into three groups: DNA modifications, non-coding RNAs and histone modifications (Merkenschlager 2010, Allis and Jenuwein 2016).

DNA methylation was the very first epigenetic mark studied. It consists of the addition of methyl (CH_3) groups to cytosine rings in the DNA (Hotchkiss, R.D. 1948). In mammals, it usually happens in regions rich in

CpG dinucleotides, most of which are methylated in humans, around 60-80% of them. Methylation is a highly dynamic mark that can be added, by methyltransferases, and erased, by demethylases, at any time and in any DNA region during the life of the cell. *De novo* methylation is carried out by DNMT3A and DNMT3B while maintenance methylation is taken care by DNMT1. DNMT1, unlike DNMT3A and B, can recognize hemi-methylated DNA during DNA replication or DNA damage and add methylation to the DNA strand lacking it. The presence of this modification in the DNA is correlated with gene repression if it occurs at transcription start sites (TSS), and gene expression if it takes place in gene bodies. This epigenetic modification is important for the repression of gene expression and X chromosome inactivation in mammals (Goldberg, Allis et al. 2007, Zhen Chen and John Y.J. Shyy 2017).

Non-coding RNAs consist of RNA sequences that are never translated into proteins. There are different types according to their size and function: small non-coding RNA, transfer RNA (tRNA), small nucleolar RNA (snRNA) and long non-coding RNA (lncRNA). Both small non-coding RNAs and long non-coding RNAs, at least 200 bases long, have been involved in the recruitment of epigenetic modifications. They are very tissue and developmental stage dependent and can function to activate or repress gene expression, as well as provide guides and scaffolds for the recruitment of chromatin modifiers to specific DNA regions. An example of their mode of action is ANRIL, an antisense noncoding RNA that binds to the INK4 gene locus and recruits the PRC1 and PRC2 repressor complexes and as a consequence shuts down gene expression (Goldberg, Allis et al. 2007,

Merkenschlager 2010, Allis and Jenuwein 2016, Zhen Chen and John Y.J. Shyy 2017).

(a) Histone modifications

Histones consist of positively charged proteins that bind to the negatively charged DNA, compacting it and giving rise to what is called chromatin. There are 5 histones: Histone 2A (H2A), Histone 2B (H2B), Histone 3 (H3) and Histone 4 (H4), all of which form the core of nucleosomes, and Histone 1 (H1), which is involved in the formation of higher-order chromatin structures. As any other protein, histone tails can be modified by the addition of functional groups called post-translational modifications (PTMs). Their presence or absence alter histone-DNA interactions affecting chromatin structure, accessibility and the subsequent recruitment of chromatin modifying proteins e.g. transcription factors or repressors (Zhen Chen and John Y.J. Shyy 2017).

While histone modifications in the tails of histones 3 and 4 have been deeply studied, histones 2A and 2B have received little attention. This is due to the fact that H3 and H4 are considered of great importance due to their high conservation in sequence among eukaryotes and they are quite stable in the chromatin, while H2A and H2B are very dynamic and are replaced from the chromatin quite often (Becker 2002).

Cross-talk among different epigenetic modifications occur in the chromatin giving rise to distinct modification patterns that will confer specific regulation to every gene. These epigenetic marks regulate one another providing regulatory cross-talk that can occur either in *cis* on the same

histone molecule, or in *trans* in other histones in the same nucleosome or different nucleosomes. The combination of these modifications give rise to what is called the "histone code", which is read by effector proteins and in turn activates different downstream functions (Strahl and Allis 2000)(Latham and Dent 2007).

Among the most studied histone modifications we find histone acetylation, methylation and phosphorylation, which are discussed below.

(i) Histone acetylation

Histone acetylation consists of the addition of acetyl groups to lysines in histone tails, adding negative charge to the positively charged histones. This gives rise to a lower affinity for the negatively charged DNA and it creates a looser nucleosome-DNA interaction, which makes protein binding to the DNA easier (Shahbazian and Grunstein 2007). This post-translation modification was found to be present in histones by D.M.P. Phillips while studying their amino acid composition (D.M.P.Phillips 1963), but it was V.G. Allfrey who showed that the most acetylated histones were lysine-rich and that the presence of this chemical group was linked to gene expression (V.G. Allfrey 1964). Today, it is known that histone acetylation has additional roles including the regulation of chromatin folding – hyperacetylation of histone tails prevents the condensation of chromatin to 30nm fibres (Garcia-Ramirez, M. 1995) – and the contribution to nucleosome assembly – *de novo* processed histones are acetylated for their correct assembly into nucleosomes by specific chaperon proteins, such as Chromatin-assembly-factor-1 (CAF1)(Smith et al. 1991)(Shahbazian and Grunstein 2007).

Acetylation is a dynamic process that is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs and HDACs are recruited to regulatory regions in the genome where they can add or remove, respectively, acetyl groups to surrounding histone tails. The existence of these two opposing enzymes allows for switches in chromatin states throughout the cell's life (Sterner et al. 2000)(Khochbin, S. 2001)(Becker 2002). Human cells' chromatin is considered to be hypoacetylated as default, where acetylation is primarily found in histones expanding through house-keeping genes, as it is the case of the β-globin loci (Michael D. Litt 2001).

The presence of acetyl groups in chromatin had always been correlated with active transcription sites. However, it has become evident that acetylation is not the only responsible for gene expression, but it is part of a more complex set of changes that include other epigenetic modifications such a phosphorylation or the rearrangement of nucleosomes in chromatin by remodeling factors. Therefore, acetylation simply marks regions of transcriptional competence (Becker 2002).

While acetylation has been extensively studied in H3 and H4, histones H2A and H2B have received little attention likely due to higher rates of exchange, as mentioned above (Jackson 1990).

Distinct acetylation patterns across the chromatin has allowed for the identification of the preferential acetylation marks for different genomic regions (Figure 1.5). While enhancers are characterised mainly by H2BK120ac, transcription start sites (TSS) are rich in H3K9ac and H3K14ac. Gene bodies, on the other hand, are mainly represented by H2AK5ac, H3K23ac, H3K14ac and H4K5ac (Rajagopal, Ernst et al. 2014). Histone 4

acetylation can occur at different residues: K5, K8, K12 and K16. Not many studies have investigated their possible roles in gene expression. However, M.F.Dion *et al.* have shown that while acetylation of K16 is strongly correlated with gene expression, for K5, K8 and K12 acetylation it is less clear (Dion, Altschuler et al. 2005).

Figure 1.5. Acetylation pattern across the gene. The illustration shows the histone acetylation marks that are more commonly associated with each of the gene components.

Acetylation at Histone 3 lysine 9 usually occurs at the TSS of a gene. L.A.Gates *et al.* showed that it has a key role in transcriptional elongation. At gene promoters, H3k4me3, always associated with regions of gene expression, helps PolII transcription initiation by acting as a docking site for TFIID – and therefore contributing to the generation of the pre-initiation complex. Additionally, it also recruits SGF29, a HAT complex subunit necessary for acetylation of H3K9 by GCN5 and PCAF. It is well known that at the beginning of gene transcription, PolII pauses at gene promoters until elongation factors are recruited to the transcription site. L.A.Gates has demonstrated that H3K9ac recruits the super elongation complex (SEC) to chromatin, releasing the transcription initiation block and allowing for transcription elongation (Gates, Shi et al. 2017). This role can be supported by the presence of H4K5ac, which may also be able to recruit SEC on its own although to a lesser extent (Latham and Dent 2007).

Little is known about the role of acetylated lysines 4 and 14 of H3. While H3K4ac seems to be necessary for the expression of a small subset of genes in yeast (Guillemette, Drogaris et al. 2011), H3K14ac has been linked to ribosomal DNA silencing and replicative aging, being K14ac absence detrimental for cellular lifespan (Xu, Su et al. 2016).

Cross-talk among acetylation, methylation and phosphorylation has been deeply studied. For instance, when a lysine residue is acetylated, it cannot be methylated anymore and vice versa. They are mutually exclusive marks and experiments with eukaryotes have shown that H3K9me opposes the functions of H3K9ac – while the former is involved in gene repression, the latter is linked to gene expression (Latham and Dent 2007). In addition to this, histone 3 post-translational modifications have been extensively studied and a lot is known about its mechanisms involved in cis cross-regulation. For instance, in eukaryotes, H3S10 phosphorylation by Ras-MAPK signalling pathways is quickly followed by the acetylation of H3K14, suggesting that H3S10P promotes H3K14ac (Peter Cheung and Allis 2000). Likewise, H3S10P stimulates H3K14ac by Gcn5 in yeast. However, the presence of this phosphorylation blocks H3K9ac as well as H3K9me1,2 but not H3K9me3 (Wan-Sheng Lo, Jeannie R. Rojas et al. 2000, Edmondson, Davie et al. 2002) (Stephen Rea and Chris P. Ponting 2000). Interestingly, H3K9me3 needs to be present for HP1 binding to histone 3, which is removed by phosphorylation of H3S10. During cell cycle progression, both H3S10P and H3K14ac must be present for HP1 removal. These data suggest that the histone code at least partially relies on the effector proteins and modifying enzymes that are present in each cell (Latham and Dent 2007).

(ii) Histone methylation

This post-translation modification was originally discovered by K. Murray when, while studying the amino acid composition of calf thymus histones by ion-exchange columns, he found a small unknown peak just after the lysine peak that corresponded to an ε-N-methyl group. This substance was seen to be present in histone lysates from all mammalian sources investigated (Murray 1964).

While acetylation occurs only on lysines, methylation of histones occurs in all basic amino acids: arginines, lysines and histidines. This modification is a bit more complex in the sense that there are several types of methylation. For instance, lysines can be mono-, di- or tri- methylated and this always happens on their ε amine group. On the other hand, arginines can be monomethylated, symmetrically dimethylated (me2s) or asymmetrically dimethylated (me2a); while histidines can only be monomethylated (Figure 1.6). These combinations of number of methylated groups plus their location on the residues are thought to be linked to the role of methylation in gene expression. A good example of this is H3K4 methylation whose monomethylation is linked to functions in the enhancer region of nearby genes while its trimethylation is linked to gene promoter functions (Bernstein et al. 2002)(Santos-Rosa et al. 2002)(Heintzman et al. 2007)(Greer and Shi 2012).

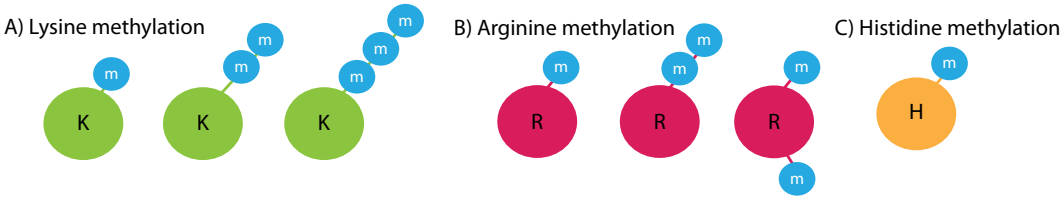


Figure 1.6. Methylation patterns of lysine, arginine and histidine. Lysines can be mono, di and trimethylated. Arginines mono and demethylated, symmetrically and asymmetrically. Histidines can only be monomethylated.

Initially, it was thought that methylation was an irreversible mark since its turnover rate is extremely low. It was not until the discovery of the first histone demethylase, LSD1 (Lysine Specific Demethylase 1), that it was determined that this modification was reversible (Shi, Lan et al. 2004). Since then, research has identified three families of methyltransferases, corresponding to SET domain proteins, Dot1 like proteins and PRMT proteins. While the former two are involved in lysine methylation and work by transferring methyl groups S-adenosylmethionine (SAM) to any lysine residue, the latter one takes care of arginine methylation. Additionally, we know of two families of lysines demethylases: amine oxidases and Jumonji C domain containing proteins, also known as iron-dependent dioxygenases (Chang et al. 2007)(Bedford and Clarke 2009, Xuejiao Tian1 2013).

Methylation is helped by the recruitment of histone modifying enzymes to specific DNA sequences such as TREs (Trithorax Group Response Elements) and PREs (Polycomb Group Response Elements). These can recruit methyltransferase complexes such as TrX (Trithorax) and PcG (Polycomb) proteins, involved in H3K4meX and H3K27me3, respectively (Figure 1.7) (Schuettengruber, Chourrout et al. 2007).

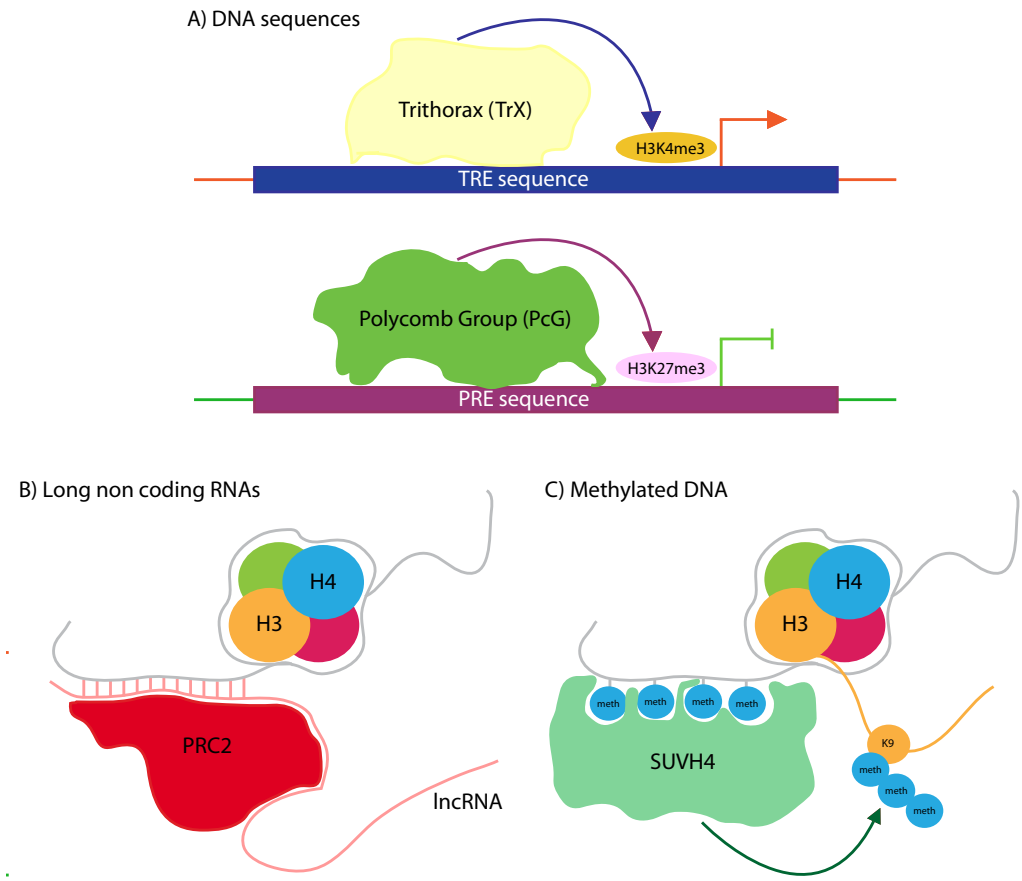


Figure 1.7. Histone methylation mechanisms. A) Histone methyltransferases or demethylases are recruited to specific sequences in the DNA. B) Long non-coding RNA bind to histone modifying enzymes and recruit them to targeted DNA regions. C) Methylated DNA serves as a docking site for histone methyltransferases.

Furthermore, lncRNAs (long non-coding RNAs) are known to directly bind to the methyltransferases complexes such as PRC2 complex (Polycomb repressive complex 2), G9a and WDR5, in order to recruit them to specific loci and affect gene expression through histone methylation (Pandey, Mondal et al. 2008). Additionally, methylation of the DNA allows for the binding of methyltransferases which will modify histone methylation, as it is the case for the H3K9 methyltransferase SUVH4, suggesting an interplay between histone and DNA methylation (Cornelia Fritsch 1999).

Like histone acetylation, there are some marks that reinforce each other while some marks exclude each other, and this can happen in *cis* or in *trans*. A good example of *cis* excluding methylation marks is H3K4me2/3, which can be bound by PHF8, which in turn demethylates H3K9me2 (Horton, Upadhyay et al. 2010). As for in *trans*, the presence of H2B monoubiquitylation is a requirement for H3K4 and H3K79 methylation (Allis 2002).

The presence of this PTM can be recognized by multiple protein types: PHD fingers, WD40 repeats, CW domains, PWWP domains, Ankyrin repeats and any protein of the Royal superfamily which comprises proteins containing chromodomains, chromo barrels, Tudor domains and MBT repeats. It is the presence or absence of these proteins that will define a different outcome to the methylation mark and the presence of the methylation mark will recruit different effector proteins. While some proteins cannot bind to lysines or arginines in their methylated state, such as BHC80, TRIM24 or UHRF1, others such as TAF3 – a component of the TFIID complex – directly binds to H3K4m3. Moreover, the phosphorylation of PolII has a decisive role in recruiting proteins that will either methylate H3 at K4, K36 or K27 to regulate transcription initiation or elongation (Vermeulen et al. 2007)(Greer and Shi 2012).

The most studied methylation marks are on lysines and include H3K4, H3K9, H3K27 and H4K20, among others. H3K4 methylation has been long linked to gene expression and active chromatin marks. It recruits proteins involved in nucleosome assembly and mobilization such as CHD1 (Chromodomain Helicase DNA-binding protein 1). However, the presence of different PTMs in surrounding histones or in the same one affect CHD1

binding to H3K4me. This is the case for H3R2me and H3T3P, which block CHD1 binding – H3T3P during mitosis ejects CHD1 from H3K4me regions but as soon as the cell reaches anaphase and H3T3P is gone, CDH1 goes back to its original position and binds to H3K4me once again (Flanagan, Mi et al. 2005). Moreover, G9a-dependent H3K4me displaces NuRD complex binding, a chromatin remodeling factor that usually associates with histone deacetylases and therefore is linked to gene repression. This methylation mark is completely opposite to H3K9me, which is strongly correlated with gene repression. While H3K4me is found primarily in euchromatic regions of the chromatin, mainly autosomal chromosomes, H3K9me is found in heterochromatin and it plays an important role in the silencing of one of the female's X chromosomes. They have antagonistic roles and so they modulate each other to some degree. H3K4me does so by preventing H3K9me through the Suv39H1 complex, although not through the Set9 protein – something that might be explained by the recruitment of additional proteins by these two different methyltransferases that will contribute to the genetic code of that region. All this said, there may be roles of H3K4me in gene inactivation that have been unexplored. Experiments in yeast point to this idea, since the methylation at this specific residue is carried out by the SET1 protein, which is known to be involved in telomere silencing (Doerks, Copley et al. 2002).

H3K4 methylation plays an active role in gene expression by recruiting TFIID to gene promoters through interactions with its TAF3 subunit and boosts gene transcription by stimulating the formation of the preinitiation complex (PIC). Additionally, H3K4me offers TFIID stabilization in TATA-less promoters (Figure 1.8) (Lauberth, Nakayama et al. 2013).

Another important role that H3K4 methylation plays is in the formation of bivalent domains necessary for embryonic development. These domains are characterised by the presence of activating, as well as repressing, histone marks that allow for a silenced chromatin in embryonic stem cells but at the same time they preserve their potential to be activated upon cell differentiation. In bivalent domains, H3K4 methylation is usually associated with trithorax proteins, keeping genes poised. These bivalent chromatin regions resolve once cells have differentiated into either regions full of H3K27 methylation – a repressive mark – or H3K4 methylation, giving cell-type specificity to those stem cells (Bernstein, Mikkelsen et al. 2006).

On the other hand, H3K9 methylation is a characteristic hallmark of heterochromatin, together with DNA methylation. This modification is largely found at centromeres (H3K9me2) and pericentric regions (H3K9me3) and is the result of the Suv39h methyltransferase, which can recruit Dnmt3b, a DNA methyltransferase, to the major satellite repeats nearby the methylated histone 3 at K9. In addition to this, Dnmt3a and Dnmt3b can be recruited to H3k9me3 residues by HP1α and HP1β and create together a repressive complex. Furthermore, gene repression may be reinforced by the binding of the methyl-CpG binding protein MeCP2 and its associated HDACs, as well as other methyltransferases (Figure 1.8) (Lehnertz, Ueda et al. 2003).

As previously mentioned, methylation at H3K9 is one of the most prominent marks in the inactive X chromosome. Indeed, X chromosome inactivation starts by the presence of H3K9me2 and H3K27me3 on the histone 3 tail and these are necessary for the maintenance of gene repression (Rougeulle, Chaumeil et al. 2004).

Methylation of histone 3 at lysines 9, 27 and histone 4 lysine 20 can repress gene transcription on their own but it has recently been reported that interacting proteins can create docking sites for repressor protein complexes. For example, H3K9me is recognized by HP1 which can recruit histone methyltransferases that will propagate this methylation to further histones and so giving rise to methylated chromatin regions that correspond to inactive areas. These areas, which are marked by H3K9me2/3, H3K27me2 and H4K20me2 are also hypoacetylated, proving that HDACs may also be involved in generating these transcriptionally inactive regions (Miao and Natarajan 2005).

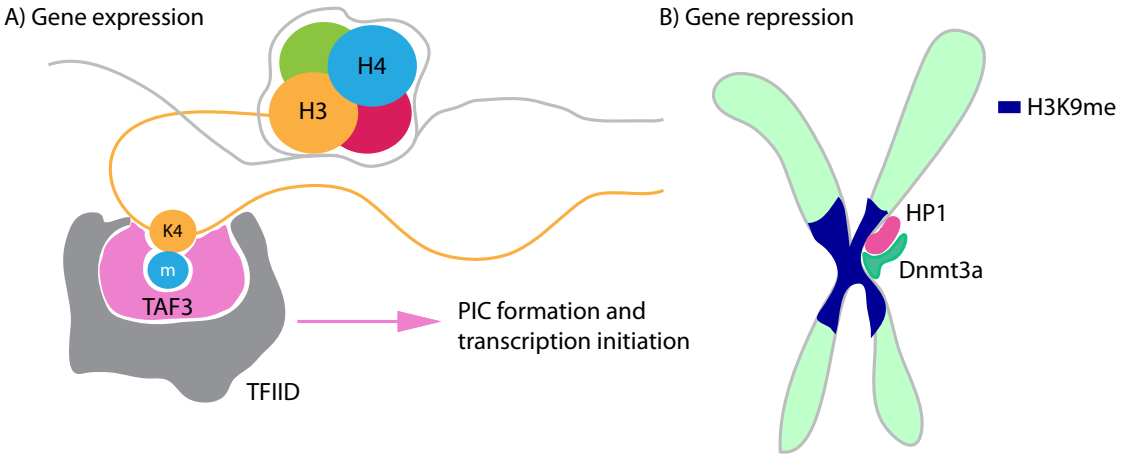


Figure 1.8. Histone methylation of H3K4 and H3K9. A) H3K4me is involved in gene activation and gene transcription. It helps recruit TFIID which allows for the formation of the PIC (Preinitiation complex) and the start of transcription. B) H3K9me is usually found in centromeres and pericentric regions. It allows HP1 docking and further recruitment of Dnmt3a, creating a repressive complex that prevents gene expression.

Histone methylation, unlike acetylation, has multiple readouts and outcomes and it really depends on the exact nature of the modification, whether, mono-, di- or tri- methylated, as well as the residue in which the PTM takes place and the proteins that associate with it that will define its function in gene transcription.

(iii) Histone phosphorylation

The presence of phosphate groups in histones was shown by M.G. Ord and L.A. Stocken (1966) when they found a phosphorylated serine on histone f1 (L.A.Stocken 1966). However, unlike histone acetylation and methylation, their study was not very popular until nearly a decade ago when it became apparent that histone phosphorylation plays an important role in affecting gene transcription, DNA repair, chromosome condensation and cell cycle progression. Phosphorylation can take place in any histone, H1, H2A, H2B, H3 and H4, but always on serine and threonine residues. Depending on where this phosphorylation occurs, the outcome will vary. For example, phosphorylation of Histone 2A at T119 is related to chromatin structure and function during mitosis while phosphorylation of Histone 3 at S10 is known to play a role in two very different mechanisms: chromosome compaction and activation of NFκB induced genes, such as c-jun and c-fos (McDonald et al. 2005) (Banerjee and Chakravarti 2011) (Rossetto, Avvakumov et al. 2012).

Histone 2A phosphorylation has been implicated in gene transcription in *in vitro* studies. Zhang and colleagues showed that MSK1 can phosphorylate H2A at serine 1 and that the presence of this phosphorylation inhibited GAL4-VP16 gene expression. Additionally, Aurora B and Rsk2 could also phosphorylate H2A S1 *in vitro*. Despite, it is unknown whether in the cell all these kinases would take care of this PTM, it is very likely that phosphorylation of this residue is directly linked to gene repression (Zhang, Griffin et al. 2004).

Histone 3 phosphorylation is the most extensively studied among all histones. We now have a vast knowledge of what phosphorylation at each

residue may be doing, as well as which enzymes are carrying them out. H3S10P has been shown to be phosphorylated by several kinases in mammalian cells: AuroraB, IKKα, Rsk2, PKB/Akt, PIM1, MSK1 and MSK2. This phosphorylation is recognized and bound by 14-3-3 domain containing proteins while its presence, prevents HP1, SRp20, ASF and SF2 binding to chromatin. H3S10P plays a key role during mitosis by allowing chromosome condensation to start, and so it has always been used as a mitosis marker. During the late G2 phase, H3S10 becomes phosphorylated in pericentriomeric regions of the chromatin. By prophase, it has already spread over the chromosomal arms and it remains intact all the way through metaphase. At anaphase, dephosphorylation starts and by telophase it is completely gone, before chromosome decondensation becomes visible (Figure 1.9) (Goto et al. 1999)(Banerjee and Chakravarti 2011). While this may be the case in mammalian cells, yeast do not need H3S10P for proper chromosome condensation since mutations of the serine to alanine have not shown any physiological defects, however, phosphorylation of hisone 2B may be compensating for the lack of S10P (Jer-Yuan Hsu, Melanie Reuben et al. 2000).

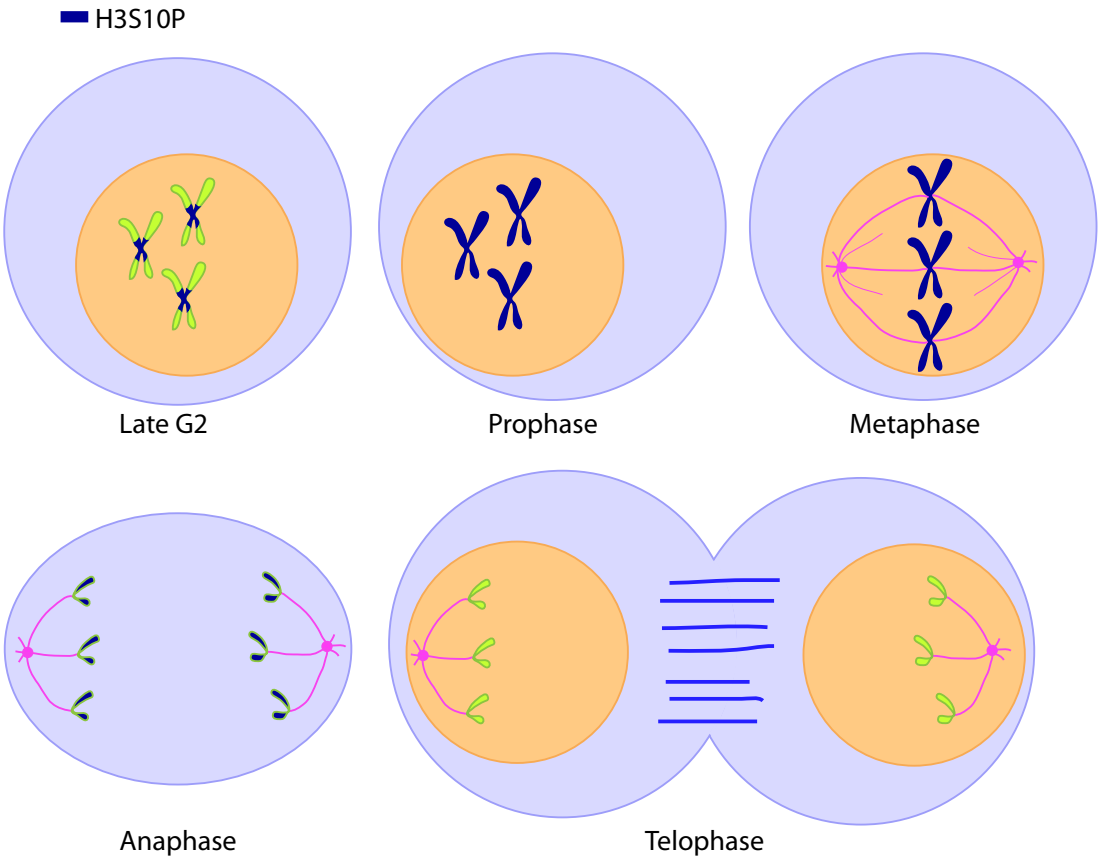


Figure 1.9. Histone 3 serine 10 phosphorylation during mitosis. H3S10P starts at late G2, by prophase is all over the chromosomes and it stays this was through metaphase. By anaphase, dephosphorylation of the residue starts and by telophase it is completely gone.

A possible mechanism by which H3S10P might be mediating chromosome condensation may be through the displacement of the HP1 protein from mitotic chromosomes and therefore facilitating chromosomal segregation. H3K9me3 is necessary for the recruitment of HP1 to chromatin, however, as soon as H3 is phosphorylated at S10, it ejects HP1, which does not need to be followed by any change in the methylation status of H3K9me3 (Fischle, Tseng et al. 2005).

Second, H3S28P is a histone mark that occurs on a similar pattern to S10P and is also thought to be involved in mitosis. This mark is carried out by the kinases Aurora B, MSK1 and MSK2 and is dephosphorylated by the

phosphatase PP1 (Goto et al. 2002)(Banerjee and Chakravarti 2011). Experiments in which a kinase-dead Aurora B was overexpressed lead to deficient levels of H3S10P and H3S28P and ended up in partial chromosomal condensation and their misalignment during metaphase (Hidemasa Goto1 2002).

Additionally, the presence of these two marks has been correlated with cellular stimulation using specific transcriptional inducers. When cells are treated with TPA (12-*O*-tertadecanoylphorbol-13-acetate), the Ras/Erk/MAPK pathway is activated resulting in activation of MSK1 and MSK2 and subsequent phosphorylation of H3S10 and H3S28. This leads to c-fos gene transcription, for which H3S10P presence is important since its absence is correlated with lower expression levels of the gene (Soloaga, A. et al. 2003).

c-fos gene transcription activation has been extensively studied. Experiments carried out by Tu, Huang et al. showed that in MEFs (mouse embryonic fibroblasts), PMA stimulation leads to IKKα/β activation of p65, which creates homodimers that travel to the cell nucleus and bind to NFκB binding sites in the c-fos gene, allowing for its expression. However, PMA stimulation of HEK293 (human embryonic kidney 293) cells did not lead to c-fos activation. This is due to the fact that the human c-fos gene does not contain an NFκB binding site while the mouse does. Additionally, TNFα (tumor necrosis factor alpha) stimulation did not induce sufficient Erk activation, which lead to a weaker c-fos gene expression (Tu, Huang et al. 2013). Moreover, Duncan *et al.* showed that during epidermal growth factor (EGF) induced c-fos gene expression but not TNFα (tumor necrosis factor alpha) induced, the IKKα kinase phosphorylates MSK1/2, which in turn

phosphorylates H3S10P at regions nearby the c-fos genes which contributes to proper gene transcription. Furthermore, MSK1/2 was shown to regulate EGF-induced IκBα promoter H3S10P even when transcription levels are low (Duncan, Anest et al. 2006). Yamamoto *et al.* showed that TNFα induced expression of NFκB responsive genes happens through IKKα recruitment together with relA and CREB to the promoters of these genes. Once there, IKKα leads H3S10P which in turns gives rise to H3K14ac (Figure 1.10) (Yumi Yamamoto and Gaynor 2003). Finally, phosphorylation of H3S10 is negatively regulated by DUSP1, also called MKP1 (mitogen activated protein kinase phosphatase 1). This protein is located in the cell nucleus and is able to dephosphorylate both tyrosine and serine/threonine residues. It has a specific affinity for H3S10P and it is unable to dephosphorylate histone 3 at Threonine 3 or Threonine 11. Additionally, mice lacking DUSP1 have been shown to have higher mortality rates when challenged with a bacterial infection than wild type mice (Kinney, Chandrasekharan et al. 2009).

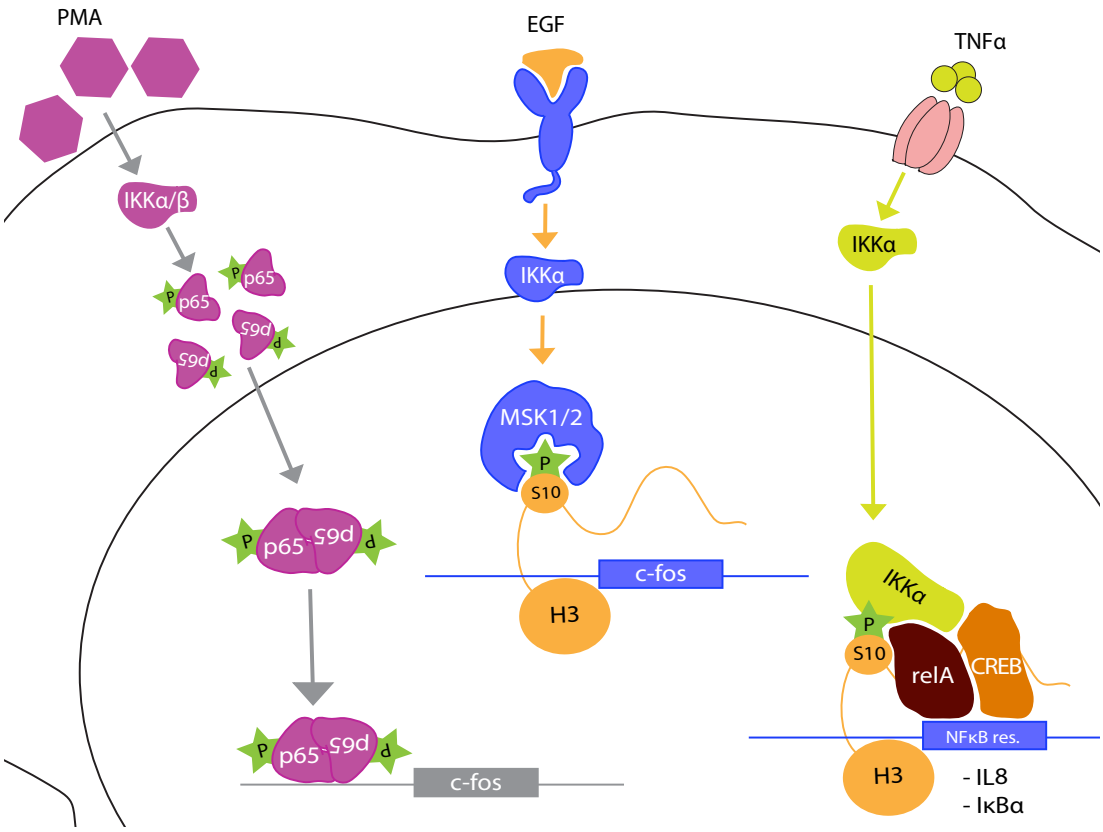


Figure 1.10. c-fos, NFκB responsive gene transcription activation and H3S10 phosphorylation. C-fos transcription can be activated via PMA through IKKα and p65 or through EGF, IKKα, MSK1/2 and subsequent H3S10P. NFκB responsive genes are activated directly through IKKα by phosphorylating H3S10 and forming a complex with relA and CREB, two transcription activators.

Transcriptional activation by H3S10P or H3S28P can occur through their binding by 14-3-3 proteins. However, it has been shown that 14-3-3 binding is more stable when H3K14ac is also present. Experiments in yeast have shown that although the presence of H3S10P leads to Gcn5 recruitment and subsequent acetylation of H3K14, H3S10P does not always precedes H3K14ac and it depends on the particular gene to be expressed. However, the presence of both of them is required for expression of genes (Walter, Clynes et al. 2008).

On the other hand, experiments in Drosophila have shown that H3S10P does not need to occur with acetylation of H3 or H4 in order to activate gene expression. Upon heat shock activation of transcription, there is a drastic increase in the hyperphosphorylation of S10 at histone 3 tails in the chromatin but during the heat shock response, the H3S10P vanishes from all the non-heat shock loci. Additionally, no acetylation of H3 or H4 tails accompanies this hyperphosphorylation (Doerks, Copley et al. 2002).

Histone 3 can be phosphorylated at some other residues such as Threonine 3. H3T3P is known to be carried out by haspin, a serine/threonine kinase, and has been implicated in mitosis as well. The presence of this modification aids to the correct positioning of the chromosome passenger complex (CPC) in the centromeres, through its binding to survivin, a protein in the CPC (Fangwei Wang 2010). The CPC is a complex involved in proper attachment of the chromosomes to microtubules, in the initiation of the spindle assembly checkpoint and in the generation of the contractile apparatus in charge of cytokinesis (Carmena, Wheelock et al. 2012).

Finally, H2A can also be phosphorylated at Serine 1. Although there is not much information about this particular residue, this phosphorylation is believed to be driven by MSK and it takes place during mitosis and S-phase, together with Histone 4 Serine 1 phosphorylation. They become visible at the same time as H3S10P and H3S28P during the cell cycle, they have a similar subcellular localization and they disappear together with S10 and S28 dephosphorylation. Additionally, these events are conserved among different organisms: worms, flies and mammals; showing their importance for mitosis progression and probably for histone deposition during S-phase (Barber, Turner et al. 2004).

To sum up, histone phosphorylation has been deeply studied for cell cycle progression and it seems to play an important role in it. However, some studies are starting to focus on the possible role that phosphorylation of certain residues have on gene transcription, as it is the case for H3S10P and H3S28P, and their interaction with other histone marks such as acetylation and methylation e.g. H3K9me3 and H3K14ac.

Section 1.03 Pathogens and host cell histone modifications

Histone modifications have been shown to be key for regulating cellular gene expression and play important roles in cell cycle progression. Modulation of histones marks can have a global effect on chromatin or give rise to finely tuned responses. They control cellular processes at the gene level, which makes them perfect targets for pathogens since this means they do not need to encode a large number of proteins for subverting the host cell upon infection. As a consequence, pathogens have evolved mechanisms to control histone marks. Up to date, research has mainly focussed on the impact that histone modulation by invading microorganisms can have on the cellular immune response: pathogens modulate histone marks that allow for the expression of genes beneficial to infection and repress those that are harmful. This way they not only dampen the cellular immune response but also contribute to their replication inside the cell leading to a successful infection. However, recent studies have also shown that some pathogens use it as a mean to generate resistance to drugs. Additionally, viruses that establish life-long latent infections in humans have been shown to modulate histone marks with a different purpose. This time, since the viral DNA is in

the cell nucleus, either integrated into the cellular genome or in an episome, it is bound by cellular histones, which are subjected to the same PTMs as those located in the cellular chromatin. As a consequence, these viruses have evolved to utilise histone modifications in order to switch their infection status: from latent to lytic, or vice versa. The most studied examples of histone modifications during infection correspond to histone acetylation, methylation and phosphorylation and are discussed below.

(a) Pathogens control host cell histone modifications

Pathogens have coevolved with their hosts for millions of years, allowing them to understand and exploit cellular mechanisms to control immune evasion. As a consequence, they have developed the best strategies to repress cellular immunity. Cytoplasmic signalling cascades leading to the activation of cellular immunity have been extensively studied since it is the first intracellular environment pathogens encounter. However, it has become more and more evident in the last decade that pathogens have also learned to control the host cell nucleus. A good way of doing this is by taking control of host cell gene expression through modulation of epigenetic marks. This allows for a global regulation of their immune response. Histone acetylation, methylation and phosphorylation have been shown to be potent mechanisms by which pathogens modulate cellular gene transcription and as a consequence it has a great therapeutic potential to treat infections that may be hard to tackle at the moment.

While there are numerous examples of how bacteria interfere with host cell chromatin marks, little to nothing is known about whether viruses also

modulate them for purposes other than switching from their latent to lytic infection phases. Here we will describe how different pathogens interact with host cell histone marks and, if possible, why they are doing it and the outcome it has for infection. Special focus will be given to histone 3 serine 10 phosphorylation, since it is a modification that has been shown to be involved in assisting NFκB transcriptional regulation during cytokine gene expression.

(i) Bacteria modulate histone marks of host chromatin

Bacteria consist of a large group of unicellular organisms that have wide tropism. They can infect protozoans, plants and animals, in which they can be restricted to a single tissue (nervous system, gastrointestinal tract, blood cells, genital tract, ect) or be more versatile and spread among different ones. Additionally, they can either live in symbiosis with their hosts or give rise to disease, leading to death in some cases. As a consequence, they have been heavily studied, specially their relationship with the immune system of the cell. In this section, we will only talk about different mechanisms that bacteria employ to subvert their hosts at the gene expression level, through modulation of host cell histone marks.

Gram-positive bacteria possess mechanisms to inject bacterial proteins to the cell before entering. Clostridium perfringens, Streptococcus pneumoniae and Listeria monocytogenes, eject the proteins perfringolysin, pneumolysin and LLO, respectively, with the aim of dampening the cellular immune response. The toxins injected by these gram-positive bacteria lead to a downregulation of H3S10 phosphorylation. Hamon *et al.* investigated a

bit more in depth the effect of LLO in modifying the cell's gene expression upon infection. Listeria monocytogenes is a bacterium that enters the host cell in a phagosome and needs the pore-forming toxin LLO (listeriolysin O) in order to be released into the cytoplasm. It is well known that the LLO belongs to the family of pore-forming toxins, known as cholesterol-dependent cytolysins (CDC), and that it activates the MAPK signalling pathway and calcium signalling in a pore-forming-dependent manner and also the NFκB pathway in a pore-forming-independent manner. However, this study showed that soon after infection and preceded by a quick increase in H3S10P, this protein on its own is able to dephosphorylate H3S10 with cells showing a 4-fold decrease when compared with phosphorylation levels in uninfected cells. Despite its mechanism of action is still unknow, this dephosphorylation is linked to an increase in H3K9ac and also a decrease in the overall acetylation levels in H3 and H4. Gene expression levels of 47,000 genes showed that upon LLO treatment, many genes change their expression pattern: 47 genes were repressed and 99 genes were activated more than 1.5 fold. RTqPCR on some of those gene targets corroborated the data: cxcl2 and dusp4 were repressed while prkdc was induced. In addition, there were some genes whose expression pattern did not change upon LLO treatment. Together, this data supports the idea that epigenetic modifications, and more specifically H3S10P, are a target for bacterial infection since they give them the control over the cellular immune response at the chromatin level. Additionally, perfrinfolysin, and pneumolysin together with other CDCs have also been shown to modify H3S10P, showing it is a conserved mechanism among pathogens for proper infection (Hamon, Batsche et al. 2007).

In addition to this, L.monocytogenes efficiently deacetylates Histone 3 at lysine 18. This deacetylation appears at 3 hours post infection and becomes more and more evident as infection progresses. Experiments involving the use of HDAC chemical inhibitors showed that treatment with NIC (nicotinamide), a sirtuin blocker, made H3K18 deacetylation impossible and specifically, 2-cyano-3-[5-(2,5-dichlorophenyl)-2-furanyl]-N-5-quinolinyl-2-propenamide (AGK2), a SIRT2 inhibitor, prevented the deacetylation of this particular lysine. Immunofluorescence and subcellular fractionation studies have shown that upon L.monocytogenes infection, SIRT2 is recruited to the cell nucleus. Additionally, a subset of SIRT2-dependent genes are downregulated, DNA binding proteins or proteins involved in transcriptional regulation: MAPK14, PIK3R3, SOS1, CAMK26 and MAP2K6. It is very likely that histone deacetylation is necessary for the latest stages of bacterial infection, bacterial replication, in order to subvert the immune response of the cell. Furthermore, two other bacteria have been reported to modulate acetylation of histones. Mycobacterium tuberculosis deacetylates histones at the promoters of the genes HLA-Dra, HLA-DRb and CIITA, downregulating their expression while Helicobacter pylori specifically deacetylates H3K23, although its implications are not known yet (Eskandarian, Impens et al. 2013).

Anaplasma phagocytophilum is a rickettsial bacteria that propagates in human neutrophils and their bone marrow progenitors, which are primary defense cells and therefore complicated hosts. As a consequence, A. phagocytophilum evolved a mechanism by which it recruits, through its protein Ankyrin A (AnkA), HDAC1 to promoters of host defense genes such as EPX, MPO, AZU1 and ELA2, and depletes them from acetylation which

leads to gene repression. In addition to this, it actively switches on the transcription of genes encoding HDAC1 and HDAC2, which is correlated with the dampening of immunity genes. Moreover, HDAC1 expression seems to have an autoregulatory mechanism and after peaking at 48 hours post infection, its transcription starts to decrease. A. phagocytophilum, together with mycobacterium, have evolved a mechanism to overtake the cellular HDACs, bring them to the gene promoters of interest and this way shut down the expression of genes that are harmful for their intracellular survival (Garcia-Garcia, Barat et al. 2009).

Pseudomonas aeruginosa, an opportunistic pathogen of airways, works in a similar way to A. phagocytophilum by inducing HDAC1 expression through the secretion of 2-aminoacetophenone, which leads to global histone hypoacetylation at H3K18. This, in turn, causes a lack of responsiveness to the stimulation with inflammatory cytokines and chemokines such as TNFα, IL1β and MCP1. Once again, this phenotype was reversed when cells were treated with HDAC inhibitors. On the same line, Escherichia coli's protein NleC, a zinc-dependent metalloprotease, binds to the cellular p300 HAT and it targets it for degradation, which results in IL8 gene repression. It can be concluded that histone acetylation is a mechanism that helps the host cell upregulate gene expression at any time during its life and as a consequence pathogens have learned to overcome that in order to suppress immune-related genes (Figure 1.11) (Grabiec and Potempa 2018).

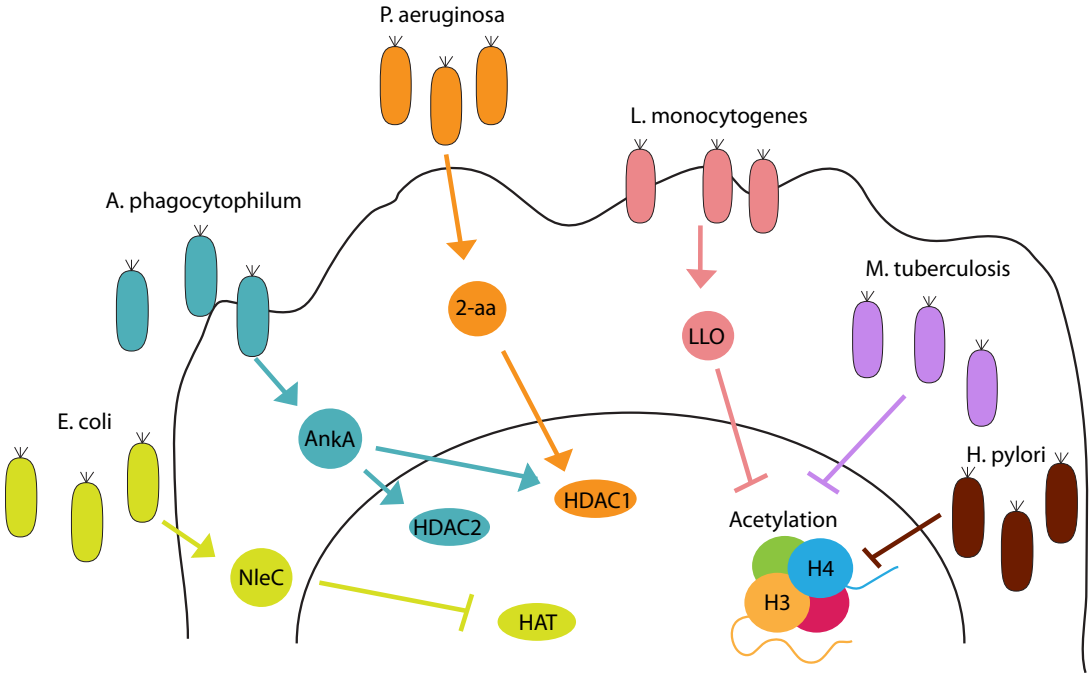


Figure 1.11. Bacteria interfere with cellular histone acetylation. The different types of bacteria induce hypoacetylation in specific genes through diverse pathways in order to suppress transcription of immune response genes. Adapted from Gabriec and Potempa, 2018.

Legionella pneumophila also regulates host cell gene expression through chromatin marks. Before the bacteria enters the cytoplasm, it secretes the protein RomA, a SET domain-containing methyltransferase that is able to methylate H3K14me3 as soon as it enters the nucleus to promote efficient bacterial replication and escape immunity. By doing this, it is decreasing the amount of H3K14ac in the genome and it is changing the default state of those sites from active gene expression to repressed upon infection. Many genes related to immune processes were affected by this and among them we find TNFα, IL6, TLR5 and Nalp3. While it is known that other pathogens methylate histones upon infection, like it is the case of Chlamydia trachomatis with H2B, H3 and H4, L. pneumophila driven H3K14me3 is a particular case. This modification has never been reported in mammalian cells and it is believed to not naturally occur, so it is unclear

why the pathogen does this. A possibility could be that the other host of this bacteria, protozoans, do use this modification to combat infection and so L. pneumophila evolved its RomA SET-domain containing protein to subvert it. Alternatively, it could as well be that the bacterium has evolved a novel way to sabotage its hosts' immune system. There is some evidence for this latter hypothesis which comes from phylogenetic analysis of the SET domain of the protein. It seems plausible that L. pneumophila obtained this SET domain protein from one of its protozoan hosts in order to target H3K4 methylation but over time, it has acquired enough mutations to make it specific towards the K14 residue in histone 3, which allows the bacteria to block acetylation at this amino acid and therefore repress gene expression (Figure 1.12) (Rolando, Sanulli et al. 2013).

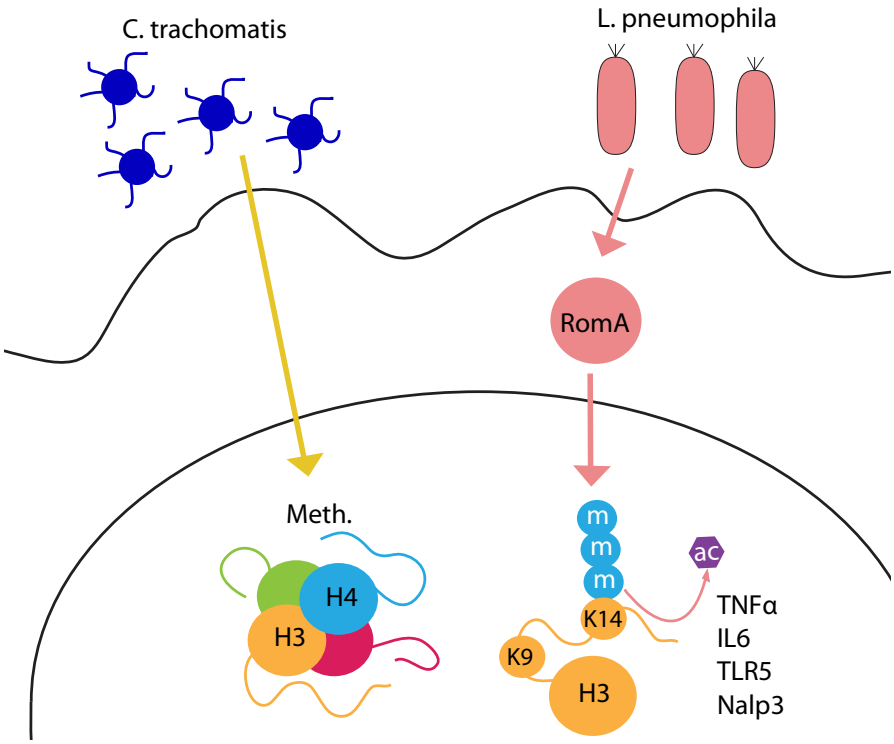


Figure 1.12. Bacteria modulate host cell histone methylation. L. pneumophila ejects its RomA protein, a methyltransferase, that trimethylates H3K14, which allows for the displacement of the acetyl group at that residue and ends up in repressing transcription of the genes nearby. C. trachomatis affects methylation of H3, H4 and H2B. Adapted from Gabriec and Potempa, 2018.

In the case of Shigella flexneri, a gram-negative bacterium, it secretes its protein OspF, which not only enters the cell's cytoplasm but also its nucleus. OspF is a dual specificity phosphatase, meaning it can dephosphorylate both serine/theronine and tyrosine residues. Arbibe et al. have shown that this bacterial protein specifically dephosphorylates mitogen-activated kinases in the nucleus and this in turn leads to dephosphorylation at H3S10 at the promoter of selected genes, preventing the transcriptional activation of NFκB-induced genes, such as CCL20 or IL8, a dendritic chemoattractant and a chemokine involved leukocyte recruitment to the site of infection, respectively. In the cell, phosphorylation of H3S10 at specific loci is triggered by either the Erk-MAPK or p38-MAPK signalling pathways. This is linked to an upregulation of immediate-early genes, such as FOS, and NFκB-responsive genes. The presence of this post-translational modification is believed to favor chromatin remodeling allowing the access of NFκB transcription factors to that particular region. An interesting finding from this study was that even though OspF was shown to directly dephosphorylate Erk and p38 inside the cell nucleus, H3S10P was only affected at specific promoter sites. This could be supported by evidence obtained from yeast experiments, in which they showed that once the MAPKs phosphorylate H3S10, they stay bound to gene promoters and this way could give rise to gene specific modulation. Additionally, H3S10P abrogation led to a decrease in H3K9me and H3K14ac, which were not directly carried out by OspF but it seems more likely to be a consequence of H3S10 dephosphorylation. Together, this data shows how a gram-negative bacteria is able to modulate the cellular immune response by targeting histone

modifications before the pathogen even enters the cell, generating a permissive environment for infection (Arbibe, Kim et al. 2007).

To sum up, bacteria have evolved proteins and mechanisms through which they take control of the cell's immune response (Figure 1.13). They modulate histone acetylation, methylation and phosphorylation at specific histone residues which allows them to suppress expression of harmful genes and allow the expression of those cellular genes that will be needed to benefit bacterial infection.

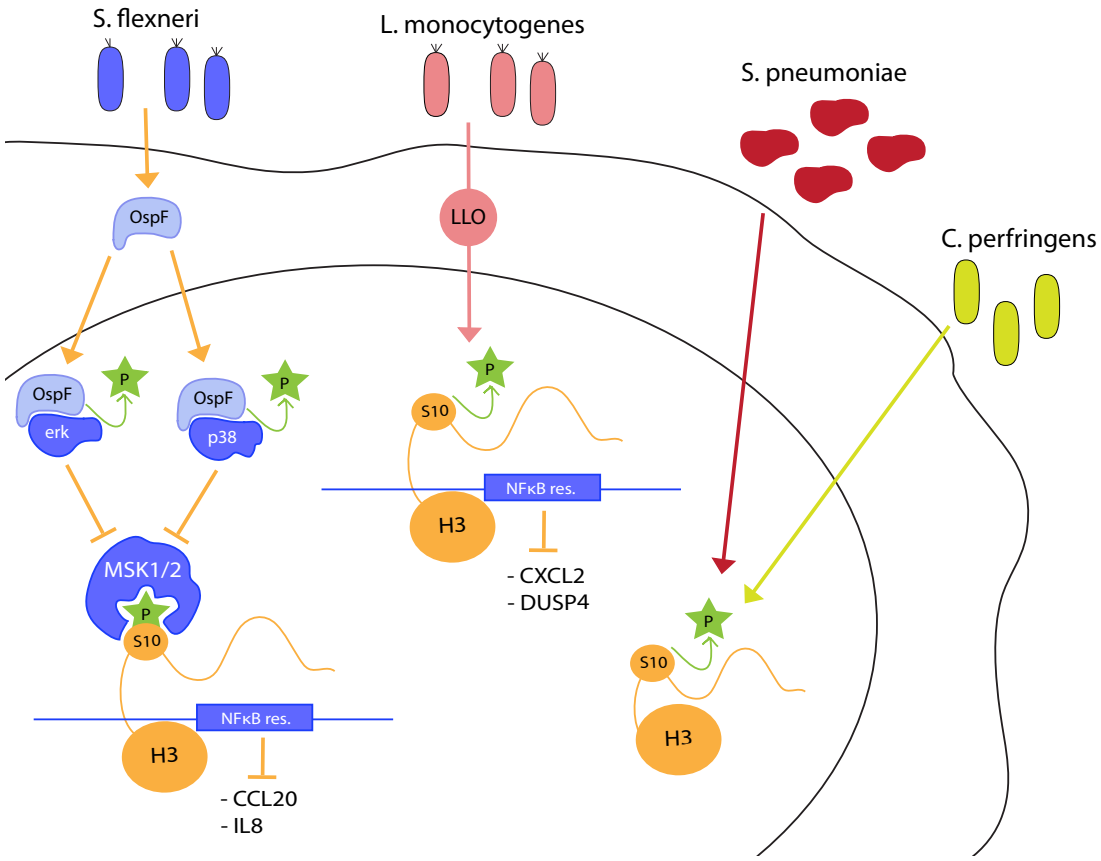


Figure 1.13. Bacteria modulate host cell histone phosphorylation. S. flexneri ejects its OspF protein into the cell cytoplasm, which then travels to the nucleus where it dephosphorylates erk and p38, which in turn prevents H3S10 phosphorylation from taking place and affects immune gene transcription. L. monocytogenes injects its LLO toxin, that by mechanisms still not understood leads to H3S10 dephosphorylation and downregulation of immune related genes. S. pneumoniae and C. perfringens also have toxins that interfere with H3S10 phosphorylation. Adapted from Gabriec and Potempa, 2018.

(ii) Parasites and fungi take over histone modifications of host cells

While parasites and fungi have not received as much attention concerning host cell histone modifications, there are some studies that show these two types of pathogens interfere with histone acetylation in the cell.

Parasites such as Toxoplasma gondii have been suggested to modify the chromatin of macrophages upon infection. Indeed, infected cells display a reduced response to IFN-γ compared to uninfected cells due to defective STAT1 binding to GAS sequences in gene promoters. A decreased histone acetylation in those regions has been observed. This leads to an impaired recruitment of chromatin remodeling complexes, which interfere with STAT1 binding and therefore transcription of the related genes. However, this defect can be recovered by using HDAC inhibitors, which in turn correlate with higher acetylation in the chromatin and upregulation of IFN-γ induced gene expression. Although the molecular mechanism is still unknown, it is clear that Toxoplasma gondii infection requires hypoacetylation of histones at specific gene promoters (Lang, Hildebrandt et al. 2012).

On the other hand, fungi may also be affecting host cell acetylation during infection since it is known that their treatment with HDAC inhibitors leads to attenuated infection. In fact, treatment of C. neoformans with the HDAC inhibitors sodium butyrate and trichostatin A (TSA) showed a defective growth of the pathogen at 37° C, in addition to decreased melanin, phospholipase and capsule polysaccharide production. Moreover, inhibition of the C. albicans genes HDA1 and RPD3, which encode HDACs, affects the fungi growth and ability to develop azole resistance. Finally, Aspergillus fumigatus is also affected by inactivation of its HDAC genes since it gives

rise to reduced germination and secondary metabolite production (Lee et al. 2009)(Li et al. 2015)(Kmetzsch 2015).

From bacteria to fungi, pathogens have evolved to specifically interact with host cell histone marks. This suggest it must be an important way to promote infection, since it has either been conserved between pathogens or evolved multiple times. There is current limited information about the mechanisms parasites and fungi employ to sabotage host chromatin marks but the little evidence there is shows acetylation is definitely key for proper infection.

(iii) Viruses modify host cell histone marks

In addition to bacteria, parasites and fungi, viruses have also developed mechanisms to modulate histone modifications. Furthermore, it is vital to remember that most viruses need to integrate their genome into the host genome, and if not, its DNA remains in the cell nucleus in the form of an episome. This means that the viral DNA is also wrapped by cellular histones and viruses not only control host cell gene expression through histone marks but also their own. This is the main difference with the rest of pathogens, where they only control epigenetics as a form of modulating host cell gene expression.

Furthermore, some studies have also shown that viral infection leads to a quick cellular response that attempts to dampen infection. Experiments carried out by B.S. Parekh et al. showed that after Sendai virus infection, histones 3 and 4 are drastically hyperacetylated in the nucleosomes within the promoter and 5’ end of the IFN-β coding region, gene that encodes a

cytokine necessary for immune activation of surrounding cells. It is the assembly of the transcription factor complex, formed by p50, p65, ATF-2, c-jun, and IRF-3, at the IFN-β enhancer region that allows for p300/CBP histone acetyltransferase to be recruited to the site and carry out its function. This way, IFN-β gene expression is activated upon infection (Maniatis 1999). Moreover, Agaloti et al. discovered that acetylation seems to occur at specific sites in these two histones: H4K8ac, H3K9ac and H3K14ac. They all seem to be carried out by the GCN5 acetyltransferase. H4K8ac was shown to be necessary for SWI/SNF recruitment, a chromatin remodeling complex that moves nucleosomes along chromatin and therefore facilitates gene transcription. On the other hand, H3K9ac and H3K14ac proved to be key for TFIID recruitment, a general transcription factor. Together, these three acetylation marks allow for IFN-β gene expression as SWI/SNF recruitment allows for nucleosome remodeling, which is essential for TFIID binding to the TATA box in the gene promoter (Figure 1.14) (Theodora Agalioti and Dimitris Thanos1 2002). This activation of IFN-β gene expression shows how the cell's immune system responds to viral infection by activating transcription of immune-related genes.

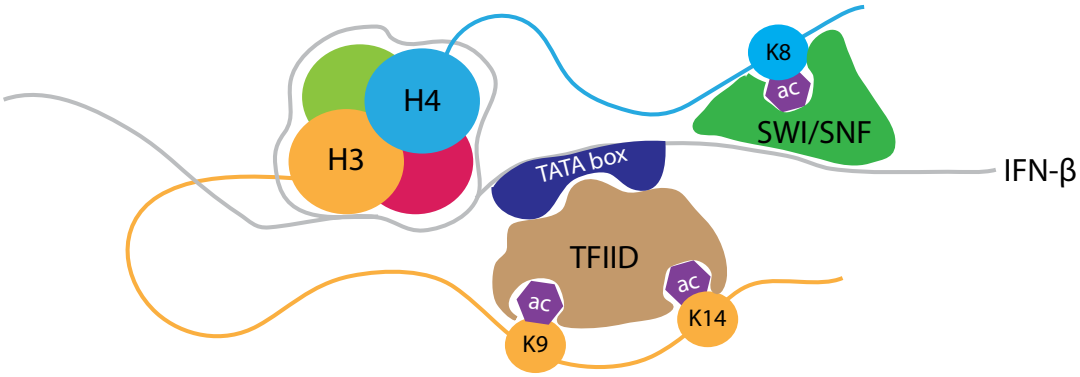


Figure 1.14. Cellular response to Sendai virus infection. As the cell recognizes the presence of the virus, it upregulates IFN-β gene expression. This is due to the acetylation of H4K8, which allows the binding of SWI/SNF and moves nucleosomes along chromatin to facilitate gene transcription. Furthermore, acetylation of H3K9 and H3K14 allows for the recruitment of the transcription factor TFIID.

On the other hand, viruses also directly control histone modifications. Such an example is Influenza virus, which interferes with host cell chromatin dynamics and epigenetic marks. This virus is known to affect methylation levels of promoters of several proinflammatory cytokines such as IL17, IL13 and CXCL6. Additionally, new research shows that Influenza also modulates histone methylation. A particularly important residue seems to be H3K79, which displays increased methylation upon viral infection. Altering H3K79 methylation levels by using inhibitors of Dot1L, the cellular enzyme responsible for the modification, reduced viral growth. The presence of this PTM is linked to gene expression, and in healthy cells it plays a role in cell cycle regulation and the DNA damage response. It is postulated that during infection it may be involved in controlling the interferon-induced cellular immune response against the virus. Another chromatin modification that Influenza modulates is H3K9me3. Upon infection, Influenza induces a type I interferon dependent upregulation of Setdb2 – histone methyltransferase belonging to the SUV39 family– that leads to increased H3K9me3 in the

CXCL1 gene, a neutrophil attractant, and subsequent transcriptional repression. This prevents neutrophil infiltration and contributes to secondary bacterial infections. As a consequence, Setdb2 modulation and H3K9me3 aid Influenza to escape the cellular immune response as well as rendering cells more susceptible to superinfection (Schliehe, Flynn et al. 2015).

As previously mentioned, some viruses use epigenetics to switch from latent to lytic infection. This is the case of Human Cytomegalovirus (HCMV), a beta-herpesvirus that establishes life-long latent infections in humans after a primary challenge, specifically in haematopoietic cells of the bone marrow and dendritic cells (DCs). In the cell nucleus, the cellular protein hDaxx silences the expression of viral immediate-early genes by binding to the promoter MIEP (major immediate-early promoter) and creating a repressive chromatin which can be counteracted by the use of HDAC inhibitors. It may be its binding to ATRX, a transcriptional repressor, that might be recruiting an army of proteins to generate a repressive chromatin state. However, HCMV combats this using two proteins: pp71, a tegument protein that blocks hDaxx and targets it for degradation inside the nucleus, and IE72, a protein involved in activating early gene expression through its binding to hDaxx at LUNA promoters (Reeves, Woodhall et al. 2010, Reeves and Sinclair 2010). Interestingly, human cytomegalovirus (HCMV) also modulates H3K79 methylation, as Influenza virus does, but the reasons for it are completely different. In this case, the virus genome is maintained as an episome in the cell nucleus, so it is wrapped by cellular histones. Therefore, by modulating this histone modification, the virus can not only control cellular immune responses but also modulate its own replication (Marcos-Villar, Diaz-Colunga et al. 2018). Moreover, early during infection, the major immediate-early

promoter (MIEP) of HCMV is bound by histones presenting repressing chromatin marks, such H3K9me2 which in turn is bound by HP1β. This repression of viral gene expression is overcome by HDAC inhibitors, for instance TSA, which ends up in an increase in Histone 4 acetylation and as a consequence in higher transcriptional activity (Groves, Reeves et al. 2009). This example accentuates once again the interplay between acetylation and methylation and how pathogens tend to methylate histones in order to prevent their acetylation and as a consequence gene expression. In addition to acetylation and methylation, there is strong evidence that HCMV might be regulating histone phosphorylation as a mechanism to escape viral latency. Kew et al. showed that for the exit of latency and establishment of a lytic infection, HCMV requires CREB to be bound to the major immediate-early promoter (MIEP). This, in turn, recruits MSK to the chromatin and leads to H3S10P and subsequent histone demethylation in the histones bound to the latent MIEP, which is followed by viral gene expression activation. It has been shown that inflammation, as well as DCs maturation or differentiation are key components for reactivation of HCMV, events for which H3S10P are important. Additionally, IL6 administration contributes to the viral exit of the latent state. Since the MIEP is composed of several motifs that make it responsive to the NFκB, CREB, AP-1 and IFN signalling pathways, it may well be that reactivation of the virus occurs through multiple pathways. In any case, H3S10P and histone acetylation remain key components necessary for latency exit of HCMV (Kew, Yuan et al. 2014). Furthermore, H3S10P has been shown to be important for HCVM viral replication. The addition to cells of BAY61-3606, an IKKα kinase inhibitor, blocks HCMV strain AD169 replication. In addition, siRNA directed

towards IKKα had a similar effect. The pharmacological or RNA targeted repression of IKKα lead to a reduction in H3S10P and acetylation of H3K9, H3K18, H3K27 as well as the trimethylation of H3K27 and H3K36 – while H3K14 acetylation levels remained unaffected. In turn, this gave rise to a decrease in the production of immediate-early proteins. Despite H3S10P is implicated in cell cycle progression, no defects were observed in cell division. As a consequence, it seems plausible that the defect in MIEP gene expression due to the decrease in H3S10P was the result of an impaired recruitment of transcription factors, such as 14-3-3 proteins that bind preferentially to H3S10P when there is also acetylation of H3K9 or H3K14, and at the same time of an impaired recruitment of repressors such as HP1, which bind to chromatin marks where H3S10P is missing but H3K9me3 is present. Additionally, H3S10P is necessary for correct chromatin partitioning to the viral compartments where viral DNA replication takes place, which could be another explanation for the phenotype observed. In conclusion, H3S10P seems to be vital for MIEP gene expression and HCMV viral replication (Ho, Donovan-Banfield et al. 2016).

Viruses modulate host cell histone acetylation, methylation and phosphorylation although in most of the reported cases it is due to modulation of their own gene expression (Figure 1.15). It has become evident the interplay that exists between acetylation and methylation and how pathogens tend to methylate histones in order to prevent their acetylation and as a consequence repress gene expression. Finally, little to nothing is known about whether viruses also modulate histone phosphorylation upon infection. However, there is strong evidence that

HCMV might be doing so as a mechanism to escape viral latency, among others.

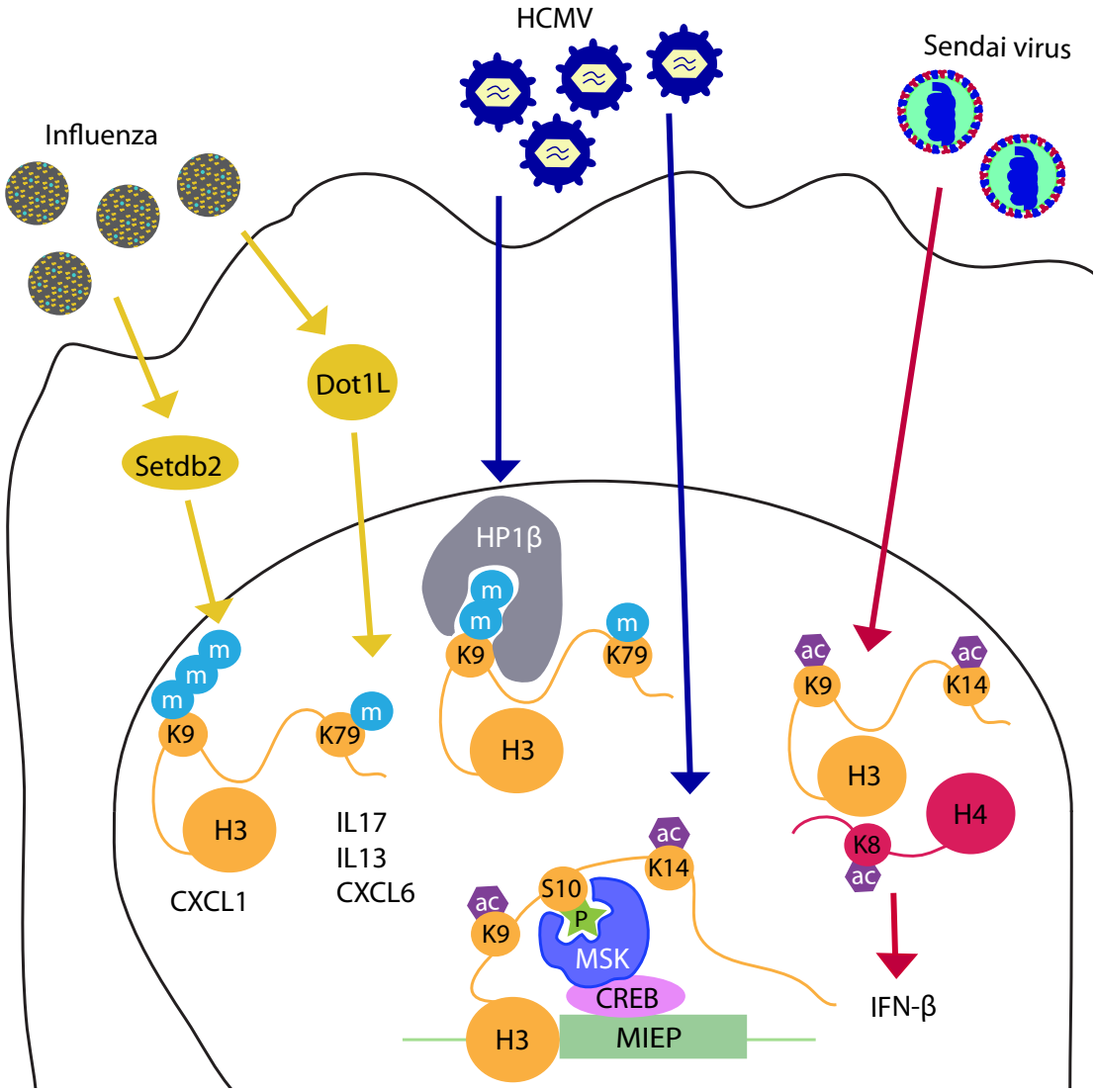


Figure 1.15. Viruses modulate histone modifications during infection. Influenza virus trimethylates H3K9 and monomethylates H3K79 in order to repress CXCL1 and IL17, IL13 and CXCL6 gene expression, respectively. During latent infection, HCMV promoters are demethylated at H3K9 and monomethylated at K79. Upon gene expression induction, CREB binds to MIEP, recruiting MSK which phosphorylates H3S10 and promotes acetylation of H3K9 and H3K14, which in turn can be bound by 14-3-3 transcription factors. Sendai virus infection results in cellular acetylation of H3K9 and H3K14 in IFN-β promoters for antiviral gene expression. Adapted from Gabriec and Potempa, 2018.

(b) Vaccinia modulates host cell histone modifications

Histone methylation modulation has been shown the be relevant for proper infection in the case of bacteria and viruses and also for cellular defense against parasites. Several methylation marks have been shown to be important so far, however, all of them have the final aim of switching off or repressing gene expression. It would be interesting to investigate whether pathogens also manipulate histone methylation with the aim of overexpressing or activating the transcription of genes that may contribute in some way to a more effective infection.

Interestingly, recent studies have focused on the role that cytoplasmic viruses may play in altering host cell epigenetics to promote their infection efficiency. Such is the case of Vaccinia virus. Despite not needing to integrate its DNA into the cells genome and replicating in the cytoplasm, recent studies have shown that several Vaccinia proteins go into the cell nucleus. In addition to this, Teferi investigated the possible role of histone methylation during Vaccinia infection and found out that H3K9me3 and H4K20me3 change drastically due to the presence of this pathogen. Both histone marks increase with the course of infection, reaching a peak at 9-12 hours post infection. The increase of these two PTMs occur in the different cell types tested but not after infection with different strains of poxviruses. Leporipoxviruses infection failed to create such chromatin marks, as opposed to Orthopoxviruses, showing that it is not a common mechanism used by all Poxviruses. Deletion and complementation experiments of the Vaccinia protein K7 show that methylation at these residues is at least partially dependent on it, although the mechanism of action is not yet understood. Additionally, while the K7 protein sequence is conserved among

viruses in the Orthopox genus, it is not found in the Leporipoxviruses used for this study, Myxoma and Shope fibroma viruses. Moreover, the gene coding for this protein is mutated in all of the ectromelia virus strains. So far, the current understanding of K7 function in the cell is only linked to its role in inhibiting NFκB activation through TLR signalling and in inhibiting the activation of the IFNβ promoter via IRF signalling. There is nothing in the protein sequence or structure that indicates it could behave as a methyltransferase. Furthermore, H3K9me3 and H4K20me3 increase upon infection does not take place in cells lacking SUV39H1 and SUV39H2, suggesting that the final effector of this methylation is a cellular protein. As K7 blocks IRAK2 and TRAF-6 function, it may be that inhibiting its downstream signalling – which involves degradation of IKBα and NFκB binding to gene promoters – enhances the formation of heterochromatin at specific gene promoters by methylating H3 at K9 and H4 at K20 (Figure 1.16) (Teferi, Desaulniers et al. 2017).

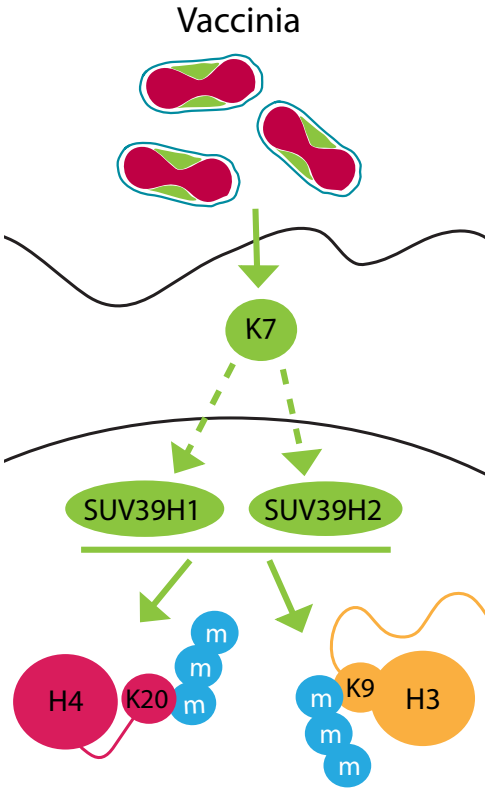


Figure 1.16. Vaccinia also modulates host cell histone methylation. VV trimethylates H4K20 and H3K9, which is initiated by the viral protein K7 but mediated by the cellular SUV39H1 and SUV39H2. Adapted from Gabriec and Potempa, 2018.

In addition to this, previous data from our lab shows that Vaccinia not only interacts with cytoplasmic proteins but also with nuclear proteins, including histones. The first piece of evidence comes from a phospho-proteomics screen carried out by Dr. Karel Novy (ETH, 2015) in collaboration with the Mercer lab. He compared uninfected with infected cells, looking for differences in the phosphorylation status of cellular proteins. This experiment showed that Vaccinia infection has an extraordinary effect on the phosphorylation of nuclear proteins (Table 1). Some of them involved in chromatin remodelling and gene expression regulation. For example, NCOR1 was found to be dephosphorylated after Vaccinia infection, while in uninfected cells it appeared as phosphorylated. This protein is known to

modulate transcriptional repression by generating complexes that promote histone deacetylation. In turn, this leads to a repressive chromatin state that blocks the access of transcription factors. Among the genes it affects we find BCL6, which in turn regulates the STAT dependent expression of Interleukin 4 (IL4). Another of the proteins whose phosphorylation decreased upon infection was MLL2. Interestingly, this protein plays an opposing function to NCOR1. MLL2 methylates K4 in H3 and this is a PTM involved in transcriptional activation. Taken together, these results show that Vaccinia is very likely to be affecting cellular gene expression by upregulating some genes while downregulating others.

Protein	Function	Phosphorylation status after VV infection vs Uninfected
HDAC2	Histone K deacetylase	Downregulated
HUWE1	Ubiquitinates core histones	
NCOR1	Forms a complex involved in histone deacetylation	
MLL2	Histone methyltransferase	
DNMT1	DNA methyltransferase	
DMAP1	DNA methyltransferase	Upregulated

Table 1. List of proteins that have been observed to change their phosphorylation status after Vaccinia Virus infection. The only ones shown are proteins involved in chromatin remodelling, such as methyltransferases, deacetylases, ubiquitinases and also a protein that forms a complex involved in deacetylation.

Second, Dr. Jason Mercer performed an siRNA screen (ETH, 2014) in which 7,000 human genes were knocked down. The goal was to figure out which cellular proteins are obligatory for Vaccinia infection. Once again, most of the hits were genes encoding cytoplasmic proteins. Among the nuclear proteins found there are transcription factors (IK, SALL2, SLIT3, HNF1A,

TCF7L2 and TFEC) as well as transcriptional repressors (HES5), nuclear proteins with a known role in the regulation of gene expression (SP110) and proteins involved in chromatin modification (SMARCA4, DPF1). Additionally, we found NUP153 (nucleoporin 153 kDa) and NUP62, HDAC5 (histone deacetylase 5) and HTATIP (lysine acetyltransferase 5). These are proteins involved in nuclear import/export and proteins involved in histone acetylation (Table 2). This screen revealed that Vaccinia needs the presence of specific host nuclear proteins in order to successfully complete infection.

Gene ID	Acronym	Gene name
3431	SP110	SP110 nuclear body protein
3550	IK	IK cytokine (down-regulator of HLA II)
6297	SALL2	Sal-like 2 (Drosophila)
6586	SLIT3	Slit homolog 3 (Drosophila)
6597	SMARCA4	SWI/SNF related, subfamily a, member 4
6927	HNF1A	HNF1 homeobox A
6934	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
8193	DPF1	D4, zinc and double PHD fingers family 1
9972	NUP153	Nucleoporin 153kDa
10014	HDAC5	Histone deacetylase 5
10524	HTATIP	K(lysine) acetyltransferase 5
22797	TFEC	Transcription factor EC
23636	NUP62	Nucleoporin 62kDa
388585	HES5	Hairy and enhancer of split 5 (Drosophila)

Table 2. Proteins that when removed from the host-cell prevent VV infection. A list of more than 180 genes was obtained from the screen. From them, these 14 hits are nuclear proteins that seem to be relevant for chromatin remodelling.

The final piece of evidence from our lab showing Vaccinia interacts with the host cell nucleus comes from a phospho-proteomics screen carried out by Mercer and colleagues. It focussed on the phosphorylation changes of cellular proteins upon infection with three different viruses: B1 mutant, F10 mutant and H1 mutant. They correspond to viruses that lack the kinases B1

or F10 or the phosphatase H1. Mass spectrometry analysis of cell nuclear and cytoplasmic fractions revealed which of the three viral proteins are necessary for the phosphorylation of each cellular protein modified upon infection. Among the nuclear targets discovered, we find proteins that belong to the RNA splicing machinery and DNA recombination machinery. In addition, the Vaccinia proteins B1, H1 and F10 were seen to interact with cellular histones: H1 with Histone 3, F10 with Histone 1, and B1, H1 and F10 with Histone 4 (Figure 1.17).

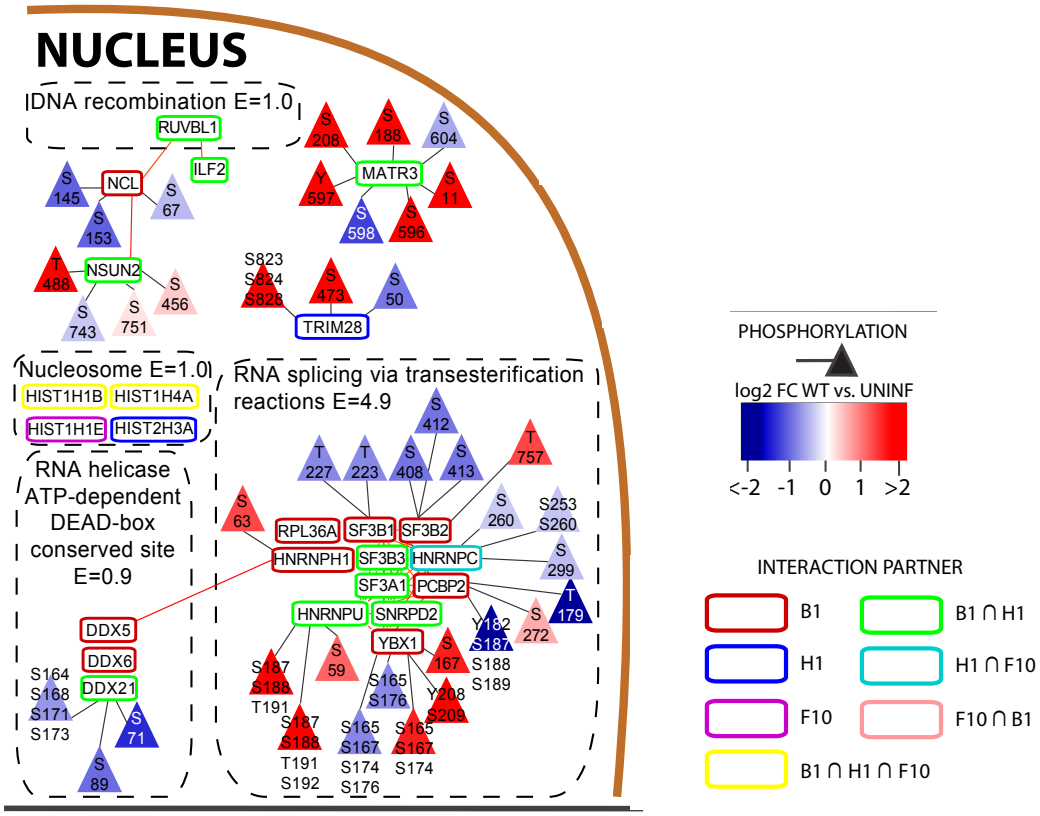


Figure 1.17. Nuclear interaction partners of the viral enzymes: B1, F10 (kinases) and H1 (phosphatase). Also, the phosphorylation change of some proteins after infection is annotated (Mercer, Unpublished).

Moreover, deep RNA sequencing experiments have shown the pattern of gene expression presented by the cell upon Vaccinia infection. They have shown that while early during infection (0-2 hours) Vaccinia expresses an

extremely low number of genes, the cell transiently increases its gene expression. This may be due to an activation of the cell immune response upon pathogen detection. It is after 2 hours post infection that there is a progressive increase in viral gene transcription while there is a progressive decrease in the cellular ones. This data shows that it is plausible that Vaccinia is indeed regulating host cell gene expression and very likely it is repressing the expression of immune related genes (Figure 1.18) (Yang et al, 2010).

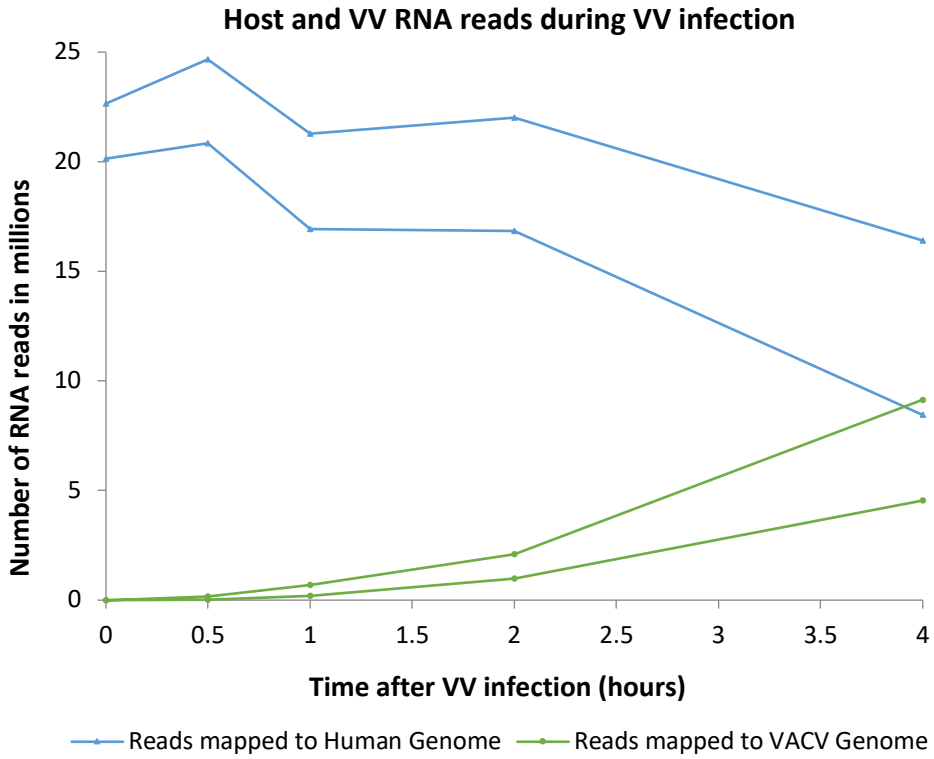


Figure 1.18. Gene expression levels of the host-cell and vaccinia virus at different time points before and after infection. As cellular gene expression decreases, viral gene expression increases (Yang, Z. *et al*. 2010).

To sum up, the phospho-proteomics interaction screen data previously obtained in the lab, performed in an unbiased fashion, plus the siRNA screen and the change in phosphorylation status of histone modifiers indicates that Vaccinia and the host cell nucleus maintain some kind of relationship.

Additionally, Teferi et al. has effectively shown that histone methylation is increased upon Vaccinia infection and that this effect is mediated by the viral protein K7.

Section 1.04 Cellular immune responses, the NFκB pathway and infection

All types of organisms possess an immune system to protect themselves from any possible pathogen. In the case of mammals, we have evolved the innate immunity, first line of defense, and the acquired immunity, a more specialised response to infection. While they both are vital for the clearance of pathogens from an organism, we will only focus on innate immunity, since our experiments were carried out *in vitro* where no adaptive immune response was possible (Akira, Uematsu et al. 2006).

Innate immunity is quickly activated after the recognition of a foreign antigen and it takes place within the infected cell, which signals to surrounding cells to alert them of the presence of a pathogen. This recognition takes place through PRRs, which are a series of germline-encoded pattern-recognition receptors that bind to PAMPs, pathogen-associated molecular patterns, which are microbial components essential for their survival that cannot be altered. There are four different families of PRR corresponding to TLRs (Toll-like receptors), CLRs (C-type lectin receptors), RLRs (Retinoic acid-inducible gene-I-like receptors) and NLRs (NOD-like receptors). When activated, they upregulate the expression of specific genes, according to the receptor activated and also the signalling cascades that it generates. However, all of those genes are involved in inflammatory

responses such as proinflammatory cytokines (TNFs, ILs), type I interferons (IFN) and chemokines (Table 3) (Takeuchi and Akira 2010).

Receptors	Location	Recognize	Genes upregulated
TLR	Membrane and intracellular compartments	Pathogen components outside the cell and in intracellular endosomes and lysosomes	Cytokines (through NFκB) and type I IFNs
CLR	Membrane	Carbohydrates in viruses, bacteria and fungi	Cytokines (through NFκB) and TLR mediated immune complexes
RLR	Cytoplasm	RNA of dsRNA viruses and dsRNA during ssRNA viral replication	Cytokines (through NFB) and type I IFNs
NLR	Cytoplasm	Bacterial peptidoglycan components	
Cytosolic DNA receptors	Cytoplasm	Foreign DNA in cell cytoplasm	Cytokines and IFNs (through STING)

Table 3. Types of PRRs, location within the cell, PAMPs specificity and gene expression activation. While TLR and CLR are membrane receptors, RLR and NLR are cytoplasmic. Adapted from Takeuchi et al. 2010.

Toll-like receptors (TLRs) are type I integral membrane glycoproteins containing an extracellular domain formed by variable leucine-rich repeat motifs and an intracellular domain homologous to the interleukin receptor 1 (IL1R) that is involved in generating the intracellular signalling cascade. TLRs were initially discovered in Drosophila, as an essential protein for embryonic development. Up to date, we know of 12 different TLRs in mammals, that recognize from pathogen lipids (TLR1, TLR2, TLR6) to nucleic acids (TLR7, TLR8, TLR9). The expression of these receptors fluctuates and can be quickly controlled in response to pathogens. Once TLRs have bound a ligand, they dimerize, undergo conformational changes and recruit adaptor molecules to their cytoplasmic domain. There are four types of adaptor proteins: MyD88, TIRAP/MAL (TIR associated protein/MyD88 adaptor like),

TICAM1 (TIR domain containing adaptor protein inducing IFNβ "TRIF"/TIR domain containing molecule 1) and TRAM (TRIF related adaptor molecule). Depending on which one is recruited to the receptor, the outcome will be different: while MyD88 leads to the expression of proinflammatory cytokines, TRIF leads to type I IFNs transcription. The expression of proinflammatory cytokines is the consequence of a long intracellular signalling cascade: TLRs are activated by the presence of a pathogen, which recruits MyD88 to its intracellular domain. If the receptors are TLR2 or TLR4, an accessory protein, TIRAP/MAL, is required for MyD88 recruitment. Then, IRAK4 and IRAK1 bind to MyD88, which allows for IRAK1 phosphorylation by IRAK4. This leads to IRAK1 binding to TRAF6, which acts as a ubiquitin ligase E3 by autoubiquitinating itself at the same time as NEMO, which targets them for degradation. Additionally, TRAF6 also recruits TAK1, which in turn phosphorylates IKK and the MAP kinase kinase 6 (MKK6), resulting in the activation of NFκB and MAP kinases which leads to the expression of proinflammatory genes. Additionally, TRL stimulation leads to IRF5, transcription factor, nuclear translocation, binding to ISRE (IFN stimulated response element) and upregulation of cytokine gene expression (Akira, Uematsu et al. 2006).

C-type lectin receptors (CLRs) have been mainly studied in their role against fungal infections. They recognize carbohydrates and comprise 17 different groups, according to phylogeny and structure. Additionally, according to their signalling pathways they can be classified in 3 groups: activator Syk-coupled CLRs with ITAM domains (immunoreceptor tyrosine-based activation motif domains), inhibitory CLRs with ITIM domains (immunoreceptor tyrosine-based inhibition motif domains) and CLRs without

clear ITAM or ITIM domains. CLRs can activate intracellular signalling indirectly through association with ITAM-containing accessory proteins or directly through their ITAM motifs. In both cases, it is followed by the phosphorylation of Syk (spleen tyrosine kinase), which binds to CARD9, Malt1 and Bcl10. This signalling leads to MAPK and NFκB activation and subsequent gene expression (Hardison et al. 2012)(Hoving, Wilson et al. 2014).

RIG-I-like receptors, or RLRs, are known for their function in recognizing viral RNA in the cell cytoplasm. There are three receptors known so far: RIG-1 (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5) and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2). They all belong to the family of DexD/H box RNA helicases and their signalling leads to expression of type I interferon and antiviral genes. In the cell cytoplasm, they bind to viral RNA through their RD (repressor domain). This leads to a conformational change that releases CARD (caspase activation and recruitment domain) from its repressor, RD, and is now able to multimerize and associate with proteins such as IPS-1, also called VISA (virus-induced signalling adapter) through which they activate IFN production and antiviral gene expression (Loo and Gale 2011).

NLRs are nucleotide-binding oligomerization domain (NOD)-like receptors specialised in detecting bacterial PAMPs in the cell cytoplasm and generates a downstream signalling that culminates in type I IFN induction. They consist of three domains: an N-terminal protein-protein interaction domain, a central nucleotide binding domain and a C-terminal leucine-rich repeat domain. This PRRs are formed by 5 subfamilies: NLRA, that contain

an acidic transactivation domain, NLRB, that contain a baculovirus inhibitor of apoptosis repeat, NLRC, that contains a CARD domain, NLRP, that contains a Pyrin domain and NLRX, that contains an unknown domain. NOD1 and NOD2, from the NLRC subfamily, are the best characterised receptors in this family. When they sense iE-DAP (g-D-glutamyl-meso-diaminopimelic acid) or MDP (muramyl dipeptide), NOD1 and NOD2 respectively, recruit the CARD domain containing proteins RICK and CARD9, which in turn stimulate MAPK and NFκB, respectively, and lead to proinflammatory gene expression (Hsu et al. 2007)(Kawai and Akira 2009).

Cytosolic DNA receptors consist of receptors that bind to DNA found in the cell cytoplasm and as a consequence activate a cellular immune response through the molecular intermediate STING. In 2007, DAIs (DNA-dependent activators of IRFs) was the first member of this family to be identified (Takaoka et al. 2007). Up to date, there are at least 10 proteins that function in a similar way, among which we find: AIM2, IFIT16, DDX41, Ku70, DNA-PK, Rad50 and cGAS. Only AIM2 and IFIT16 share a characteristic structure, formed by a signalling domain (PYRIN domain) and a DNA binding domain (HIN200 domain), and as a result they give rise to a new PRR subgroup called ALRs or AIM2-like receptors (Dempsey et al. 2015). Nevertheless, all cytosolic DNA receptors must interact with STING in order to activate the immune response, whether that leads to IFN or NFκB activation. STING is a signalling adaptor molecule that plays a role in cytosolic DNA sensing – viral, bacterial or of self-origin, in the case of autoimmune diseases – by alerting the cell through immune signalling activation (Burdette et al. 2013). A critical DNA sensor is cGAS, or cGAMP synthase, since it rapidly synthesises cGAMP or cyclic-GMP-AMP when

mammalian cells are stimulated with DNA, a molecule that leads to STING activation by dimerization, phosphorylation and subsequent translocation to a perinuclear region. Enzymatic activation of cGAS occurs through direct binding to free cytosolic DNA – Figure 1.19 (Wu et al. 2013). In addition to this, cGAS is of key importance for acquiring immunity towards invading pathogens since the cGAMP produced in virally infected cells can be transported through gap junctions to neighboring uninfected cells, conferring them immunity against the pathogen (Ablasser et al. 2013).

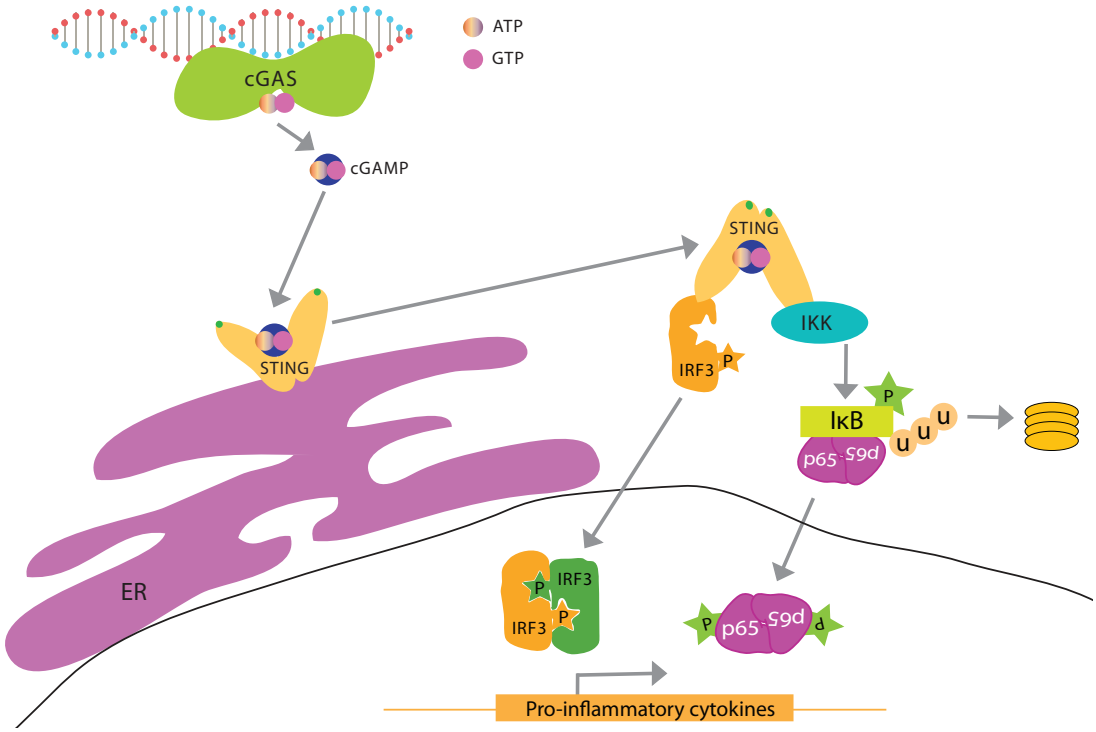


Figure 1.19. Detection of cytosolic DNA. cGAS binds to free cytosolic DNA which catalysis the synthesis of cGAMP from ATP and GTP. cGAMP then binds to STING, activating it and leading to the subsequent activation of pro-inflammatory cytokine gene expression through IRF and NFκB.

All these PRRs, recognize foreign molecules, proteins and pathogens in the organism and lead to the expression of cytokines. These consist of small nonstructural proteins that are secreted by a cell in response to a stimulus

and act on itself and other cells, signalling in an autrocrine, paracrine and endocrine fashion. They were initially discovered to influence immune responses but it is now known that they are also involved in nonimmunologic roles, such as embryonic development, cognitive functions, aging, stem cell differentiation, vaccine efficacy and allograft rejection (Dinarello 2000, Leonard and Lin 2000, Dinarello 2007). They can be classified into main groups: interleukins (ILs), tumor necrosis factors (TNFs), chemokines and interferons (IFNs). Among them, the principal proinflammatory cytokines are: interleukin 1 (IL1), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα) and the chemokine CXCL8, which act to activate the immune system and defend cells and organisms from invading pathogens, among others (Turner, 2014). Cytokines signal through 5 different types of receptors, some of which will be discussed in more detail below: Immunoglobulin superfamily receptors (IgSF), formed by immunoglobulin fold domains: two antiparallel and opposing β-sheets connected in a specific manner (Dermody, Kirchner et al. 2009); Type I cytokine receptors, membrane proteins with an N-terminal extracellular domain and a C-terminal intracellular domain (Wang, Lupardus et al. 2009); Type II cytokine receptors, transmembrane proteins that form heterodimeric receptors mainly bound by interferons (Renauld 2003); TNF receptors, transmembrane proteins with an ectodomain and an intracellular one that lacks enzymatic activity and therefore relies on the recruitment of proteins for the generation of signalling cascades (Al-Lamki and Mayadas 2015, Ward-Kavanagh, Lin et al. 2016) and finally chemokine receptors, which consist of 7 transmembrane-spanning GPCRs (Finn 2000).

Interleukins are a vast group of cytokines whose main function is to transmit messages among leukocytes, allowing for leukocyte communication. Its first member, IL1, was discovered in 1977 but it was not named until 1979 in the Second International Lymphokine workshop, held in Switzerland (Duff 1990) (Akdis, Burgler et al. 2011). Currently, there are 33 different interleukins known, divided into at least 14 families. They are expressed by many cell types and they possess proinflammatory as well as anti-inflammatory functions (Turner, 2014). The IL1 family is the most investigated family and contains 11 members, IL1α and IL1β being of key importance for inflammatory processes. Although each of the IL1 family members are encoded by different genes, their proteins have shared functionality (Dinarello 2007). IL1α and IL1β are key proinflammatory cytokines. While IL1α is expressed constitutively in cells, IL1β is primarily expressed as a response to infection through the stimulation of TLRs and NLRs. IL1β is synthesized as a precursor protein that needs to be cleaved by caspase 1 or ICE (IL-1 converting enzyme) while IL1α is always active. They both bind to and signal through IL-1R1 and IL-1R2, which are TLRs that activate MAPK and NFκB through MyD88, as explained earlier in this chapter, and lead to further cytokine expression (Figure 1.19) (Turner, 2014).

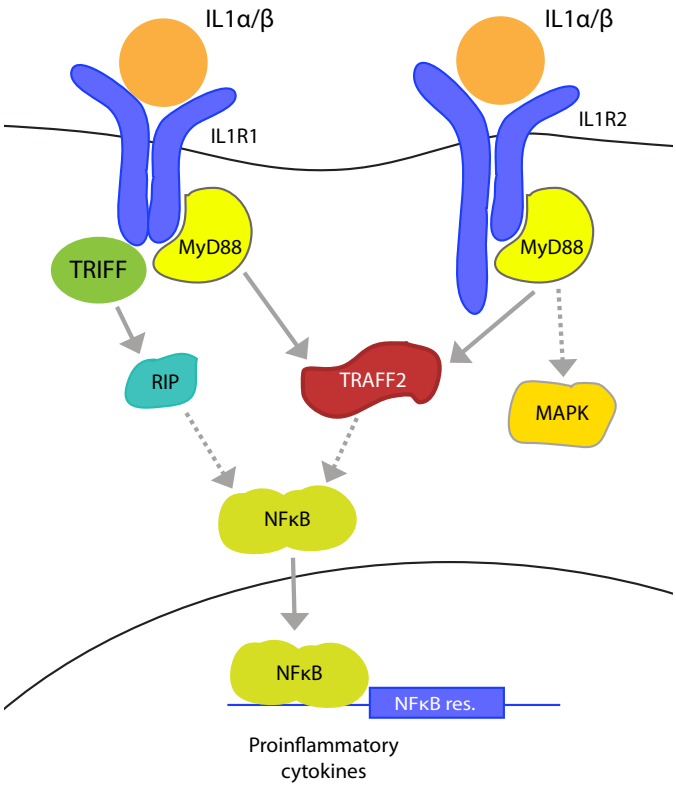


Figure 1.20. **Interleukin signalling. IL1α and IL1β signal through the same receptors, IL1R1 and IL1R2.** While IL1R1 can activate downstream signalling either through TRIFF or MyD88, IL1R2 only does it through MyD88 recruitment. Both pathways end up in the activation of NFκB and the expression of proinflammatory cytokines.

On the other hand, interleukins belonging to the interleukin family 6, IL6 and IL11, signal through a very different receptor and downstream signalling. IL6 is key for haematopoiesis and B-cell maturation. It's signalling goes through the STAT (Signal transducer and activator of transcription) family of transcription factors. IL6 receptor binding triggers JAK (Janus family tyrosine kinase) auto-phosphorylation and cross-phosphorylation of the cytoplasmic tail of the receptor. Phosphorylation of the receptor's intracellular domain acts as docking sites for MAPKs (Erk, Junk, p38) and STATs. The latter ones form homo or heterodimers, which translocate into the nucleus and bind to GAS (interferon gamma activated sequence) in the

case of the Stat1-Stat3 heterodimer, and ISREs (IFN-stimulated response elements) in the case of the Stat1-Stat2 heterodimer (Figure 1.20). In this way, they activate the expression of antiviral related genes (Renauld 2003).

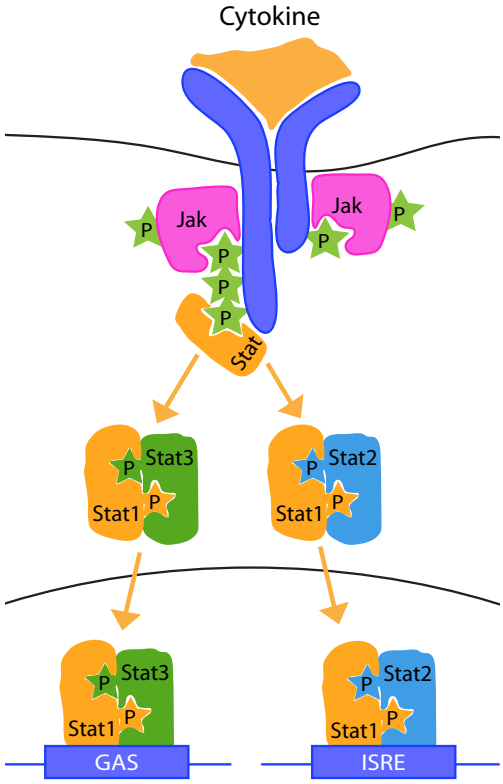


Figure 1.21. Cytokine signalling through STAT proteins. Upon cytokine binding to the IL6 receptor, the intracellular portion of the receptor gets phosphorylated, allowing for Jak kinases docking and phosphorylation of Stats. These then form dimers which will travel to the nucleus and bind GAS or ISRE sequences, activating proinflammatory cytokine gene expression. Adapted from Turner, 2014.

Tumor necrosis factor alpha (TNF) is a 34 kDa pleiotrophic cytokine formed by a homotrimer of type II transmembrane proteins mainly produced by immune cells. They work as membrane-bound proteins as well as in their soluble form, which is a cleaved version, by ADAM17 or TACE (TNFα converting enzyme), of only 17 kDa (Black et al. 1997)(Al-Lamki and Mayadas 2015). There are different types of TNF molecules, among which TNFα and TNFβ are the most studied. TNFα was discovered in 1970 as an

endotoxin-induced serum factor produced by monocytes in charge of the necrosis of some tumors, while TNFβ as a tumor necrosis factor produced by lymphocytes. Since TNFα is the prototypic member of the TNF superfamily, we will only discuss its intracellular signalling cascade in detail. This cytokine is known to have a proinflammatory function in the immune system by allowing cytokine production, activation of transcription of adhesion molecules, stimulation of cell proliferation and tumor necrosis, but it also mediates other roles such as apoptosis, lipid metabolism, coagulation, insulin resistance and endothelial cell function. This protein is originally produced as a precursor transmembrane protein of 26 kDa called mTNFα that once it reaches the cellular membrane it forms non-covalent trimers. These are cleaved by TACE which gives rise to their extracellular soluble version, sTNFα also in trimers, which can bind to TNF receptors and activate them. Their intracellular domain is processed by SPPL2a and SPPL2b, two signal peptide peptidases, and it translocates into the nucleus where it activates proinflammatory cytokine signalling, specially IL12. The receptors that mTNFα and sTNFα bind to are TNFR1, present in all cell types and main mediator of proinflammatory cytokine activation, and TNFR2, expressed mainly in leukocytes and endothelial cells. These two receptors are formed by a single transmembrane domain, an extracellular domain, which is cysteine rich and has a PLAD domain (pre-ligand binding assembly domain) necessary for trimerization, and an intracellular death domain which is the one that differs between the two, allowing for diverse signalling cascades. TNFR1 contains, intracellularly, a death domain (DD) that can activate apoptotic pathways inside the cell through the formation of Complex II, in addition to cytokine signalling through complex I. While TNFR1 needs to

recruit TRADD for signal transduction in both instances, TNFR2 can directly activate TRAF2 (TNFR associated factor 2), since it lacks the DD domain but instead it counts with a TRAF2 binding domain. TNFR1 has a higher affinity for sTNFα and is a more potent activator of NFκB than TNFR2, which has a higher affinity for mTNFα (Figure 1.21)(Turner, 2014). The TNF family of cytokines signals through a completely different set of receptors and adaptor proteins than interleukins but they have the same final outcome: activation of MAPK, NFκB and cytokine gene expression.

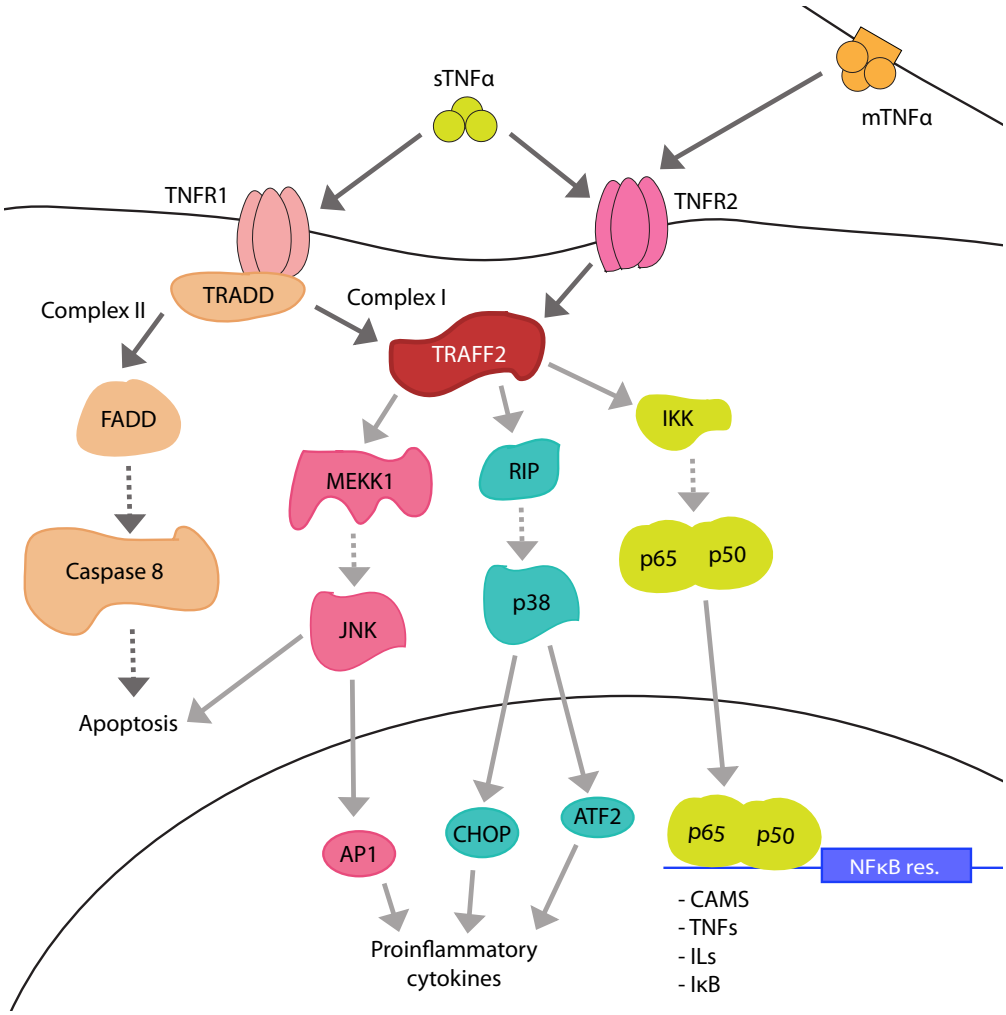


Figure 1.22. TNFα signalling and gene expression. TNFα can be membrane bound (mTNFα) or in soluble form (sTNFα). While the former binds preferentially to TNFR2, the latter can bind both TNFR1 and TNFR2. TNFR1 then signals through the recruitment of TRADD, either towards cell apoptosis or towards the upregulation of cytokine gene expression. TNFR2 signals directly through TRAFF2, in order to activate the transcription of immune related genes. Adapted from Turner, 2014.

Chemokines are small peptides, of around 8 to 12 kDa, that activate leukocytes and recruit some nonhematopoietic cells to the site of infection. They can be classified in 4 groups according to the location of their 4 conserved cysteine residues on the N-terminal: CXC, CC, C and CX3C. CXCL8 was the very first chemokine discovered almost a decade ago. Currently, we know of 44 different chemokines and 23 different receptors. Their receptors consist of GPCRs, formed by an acidic extracellular N-terminus, an intracellular C-terminus rich in serine and threonine residues available for phosphorylation and the seven helical transmembrane domains. CXCL8, originally known as IL8, is the most important chemokine involved in proinflammatory responses and therefore the most studied. It is key for endothelial cell angiogenesis and recruitment of neutrophils, monocytes, lymphocytes, basophils and eosinophils. This chemokine can be present in monomeric or dimeric forms. Dimers form due to high chemokine concentrations and need to be bound by GAGs (glycosaminoglycans) in order to be able to bind to receptors and signal. CXCL8 binds to one of its two receptors: CXCR1 or CXCR2 (Figure 1.22). Once bound, the G proteins, $G\alpha$, $G\beta$ and $G\gamma$, in the intracellular domain are activated allowing for the dissociation from one another. This way, $G\alpha$ goes on to activate the membrane bound AC (adenylate cyclase), which in turn activates PKA (protein kinase A) and downstream signalling through ERK. On the other side, the $G\beta\gamma$ dimer activates PLCβ (phospholipase β), which leads to IP3 (inositol 3,4,5-triphosphate) and DAG (diacylglycerol) production. IP3 induces degranulation while DAG then activates MAPK signalling (Turner, 2014)(Marco Baggiolini 1997, Finn 2000).

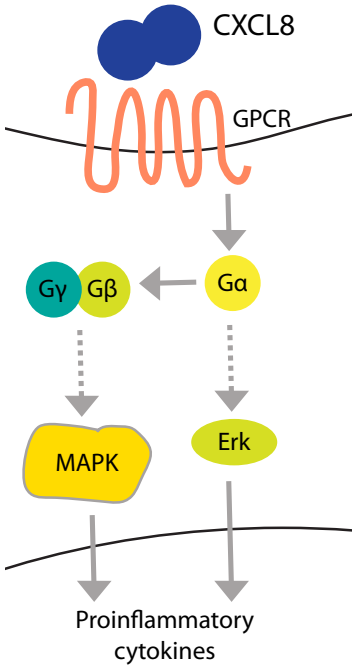


Figure 1.23. Chemokine signalling. Chemokines bind to GPCR receptors and activate their intracellular signalling that goes through the proteins Gα, Gβ Gγ that in turn activate MAPK and Erk and this leads to proinflammatory cytokine gene expression.

Finally, it is also important to mention that despite the fact that I have only described the proinflammatory cytokines in detail, there are also anti-inflammatory ones which act to repress the signalling and effects of the former. In fact, some cytokines will have dual roles, functioning in inflammatory processes as well as anti-inflammatory ones. This is the case of IL4, IL10 or IL13 that are strong activators of B lymphocytes and at the same time they repress the expression of proinflammatory cytokines: IL1β, TNFα and some chemokines (Dinarello 2000).

Cells are well equipped for protecting themselves and the organism from potential threads, whether these are bacteria, viruses, fungi or parasites. They count with a very specialised and varied number of cellular receptors (PRRs) that recognize a wide array of extracellular and intracellular

PAMPs. Additionally, they signal through diverse pathways in order to give the most appropriate response for each corresponding insult, which ends up in the induction of specialised gene expression. In the case of infection, the genes upregulated correspond to cytokines, whose role is to alert surrounding cells. They do this in a specific way, according to their nature and the receptor they bind to in the receiving cell. The outcome can be diverse and entail processes from angiogenesis to apoptotic pathways. However, in the context of infection, the principal role of cytokines is to activate innate immunity by the secretion of proinflammatory cytokines. This commonly takes place through MAPK, Stat and NFκB. We have a special interest in the NFκB signalling since it is a pathway that has been extensively researched in Vaccinia infection, as the virus has evolved a large number of proteins to repress NFκB signalling. As a consequence, we will describe this pathway in detail, to be able to understand how it actually works, which other pathogens target it and by which means.

NFκB, or nuclear factor kappa-B, is a transcription factor that is expressed constitutively in all cell types. It regulates more than 500 genes involved in inflammation and immunity, such as nNos, ILs, TNFs, IFNs; in cell survival and apoptosis, as HSP90, iNos, Bcl2 and caspases; in cell cycle progression, such as cyclins and cdks; and in processes such as cell differentiation, angiogenesis, metastasis and cellular transformation. NFκB was initially discovered, more than 25 years ago, for its role in activating the expression of the kappa light chain in B cells (Pahl 1999, Israel 2010, Hayden and Ghosh 2012) (Gupta, Sundaram et al. 2010). This transcription factor is sequestered in the cytoplasm of cells, until needed. For its activation, cytokines play a key role, as described above. Signalling can

come from either extracellular receptors, TNFRs and TLRs, as well as intracellular ones, NLRs. While the signalling cascades are different for each receptor type (Figure 1.23), they all converge in the modulation of IKKs (inhibitor of κB kinase). NFκB consists of a dimer of two of the following proteins: p65 (RelA), cRel and RelB, all of which are synthesized in an active form, and p105 (NFκB1) and p100 (NFκB2), which are initially inactive due to the presence of seven tandem ankyrin repeats involved in protein-protein interactions that hinder their own NLS (nuclear localization signal). These ankyrin repeats are located on their C-terminus and must be cleaved to give rise to their active forms p50 and p52, respectively (Smale 2012)(Zhang, Lenardo et al. 2017). These five proteins contain an RHD (Rel homology domain) in their N-terminus, which allows them to bind to the DNA, to form dimers and to bind to inhibitory proteins (Zhang, Lenardo et al. 2017). Additionally, p65, cRel and RelB also have a TAD (trans-activation domain) in their C-terminus, that is necessary for transcription initiation. Even if p52 and p50 lack this domain, they can always form heterodimers with other NFκB subunits that do contain it, in cases where their role is to activate gene expression. On the other hand, dimers of p50 and p52 can negatively regulate transcription of NFκB induced genes by preventing the binding of NFκB subunits that do contain a TAD domain. As previously mentioned, these NFκB dimers are located in the cytosol when inactive. It is important to mention that NFκB signals through two very distinct pathways: the canonical one and the non-canonical or alternative. In the canonical pathway, NFκB dimers are retained in the cytoplasm due to their binding to IκB (inhibitor of kappa B), which is a protein that contains a CLS (cytoplasmic localization domain) and when bound to NFκB it hinders its NLS through its

ankyrin repeat domain, therefore keeping the transcription factor in the cytoplasm. For canonical NFκB activation, degradation of IκB is vital. This is mediated by the previously mentioned IKKs e.g. IKKβ, which phosphorylates the destruction box serine residues on IκB, which in turn allows for βTrCP binding to the protein and recruitment of SCRF (Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box), an E3 ligase, and also UbcH5, an E2 enzyme, that together polyubiquitinate IκB on its K48 residue. This leads to IκB proteasomal degradation and therefore the release of the NFκB dimers, whose NLS sequence is now exposed, and they are translocated into the nucleus. All this occurs as a consequence of the prior phosphorylation and therefore activation of IKKβ, which in its inactive state is bound in a complex to IKKα and NEMO (NFκB essential modulator), called the IKK complex. In turn, NEMO is a regulatory protein whose function is still not completely understood. It is a 48 kDa protein necessary for NFκB activation in the canonical pathway, it lacks kinase activity but it is formed by a helix-loop-helix and a leucine zipper domain, both essential for protein-protein interactions. Some studies suggest that NEMO may be important in recruiting proteins involved in the phosphorylation of IKKs: RIP (receptor interacting protein) recruitment to NEMO induces its oligomerization, which in turn frees and leads to IKKs activation. Additionally, it has been noted that NEMO can be phosphorylated at Serine 68, which prevents its binding to IKKs and therefore contributes to their activation. K63-linked polyubiquitin chains also seem to play a role in NEMO dissociation from the IKK dimer and for IKK activation: they allow for the ubiquitination of NEMO, which in turn recruits TAK1 (transforming growth factor beta activated kinase 1), which phosphorylates and activates IKKs. The non-canonical

pathway usually signals through the p100-RelB dimer. In this pathway, IKKs do not form a complex with NEMO. Despite that, they still need to be phosphorylated to become active kinases and this is done by NIK (NFκB inducing kinase), also called MAP3K14. NIK function is obtained by its stabilization. In unstimulated cells, TRAF2 (TNF receptor associated factor 2), TRAF3 (TNF receptor associated factor 3) and cIAP1/2 (cellular inhibitor of apoptosis 1 and 2) form a complex that target NIK for degradation through polyubiquitination. Upon receptor activation, TRAF2 and TRAF3 are degraded, which allows for NIK stabilization and phosphorylation of IKKα. This protein in turn phosphorylates p100, which allows for its polyubiquitination and partial proteosomal degradation to p52. Now, the p52-RelB dimers have exposed NLS sequences and are translocated into the nucleus (Verma 2004, Israel 2010, Hayden and Ghosh 2012, Cildir, Low et al. 2016, Sun 2017). Once in the nucleus, the NFκB dimers can bind to their target sequence in the DNA, called κB segments or sites, which will vary according to the subunits forming the dimer. Once there, they can either activate gene expression or repress it, in the case of dimers that lack a TAD domain. Cytokine induced NFκB activation results in the expression of proinflammatory cytokines and genes involved in immunomodulation, such as CCL5 and ICAM1 (J.R. Matthews 1995). Among the NFκB responsive genes we find proinflammatory cytokines such as CCL5, ICAM1, VCAM1, IL6, IL8 and E-selectin. Their transcription can be stimulated by IL1β and TNFα in several cell types. Gene expression happens as a consequence of NFκB dimers binding to regulatory sequences of these genes. In the case of CCL5, it is known that it has upstream sequences for the binding of several transcription factors, including AP1 and IL6, apart from the NFκB one. In

addition to this, mutations in the NFκB binding sequence leads to a decrease in CCL5 gene expression upon cytokine stimulation (Shinsaku Sakurada 1996, Xia, Liu et al. 2001, Hirano, Komura et al. 2003).

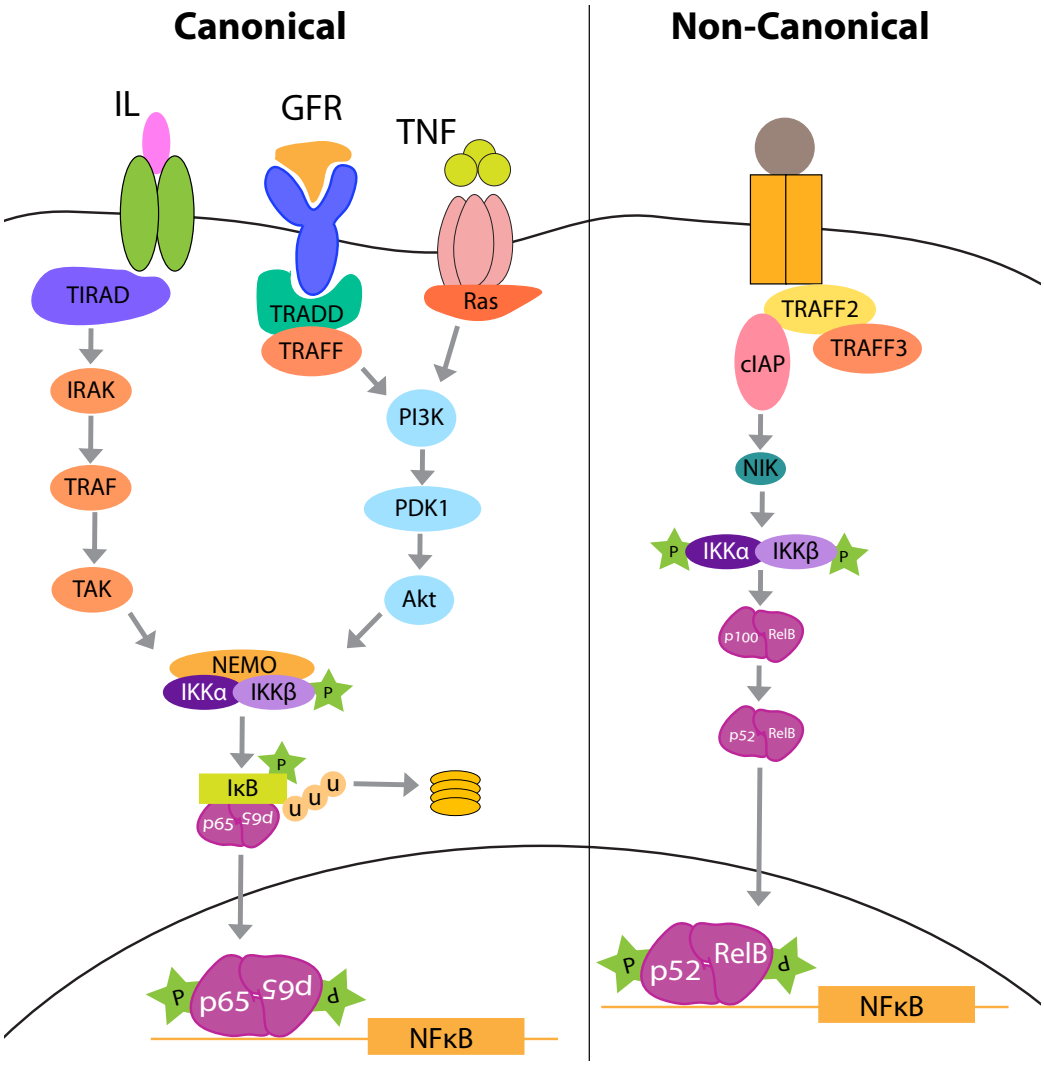


Figure 1.24. Canonical and non-canonical pathways of NFκB signalling. While the canonical pathway goes through NEMO, the non-canonical goes through NIK.

While the role IKKβ in NFκB activation is clear: it phosphorylates IκB and targets it for degradation freeing NFκB dimers, IKKα remains a mystery. However, recent data shows that this protein may be playing a role in gene activation upon cytokine stimulation. Mouse embryo fibroblasts lacking IKKα show a defect in the transcription of NFκB induced genes. It is known that

this protein travels to the nucleus of the cell, once the repressor complex, formed by NEMO, IKKα and IKKβ, has been disassembled upon external stimuli. Additionally, ChIP experiments have shown IKKα binding to promoter regions of the NFκB responsive genes and the absence of this protein leads to a dysregulation of those genes, such as IL6, Mip2 and IκBα. Furthermore, it correlates with the absence of H3S10P on those sites. Yamamoto *et al.* showed that IKKα interacts with CREB-binding proteins and RelA and together they are recruited to gene promoters where IKKα is involved in H3S10P and H3 acetylation, contributing to gene expression (Vasiliki Anest et al. 2003)(Yamamoto, Verma et al. 2003).

As mentioned above, NFκB can signal through the canonical and the non-canonical pathways. While the canonical one relies on NEMO and usually signals through p65-p50 dimers, the non-canonical one does not involve NEMO but cIAP and NIK instead, and it usually signals through p100-RelB dimers. The response obtained by each of the pathways also varies: canonical signalling has a fast activation as well as deactivation, it takes place within minutes from the stimulus, while non-canonical signalling is slower, it does not get activated until hours after stimulation (Cildir, Low et al. 2016).

NFκB signalling is therefore key for cell defense against invading pathogens. Bacteria and viruses have evolved to manipulate this signalling for their own advantage. While all bacteria known do suppress NFκB pathways, viruses, due to their different nature, have learned to activate it as well as repress it. The mechanisms employed by bacteria vary greatly, from avoiding TLR triggering to the modification of histone marks at NFκB induced genes. Avoidance of TLR triggering can be achieved through the

presence of an external capsule, as it is the case for Enterococcus faecalis and Staphylococcus aureus, that impede the binding of TLRs to their PAMPs and therefore escape recognition. Another way of escaping TLRs is by constantly modifying bacterial PAMPs as Salmonella typhimurium, Pseudomonas aeruginosa and Mycobacterium tuberculosis do. Additionally, some bacteria secrete molecules that interfere with the binding of TLRs to their PAMPs. A good example of this is Staphilococcus aureus, which secretes SSLs (staphylococcal superantigen-like proteins) that compete with the bacterium PAMPs binding of TLR2. Finally, bacteria have also learned to activate parallel cellular signalling that leads to intracellular inactivation of NFκB signalling, such as Porphiromonas gingivalis. This bacterium activates TLR2 signalling through its fimbriae but this PAMP also activates CXCR4, which counteracts TLR2 activation. Inside the cell cytoplasm, bacteria can interact with proteins of the signalling cascade. For instance, Brucella species have a protein called TcpB/Btp1 that mimics TIRAP/MAL binding to TLRs and at the same time induce MAL/TIRAP ubiquitination and degradation, shutting down NFκB signalling through these receptors. Additionally, bacteria can also interact with the direct activators and inhibitors of NFκB: IKKs and IκB, respectively. Such is the case of Yersinia, that acetylates IKKs on a threonine residue located inside their activation loop and this prevents their phosphorylation and subsequent activation. On the other hand, Shigella flexneri contains its OspG protein that interacts with UbcH5, an E2 ubiquitin ligase that prevents the polyubiquitination and degradation of IκB. Finally, recent studies have shown that bacteria can also influence NFκB gene expression directly, by modifying histone marks. Phosphorylation of H3S10P creates an open chromatin and helps NFκB bind

to promoters or genes such as IL8 or IL10. Shigella flexneri injects into the cell its phosphatase OspF, that travels to the nucleus and dephosphorylates MAP kinases, leading to a decrease in H3S10P which leads to a decrease in IL8 gene expression. Additionally, E. coli targets p300/CBP, an NFκB coactivator that acetylates surrounding histones leading to a more open chromatin that helps NFκB binding. It does it through its NleC protein that binds to p300 and induces its degradation. Finally, some NFκB dimers do not contain a TAD domain and therefore act as repressors. As a consequence, bacteria such as Bortedella pertussis enhance the nuclear translocation of those subunits, which bind to NFκB inducible genes and repress them (Nagamatsu et al. 2009)(Johannessen, Askarian et al. 2013, Reddick and Alto 2014).

Viruses, however, have evolved different mechanisms for interacting with NFκB signalling. Some viruses, such as HIV (human immunodeficiency virus), Sindbis virus, Sendai virus and African Swine fever, upregulate this pathway in order to promote viral replication and prevent cellular apoptosis. Additionally, these viruses contain NFκB binding sequences upstream of their own genes, so when they upregulate the pathways that lead to their own gene transcription. NFκB activation by HIV is necessary at low levels and allows the virus to give rise to a chronic infection. This is mediated by the viral proteins Tat, Vpr and Nef. While Tat increases the DNA binding ability of NFκB and induces the expression of IL10, IL6 and TNFα, which promotes HIV replication at early stages; Vpr and Nef are viral late proteins that are known to stimulate HIV1 gene expression. Sindbis virus, in turn, activates NFκB to induce apoptosis in order to contribute to viral spread through phagocytic cells. Sendai virus stimulates IKK activation which ends up in

persistent NFκB induction and expression of IL8. Finally, African Swine fever virus induces cell apoptosis through its protein A224L that interacts with TRAF2 and IKKs, leading to NFκB activation through Complex II. Some other viruses repress NFκB in order to stop immune gene expression, as in the case of Poxviruses. The complex mechanism by which Poxviruses inhibit this pathway is exemplified below by Vaccinia virus. Interestingly, there is a third group of viruses that switch from activating the pathway to repressing in, in the switch from their latent to lytic cycles, respectively. This group is formed by viruses like Kaposi's-sarcoma associated herpesvirus (KSHV), Epstein-barr virus (EBV), adenoviruses and human cytomegalovirus (HCMV). Both KSHV and EBV are human oncogenic gammaherpesviruses that induce NFκB activation during their latent phases of infection while they repress it during the lytic one. The persistent activation of the pathway soon after infection allows these viruses to make cells refractory to apoptosis and become immortalised. While KSHV activates NFκB through NEMO binding to its protein K13, EBV utilises its glycoprotein gp350 or its gp250 isoform to bind to TLR2 and CD21 receptors and stimulate signalling. Inhibition of NFκB occurs through EBNA1, in the case of EBV, which prevents IKKs phosphorylation and downstream signalling. KSHV is able to inhibit the pathway through the same protein it activates it: K13. This KSHV protein activates classical NFκB activity, blocks the viral lytic cycle and upregulates the expression of miR-146a. It is the latter that represses the expression of the CXCR4 receptor reducing NFκB induction. Finally, HCMV induces several waves of NFκB activation and uses it to exit the latent infection phase by promoting immediate-early gene expression. The first wave occurs as a consequence of viral entry and is mediated by its envelope glycoprpoteins

gB and gH. The second one has the aim of activating HCMV immediate-early genes, whose promoters can be bound by the transcription factor. And finally, the third wave is driven by the early viral proteins IE1-72, IE2-55 and IE2-86 (John Hiscott 2001, M.Gabriella Santoro 2003, Rahman and McFadden 2011).

The NFκB signalling pathway is a complex pathway that can be activated by numerous ligands and through many different receptors. It is key for the cell response to infection. As a consequence, bacteria and viruses have learned to modulate it over the years, whether that is by preventing its activation, by fighting its activation in direct or indirect ways, or by accommodating their needs to it, e.g. acquiring NFκB binding sites in viral genomes for gene expression.

Section 1.05 The NFκB pathway and Vaccinia virus

Between 30% and 50% of Vaccinia's genome (~200 genes) encode immunomodulatory proteins. The Vaccinia genome is composed of a central region of conserved genes, around 100 kb, required for viral replication and terminal regions encoding virulence factors and immunomodulatory proteins. Generally, these proteins are expressed early during infection, although there are some exceptions. Some seem to have redundant roles, however, their deletion showed to be detrimental for viral infection indicating they all are essential and function in different ways to suppress host cell immunity. Additionally, some of the proteins have more than one function (Smith, Benfield et al. 2013, Bidgood and Mercer 2015).

Vaccinia modulation of the innate immune response has been extensively studied. Currently there is a great understanding of which viral

proteins are involved in it, although the precise function of each of them is not fully understood. Vaccinia blocks cellular immunity by interfering with cytokine, chemokine and interferon receptor binging as well as their intracellular signalling through the IRF and NFκB pathways (Smith, Benfield et al. 2013).

Cytokine signalling through IL1β is targeted by the Vaccinia proteins F1, B15 and B13. While F1 and B13 are involved in repressing cellular apoptosis, B15 is necessary for proinflammatory cytokine expression. F1 binds to NLRP1 (nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein 1) through which it prevents caspases activation and IL1β secretion. B15 directly binds to IL1β and inhibits cytokine binding to its receptor. B13 interacts with caspase 1 and blocks pro-IL1β cleavage and subsequent activation, which blocks cytokine signalling through IL1 and IL18 (Veyer, Carrara et al. 2017). The Vaccinia protein C12 blocks IL18 signalling by directly binding to IL18 and preventing receptor binding. Finally, TNFα signalling is also important for Vaccinia infection. However, despite most strains encode vTNFR genes, no proteins interfering with this cytokine have been discovered to date (Howard, S. 1991).

Chemokine signalling is taken care of by the viral proteins CC1, A41, B7 and B23. While CC1 binds to CC-type chemokines and prevents binding to their receptors, A41 simply acts by disrupting chemokine concentration gradients. Finally, B7 and B23 are of unknown activity but are predicted to bind to chemokines based on their structure similarity to the Cowpox protein V216 (Smith, Benfield et al. 2013).

Interferon signalling can be blocked extracellularly at receptor binding as well as intracellularly. Vaccinia proteins B8 and B18 are secreted and

respectively sequester IFNα and IFNγ in the extracellular space to prevent binding to IFNRs in uninfected cells. Once TLRs have been bound by interferon and the signal cascade inside the cell is activated, Vaccinia employs H1 phosphatase to stop it. H1 dephosphorylates Stat1 and Stat2 and this prevents their nuclear translocation and binding of ISRE and GAS sequences upstream of immune related genes. Additionally, interferon signalling is also prevented by D9 and D10, through the degradation of cellular mRNAs and subsequent reduction of protein synthesis – among them immune related proteins (Najarro, Traktman et al. 2001, Smith, Benfield et al. 2013).

Vaccinia has evolved to strongly repress IFR and NFκB signalling pathways (Figure 1.24). On the one hand, in infected cells, the focus of the virus is to repress IRFs, mainly IRF3 and IRF7, to prevent their nuclear translocation and therefore the activation of immune related genes. This is achieved by the Vaccinia proteins C6, K7, A46, N2 and C16. C6 binds to the TBK1 (TANK binding kinase 1) adaptor proteins NAP1 (NAK associated protein 1), TANK (TRAF Family Member Associated NFKB Activator) and SINTBAD (similar to NAP1 TBK1 adaptor). This in turn prevents IRF3/IRF7 activation, nuclear translocation and transcriptional expression of immune related genes. K7 and A46 viral proteins bind to DDX3 and TRFI, TRAM, MAL and MyD88, to prevent IRF3 activation and downstream signalling. The Vaccinia protein N2 is known to interfere with this pathway after IRF3 activation and nuclear translocation but its mode of action is unknown. C16 and E3 block IRF3 activation by preventing cellular recognition of dsDNA. Vaccinia employs 9 known proteins to repress NFκB signal transduction, them being A46, A49, A52, B14, C4, K1, K7, M2 and N1. From them, A46,

A52 and K7 inhibit NFκB only when activated via TLRs and IL1β but not TNFα. The rest of them are able to inhibit the signalling whether it comes from IL1β or TNFα activation. Starting with A46, it binds to the TLR adapting proteins MyD88, MAL, TRIF and TRAM, and inhibits the activation of MAPK, NFκB dimers and IRF3. A52 is a protein belonging to the Bcl2 family, it binds to IRAK2 and TRAF6 at the receptor level and prevents downstream signalling. K7, another Bcl2 protein, prevents TLR induced NFκB signalling by binding IRAK2 and TRAF6. B14 takes care of IKKβ by preventing its phosphorylation and therefore activation. A49 is in charge of stabilizing IκB phosphorylation by binding to βTrCP and preventing IκB ubiquitination and subsequent degradation. N1 is another protein that has been suggested to interact with IκB though this is not clear. Additionally, it has been reported that N1 dimerization is key for inhibition. The viral protein M2 acts through prevention of Erk2 phosphorylation and p65 nuclear translocation. Finally, C4 and K1 are two proteins of unknown function that have been shown to play a role in NFκB repression downstream of the IKK complex and IκB repressor, respectively (Smith, Benfield et al. 2013)(Sumner, Maluquer de Motes et al. 2014).

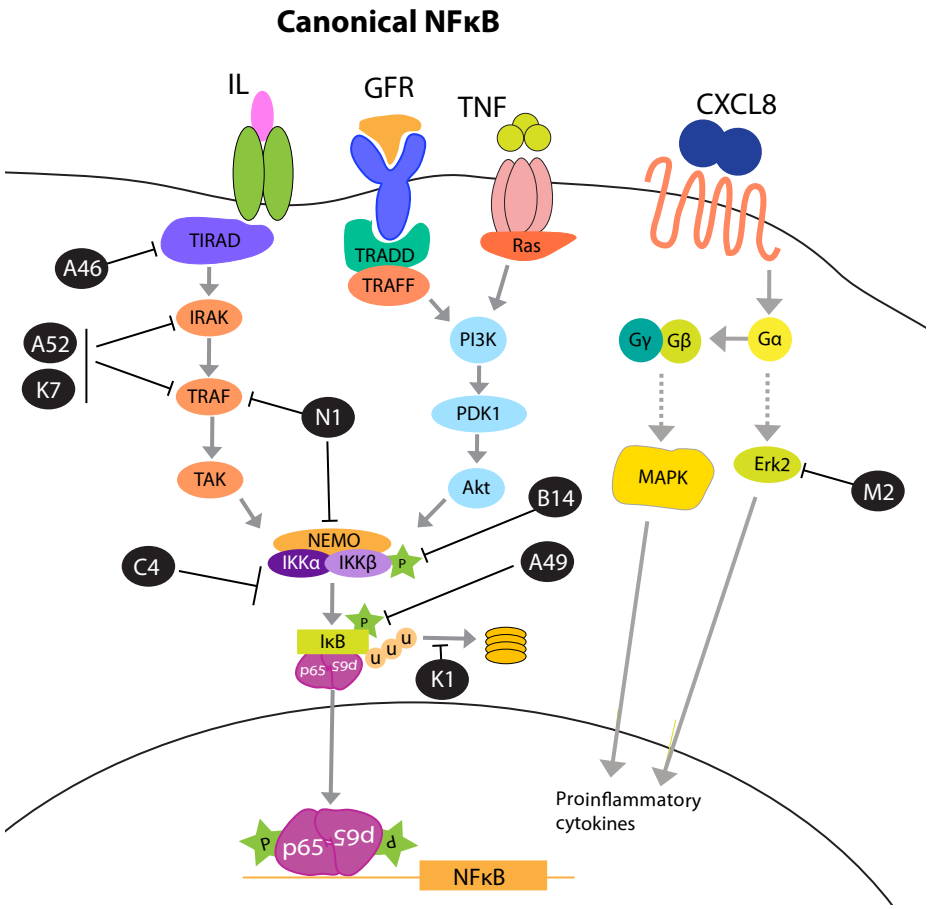


Figure 1.25. Vaccinia virus inhibits NFκB signalling. All the current known Vaccinia proteins (black) and the process they block in the signalling pathway are shown.

Even though Vaccinia encodes 9 known viral proteins that target NFκB signalling, Smith *et al.* showed that our knowledge is incomplete and that there must be at least one more unknown Vaccinia protein that interferes with this pathway. Experiments carried out with the mutant virus vv811ΔA49. Which lacks 56 open reading frames of Vaccinia's genome including the 9 viral proteins in charge of NFκB repression, showed that NFκB dependent gene transcription was still partially repressed upon infection. When cells were infected with this mutant and stimulated with either IL1β or TNFα, nuclear translocation of the NFκB dimer was not blocked as seen with WT Vaccinia. However, the virus was still able to repress NFκB

responsive genes independent of late viral gene expression. As a consequence, Vaccinia must encode another protein involved in the antiviral response, that is encoded outside of the 56 reading frames of vv811ΔA49, is expressed early or pre-packaged in virions, and acts inside the cell nucleus (Sumner, Maluquer de Motes et al. 2014).

So far, we know that one-third to one-half of Vaccinia's genome encodes viral proteins necessary for immune regulation. The virus is able to shut down cellular innate immunity effectively through interference with cytokines, chemokines and interferons as well as their intracellular signalling pathways through IRF and NFκB transcription factors. Many of the proteins involved act on the same cellular proteins, however, their roles do not seem to be redundant. Additionally, some of the proteins are known to play a role despite their mode of action not being fully understood. Finally, at least one more protein remains unidentified that seems to be important for the repression of NFκB signalling in the cell nucleus.

Section 1.06 Aims of this thesis

Pathogens have been shown to modulate accessibility of transcription factors to chromatin through modification of the cell's histone code upon infection. This has been shown to be crucial for regulating not only cellular gene expression but also pathogen gene expression, in the case of some viruses. Prior to the publication of Teferi et al. where they show Vaccinia is involved in H3K9me3 and H4k20me3, we had data in the lab suggesting Vaccinia's involvement in nuclear processes. This data showed that Vaccinia is interacting with the cell nucleus and that it is modulating phosphorylation

of several histone modifiers, involved in histone acetylation and methylation. Additionally, successful Vaccinia infection requires the presence of some histone deacetylases, acetyltransferases, transcription factors and nucleoporins. Finally, we have evidence that Vaccinia interacts with cellular histones through its kinases B1 and F10 and its phosphatase H1.

The goal of my thesis was to shed some light on the interactions between Vaccinia and the cell nucleus. First, by understanding how Vaccinia impacts the histone code of the cell it infects by investigating changes in histone acetylation, methylation and phosphorylation. Second, to understand whether any of the 3 viral enzymes, B1, H1 and F10, lead to the formation of specific chromatin remodelling marks and if so, whether they are involved in dampening the cellular immune response to promote viral infection. Finally, to gain a global overview of the nature of Vaccinia-host nucleus interactions, quantitative mass spectrometry was employed to assess which viral and cellular proteins shuttle into and out of the cell nucleus during the course of infection.

Chapter II. Materials and methods

Section 2.01 Cells and Cell culture

A549 cells (ATCC®) and BSC40 cells (from Dr. Paula Traktman) were used for experiments. They were kept in DMEM medium (Gibco #41965-039) containing 10% FBS (Gibco #10500-064), 1% Glutamax (Gibco #35050-038), 1% Penicillin-Streptomycin (Sigma #P0781), 1% Sodium Pyruvate (Thermo Fisher #11360070) and 1% non-essential amino acids (Gibco #11140-035). Cells were split using PBS and trypsin produced in house. Infected cells were kept in a separate incubator from uninfected cells, at 37 °C as well. The incubators used were from Eppendorf/New Brunswick Scientific #Galaxy 170R.

Section 2.02 Viruses

The viruses used in this work are summarised in the table below.

Strain	Virus	Mutation/Recombinant	Origin
Western Reserve (WR)	**WT**	N/A	-
	SH-B1 (WR116)	N terminus tagged SH E/L eGFP in TK locus	Mohammedyaseen Syedbasha and Florian Schmidt
	H1-SH (WR118)	C terminus tagged SH E/L eGFP in TK locus	
	F10-SH (WR118)	C terminus tagged SH E/L eGFP in TK locus	
	B1 ts (WR081)	31°C permissive 39.7°C tiny plaques	R. Condit
	H1- (WR013)	Inducible lacH1 under IPTG control made in the absence of IPTG	Moss, B. & Resch, W. 2005

Strain	Virus	Mutation/Recombinant	Origin
Western Reserve (WR)	**F10 ind. (WR015)**	Inducible lacF10 under IPTG control	Moss, B et al. 2004
	Early eGFP (WR027)	eGFP under early promoter in TK locus	Florian Schmidt
	Late eGFP	eGFP under late promoter in TK locus	
Copenhagen (CP)	**WT**	N/A	David Evans
	Vv811	Lacks 55 ORFs on left and right side of the genome	
Copenhagen (CP)	**∆A49**	Early/ Late eGFP in A49 locus	Corina Beerli
	H1+	Earl/Late mcherry in H1 locus Lac and promoter H1-HA in A49 locus	
	H1-	H1+ grown in the absence of IPTG	

Section 2.03 MOI in each experiment

The MOIs used in each experiment are detailed below.

Experiment	Virus	MOI
Histone screen (WB)	WR - WT	10
Mass Spectrometry		
Imaging Vaccinia proteins	SH - B1	2-10
	H1- SH	
	F10- SH	
Subcellular fractionations	SH -B1	5 -10
	H1-SH	3.5 - 10
	F10-SH	5 - 10
Mutants experiments	B1 ts	10
	H1-	
	F10 ind.	
Drug inhibitors	Early eGFP	1
	Late eGFP	
P65 imaging	WR - WT	5
	WR – H1-	
	CP - WT	
	CP – vv811	
	CP – ∆A49	
	CP – H1+	
	CP – H1-	
RTqPCR	WR - WT	0.5
	WR – H1-	
	CP - WT	5
	CP – vv811	
	CP – ∆A49	
	CP – H1+	
	CP – H1-	

Section 2.04 Virus production

BSC40 cells (ATCC®) were used for this purpose. Per virus produced, twenty 15 cm dishes were infected, each with 10 ml of cell medium containing 10 µl of the virus stock, either crude extract or sedimented virus. Plates were returned to the incubator and after 1 hour, 20 ml of fresh cell medium was added. Cultures were harvested 48 hours post infection by scrapping the cells and subsequently spinning them down at 300 x g for 10 minutes in a benchtop centrifuge (Beckman Coulter #Allegra X-30R). The virus was then sedimented and, in some cases, band-purified.

Section 2.05 Virus sedimentation

The infected cell pellet was resuspended in 12 ml of 10 mM Tris pH 9.0 and left on ice for 5 minutes. Cells were then disrupted by a tight douncer (25 strokes), transferred to a 15 ml Falcon tube and span down in the benchtop centrifuge at 300 x g for 10 minutes at 4 °C. The supernatant was then transferred to a new Falcon tube and spun down as before. An SW32 tube (Beckman Coulter #344060) was filled with 16 ml of 36% sucrose in 20 mM Tris pH 9.0 and the cytosolic extract (last supernatant, ~10ml) was loaded on top of that. Finally, 20 ml of 10 mM Tris pH 9.0. were added and 2 ml of mineral oil was used to seal the virus. The filled SW32 was then spun down in an ultracentrifuge (Beckman Coulter #Optima L70) at 38,000 x g for 1 hour and 20 minutes at 4 °C. Afterwards, the supernatant was aspirated and the pellet was resuspended in 1-2 ml of 1 mM Tris. The virus was then ready and stored at -80 °C or used for further band purification.

Section 2.06 Virus band purification

The middle of an SW41 tube (Beckman Coulter #344062) was marked. The 25%-40% sucrose gradient was made by filling an SW41 tube with 6 ml of 25% sucrose in 10 mM Tris and underlaying it with 40% sucrose in 10 mM Tris. The gradient was prepared by using the Gradient maker (Gradient Master 108, BIOCOMP) for 3 minutes, at 81.5° and 18 rpm. The sedimented virus was vortexed and sonnicated twice, loaded onto the sucrose gradient and spun down at 16,500 x g for 52 minutes at 4 °C in the ultracentrifuge. Using a 21G needle, the white band formed in the middle of the tube (virus particles) was aspirated. Finally, 12 ml of 1 mM Tris were added to the virus, transferred to an SW41 tube (Beckman Coulter #344062) and spun down at 53,000 x g for 40 minutes at 4 °C in the ultracentrifuge. Then, the supernatant was aspirated and the pellet was resuspended in 1-2 ml of 1 mM Tris, aliquoted and stored at -80 °C.

Section 2.07 Viral titre determination

For this purpose, we used confluent BSC40 or A549 cells on 6 well plates (Thermo Scientific #140675). First, the virus stock was thawed, sonnicated (Wolflabs #XUBA1) for 15 seconds and then vortexed (Scientific industries #Vortex Genie-2) for another 15 seconds. This was repeated 3 times in total. The virus was then diluted in 10-fold serial dilutions, starting by adding 2 µl of stock virus to 998 µl of DMEM and counting this as your -3 dilution. The medium was aspirated from the 6 well plates and 500 µl of the dilutions were added to the corresponding well. The plates were put back in the incubator at 37 °C and rocked every 10 minutes to ensure even

dispersal. After 1 hour, the virus was aspirated and the cells were fed with 2 ml of fresh medium. Plates were incubated for 48 hours at 37 °C. They were then fixed and stained with 0.1% crystal violet (Sigma #C0775) in 3.7% formaldehyde (Sigma #47608-1L-F) for 20 minutes at room temperature. Finally, the fix/stain was poured off and wells were rinsed with H2O and dried overnight. The following day, the plaques were counted and the titre was calculated according to the dilution of the well counted. MOIs were based on the cell line infected for each experiment.

Section 2.08 Viral infection

A549 cells were seeded 1 to 2 days before the experiment was carried out. The viral infection was carried out by thawing the required virus and sonnicating it for 15 seconds followed by vortexing for another 15 seconds. This step was repeated 3 times in total. The virus was then diluted to the indicated MOIs. The medium was aspirated from the cells and the diluted virus was added, this was the medium used for adsorption. Plates were left for 30 minutes to 1 hour at room temperature to allow virus binding to the cell membrane but prevent virus entry, and therefore allow for a more synchronous infection. After that, the virus was aspirated and the cells were fed with fresh medium. Plates were then placed in a 37 °C incubator, were the virus can enter the cells, now in a more synchronised manner. Cells were harvested at the indicated time points and using different methods, described below.

*Approximate medium volumes

Plate	Medium for adsorption	Fresh medium
12 well	0.1 – 0.3 ml	1 ml
6 well	0.5 -1 ml	2 ml
60 mm	1 – 3 ml	3 ml
100 mm	4 - 5 ml	10 ml
150 mm	4 - 10 ml	25 ml

Section 2.09 Inhibitor drug assays and viral infections

A549 cells were seeded 1 to 2 days prior to the experiment. On the experiment day, they were pre-treated with either BCI - NSC 150117 (Axon MedChem #2178) or SB 747651 tetrahydrochloride (Axon MedChem #1897)* diluted in medium to the indicated concentrations, for 30 minutes to 1 hour. The medium was aspirated and the diluted virus in medium (MOI 1) containing the indicated concentration of drug was added. The virus was left to bind to the cells at room temperature for 30 minutes to 1 hour and then, the medium was aspirated and replaced with medium containing only the diluted drug. The cells were then harvested, according to the procedure they will be analysed with, at the desired timepoints.

* Drugs

Drug	Function
BCI - NSC 150117	Allosteric inhibitor of DUSP1 and DUSP6
SB 747651 tetrahydrochloride	MSK1 inhibitor

Section 2.10 Harvesting cells for Western blotting

Once the medium was aspirated, PBS was added to the well or dish – volume according to plate used. Cells were scrapped off the dish, transferred to a 1.5 ml eppendorf tube and spun down at 300 x g for 5 minutes in a

table top centrifuge (Eppendorf #5424R). The supernatant was aspirated and the cell pellets were kept at -80 °C. A 1x mix of DTT and SDS-PAGE loading buffer (cell signaling pack #7722S) was made. This was added to the cell pellet – volumes according to plate size*. The pellets were broken down by pipetting up and down several times and the samples were sonicated for 20 to 40 minutes in an ultrasonnic bath (Wolflabs #XUBA1).

*Approximate buffer volumes

Plate	DTT + buffer
12 well	50 – 100 µl
6 well	100 – 200 µl
60 mm	200 – 400 µl
100 mm	400 – 800 µl
150 mm	

Section 2.11 Subcellular fractionation

Cells were fractionated using the Qiagen Qproteome Cell Compartment Kit (Qiagen #37502) and the nuclear and cytosolic fractions were kept for analysis. The infected cells were scraped, spun down at 300 x g for 5 minutes then resuspended in ice cold PBS and centrifuged at 500 x g for 10 minutes at 4°C. This step was carried out twice. Then, lysis buffer containing protease inhibitor solution (10µl per every 1ml of lysis buffer) was added and cells were incubated in an end-over-end shaker for 10 minutes at 4°C. Samples were then centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was frozen as it consists of the cytosolic proteins fraction. The pellet was resuspended in ice-cold extraction buffer CE2 (also containing 10µl protease inhibitor per 1ml of buffer) and incubated in an end-over-end shaker at 4°C for 30 minutes. After this, the suspension was

centrifuged at 6000 x g for 10 minutes at 4°C. The supernatant contains the membrane proteins and it was frozen as a separate fraction. 13µl of water containing 7µl of Benzonase nuclease was added to the pellet, which was carefully resuspended by flicking the tube and incubated at room temperature (15°C – 25°C) for 15 minutes. Ice-cold extraction buffer CE3 (containing 10µl of protease inhibitor per 1ml of buffer) was added into the tube and incubated in an end-over-end shaker for 10 minutes at 4°C. Samples were then centrifuged at 6800 x g for 10 minutes at 4°C. The supernatant consists of the fraction containing the nuclear proteins, which was immediately frozen. Finally, the pellet contains the cytoskeletal proteins that can be resuspended in extraction buffer CE4. The protein yield of every sample was determined by spectrophotometer (Thermo Scientific #Nanodrop 1000).

Section 2.12 Histone purification

Histones were purified from A549 cells using the Active Motif Histone Purification Kit (Active Motif #40025), which preserves post-translational modifications. Infected cells were washed twice with warm serum-free medium and then ice-cold extraction buffer was added. Cells were scraped and transferred to a 15ml falcon, where they were pipetted up and down to homogenise them into solution. The cells were left in the extraction buffer from 2 hours to overnight in an end-over-end shaker at 4°C. After that, cell extracts were transferred into fresh tubes and centrifuged in a microcentrifuge at maximum speed for 5 minutes at 4°C. The supernatant, which contains the histones, was transferred to a new tube. At this step,

histones can be frozen down at -80°C or continue with the protocol. The crude histones were then neutralised by adding ¼ volume of 5x neutralization buffer – if after checking the pH they are still acidic then additional neutralization buffer was added until they reached pH 8. By this time, we have already prepared the column for purification*. With the bottom cap secured in the column, 0.5ml of the sample was added and the column matrix was resuspended and mixed with the sample – preventing air bubbles appearing. The resin was then left to settle for 5 minutes. The bottom cap was then removed and the column placed in a 15ml falcon. 3ml more of the samples were added, without disrupting the matrix this time. The column was centrifuged at 50 x g for 3 minutes at 4°C. These last 2 steps were repeated until the whole samples was passed through the column. At this stage, the histones were retained by the column matrix. The flow through was either kept or discarded. 9ml of histone wash buffer were run through the column and centrifuged as previously described. The second and third washes were kept. Then, all core histones were eluted by adding H3/H4 elution buffer and centrifuging at 50 x g for 3 minutes at 4°C – the sample was then transferred from the 15ml falcon to a 1.5ml Eppendorf. This was repeated a total of 4 times. Eluates were not pulled together. Histone proteins are ready to be used but they contain high levels of salts, which were removed by precipitating histones overnight at 4°C with 4% perchloric acid final concentration. The next day, samples were centrifuged in a microcentrifuge at maximum speed for 1 hour at 4°C. The pellets were hen washed twice with 4% perchloric acid and spun down in a microcentrifuge at maximum RCF for 5 minutes at 4°C, the supernatant was removed without disrupting the pellet and the pellet was washed twice with

0.2% HCl. Then washed twice with 100% acetone and finally left to air dry until the pellet is completely dry. The histones were then resuspended in sterile water, by flicking the tube, followed by vortexing and centrifuging briefly. The protein concentration was measured at Nanodrop at 280nm and stored at -80°C. The column was washed according to the manual's instructions in order to keep it for future uses.

*Preparing column for purification: The column was placed on a stand and the bottom cap was secured. Using a Pasteur pipette, the filter disc was pushed to the bottom of the column. 1ml of sterile water was added and the top water level was marked on the column – the water was then left to drain. Purification resin was added up to the mark previously made. The resin was left to settle in the column for 10 minutes. Then, 9ml of sterile water were used to clean the column, adding 3ml at a time and then centrifuging for 3 minutes at 50 x g at 4°C. Finally, the column was equilibrated with 9ml of equilibration buffer, centrifuging as before.

Section 2.13 Western blotting

Pre-cast 4-12% Bis Tris Plus gels (Thermo Fisher) were run in Bolt™ Mini Gel Tanks (Thermo Fisher Scientific #A25977), using Thermo Fisher Scientific running buffer (#B0002) according to their user manual. Protein transfers were performed in Mini blot modules (Thermo Fisher #B1000), by soaking Pre-cut nitrocellulose 0.2 µm pore membranes (Life Technologies #LC2000) and equilibrating the pre-cast gels in Thermo Scientific transfer buffer (#BT00061). Gels were transferred at 15 V for 30 minutes. After that, membranes were blocked at room temperature for 10 – 60 minutes in 5%

milk (Sigma # 70166)/ TBST, made from TBS (Abcam #Ab64248) and Tween 20 (Sigma #P1379). Finally, the membranes were incubated overnight at 4 °C in primary antibodies*[1] diluted in 5% BSA (Sigma #A7906), after a quick TBST wash. The following day, membranes were washed 3 times in TBST and incubated with HRP linked secondary antibodies*[2] at room temperature for at least 1 hour. Membranes were washed again 3 times in TBST and developed in a luminescent image analyser (GE Healthcare #ImageQuant LAS 4000mini) using Luminata Forte (Merck #WBLUR0100).

*[1] Primary antibodies

Antibody	Catalog number	Company	Dilution	Species
H2A	12349	Cell signaling	1:1000	Rabbit
H2A S1P	ab177309	Sigma Aldrich	1:500	
H2A K5 Ac	2576S	Cell signaling	1:1000	
H2B	12364			
H2B K5 Ac	12799			
H4	13919			
H4 S1P	ab177309	Sigma Aldrich	1:500	
H4 K8 Ac	2594	Cell signaling	1:1000	
H3	4499 & 9715			
H3 S10P	9701			
H3 T3P	13576			
H3 K4 me3	9751			
H3 K9 me3	13969			
H3 K9 Ac	9649			
H3 S28P	9713	Cell signaling	1:500	
H3 K14 Ac	7627			
H3 K4 Ac	ab176799	Abcam		
Erk1/2	4370	Cell signaling	1:1000	
pErk1/2	9102			
p38	9212			
P-p38	9211			
Msk1	3489			
pMsk1	9595			
Anti-HA	901502	BioLegend	1:1000	Mouse

*[2] Secondary antibodies

Antibody	Company	Catalog number	Concentration
HRP rabbit	Cell signaling	7074	1:1000
HRP mouse	Cell signaling	7076	

Section 2.14 Immunofluorescence protocol

Cells were seeded onto VWR coverslips (VWR #631-0149) for confocal imaging and onto Zeiss coverslips (Zeiss #0109030091) for Super-resolution microscopy. After infection at the stated MOIs, coverslips were transferred to a dish containing 4% PFA (diluted from Sigma # 47608-1L-F) in order to fix the cells. After 30 minutes, the 4% PFA was removed and PBS was added. The cells were then kept at 4 °C until stained. If the viruses expressed fluorescent proteins, the plates were wrapped in aluminum foil. Cells were washed once with PBS and subsequently permeabilised for 20 – 30 minutes with 0.1% Triton X-100 (Sigma #X-100) in PBS. Cells were then blocked for 30 minutes – 1 hour with 10% FBS in 0.1% Triton X-100. Next, primary antibodies were diluted in blocking buffer and added to the cells for 1 – 3 hours at room temperature. Finally, cells were washed three times in PBS and the secondary antibodies and Hoechst (1:2000 – 1:10000) (Invitrogen #H3570), diluted in blocking buffer, were added for 1 hour at room temperature. Cells were washed three more times with PBS and the coverslips mounted on slides (Thermo Fisher #ISO 8037/1) using either Immu-mount (Thermo Fisher #9990402) or Vectashield (Vector #H1000), if the cells were going to be imaged by Super-resolution microscopy.

*Primary and secondary antibodies

Antibody		**Company**	**Catalog nº**	**Concentration**
Primary	HA.11	BioLegend	901502	1:500
	p65	Cell signaling	8242S	1:400
	A27	-	-	1:2000
Secondary	Mouse 488	Invitrogen	A11029	1:400
	Mouse 592		A11005	1:200

Section 2.15 Flow cytometry analysis

Cells were seeded in 12 well plates and infected with Early eGFP or Late eGFP virus at MOI 1. Cells were harvested at 4 hours after infection for early gene expression and 6 hours for late. Cells were washed with PBS and then 200 µl of trypsin were added. When cells were detached from the bottom of the dish, 1.6ml of 10% FBS and 200 µl of 20% PFA were added. Cells were then transferred to a 1.5 ml Eppendorf tube and immediately used for flow cytometry (Merck #Guava easyCyte single sample flow cytometer).

FACS data was analysed using InCyte 2.7 Software for Guava® EasyCyte (Merck #0500-4120). Cells were gated and only healthy cells taken into account (Figure 2.1a). Uninfected cells had a fluorescence between 1*10^0 to 1*10^2, while infected cells had a fluorescence in between 1*10^4 and 1*10^5. Fluorescence was plotted on a logarithmic scale. The ratio of green cells (infected – R4 in Figure 2.1b) over the ratio of non-green cells (non-infected) was calculated. Then, using our uninfected sample as baseline for background fluorescence, we normalised our data (Figure 2.1b). Finally, we presented the data as percent of infected cells.

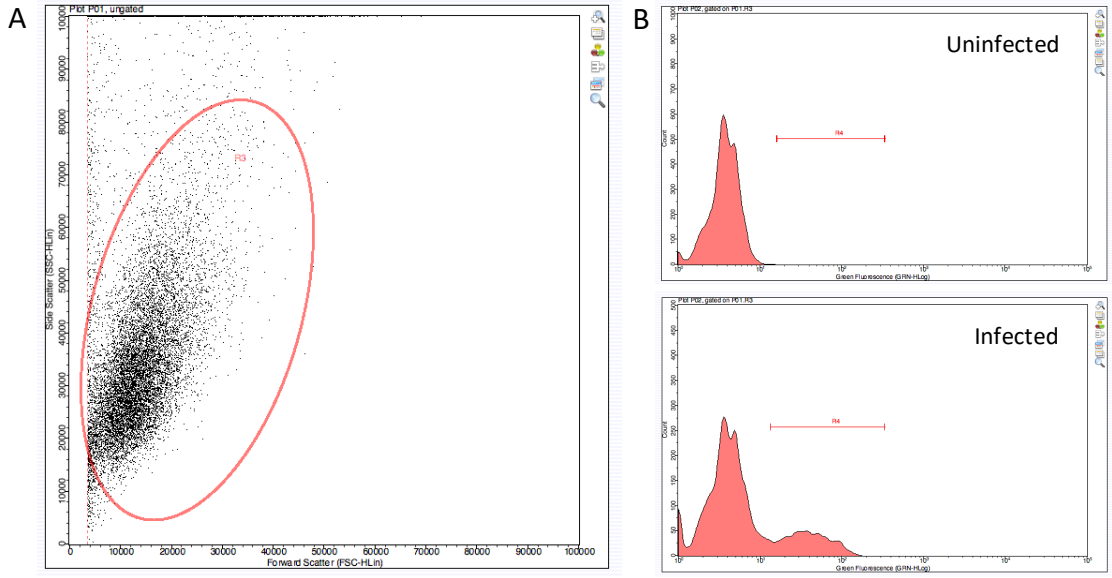


Figure 2.1. Analysis of the data obtained by flow cytometry. A) Gating of healthy cells. B) Fluorescence intensity of the cells in an uninfected and an infected sample.

Section 2.16 Vaccinia protein H1 purification

Our Vaccinia H1 protein was purified from XL1-Blue Competent Cells (Stratagene #200249). 50µl of our Tetracycline resistant bacteria was transformed with the His-H1 plasmid, carrying Ampicillin resistance. This was done by thawing the bacteria on ice and aliquoting 50µl of them into pre-chilled tubes, to which 0.85µl of beta-mercaptoethanol was added. The cells were then incubated on ice for 10 minutes and then the plasmid was added. Cells were then incubated on ice for 30 minutes minimum. A 45 seconds heat pulse at 42°C and 2 minutes incubation on ice followed. Then, cells were quickly resuspended in 1ml of pre-warmed LB and were incubated at 37°C for 1 hour, shaking at 225-250rpm to help them recover. After this, cells were grown in 30ml LB overnight at 37°C and shaking 225-250rpm. The following day, 500ml of LB was inoculated with the 30ml culture of bacteria and were incubated at 30°C shaking 225-250rpm. Once their OD600

reached 0.4-0.6, they were induced with 1mM IPTG. In some cases, 1ml of bacteria were harvested at 0h after IPTG induction. The rest of the culture was harvested 6 hours post IPTG administration by centrifuging at 11,000 x g for 2 minutes. The cell pellets were kept and frozen at -80°C. The following day, the frozen samples were thawed on ice and resuspended in 20ml of Buffer A*, containing 20mM Imidazole (Sigma #56750 – 100g). They were then sonicated at level 6 in a sonnicator (VWR Scientific, Branson 450 Sonifier) with 15 x 15 seconds pulses on ice. Subsequently, samples were spun down at 4,000 x g for 15 minutes at 4°C. Supernatant was transferred into a new 50ml Falcon and spun down in the same way. This time, the crude extract was filtered through a 0.22 μm filter (Sarstedt #92.189.121). In the cold room at 4°C, protein columns containing Ni-NTA agarose beads (Qiagen #30210) were equilibrated with 20ml of 20mM Imidazole. The bacterial cell lysates were then run through the columns. Then, elution of the purified protein was done by adding Buffer B* with increasing Imidazole concentration: 50mM, 100mM, 150mM and 200mM – of each, 3 ml were added to the columns and 3 separate fractions were kept for each concentration (of ≈1ml each). An SDS-PAGE pre-cast 4-12% Bis Tris Plus gel (Thermo Fisher) was run with our purified protein and a control protein as well (BSA) and stained with Simply Blue Safestain (Thermo Fisher #LC6060). Once the gels were stained, we could visualise which eluates contained our H1 protein and determine their concentration by comparison with our BSA control. Finally, the fractions rich in H1 were dialysed overnight at 4°C in 1-2 litres of Dialysis buffer*. The next day, the dialysed protein was measured in Nanodrop at 280nm to get to know its concentration and aliquoted and stored at -80°C until needed.

* Buffers used for the protein purification.

	Buffer A	Buffer B	Dialysis Buffer
Sodium chloride	500mM	500mM	500mM
Na2HPO4 *H2O	50mM	50mM	50mM
Imidazole	20mM	50mM-200mM	-
Tween 20	0.1%	0.1%	0.1%
pH	8.0	8.0	-

Section 2.17 In vitro de-phosphorylation assay

This assay was carried out following the steps described by Liu, K., Lemon, B. and Traktman, P. in *The Dual-Specificity Phosphatase Encoded by Vaccinia Virus, VH1, Is Essential for Viral Transcription In Vivo and In Vitro* (Journal of Virology, Vol. 69, No. 12, p7823-7834). In our case, we used 1 - 1.5 µg of purified H1 protein and 0.2 µg of purified H3. We used 0.4 µg DUSP1 (MBL #CY-E1373) as a positive control and no enzyme added as negative control. The proteins were thawed on ice, diluted in sterile water and added to the reaction tube (1.5ml Eppendorf) in the following order: buffer, histone 3 and finally the H1 or DUSP1. The tubes were quickly spun down and put in a heating block at 37°C for 90-120 minutes. Quickly after that, the samples were heated to 100°C to stop the reaction and DTT and SDS-PAGE loading buffer (cell signaling pack #7722S) were added to a final concentration of 1x. Samples were then used for Western blotting, as described in section 2.13.

* Buffers used

Reagents	Control buffer (2x)	Active buffer (2x)
100 mM Tris	✓	✓
100 mM DTT	-	✓
0.10% NP-40	-	✓
ddH2O	✓	✓
pH	8	8

* Reaction

	- control	+ control	H1
Buffer	20 µl	20 µl	20 µl
ddH2O	15 µl	10 µl	10 µl
Histone 3	5 µl	5 µl	5 µl
DUSP1	-	5 µl	-
H1	-	-	5 µl
Final Vol.	40 µl	40 µl	40 µl

Section 2.18 Generation of recombinant vectors

H1 vectors were purchased and amplified. The vectors used were purchased in Life Technologies (GeneArt), being H1L.HA tagged (Project number 2017ABUBPP) and H1-HA tagged with a C110S mutation (Project number 2017ABUBPP), using Vaccinia WR strain for the protein sequences. They were codon optimised and a Kozak sequence was added. The H1 protein DNA sequence was tagged in its N-terminus by HA and the whole construct was put under the control of a CMV promoter. They were amplified by transforming bacterial DH5α (Thermo Scientific # 18263012) cells with 5 ng of the purified plasmid.

Section 2.19 Vector amplification

DH5α cells were thaw on ice and 50 µl were pipetted into a 1.5 ml Eppendorf tube. The DNA was then added to the cells, in volumes ranging from 1 µl to 15 µl, and they were left on ice for a minimum of 30 minutes. Bacteria were then heat shocked at 42 °C for 45 seconds and recovered by adding 250 µl of LB broth and leaving them shaking in a 37 °C incubator for 1 hour. Then, 200 – 250 µl of bacteria were plated on plates containing the necessary antibiotic. The following day, a bacterial colony was selected and grown on either 5ml or 300 ml of LB broth, shaking overnight at 37 °C. The next day, the DNA was purified from bacteria by running a QIAprep Spin Miniprep Kit (Qiagen #27106), if 5 ml were incubated. If 300 ml of LB was used, the DNA was purified 2 days after inoculation by running a Purelink HiPure plasmid filter Maxiprep kit (Invitrogen #210017).

Section 2.20 Cell transfection

Cells were seeded 1 to 2 days prior to transfection. A mix of Opti-MEM (Gibco) Lipofectamine 2000 (Invitrogen # 11668019) and DNA* was made to a final volume of 250µl per well of a 6 well plate. It was let to sit for ≈ 5 minutes at room temperature and then added in a drop wise manner to the cells, on top of the medium. Cells were returned to the incubator and harvested 20 – 24 hours after transfection and used for Western blot.

* Transfection mix

Reagents	Vector used	
	H1 -	**H1.wt**
Opti-MEM	239 µl	239 µl
Lipofectamine 2000	9 µl	9 µl
DNA	2 µl of 0.47 µg/µl	2 µl of 1.5 µg/µl
Final volume	250 µl	250 µl

Section 2.21 Imaging p65 nuclear translocation

A549 cells were seeded in 96 well CellCarrier-96 Ultra plates (Perkin-Elmer #6055302) plate 1 day before the experiment was carried out. Cells were infected with the indicated viruses Western Reserve WT or H1- or Copenhagen WT, vv811, vv811ΔA49, vv811ΔA49H1+ or vv811ΔA49H1- at an MOI of 5. They were immediately placed at 37 °C and 30 minutes after infection, the virus was removed and cells were fed with fresh medium. 6 hours after infection cells were stimulated with 50ng/ml of TNFα or 25ng/ml of IL1β for 30 minutes. Following this, cells were fixed for 30 minutes with 4% PFA and stored at 4 °C until staining for p65 was carried out as described in "Section 2.14 Immunofluorescence protocol". Imaging was carried out by Dr. Janos Kriston-Vizi. Image analysis was performed by Dr. Artur Yakimovich using a custom-made workflow based on CellProfiler (Carpenter et al., 2006) and KNIME (Fillbrunn et al., 2017) software. In this workflow, using Hoechst 33342 staining signal the cell nuclei and the surrounding cytoplasm were detected using automated thresholding algorithm. The number of cells in each condition was recorded, as well as the staining intensities for Hoechst, p65 and A27 or E/L EGFP for both nucleus and cytoplasm. The per-cell p65 nuclear to cytoplasmic ratios were calculated in

each of the technical replicates (2 per biological repeat) and in the 3 biological repeats. These values were then plotted and compared among the different conditions.

Section 2.22 Quantifying cytokine expression by RTqPCR

A549 cells were seeded in 6 well plates 1 or 2 days before the experiment was carried out. Cells were infected with the corresponding virus Western Reserve WT or H1- or Copenhagen WT, vv811, vv811ΔA49, vv811ΔA49H1+ or vv811ΔA49H1- at an MOI of 0.5 or 5. They were place at the 37 °C for 30 minutes, the virus was removed and cells were fed with fresh medium. 6 hours after infection cells were stimulated with 50ng/ml of TNFα or 25ng/ml of IL1β for 4 hours. Cells were then using the RNeasy Plus Mini Kit (Qiagen #74134) as follows. In the virus hood, medium from infected cells was aspirated and 350µl of beta-mercaptoethanol containing RLT buffer was added per well (30µl of beta-mercaptoethanol per 3ml of RLT). Cells were then scraped, transferred to a 1.5ml tube and quickly vortexed. The samples were then passed 5 times through a 20-gauge needle, using a 1ml syringe. The lysate was then pipetted into a gDNA Eliminator spin column and centrifuged at 8,000 x g for 30 seconds. The flow through was kept and 350µl of 70% ethanol was added to it – mixed well by pipetting. The 700µl samples were then transferred to an RNeasy spin column. In the wet lab, the samples were centrifuged at 8,000 x g for 15 seconds, this time discarding the flow through. 700µl of RW1 buffer was added to each column and centrifuged again at 8,000 x g for 15 seconds. 500µl of RPE buffer was added to the columns and once again, centrifuged

at 8,000 x g for 15 seconds. As before, 500µl of RPE buffer was added to the columns but this time centrifuged at 8000g but for 2 minutes. The columns were then placed on clean 1.5ml tubes, 30µl of RNase-free water was added and they centrifuged at 8,000 x g for 1 minute. Finally, the RNA concentration was measured using a spectrophotometer (Thermo Scientific #Nanodrop 1000). The samples were then stored at -20°C until they were processed for RTqPCR.

A 2 step RTqPCR was performed in collaboration with Dr. Moona Huttunen or Dr. Susanna Bidgood. Firstly, the volume of RNA needed for each sample was calculated – to use 500ng of RNA. A master mix*[1] containing Oligo(dT) from Thermo Fisher #18418012, dNTPs (Thermo Fisher #R0192) and nuclease free water (Qiagen #129114) was made and 8µl of it pipetted into each PCR tube (VWR #732-1517). Then, the 500ng of RNA were added and topped up with nuclease-free water to a final volume of 12µl. Samples were quickly spun down to make sure all the sample was at the bottom of the tube and then a PCR program at 65 °C for 5 minutes followed by a quick chill was run. Samples were spun down once again to collect everything at the bottom of the tube and a second master mix*[2] was made of which 7µl of it is pipetted into each tube, mixed and spun down. A PCR program at 42 °C for 2 minutes was run and samples were subsequently spun down quickly. Following this, 1µl of SuperScript II reverse transcriptase (Thermo Fisher #18064014) was added to each tube, which were then mixed and spun down. Another PCR program at 42 °C for 50 minutes followed by 70°C for 15 minutes was run. Samples at this stage were either frozen or used for the following steps. The last step consisted in expanding the cDNA. For this, our cDNA was diluted 1:5 (20µl cDNA + 80µl water) and

a third master mix*[3] was made. Finally, 2µl of diluted cDNA was added to the appropriate well of a 96 well plate. Then, 18µl of master mix was pipetted. The plates were closed with their lids and spun down for 3 minutes at 300 x g. The qPCR program was run according to MESA Blue qPCR MasterMix Plus for SYBR Assay (Eurogentec #**-SY2X-03+WOUB) instructions. Expression of the genes was normalized to that of an internal control, GAPDH as in Sumner, R. et al. 2014.

*[1] Master mix

Reagent	Volume
Oligo(dT)	8µl
dNTPs	8µl
Nuclease free water	48µl

*[2] Second master mix

Reagent	Volume
Water	8µl
5x buffer	32µl
DTT	16µl

*[3] Third master mix

Reagent	Volume
Water	152µl
Forward primer	4µl
Reverse primer	4µl
MESA	200µl

Chapter III. Histone Acetylation, Methylation and VV infection

As previously discussed, histone acetylation plays an important role in gene transcription while histone methylation is more variable and its outputs will depend on the exact nature of the modification and in which histone and residue it takes place. While Histones 3 and 4 have been extensively investigated, histones 2A and 2B have been left behind due to their high dynamics in chromatin and so they still remain of great interest (Becker 2002).

Sendai virus studies suggest that histone acetylation is a mechanism employed by the cell to upregulate immune-related genes. Specifically, they observed increased acetylation after infection in H3K9, H3K14 and H4K8 (Maniatis 1999). These three residues could potentially be the key to activating IFN-β gene expression in response to infection. A wide range of pathogens employ multiple efforts to get rid of histone acetylation upon infection, corroborating that this mark may be of great importance for host subversion. Listeria monocytogenes and Anaplasma phagocytophilum secrete proteins into the host cytoplasm, LLO and AnkA respectively, that target H3K18 acetylation; similarly to Pseudomonas aeruginosa (Hamon, Batsche et al. 2007) (Garcia-Garcia, Barat et al. 2009) (Grabiec and Potempa 2018). Adenoviruses also focus on H3K18 acetylation and they do so by sequestering CBP/p300 complex, involved in its acetylation (Eskandarian, Impens et al. 2013). On a similar note, Escherichia coli protein NleC binds to p300 and targets it for degradation repressing this way IL8 gene expression (Grabiec and Potempa 2018). Helicobacter pylori decreases

H3K23 acetylation upon infection and Mycobacterium tuberculosis and Toxoplasma gondii have been reported to decrease histone acetylation and this to correlate to a decrease in the expression of immune related genes such as HLA-dra, CIITA and STAT1 induced genes (Eskandarian, Impens et al. 2013) (Lang, Hildebrandt et al. 2012). Finally, deletion of Candida albicans and Aspergillus fumigatus HDAC genes has been shown to have an effect on the fungi's growth (Lee et al. 2009)(Li et al. 2015) (Kmetzsch 2015).

Viruses have also evolved to modulate host cell histone methylation to actively repress immune-related genes and activate genes necessary for infection. Such is the case of Influenza, which trimethylates H3K9 located in the promoter of the CXCL1 gene and this way it prevents its transcription. Moreover, it increases monomethylation of H3K79, allowing the expression of cellular genes that are necessary for viral growth (Marcos-Villar, Diaz-Colunga et al. 2018).

Summarizing, modulating histone acetylation and methylation are widespread mechanism employed by many pathogens to overcome the cell's hostile intracellular environment (Figure 3.1).

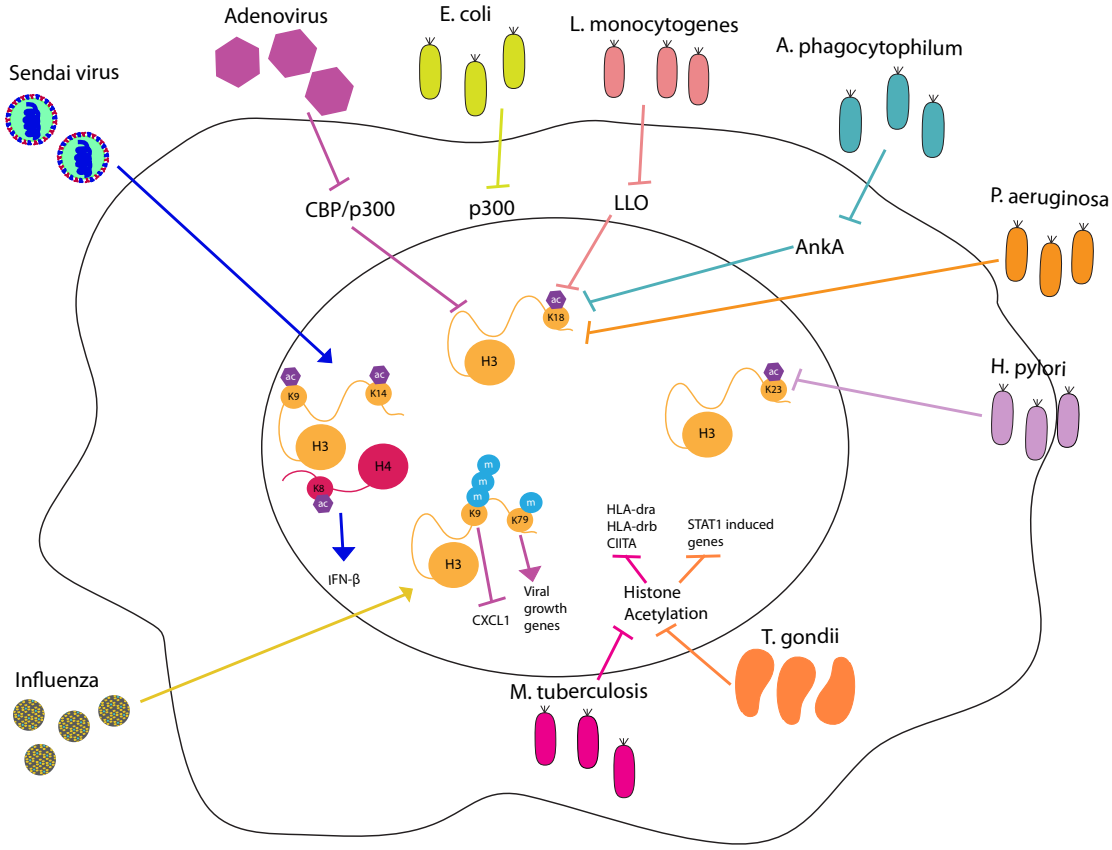


Figure 3.1. Summary of the histone modifications known to be affected by bacteria and viruses. Sendai virus infection leads to upregulation of the cell immune related genes by H3 acetylation at K9 and K14. Adenovirus, E.coli, L.monocytogenes, A.phagocytophilum, and P.aeruginosa get rid of H3 K18 acetylation. H.pylori removes H3K23 acetylation. M. tuberculosis and T. gondii modulate histone acetylation to prevent expression of HLA-dra, HLA-drb, CIITA and STAT1 dependent genes, respectively. Finally, Influenza promotes H3K9me3 to repress CXCL1 gene expression and also K79me to allow the expression of genes necessary for viral growth. Adapted from Gabriec and Potempa, 2018.

Section 3.01 Vaccinia modulates cellular histone acetylation and methylation

Up to date, no studies have focused on determining the impact that Vaccinia infection has on cellular histone acetylation. However, Teferi did investigate how Vaccinia affected methylation of two specific residues: H3K9me3 and H4K20me3. Both of them are increased upon infection, and

H3K9me3 is believed to be mediated by the Vaccinia protein K7 through SUV39H1 and SUV39H2 (Teferi, Desaulniers et al. 2017). In addition to this, data from our lab suggested the virus is interacting with cellular proteins involved in histone acetylation and methylation. Dr. Karel Novy showed that HDAC2, a histone deacetylase; NCOR1, a transcriptional coregulator that recruits HDACs; and SET, a protein that prevents Histone 4 acetylation by binding to HATs, change their phosphorylation pattern upon Vaccinia infection. The phosphorylation state of methyltransferases, DNMMT1, MLL2, SETDB1 and SUV39H1, also changes upon Vaccinia infection, indicating that the virus is actively modulating host cells at the chromatin level. Finally, the siRNA screen carried out by Dr. Jason Mercer showed that Vaccinia is incapable of infecting human cells if they lack HDAC5 and HTATIP, proteins involved in histone deacetylation and acetylation, respectively.

With regard to acetylation and methylation, here I screened several histone acetylation marks in all four core histones: H2A K5ac, H2B K5ac, H3 K14ac, H3 K4ac, H3K4me3, H3 K9ac, H3 K9me3 and H4 K8ac. For this, I infected A549 cells with MOI 10, harvested at different times during infection and performed a western blot on them. All the data presented in this chapter was generated by me.

Section 3.02 H2A K5 acetylation

H2A K5 is a modification that seems to be present in the body of the gene. It is usually accompanied by other modifications of Histone 3, such as H3K14ac. Since very little information is known about its possible role during infection, I decided to investigate whether Vaccinia infection may be modulating it and if so, to which extent. For this, I infected A549 cells, which

is the closest cell line to lung epithelial cells – a primary tissue infected by Vaccinia– with Western Reserve and studied acetylation at critical times for viral infection. These correspond to two early time points: 30 minutes, the time by which Vaccinia is entering the cell, and 1 hour, when Vaccinia is in the cytoplasm and early gene expression has just started. Then we have two intermediate timepoints: 2 hours which marks the initiation of genome replication and it peaks at 4 hours. Finally, two late time points: 6 hours, which marks the onset of late gene expression, when virion morphogenesis has begun to produce the first set of newly infectious MVs and, and 8 hours when the first cycle of Vaccinia infection has been completed and EVs are released to infect surrounding cells.

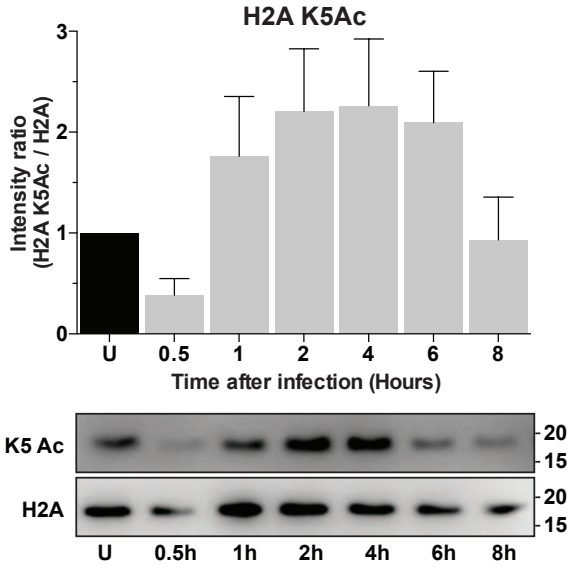


Figure 3.2. Histone acetylation of H2A K5 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.064, U-1h = 0.3265, U-2h = 0.1918, U-4h = 0.1978, U-6h = 0.1664, U-8h = 0.886.

Upon Vaccinia entry, we see a dramatic loss of any acetylation that was present in uninfected cells (Figure 3.2). However, at 1 hour post

infection, there seems to be an increase that continues through the infection cycle, peaking at 4 hours post infection. Once late gene expression starts, at 6 hours, acetylation of this residue drops back down until, at 8 hours, it reaches the levels seen at 30 minutes post infection.

Section 3.03 H2B K5 acetylation

H2B K5 is another histone mark that has received little attention. It has been reported to be present at gene bodies and form part of the histone code necessary for gene expression (Rajagopal, Ernst et al. 2014) . Yet, no information about the specific role H2B K5 acetylation has been reported. Its understudy made it an attractive mark to be investigated. As above, we decided to have a closer look at what happens to this histone acetylation mark during Vaccinia infection.

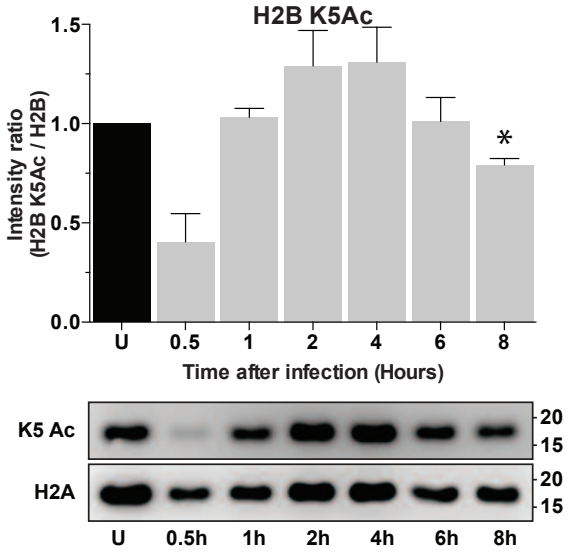


Figure 3.3. Histone acetylation of H2B K5 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.0528, U-1h = 0.5418, U-2h = 0.2449, U-4h = 0.2226, U-6h = 0.9349, U-8h = 0.0241.

The pattern of H2B K5 acetylation mirrors that of H2A K5 (Figure 3.2). It drops to less than half of the acetylation level seen in uninfected cells by 30 minutes post infection, then quickly comes back up to uninfected levels 30 minutes later, corresponding to our 1 hour timepoint. Acetylation continues to increase, peaking at 2-4 hours post infection, followed by a loss of the modification back down to less than uninfected levels at 8 hours.

Section 3.04 H3 K14 acetylation

H3 K14 is a mark that has been heavily studied because of its implications in gene transcription activation. Firstly, it has been stipulated that it is not only present at and marks transcription start sites (TSS) but also for gene bodies. Secondly, in yeast, the presence of this PTM is linked to ribosomal DNA silencing and replicative aging, K14 acetylation being necessary for the lifespan of cells. Thirdly, H3K14ac, together with H3K9ac, is able to recruit transcription factor initiators such as TFIID and therefore contribute to gene expression activation. Finally, experiments using Sendai virus have shown that cells need to acetylate this residue in order to express the IFN-β gene, and therefore activate cellular immunity.

When Vaccinia infected cells were analysed for this mark, similar to what happened with H2A and H2B acetylation, soon after infection there is a fast decrease in the acetylation level (Figure 3.4). However, unlike the previous marks this level of acetylation remained largely unchanged between 1 and 8 hours post infection.

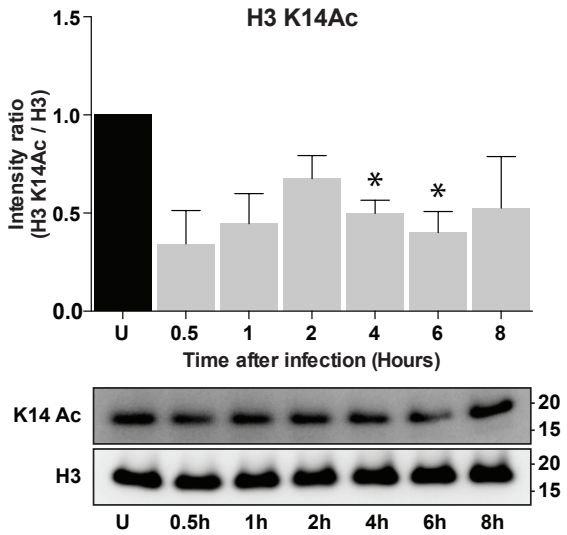


Figure 3.4. Histone acetylation of H3 K14 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.0605, U-1h = 0.689, U-2h = 0.1086, U-4h = 0.0174, U-6h = 0.0305, U-8h = 0.2116.

Section 3.05 H3 K4 acetylation and methylation

H3 K4 is a histone residue that has received the attention of many studies. It can be acetylated as well as methylated. H3 K4ac has been linked to the expression of a subset of genes in yeast. H3 K4 me3 is of great importance for the recruitment of TFIID and the formation of the preinitiation complex, it is mainly located in euchromatin and it enhances H3 K9ac by recruiting HAT such as GCN5 and PCAF. Additionally, H3 K4me3 antagonises and prevents the trimethylation of H3 k9, a repressive mark.

Starting with H3 K4 acetylation (Figure 3.5), we see an initial drop at 30 minutes post infection in the PTM as we saw previously with acetylation at H2A K5, H2B K5 and H3 K14. From then onwards, acetylation increases constantly overtime, peaking and reaching higher levels than in uninfected cells at 6 hours post infection and then falling to uninfected levels by 8 hours post infection.

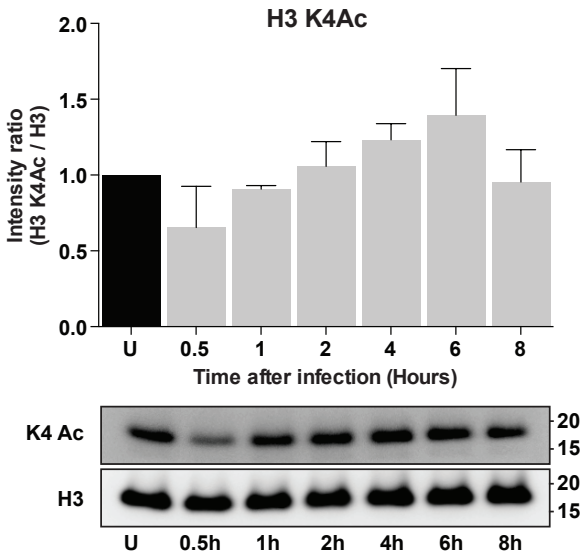


Figure 3.5. Histone acetylation of H3 K4 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.3311, U-1h = 0.0535, U-2h = 0.7598, U-4h = 0.1624, U-6h = 0.3284, U-8h = 0.8513.

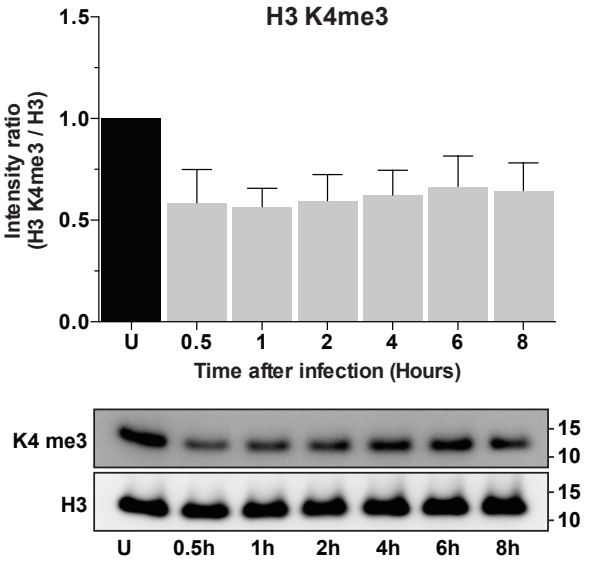


Figure 3.6. Histone trimethylation of H3 K4 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.1593, U-1h = 0.0543, U-2h = 0.1638, U-4h = 0.1880, U-6h = 0.2752, U-8h = 0.1892.

Now, looking at trimethylation of H3 K4 (Figure 3.6), we find a very subtle trend that correlates with what was seen in H3 K4 acetylation but this

time, the methylation levels are halved of the those found in uninfected cells. We suggest then, that H3 K4 is predominantly acetylated during viral infection and as a consequence, it loses its methylation.

Section 3.06 H3 K9 acetylation and methylation

These two histone marks have also been deeply studied. While H3K9me2 is well known to play a role in gene repression and heterochromatin formation, it is vital for X chromosome inactivation by recruitment of Dnmt3a/b and HP1α/β; H3K9me3 is a mark for gene repression in autosomal chromosomes. H3K9ac, on the other hand, is a mark for gene expression and together with H3K14ac is able to recruit TFIID complex for transcription initiation. Additionally, H3K9me3 and H3K9ac antagonise each other giving rise this way to a chromatin full of trimethylation or acetylation of the residue in the neighboring histones and creating a repressed or active region, respectively. Interestingly, H3K9ac has been reported to increase its acetylation levels upon Sendai virus infection in order to allow for IFN-β gene expression. Moreover, Teferi et al. found in their experiments that infection with Vaccinia virus leads to increased H3K9me3 over time.

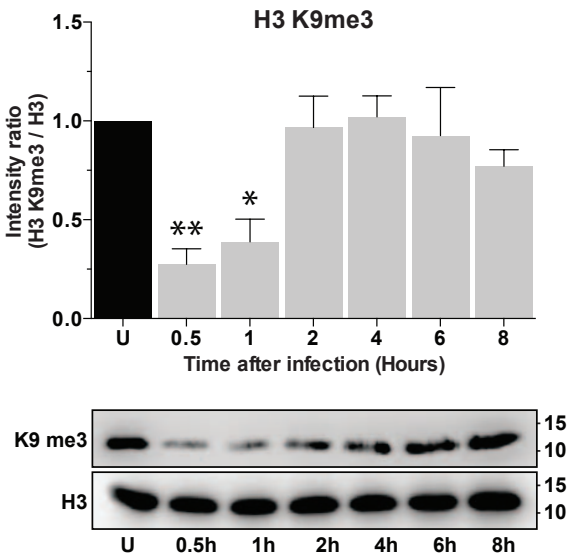


Figure 3.7. Histone trimethylation of H3 K9 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.0092, U-1h = 0.0296, U-2h = 0.7895, U-4h = 0.9858, U-6h = 0.7924, U-8h = 0.1144.

We decided to check this and also to investigate whether this increase in trimethylation of the residue would be correlated with a decrease in its acetylation (Figure 3.7). We can see, that the same way Teferi et al. observed, Vaccinia infection leads to an increase in H3K9me3 over the timecourse studied. Initially, at 30 minutes post infection, we see a sudden decrease in methylation respect to uninfected levels. At 1 hour this methylation starts to increase constantly over time.

In the light of this data, we next decided to look at histone acetylation at this residue since we would expect a decrease in this mark due to the presence of a methyl group in this location (Figure 3.8). Contrary to what we hypothesised, acetylation at H3K9 also increases gradually during Vaccinia infection. As every other mark investigated, it drops at 30 minutes after infection compared to uninfected levels but then it not only recovers

overtime but reaches acetylation levels higher than that present in uninfected cells.

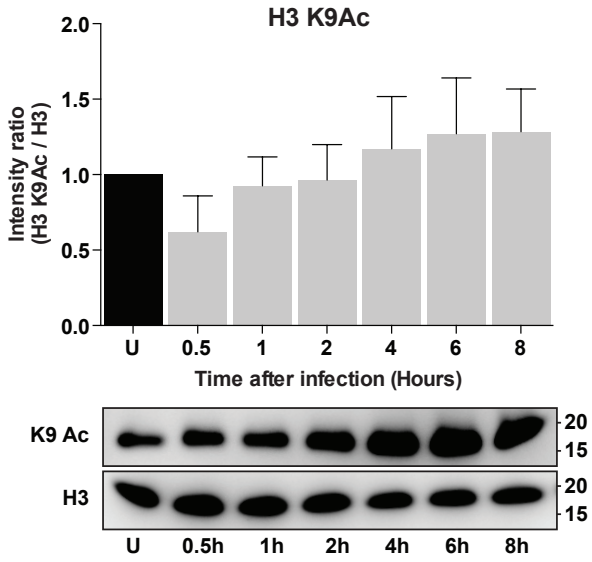


Figure 3.8. Histone acetylation of H3 K9 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.2529, U-1h = 0.7365, U-2h = 0.8926, U-4h = 0.6712, U-6h = 0.5410, U-8h = 0.4208.

The fact that both trimethylation and acetylation increase in this residue overtime implies that these changes are likely occurring in different subsets of histones, or that there are dynamic changes in H3 trimethylation and acetylation during vaccinia infection.

Section 3.07 H4 K8 acetylation

Finally, H4 K8 acetylation is involved in gene expression by recruiting a nucleosome remodeling complex, SWI/SNF, that moves nucleosomes in an ATP-dependent manner allowing chromatin accessibility. Furthermore, H4K8 acetylation levels have been shown to increase upon Sendai virus infection as a cellular mechanism leading to IFN-β gene expression.

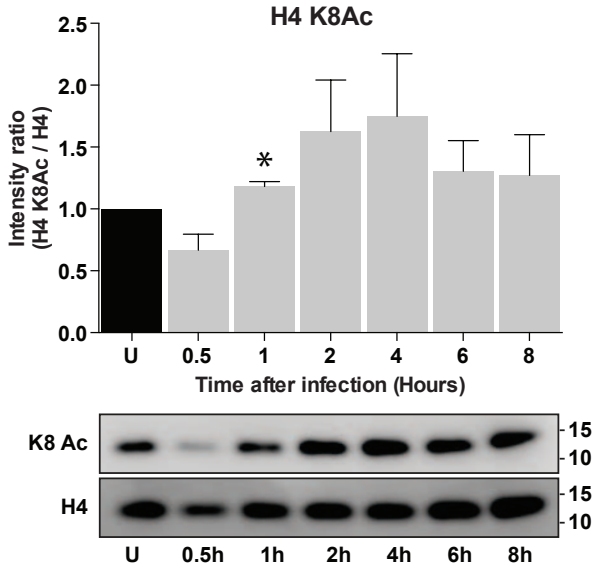


Figure 3.9. Histone acetylation of H4 K8 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.1235, U-1h = 0.0458, U-2h = 0.2726, U-4h = 0.2772, U-6h = 0.3419, U-8h = 0.4937.

As with all other marks investigated, acetylation at H4K8 starts with an initial drop at 30 minutes after infection (Figure 3.9). From then on vaccinia drives acetylation reaching a peak at 4 hours after infection. This level of acetylation is then maintained throughout the time-course of infection.

Section 3.08 Conclusion

From these experiments we can conclude that vaccinia infection modulates the acetylation and methylation of cellular histones. The general trend, with two exceptions, H3K14ac and H3K4me3, indicates that vaccinia infection results in the upregulation of both types of histone marks.

The marks investigated can be categorised in 3 groups according to their specific patterns. In the first group we find H2AK5ac, H2BK5ac and

H4K8ac. These modifications decrease 30 minutes after infection, then increase to higher levels than the uninfected control at 2-4 hours post infection, and finally decrease once again to levels similar or lower to that present in uninfected cells.

The second group includes H3K4ac, H3K9ac and H3K9me3. These modifications, as seen in group 1, drop at 30 minutes after infection and increase over time until reaching a peak at 6 hours post infection. Once they have peaked they maintain similar levels of acetylation or methylation as uninfected cells for the duration of infection.

The third group comprises only two modifications, H3K14ac and H3K4me3. In both cases, the modifications levels drop 30 minutes after infection then, as opposed to groups 1 and 2, they remain down throughout infection.

Collectively, these results indicate that vaccinia virus infection leads to dysregulation of acetylation and methylation in specific histone marks (Figure 3.10). As no histone acetyltransferases (HATs), methyltransferases, histone deacetylases (HDACs) or demethylases have been found in the vaccinia genome, I suspect the virus is hijacking or modifying cellular enzymes to achieve these modifications. Further investigations will need to confirm whether these changes are due to cellular proteins taken over by the virus to dampen the cellular immune response, to upregulate genes necessary for viral replication or propagation, or they are the result of the cell responding to infection by activating immune-related genes and signalling pathways.

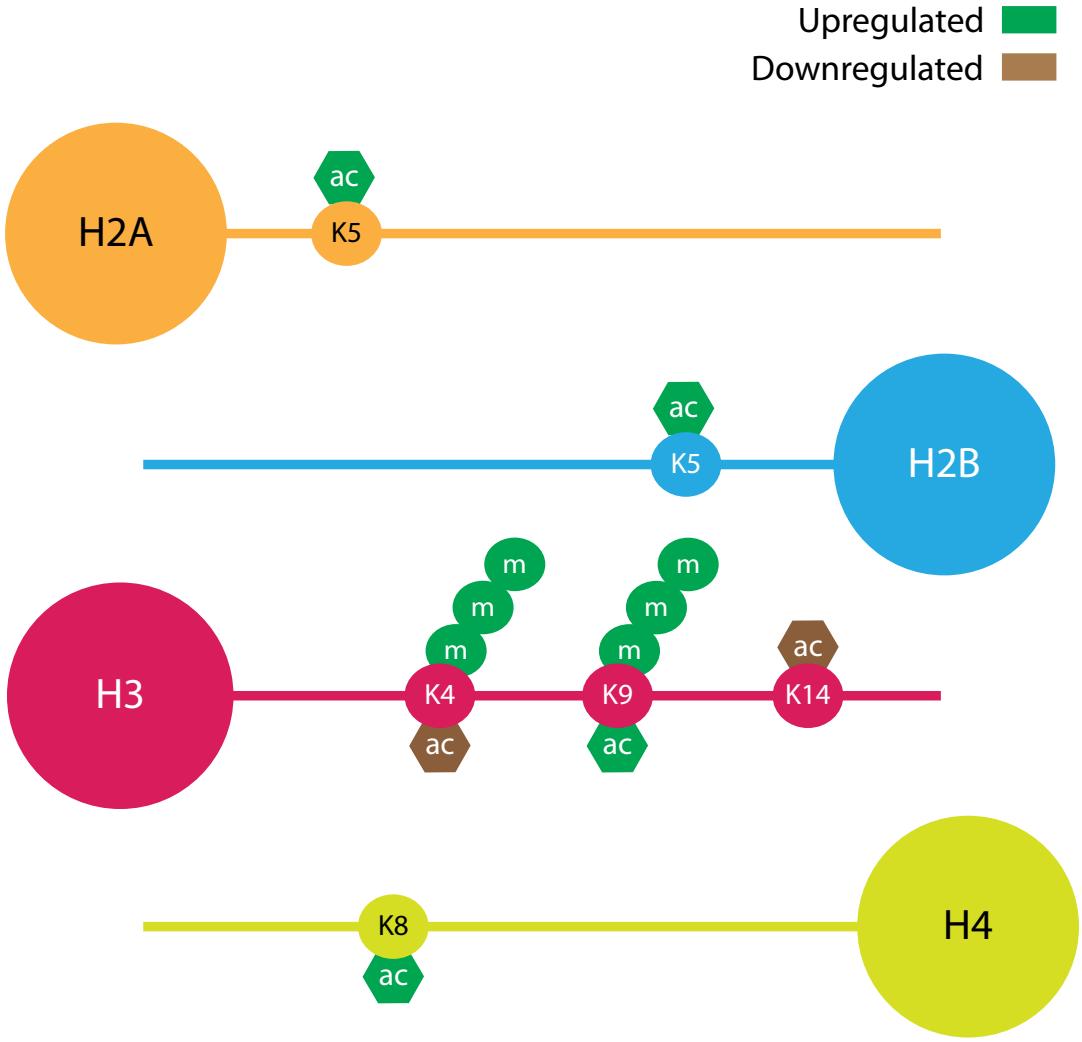


Figure 3.10. Histone acetylation and methylation as modulated by Vaccinia virus. Acetylation at H2AK5, H2BK5, H4K8 and methylation at H3K4me3 and H3K9me3 are upregulated. Acetylation at H3K4 and H3K14 are downregulated during Vaccinia infection.

Chapter IV. Histone Phosphorylation and Vaccinia infection

Histone phosphorylation is an epigenetic mark that has not received as much attention as acetylation or methylation until recently. Despite that fact that all histones can be phosphorylated, research has mainly focused on histone 3 phosphorylation. Despite most of the PTMs studied are related to cell cycle progression and chromosomal condensation, some of them have also been linked to transcriptional activation (Banerjee and Chakravarti 2011). Phosphorylation of H3S10 and H3S28 through MSK1 and MSK2 is involved in c-fos gene expression. The presence of H3S10P in chromatin is usually accompanied by the acetylation of H3K14, and although in some instances H3S10P recruits Gcn5 to acetylate H3K14, this may not always be the case (Walter, Clynes et al. 2008).

Phosphorylation of histone 3 at serine 10 is important for the expression of some immune-related genes. For this reason, bacteria have evolved mechanisms to modulate it during infection. Shigella flexneri, a gram-negative bacterium, secretes its dual specificity phosphatase OspF before it enters the cells. This protein, travels all the way to the nucleus where it directly targets H3S10P, by dephosphorylating erk and p38, at the promoter of specific genes and this way represses the expression of NFκB-induced genes, such as CCL20 or IL8 (Arbibe, Kim et al. 2007). Gram-positive bacteria, such as Listeria monocytogenes, have similar mechanisms to modulate the phosphorylation of this histone residue. In this case, the secreted toxin is LLO, whose presence leads to a massive reduction in H3S10P in the host cell. Additionally, this dephosphorylation is accompanied

by an increase in H3K9ac and also a decrease H3 and H4 overall acetylation, which all contribute to the formation of a more repressive chromatin (Hamon, Batsche et al. 2007).

Although histone phosphorylation remains understudied, it seems clear its importance for cell cycle progression as well as gene expression of specific genes. Additionally, different types of bacteria target it as a way of stopping the cellular immune response upon infection (Figure 4.1).

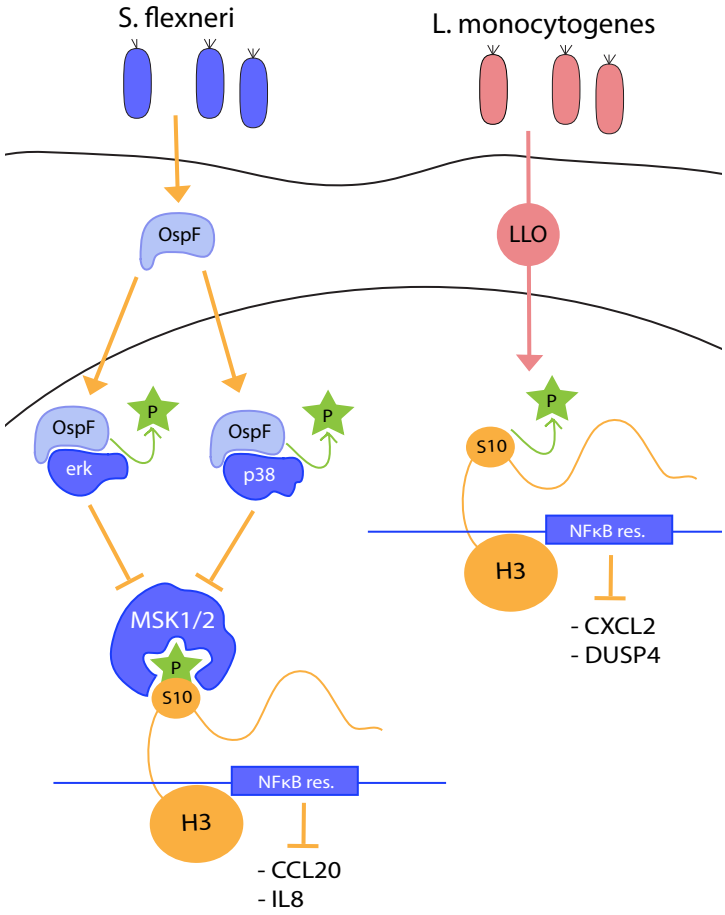


Figure 4.1. Histone phosphorylation is affected by bacteria infection. Shigella flexneri ejects its OspF protein into the cell cytoplasm, which travels to the nucleus where it directly dephosphorylates erk and p38 which leads to H3S10 dephosphorylation. Legionella pneumophila also injects a toxin, LLO, which targets H3S10P, although its mechanism of action is still unknown. Adapted from Gabriec and Potempa, 2018.

Viruses have also been shown to modulate this modification although for completely different reasons. For instance, HCMV sends its genome into the cell nucleus where it stays in the form of an episome during the latent phase of infection. This small piece of DNA is also wrapped by cellular histones that are subjected to the same PTMs as the host's genome. In order to exit the latent phase of infection and become lytic, the virus needs to activate gene expression at the MIEP (major immediate-early promoter) which is done through H3S10P of its surrounding histones (Kew, Yuan et al. 2014). Additionally, H3S10P is vital for HCMV replication since it may play a role in correct chromatin partitioning (Ho, Donovan-Banfield et al. 2016).

Phosphorylation was of more interest to us than acetylation and methylation, because vaccinia encodes 3 essential phospho-enzymes: 2 kinases (B1 and F10), and 1 phosphatase (H1). B1 is a 34 kDa serine/threonine kinase which is expressed early during viral infection and is necessary for viral DNA replication. Its function has been assessed mainly through the use of mutants in different cell lines and the degree to which it confers a replication problem is cell line dependent as a cellular homolog, VRK1, can substitute for B1 when expressed from the viral genome (RACHELE REMPEL 1990, Traktman 1992). In addition, B1 serves to counteract the antiviral restriction factor barrier to autointegration factor 1 (BAF1) (Matthew S. Wiebe 2007). During vaccinia infection cytoplasmic BAF1 targets the viral replication sites in an attempt to block Vaccinia DNA replication. B1 phosphorylates BAF1 to counteract its anti-viral action and allow for the cytoplasmic replication of vaccinia DNA. F10 is another viral kinase that can phosphorylate tyrosine residues as well as threonine/serine ones. It has a predicted molecular weight of 52 kDa, it is expressed late

during viral infection. F10 is essential for the diversion and remodeling of cellular membranes during early phases of virion morphogenesis (Punjabi and Traktman 2005, Greseth, Carter et al. 2017). The H1 phosphatase is a 20 kDa viral protein that can dephosphorylate serine/threonine and tyrosine residues. It is expressed late during infection and is encapsidated in the virus lateral bodies. H1 is known to play a role in viral transcription, as viruses lacking H1 show an early transcriptional defect in the next round of infection (Ke Liu 1995)(Novy, Kilcher et al. 2018). Additionally, expressed H1 is involved in the dephosphorylation of the viral protein A17, necessary for the early stages of immature virion morphogenesis, as when H1 is repressed, A17 is hyperphosphorylated which correlates with an arrest in virus morphogenesis (M. Derrien 1999). Finally, the H1 that is packaged into lateral bodies also has an important role during the viral lifecycle. It is needed to suppress cellular immune signalling through the dephosphorylation of Stat1. Upon IFNγ induction, H1 prevents Stat1 phosphorylation therefore blocking its nuclear translocation. This leads to the dampening of the cellular immune response through repression of IFNγ induced genes (Najarro, Traktman et al. 2001, Schmidt, Bleck et al. 2013).

Given that vaccinia encodes these dual specificity enzymes, two of which have been previously shown to engage in blocking anti-viral cell responses, we decided to investigate histone phosphorylation upon Vaccinia infection and the most evident phosphorylation changes were followed up by using the corresponding mutant virus. All the data presented in this chapter was generated by me, infecting A549 cells at the stated MOIs and with the stated viruses. Purification of the Vaccinia H1 phosphatase was done by me or Corina Beerli.

Section 4.01 Vaccinia modulates cellular histone phosphorylation

Vaccinia infection has been extensively studied, it has already been reported to drive histone methylation upon infection (Teferi, Desaulniers et al. 2017). However, there is no data in regard to histone phosphorylation. Previous data in our lab, obtained by Dr. Karel Novy, showed that B1, F10 and H1 interact with host cell histones using tandem affinity mass spectrometry. As a consequence, we decided to investigate if Vaccinia was modulating histone phosphorylation. For this purpose, I screened several histone phosphorylation marks: H2A S1P, H3 S28P, H3 T3P and H3 S10P.

Section 4.02 H2A S1 phosphorylation

H2A S1P has been shown to be involved in gene expression. *In vitro* studies have demonstrated that the presence of this phosphorylation prevents the expression of the genes nearby (Zhang, Griffin et al. 2004). I infected A549 cells with wild type vaccinia at MOI 10 and harvested cells at different times during infection. Western blots were then performed for H2A S1P and total H2A levels.

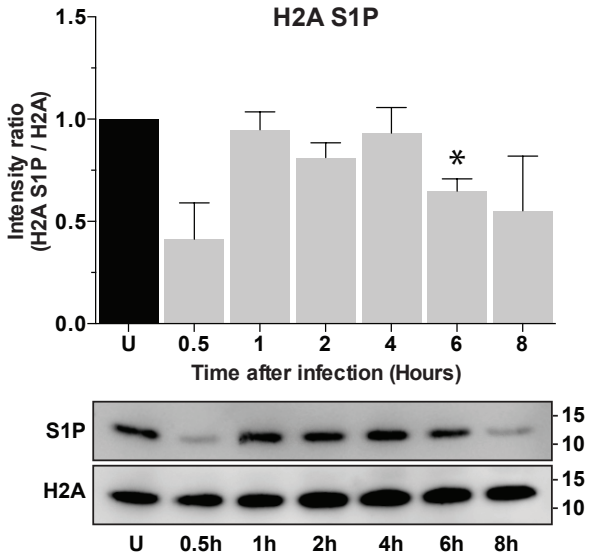


Figure 4.2. Histone phosphorylation of H2A S1 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.0799, U-1h = 0.6117, U-2h = 0.1234, U-4h = 0.6396, U-6h = 0.0275, U-8h = 0.2358.

At 30 minutes post Vaccinia entry into the host cell cytoplasm, we see a drastic drop of S1P phosphorylation (Figure 4.2). However, at 1 hour post infection, this phosphorylation comes back to uninfected levels and stays more or less stable throughout the course of infection.

Section 4.03 H3 S28 phosphorylation

H3 S28 phosphorylation is important for cell cycle progression but also for gene expression. Its presence in the histone tail, together with H3S10P, is necessary for c-fos gene expression upon Ras/Erk/MAPK induction (Soloaga, A. et al. 2003). We decided to test the effects that Vaccinia infection would have upon this mark. Again, A549 cells were infected with wild type vaccinia at MOI 10 and cells harvested at different times during infection. Western blots were then performed for H3 S28P and total H3 levels.

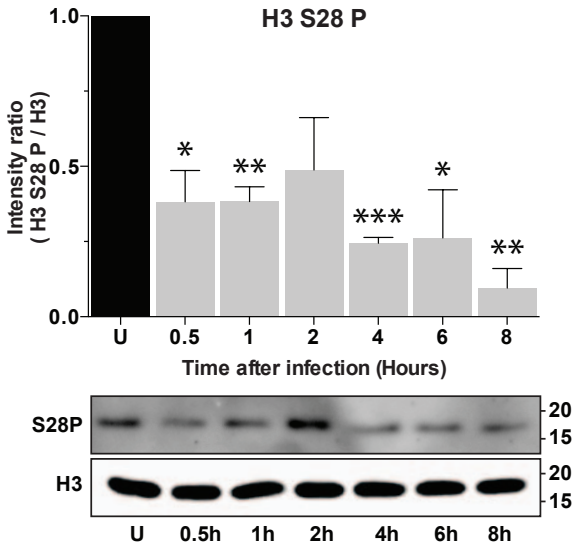


Figure 4.3. Histone phosphorylation of H3 S28 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.027, U-1h = 0.0064, U-2h = 0.0985, U-4h = 0.0007, U-6h = 0.0439, U-8h = 0.0052.

The pattern of H3 S28 phosphorylation is completely different to that of H2A S1P (Figure 4.3). At 30 minutes post infection, this mark drops by more than 50% relative to uninfected cells. It remains at this level until 4 hours post infection, at which time it takes another drop to ~25% of uninfected levels, where it remains throughout the rest of the infection timecourse.

Section 4.04 H3 T3 phosphorylation

H3 T3 phosphorylation is an important PTM in the cell cycle since it is required for the proper attachment of microtubules to the centromeres of chromosomes. However, so far, there is no data linking this mark with gene expression. A549 cells were infected with wild type vaccinia at MOI 10 and cells harvested at different times during infection. Western blots were then performed for H3 3P and total H3 levels.

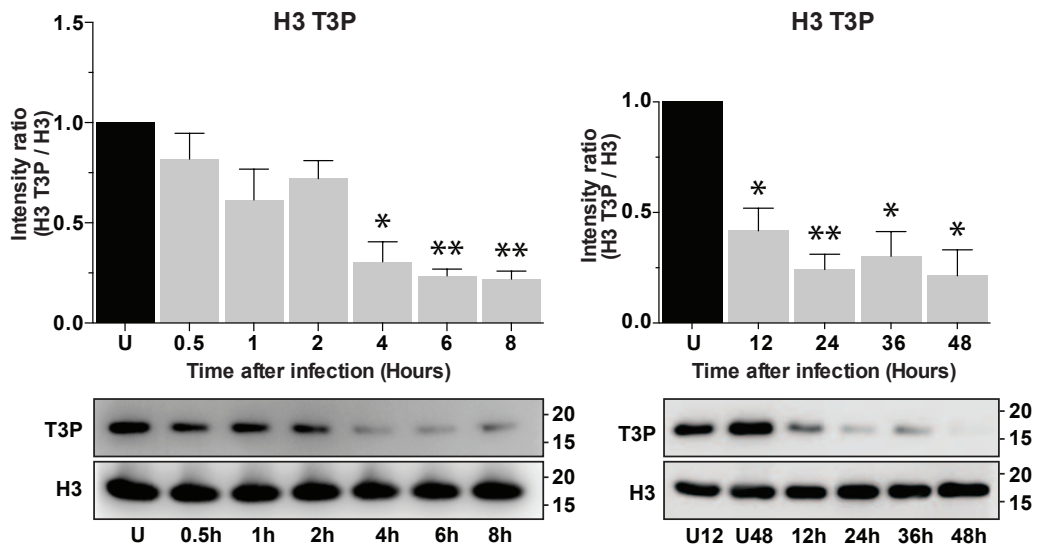


Figure 4.4. Histone phosphorylation of H3 T3 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.2917, U-1h = 0.1286, U-2h = 0.0891, U-4h = 0.0199, U-6h = 0.0018, U-8h = 0.0026, U-12h = 0.0295, U-24h = 0.0083, U-36h = 0.0248, U-48h = 0.0215.

In contrast to most of the modifications investigated, H3 T3 phosphorylation does not suffer from a decrease at 30 minutes post infection. Instead, it stays at nearly uninfected levels until 4 hours post infection, where a large drop in phosphorylation, to 25% of uninfected cells is observed. This level of H3 T3 phosphorylation is maintained out to 8 hours post infection. As such, we wondered whether this mark would remain down as vaccinia infection progresses. The time course was expanded to 12 hours, 24 hours, 36 hours and 48 hours. We find that the repression of H3 T3P by Vaccinia is maintained throughout the infection process as late as 48 hours, a time point when virus has been propagated and spread throughout the entire cell culture.

Section 4.05 H3 S10 phosphorylation

Finally, we decided to look into H3 S10 phosphorylation. This modification was of special interest to us since it has been linked to the expression of immune related genes in the cell. This phosphorylation is carried out by MSK1 and MSK2 in the cell and goes through the Ras/Erk/MAPK pathway. Bacteria target different components of its activation pathway to generate a loss in H3 S10 phosphorylation which in turn leads to repression of genes such as IL8, CCL20 and CXCL2. Due to the relevance that this modification has for infection, we decided to check whether Vaccinia would be interfering with it as well.

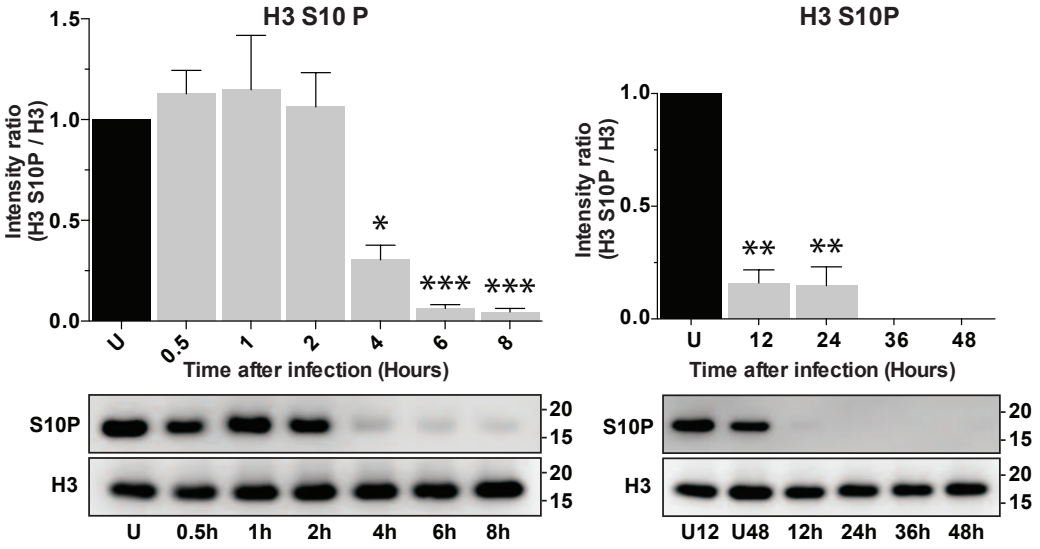


Figure 4.5. Histone phosphorylation of H3 S10 during Vaccinia infection. A549 cells infected at MOI 10 with WR strain. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values: U-0.5h = 0.2936, U-1h = 0.5199, U-2h = 0.2807, U-4h = 0.0693, U-6h = 0.0043, U-8h = 0.006, U-12h = 0.0049, U-24h = 0.0095, U-36h = N/A, U-48h = N/A.

Again, 549 cells were infected with wild type vaccinia at MOI 10 and harvested at different times during infection. Western blots were then performed for H3 S10P and total H3 levels. Upon Vaccinia infection, we

observe a slight increase in phosphorylation compared to uninfected cells out to 2 hours post infection. However, at 4 hours post infection, H3 S10P levels drop massively to ~50% of uninfected levels. By 6 hours post infection, H3 S10P levels have dropped to ~10% of uninfected levels where it remains. Once again, we addressed whether this suppression of phosphorylation would remain stable as infection progresses. The time course was expanded to 12 hours, 24 hours, 36 hours and 48 hours. We observe that H3 S10P levels at the 12 and 24 hours timepoints remain around 10%, while there is a complete diminution to undetectable levels at 36 and 48 hours post infection.

Section 4.06 Vaccinia H1 phosphatase and H3 S10 phosphorylation

Our western blot screen of histone phospho-modifications during Vaccinia infection indicated that H3 was a major target during infection. Vaccinia infection had the greatest impact on H3 S10. As modification of this residue has been reported to be used by other pathogens to modulate gene expression of immune-related genes, we decided to focus on H3 S10 in order to determine the mechanism by which Vaccinia drives its dephosphorylation. As Vaccinia encodes its own phosphatase, it was natural that we investigate the role of H1 on this modification. For this I used a mutant virus in which H1 expression is controlled by a lac operon. As a consequence, H1 is only expressed when IPTG is added (M. Derrien 1999). WT virions and virions that lack H1, referred to from here on as H1(-) virions, were produced. A549 cells were then infected with an equivalent number of WT or H1(-) virions

corresponding to a WT MOI of 2. At various time points the cells were harvested and western blots were performed for H3 S10P and total H3.

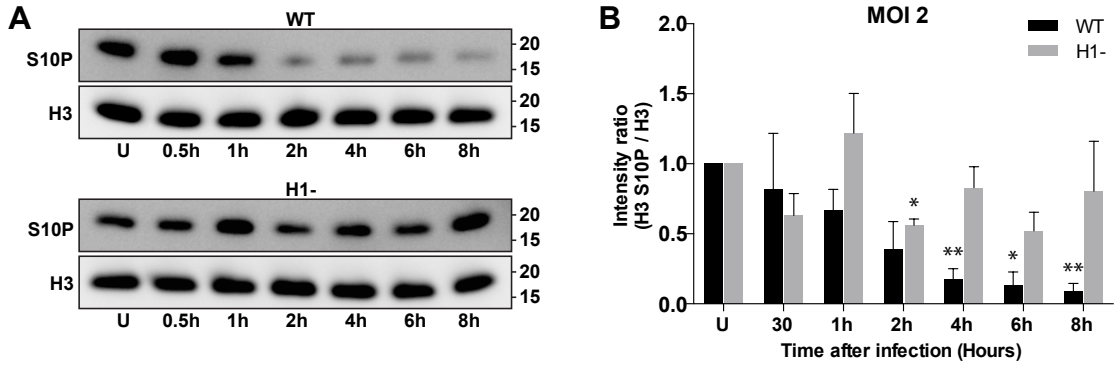


Figure 4.6. H3 S10 upon Vaccinia infection with WT and H1- viruses. A549 cells infected at MOI 2 with WR strain and H1-. 3 repeats of each experiment were quantified and plotted with their SEM values. Paired t-test, p values for WT: U-0.5 = 0.6927, U-1h = 0.1529, U-2h = 0.0894, U-4h = 0.0077, U-6h = 0.0112, U-8h = 0.0038, p values for H1-: U-0.5h = 0.1439, U-1h = 0.5194, U-2h = 0.0106, U-4h = 0.3709, U-6h = 0.0699, U-8h = 0.6377.

When infecting our cells with H1- virus, phosphorylation at H3 S10 stays present through the whole infection cycle compared to our WT infected cells, in which, as before, H3 S10P drastically decreases at 4 hours post infection (Figure 4.6). While the amount of H3 S10 phosphorylation in cells infected with H1- decreases relative to uninfected cells, it never reaches the dephosphorylation levels seen in our WT virus. We attribute this residual dephosphorylation to remaining H1 phosphatase within the H1- virions (Novy, Kilcher et al. 2018).

Since Vaccinia H1 is affecting H3 S10P, this viral phosphatase must go into the cell nucleus to carry out its function. In order to confirm this, we imaged A549 cells infected with a virus expressing a tagged H1-SH by using super-resolution microscopy. Additionally, infected cells were fractionated

and blotted the nuclear and cytoplasmic fractions in the search of H1-SH (Figure 4.7).

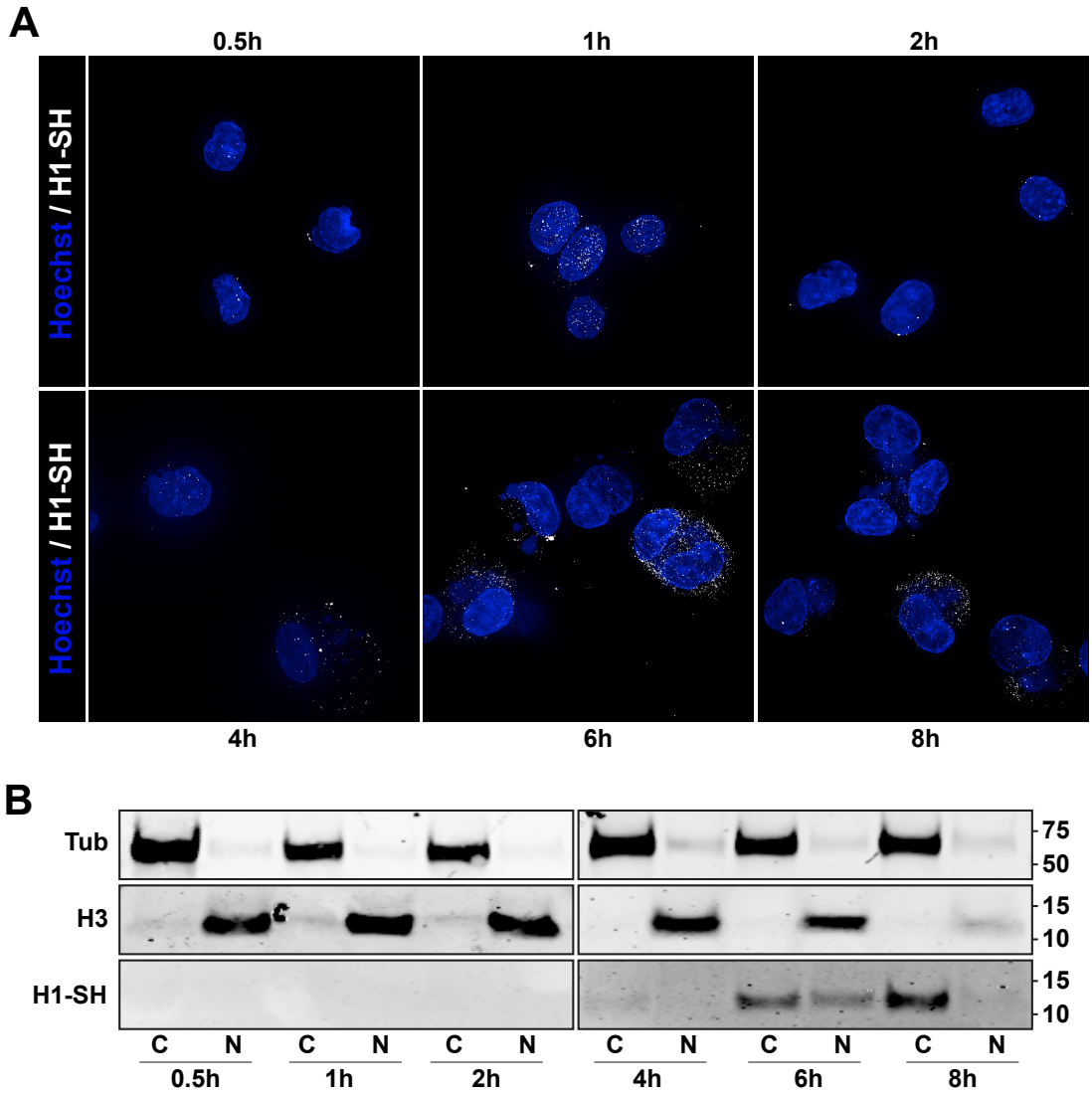


Figure 4.7. Vaccinia H1 phosphatase goes into the cell nucleus. A) Super-resolution microscopy showing H1 localization in the cell during the timecourse of infection (n=1). B) Western blot of nuclear and cytoplasmic fractions of A549 cells infected with tagged H1-SH showing H1 presence in the nucleus (n=1).

H1 was found to be in the cell nucleus as early as 1 hour after infection and peak at 6 and 8 hours post infection, together with late gene expression, as it is a viral protein expressed late in the infection cycle. Regarding Western blotting, we also were able to see H1 in the nuclear fractions.

However, it was not visible until 6 hours post infection, which is probably due to the low levels of protein present in the early timepoints. Moreover, analysis of the H1 protein sequence showed that it carries a bipartite nucleo-cytoplasmic sequence (Figure 4.8). Taken together, this preliminary data shows that Vaccinia H1 protein goes into the cell nucleus during infection. This data is consistent with Karel Novy phospho-proteomics interaction screen in which he showed that Vaccinia H1 interacts with the nuclear H3.

	H1	Score
36 -	GNYKNAMDAPSSEVKFKYVLNLTMDKYTLP	- 63 **3.2**

Figure 4.8. Vaccinia H1 phosphatase contains a bipartite nucleo-cytoplasmic sequence. Analysis using the cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) showed the sequence above corresponds to a nuclear-cytoplasmic sequence; where scores of 8, 9, or 10 mean the proteins are exclusively localised to the nucleus, scores of 7 or 8 partially localised to the nucleus, scores of 3, 4, or 5 localised to both the nucleus and the cytoplasm and scores of 1 or 2 localised only to the cytoplasm.

While these results indicated that H1 is required for Vaccinia-mediated H3 S10 dephosphorylation, whether H1 acts directly on H3 S10 or via modification of a cellular kinase could not be determined through these assays.

Therefore, we decided to carry out an *in vitro* dephosphorylation assay to determine if H1 could act directly on histone 3. For this purpose, H1 was purified from bacterial cells, and histone 3 was purified from A549 cells. Prior to the phosphatase assay, the purified histone 3 was assessed by western blot to determine if the purified histones retained their S10 phosphorylation.

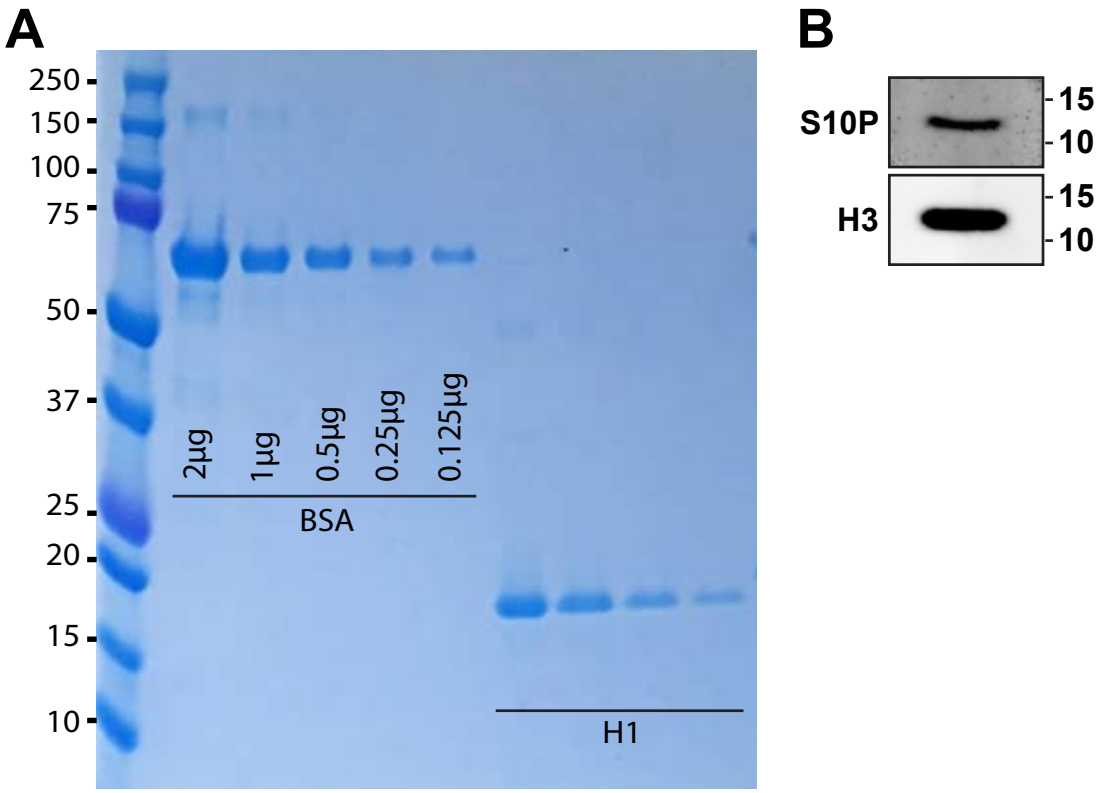


Figure 4.9. Purification of Vaccinia H1 and cellular H3. Purifications were carried out as described in materials and methods. A) Vaccinia H1 is diluted and compared to known concentrations of BSA to elucidate its concentration. B) Purified Histone 3 is blotted confirming its phosphorylation.

H1 was successfully purified to high homogeneity as seen in Figure 4.9. We obtained concentrations of 25ng/µl as calculated against to our BSA control. Purification of histone 3 was also a success since the histone conserved its phosphorylation. An *in vitro* phosphorylation assay was then carried out using these two purified proteins. Two control reactions were performed with each assay. DUSP1, which is a phosphatase known to dephosphorylate H3S10 was used as a positive control for H3 S10 dephosphorylation, and as H1 requires the addition of DTT for its activity, an inactive DTT minus buffer control was used a negative control.

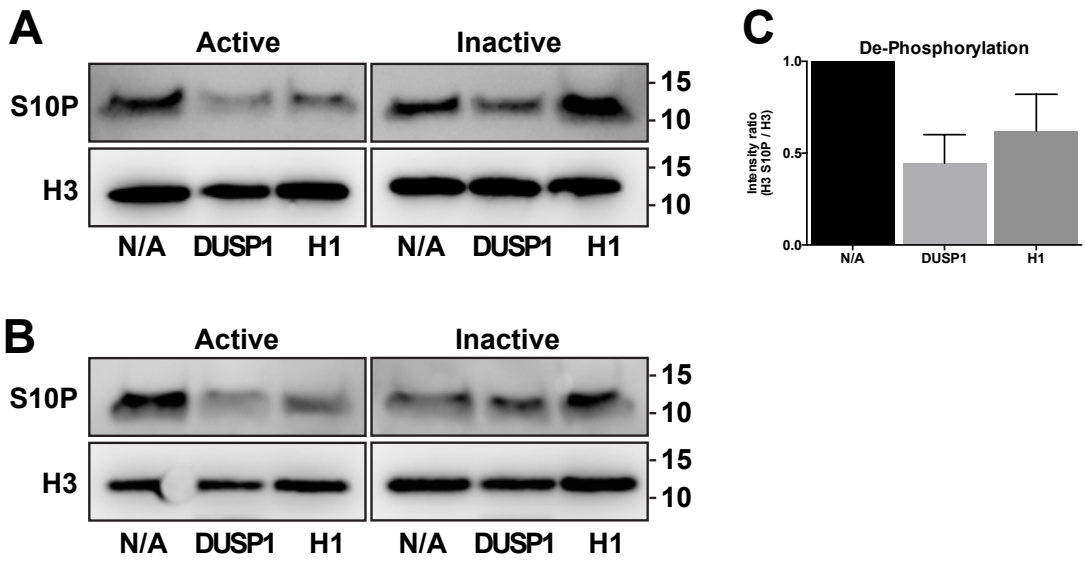
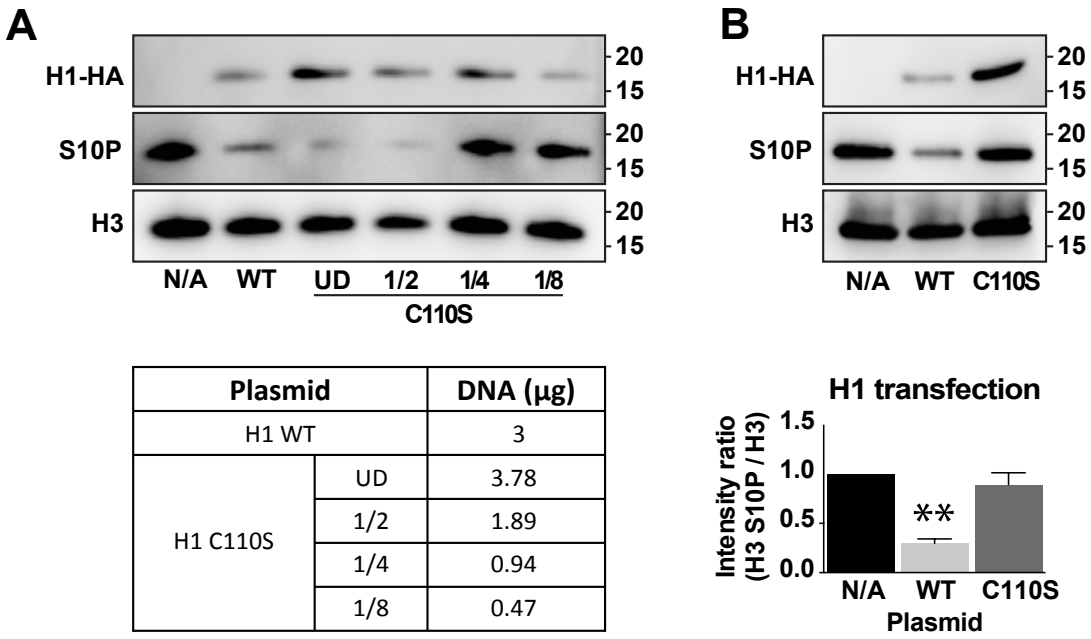


Figure 4.10. *In vitro* dephosphorylation of Histone 3 serine 10. Two repeats of the dephosphorylation assay are shown. A, B) Active and inactive buffers are shown in which H3 was incubated with N/A (no protein added) DUSP1 or H1. C) Quantification of the *in vitro* assays shown in A and B. Paired t-test, p values for DUSP1: 0.1734 and H1: 0.3084.

Experiments involving the *in vitro* dephosphorylation were challenging. More than 16 repeats were carried out. Several rounds were necessary for the purification of H1 and H3 and new DUSP1 needed to be acquired. At some point, it was not possible to see effects with the DUSP1 control protein. Buffers were made fresh many times, and pH and DTT concentrations adjusted carefully for successive repeats. Despite all efforts, only two of the repeats in which all controls worked, showed a H1 phosphatase activity towards H3 S10, as shown in Figure 4.10.

As another approach, we decided to test whether transfecting H1 in the absence of the virus could replicate the effect we saw on H3S10P upon viral infection. For this purpose, A549 cells were transfected with plasmids expressing H1 WT as well as H1 C110S, a mutation in the residue located in H1's catalytic site which is vital for its phosphatase activity. Both of these

proteins contained a HA tag at their N-terminus that could be used for their detection in western blotting (Figure 4.11).



Plasmid		DNA (µg)
H1 WT		3
H1 C110S	UD	3.78
	1/2	1.89
	1/4	0.94
	1/8	0.47

Figure 4.11. A549 cell transfection with H1 WT and H1 C110S. Transfected H1 WT and H1 C110S were detected with a HA antibody. A) Transfection optimization. Cells were transfected with H1 WT and also with varying amounts of H1 C110S. Amounts shown in the table. B) Transfection of A549 cells with our H1 WT, using 3µg, and H1 C110S, using 0.94µg. quantification of the amount of H3S10 phosphorylation is shown in the graph. Paired t-test, p values for WT: 0.0044 and C110S: 0.4652.

Transfection of A549 cells showed that using the same amount of H1 WT and H1 C110S gave rise to different amounts of protein expression as detected by the western blot directed against the HA tag on these proteins. H1 C110S was expressed at much higher levels than H1 WT. As previously seen in our lab, overexpression of WT H1 was deleterious to cells. For this reason, an optimization experiment was carried out. Using this, we determined that transfection of 1/4 or 1/8 of the amount of H1 C110S plasmid yielded comparable protein amounts to the wild type H1 plasmid. As such, assays were carried out using 3µg of H1 WT and 0.94µg H1 C110S for transfection. Figure 4.11 panel B shows that H1 WT was clearly able to

induce the dephosphorylation of H3 S10P while H1 C110S was not. Quantifications show that transfection with H1 WT leads to a decrease in H3 S10P by more than 50% of that found in untransfected cells, while H1 C110S makes no significant difference to the phosphorylation status of H3 S10.

Section 4.07 Conclusion

Collectively the results demonstrate that vaccinia infection is affecting the phosphorylation status of cellular histones. Despite an overall trend towards decreased phosphorylation, H2A S1 was maintained more or less stable throughout the time course. H3 S28P decreased early on during infection and remined low throughout the time course investigated. H3 T3 and H3 S10 phosphorylation showed similar kinetics, disappearing at 4 hours after infection (Figure 4.12).

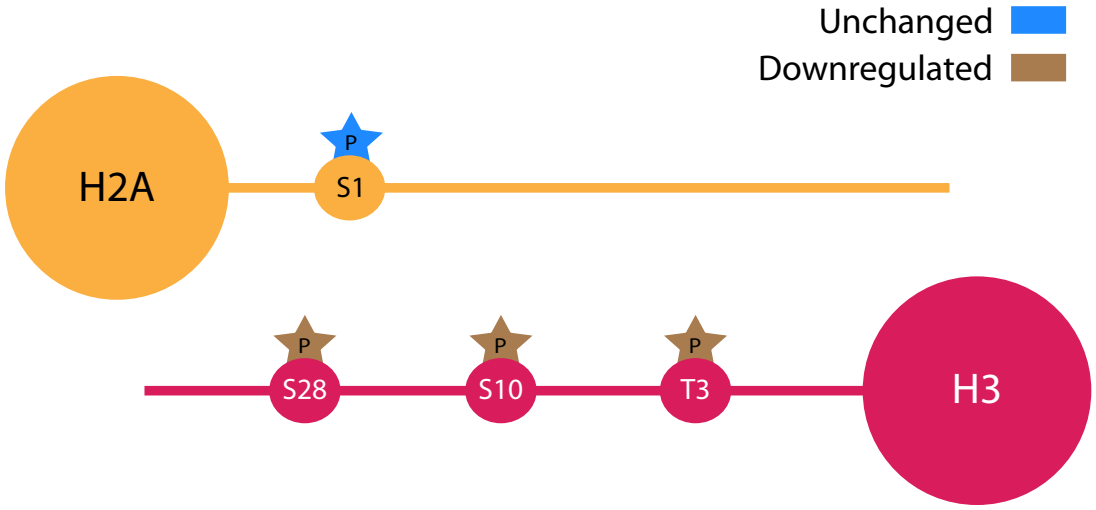


Figure 4.12. Histone phosphorylation as modulated by Vaccinia virus. Phosphorylation at H2A S1 stays unchanged, while H3 S28, H3 T3 and H3 S10 decrease upon Vaccinia infection.

Additionally, we have identified Vaccinia H1 phosphatase as the viral factor responsible for the dephosphorylation of histone 3 at serine 10.

Infection with H1 deficient viruses as well as transfection of H1 in the absence of infection showed clear links between the phospho-status of H3 S10 and the presence of this viral phosphatase. Although preliminary, *in vitro* dephosphorylation assays, combined with the previous H1 interaction data by Novy *et al.* suggest that H3 S10 is a direct substrate of H1. The consequences of this will be explored in the following chapters.

Chapter V. Vaccinia affects the cellular immune response through H3S10 modification

Although I found that H3S10 phosphorylation is drastically affected by Vaccinia infection, the impact of this modification on viral infection remained unknown. H3S10 phosphorylation has been implicated in cell cycle progression and it is thought to have a role in chromosome condensation (Johansen et al. 2006)(Houben et al. 2007)(Banerjee and Chakravarti 2011). It has also been shown to play a role in controlling gene expression, by generating a permissive chromatin state which allows for transcription factor binding. H3S10 phosphorylation can be stimulated by multiple pathways. TPA stimulation, for instance, leads to MSK1 and MSK2 dependent H3S10 phosphorylation and subsequent c-fos gene expression (Ana Soloaga and C.Arthur2 2003). EGFR stimulation in turn, leads to IKKα activation and MSK1 and subsequent H3S10 phosphorylation (Duncan, Anest et al. 2006). Finally, TNFα induces an IKKα dependent H3S10 phosphorylation at NFκB responsive genes (Yumi Yamamoto and Gaynor 2003).

Additionally, dephosphorylation of this mark has been reported by infection of some other pathogens (Figure 5.1): Streptococcus pneumoniae, Clostridium perfringens, Shigella flexneri and Listeria monocytogenes. They interfere with H3S10 as a way of dampening the cellular immune response. For example, by preventing H3S10P, Shigella flexneri is able to repress the expression of the CXCL20 and IL8 genes, both NFκB responsive genes. Listeria monocytogenes directly dephosphorylates this site, to prevent the expression of other NFκB responsive genes, namely CXCL2 and DUSP4 (Arbibe, Kim et al. 2007) (Hamon, Batsche et al. 2007).

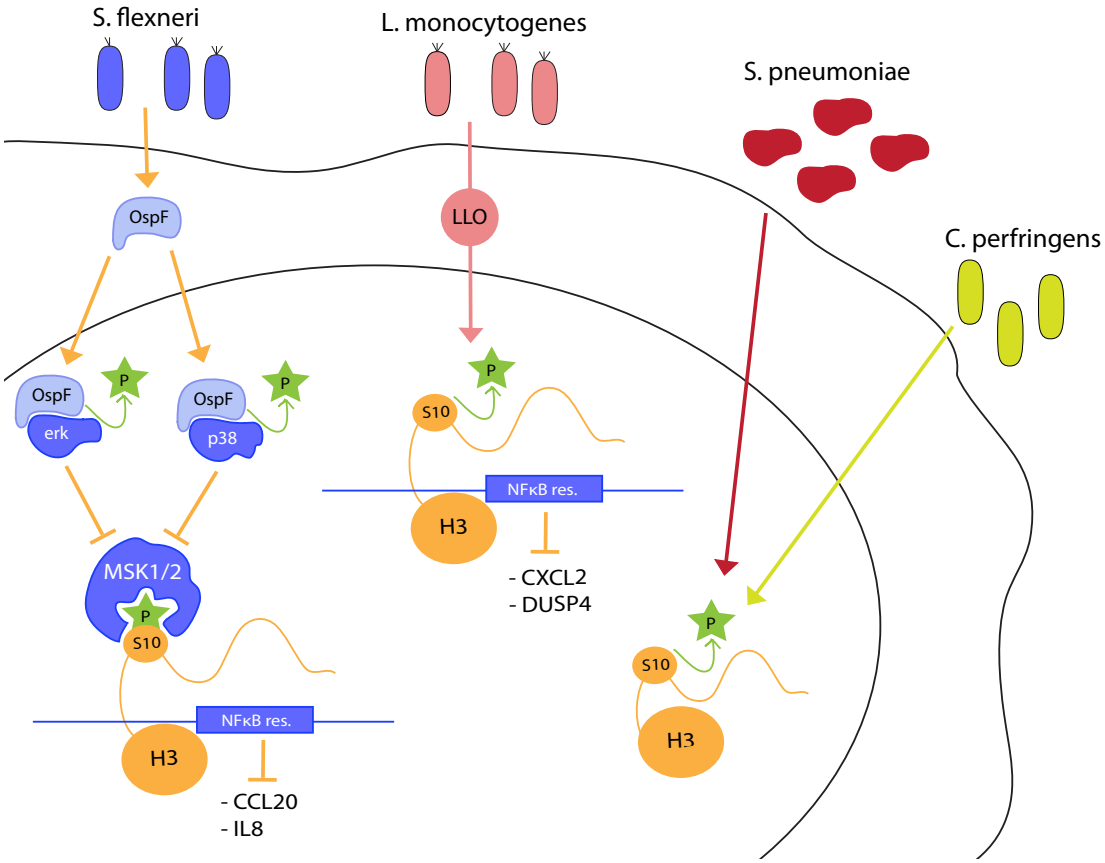


Figure 5.1. Bacteria modulate H3S10P in order to dampen the cellular immune response. S. flexneri ejects its OspF protein into the cell cytoplasm, which then travels to the nucleus where it dephosphorylates erk and p38, which in turn prevent H3S10 phosphorylation from taking place and affect immune gene transcription. L. monocytogenes injects its LLO toxin, that by mechanisms still ununderstood leads to H3S10 dephosphorylation and downregulation of immune related genes. S. pneumoniae and C. perfringens also have toxins that interfere with H3S10 phosphorylation. Adapted from Gabriec and Potempa, 2018.

Like these bacterial pathogens, we hypothesised that Vaccinia may also be interfering with H3S10 phosphorylation to block the cellular immune response, to benefit viral infection.

All work presented in this chapter was performed by me with the exception of high throughput imaging and analysis done with the help of Dr. Janos Kriston-Vizi and Dr. Artur Yakimovich, RTqPCR with the help of Dr.

Moona Huttunen or Dr. Susanna Bidgood and the construction of mutant viruses vv811ΔA49 and vv811ΔA49H1+/- by Corina Beerli.

Section 5.01 H3S10 de-phosphorylation benefits Vaccinia infection

Considering the dramatic effect Vaccinia infection has on H3S10 phosphorylation, we hypothesised it may be important for successful infection. In order to test this, I set out to alter the phosphorylation status of H3S10 by inhibiting the cellular enzymes that control its phosphorylation and dephosphorylation and ask how this impacts Vaccinia infection. For this I used an inhibitor (BCI) of DUSP1, the cellular phosphatase that dephosphorylates H3S10, and an inhibitor (SB) of MSK1, one of the kinases that phosphorylates H3S10. To assure the inhibitors could be used to modulate H3S10 phosphorylation, A549 cells were pre-treated with BCI or SB, and left uninfected or were infected with WT Vaccinia for 4 hours or 6 hours. Cells were harvested and western blots were performed for H3S10 and total H3 (Figure 5.2). As expected inhibition of DUSP1 using BCI led to an increase in cellular H3S10P levels, while inhibition of MSK1 led to little impact on H3S10 levels of phosphorylation. Importantly, the patterns of H3S10 phosphorylation seen in the presence of these inhibitors was not altered by Vaccinia infection allowing me to test the impact of altered H3S10 phosphorylation on Vaccinia infection.

A549 cells pretreated with BCI or SB were infected using Vaccinia virus recombinants that express EGFP under either an early or a later viral promoter. 4-6 hours after infection cells were harvested and the number of

infected cells was quantified by flow cytometry. The increased H3S10 phosphorylation levels seen in the presence of BCI, the DUSP1 inhibitor, correlated with a decrease in the proportion of cells expressing early and late GFP. Conversely, the unchanged levels present in cells treated with MSK1 inhibitor correlated with a higher number of cells expressing early and late GFP (Figure 5.2). It is important to mention that the effects on late gene expression with MSK1 was merely a trend and not as striking as what was seen with early gene expression.

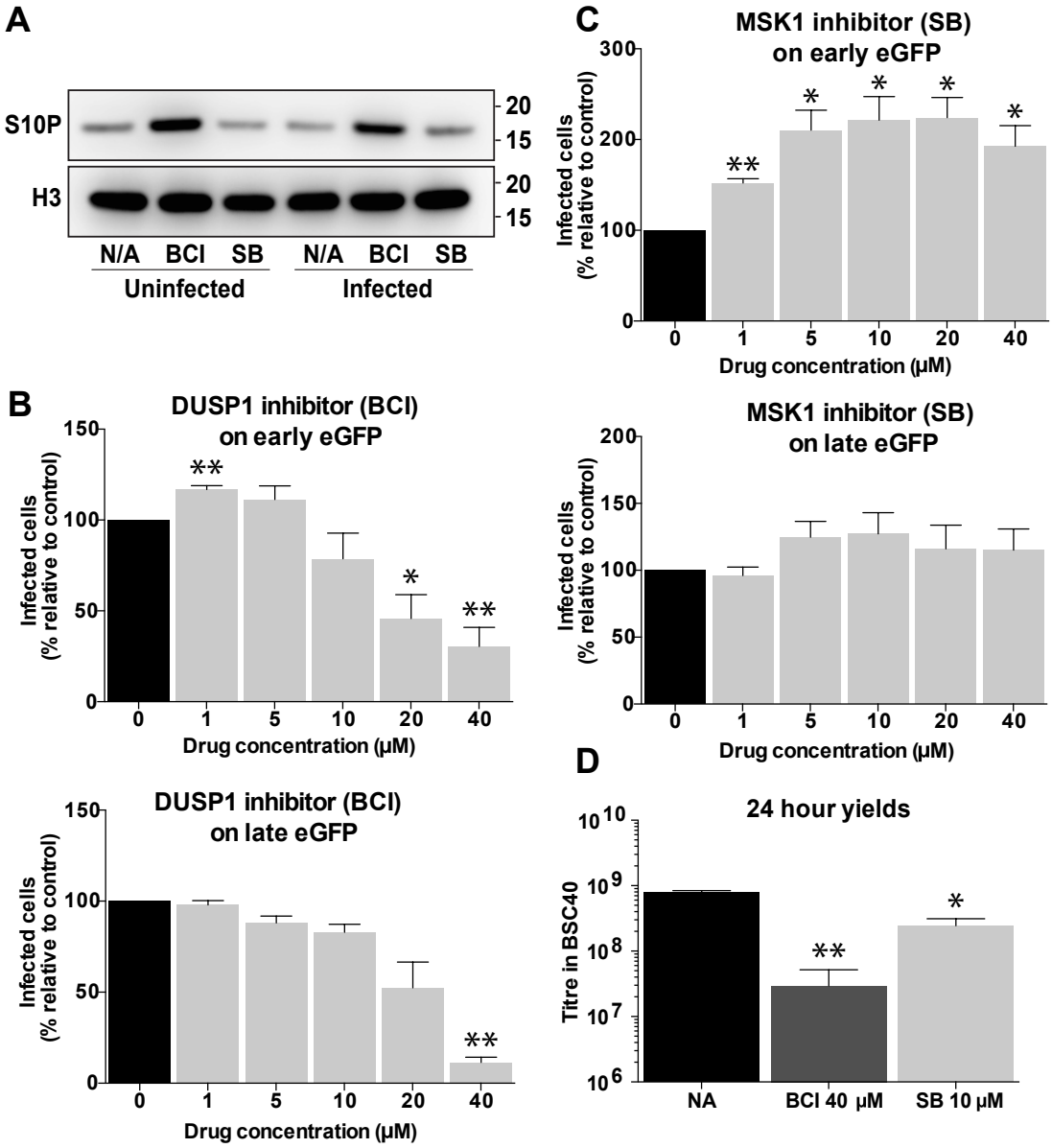


Figure 5.2. DUSP1 and MSK1 drug inhibitors have an effect on Vaccinia infection. A) Western blot of A549 cells treated with BCI, SB or nothing (N/A) for 4 hours. B,C) Percent of infected cells upon BCI and SB treatment and as a readout of early or late GFP. Early GFP experiments were measured 4 hours post infection while late GFP was measured 6 hours after infection. D) 24 hours yield of cells treated with BCI, SB or nothing (N/A) and infected with WT Vaccinia. Paired t-test, p values for BCI on early GFP: N/A-1μM = 0.0041, N/A-5μM = 0.2336, N/A-10μM = 0.2289, N/A-20μM = 0.0257, N/A-40μM = 0.0068. Paired t-test, p values for BCI on late GFP: N/A-1μM = 0.4749, N/A-5μM = 0.0833, N/A-10μM = 0.0581, N/A-20μM = 0.0778, N/A-40μM = 0.0011. Paired t-test, p values for SB on early GFP: N/A-1μM = 0.0016, N/A-5μM = 0.0157, N/A-10μM = 0.0182, N/A-20μM = 0.0112, N/A-40μM = 0.0267. Paired t-test, p values for SB on late GFP: N/A-1μM = 0.6014, N/A-5μM = 0.1715, N/A-10μM = 0.2286, N/A-20μM = 0.4585, N/A-40μM = 0.4410. Paired t-test, p values for 24 hours yield: N/A-BCI 40μM = 0.0034, N/A-SB 10μM = 0.0367.

Next, I asked if these altered levels of H3S10 phosphorylation had any impact on the amount of infectious viral particles produced. For this, cells were treated with the inhibitors at the concentration that gave the strongest phenotype in flow cytometry (40μM for BCI and 10μM for SB). 24 hours after infection cells were harvested and assessed for productive virus yield by plaque assay. In agreement with the flow cytometry results I saw a nearly 2-log decrease in virus yield in the presence of BCI. Collectively, this data shows that when H3S10 remains phosphorylated during the course of Vaccinia infection, both the number of infected cells that express early and late genes as well as the amount of infectious virus produced is dramatically reduced. On the other hand, when H3S10 phosphorylation remains low, the early stages of Vaccinia infection appear more robust, an effect that tapers off later during infection and does not translate to increased virus yields. Together, these results suggest that dephosphorylation of H3S10 by Vaccinia is essential for successful infection and virus production.

Section 5.02 Vaccinia mutants allow p65 nuclear translocation during infection

Since H3S10 dephosphorylation appears to be important for Vaccinia infection, we wanted to investigate further whether this had something to do with dampening cellular immune responses through NFκB, as it has been reported to be the case for some other pathogens. Vaccinia is known to have 9 viral proteins targeting this pathway, however, it was reported that there is at least one more encoded by the virus. Smith et al. carried out

experiments in which they knocked out all 9 known NFκB inhibitors in Vaccinia virus, but NFκB dependent gene expression was still blocked by Vaccinia infection. As infection with this virus, vv811ΔA49, resulted in p65 translocation into the nucleus, they concluded that the missing NFκB inhibitor must be acting there (Smith, Benfield et al. 2013, Sumner, Maluquer de Motes et al. 2014).

To better understand whether the H1 phosphatase could be involved in repressing the immune response of the cell through H3S10 dephosphorylation, we first addressed whether there was any p65 nuclear translocation upon infection with our WT and H1- Western Reserve (WR) viruses. For this, I infected A549 cells with either virus at MOI 5, followed by stimulation of the cells with TNFα or IL1β. After 6.30 hours cells were fixed, stained for p65 imaged and analysed for p65 nuclear translocation.

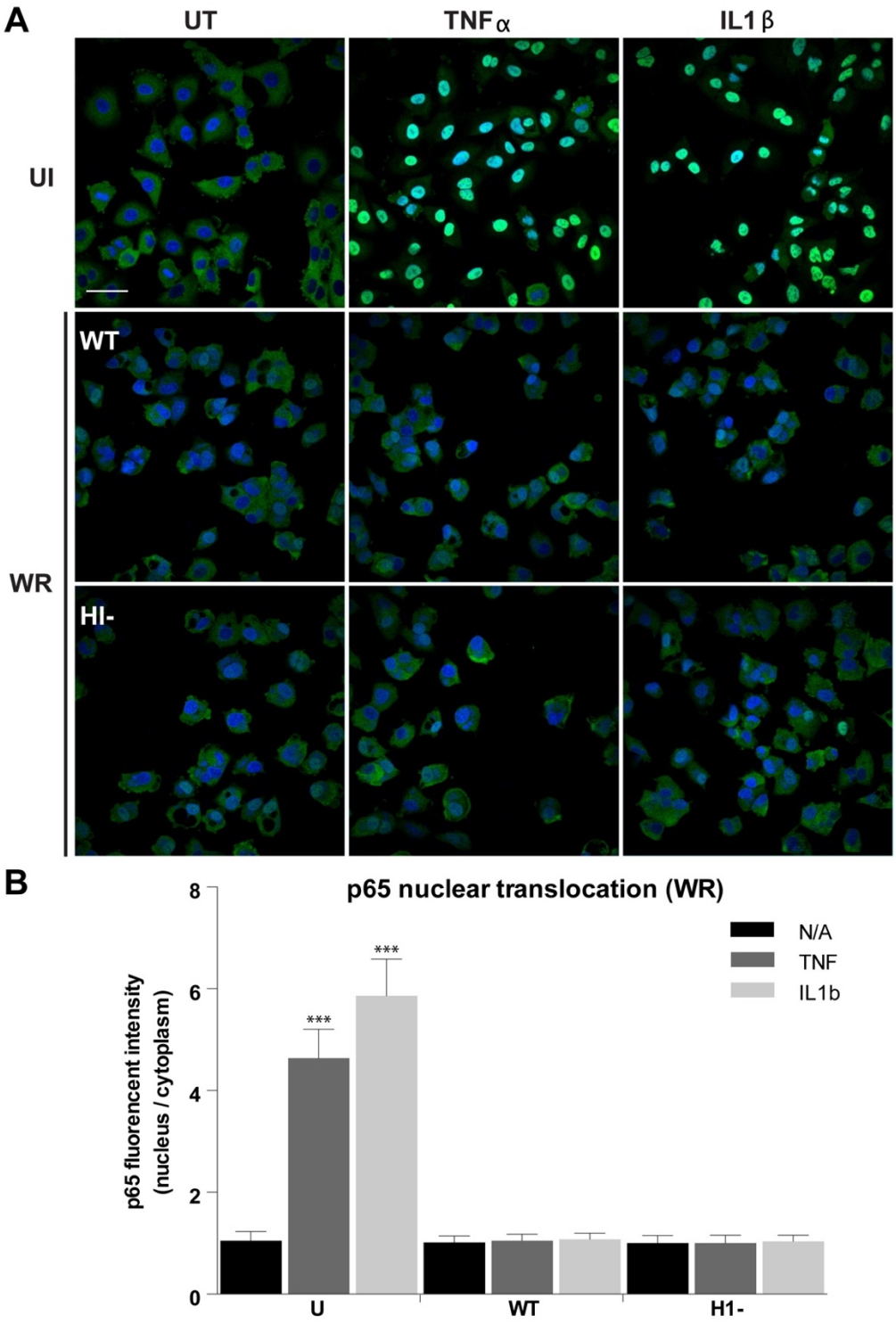


Figure 5.3. p65 staining in cells infected with WT and H1-. A) Images showing p65 localization inside the cell upon TNFα or IL1β stimulation in uninfected, WT or H1- infected cells. B) Quantification of the p65 fluorescence intensity in both nucleus and cytoplasm. It shows the ratio of the nuclear intensity over the cytoplasmic one in uninfected cells, WT and H1- infected cells. Paired t-test, p values for U-N/A vs U-TNF: 0.0003, U-N/A vs U-IL1b: 0.0003, U-N/A vs WT-N/A: 0.5853, U-N/A vs WT-TNF: 0.9735, U-N/A vs WT-IL1b: 0.6944, U-N/A vs H1-: 0.2451, U-N/A vs H1-TNF: 0.3809 and U-N/A vs H1- IL1b: 0.7698.

As expected, stimulation of uninfected cells with TNFα or IL1β led to p65 nuclear translocation. Upon Vaccinia infection with WT (WR) and H1- (WR), we observed that p65 remained in the cell cytoplasm, as expected given that the full repertoire of known NFκB inhibitors remained intact in these viruses. Next, we decided to repeat these experiments using the vv811ΔA49 mutant which allows for p65 nuclear translocation. Again, A549 cells were infected at MOI 5, stimulated with TNFα or IL1β, and analysed for p65 nuclear translocation. For these experiments, WT (Copenhagen strain) and vv811, both of which still block p65 nuclear translocation, were used as controls.

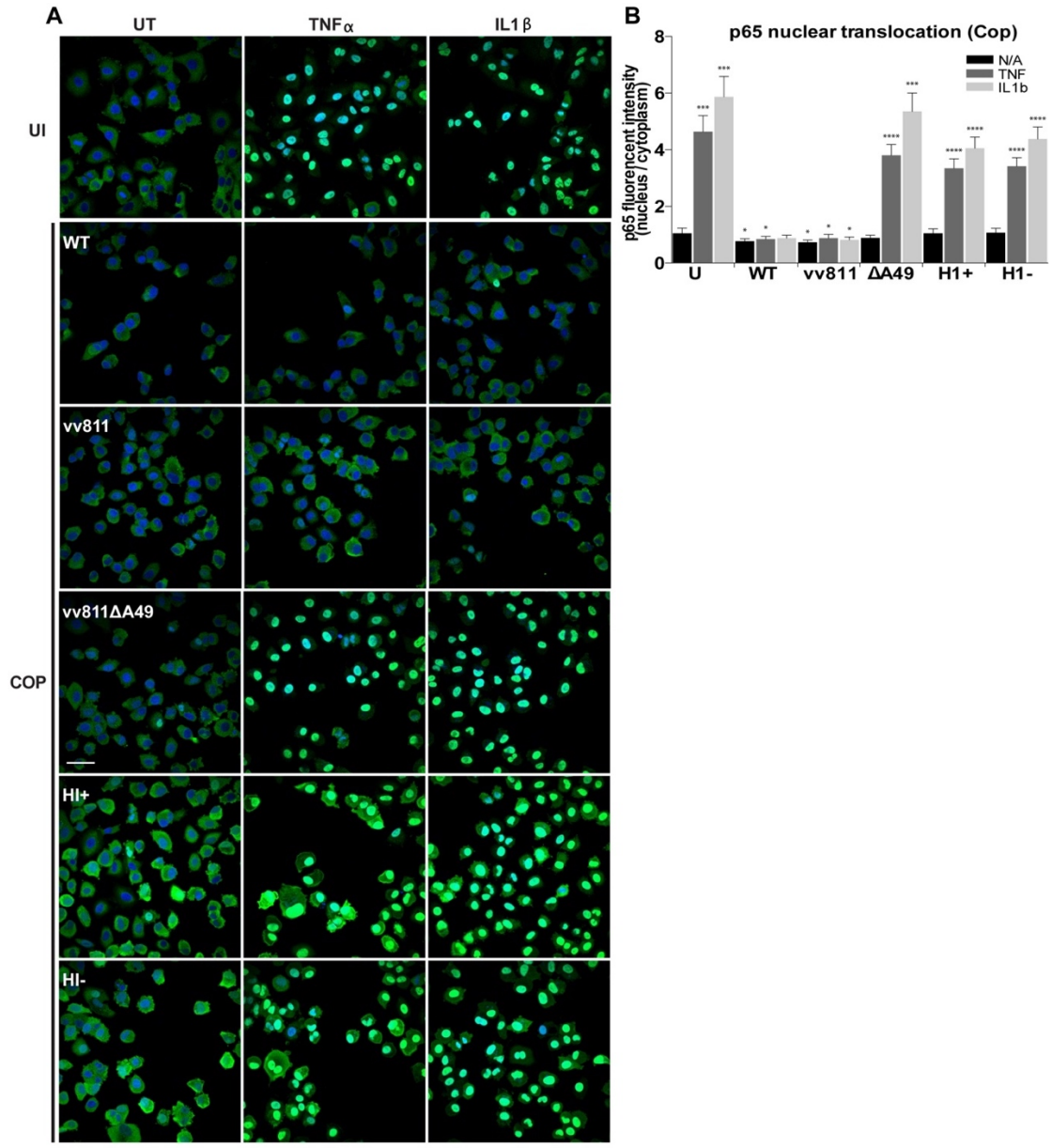


Figure 5.4. p65 staining in cells infected with WT, vv811, vv811ΔA49 and vv811ΔA49H1+/-. A) Images showing p65 localization inside the cell upon TNFα or IL1β stimulation in uninfected, WT, vv811, vv811ΔA49, vv811ΔA49H1+ and vv811ΔA49H1- infected cells. B) Quantification of the p65 fluorescence intensity in both nucleus and cytoplasm. It shows the ratio of the nuclear intensity over the cytoplasmic one in uninfected cells, WT, vv811, vv811ΔA49, vv811ΔA49H1+ and vv811ΔA49H1- infected cells. Paired t-test, p values for U-N/A vs U-TNF: 0.0003, U-N/A vs U-IL1b: 0.0003, U-N/A vs WT-N/A: 0.0396, U-N/A vs WT-TNF: 0.0455, U-N/A vs WT-IL1b: 0.0772, U-N/A vs vv811-N/A: 0.0267, U-N/A vs vv811-TNF: 0.0323, U-N/A vs vv811-IL1b: 0.0294, U-N/A vs ΔA49-N/A: 0.0854, U-N/A vs ΔA49-TNF: <0.0001, U-N/A vs ΔA49-IL1b: 0.0003, U-N/A vs H1+: 0.9652, U-N/A vs H1+TNF: <0.0001, U-N/A vs H1+IL1b: <0.0001, U-N/A vs H1-: 0.7154, U-N/A vs H1-TNF: <0.0001 and U-N/A vs H1-IL1b: <0.0001.

As reported, we only observed p65 nuclear translocation upon cytokine stimulation in uninfected cells and in cells infected with vv811ΔA49. Both WT Copenhagen and VV811 were still capable of blocking p65 from entering the nucleus.

In order to assess the potential role of the H1 phosphatase in shunting the host cell NFκB response downstream of p65 nuclear translocation we generated an IPTG inducible H1 virus in the vv811ΔA49 background. This virus vv811ΔA49indH1, produces H1 in the presence of IPTG (vv811ΔA49H1+) and in the absence of IPTG (vv811ΔA49H1-) H1 is not expressed.

When cells were infected with vv811ΔA49indH1 in the presence or absence of IPTG, and cells stimulated with TNFα or IL1β, p65 was translocated to the nucleus. This indicated that H1 plays no cytoplasmic role in shunting the cellular NFκB response to Vaccinia infection.

Section 5.03 H3S10 de-phosphorylation might repress NFκB gene expression

In order to understand whether the absence of H1, and thus an absence of H3S10 dephosphorylation would result in an increase in cytokine gene expression upon Vaccinia infection, we carried out a set of RTqPCR experiments in which we looked at CCL5, CCL2 and ICAM1. A549 cells were infected with WR WT and H1- viruses at an MOI of 0.5 for 6 hours followed by stimulation with either IL1β or TNFα for 4 hours. Cells were then harvested and RNA from each sample prepared for RTqPCR to assess CCL5, CCL2, ICAM1 cytokine gene expression at the mRNA level, using GAPDH to

normalize expression levels (Figure 5.5). RTqPCR was included for Vaccinia DNA pol to control for the transcription defect of H1 (-) virions.

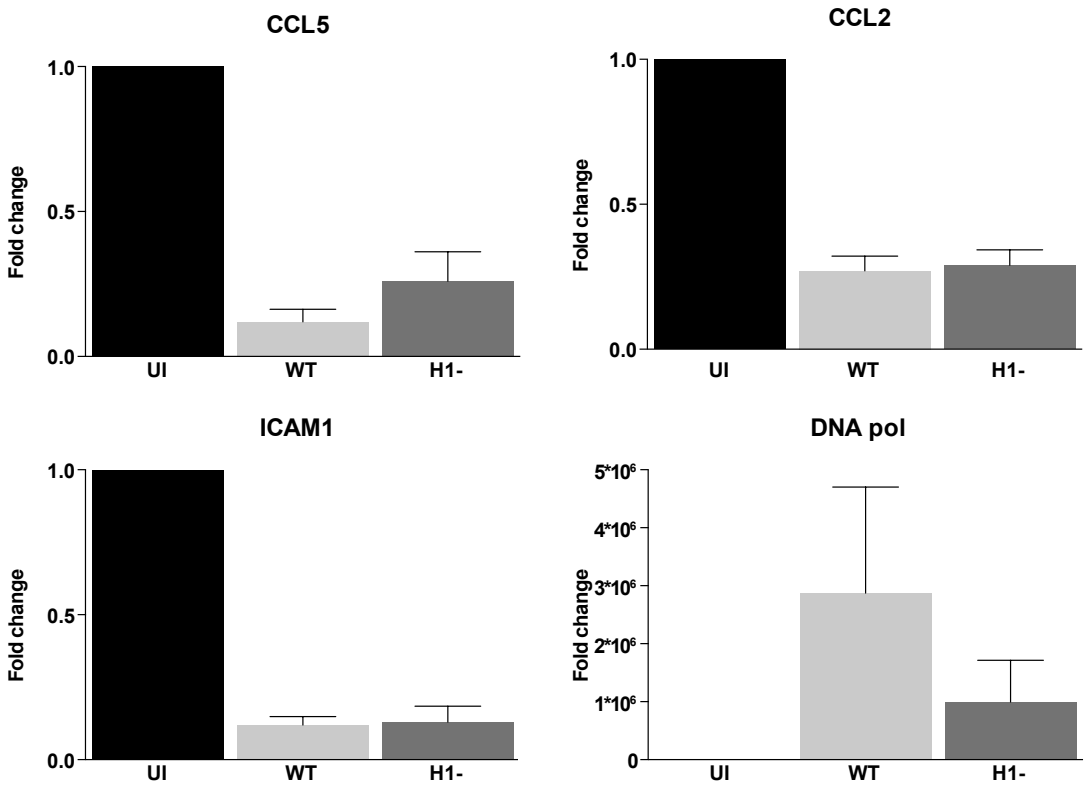


Figure 5.5. Cytokine gene expression upon stimulation with IL1β in WT and H1- infected cells. CCL5, CCL2, ICAM1 and viral DNA polymerase level of expression, as expressed by a fold induction from stimulated uninfected cells. Paired t-test, p values for WT vs H1-: CCL5 = 0.1191, CCL2 = 0.7699, ICAM1 = 0.8336, DAN pol = 0.1922.

Upon IL1β stimulation, we found that expression of CCL5, CCL2 and ICAM1 were all upregulated in uninfected samples. Infection with either WT WR or H1(-) viruses were capable of shunting IL1β-mediated upregulation of these cytokines.

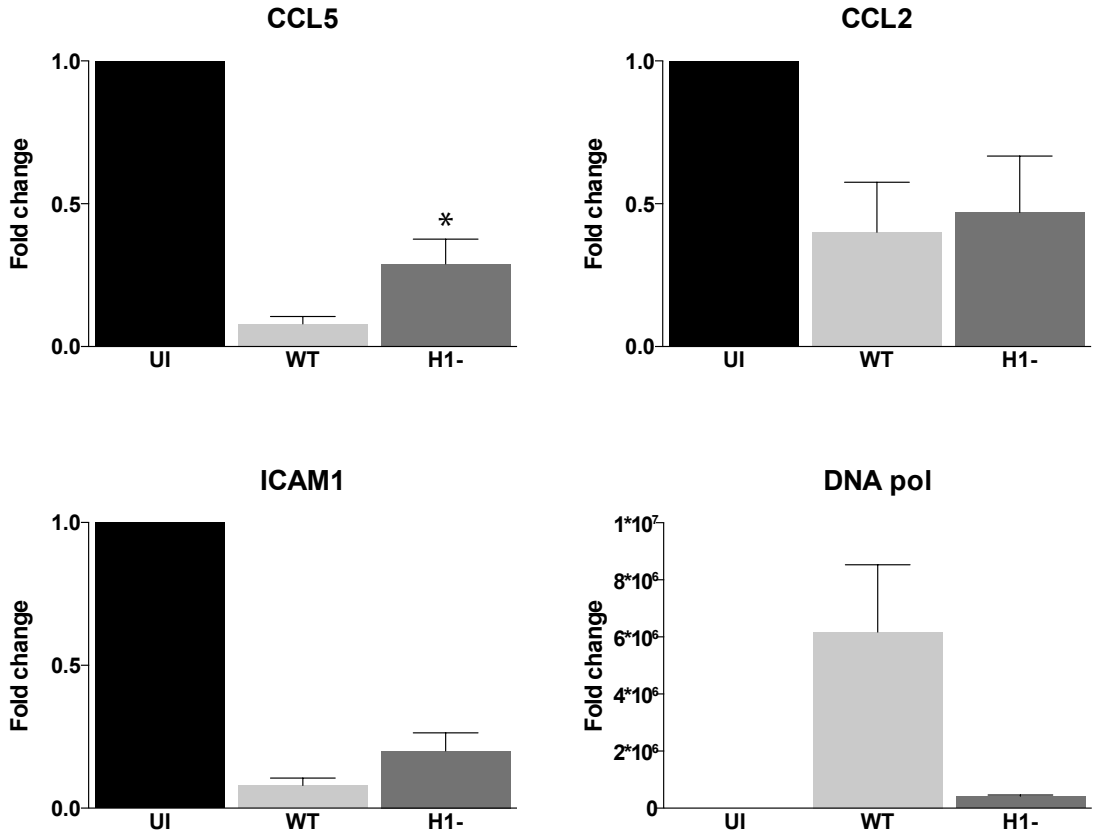


Figure 5.6. Cytokine gene expression upon stimulation with TNFα in WT and H1- infected cells. CCL5, CCL2, ICAM1 and viral DNA polymerase level of expression, as expressed by a fold induction from stimulated uninfected cells. Paired t-test, p values for WT vs H1-: CCL5 = 0.0436, CCL2 = 0.6057, ICAM1 = 0.0684, DNA pol = 0.2427.

When the same set of experiments was carried out with TNFα we found that in each case, there was a trend: when infecting with H1- the expression levels of CCL5, CCL2 and ICAM1 were higher, although not statistically significant (Figure 5.6). As a reminder, the WR strain contains the full known set of 9 NFκB inhibitors, so even this minor boost in TNFα-mediated cytokine expression suggested to me that H1 may be partially responsible for the expression of these cytokines during Vaccinia infection.

Now that it was clear that when infecting cells with vv811ΔA49indH1(+ or -) virus, p65 was translocated into the nucleus, and that deletion of H1 from WR had a small but consistent impact on TNFα-mediated cytokine gene

expression, I turned my attention back to vv811ΔA49H1- and focused only on TNFα-activated cytokines that can be regulated by H3S10 phosphorylation, namely CCL5 and ICAM1 (Mich&le Fattal-German 1998)(Blaber 2003) (Ahn, Huang et al. 2007) (Seidel, Roth et al. 2011).

For these experiments vv811ΔA49H1- was produced in the absence of IPTG to assure a reduced amount of H1 was packaged into virions. Cells were infected with WT Copenhagen, vv811, vv811ΔA49, vv811ΔA49H1- at MOI 5 and stimulated with TNFα for 4 hours, 6 hours after infection. Cells were harvested and RNA prepared for RTqPCR.

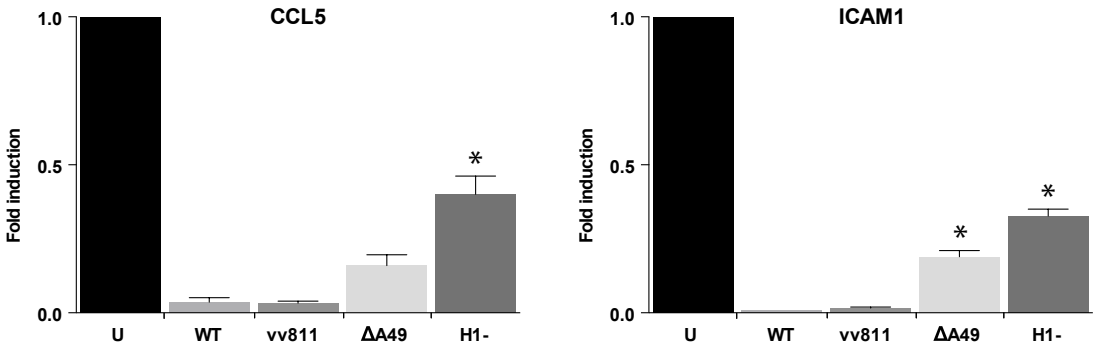


Figure 5.7. Cytokine gene expression upon stimulation with TNFα in WT, vv811, vv811ΔA49, vv811ΔA49H1- made in the absence of IPTG. CCL5 and ICAM1 level of expression, as expressed by a fold induction from stimulated uninfected cells. Paired t-test, p values for CCL5: vv811 vs WT = 0.7418, ΔA49 vs vv811 = 0.0576, H1- vs ΔA49 = 0.0119 and for ICAM1: vv811 vs WT = 0.1835, ΔA49 vs vv811 = 0.0134 and H1- vs ΔA49 = 0.0279.

As seen in preceding experiments, stimulation of A549 cells led to increased levels of CCL5 and ICAM1 gene expression (Figure 5.7). As expected WT Copenhagen and vv811 both effectively block cytokine induction, while vv811ΔA49 is reduced in its ability to repress CCL5 and ICAM1 gene expression. Further removal of H1 from the Vaccinia genome

resulted in a further reduction in VACVs ability to repress TNFα-mediated CCL5 and ICAM1 gene expression.

To sum up, we have shown that WT Vaccinia blocks p65 nuclear translocation and shuts down both IL1β and TNFα mediated cytokine production. Consistent with previous reports, I show that when all known NFκB inhibitors are removed from the virus, Vaccinia can no longer shunt p65 nuclear translocation, while its ability to suppress cytokine production remains largely intact. Further removal of the H1 phosphatase showed a further reduction in the viruses ability to block production of CCL5 and ICAM1, two cytokines whose expression is influenced by H3S10 phosphorylation. Taken together with my data in chapter IV showing that the H1 phosphatase can enter the host cell nucleus and is responsible for H3S10 dephosphorylation; these results indicate that the Vaccinia H1 phosphatase acts as the 10^{th} NFκB repressor encoded by vaccinia and the first that acts at the level of histone modification.

Chapter VI. Discussion

By performing a broad screen of histone modifications during Vaccinia infection I have identified virus-mediated changes in histone acetylation, methylation and phosphorylation. I found that there was not simply a global de-regulation of the histone code, but rather subsets or patterns of regulation, with some being increased upon infection and others decreased (Figure 6.1). While it is clear these changes in the histone code are occurring upon infection, there is nothing known about them, whether they are driven via a cell response to infection or by the virus, in which gene promoters they occur, or how they may be influencing gene expression. Strikingly, I identified major changes in Histone 3. By 4 hours post infection the phosphorylation levels of three residues: T3, S10 and S28, were all found to undergo dramatic de-phosphorylation and to remain that way until at least 48 hours post infection, for T3 and S10. Focusing on H3 S10, I identified one viral protein involved in this: the Vaccinia phosphatase H1. Confirming previous data from our lab which indicated that H1 interacts with Histone 3 (Novy et al. unpublished), I could show that the H1 phosphatase can enter the cell nucleus. Using a virus inducible for H1 expression, I showed that in the absence of this phosphatase, the virus can no longer dephosphorylate H3 S10. In addition, I could show that transient expression of H1 in uninfected cells leads to a reduction in the H3 S10 phosphorylation while catalytically inactive H1 did not. Although preliminary, *in vitro* dephosphorylation assays suggest that H1 is acting on histone 3 directly. Furthermore, by modifying this histone mark, we could show that if H3 S10

remains phosphorylated during infection, this appears to be deleterious to virus gene expression and productive virus yield. Finally, I was able to link H1-mediated H3 S10 dephosphorylation to Vaccinia's ability to block cytokine gene expression using several mutant viruses lacking the Vaccinia-encoded NFκB inhibitor.

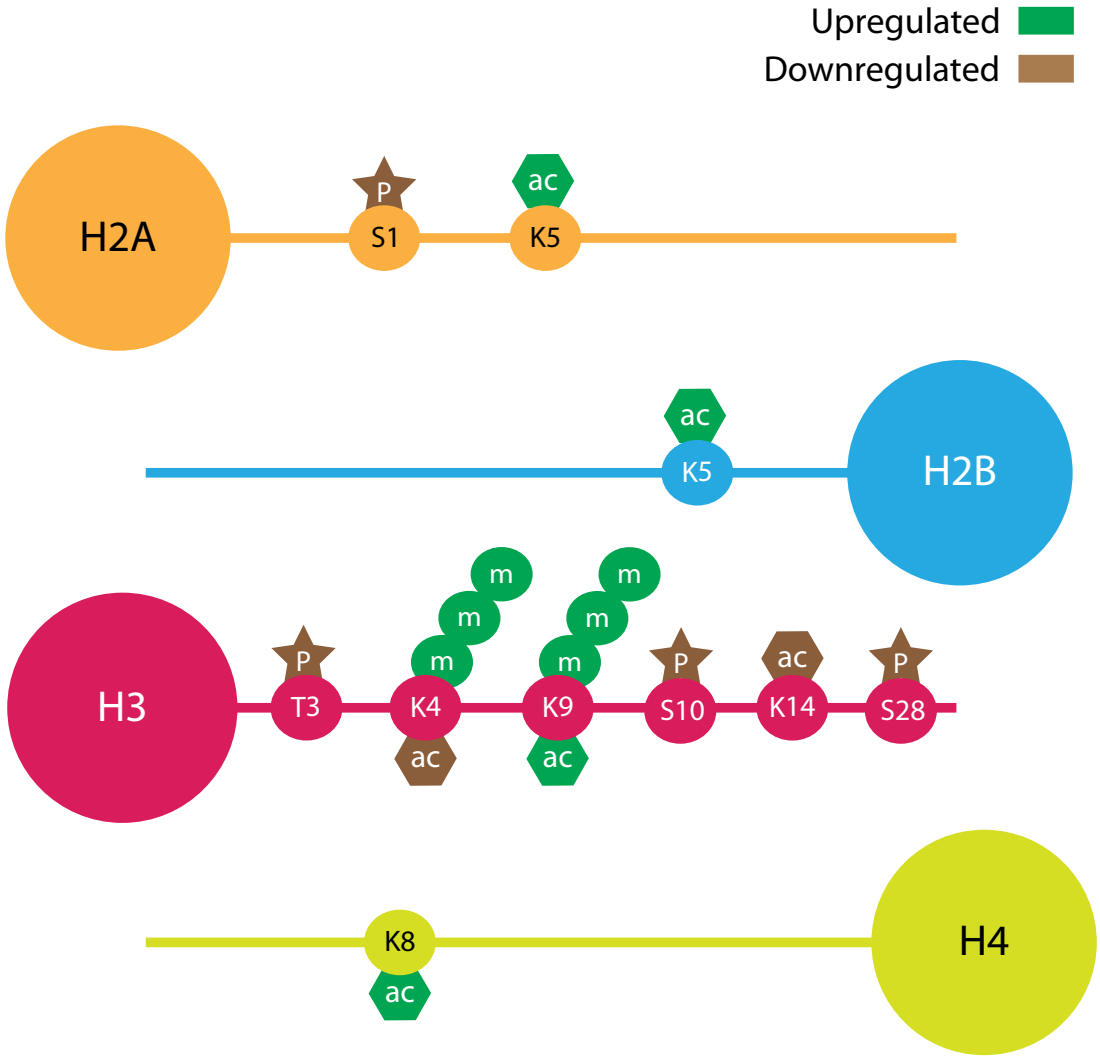


Figure 6.1. Vaccinia infection leads to changes in histone modifications. A diagrammatic representation of acetylation, methylation and phosphorylation changes that occur upon Vaccinia infection.

To date, Smith et al. had shown that despite Vaccinia having 9 viral proteins dedicated to suppressing the cellular immune response through the NFκB pathway, there is at least, a 10th one and this one must be acting early

during infection and based on p65 nuclear translocation, doing so from inside the cell nucleus (Smith, Benfield et al. 2013). The H1 phosphatase has been previously linked to dephosphorylation of cytoplasmic STAT1 to block its INF-γ-mediated nuclear translocation and antiviral response (Najarro, Traktman et al. 2001, Schmidt, Bleck et al. 2013). Here I show that H1 can enter the host cell nucleus to shut down TNFα mediated antiviral responses through modification of Histone 3 (Figure 6.2). The fact that H1 acts in the cytoplasm dephosphorylating Stat1 and in the nucleus dephosphorylating H3 S10 explains the presence of a bipartite nucleo-cytoplasmic signalling sequence within this protein.

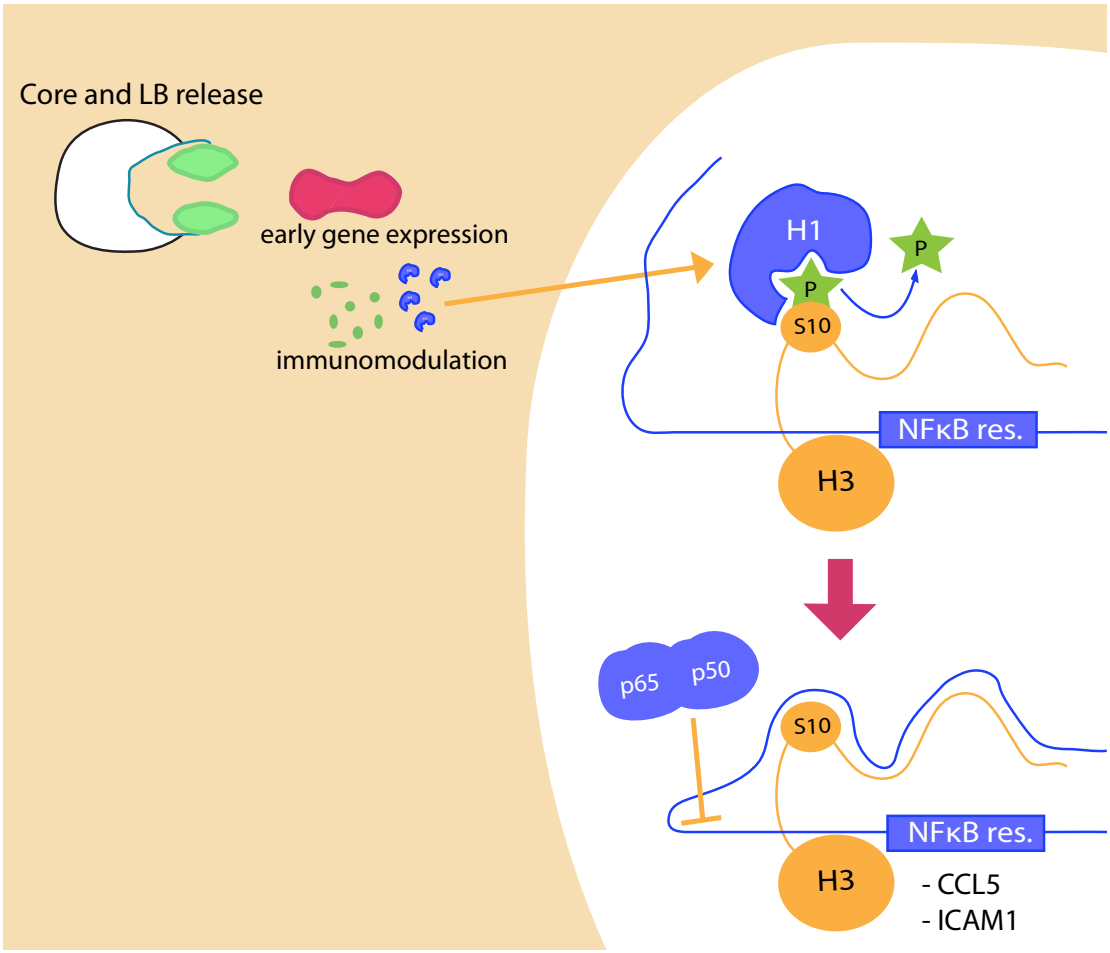


Figure 6.2. Vaccinia H1 affects H3 S10P. The viral phosphatase that comes within the lateral bodies of the virion, enters the cell nucleus early during infection and dephosphorylates H3 S10, repressing the expression of CCL5 and ICAM1, NFκB responsive cytokines stimulated with TNFα.

I have therefore identified H1 as the 10th viral protein involved in repressing the NFκB pathway upon Vaccinia infection. Interestingly, as the cytokine gene expression experiments showed, there must be at least one more virus encoded repressor of this pathway, as knocking down all the currently known Vaccinia proteins involved in NFκB repression, including H1, did not lead to complete recovery of the cytokine expression levels seen in uninfected cells. Having now constructed a virus which lacks all 10 of these genes, it would be of future interest to use this as a tool to identify the remining inhibitors using a viral gene depletion or knock-out screening strategy.

Section 6.01 Histone acetylation, methylation and phosphorylation upon Vaccinia infection

Histone marks have been extensively studied in the context of gene expression and more and more research is focussing on their impact during pathogen invasion. Bacteria, fungi, parasites and viruses have been shown to modulate or have an impact on histone modifications, as means of dampening cell immunity, promoting their replication inside the cell, switching from latent to lytic gene expression or simply because their entry to the cytoplasm is detected by the cell and it upregulates the cellular immune response. As presented in this work, Vaccinia infection also affects histone marks. I have observed an increase in the acetylation levels of H2A K5, H2B K5, H4 K8, H3 K4 and H3 K9. Despite not much is known about the role of acetylation in histones 2A and 2B, H2A K5 acetylation has been shown to be present mainly in gene bodies (Rajagopal, Ernst et al. 2014). It is clear

that H4 K8 and H3 K9 acetylation are both related to gene expression. While H3 K9ac is a mark for transcriptional start sites and plays a role in transcriptional elongation, H4 K8ac allows for the recruitment of chromatin modifying proteins such as SWI/SNF, that moves nucleosomes along chromatin. Additionally, the increase in H3 K4 acetylation could be related to gene expression in the sense that it serves to block methylation of the residue (Theodora Agalioti and Dimitris Thanos 2002). The fact that Vaccinia infection is increasing the levels of these marks could mean two things: either the virus is actively doing so in order to express genes that will be beneficial for infection or the cell is upregulating them to generate an immune response against the invading pathogen. In any case, the outcome of these modifications is clear: upregulation of a subset of cellular genes. Conversely, upon Vaccinia infection, there is one acetylation mark that is decreased: H3 K14. This mark has been shown to be present in both transcriptional start sites as well as gene bodies. Although there is not a strong correlation with gene expression, it is involved in the histone code together with other chromatin marks that will be discussed below (Rajagopal, Ernst et al. 2014). Interestingly, all bacteria studied, E. coli, A. phagocytophilum, P. aeruginosa, L. monocytogenes, M. tuberculosis and H. pylori, target histone acetylation with the aim of reducing it. On the other hand, viral infection leads to the upregulation of some acetylation marks as it is the case of HCMV with H3 K9ac and H3 K14ac in the chromatin region surrounding the viral MIEP for the expression of its own genes (Ho, Donovan-Banfield et al. 2016).

In regard to methylation, I only studied two sites: H3 K4 trimethylation, that is decreased upon infection, and H3 K9 trimethylation, that increases

upon infection. They both have been vastly discussed in the literature and their roles in gene expression are quite well understood. In the case of H3 K4me3, it serves as a docking site for the transcription factor TFIID, which in turn allows for the formation of the pre-initiation complex and helps PolII start transcription (Greer and Shi 2012) (Lauberth, Nakayama et al. 2013). Despite H3 K4me3 has been reported to recruit HATs that acetylate H3 K9ac, I observe opposing trends with regards of these two marks: an increase in H3 K9ac despite there is a decrease in H3 k4me3. This is could mean that while trimethylation at the residue may be either cellularly driven or virally driven, the acetylation at H3 K9 is most likely to be driven by Vaccinia, as cross-talk between H3 K4m3 and H3 K9ac is not possible in the absence of H3 K4me3 (Gates, Shi et al. 2017). Regarding the other methylation mark studied, H3 K9me3, I also observe an increase upon Vaccinia infection. Since I also observed an increase in H3 K9ac, these two marks must be occurring in different genes and therefore regulating the expression of selected cellular genes in opposite directions: the genes surrounded by H3 K9ac will be expressed while the genes nearby H3 K9me3 will be supressed. Histone methylation is a slightly more complex mark than acetylation, since depending on the number of methyl groups added and the residue in which it takes place, it has different outcomes. As a consequence, pathogens have learned to modulate it in several ways. L. pneumophila increases H3 K14me3 to prevent its acetylation and so repress immune related genes and Influenza represses cytokine induced genes by upregulating trimethylation of H3 K79, in addition to H3 K9 (Rolando, Sanulli et al. 2013). HCMV also uses methylation as a way to stay in the latent phase of infection through H3 K79me and H3 K9me2, which can be bound by HP1 proteins and create

a closed chromatin conformation (Marcos-Villar, Diaz-Colunga et al. 2018). Finally, Vaccinia has already been reported to modulate histone methylation. They showed that upon infection H4 K20me3 and H3 K9me3 increase, supporting the data presented here (Teferi, Desaulniers et al. 2017).

Finally, I also looked into histone phosphorylation. All of the marks studied, H2A S1, H3 T3, H3 S10 and H3 S28 all dropped upon infection, although to different levels. H3 T3 and H3 S28 phosphorylation have been deeply studied with regard to their link to cell cycle control (Flanagan, Mi et al. 2005) (Hidemasa Goto 2002). Additionally, H3 S28 phosphorylation has also been mentioned as an activator of gene expression in genes such as c-fos (Soloaga, A. et al. 2003).

As such, the decrease I see upon infection must be then linked to the repression of a large group of cellular genes. H2 S1P is always been linked to repression of gene expression (Barber, Turner et al. 2004). Since its phosphorylation levels decrease very early post infection, and then again at 8 hours, it may be that only a subset of genes under its control are upregulated by Vaccinia infection.

Lastly, the most important mark studied: H3 S10 phosphorylation. Despite having a role in cell cycle progression by allowing for chromatin condensation, increasing amounts of research have focussed on its role for gene expression, especially in cytokine transcription during bacterial infection. Different stimulation pathways lead to phosphorylation at H3 S10. In the case of c-fos gene expression, TPA stimulation goes through Ras-Erk signalling and MSK1 and MSK2 phosphorylation of the residue (Tu, Huang et al. 2013). On the contrary, EGF stimulation goes through IKKα, that in turn activates MSK1 and MSK2 and leads to H3 S10 phosphorylation

(Duncan, Anest et al. 2006). In the case of cytokine gene expression, TNFα stimulation leads to IKKα activation, nuclear translocation and formation of a complex with RelA and CREB, which drive H3 S10 phosphorylation (Yumi Yamamoto and Gaynor 2003). Upon Vaccinia infection, I observed a drastic decrease in H3 S10 phosphorylation. Additionally, the presence of this mark is followed by H3 K14ac, a mark that during Vaccinia infection is also reduced. Consistent with H3 K14ac's role in preventing H3 K9ac and H3 K9me3, its decrease during infection correlates with an increase in these marks. Together, they seem to be a major contributing part of the histone 3 code modification seen upon Vaccinia infection (Figure 6.3). It will be of future interest to determine the purpose of these other marks in H3 that occur upon Vaccinia infection.

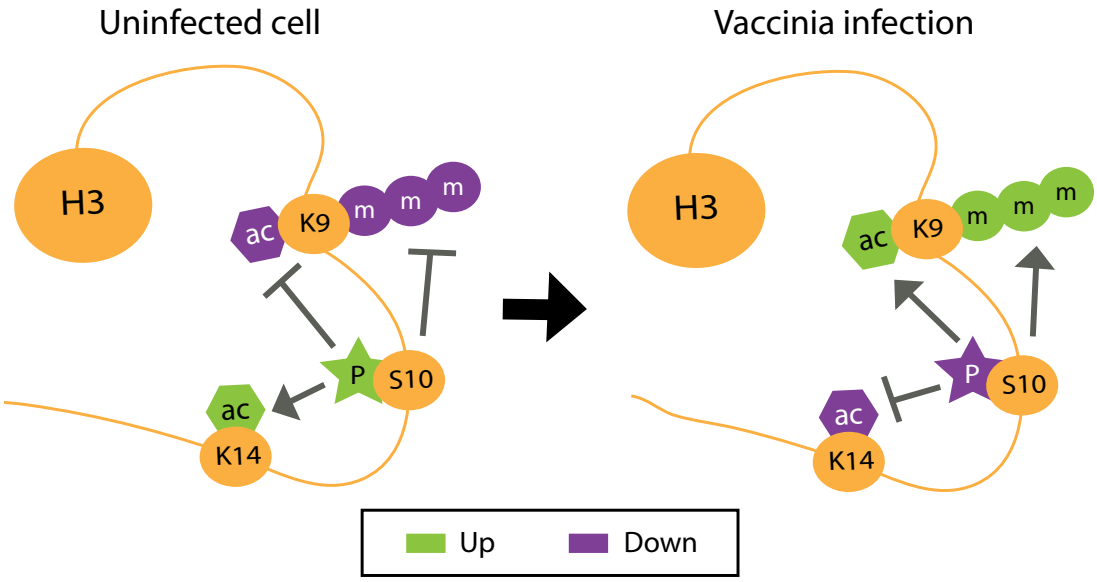


Figure 6.3. H3 histone code upon Vaccinia infection. Upon Vaccinia infection, H3 S10P decreases drastically, which leads to a reduction in H3 K14ac and to an increase in H3 K9ac and H3 K9me3.

Section 6.02 Dephosphorylation of H3S10 is driven by Vaccinia H1 and contributes to cytokine gene expression

Dephosphorylation of H3 S10 is heavily affected by Vaccinia infection. As a consequence, it was hypothesised that it must be important for infection progression. Experiments carried out using inhibitors against the cellular phosphatase (DUSP1) and kinase (MSK1), which control the phospho-status of H3 S10, show that dephosphorylation of H3 S10 is key to successful infection. If phosphorylation is present during infection, the proportion of cells that are infected and can carry out early viral gene expression decreases with increasing drug concentrations: 50% reduction in infection with BCI. Similar effects are observed with late gene expression. On the other hand, if MSK1 function is blocked during infection and H3 S10P is repressed, I observed an increase in the proportion of cells that get infected and are able to go through early viral gene expression: a two-fold increase in early gene expression with SB. In this case, late viral gene expression was also increased although it did not reach statistically significant levels. The number of infectious particles that Vaccinia can produce in the presence of these two drugs was also assessed, through 24-hour yields. In the case of DUSP1 inhibition, I observed a decrease in the number of infectious particles, as expected by the reduced proportion of cells that underwent early and late viral gene expression. However, regarding MSK1 inhibition, I also observed a minor decrease in the number of infectious particles, although the number was not as low as for DUSP1 inhibition. This correlates with the fact that upon late viral gene expression, we observed a decrease in the proportion of cells infected compared to early gene expression and could indicate that the presence of this drug is toxic for cells and is more

evident the longer they are incubated in it. Therefore, these experiments showed that Vaccinia's ability to infect cells is affected by the presence of H3 S10 with phosphorylation of the residue being detrimental to the virus and dephosphorylation contributing to Vaccinia infection, at the level of early viral gene expression.

The vaccinia lifecycle largely relies on dynamic phosphorylation driven by its 3 encoded dual-specificity enzymes the kinases B1 and F10 and the phosphatase H1 (Novy, Kilcher et al. 2018). Having defined that Vaccinia dephosphorylates H3 S10, that its phosphatase H1 has been detected as a histone 3 interactor by mass spectrometry, that H1 contains a bipartite nucleo-cytoplasmic localization signal and the protein can be detected in the cell nucleus, the next logical step was to test whether H1 was involved in the dephosphorylation of H3 S10 during infection. For this, viruses lacking the H1 protein were used for infection and the levels of H3 S10P were assessed. In the absence of H1 the levels of H3 S10 phosphorylation relative to those found in WT virus infected cells was clearly higher. This indicated that the virus had a defect in dephosphorylating this histone in the absence of H1. A minor decrease in phosphorylation relative to uninfected levels could be observed and explained by the residual H1 that is known to be packaged and expressed in the mutant virions (Novy, Kilcher et al. 2018). In addition to this, preliminary data from *in vitro* dephosphorylation assays show that H1 may be directly dephosphorylating this residue. Furthermore, cells expressing an active H1 or a catalytic inactive H1 clearly showed that this phosphatase leads to H3 S10 dephosphorylation in cells in the absence of infection. Once it was clear H1 is modulating H3 S10P upon Vaccinia infection, it was important to understand the purpose of this

dephosphorylation. Research has shown that infection by bacterial pathogens leads to H3 S10P dephosphorylation in order to repress genes involved in immune signalling. Since Vaccinia is known to have 9 protein repressors of the NFκB pathway, the first step was to figure out which Vaccinia mutants allow for p65 nuclear translocation, as it appeared H1 is acting there. Previous work gave us a lead: only when removing all 9 NFκB inhibitors from the virus can p65 enter the cell nucleus. Using this parental virus, vv811ΔA49, we generated a virus that also is inducible for H1, vv811ΔA49indH1, in order to compare the ability of the various viruses to supress cytokine expression levels. Preliminary data was first obtained by checking cytokine gene expression in WR WT versus H1- viruses. H1- viruses did not repress the expression of CCL5 and ICAM1 as efficiently as WT when stimulating with TNFα. Since these results were promising, experiments using vv811ΔA49 and vv811ΔA49H1- followed. In this case, the results were clear: vv811ΔA49H1- leads to higher levels of CCL5 and ICAM1 gene expression when compared to vv811ΔA49. The repression of the expression of these two chemokines, CCL5 and ICAM1, could be an attempt to prevent the recruitment of immune cells to sites of Vaccinia infection. While CCL5 has been implicated in the migration and recruitment of T-cells, dendritic cells, eosinophils, NK cells, mast cells and basophils; ICAM1 has been related to the recruitment of lymphocytes to the areas of infection in the cell. Additionally, CCL5 activates NFκB signalling in the target cells, leading to a cascade of proinflammatory cytokine and chemokine expression to fight the infection (Marques, Guabiraba et al. 2013)(Long 2011).

While I was able to look at a focused set of TNFα H3S10 regulated genes, it would be of great interest to gain a global understanding of how

dephosphorylation of H3S10 by Vaccinia H1 regulates cellular gene expression during infection. Follow-up work should be aimed at comparative RNA sequencing analysis of host cell gene expression in uninfected, WT and vv811ΔA49H1- infected cells under TNFα-stimulated conditions.

Section 6.03 Vaccinia virus modifies host cell chromatin for successful infection

Vaccinia is an ancient virus that has co-evolved with its hosts for thousands of years and as a result it has developed the best strategies to subvert the invading organism (Schrick et al. 2017). Once the virus enters the cell's cytoplasm, and therefore becomes vulnerable, it creates a defence line by replicating its genome in isolated compartments (Bidgood and Mercer 2015). However, it does need external resources found in the cell and, as a consequence, it has learned to interact with it. There is evidence that cellular proteins are recruited to viral factories as well as some viral proteins are transported into the cell nucleus (Oh and Broyles 2005) (Postigo et al. 2017). However, this is the first study that shows a Vaccinia enzyme shuttling to the cell nucleus during infection with the aim of modifying a specific histone mark at the host cell chromatin in order to repress cytokine expression and so to shut down the cellular immune response. This presents a novel mechanism by which this virus targets the cell defences and suggests that the interactions between Vaccinia and the host cell nucleus may be more important than previously thought. Additionally, this study is the first one to show a comprehensive research of host cell chromatin changes upon Vaccinia infection, looking at different types of histone

modifications in all four core histones. Most of the residues investigated change their status upon infection pointing out that either the virus or the cell is trying to achieve a different pattern of cellular expression to face this challenge. Collectively, these data indicate the importance that controlling cellular proteins at the gene level has during viral infection since it can completely prevent the presence of those proteins in the cell.

Previously, it was known that the H1 viral protein is a phosphatase capable of dephosphorylating serine/threonine and tyrosine residues, that is in the cell cytoplasm during infection, that plays an important role in dephosphorylating viral proteins in order to reset them and make them active for the next round of infection and it is required for viral DNA replication (Najarro, Traktman et al. 2001)(Ke Lie 1995) (Novy, Kilcher et al. 2018). Here I have showed that the H1 phosphatase goes into the cell nucleus and that it shuttles between the nucleus and the cytoplasm, data supported by that obtained by Dr. Susanna Bidgood in the lab (Chapter X. Appendix, page 216). As soon as the virus enters the cell, the H1 phosphatase goes into the cell nucleus and it is not until 4 hours post infection that it can also be seen in the cell cytoplasm. Since this protein is expressed during late viral gene expression, and that does not take place until 6 hours post infection, at 4 hours some of the H1 protein that is in the nucleus must be getting into the cytoplasm. This supports the fact that the most drastic dephosphorylation of H3 at S10 is observed then as the result that most H1 has completed its function in the cell nucleus: dephosphorylation of H3 S10. However, not all H1 leaves the nucleus probably just to maintain the phosphorylation at this histone residue down throughout the rest of infection. Alternatively, it could be speculated that

the dephosphorylation of H3 S10 does not take place until 4 hours post infection because H1 requires another viral protein, a cofactor, that is not produced until then, although it is not very likely since transfection experiments showed that WT H1 on its own lead to H3 S10 dephosphorylation. Nevertheless, in future experiments, it would be ideal to block gene expression and see whether the drop in H3 S10 phosphorylation at 4 hours still occurs in the absence of newly produced viral proteins.

The H1 phosphatase is conserved in all Poxviruses tested up to date, from Raccoonpox virus to Variola virus. Additionally, it has a strikingly high sequence similarity between the two most divergent poxvirus: Raccoonpox virus and Vaccinia virus, which are 89% similar at the DNA level and 92% similar at the amino acid level. This suggests that the degree of similarity in the rest of Poxviruses must be extremely high, pointing out a vital role for this phosphatase during viral infection (Hakes, D.J et al. 1993). It would be interesting to see whether infection with other Poxviruses leads to H3 S10 dephosphorylation and whether infection with their H1- mutant version would affect it.

Upon infection, cells switch on pathways to combat the virus by expressing an array of cytokines. These not only alert neighbouring cells of the presence of a pathogen but also activate immune cells, that are the ones who will get rid off the intruder. Vaccinia, by dephosphorylating H3 S10, is blocking the expression of CCL5, a chemokine involved in leukocyte, T cell, monocyte, dendritic cell […] recruitment and ICAM1, a cell surface protein expressed by endothelial cells and immune cells, which is necessary for cell migration (Appay, V. et al. 2001) (Ramos, T.N. et al. 2014). *In vivo* experiments using Vaccinia virus deficient for H1 could show what is the

impact that the suppression of these two cytokines have for successful infection, whether CCL5 and ICAM1 allow for immune cells to be recruited to the site of infection or whether T cells homing to the lymph nodes is impaired. Additionally, the effect of these cytokines could be preventing viral spread, since the dephosphorylation of H3 S10 does not occur until 4 hours post infection it could be speculated that it is a way to shut down the immune response of neighbouring cells to allow for subsequent rounds of infection and therefore viral spread. Finally, these experiments were carried out in A549s, however, each cell type expresses a different array of cytokines when encountering a pathogen. As a consequence, it could be possible that infecting a cell type that does not upregulate CCL5 and ICAM1 expression in response to Vaccinia infection would allow for Vaccinia to replicate normally in the presence of H3 S10 phosphorylation. On the other hand, Modified Vaccinia virus Ankara (MVA) has been reported to increase the expression of some cytokines during infection while WR Vaccinia does not. Among these cytokines we find CXCL2, CCL2 and CCL5 (Lehmann, M.H. et al. 2015). In the light of this, future research could aim at figuring out whether the H1 protein is missing or truncated in MVA and find out whether there is a dephosphorylation of H3 S10 during infection with this modified virus.

Vaccinia is a complex virus due to the large number of genes and proteins it encodes, from which a 5% is destined to repress the cellular immune response. The fact that the H1 phosphatase also contributes to the viral defence mechanism against the cell and when missing together with all the other 9 genes known to be involved in this, cellular cytokines gene expression is still repressed by viral infection. This shows there are more proteins still unknown that control the immune response of the cell, pointing

out the complexity of this virus that with only 1% of genome – when compared to the genome of mammalian cells – can overcome not only a single cell but a whole organism.

Chapter VII. Limitations

As with all projects there are some limitations concerning this project. One limitation was the variation obtained between western blot repeats with some of the histone marks. While these experiments show a trend of histone modification upon Vaccinia infection, not all were statistically significant. This could be due to varying levels of each of the modifications present in the cells before infection or the variability of synchronised infection. A second limitation is in regard to the inhibitors used to modulate H3 S10 phosphorylation. As DUSP1 is the cellular homolog of the Vaccinia phosphatase H1, it could very well be that the DUSP1 inhibitor is also inhibiting H1 and this is what leads to a decrease in infection. However, since the drug is known to be an allosteric inhibitor of DUSP1 and H1 is ~35% percent identical, this is not likely to be the case. In this experiment, the MSK1 inhibitor showed increased infection at early timepoints but failed to do so after 24 hours. This could be due to cell toxicity, but it is more likely due to the fact that at the MOI used for these experiments all cells are infected within the culture dish, thus looking for increased output is difficult. Nevertheless, the administration of BCI (DUSP1 inhibitor) and SB (MSK1 inhibitor) could have affected cell viability since they target the MAPK-ERK signalling pathway, which is involved in the cellular response to environmental stress as well as in the negative regulation of cell proliferation (Balla, U.S. et al 2002). If cell viability was compromised by the use of the inhibitor, Vaccinia would have encountered problems to infect cells leading to a lower proportion of cells infected. Therefore, the readout of our flow

cytometry experiments and our 24-hour yields could be showing a decreased infectivity due to toxicity instead of due to high levels of phosphorylation at H3 S10. As a consequence, cell toxicity assays should be used in the future to check for this. Additionally, the data already obtained could be re-analysed and instead of looking at the proportion of cells that are GFP positive, the intensity of GFP fluorescence could be assessed which would indicate the ability for Vaccinia to infect treated cells in comparison to untreated cells. In this case, since we are only looking at the population of cells that are infected, toxicity by drug inhibitors can be ruled out. The third limitation is the *in vitro* dephosphorylation assays. While performed nearly 20 times, only 2 representative experiments worked consistently, i.e. the positive control reaction showed H3S10 dephosphorylation. This suggests that some fundamental component of the experiment, for example the reaction buffer, is not functioning as expected. Finally, regarding cell transfections, I consistently observed differences in the amount of expression that could be achieved between H1 WT and the H1 C110S mutant. Interestingly, I saw that when vastly over-expressed, H1 C110S could mimic H1 WT and somehow drive H3S10 dephosphorylation. Given that H1 is only active as a dimer and C110S is catalytically inactive, these results suggest that when vast amounts are present this protein may interact with a cellular protein, perhaps DUSP1, leading to H3 S10 dephosphorylation.

Chapter VIII. Conclusion

Unlike most viruses, Vaccinia replicates in the cell cytoplasm. Thus, its interactions with the cell nucleus had been ignored for decades. This project has shed light on the fact that Vaccinia infection leads to major histone modifications, at least some of which are virus driven. I demonstrate that the virus encoded phosphatase H1 can enter the host nucleus and dephosphorylate cellular Histone 3 at serine 10 in order to affect the expression of anti-viral genes under the control of TNFα activation. I further show that dephosphorylation of this residue is not only vital for successful Vaccinia infection, but it is also important for repressing the immune signalling of the cell during infection. This indicated that the viral phosphatase H1 is a novel NFκB inhibitor encoded by Vaccinia. Thus, through this work I have uncovered an important new function of the Vaccinia virus phosphatase, H1, and expanded the known repertoire of Vaccinia virus NFκB inhibitors from 9 to 10 (Figure 8.4).

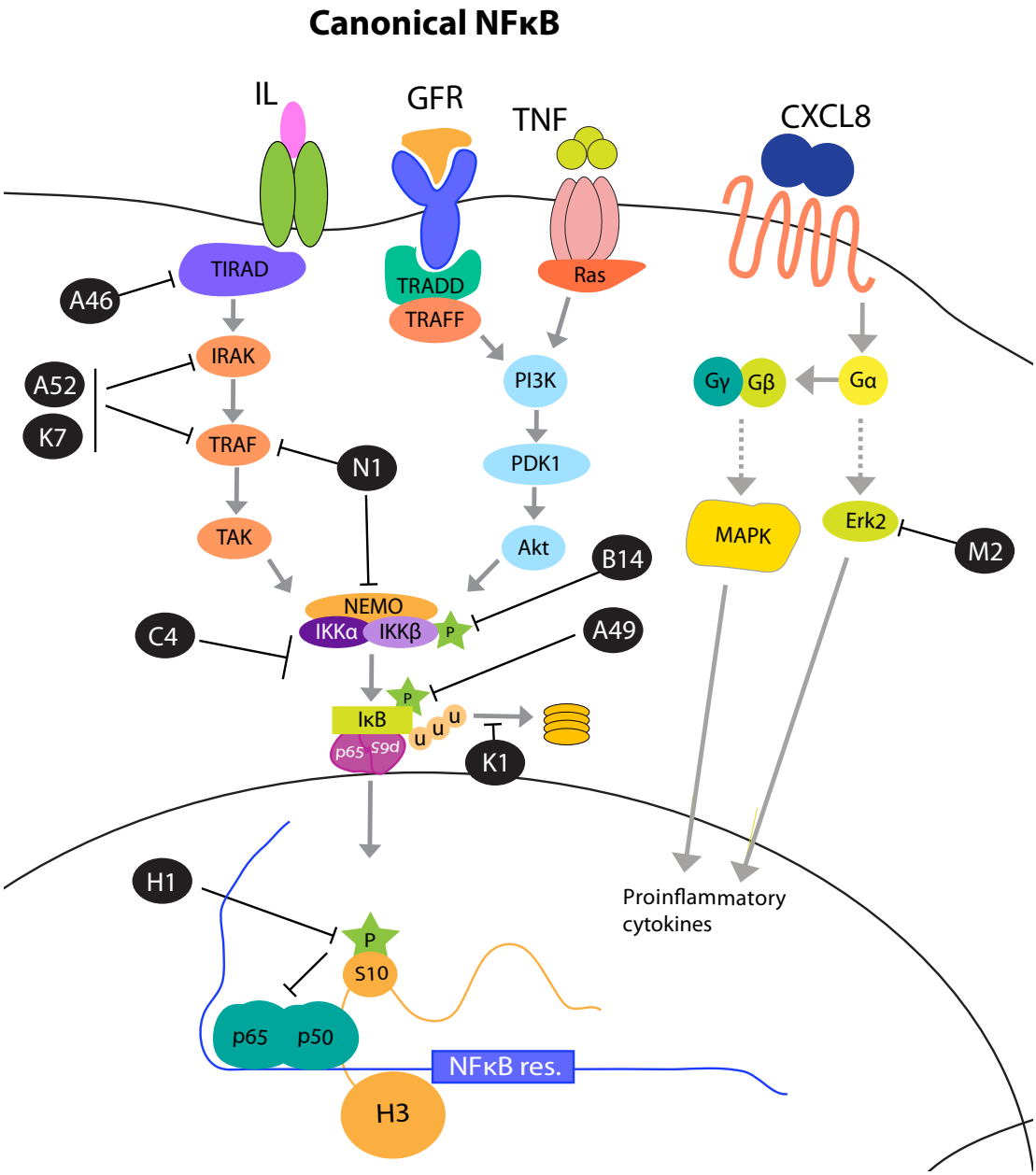


Figure 8.4. Vaccinia has 10 NFκB inhibitors. Upon Vaccinia infection, the different NFκB inhibitors block the pathway at multiple different points. The viral phosphatase H1 does it in the cell nucleus, through the dephosphorylation of H3S10 after TNFα stimulation.

Chapter IX. References

Ablasser, A. et al. (2013). “Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP.“ Nature 503 (7477): 530-534.

Ahn, S. H., R. L. Diaz, M. Grunstein and C. D. Allis (2006). "Histone H2B deacetylation at lysine 11 is required for yeast apoptosis induced by phosphorylation of H2B at serine 10." Mol Cell **24**(2): 211-220.

Akdis, M., S. Burgler, R. Crameri, T. Eiwegger, H. Fujita, E. Gomez, S. Klunker, N. Meyer, L. O'Mahony, O. Palomares, C. Rhyner, N. Ouaked, A. Schaffartzik, W. Van De Veen, S. Zeller, M. Zimmermann and C. A. Akdis (2011). "Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases." J Allergy Clin Immunol **127**(3): 701-721 e701-770.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Al-Lamki, R. S. and T. N. Mayadas (2015). "TNF receptors: signaling pathways and contribution to renal dysfunction." Kidney Int **87**(2): 281-296.

Allis, C. D. and T. Jenuwein (2016). "The molecular hallmarks of epigenetic control." Nat Rev Genet **17**(8): 487-500.

Allis, Z.-W. S. C. D. (2002). "Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast." Nature **418**: 104-108.

Ana Soloaga, S. T., Giselle R.Wiggin, Navita Rampersaud1, Mark H.Dyson, Catherine A.Hazzalin, Louis C.Mahadevan and J. S. C.Arthur (2003). "MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14." The EMBO Journal **22**: 2788±2797.

Appay, V. and Rowland-Jones, S. (2001). “ RANTES: a versatile and controversial chemokine”. Trends in Immunology 22 (2): 83-87.

Arbibe, L., D. W. Kim, E. Batsche, T. Pedron, B. Mateescu, C. Muchardt, C. Parsot and P. J. Sansonetti (2007). "An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses." Nat Immunol **8**(1): 47-56.

Balla, U.S. et al (2002). “MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signalling network”. Science 297: 1018-1023.

Banerjee, T. and D. Chakravarti (2011). "A peek into the complex realm of histone phosphorylation." Mol Cell Biol **31**(24): 4858-4873.

Barber, C. M., F. B. Turner, Y. Wang, K. Hagstrom, S. D. Taverna, S. Mollah, B. Ueberheide, B. J. Meyer, D. F. Hunt, P. Cheung and C. D. Allis (2004). "The enhancement of histone H4 and H2A serine 1 phosphorylation during mitosis and S-phase is evolutionarily conserved." Chromosoma **112**(7): 360-371.

Becker, A. E. P. B. (2002). "Histone acetylation: a switch between repressive and permissive chromatin." EMBO reports **3**(3): 224-229.

Bedford, M. T. and S. G. Clarke (2009). "Protein arginine methylation in mammals: who, what, and why." Mol Cell **33**(1): 1-13.

Behbehani, A. M. (1983). "The Smallpox Story: Life and Death of an Old Disease." Microbiological reviews **47**: 455-509.

Bernstein BE, et al. (2002). “Methylation of histone H3 Lys 4 in coding regions of active genes.” Proc Natl Acad Sci. 99:8695–700.

Bernstein, B. E., T. S. Mikkelsen, X. Xie, M. Kamal, D. J. Huebert, J. Cuff, B. Fry, A. Meissner, M. Wernig, K. Plath, R. Jaenisch, A. Wagschal, R. Feil, S. L. Schreiber and E. S. Lander (2006). "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." Cell **125**(2): 315-326.

Bidgood, S. R. and J. Mercer (2015). "Cloak and Dagger: Alternative Immune Evasion and Modulation Strategies of Poxviruses." Viruses **7**(8): 4800-4825.

Blaber, R. (2003). "Selective Regulation of ICAM-1 and RANTES Gene Expression after ICAM-1 Ligation on Human Renal Fibroblasts." Journal of the American Society of Nephrology **14**(1): 116-127.

Black RA, Rauch CT, Kozlosky CJ et al. (1997). “A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells.” Nature 385: 729–733.

Burdette, D.L. et al. (2013). “STING and the innate immune response to nucleic acids in the cytosol.” Nat. Immunology 14(1): 19-26.

Carmena, M., M. Wheelock, H. Funabiki and W. C. Earnshaw (2012). "The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis." Nat Rev Mol Cell Biol **13**(12): 789-803.

Chang, B., Chen, Y., Zhao, Y., and Bruick, R.K. (2007). “JMJD6 is a histone arginine demethylase.” Science 318, 444–447.

Chang, S. J., A. C. Shih, Y. L. Tang and W. Chang (2012). "Vaccinia mature virus fusion regulator A26 protein binds to A16 and G9 proteins of the viral entry fusion complex and dissociates from mature virions at low pH." J Virol **86**(7): 3809-3818.

Cildir, G., K. C. Low and V. Tergaonkar (2016). "Noncanonical NF-kappaB Signaling in Health and Disease." Trends Mol Med **22**(5): 414-429.

Cornelia Fritsch1, J. L. B., Judith A. Kassis2 and Jürg Müller1,* (1999). "The DNA-binding Polycomb group protein Pleiohomeotic mediates silencing of a Drosophila homeotic gene." Development **126**: 3905-3913.

D.M. Prescott, J. K. a. J. B. K. (1971). "Replication of Vaccinia Virus DNA in Enucleated L-cells." J. Mol. Biol. **59**: 505-508.

D.M.P.PHILLIPS (1963). "The Presence of Acetyl Groups in Histones." Biochemical Journal **87**: 258-263.

Dempsey, A. and Bowie, A.G. (2015). “Innate immune recognition of DNA: A recent history”. Virology 479-480: 146-152.

Dermody, T. S., E. Kirchner, K. M. Guglielmi and T. Stehle (2009). "Immunoglobulin superfamily virus receptors and the evolution of adaptive immunity." PLoS Pathog **5**(11): e1000481.

Dinarello, C. A. (2000). "Proinflammatory Cytokines." Chest **118**(2): 503-508.

Dinarello, C. A. (2007). "Historical insights into cytokines." Eur J Immunol **37 Suppl 1**: S34-45.

Dion, M. F., S. J. Altschuler, L. F. Wu and O. J. Rando (2005). "Genomic characterization reveals a simple histone H4 acetylation code." Proc Natl Acad Sci U S A **102**(15): 5501-5506.

Doellinger, J., L. Schaade and A. Nitsche (2015). "Comparison of the Cowpox Virus and Vaccinia Virus Mature Virion Proteome: Analysis of the Species- and Strain-Specific Proteome." PLoS One **10**(11): e0141527.

Doerks, T., R. R. Copley, J. Schultz, C. P. Ponting and P. Bork (2002). "Systematic identification of novel protein domain families associated with nuclear functions." Genome Res **12**(1): 47-56.

Duff, F. S. d. G. a. G. W. (1990). "Interleukin 1: the first interleukin." Immunlogy Today **11**(1): 13-20.

Duncan, E. A., V. Anest, P. Cogswell and A. S. Baldwin (2006). "The kinases MSK1 and MSK2 are required for epidermal growth factor-induced, but not tumor necrosis factor-induced, histone H3 Ser10 phosphorylation." J Biol Chem **281**(18): 12521-12525.

Edmondson, D. G., J. K. Davie, J. Zhou, B. Mirnikjoo, K. Tatchell and S. Y. Dent (2002). "Site-specific loss of acetylation upon phosphorylation of histone H3." J Biol Chem **277**(33): 29496-29502.

Eskandarian, H. A., F. Impens, M. A. Nahori, G. Soubigou, J. Y. Coppee, P. Cossart and M. A. Hamon (2013). "A role for SIRT2-dependent histone H3K18 deacetylation in bacterial infection." Science **341**(6145): 1238858.

Esparza, J., L. Schrick, C. R. Damaso, and A. Nitsche. 2017. "Equination (inoculation of horsepox): An early alternative to vaccination (inoculation of cowpox) and the potential role of horsepox virus in the origin of the smallpox vaccine." Vaccine 35 (52):7222-7230.

Fangwei Wang, J. D., John R. Daum, Ewa Niedzialkowska, Budhaditya Banerjee, P. Todd Stukenberg, Gary J. Gorbsky, Jonathan M. G. Higgins (2010). "Histone H3 Thr-3 Phosphorylation by Haspin Positions Aurora B at Centromeres in Mitosis." Science **330**: 231-235.

Finn, C. M. a. A. (2000). "Chemokine receptors and their role in inflammation and infectious diseases." Blood **95**(10): 3032-3043.

Fischle, W., B. S. Tseng, H. L. Dormann, B. M. Ueberheide, B. A. Garcia, J. Shabanowitz, D. F. Hunt, H. Funabiki and C. D. Allis (2005). "Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation." Nature **438**(7071): 1116-1122.

Flanagan, J. F., L. Z. Mi, M. Chruszcz, M. Cymborowski, K. L. Clines, Y. Kim, W. Minor, F. Rastinejad and S. Khorasanizadeh (2005). "Double chromodomains cooperate to recognize the methylated histone H3 tail." Nature **438**(7071): 1181-1185.

FOLLETT, T. H. P. A. E. A. C. (1974). "VacciniaVirusReplicationinEnucleateBSC-1 Cells:Particle ProductionandSynthesisofViralDNA andProteins." Journal of virology **13**(2): 488-493.

Forbat, E., F. Al-Niaimi and F. R. Ali (2017). "Molluscum Contagiosum: Review and Update on Management." Pediatr Dermatol **34**(5): 504-515.

Garcia-Garcia, J. C., N. C. Barat, S. J. Trembley and J. S. Dumler (2009). "Epigenetic silencing of host cell defense genes enhances intracellular survival of the rickettsial pathogen Anaplasma phagocytophilum." PLoS Pathog **5**(6): e1000488.

Gates, L. A., J. Shi, A. D. Rohira, Q. Feng, B. Zhu, M. T. Bedford, C. A. Sagum, S. Y. Jung, J. Qin, M. J. Tsai, S. Y. Tsai, W. Li, C. E. Foulds and B. W. O'Malley (2017). "Acetylation on histone H3 lysine 9 mediates a switch from transcription initiation to elongation." J Biol Chem **292**(35): 14456-14472.

Goldberg, A. D., C. D. Allis and E. Bernstein (2007). "Epigenetics: a landscape takes shape." Cell **128**(4): 635-638.

Goto, H., et al. (1999). “Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation.” J. Biol. Chem. 274:25543–25549.

Goto, H., Y. Yasui, E. A. Nigg, and M. Inagaki. (2002). “Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation.” Genes Cells 7:11–17.

Grabiec, A. M. and J. Potempa (2018). "Epigenetic regulation in bacterial infections: targeting histone deacetylases." Crit Rev Microbiol **44**(3): 336-350.

Greer, E. L. and Y. Shi (2012). "Histone methylation: a dynamic mark in health, disease and inheritance." Nat Rev Genet **13**(5): 343-357.

Greseth, M. D., D. C. Carter, S. S. Terhune and P. Traktman (2017). "Proteomic Screen for Cellular Targets of the Vaccinia Virus F10 Protein Kinase Reveals that Phosphorylation of mDia Regulates Stress Fiber Formation." Mol Cell Proteomics **16**(4 suppl 1): S124-S143.

Groves, I. J., M. B. Reeves and J. H. Sinclair (2009). "Lytic infection of permissive cells with human cytomegalovirus is regulated by an intrinsic 'pre-immediate-early' repression of viral gene expression mediated by histone post-translational modification." Journal of General Virology **90**(10): 2364-2374.

Guillemette, B., P. Drogaris, H. H. Lin, H. Armstrong, K. Hiragami-Hamada, A. Imhof, E. Bonneil, P. Thibault, A. Verreault and R. J. Festenstein (2011). "H3 lysine 4 is acetylated at active gene promoters and is regulated by H3 lysine 4 methylation." PLoS Genet **7**(3): e1001354.

Gupta, S. C., C. Sundaram, S. Reuter and B. B. Aggarwal (2010). "Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy." Biochim Biophys Acta **1799**(10-12): 775-787.

Hakes, D.J., Martell, K.J., Zhao, W., Massung, R.F., Esposito, J.J. and Dixon, J.E. (1993). “A protein phosphatase related to the vaccinia virus VH1 is encoded in the genomes of several orthopoxviruses and a baculovirus.” Proc. Natl. Acad. Sci. 90: 4017-4021.

Hamon, M. A., E. Batsche, B. Regnault, T. N. Tham, S. Seveau, C. Muchardt and P. Cossart (2007). "Histone modifications induced by a family of bacterial toxins." Proc Natl Acad Sci U S A **104**(33): 13467-13472.

Hardison SE, Brown GD (2012). “C-type lectin receptors orchestrate antifungal immunity.” Nat Immunol 13: 817–822.

Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-234.

Heintzman ND, et al. (2007). “Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome.” Nat Genet. 39:311–8.

Hidemasa Goto, a., Yoshihiro Yasui, Erich A. Nigg and Masaki Inagaki, (2002). "Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation." Genes to cells **7**: 11-17.

Hirano, F., K. Komura, E. Fukawa and I. Makino (2003). "Tumor necrosis factor α (TNF-α)-induced RANTES chemokine expression via activation of NF-κB and p38 MAP kinase: roles of TNF-α in alcoholic liver diseases." Journal of Hepatology **38**(4): 483-489.

Ho, C. M., I. Z. Donovan-Banfield, L. Tan, T. Zhang, N. S. Gray and B. L. Strang (2016). "Inhibition of IKKalpha by BAY61-3606 Reveals IKKalpha-Dependent Histone H3 Phosphorylation in Human Cytomegalovirus Infected Cells." PLoS One **11**(3): e0150339.

Hotchkiss, R. D. (1948). “The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography.” J. Biol. Chem. 175, 315–332.

Horton, J. R., A. K. Upadhyay, H. H. Qi, X. Zhang, Y. Shi and X. Cheng (2010). "Enzymatic and structural insights for substrate specificity of a family of jumonji histone lysine demethylases." Nat Struct Mol Biol **17**(1): 38-43.

Houben, A., et al. (2007). “Phosphorylation of histone H3 in plants—a dynamic affair.” Biochim. Biophys. Acta 1769:308–315.

Hoving, J. C., G. J. Wilson and G. D. Brown (2014). "Signalling C-type lectin receptors, microbial recognition and immunity." Cell Microbiol **16**(2): 185-194.

Hughes, A. L., S. Irausquin and R. Friedman (2010). "The evolutionary biology of poxviruses." Infect Genet Evol **10**(1): 50-59.

Hsu, Y. M., Zhang, Y., You, Y. et al. (2007). “The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens.” Nat. Immunol. 8:198.

Israel, A. (2010). "The IKK complex, a central regulator of NF-kappaB activation." Cold Spring Harb Perspect Biol **2**(3): a000158.

Jackson, V. (1990). "In Vivo Studies on the Dynamics of Histone-DNA Interaction: Evidence for Nucleosome Dissolution during Replication and Transcription and a Low Level of Dissolution Independent of Both1." Biochemistry **29**: 719-731.

Jer-Yuan Hsu, Zu-Wen Sun, Xiumin Li, K. T. Melanie Reuben, Douglas K. Bishop, Jeremy M. Grushcow, Cynthia J. Brame, Jennifer A. Caldwell, and R. L. Donald F. Hunt, M. Mitchell Smith and C. David Allis (2000). "Mitotic Phosphorylation of Histone H3 Is Governed by Ipl1/aurora Kinase and Glc7/PP1 Phosphatase in Budding Yeast and Nematodes." Cell **102**: 279–291.

Johansen, K. M., and J. Johansen. (2006). "Regulation of chromatin structure by histone H3S10 phosphorylation". Chromosome Res. 14:393–404.

Johannessen, M., F. Askarian, M. Sangvik and J. E. Sollid (2013). "Bacterial interference with canonical NFkappaB signalling." Microbiology **159**(Pt 10): 2001-2013.

John Hiscott, H. K., and Pierre Génin (2001). "Hostile takeovers: viral appropriation of the NF-κB pathway." The Journal of Clinical Investigation **107**(2): 143-151.

J.R. Matthews, R. T. H. (1995). "Regulation of the DNA Binding Activity of NF-KB." Int. J. Biochem. Cell Biol. **27**(9): 865-879.

Kawai T, Takahashi K, et al. (2005). "IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction." Nat Immunol. 6(10):981–988.

Kawai, T. and S. Akira (2009). "The roles of TLRs, RLRs and NLRs in pathogen recognition." Int Immunol **21**(4): 317-337.

Khochbin, s. Verdel, A., Lemercier, C. and Seigneurin-Berny, D. (2001). "Functional significance of histone deacetylase diversity." Curr. Opin. Genet. Dev. 11: 162-166.

Ke Liu, B. L., and Paula Traktman (1995). "The Dual-Specificity Phosphatase Encoded by Vaccinia Virus, VH1, Is Essential for Viral Transcription In Vivo and In Vitro." Journal of Virology **69**(12): 7823-7834.

Kew, V. G., J. Yuan, J. Meier and M. B. Reeves (2014). "Mitogen and stress activated kinases act co-operatively with CREB during the induction of human cytomegalovirus immediate-early gene expression from latency." PLoS Pathog **10**(6): e1004195.

Kinney, C. M., U. M. Chandrasekharan, L. Yang, J. Shen, M. Kinter, M. S. McDermott and P. E. DiCorleto (2009). "Histone H3 as a novel substrate for MAP kinase phosphatase-1." Am J Physiol Cell Physiol **296**(2): C242-249.

Kmetzsch, L. (2015). "Histone deacetylases: Targets for antifungal drug development." Virulence **6**(6): 535-536.

L.A.Stocken, M. G. O. A. (1966). "Metabolic Properties ofHistones from Rat Liver and Thymus Gland." Biochem. Journal **98**: 888-897.

Laliberte, J. P., A. S. Weisberg and B. Moss (2011). "The membrane fusion step of vaccinia virus entry is cooperatively mediated by multiple viral proteins and host cell components." PLoS Pathog **7**(12): e1002446.

Lang, C., A. Hildebrandt, F. Brand, L. Opitz, H. Dihazi and C. G. Luder (2012). "Impaired chromatin remodelling at STAT1-regulated promoters leads to global unresponsiveness of Toxoplasma gondii-infected macrophages to IFN-gamma." PLoS Pathog **8**(1): e1002483.
Latham, J. A. and S. Y. Dent (2007). "Cross-regulation of histone modifications." Nat Struct Mol Biol **14**(11): 1017-1024.

Lauberth, S. M., T. Nakayama, X. Wu, A. L. Ferris, Z. Tang, S. H. Hughes and R. G. Roeder (2013). "H3K4me3 interactions with TAF3 regulate preinitiation complex assembly and selective gene activation." Cell **152**(5): 1021-1036.

Lee I, Oh J.H., Shwab E.K., Dagenais T.R., Andes D., Keller N.P. (2009). “HdaA, a class 2 histone deacetylase of Aspergillus fumigatus, affects germination and secondary metabolite production.” Fungal Genet Biol 46:782-90.

Lehnertz, B., Y. Ueda, A. A. H. A. Derijck, U. Braunschweig, L. Perez-Burgos, S. Kubicek, T. Chen, E. Li, T. Jenuwein and A. H. F. M. Peters (2003). "Suv39h-Mediated Histone H3 Lysine 9 Methylation Directs DNA Methylation to Major Satellite Repeats at Pericentric Heterochromatin." Current Biology **13**(14): 1192-1200.

Lehmann, M.H., Price, P.J.R., Brandmuller, C. and Sutter, G. (2015). “Modified Vaccinia virus Ankara but not Vaccinia virus induces chemokine expression in cells of the monocyte/macrophage lineage.” Virology journal 201512 (21): 1-6.

Leonard, W. J. and J.-X. Lin (2000). "Cytokine receptor signaling pathways." Journal of Allergy and Clinical Immunology **105**(5): 877-888.

Li X, Cai Q, Mei H, Zhou X, Shen Y, Li D, Liu W. (2015). “The Rpd3/Hda1 family of histone deacetylases regulates azole resistance in Candida albicans.” J Antimicrob Chemother 70:1993-2003.

Long, E. O. (2011). "ICAM-1: Getting a Grip on Leukocyte Adhesion." The Journal of Immunology **186**(9): 5021-5023.

Loo, Y. M. and M. Gale, Jr. (2011). "Immune signaling by RIG-I-like receptors." Immunity **34**(5): 680-692.

M. Derrien, A. P., M. Khanna, O. Grubisha, and P. Traktman (1999). "Tyrosine Phosphorylation of A17 during Vaccinia Virus Infection: Involvement of the H1 Phosphatase and the F10 Kinase." Journal of Virology **73**(9): 7287-7296.

Marques, R. E., R. Guabiraba, R. C. Russo and M. M. Teixeira (2013). "Targeting CCL5 in inflammation." Expert Opin Ther Targets **17**(12): 1439-1460.

M. Gabriella Santoro, A. R. a. C. A. (2003). "NF-kB and virus infection: who controls whom." The EMBO journal **22**(11): 2552-2560.

Maniatis, B. S. P. a. T. (1999). "Virus Infection Leads to Localized Hyperacetylation of Histones H3 and H4 at the IFN-b Promoter." Molecular cell **3**: 125-129.

Marco Baggiolini, B. D., and Bernhard Moser (1997). "HUMAN CHEMOKINES: An Update." Annu. Rev. Immunol. **15**: 675-705.

Marcos-Villar, L., J. Diaz-Colunga, J. Sandoval, N. Zamarreno, S. Landeras-Bueno, M. Esteller, A. Falcon and A. Nieto (2018). "Epigenetic control of influenza virus: role of H3K79 methylation in interferon-induced antiviral response." Sci Rep **8**(1): 1230.

Marta Garcia-Ramirez, C. R. a. J. A. (1995). "Modulation of Chromatin Folding by Histone Acetylation." The Journal of Biological Chemistry **270**: 17923-17928.

Matthew S. Wiebe, P. T. (2007). "Barrier to Autointegration Factor is a Host Defense Against Poxvirus Replication that is Overcome by the Viral B1 Kinase." Cell Host Microbe: 187-197.

McFadden, G. (2005). "Poxvirus tropism." Nat Rev Microbiol **3**(3): 201-213.

Macdonald, N., Welburn, J.P., Noble, M.E., Nguyen, A., Yaffe, M.B., Clynes, D., Moggs, J.G., Orphanides, G., Thomson, S., Edmunds, J.W., et al. (2005). “Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3.” Mol. Cell 20, 199–211.

Medaglia, M. L. G., N. Moussatche, A. Nitsche, P. W. Dabrowski, Y. Li, I. K. Damon, C. G. O. Lucas, L. B. Arruda, and C. R. Damaso (2015). "Genomic Analysis, Phenotype, and Virulence of the Historical Brazilian Smallpox Vaccine Strain IOC: Implications for the Origins and Evolutionary Relationships of Vaccinia Virus." Journal of Virology 89 (23):11909-11925.

Merkenschlager, A. G. F. M. (2010). "Fresh powder on Waddington’s slopes." EMBo reports **11**: 490-492.

Miao, F. and R. Natarajan (2005). "Mapping global histone methylation patterns in the coding regions of human genes." Mol Cell Biol **25**(11): 4650-4661.

Michael D. Litt, M. S., Felix Recillas-Targa, Marie-Noelle Prioleau and Gary Felsenfeld (2001). "Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci." The EMBO Journal **20**(9): 2224-2235.

Michelle Fattal-German, F. L. R. L., Jacques Cerrina, Florence Lecerf and Sonia Berrih-Aknin (1998). "Expression and modulation of ICAM-1, TNF-CRand FWNTES in human alveolar
macrophages from lung-transplant recipients in oifro." Transplant Immunology **6**: 183-192.

Murray, K. (1964). "The Occurrence of iE-N-Methyl Lysine in Histones." Biochemistry **3**(1): 10-15.

Nagamatsu, K., Kuwae, A., Konaka, T., Nagai, S., Yoshida, S., Eguchi, M., Watanabe, M., Mimuro, H., Koyasu, S. & Abe, A. (2009). “Bordetella evades the host immune system by inducing IL-10 through a type III effector, BopN.” J Exp Med 206, 3073–3088.

Najarro, P., P. Traktman and J. A. Lewis (2001). "Vaccinia virus blocks gamma interferon signal transduction: viral VH1 phosphatase reverses Stat1 activation." J Virol **75**(7): 3185-3196.

Noyce, R. S., S. Lederman, and D. H. Evans. 2018. "Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments." Plos One 13 (1). doi: ARTN e0188453.

Novy, K., S. Kilcher, U. Omasits, C. K. E. Bleck, C. Beerli, J. Vowinckel, C. K. Martin, M. Syedbasha, A. Maiolica, I. White, J. Mercer and B. Wollscheid (2018). "Proteotype profiling unmasks a viral signalling network essential for poxvirus assembly and transcriptional competence." Nat Microbiol **3**(5): 588-599.

Oh, J. and S. S. Broyles (2005). "Host cell nuclear proteins are recruited to cytoplasmic vaccinia virus replication complexes." J Virol **79**(20): 12852-12860.

Oliveira, G. P., R. A. L. Rodrigues, M. T. Lima, B. P. Drumond and J. S. Abrahao (2017). "Poxvirus Host Range Genes and Virus-Host Spectrum: A Critical Review." Viruses **9**(11).
Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kB transcription factors." Oncogene **18**: 6853-6866.

Pandey, R. R., T. Mondal, F. Mohammad, S. Enroth, L. Redrup, J. Komorowski, T. Nagano, D. Mancini-Dinardo and C. Kanduri (2008). "Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation." Mol Cell **32**(2): 232-246.

Peter Cheung, K. G. T., Wang L. Cheung, Paolo Sassone-Corsi, John M. Denu, and a. C. D. Allis (2000). "Synergistic Coupling of Histone H3 Phosphorylation and Acetylation in Response to Epidermal Growth Factor Stimulation." Molecular Cell **5**: 905-915.

Postigo, A., A. E. Ramsden, M. Howell and M. Way (2017). "Cytoplasmic ATR Activation Promotes Vaccinia Virus Genome Replication." Cell Rep **19**(5): 1022-1032.

Punjabi, A. and P. Traktman (2005). "Cell biological and functional characterization of the vaccinia virus F10 kinase: implications for the mechanism of virion morphogenesis." J Virol **79**(4): 2171-2190.

Rachele Rempel, M. A., Elizabeth Evans, and Paula Traktmanl (1990). "Temperature-Sensitive Vaccinia Virus Mutants Identify a Gene with an EssentialRoleinViralReplication." Journal of Virology **64**(2): 574-583.

Rahman, M. M. and G. McFadden (2011). "Modulation of NF-kappaB signalling by microbial pathogens." Nat Rev Microbiol **9**(4): 291-306.

Rajagopal, N., J. Ernst, P. Ray, J. Wu, M. Zhang, M. Kellis and B. Ren (2014). "Distinct and predictive histone lysine acetylation patterns at promoters, enhancers, and gene bodies." G3 (Bethesda) **4**(11): 2051-2063.

Ramos, T.N., Bullard, D.C. and Barnum, S.R. (2014). "ICAM1: Isoforms and Phenotypes." J. Immunol. 192 (10): 4469-4474.

Reddick, L. E. and N. M. Alto (2014). "Bacteria fighting back: how pathogens target and subvert the host innate immune system." Mol Cell **54**(2): 321-328.

Reeves, M., D. Woodhall, T. Compton and J. Sinclair (2010). "Human cytomegalovirus IE72 protein interacts with the transcriptional repressor hDaxx to regulate LUNA gene expression during lytic infection." J Virol **84**(14): 7185-7194.

Reeves, M. B. and J. H. Sinclair (2010). "Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter." J Gen Virol **91**(Pt 3): 599-604.

Renauld, J. C. (2003). "Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators." Nat Rev Immunol **3**(8): 667-676.

Rolando, M., S. Sanulli, C. Rusniok, L. Gomez-Valero, C. Bertholet, T. Sahr, R. Margueron and C. Buchrieser (2013). "Legionella pneumophila effector RomA uniquely modifies host chromatin to repress gene expression and promote intracellular bacterial replication." Cell Host Microbe **13**(4): 395-405.

Rossetto, D., N. Avvakumov and J. Cote (2012). "Histone phosphorylation: a chromatin modification involved in diverse nuclear events." Epigenetics **7**(10): 1098-1108.
Rougeulle, C., J. Chaumeil, K. Sarma, C. D. Allis, D. Reinberg, P. Avner and E. Heard (2004). "Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome." Mol Cell Biol **24**(12): 5475-5484.

Santos-Rosa H, et al. (2002). "Active genes are tri-methylated at K4 of histone H3." Nature. 419:407– 11.

Schliehe, C., E. K. Flynn, B. Vilagos, U. Richson, S. Swaminanthan, B. Bosnjak, L. Bauer, R. K. Kandasamy, I. M. Griesshammer, L. Kosack, F. Schmitz, V. Litvak, J. Sissons, A. Lercher, A. Bhattacharya, K. Khamina, A. L. Trivett, L. Tessarollo, I. Mesteri, A. Hladik, D. Merkler, S. Kubicek, S. Knapp, M. M. Epstein, D. E. Symer, A. Aderem and A. Bergthaler (2015). "The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection." Nat Immunol **16**(1): 67-74.

Schmidt, F. I., C. K. Bleck, L. Reh, K. Novy, B. Wollscheid, A. Helenius, H. Stahlberg and J. Mercer (2013). "Vaccinia virus entry is followed by core activation and proteasome-mediated release of the immunomodulatory effector VH1 from lateral bodies." Cell Rep **4**(3): 464-476.

Schrick, L., S. H. Tausch, P. W. Dabrowski, C. R. Damaso, J. Esparza, and A. Nitsche (2017). "An Early American Smallpox Vaccine Based on Horsepox." New England Journal of Medicine 377 (15):1491-1492.

Schuettengruber, B., D. Chourrout, M. Vervoort, B. Leblanc and G. Cavalli (2007). "Genome regulation by polycomb and trithorax proteins." Cell **128**(4): 735-745.

Seidel, P., M. Roth, Q. Ge, I. Merfort, T. S'Ng C and A. J. Ammit (2011). "IkappaBalpha glutathionylation and reduced histone H3 phosphorylation inhibit eotaxin and RANTES." Eur Respir J **38**(6): 1444-1452.

Shahbazian, M. D. and M. Grunstein (2007). "Functions of site-specific histone acetylation and deacetylation." Annu Rev Biochem **76**: 75-100.

Shi, Y., F. Lan, C. Matson, P. Mulligan, J. R. Whetstine, P. A. Cole, R. A. Casero and Y. Shi (2004). "Histone demethylation mediated by the nuclear amine oxidase homolog LSD1." Cell **119**(7): 941-953.

Shinsaku Sakurada, T. K. a. T. O. (1996). "Induction of cytokines and ICAM-1 by proinflammatory cytokines in primary rheumatoid synovial fibroblasts and inhibition by A/-acetyl-i_-cysteine and aspirin." International immunology **8**(10): 1483-1493.

Smith, G. L., C. T. Benfield, C. Maluquer de Motes, M. Mazzon, S. W. Ember, B. J. Ferguson and R. P. Sumner (2013). "Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity." J Gen Virol **94**(Pt 11): 2367-2392.

Rea, S., O'Carroll, D., Strahl, B., Sun, Z., Schmid, M., Opravil, S., Mechtler, K. and Ponting, C. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature **406**: 593-599.

Sterner, D.E. and Berger, S.L. (2000). “Acetylation of histones and transcription-related factors.” Microbiol. Mol. Biol. Rev. 64: 435-459.

Strahl, B.D. & Allis, C.D (2000) “The language of covalent histone modifications.” Nature 403, 41–45

Stuart, J. H., R. P. Sumner, Y. Lu, J. S. Snowden and G. L. Smith (2016). "Vaccinia Virus Protein C6 Inhibits Type I IFN Signalling in the Nucleus and Binds to the Transactivation Domain of STAT2." PLoS Pathog **12**(12): e1005955.

Sumner, R. P., C. Maluquer de Motes, D. L. Veyer and G. L. Smith (2014). "Vaccinia virus inhibits NF-kappaB-dependent gene expression downstream of p65 translocation." J Virol **88**(6): 3092-3102.

Sun, S. C. (2017). "The non-canonical NF-kappaB pathway in immunity and inflammation." Nat Rev Immunol **17**(9): 545-558.

Susan T. Howard, Y. S. C., and Geoffrey L. Smith (1991). "Vaccinia Virus Homologues of the Shope Fibroma Virus Inverted Terminal Repeat Proteins and a Discontinuous ORF Related to the Tumor Necrosis Factor Receptor Family." Virology **180**: 633-647.

Smale, S.T. (2012). “Dimer-specific regulatory mechanisms within the NF-kB family of transcription factors.” Immunol. Rev. 246, 193–204.

Smith, S. and Stillman, B. (1991). “Stepwise assembly of chromatin during DNA replication in vitro”. The EMBO journal 10 (4): 971-980.

Smith, G. L., C. T. Benfield, C. Maluquer de Motes, M. Mazzon, S. W. Ember, B. J. Ferguson and R. P. Sumner (2013). "Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity." J Gen Virol **94**(Pt 11): 2367-2392.

Takaoka, A. et al. (2007). “DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response”. Nature 448 (7152): 501-505.

Takeuchi, O. and S. Akira (2010). "Pattern recognition receptors and inflammation." Cell **140**(6): 805-820.

Teferi, W. M., M. A. Desaulniers, R. S. Noyce, M. Shenouda, B. Umer and D. H. Evans (2017). "The vaccinia virus K7 protein promotes histone methylation associated with heterochromatin formation." PLoS One **12**(3): e0173056.

Theodora Agalioti, G. C., and Dimitris Thanos (2002). "Deciphering the Transcriptional Histone Acetylation Code for a Human Gene." Cell **111**: 381-392.

Traktman, R. E. R. a. P. (1992). "Vaccinia Virus Bi Kinase: Phenotypic Analysis of Temperature-Sensitive Mutants and Enzymatic Characterization of Recombinant Proteins." Journal of Virology **66**(7): 4413-4426.

Tu, Y. C., D. Y. Huang, S. G. Shiah, J. S. Wang and W. W. Lin (2013). "Regulation of c-Fos gene expression by NF-kappaB: a p65 homodimer binding site in mouse embryonic fibroblasts but not human HEK293 cells." PLoS One **8**(12): e84062.

V. G. Allfrey, R. F., and A. E. Mirsky (1964). "Acetylation And Methylation Of Histones And Their Possible Role In The Regulation Of RNA Synthesis." Biochemistry **51**: 786-794.

Vasiliki Anest, J. L. H., Patricia C. Cogswell, and B. D. S. A. S. B. Kris A. Steinbrecher (2003). "A nucleosomal function for IkB kinase-a in NF-kB-dependent gene expression." Nature **423**: 659-663.

Verma, I. M. (2004). "Nuclear factor (NF)-kappaB proteins: therapeutic targets." Ann Rheum Dis **63 Suppl 2**: ii57-ii61.

Vermeulen M, et al. (2007). “Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4.” Cell. 131:58–69.

Veyer, D. L., G. Carrara, C. Maluquer de Motes and G. L. Smith (2017). "Vaccinia virus evasion of regulated cell death." Immunol Lett **186**: 68-80.

Walter, W., D. Clynes, Y. Tang, R. Marmorstein, J. Mellor and S. L. Berger (2008). "14-3-3 interaction with histone H3 involves a dual modification pattern of phosphoacetylation." Mol Cell Biol **28**(8): 2840-2849.

Wan-Sheng Lo, R. C. T., L. D. Jeannie R. Rojas, Jer-Yuan Hsu, C. David Allis, Ronen Marmorstein, and a. S. L. Berger (2000). "Phosphorylation of Serine 10 in Histone H3 Is Functionally Linked In Vitro and In Vivo to Gcn5-Mediated Acetylation at Lysine 14." Molecular Cell **5**: 917–926.

Wang, X., P. Lupardus, S. L. Laporte and K. C. Garcia (2009). "Structural biology of shared cytokine receptors." Annu Rev Immunol **27**: 29-60.

Ward-Kavanagh, L. K., W. W. Lin, J. R. Sedy and C. F. Ware (2016). "The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses." Immunity **44**(5): 1005-1019.
Weaver, J. R. and S. N. Isaacs (2008). "Monkeypox virus and insights into its immunomodulatory proteins." Immunol Rev **225**: 96-113.

Wu, j. et al. (2013). “ Cyclic GMP-AMP is an endogenous second messenger in innate immune signalling by cytosolic DNA.” Science 339 (6121): 826-830.

Xia, Y. F., L. P. Liu, C. P. Zhong and J. G. Geng (2001). "NF-kappaB activation for constitutive expression of VCAM-1 and ICAM-1 on B lymphocytes and plasma cells." Biochem Biophys Res Commun **289**(4): 851-856.

Xu, H. H., T. Su and Y. Xue (2016). "Histone H3 N-terminal acetylation sites especially K14 are important for rDNA silencing and aging." Sci Rep **6**: 21900.

Xuejiao Tian, Saiyang Zhang, Hong-Min Liu, Yan-Bing Zhang, Christopher A Blair, Dan Mercola, Paolo Sassone-Corsi, and Xiaolin Zi, (2013). "Histone Lysine-Specific Methyltransferases and Demethylases in Carcinogenesis: New Targets for Cancer Therapy and Prevention." Current Cancer Drug Targets **13**(5): 558-579.

Yamamoto, Y., U. N. Verma, S. Prajapati, Y. T. Kwak and R. B. Gaynor (2003). "Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression." Nature **423**(6940): 655-659.

Yoneyama, M. and Fujita, T. (2008). “Structural mechanism of RNA recognition by the RIG-I-like receptors.” Immunity 29: 178-181.

Yumi Yamamoto, U. N. V., Shashi Prajapati, Youn-Tae Kwak and R. B. Gaynor (2003). "Histone H3 phosphorylation by IKK-a is critical for cytokine-induced gene expression." Nature **423**: 655-659.

Zhang, Q., M. J. Lenardo and D. Baltimore (2017). "30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology." Cell **168**(1-2): 37-57.

Zhang, Y., K. Griffin, N. Mondal and J. D. Parvin (2004). "Phosphorylation of histone H2A inhibits transcription on chromatin templates." J Biol Chem **279**(21): 21866-21872.

Zhen Chen, S. L., Shankar Subramaniam, and a. S. C. John Y.-J. Shyy (2017). "Epigenetic Regulation: A New Frontier for Biomedical Engineers." Annu. Rev. Biomed. Eng. **19**: 195-219.

Chapter X. Appendix

Subcellular fractionation of A549 cells infected with WR Vaccinia, that contains the H1 protein tagged with HA, at MOI 50. Three repeats were carried out by Dr. Susanna Bidgood using the Subcellular fractionation kit described in Section 2. Materials and Methods followed by TCA precipitation of the proteins.

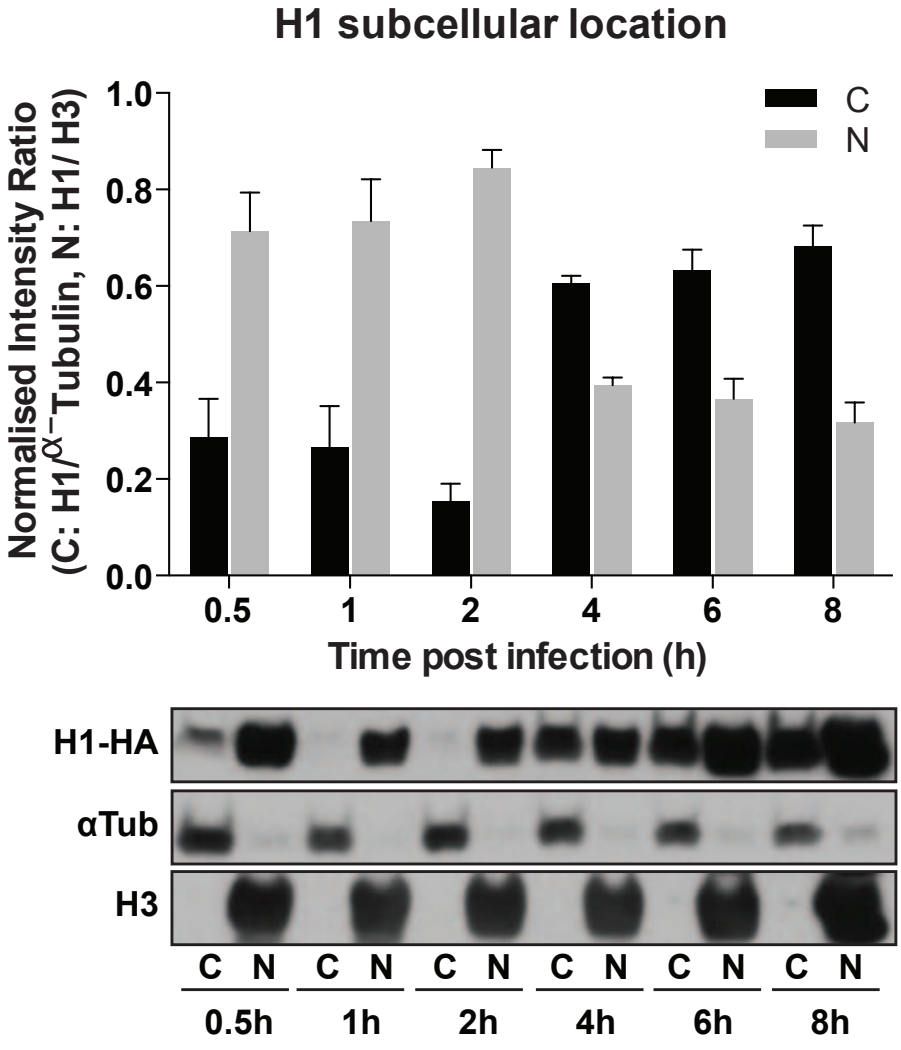


Figure 10.1. H1 subcellular localization. H1 localization in the cell cytoplasm (C) and cell nucleus (N) are shown in a timecourse experiment from 0.5 hours to 8 hours post infection. The quantification can be seen on top of the Western blot (n=3).