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Human nuclear PYHIN proteins inhibit LTR-driven gene expression by sequestering the host transcription factor Sp1

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Abbreviations

+	Positive
°C	Degree Celsius
µ	Micro
3’	3 prime
5’	5 prime
Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
AIM2	Absent in Melanoma 2
ALR	AIM2-like receptors
ASC	Apoptosis-associated speck-like protein containing a CARD
ATCC	American Type Culture Collection
BFP	Blue fluorescent protein
bp	Base pairs
BTD	Buttonhead domain
$CaCl_2$	Calcium chloride
CARD	Caspase activation domain
Cas9	CRISPR associated protein 9
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDK9	Cyclin-dependent kinase
cDNA	Complementary DNA
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP synthase
ChIP-seq	Chromatin immunoprecipitation and sequencing
CHKV	Chikungunya virus
c-Myc	Cellular Myelocystomatosis
CO_2	Carbon dioxide
Co-IP	Co-Immunoprecipitation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle threshold
CTD	C-terminal domain
CXCR4	C-X-C chemokine receptor type 4
CycT1	Cyclin T1
DDF	Death domain fold
ddH_2O	Double distilled water
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco’s phosphate buffered saline

dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
fw	Forward
Gag	Group-specific antigen
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBP	Guanylate binding protein
h.p.i.	Hours post infection
h.p.t.	Hours post transfection
H_2SO_4	Sulfuric acid
H3K93me	Tri-methylated lysin 9 of histone 3
HA	Hemagglutinin
HB	Hypotonic buffer
HBS	HEPES buffered saline
HBV	Hepatitis B virus
HCMV	Human Cytomegalovirus
HDAC1	Histone deacetylase 1
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIN	Hematopoietic interferon-inducible nuclear protein domain
HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus type 1
hTERT	Human telomerase reverse transcriptase
IFI16	Interferon-γ-inducible protein 16
IFIX	Interferon-inducible protein X
IFN	Interferon
IL	Interleukin
IMC	Infectious molecular clone
IRES	Internal ribosome entry site
IST	Inducer of short transcripts
IκBα	Inhibitor of kappaB alpha
KCl	Potassium chloride
KD	Knockdown
kDa	Kilo Daltons
KEGG	Kyoto encyclopedia of genes and genomes
KH_2PO_4	Potassium dihydrogenphosphate

KLF	Krüppel-like factor
KO	Knockout
KSHV	Kaposi's sarcoma-associated herpesvirus
l	Liter
LTR	Long Terminal Repeat
M	Molarity (g/mol)
m	Milli, meter
M-CSF	Macrophage colony-stimulating factor
MDMs	Monocyte-derived macrophages
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHV-68	Murine gammaherpesvirus 68
MIEP	Major Immediate Early promoter
MNDA	Myeloid Nuclear Differentiation Antigen
MOI	Multiplicity of infection
NaCl	Sodium chloride
Nef	Negative factor
NF-κB	Nuclear Factor-κB
NLS	Nuclear localization signal
nm	Nanometer
NP-40	Nonidet P-40
Nt	Nucleotide(s)
O/N	Overnight
Opti-MEM	Improved Minimal Essential Medium
ORF	Open reading frame
p	p-value
p24	HIV capsid protein
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PFA	Paraformaldehyde
PIB	Phosphatase inhibitor buffer
PLL	Poly-L-Lysine
Pol II	RNA polymerase type II
polyA	Polyadenylation
POP	Pyrin domain-only protein
P-TEFb	Positive transcription elongation factor b
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
qRT-PCR	Real-time reverse transcription-PCR
R	Regulatory region

rev	Reverse
RLU/s	Relative light unit per second
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RTA	R transactivator
rtTA	Reverse tetracycline-controlled transactivator
S.O.C.	Super Optimal broth with Catabolite repression
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of the mean
sgRNA	Single guide RNA
siRNA	Small interfering RNA
Sp	Specificity protein
SREBP1	Sterol Regulatory Element Binding Protein 1
ssDNA	Single-stranded DNA
ssRNA (+)	Single-stranded RNA with positive orientation
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAF	TBP associated cofactors
TAR	Transactivation response region
Tat	Transactivator of transcription
TBP	TATA-binding protein
TF	Transcription factor
TRIM	Tripartite motif-containing protein
TSS	Transcription start site
TZM-bl	Tranzyme Inc., β-galactosidase and luciferase
U3	Unique 3’ region
U5	Unique 5’ region
VSV-G	Vesicular Stomatitis Virus Glycoprotein
WT	Wild type
ZIKV	Zika virus
Δ	Deletion

Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

Amino acids

Ala	Alanine
Cys	Cysteine
His	Histidine
Lys	Lysine
Ser	Serine

1 Introduction

1.1 The human immunodeficiency virus

Human immunodeficiency virus (HIV) is the etiological agent of the acquired immunodeficiency syndrome (AIDS). Originally described as an emerging disease in 1981, primarily found in male homosexuals and drug abusers (Gottlieb et al., 1981; Masur et al., 1981), AIDS rapidly turned into a catastrophic pandemic, causing the death of nearly 32 million individuals until today (WHO – HIV/AIDS Key Facts; UNAIDS – 2019 fact sheet). Just by the end of 2018, roughly 37.9 million people were reported to be infected and 1.7 million new infections occurred throughout that year (WHO – HIV/AIDS Key Facts; UNAIDS – 2019 fact sheet). AIDS embraces a variety of clinical manifestations that are typical of the later stages of infection, characterized by the progressive loss of $CD4^+$ T lymphocytes and a general weakening of the immune system, rendering the host more susceptible to life-threatening opportunistic infections (Gottlieb et al., 1981; Masur et al., 1981). Subsequent studies revealed that the disease is caused by a retrovirus (Barré-Sinoussi et al., 1983), and the term human immunodeficiency virus was established soon thereafter. Phylogenetic analyses showed that HIV evolved as a result of several independent zoonotic transmissions of related simian immunodeficiency viruses (SIVs) present non-human primates (Gao et al., 1999; Van Heuverswyn et al., 2006; Hirsch et al., 1989). Such events gave rise to multiple HIV lineages. HIV type 1 (HIV-1) groups M, the one responsible for the global pandemic, and N are derived from SIVs infecting chimpanzees (*Pan troglodites*) (Keele et al., 2006). The precursors of HIV-1 groups O and N are instead SIVs found in gorillas (*Gorilla gorilla*), which emerged themselves as a cross-species transmission of lentiviruses present in chimpanzees (Van Heuverswyn et al., 2006; Takehisa et al., 2009). Nine groups (A-H) of HIV type 2 (HIV-2) have all emerged from independent transmission of SIV naturally hosted in sooty mangabey monkeys (*Cercocebus atys*) (Ayouba et al., 2013; Gao et al., 1992; Hirsch et al., 1989). In contrast to the global spread of HIV-1 group M, HIV-2 subtypes remained endemic in West Africa (Lemey et al., 2003).

The HIV replication cycle (Fig. 1) begins with the attachment of the viral surface envelope (Env) glycoproteins to the host cell receptor, namely the cluster of differentiation 4 (CD4). On the surface of an infectious viral particle, Env consists of two subunits, named gp120 and gp41. Gp120 mediates the initial attachment to CD4 and subsequently to either the C-C chemokine receptor 5 (CCR5) or the C-X-C chemokine receptor 4 (CXCR4) coreceptors. This binding triggers the exposure of gp41, which penetrates the host cell plasma membrane

and ties it to the viral envelope, leading to the formation of a fusion pore through which the viral capsid is delivered in the host cytoplasm (Wilen *et al.*, 2012). The conical capsid shell contains two copies of the viral genome expressed as single stranded RNA strands with positive orientation (ssRNA (+)), complexed with the viral proteins nucleocapsid, reverse transcriptase and integrase. The release of the capsid into the cytoplasm lays the groundwork for reverse transcription, a unique step within the retroviral replication cycle that converts the ssRNA (+) genome into linear double stranded DNA (dsDNA) (Hu and Hughes, 2012). Reverse transcription initiates uncoating of the viral capsid (Cosnefroy *et al.*, 2016; Rankovic *et al.*, 2017), and these two steps are tightly regulated in order to avoid detection of intermediate reverse transcription products by cytosolic nucleic acid sensors (Sauter and Kirchhoff, 2016). Upon completion of reverse transcription, the partially disassembled capsid docks at the nuclear pore through which the viral dsDNA is transported into the nucleus, where the viral integrase will irreversibly insert the viral genome into actively transcribed regions of the host chromosome (Craigie and Bushman, 2012).

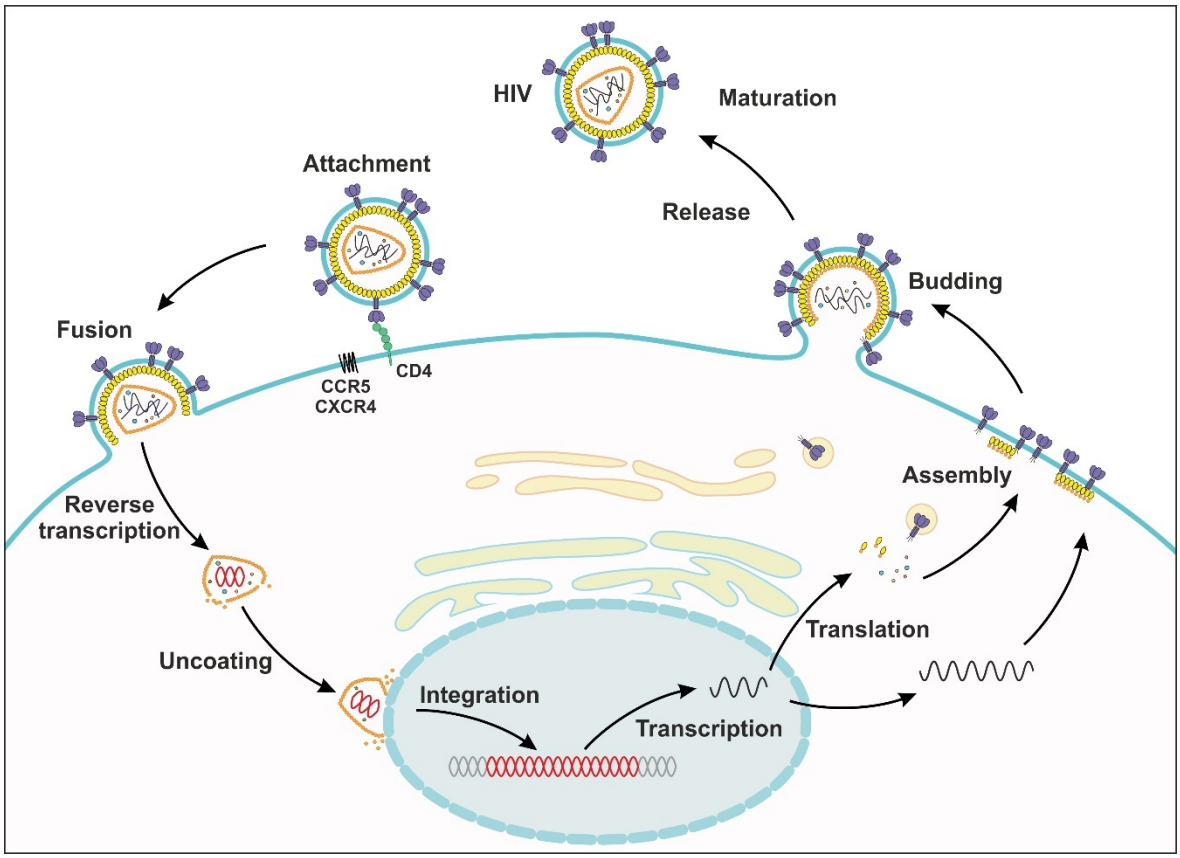

Figure 1. Overview of HIV replication (modified from E. Braun (source not published) with permission from E. Braun)**.** Infectious virions attach to the host receptor CD4 via their envelope glycoproteins and either CCR5 or CXCR4 as co-receptor. Upon fusion of viral and host cell membranes, the viral capsid is released into the cytoplasm where reverse transcription and partial uncoating occur. The newly synthetized viral DNA is

subsequently transported into the nucleus, integrated in the host chromosome, and viral gene expression is activated. Viral transcripts are either spliced and translated into proteins or remain unspliced and will become the progeny genomic RNA. Newly forming virions assemble and bud at the plasma membrane and are ultimately released into the extracellular environment, where they mature into fully infectious viral particles.

The integrated provirus provides the template for viral gene expression and viral transcripts are exported into the cytosol where they will be either translated into proteins or - in the case of the genomic RNA - incorporated into the nascent viral particles (Freed, 2015). The completion of the viral replication cycle occurs at the plasma membrane, where the group-specific antigen (Gag)/polymerase (GagPol) facilitates the incorporation of Env into newly forming virions and the recruitment of host machineries required to complete the budding process. In the newly released virion, the protease encoded within the GagPol precursor cleaves the GagPol and Gag polyproteins into the fully processed structural and enzymatic components, required for viral particle infectivity.

The discovery and characterization of unique viral enzymes such as the reverse transcriptase or integrase paved the way for the development of specific pharmacological inhibitors. The combinatorial delivery of drugs simultaneously targeting at least two viral enzymes known as the highly active anti-retroviral therapy (HAART) marked a significant improvement in the treatment of the infection, lowering the plasma viral load below detection limits and restoring the number of $CD4^+$ T lymphocytes (Arts and Hazuda, 2012). However, current treatment is still limited to controlling active replication of the virus, and the ability of HIV to establish a lifelong latent infection in $CD4^+$ T lymphocytes and cells of the myeloid lineage represents the major hurdle to achieve complete eradication of AIDS (Datta et al., 2016).

1.2 Regulation of HIV-1 gene expression by host and viral proteins

One common characteristic within the genomic architecture of retroviruses is the presence of two repetitive sequences termed long terminal repeats (LTR) at both ends of the genome. These LTRs are generated as a result of reverse transcription, hence the full-length LTR exists only in the dsDNA form of the viral DNA. This is because in the genomic RNA the 5’ LTR is only partially transcribed, as the region upstream the transcription start site (TSS) is occupied by host transcription factors. The HIV-1 LTR (Fig. 2A) shares its typical structure with other retroviral LTRs, harbouring a repeated region (R) flanked by two untranslated regions (U3 and U5) located upstream and downstream, respectively. Despite

having the same sequence, in the context of the integrated proviral DNA the 5' LTR is responsible for the initiation of viral gene expression while the 3' LTR provides the signals for termination and polyadenylation of viral mRNAs (Guntaka, 1993). The U3 region of the 5' LTR contains several *cis* regulatory elements that provide binding sites for host transcription factors. The core promoter is located upstream of the R region and consists of three tandem specificity protein 1 (Sp1)-binding sites (Jones et al., 1986) followed by a TATA box (Garcia et al., 1989) and two potent initiator elements (INR) (Zenzie-Gregory et al., 1993). All these elements cooperatively promote the binding of the initiation factor TFIID, consisting of the TATA-binding protein (TBP) and its associated cofactors (TAF) to the LTR (Rittner et al., 1995). In addition to the core promoter, the HIV-1 LTR contains an upstream enhancer region encompassing two or three tandem NF-κB binding sites (Nabel and Baltimore, 1987). These are required to support HIV-1 replication in activated $CD4^+$ T lymphocytes (Alcamí et al., 1995) via the synergistic interaction between NF-κB and Sp1 (Perkins et al., 1993). Although the core promoter alone is capable of generating transcription products *in vitro* (Rittner et al., 1995), LTR-directed transcription itself is very inefficient *in vivo* and does not proceed past the first 60 nucleotides (Kao et al., 1987). This is due to the presence of two inducer of short transcripts (IST), which trigger the prompt accumulation of non-polyadenylated, short RNA molecules (Ratnasabapathy *et al.*, 1990; Sheldon *et al.*, 1993). To overcome this transcriptional hurdle, HIV-1 encodes for a potent *trans*-acting transcriptional factor (Tat) (Sodroski et al., 1985), which acts by binding to a specific *trans*-acting responsive region (TAR) (Muesing *et al.*, 1987). The TAR is a regulatory element in all viral RNA transcripts that forms a highly stable, nuclease resistant, stem-loop structure (Berkhout *et al.*, 1989; Selby *et al.*, 1989) to which Tat binds with high affinity (Dingwall et al., 1989, 1990). Binding of Tat to the TAR loop requires the recruitment of the positive transcriptional elongation factor b (P-TEFb) (Garber *et al.*, 1998; Wei *et al.*, 1998). This is a heterodimeric complex consisting of the cyclin-dependent kinase 9 (CDK9) (Zhu et al., 1997) and the regulatory subunit Cyclin T (CycT1) (T for Tat) (Wei et al., 1998). Binding of Tat to P-TEFb induces conformational changes within CDK9 that activate its kinase activity (Tahirov et al., 2010; Wei et al., 1998) and stimulates transcriptional elongation by promoting hyperphosphorylation of the C-terminal domain (CTD) of the RNA Pol II (Kim et al., 2002; Marshall and Price, 1995; Marshall et al., 1996) (Fig 2B).

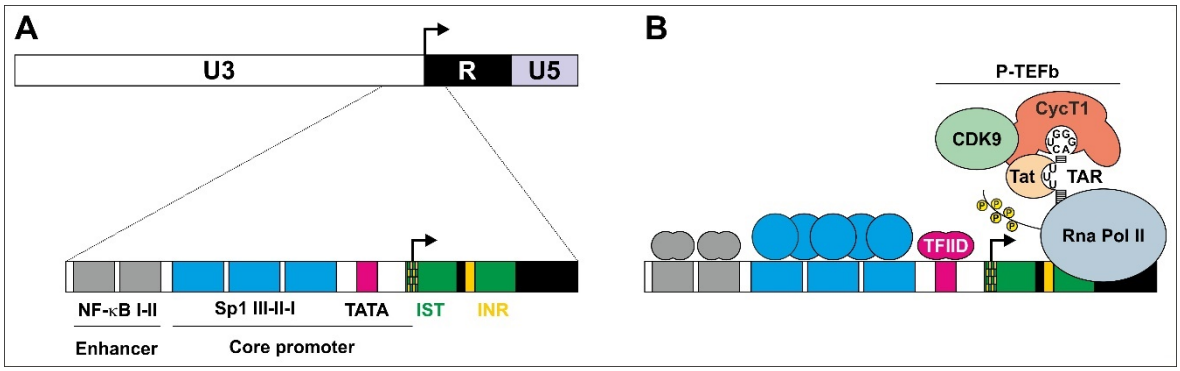

Figure 2. Structure and transcriptional regulation of the HIV-1 proviral long terminal repeat (LTR). (A) The HIV-1 LTR consists of three main domains: R (repeat) is a 96-nucleotide (nt) sequence present in both the 5' and 3' LTR; U5 is an 84-nt long fragment located downstream of the R region, and U3 is a more than 450-nt long segment situated immediately upstream of R. The transcription start site (black arrow) marks the boundary between U3 and R regions. The magnified section between the dotted lines illustrates the organization of the main *cis*-acting elements of the HIV-1 LTR: the two nuclear factor-κB (NF-κB) sites forming the enhancer region, the three specificity protein 1 (Sp1)-binding elements and the TATA box, composing the core promoter. The two initiator elements (INR) and the inducer of short transcripts (IST) located in the R region are also shown. **(B)** Upon integration into the host chromosome, NF-κB and Sp1 cooperatively interact to bind to the proviral LTR and initiate transcription. Sp1 recruits the transcription factor TFIID to the TATA box, which in turn facilitates the accommodation of the RNA polymerase type II on the transcription start site. Synthesis of viral RNAs, however, does not proceed past the first 60 nt and results in the accumulation of prematurely terminated transcripts, which rapidly fold into a stable stem-loop structure (TAR). Tat expression promotes binding of the positive transcription elongation factor b (P-TEFb) to the TAR bulge, an event that induces activating conformational changes within the cyclin-dependent kinase 9 (CDK9). The activated CDK9 subsequently enhances phosphorylation of the C-terminal domain (CTD) of the RNA polymerase, inducing transcriptional elongation and production of longer viral RNA species (Nchioua et al., 2020).

1.3 The human Pyrin and HIN domain-containing protein family

Pyrin and HIN domain-containing (PYHIN) family members, also known as the Absent in melanoma 2-like receptors (ALR), are mammalian proteins defined by an N-terminal Pyrin domain (PYD) and at least one C-terminal hematopoietic interferon-inducible nuclear domain with 200 amino acid repeats (HIN-200 or HIN) (Connolly and Bowie, 2014). The PYD domain belongs to the superfamily of the death domain fold (DDF) and possesses an alpha-helical-based folding allowing homo- or heterotypic interactions with other PYD containing proteins, regulating important cellular processes ranging from cell cycle regulation to apoptosis (Stehlik, 2007). The HIN domain is unique to the PYHIN family and is involved in binding DNA in a non-sequence specific fashion, due to the presence of two

tandem oligonucleotide/oligosaccharide-binding (OB) folds (Albrecht *et al.*, 2005; Shaw and Liu, 2014). HIN domains have been divided into three subfamilies designated HIN-A, -B and -C, based on the presence of specific amino acidic residues which follow the conserved MFHATVAT motif. (Ludlow et al., 2005). Humans encode four PYHIN proteins: γ-interferon (IFN)-inducible protein 16 (IFI16), IFN-Inducible protein X (IFIX) also known as the Pyrin and HIN domain-containing protein 1 (PYHIN1), Myeloid Nuclear Differentiation Antigen (MNDA) and Absent in melanoma 2 (AIM2) (Connolly and Bowie, 2014). Amongst them, only IFI16 harbours two HIN domains separated by a spacer region, whose varying length regulated by alternate splicing gives rise to three protein isoforms (Johnstone et al., 1998a). One important feature regulating the functions of PYHIN proteins is their subcellular localisation: while AIM2 is predominantly found in the cytosol, IFI16, IFIX and MNDA are located in the nucleus (Briggs et al., 2001; Diner et al., 2015; Ding et al., 2004; Duhl et al., 1989; Hornung et al., 2009; Khare et al., 2014) due to the presence of a bipartite nuclear localisation signal (NLS) located downstream of the PYD of IFI16 (Li et al., 2012). Based on sequence homologies to IFI16, IFIX and MNDA harbour at least one NLS-like sequence (Li et al., 2012). However, the role of these motifs in conferring nuclear localization to the latter two PYHIN proteins has not been experimentally confirmed (Fig. 3). Early studies on PYHIN proteins aimed at elucidating their involvement in basic cellular processes. However, the finding that *PYHIN* genes cluster within an IFN-inducible locus (Landolfo *et al.*, 1998; Ludlow *et al.*, 2005) suggested that PYHIN proteins could play a role in the innate immune response against pathogens.

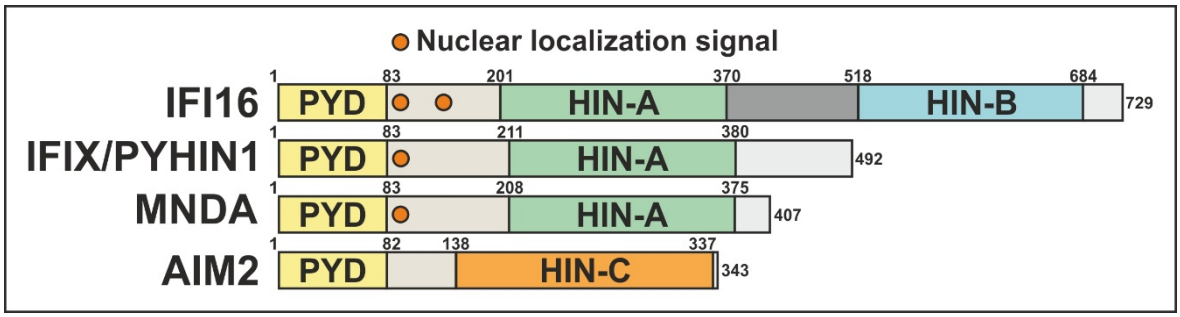

Figure 3. The human PYHIN protein family. PYHIN family members share a Pyrin domain at the N-terminus, important in regulating multiple cellular processes via protein-protein interactions. The C-terminal HIN domain allows DNA binding in a sequence-independent manner due to the presence of oligonucleotide/oligosaccharide-binding (OB) folds. While IFI16 has a bipartite nuclear localisation signal, IFIX and MNDA do have at least one NLS-like sequence.

1.3.1 PYHIN proteins as sensors of foreign DNA

It is long known that exogenous DNA elicits strong immunostimulatory responses (Isaacs *et al.*, 1963), and Toll-like receptors (TLRs) has been identified as sensors of foreign nucleic acids in macrophages and dendritic cells as (Akira and Takeda, 2004; Janeway and Medzhitov, 2002). However, it soon became evident that other cytosolic sensors detect exogenous or aberrant DNA in other cell types (Hochrein et al., 2004; Ishii et al., 2006; Okabe et al., 2005). Soon afterwards, AIM2 was identified as one of the cellular proteins responsible for sensing cytosolic DNA (Hornung et al., 2009).

AIM2 is physiologically localized to the cytosol at low concentrations, forming small oligomers that fail to activate downstream signalling (Morrone et al., 2015). Upon binding to cytosolic DNA, AIM2 triggers the inflammasome response by forming filamentous structures with the downstream adaptor apoptosis-associated speck-like protein containing a caspase activation domain (ASC) mediated by the PYD (Hornung et al., 2009; Morrone et al., 2015). The assembly of such large filaments provides multiple sites for the recruitment and activation of caspase-1, further amplifying inflammasome signalling (Dick et al., 2016). Activated caspase-1 promotes the proteolytic activation of proIL-1β and proIL-18, the precursors of two inflammatory cytokines (Hornung et al., 2009), as well as pyroptotic cell death. Caspase-1-mediated pyroptosis differs from caspases-triggered apoptosis in its disruption of membranes, leading to the release of cytosolic content in the extracellular milieu and exposing a plethora of inflammatory signals that further propagate the immune response (Man *et al.*, 2017). AIM2 has been shown to sense microbial DNA derived from *Francisella*, *Mycobacterium*, *Streptococcus*, *Staphylococcus*, *Listeria*, *Aspergillus* and *Plasmodium* species, as well as murine cytomegalovirus, vaccinia virus (VACV) and human papillomavirus (HPV) (Man *et al.*, 2016; Lugrin and Martinon, 2018). (Fig. 4).

IFI16 has also been extensively investigated for its role as an innate DNA sensor (Unterholzner et al., 2010). It is able to detect non-host nucleic acids in both the cytosol (Horan et al., 2013; Jakobsen et al., 2013; Monroe et al., 2014; Unterholzner et al., 2010; Wei et al., 2003) and the nucleus (Kerur *et al.*, 2011; Orzalli *et al.*, 2012; Ansari *et al.*, 2013). The latter finding abolished the dogma that foreign DNA is detected exclusively in a compartmentalization-dependent manner, and recent studies suggest that IFI16 distinguishes host and foreign nucleic acids based on its accessibility and its oligomerization (Stratmann et al., 2015). Upon recognition of long fragments of naked DNA in the nucleus, multiple IFI16 molecules aggregate and form clusters, held together by PYD-mediated

oligomerization (Morrone et al., 2014). Such oligomers subsequently translocate to the cytosol via acetylation of the bipartite NLS (Li *et al.*, 2012; Ansari *et al.*, 2015). There, IFI16 cooperates with another cytosolic sensor, the cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) and potentiate the stimulator of interferon gene (STING)-mediated activation of IFN expression (Almine et al., 2017; Jakobsen et al., 2013; Jønsson et al., 2017; Orzalli et al., 2015). Upon activation, STING relocates from the endoplasmic reticulum (ER) to the Golgi apparatus where it interacts with the TANK-binding kinase 1 (TBK1) (Ishikawa et al., 2009), a kinase that phosphorylates and activates the transcription factor interferon regulatory factor 3 (IRF3) (Ishikawa and Barber, 2008). Phosphorylated IRF3 then dimerizes and translocates to the nucleus where it promotes transcriptional activation of type I interferon (Agalioti et al., 2000) (Fig. 4). Even though IFI16 has been shown to recognize a variety of virus-derived DNA species (Dell'Oste et al., 2015), its role in sensing viral replication is controversial (Diner et al., 2016).

Besides recognizing and binding to dsDNA (Jin et al., 2013), IFI16 may also detect ssDNA species derived from intermediate products of reverse transcription in primary macrophages (Jakobsen et al., 2013). In collaborative studies, our group showed that IFI16 boosts STING-dependent sensing of HIV-1 replication in macrophages (Jønsson et al., 2017). In activated $CD4^+$ T lymphocytes, IFI16 was found to colocalize with cytoplasmic DNA and STING, but failed to induce an IFN response (Berg et al., 2014). Conversely, IFI16 has been shown to activate the inflammasome response and cause pyroptotic cell death in resting $CD4^+$ T lymphocytes abortively infected by HIV-1 (Monroe et al., 2014). Thus, IFI16 may contribute to the loss of $CD4^+$ T lymphocytes, a hallmark of AIDS. In addition, findings of our group suggested that IFI16 could have a direct antiviral activity that is independent of its DNA binding and sensing functions (McLaren et al., 2015). These results were confirmed and further expanded with the finding that IFI16 silences viral gene expression by targeting the host transcription factor Sp1 (Hotter et al., 2019).

The role of IFIX and MNDA in DNA sensing is only poorly understood. Initial reports showed that IFIX triggers IFN-β expression in HEK293 cells (Diner et al., 2015). More recent data suggest that IFIX acts as an effector of the innate immune response by activating the promoters of inflammatory cytokines (Massa et al., 2020). MNDA has also been shown to bind DNA (Li *et al.*, 2014), but whether it plays a role in sensing, still remains an open question. Notably, a recent study showed that the removal of the entire murine *PYHIN* locus has no effect on viral DNA sensing and IFN production (Gray et al., 2016). Together with

our data showing that *PYHIN* knockout (KO) mice display higher viremia during acute Friend virus infection compared to wild type mice (Hotter et al., 2019), these findings suggest that PYHIN proteins have a more direct antiviral function.

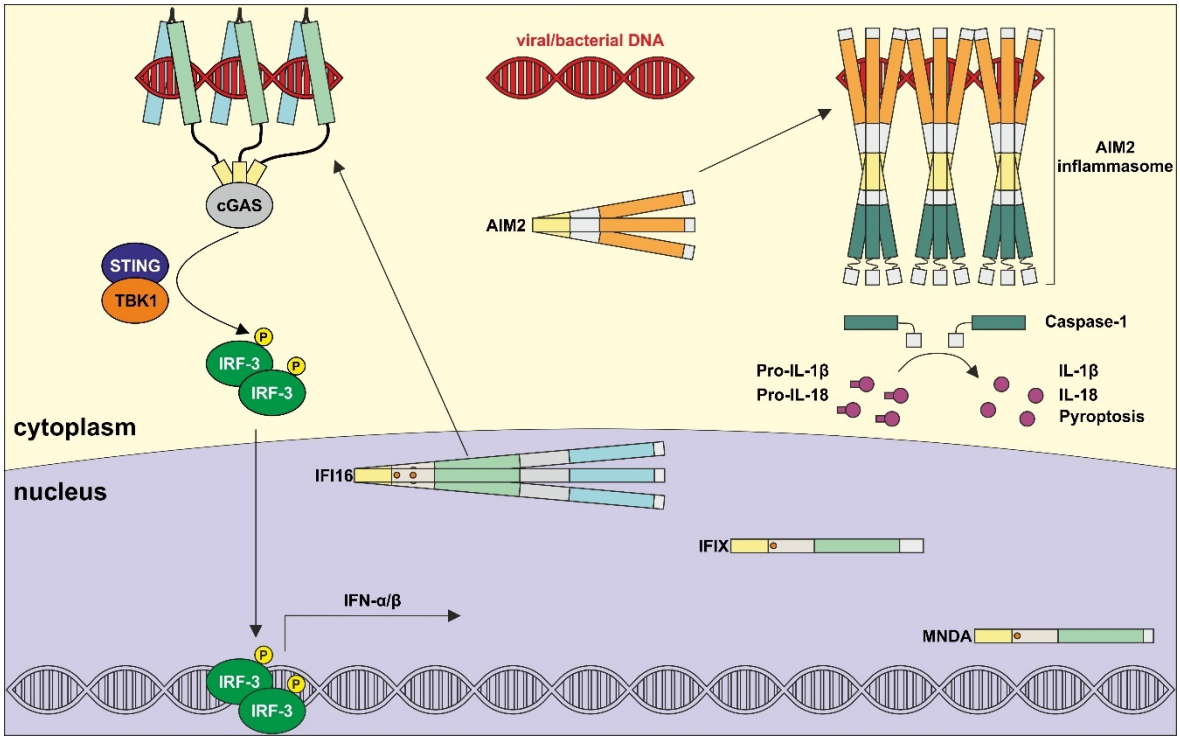

Figure 4. AIM2 and IFI16 are sensors of exogenous DNA. AIM2 is normally located in the cytosol as small oligomers. Upon binding of DNA, multiple AIM2 clusters assemble on the nucleic acids, leading to oligomerization and recruitment of the adaptor ASC via PYD-PYD interactions. Oligomerization of ASC leads to the formation of fibrillar supramolecular structures that promote Caspase-1 recruitment via CARD-CARD interactions and its subsequent activation. Caspase oligomerization on the adaptor ASC filaments generates a multitude of active sites leading to the proteolytic maturation of IL-1β and IL-18 and pyroptosis. IFI16 predominantly exhibits a nuclear localization. When sensing virus-derived DNA, IFI16 relocates to the cytosol where it oligomerizes and cooperates with cGAS to activate the STING signalling cascade. Both production of cGAMP and its interaction with IFI16 induce STING shuttling to the Golgi, where the latter interacts with TBK1 and promotes phosphorylation of IRF3. Upon phosphorylation, IRF3 dimerizes and migrates to the nucleus to promote transcription of type I interferon.

1.3.2 PYHIN proteins as antiviral restriction factors

Restriction factors are germline encoded proteins that contribute to the first line of defence against viral infections. Although the precise definition is under debate, restriction factors usually show several hallmarks: (1) they are often constitutively expressed in tissues, and their expression can be further upregulated by IFNs; (2) they can directly or indirectly interfere with the viral replication cycle; (3) viruses evolved strategies to evade, counteract

or even hijack their function; (4) as a result of the constant evolutionary arms race between host and pathogen, the open reading frames (ORFs) of restriction factors frequently exhibit signatures of positive selection (Kluge *et al.*, 2015).

Multiple studies pointed out that IFI16 exerts antiviral activity (Table 1) independently of its sensing function (Lo Cigno et al., 2015; Hotter et al., 2019; Johnson et al., 2014). For example, endogenous IFI16 was shown to restrict replication of herpes-simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), Kaposi's sarcoma-associated herpesvirus (KSHV), human papillomavirus (HPV), hepatitis B virus (HBV) and HIV-1 at a transcriptional level. IFI16 may achieve this by promoting the formation of repressive chromatin on viral promoters or by reducing the binding of host transcription factors required for efficient viral gene expression, such as Sp1 or signal transducer and activator of transcription (STAT) (Gariano *et al.*, 2012; Johnson *et al.*, 2014; Lo Cigno *et al.*, 2015; Merkl *et al.*, 2018; Hotter *et al.*, 2019; Merkl and Knipe, 2019; Roy *et al.*, 2019; Yang *et al.*, 2019). Recently, IFI16 has also been shown to inhibit replication of two RNA viruses, Zika (ZIKV) and Chikungunya, (CHKV) in primary foreskin fibroblasts (Wichit et al., 2019). In the latter case, IFI16 was found to bind the incoming chikungunya virus genome, and its antiviral activity was independent of interferon (Kim et al., 2020). Its precise antiviral mechanism remains although elusive.

In contrast to IFI16, little information is available on the antiviral activity of the other two nuclear PYHIN family members. MNDA was shown to inhibit replication of the murid gammaherpesvirus-68 (MHV-68), potentially by reducing the basal activity of the viral R transactivator (RTA)-gene promoter (Liu et al., 2012), while IFIX has been recently described to suppress HSV-1 transcription (Crow and Cristea, 2017). In both cases, however, the mechanism through which MNDA and IFIX exert their antiviral activity is unknown (Fig. 5).

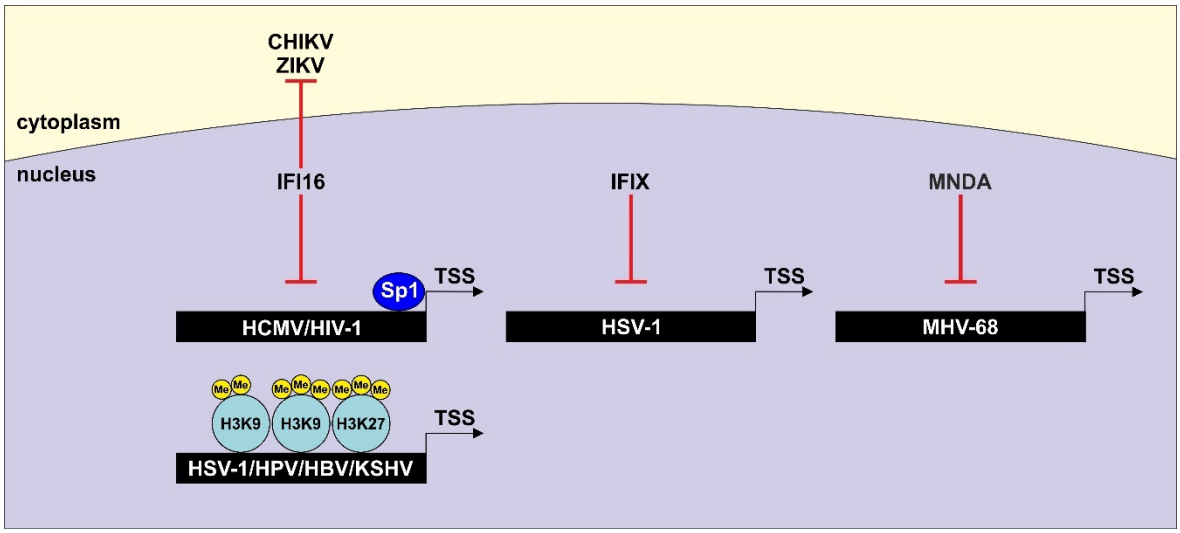

Figure 5. Nuclear PYHIN proteins inhibit transcription of DNA viruses. IFI16 possesses a broad antiviral activity against multiple nuclear-replicating viruses. In the context of human cytomegalovirus (HCMV) or human immunodeficiency virus (HIV), IFI16 inhibits viral gene expression by reducing the availability of the host transcription factor Sp1. Upon herpes simplex virus type 1 (HSV-1), human papillomavirus (HPV), hepatitis B virus (HBV) or Kaposi's associated herpes virus (KSHV) infection, IFI16 silences viral transcription by inducing the formation of repressive heterochromatin (either H3K9 di- or tri-methylated or H3K27 tri-methylated) on viral promoters. Additionally, IFI16 has been shown to repress replication of two cytoplasmic RNA viruses, Zika and Chikungunya, even though the precise mechanism remains unknown. The antiviral activity of IFIX and MNDA has already been reported for HSV-1 and the murid gammaherpesvirus 68 (MHV-68), respectively, but their *modus operandi* is far from being elucidated.

Table 1: Antiviral activities of human nuclear PYHIN proteins

PYHIN protein	Virus targeted	Proposed mechanism	Reference
IFI16	HSV-1	Assembles filamentous structures on the replicating DNA and promotes epigenetic silencing of viral genes	(Johnson et al., 2014; Merkl and Knipe, 2019; Merkl et al., 2018)
	HCMV	Reduces Sp1 binding to the *UL54* gene promoter, suppressing viral DNA replication and gene expression	(Gariano et al., 2012)
	HPV	Induces formation of repressive chromatin on viral promoters and potentially interferes with Sp1-driven gene expression	(Lo Cigno et al., 2015)
	HBV	Inhibits binding of STAT1/STAT2 to the	(Yang et al., 2019)

PYHIN protein	Virus targeted	Proposed mechanism	Reference
		enhancer 1/*X* gene promoter and enhances formation of repressive chromatin	
	KSHV	Associates to the viral genome and promotes tri-methylation of lysin 9 of histone H3 (H3K93me), leading to epigenetic repression and transcriptional silencing	(Roy et al., 2019)
	HIV-1	Competes with DNA and reduces the availability of Sp1 for viral gene expression	(Hotter et al., 2019)
	ZIKV	Inhibits viral genome replication and infectious virion production	(Wichit et al., 2019)
	CHKV	Binds to the incoming viral RNA and inhibits viral replication and maturation	(Kim et al., 2020)
IFIX	HSV-1	Binds to the viral DNA and inhibits viral gene expression	(Crow and Cristea, 2017; Diner et al., 2015)
MNDA	MHV-68	Reduces expression of immediate-early genes	(Liu et al., 2012)

1.4 The specificity protein 1

The transcription factor specificity protein 1 (Sp1) is the founder member of the Specificity protein/Krüppel-like factor (Sp/KLF) family of transcription factors. Nowadays it includes 26 members (van Vliet et al., 2006), all sharing a conserved DNA binding domain (DBD) either at or close to the C-terminus (Philipsen and Suske, 1999). The DBD consists of three tandem Cys2-His2-type zinc fingers, each containing two cysteines and two histidines coordinating one zinc cation (Kadonaga et al., 1987). The zinc fingers are folded in two anti-parallel beta-sheets and one alpha helix, the latter directly involved in establishing non-covalent interactions with the DNA (Narayan *et al.*, 1997; Oka *et al.*, 2004). According to studies regarding zinc fingers binding to nucleic acids (Pavletich and Pabo, 1991), three amino acids within each zinc finger have been identified as crucial for contacting specific bases on the major groove of the DNA (Fig. 6A). Such amino acids are conserved amongst five other members of the Sp/KLF family, namely Sp3, Sp4, KLF9, KLF10 and KLF11 (Philipsen and Suske, 1999). Consistently, the aforementioned transcription factors recognize the classical Sp1-binding elements with very similar affinity (Cook et al., 1998;

Hagen et al., 1992; Sogawa et al., 1993). The canonical Sp1-binding site has been defined as the “GC” box (5’-GGGGCGGGG-3’) and represents the sequence to which the three zinc fingers bind with the highest affinity. However, Sp1 tolerates some sequence variability and binds with slightly lower affinity to “GT” (5’-GGTGTGGGG-3’) and “CACC” (5’-CTGACTCCACCCCTGAGG-3’) boxes (Kadonaga et al., 1986; Nagaoka et al., 2001; Yu et al., 1991). Chromosome mapping analysis predicted at least 12.000 Sp1-binding sites in the human genome (Cawley et al., 2004). This number has been confirmed by chromatin-immunoprecipitation and sequencing (ChIP-seq) data (Rouillard et al., 2016). Curiously, many viruses also exploit the transactivation potential of Sp1 to regulate their gene expression (Dynan and Tjian, 1983; Jones et al., 1985, 1986).

To modulate host and viral gene expression, Sp1 possesses one N-terminal inhibitory domain (Murata et al., 1994) and two transactivator domains designated A and B. Both contain a glutamine-rich region (Courey and Tjian, 1988) downstream of a serine/threonine cluster (Kadonaga et al., 1987) harboring sites of regulatory post-translational modifications (PTMs). These include phosphorylation, acetylation and glycosylation (Chang and Hung, 2012; Tan and Khachigian, 2009). The inhibitory domain comprises a sequence termed Sp-box, which is highly conserved amongst members of the Sp-subfamily of transcription factors (Harrison et al., 2000). Its exact function remains unknown. However, the finding that the Sp-box lies close to an endoproteolytic cleavage site (Su et al., 1999) suggests a potential role in regulating protein stability via proteasomal degradation (O’Connor et al., 2016). The C domain, a highly charged region potentially regulating the interaction with specific transcription factors, is located between the transactivator domain B and the zinc fingers (Yieh *et al.*, 1995). The C-domain contains another highly conserved motif called Buttonhead (BTD) (Wimmer et al., 1993), involved in mediating synergistic transcriptional activation with the sterol regulatory element binding protein 1 (SREBP1) (Athanikar et al., 1997; Yieh et al., 1995). At the very C-terminus, Sp1 further contains a unique D domain, which is crucial for mediating the synergistic activation of the promoter (Pascal and Tjian, 1991) (Fig. 6B). Sp1 is mostly known as activator of transcription (Kadonaga *et al.*, 1986; Courey and Tjian, 1988). Sp1 may directly recruit the RNA polymerase type II (RNA Pol II) to the promoter by interacting with the TATA-binding protein (TBP) (Emili *et al.*, 1994), the TBP-associated factor 4 (TAF4) (Saluja *et al.*, 1998) and the general transcription factor TFIIB (Roberts et al., 1995). However, Sp1 regulates numerous host genes (Cawley et al., 2004; Rouillard et al., 2016) and several studies reported mechanisms through which cells

modulate its transcriptional activity, ranging from post translational modifications to interactions with other proteins (Wierstra, 2008; O'Connor et al., 2016).

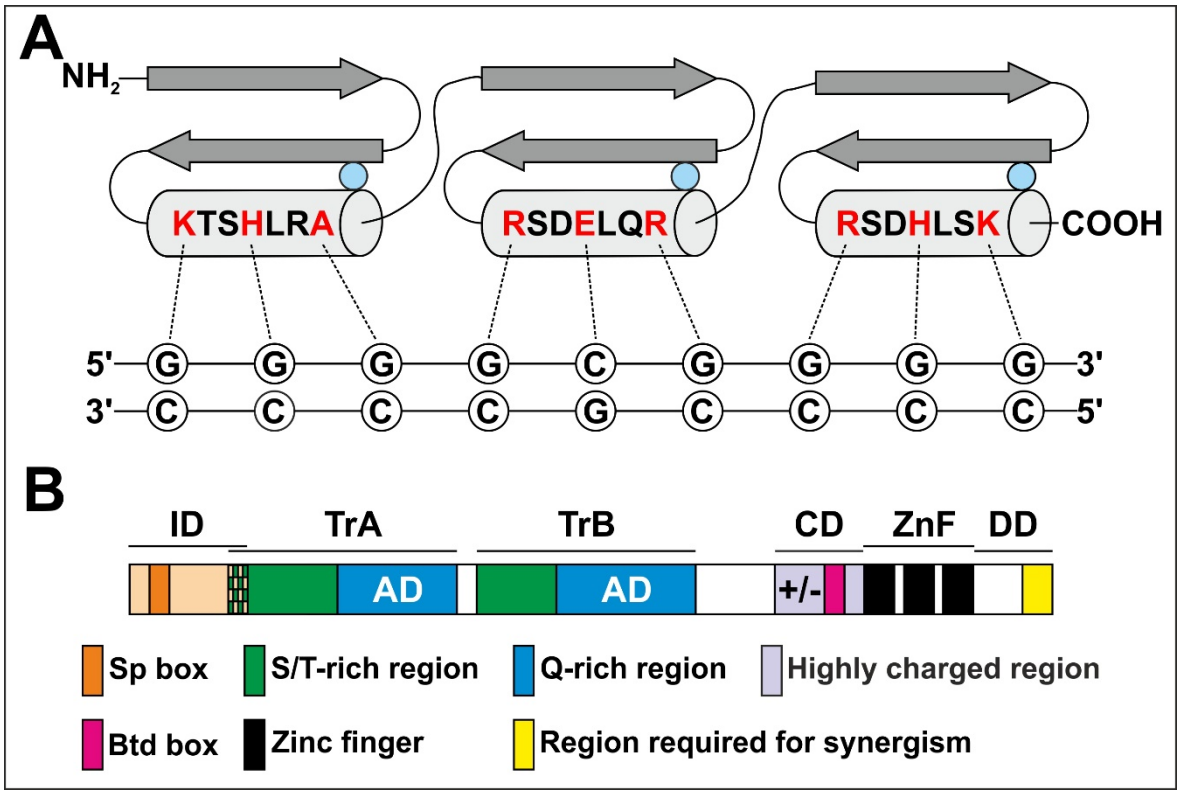

Figure 6. Zinc fingers binding model and functional domains of Sp1. (A) The three zinc fingers characterize all members of the Sp/KLF family of transcription factors. Each zinc finger adopts a well-defined structure consisting of two antiparallel beta-sheets (grey arrows) and one alpha helix (light grey cylinder), folded in order to coordinate one zinc cation (light blue circle) via two cysteines and two histidines. In the outermost alpha helix there are specific amino acids (highlighted in red), whose side chains mediate non-covalent interactions with the nucleic acids of the target sequence. **(B)** Sp1 negatively or positively modulates gene expression via an N-terminal inhibitory domain (ID) and two transactivation domains (TrA and TrB), respectively. Each transactivation domain consists of a putative regulatory serine/threonine-rich region (green) adjacent to a glutamine-rich domain (blue), responsible for the proper transcriptional activation. The C-domain (CD – light purple) harbours a highly charged region and is located upstream of the three zinc-fingers (ZnF), responsible for DNA binding. The C-terminal D-domain (DD) (yellow) is required for the synergistic transactivation of the target promoter.

2 Scientific aims

Human PYHIN proteins (IFI16, IFIX, MNDA and AIM2) are emerging as important mediators of the cell intrinsic immunity. AIM2 and IFI16 in particular are mostly known for their ability to detect pathogen-derived DNA and to trigger the inflammasome and type-I interferon response, respectively (Connolly and Bowie, 2014). In the context of HIV-1, IFI16 has been shown to sense viral-derived DNA species in primary macrophages and abortively infected resting $CD4^+$ T lymphocytes (Jakobsen et al., 2013; Monroe et al., 2014). Recent evidence additionally indicates that endogenous IFI16 acts as a viral restriction factor which directly inhibits replication of herpes and papillomaviruses by silencing viral gene expression (Lo Cigno et al., 2015; Dell'Oste et al., 2015). By combining evolutionary genomics, transcriptomics and HIV-host cell protein interaction databases in a genome wide *in silico* screen, IFI16 additionally emerged as a putative HIV-1 restriction factor (McLaren et al., 2015). Preliminary data suggested that IFI16 inhibits LTR-driven gene expression independently of its DNA-sensing function (McLaren et al., 2015). Very little information was however available about the role of the remaining two PYHIN proteins. MNDA was shown to reduce replication of murine gammaherpesvirus 68 (MHV-68) (Liu et al., 2012), while IFIX was described as a putative restriction factor acting against herpes simplex type 1 (HSV-1) (Diner et al., 2015).

The aims of my studies were to better understand the mechanism through which IFI16 exerts its antiretroviral function against several HIV-1 primary isolates and to investigate whether other members of the PYHIN family share the same activity.

3 Materials and methods

3.1 Material

3.1.1 Cell culture

3.1.1.1 Eukaryotic cell lines

Cell line	Description
HEK293T	Human embryonic kidney (HEK) cell line used for transient transfection; transformed by human adenovirus type 5 and stably expressing the simian virus 40 (SV40) large T antigen, which allows the replication of plasmids containing the SV40 origin of replication (DuBridge et al., 1987). Obtained via the ATCC (#CRL-3216).
TZM-bl	HeLa cell derivative used as reporter cell line for HIV infection; stably expressing human CD4, CCR5 and CXCR4 as well as firefly luciferase and *Escherichia coli*-derived β-galactosidase under the control of the HIV-1 long terminal repeat (LTR) promoter (Platt et al., 1998; Wei et al., 2002). Obtained via the NIH AIDS Reagent Program (#8129).

3.1.1.2 Primary cells

Cells	Origin
$CD4^+$ T lymphocytes	Buffy coats of healthy donors were obtained from the German Red Cross Blood Donor Service Baden-Württemberg at Ulm University. $CD4^+$ T cells were negatively isolated by using the RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail (STEMCELL Technologies) and Biocoll Separating Solution (Biochrom GmbH) by gradient centrifugation.
MDMs	Monocyte-derived macrophages (MDMs) were obtained by stimulation of human peripheral blood mononuclear cells (PBMCs) with recombinant human macrophage colony-stimulating factor (M-CSF) (R&D system) and human serum (Sigma Aldrich). PBMCs were isolated from buffy coats of healthy donors with the Biocoll Separating Solution (Biochrom GmbH) by gradient centrifugation.

3.1.1.3 Bacteria

Strain	Description
Escherichia coli XL-2 blue	endA1 supE44 thi-1 recA1 gyrA96 relA1 lac F´ proAB lacIqZΔM15 Tn10 (Tetr)
Escherichia coli XL-2 blue MRF'	endA1 supE44 thi-1 recA1 gyrA96 relA1 lac F´ proAB lacIqZΔM15 Tn10 (Tetr) McrA-, McrCB-, McrF-, Mrr-, HsdR-

3.1.2 Media

3.1.2.1 Eukaryotic cell culture

Medium	Manufacturer
DMEM	Dulbecco's Modified Eagle Medium Gibco/Life Technologies
RPMI-1640	Roswell Park Memorial Institute Medium Gibco/Life Technologies
Opti-MEM	Improved Minimal Essential Medium Gibco/Life Technologies
MEM	Minimal Essential Medium Gibco/Life Technologies

3.1.2.2 Bacterial culture

Medium	Manufacturer/Composition
LB medium	Lysogeny broth (LB) medium 10 g/l Bacto Tryptone, 5 g/l Bacto Yeast Extract, 10 g/l NaCl in distilled water; supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml)
LB agar	16 g/L Bactro Agar in LB medium; supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml)
S.O.C. Medium	Super Optimal broth with Catabolite repression Invitrogen/Life Technologies 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose
SOC Outgrowth Medium	Super Optimal broth with Catabolite repression New England Biolabs 2% Vegetable Peptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose

3.1.3 Nucleic acids

3.1.3.1 Expression vectors and proviral constructs

Construct	Description
pCG_PYHIN/Sp1 IRES BFP	Expression vector based on the human cytomegalovirus (CMV) immediate early (IE) promoter containing an *ampR* gene (Tanaka and Herr, 1990). Unique XbaI and MluI restriction sites were used to insert open reading frames (ORFs) encoding C-terminally HA-tagged wild type or truncated human IFI16, PYHIN1, MNDA and AIM2, C-terminally HA-tagged IFI16/AIM2 chimeras and C-terminally FLAG-tagged wild type and mutants human Sp1. The unique restriction site BamHI was used to insert an internal ribosome entry site (IRES) and the coding sequence for the blue fluorescent protein (BFP).

Construct	Description
pBR_HIV-1 NL4-3	Modified pBR322 expression vector harboring *tetR* and *ampR* genes (Bolivar et al., 1977; Watson, 1988). The construct encodes the full provirus of HIV-1 group M subtype B NL4-3, purchased from NIH (#114) (Adachi et al., 1986).
pBR_HIV-1 NL4-3 IRES eGFP	Modified version of the pBR_HIV-1 NL4-3 proviral construct, in which the *nef*/LTR overlap was removed (Münch et al., 2005) and the eGFP introduced between *nef* and the 3'-LTR is co-expressed via an IRES (Schindler *et al.*, 2005).
pHIT/G	Expression plasmid encoding for the Vesicular Stomatitis Virus glycoprotein (VSV-G) (Fouchier et al., 1997) under the control of a CMV promoter (Soneoka et al., 1995).
pHIV-1 AD8	Modified pBR322 expression vector encoding the full provirus of HIV-1 group M subtype B AD8 (Theodore et al., 1996), a macrophage-tropic infectious molecular clone carrying a G-to-A mutation in the ATG of *vpu* (Myers et al., 1997) resulting in higher *env* expression levels (Schubert et al., 1999). A kind gift of Vicente Planelles (Zimmerman et al., 2006).
pHIV-1 M LAI1 wild type	Modified pKP59 plasmid derived from the pBR322 vector (Mounts et al., 1989) encoding the full length replication competent HIV-1 group M subtype B LAI clone. The construct harbours an additional 34 nt from the *nef* gene upstream of the 5' LTR as a result of a duplication (Peden et al., 1991). It was kindly provided by Atze Das and Ben Berkhout (Das et al., 2011).
pHIV-1 M LAI1 rtTA-2d15tetO	Modified version of the pHIV-1 group M LAI1 proviral construct with 5 point mutations introduced to inactivate the TAR loop (Verhoef et al., 2001) and a Y26A amino acid substitution in Tat which drastically reduces Tat function and viral replication (Verhoef et al., 1997). To induce viral gene expression, the *nef* reading frame was replaced with the reverse tetracycline-controlled transactivator (rtTA) gene (Gossen et al., 1995; Urlinger et al., 2000) and 2 *tetO* operator elements in the sense orientation were introduced upstream of the Sp1 binding sites in the 5' and 3' LTR (Verhoef et al., 2001).
pHIV-1 M LAI1 rtTA-2d15tetO-TAR-ER3	Modified version of the pHIV-1 group M LAI1 proviral construct in which the TAR loop has been replaced with a non-related ER3 sequence (Das et al., 2007) and the Tat protein has been inactivated by the Y26A mutation (Verhoef et al., 2001).
pHIV-1 M LAI1 rtTA-2d15tetO-TATstop(mTat2)	Modified version of the pHIV-1 group M LAI1 proviral construct harbouring two translational stop codons at positions 15 and 17 in the Tat reading frame (Das et al., 2011) and in which the TAR loop has been inactivated by 5 point mutations (Verhoef et al., 2001).

Construct	Description
pHIV-1 M LAI1 rtTA-tetOcmv-TATwt	Modified version of the pHIV-1 group M LAI1 proviral construct in which the U3 region and the Sp1 binding sites were replaced by 7 *tetO* operator elements and the 30-bp minimal promoter of CMV IE promoter and the TAR was substituted with a 33-nt hairpin-forming sequence (Das et al., 2011).
pHIV-1 M LAI1 rtTA-tetOcmv-TATstop	Proviral construct derived from the pHIV-1 M LAI1 rtTA-tetOcmv-TATwt backbone in which the three Sp1 binding sites have been re-introduced in both 5' and 3' LTR and subsequently inactivated by random mutagenesis, and Tat protein expression was suppressed by two translational stop codons (Das et al., 2011).
pHIV-1 M LAI1 rtTA-tetOcmv-Sp1-TATwt	Proviral construct derived from the pHIV-1 M LAI1 rtTA-tetOcmv-TATwt backbone in which the three Sp1 binding sites have been re-introduced in both 5' and 3' LTR (Das et al., 2011).
pHIV-1 M LAI1 rtTA-tetOcmv-Sp1-TATstop	Proviral construct derived from the pHIV-1 M LAI1 rtTA-tetOcmv-TATwt backbone in which the three Sp1 binding sites have been re-introduced in both 5' and 3' LTR and Tat protein expression was suppressed by two translational stop codons (Das et al., 2011).
pCR-XL-TOPO_HIV-1 M subtype B CH058.c (transmitted founder virus)	Proviral construct encoding the infectious molecular clone of transmitted founder virus CH058.c. It was a kind gift of B. Hahn (Ochsenbauer et al., 2012)
pCR-XL-TOPO HIV-1 M subtype B CH040.c (transmitted founder virus)	Proviral construct encoding the infectious molecular clone of transmitted founder CH040.c. It was a kind gift of B. Hahn (Ochsenbauer et al., 2012)
pCR-XL TOPO_HIV-1 M subtype C CH042 (transmitted founder)	Proviral construct encoding the infectious molecular clone of transmitted founder CH042.c. It was a kind gift of B. Hahn (Freel et al., 2012)

3.1.3.2 Primers used to generate pCG_IRES_BFP expression constructs

Construct name	Primer name	Primer sequence
IFI16	IFI16 XbaI fw	CGTCTAGACCATGGGAAAAAAATACAAGAACATTGTTC
	IFI16 MluI rev	CTACGCGTTTAGAAGAAAAAGTCTGGTGAAGTTTCCATAC
IFI16 C-HA	IFI16 C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATCGTATGGGTAGAAGAAAAAGTCTGGTGAAGTTTCC
IFIX	IFIX XbaI fw	CGTCTAGACCATGGCAAATAACTACAAG

Construct name	Primer name	Primer sequence
	IFIX C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTAGGAACTGCTGGATGG
MNDA	MNDA XbaI fw	CGTCTAGACCATGGTGAATGAATACAAG
	MNDA C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTAATTAACATTCATTGGTCCTT C
	Remove internal XbaI fw	GCCTGTCTTGACAAACTAATAGAAC
	Remove internal XbaI rev	GTTCTATTAGTTTGTCAAGACAGGC
IFIX PYD+linker	IFIX XbaI fw	CGTCTAGACCATGGCAAATAACTACAAG
	IFIX PYD+linker C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTACAATGGTTTTAGGCTCTCAG
IFIX PYD only	IFIX XbaI fw	CGTCTAGACCATGGCAAATAACTACAAG
	IFIX PYD only C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTATTTTAACTTTTCTCTTTTAAG AGTTTC
MNDA PYD+linker	MNDA XbaI fw	CGTCTAGACCATGGTGAATGAATACAAG
	MNDA PYD+linker C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTACTGGGTTTCCTGATTCG
MNDA PYD only	MNDA XbaI fw	CGTCTAGACCATGGTGAATGAATACAAG
	MNDA PYD only C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTATTTTGACTTCTCTTTTCGAA G
AIM2 PYD+linker	AIM2 XbaI fw	CGTCTAGACCATGGAGAGTAAATACAAG G
	AIM2 PYD+linker C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTACTGGGCCACCATCTG
AIM2 PYD only	AIM2 XbaI fw	CGTCTAGACCATGGAGAGTAAATACAAG G
	AIM2 PYD only C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTATTTCTCCTTCTCCTCCTG
IFI16-AIM2 chimeras	IFI16 XbaI fw	CGTCTAGACCATGGGAAAAAAATACAAG AACATTGTTC
	IFI16 PYD+AIM2 linker overhang rev	CGATTTGTATTGCTTATCAACTTTTAACTT TTCTTTTTTAAGAGTTTC
	IFI16 HinA+AIM2 linker overhang fw	GCCTGAACAGAAACAGGCCAAATGTCAG GTAAC
	IFI16 C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTAGAAGAAAAAGTCTGGTGAA GTTTCC
	AIM2 linker + IFI16 PYD overhang fw	GAAACTCTTAAAAAAGAAAAGTTAAAAG TTGATAAGCAATACAAATCG
	AIM2 linker + IFI16 HinA overhang rev	GTTACCTGACATTTGGCCTGTTTCTGTTC AGGC

Construct name	Primer name	Primer sequence
	AIM2 XbaI fw	CGTCTAGACCATGGAGAGTAAATACAAG G
	AIM2 PYD+IFI16 linker overhang rev	GATAGGGCTGGTCCTTTTACTTTCTCCTT CTCCTCCTG
	AIM2 HinC+IFI16 linker overhang fw	GAGAACCCGAAAACAGTGATGGTGGCCC AGC
	AIM2 C-HA MluI rev	CTACGCGTTAAGCGTAATCTGGAACATC GTATGGGTATGTTTTTTTTTGGCCTTAAT AAC
	IFI16 linker+AIM2 PYD overhang fw	CAGGAGGAGAAGGAGAAAGTAAAAGGA CCAGCCCTATC
	IFI16 linker+AIM2 HinC overhang rev	GCTGGGCCACCATCACTGTTTTCGGGTTC TC
Sp1 C-FLAG	Sp1 HA_to_FLAG fw	GATGATGATAAATGAACGCGTCGGATCC TG
	Sp1 HA_to_FLAG rev	ATCTTTATAATCGAAGCCATTGCCACTGA TATTAATG
Sp1 ΔID	Sp1 ΔID fw	CCGAGTCAGTCAGGGGGA
	Sp1 ΔID rev	CATGGTCTAGAAGGCCTACG
Sp1 ΔTrA	Sp1 ΔTrA fw	TATGTGACCAATGTACCAGTGG
	Sp1 ΔTrA rev	GCCCTGGGAGTTGTTGCT
Sp1 ΔTrB	Sp1 ΔTrB fw	AGCAGCAGCAACACCACT
	Sp1 ΔTrB rev	CTGAGTCTGTCCTGAGAGTAC
Sp1 ΔCD	Sp1 ΔCD fw	GACAGTGAAGGAAGGGGC
	Sp1 ΔCD rev	GGTCTGCCCCAAGGAAAC
Sp1 ΔZnF	Sp1 ΔZnF fw	CAGAATAAGAAGGGAGGCCCAGG
	Sp1 ΔZnF rev	TCCCCCGAGCCCCTTCCT
Sp1 ΔDD	Sp1 ΔDD fw	GATTATAAAGATGATGATGATAAATG
	Sp1 ΔDD rev	GTGGGTCTTGATATGTTTTG

3.1.3.3 siRNAs

siRNA target	Manufacturer	siRNA sequence
IFI16	Dharmacon #LQ-020004-00-0005	CAGCGUAACUCCUAAAAUCdTdT; GGAGUAAGGUGUCCGAGGAdTdT; GGACCAGCCCUAUCAAGAAdTdT; GAUCUGUAAUUCAUAGUCAdTdT;
IFIX	Eurofins MWG	GCAACCGUCUCACAGCUAAdTdT
MNDA	Dharmacon #LQ-010525-00-0005	CAGUAAGACUCAAUAUUUCdTdT; GUGUAUGGGUUGUUUAUGUdTdT; GUAAGGAAGAAGGUCAUUAdTdT; GCAACAGCGCCAUUUAAAUdTdT
AIM2	Eurofins MWG	UAUGGUGCUAUGAACUCCAGAUGUCdTdT; UUUCAGCUUGACUUAGUGGCUUUGGdTdT
Non-targeting control	Eurofins MWG	UUCUCCGAACGUGUCACGUdTdT

3.1.3.4 sgRNA

sgRNA target	Manufacturer	sgRNA sequence
IFI16	IDT	GACCAGCCCTATCAAGAAAG
Non-targeting control	IDT	ACGGAGGCTAAGCGTCGCAA

3.1.3.5 Primers and probes for qRT-PCR

Name	Sequence
Long LTR fw	GCCTCAATAAAGCTTGCCTTGA
Long LTR rev	GGGCGCCACTGCTAGAGA
Long LTR probe	6FAM-CCAGAGTCACACAACAGACGGGCACA-BBQ
Nef fw	GGTGGGAGCAGYATCTCGAGA
Nef rev	TGTAAGTCATTGGTCTTAAAGGTACCTGAGG
Nef probe	6FAM-TGCTTCYAGCCAGGCACAAKCAGCATT-BBQ
PolyA fw	GCCCTCAGATGCTRCATATAA
PolyA rev	TTTTTTTTTTTTTTTTTTTTTTTTTTGAAG
PolyA probe	6FAM-TGCCTGTACTGGGTCTCTCTGGTTAG-BBQ

3.1.4 Kits and reagents

Kit	Manufacturer
Amaxa™ 4D-Nucleofactor™ Human Activated Cell P3 Lonza Kit	Lonza
DNA Ligation Kit Ver 2.1	Takara Bio Inc.
DNA-free™ DNA Removal Kit	ThermoFisher
GalScreen	Applied Bioscience
GAPDH Endogenous Control (VIC/TAMRA)	ThermoFisher
Mini DNA Preparation Kit	Qiagen
Phusion High-Fidelty PCR Kit	Thermo Scientific
Pierce Gold BCA Protein Assay Kit	ThermoFisher
PrimeScript RT Reagent Kit	TAKARA
Q5® High-Fidelty PCR Kit	New England Biolabs
RNeasy Plus Mini Kit	QIAGEN
Signal Enhancer HIKARI Solution for Western Blotting and ELISA	Nacalai Tesque, Inc
Silica Beads DNA Gel Extraction Kit	ThermoFisher
TaqMan™ Fast Universal PCR Master Mix	ThermoFisher
TransAM Sp1 Transcription Factor Assay Kit	ActiveMotif
UltraClean 15 DNA Purification Kit	MO BIO Laboratories Inc.
Wizard® Plus Midipreps DNA Purification System	Promega

Reagent	Manufacturer
1,4-diazabicyclo-(2,2,2)-octane (DABCO)	Carl Roth GmbH & Co. KG
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
4X Protein Sample Loading Buffer	LI-COR
Agarose	Sigma-Aldrich
Bacto Agar	BD Biosciences
Bacto Tryptone	BD Biosciences
Bacto Yeast Extract	BD Biosciences
Benzonase Nuclease	Sigma-Aldrich
Biocoll Separating Solution	Biochrom GmbH
BlueStar Prestained Protein Marker	Nippon genetics Co. Ltd.
Calcium chloride ($CaCl_2$) dihydrate	Sigma-Aldrich
Complete ULTRA Tablets, Mini EDTA-free	Roche
Disodium phosphate (Na_2HPO_4)	Sigma-Aldrich
Doxycycline	Sigma Aldrich
Dulbecco's phosphate buffered saline (DPBS) [-]$CaCl_2$ [-]$MgCl_2$	Gibco/Life Technologies
Dynabeads Human T-Activator CD3/CD28	ThermoFisher
Ethanol	Dispensary
Ethidium bromide	AppliChem GmbH
Ethylendiaminetetraacetate (EDTA)	Biochrom GmbH
FACS Flow Solution	BD Biosciences
FACS Shutdown Solution	BD Biosciences
Fetal Calf Serum (FCS)	Gibco/Life Technologies
Fluorescence-activated Cell Sorting (FACS) Clean Solution	BD Biosciences
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
Hoechst 33342 Solution	ThermoFisher
Human Interferon α 2a (IFNα 2a)	Pbl assay science
Human Interferon γ (IFNγ)	Sigma-Aldrich
Human Interleukin 2 (IL-2)	Miltenyi Biotec GmbH
Human macrophage colony-stimulating factor (M-CSF)	R&D Systems
Human Serum	Sigma Aldrich
Hydrochloric acid (HCl)	VWR
Isopropanol	Dispensary
Kanamycin	Invitrogen/Life Technologies
L-glutamine	Gibco/Life Technologies PAN-Biotech
Lipofectamine RNAiMAX Transfection Reagent	Thermo Scientific
Loading dye	Carl Roth GmbH & Co. KG
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich
Methanol	Sigma-Aldrich
Molecular weight size marker '1KB plus DNA ladder'	Invitrogen/Life Technologies

Reagent	Manufacturer
NEBuffers	New England Biolabs Inc.
Nonidet P-40	USB
NuPAGE MES SDS Running Buffer (20x)	Invitrogen/Life Technologies
Paraformaldehyde (PFA)	Merck Millipore
Penicillin-Streptomycin	Gibco/Life Technologies PAN-Biotech
Pierce Protein A/G Magnetic Beads	ThermoFisher
Potassium chloride (KCl)	AppliChem GmbH
Potassium dihydrogenphosphate	AppliChem GmbH
RNase away	Molecular Bio Products
RosetteSep™ Human CD4+ T Cell Enrichment Cocktail	Stem Cell Technologies
Skimmed Milk Powder	Sigma-Aldrich
Sodium chloride (NaCl)	Millipore
Sodium dodecyl sulfate (SDS)	Fluka
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich
Tris	AppliChem GmbH
Triton X-100	Sigma-Aldrich
Tween-20	Sigma-Aldrich
β-glycerophosphate	Calbiochem
β-mercaptoethanol	Sigma-Aldrich

3.1.5 Enzymes

Enzyme	Manufacturer
HiFi Cas9 Nuclease V3	IDT
Restriction endonucleases	New England Biolabs Inc.
Trypsin/EDTA	PAN-Biotech
Trypsin-EDTA (10x)	Gibco/Life Technologies

3.1.6 Solutions and buffers

Solution/buffer	Composition
For transfection	
HEPES buffered saline (HBS) 2x	5.94 % HEPES (w/v), 8.18 % NaCl (w/v), 0.25 % Na_2HPO_4 x $2H_2O$ (w/v) in distilled water for 10x HBS; for 2x HBS, the 10x stock was diluted in distilled water, pH 7.12, sterilized by filtration
$CaCl_2$	2 M $CaCl_2$ in distilled water, sterilized by filtration
For p24 ELISA	
Blocking buffer	10 % FCS (v/v) in PBS

Solution/buffer	Composition
Lysis buffer	10 % Triton X-100 (v/v) in DPBS
Sample diluent	10 % FCS (v/v) in PBS
Washing buffer	0.05 % Tween-20 (v/v) in DPBS
Antibody buffer	10 % FCS (v/v), 0.05 % Tween-20 (v/v) in DPBS
For flow cytometry	
FACS buffer	1 % FCS (v/v) in DPBS
Fixing solution	2 % PFA (v/v) in DPBS
For Western Blot	
Antibody buffer	1 % skimmed milk powder (w/v), 0.2 % Tween-20 (v/v) in PBS
Blocking buffer	5 % skimmed milk powder (w/v) in PBS
Co-IP lysis buffer	150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 0.5 mM Na_3VO_4, 0.5 mM NaF, 0.1 % Nonidet P-40 (v/v) in distilled water, pH 7.5, Complete ULTRA protease inhibitor cocktail tablet (1 pill/10 ml lysis buffer added directly before use)
Running buffer	1x NuPAGE MES SDS Running Buffer in distilled water
Transfer buffer	47.9 mM Tris, 38.6 mM glycine, 1.3 mM SDS, 20 % methanol (v/v) in distilled water, pH 8.3
Washing buffer	0.2 % Tween-20 (v/v) in PBS
For RNA isolation	
Lysis buffer	1 % β-mercaptoethanol (v/v) in Buffer RLT Plus
For Co-IP	
Nonidet P-40 washing buffer	50 mM HEPES, 300 mM NaCl, 0.5 % Nonidet P-40 (v/v) in distilled water, pH 7.4
For Sp1-activity assay	
Hypotonic buffer (HB)	20 mM HEPES (w/v), 5 mM NaF (w/v), 0.1 mM EDTA (v/v)
Nonidet-P40 solution	10 % Nonidet-P40 (v/v), 90 % distilled H_2O (v/v)
Phosphatase inhibitor buffer (PIB)	125 mM NaF (w/v), 250 mM β-glycerophosphate (w/v), 25 mM $NaVO_3$ (w/v)
Other	
5x PBS	140 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 in distilled water, pH 7
10x Poly-L-Lysine (PLL) stock	0.1 mg/ml (w/v) in DPBS

3.1.7 Antibodies

Antibody and dilution	Manufacturer
For p24 ELISA	
Goat polyclonal anti-rabbit IgG (Fc), HRP-conjugated	Dianova (#111-035-008)
Mouse monoclonal anti-p24 (MAK183)	ExBIO (#11-CM006-BULK)

Antibody and dilution	Manufacturer
Rabbit anti-p24 serum derived from immunized rabbits	Eurogentec
For Western Blot	
Mouse monoclonal anti-IFI16, 1:150	Santa Cruz (#sc-8023)
Rabbit polyclonal anti-IFIX, 1:1000	ProSci (#61-908)
Mouse monoclonal anti-MNDA, 1:100	Santa Cruz (#sc-390739)
Mouse monoclonal anti-AIM2, 1:100	Santa Cruz (#sc-515514)
Rabbit polyclonal anti-GFP/BFP, 1:10000	Abcam (#ab290)
Mouse monoclonal anti-FLAG, 1:1000	Sigma Aldrich (#F1804)
Mouse monoclonal anti-HA, 1:1000	Abcam (#ab18181)
Rabbit monoclonal anti-HA, 1:1000	Cell signaling (#3724S)
Mouse monoclonal anti-GAPDH, 1:200	Santa Cruz (#sc-365062)
Rat monoclonal anti-GAPDH, 1:1000	Biolegend (#607902)
Goat polyclonal anti-Sp1, 1:1000	Abcam (#ab157123)
IRDye 680RD Goat anti-Mouse IgG (H + L), 1:20000	LI-COR (#925-68070)
IRDye 680RD Goat anti-Rabbit IgG (H + L), 1:20000	LI-COR (#925-68071)
IRDye 680RD Goat anti-Rat IgG (H + L), 1:20000	LI-COR (#926-68076)
IRDye 680RD Donkey anti-Goat IgG (H + L), 1:20000	LI-COR (#926-68076)
IRDye 800RD Goat anti-Mouse IgG (H + L), 1:20000	LI-COR (#926-32210)
IRDye 800RD Goat anti-Rabbit IgG (H + L), 1:20000	LI-COR (#926-32211)
IRDye 800RD Goat anti-Rat IgG (H + L), 1:20000	LI-COR (#925-32219)
IRDye 800RD Donkey anti-Goat IgG (H + L), 1:20000	LI-COR (#925-32214)
For Co-IP	
Mouse monoclonal anti-HA, 1:400	Abcam (#ab18181)

3.1.8 Consumables

Consumable	Manufacturer
Amicon® Ultra – 15 Centrifugal Filters	Merck Millipore
C-Chip Disposable Hemocytometer, Neubauer Improved	Nano EnTek Inc
Cell culture flasks	Sarstedt AG & Co. KG
Cell culture plates	Sarstedt AG & Co. KG
Combitips plus/advanced	Eppendorf AG
Cover glasses	VWR

ELISA plate High Bind. F	Sarstedt AG & Co. KG
Filter tips (10, 1000 µl)	Nerbe plus GmbH
Filter tips (20, 200 µl)	Sarstedt AG & Co. KG
Filtropur V50 0.45 µm Vacuum Filter	Sarstedt AG & Co. KG
Gel loading tips	Santa Cruz Biotechnology, Inc.
Immobilon-FL Transfer Membrane	Merck Millipore
Microscope slides	VWR
Nunc Cell-Culture Treated Multidishes	VWR
Nunc white polystyrene 96-well Microwell plates	Nunclon/Thermo Scientific
NuPAGE Novex Bis-Tris gels	Invitrogen/Life Technologies
Polystyrene 5 ml round-bottom tubes	Falcon/Corning, Inc.
Reaction tubes (0.25, 1.5, 2 ml)	Sarstedt AG & Co. KG
RNase-Free 1.5 ml Microfuge Tubes	Ambion
Sealing tape	Thermo Scientific
Serological pipettes (5, 10, 25 ml)	Sarstedt AG & Co. KG
Tubes (15, 50 ml)	Sarstedt AG & Co. KG
Whatman chromatography paper 2 mm CHR	GE Healthcare

3.1.9 Equipment

Equipment	**Manufacturer**
Bacteria incubator BD 720	BINDER
Bacteria incubator Certomat IS	Sartorius AG
Bacteria incubator RS 422	BINDER
Centrifuge 5417C	Eppendorf AG
Centrifuge 5417R	Eppendorf AG
Centrifuge 5427R	Eppendorf AG
Centrifuge 5430	Eppendorf AG
Centrifuge 5810R	Eppendorf AG
Compact Digital MicroPlate Shaker	Thermo Scientific
CO_2 incubator HERAcell 240	Heraeus
CO_2 incubator Steri-Cult 3311	Thermo Scientific
FACSCanto II	BD Biosciences
Flex cycler	Analitik Jena AG
Gel Doc XR+	Bio-Rad Laboratories GmbH
HERAsafe HS18	Thermo Scientific
LI-COR Odyssey Imager	LI-COR Biosciences GmbH
Mini-Sub GT cell	Bio-Rad Laboratories GmbH
Model 200/2.0 Power Supply	Bio-Rad Laboratories GmbH
NanoDrop 2000	Thermo Scientific
Orbital shaker	GFL
Orion microplate luminometer	Berthold
Orion II Microplate luminometer	Titertek-Berthold

Equipment	Manufacturer
Power Pac HC	Bio-Rad Laboratories GmbH
Rotary Tube Mixer RSM7	Ratek Instruments Pty Ltd
StepOnePlus™ Real-Time PCR System	Applied Biosystems
Stuart tube roller mixer SRT6D	Sigma-Aldrich
The belly dancer/Hybridization water bath	Stovall Life Science, Inc.
Thermomixer compact/comfort	Eppendorf AG
Trans-Blot SD Semi-Dry Transfer Cell	Bio-Rad Laboratories GmbH
UV Screen	Syngene International
VMax kinetic ELISA microplate reader	Molecular Devices, LLC
Veriti™ 96-Well Thermal Cycler	Applied Biosystems
XCell SureLock Electrophoresis cell	Thermo Scientific
XCell4 SureLock Midi-Cell	Thermo Scientific

3.1.10 Software

Software	Source
BD FACSDiva	BD Biosciences
CorelDRAW 2017	Corel Corporation
Enrichr	https://amp.pharm.mssm.edu/Enrichr/
Extended Nucleic Acid Sequence Massager	https://www.cmbn.no/tonjum/seqMassager-saf.htm
GraphPad Prism 7	GraphPad Software, Inc.
Image Lab 5.2.1	Bio-Rad Laboratories GmbH
Image Studio Lite Ver 5.0	LI-COR Biosciences GmbH
ImageJ	http://imagej.nih.gov/ij/
Microsoft Office 2016	Microsoft Corporation
Multalin Ver 5.4.1	http://multalin.toulouse.inra.fr
Nanodrop 2000	Thermo Scientific
NEBaseChanger® v. 1.2.9	https://nebasechanger.neb.com/
Odyssey Image Studio	LI-COR Biosciences GmbH
Simplicity 4.02	Titertek-Berthold
SnapGene® 4.3.8.1	GSL Biotech LLC
SoftMax Pro 5.4	Molecular Devices, LLC
StepOne™	ThermoFisher

3.1.11 Other External facilities

Company	Services provided
Eurofins Genomics	siRNAs, DNA sequencing
Microsynth AG SeqLab	DNA sequencing
Biomers	DNA oligo synthesis
TIB MOLBIOL Syntheselabor GmbH	qPCR probe synthesis

3.2 Methods

3.2.1 Cloning

3.2.1.1 Primers

Cloning primers were designed using either SnapGene® or NEBaseChanger®, and effective binding of the primers to the target sequence was assessed with Multalin. Oligos were synthetized via Biomers and resuspended in H_2O HPLC Gradient grade to achieve a final concentration of 100 pmol/µl. Primers were stored at -20°C and diluted 1:10 (v/v) in H_2O HPLC Gradient grade before performing the polymerase chain reaction (PCR).

3.2.1.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out using either the Phusion High-Fidelity DNA Polymerase or the Q5® High-Fidelity DNA Polymerase, according to the manufacturers' instructions. A total of 35 thermal cycles per PCR were performed in either a Veriti™ 96-Well Thermal Cycler (Applied Biosystems) or in a Flex cycler (Analytik Jena) in a total volume of 50 µl. To validate the correctness of the reaction, 25 µl of product were separated in agarose gels (either 0.7 % or 1 % agarose (w/v) in 1x TAE buffer) with the addition of ethidium bromide (0.2 µg/ml). After 25 minutes of electrophoresis at 140 V, DNA bands were visualized via the Gel Doc XR+ (Bio-Rad Laboratories GmbH) detection system.

3.2.1.3 Enzymatic digestion

PCR products and DNA constructs were digested with enzymes and buffers provided by New England BioLabs according to the manufacturer's instructions.

3.2.1.4 DNA extraction from agarose gel

Upon digestion and separation in an agarose gel (see section 3.2.1.2 for further details), DNA fragments were excised using a UV Screen (Syngene International). DNA was purified from the gel blocks with the UltraClean 15 DNA Purification Kit according to the manufacturer's instruction.

3.2.1.5 Ligation reaction

Insert and vector DNA purified from the agarose gels were mixed and ligated via the DNA Ligation Kit 2.1 (Takara Bio Inc.). Ligation reaction was performed overnight (O/N) at 16 °C. For PCR performed with the Q5® High-Fidelty PCR Kit, ligation was performed according to the manufacturer's instructions.

3.2.1.6 Bacterial transformation

To amplify expression vectors or recombination-prone proviral constructs, *Escherichia coli* XL-2 blue or *Escherichia coli* XL-2 blue MRF' strains were used, respectively. Bacteria cells were thawed for 10 minutes on ice before mixing 1 µg plasmid DNA or 5 µl ligation product with 10 µl or 20 µl of chemically competent bacteria, respectively. The bacteria-DNA mix was incubated on ice for 20 min followed by a heat-shock at 42 °C for 30 sec. After incubation on ice for 2.5 min, 100 µl (normal transformation) or 200 µl (transformation of ligation products) S.O.C. Medium was added and the transformation mixture was incubated at either 37 °C for 30 min (XL-2 blue) or at 30 °C for 90 min (XL-2 blue MRF') on a Thermomixer compact/comfort (Eppendorf AG). Either 35 µl (normal transformation) or the whole mixture (transformation of ligation products) were plated on LB agar plates containing ampicillin (100 µg/ml) or kanamycin (50 µg/ml). Bacteria were incubated at 37 °C O/N (XL-2 blue) or at 30 °C for 24 h (XL-2 blue MRF') and single-bacteria colonies were inoculated in the desired amount of LB medium supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

3.2.1.7 Plasmid DNA purification

Plasmid DNA for cloning and sequencing was extracted from 5 ml bacterial cultures with the Mini DNA Preparation Kit (Qiagen) following the manufacturer's instructions. Plasmid DNA for transfection of eukaryotic cells was isolated from 150 ml bacterial cultures using the Wizard Plus Midi DNA Preparation Kit (Promega) according to the manufacturer's instructions. The NanoDrop 2000 spectrophotometer (Thermo Scientific) was blanked with 1 µl of H_2O HPLC Gradient grade and final DNA concentration and purity was assessed with 1 µl of plasmid DNA and the NanoDrop 2000 software.

3.2.1.8 DNA sequencing

To assess correctness of the cloning, plasmid DNA was shipped to either Microsynth AG Seqlab or Eurofins MWG GmbH. Sequencing reaction mixtures were prepared according to the provider's instructions.

3.2.2 Eukaryotic cell culture

HEK293T and TZM-bl cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with heat-inactivated fetal calf serum (FCS) (10 % (v/v)), L-glutamine (2 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml). Cells were split regularly by rinsing of the flasks with DPBS followed by Trypsin-EDTA (1.5 – 2 ml) incubation at 37 °C for 2 – 15 minutes and resuspension in fresh DMEM.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy human donors using the Biocoll Separating Solution (Biochrom GmbH), while $CD4^+$ T lymphocytes were isolated via negative selection with the RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail (STEMCELL Technologies) followed by Biocoll gradient centrifugation according to the manufacturer's instructions. $CD4^+$ T lymphocytes were kept in culture in RPMI-1640 medium supplemented with heat-inactivated FCS (10 % (v/v)), L-glutamine (2 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml).

To isolate monocyte-derived macrophages (MDMs), PBMCs were differentiated with recombinant human macrophage colony-stimulating factor (M-CSF) (15 ng/ml) and heat-inactivated human serum (10% (v/v)) in RPMI-1640 medium supplemented with FCS (10 % (v/v)), L-glutamine (2 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml) for 3 days. On day 4 post-stimulation, the culture medium was replaced with fresh DMEM supplemented with M-CSF and heat-inactivated human serum (10 % (v/v)) and kept in culture for 3 additional days.

Cell cultures were maintained at 37 °C, in an environment containing 90 % relative humidity and 5 % CO_2.

3.2.3 Transient transfection

HEK293T cells were transiently transfected via the calcium-phosphate method. Approximately $7x10^5$ cells were seeded in 2 ml supplemented DMEM per well of a 6-well plate, in order to achieve about 50-60 % confluency at the moment of transfection. Transfection mixes were prepared by mixing 5 µg plasmid DNA with 13 µl 2 M $CaCl_2$ and left to rest at room temperature (RT) while the culture medium was replaced with 2 ml fresh DMEM. Transfection mixes' total volume was adjusted to 100 µl with the addition of H_2O HPLC Gradient grade. Finally, 100 µl 2x HBS was added in a dropwise manner and the resulting mixture was further resuspended via a 10-times pipetting step before adding it dropwise to the cell culture medium. To generate HIV-1 stocks, cells were transfected exclusively with 5 µg of proviral construct and, if indicated, 1 µg of an expression plasmid for the vesicular stomatitis virus glycoprotein (VSV-G) was added to the transfection mix (Fouchier et al., 1997). Prior to spinoculation, virus stocks were concentrated by a 10-fold factor using Amicon® Ultra – 15 Centrifugal filteres (Merck Millipore). Whenever increasing amount of pCG-based expression constructs were used for a titration experiment, an empty vector control plasmid was added to keep the total DNA amount constant. Between 8 and 16 h post transfection, the cell culture medium was replaced with 2 ml fresh DMEM.

Between 40 and 48 hours post transfection, cell culture supernatants were harvested in sterile 2 ml reaction tubes, centrifuged at 3.000 rpm for 3 min and transferred into fresh 2 ml reaction tubes. Cells were rinsed once with 1x DPBS and harvested by mechanical washing.

3.2.4 qRT-PCR

Real-time reverse transcription-PCR (qRT-PCR) was used to quantify the total viral transcript levels. Transfected HEK293T cells were rinsed once with 1x DPBS, collected in 1.5 ml reaction tubes and lysed in 350 µl Buffer LRT Plus supplemented with 1 % β-mercaptoethanol (v/v). Transduced $CD4^+$ T lymphocytes were instead pelleted in a V bottom-shaped 96-well plate (Sarstedt AG & Co. KG) and lysed in 100 µl Buffer RLT Plus supplemented with 1 % β-mercaptoethanol (v/v). Samples were kept on ice and total RNA isolation was performed using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instruction. To avoid any quantification bias due to the non-exon-spanning nature of the primers and probes, residual genomic DNA was removed with the DNA-free™ DNA Removal Kit (Thermo Scientific) according to the manufacturer's instructions. Purified RNA concentrations were determined via the NanoDrop 2000 spectrophotometer and equal RNA amounts were used to synthetize cDNA with the PrimeScript RT Reagent Kit. Each reaction was performed with random hexamers and oligo dT primers with a Veriti™ 96-Well Thermal Cycler (Applied Biosystems). To validate the complete removal of genomic DNA, control reactions were performed without reverse transcriptase. Resulting cDNA was subjected to qRT-PCR using TaqMan™ Fast Universal PCR Master Mix using the StepOnePlus™ Real-Time PCR System in multiplex reactions with GAPDH primers and probes (ThermoFisher) as internal control. Total amount of viral transcripts was quantified using the ΔΔCt method. Viral primers and probes were designed as described (Yukl et al., 2018) to measure proximal elongation (R-U5/*gag* or Long LTR), distal elongation (*nef*) and termination and maturation of viral transcripts (U3-polyA). Primers and fluorescent probes are listed in Table S2.

3.2.5 Flow cytometry

Flow cytometry was used to assess the modulation of LTR-driven enhanced green fluorescence protein (eGFP) expression by PYHIN proteins. HEK293T cells were cotransfected with expression vectors for PYHIN proteins co-expressing BFP via an IRES and HIV-1 NL4-3 proviral constructs co-expressing eGFP via an IRES. At 48 hours post transfection, cells were washed in FACS buffer and fixed in 2 % PFA for 30 min at 4 °C. Mean fluorescence intensities (MFI) of eGFP in the total BFP positive (+) population was

determined with the FACSCanto II (BD Biosciences). Flow cytometry data were analyzed with the BD FACSDiva software.

3.2.6 Quantification of infectious HIV-1 yield

Infectious virus yield from transfected HEK293T cells, infected macrophages or transduced $CD4^+$ T lymphocytes was measured via the TZM-bl reporter cell system. TZM-bl cells are HeLa-derived naturally expressing CXCR4 cells that have been transduced with lentiviral vectors to stably express CD4 and CCR5, to render them susceptible to HIV-1 infection (Platt et al., 1998). TZM-bl cells have been further genetically engineered with the stable insertion of a lentiviral vector expressing the *Escherichia coli* β-galactosidase (β-gal) gene, whose promoter is activated by the viral transactivator of transcription (Tat) (Wei et al., 2002).

To quantify infectious HIV-1 yield, $6x10^3$ TZM-bl reporter cells were seeded in 100 µl supplemented DMEM per well 24 h prior to infection. Infection of TZM-bl reporter cells was performed in technical triplicate by addition of 0.1-100 µl supernatants. Three days post infection, cell culture supernatants were removed and cells were lysed in 40 µl Gal-Screen substrate (Applied Biosystem or Life Technologies) and incubated at RT for 30 min. Afterwards, 35 µl of the cell lysates were transferred to white 96-well Microwell plates (Nunc) and β-gal-derived luminescence was quantified as relative light units per second (RLU/s) via an Orion II Microplate luminometer (Titertek-Berthold).

3.2.7 p24 ELISA

To investigate the role of the U3-LTR, Sp1 binding sites, TAR loop and the viral Tat protein in the sensitivity against IFI16, IFIX and MNDA, we made use of previously published HIV-rtTA constructs, harbouring an internal Tet-On gene regulation system to activate transcription (Das et al., 2011). To activate viral gene expression, doxycycline (Sigma Aldrich) was added to the cell culture medium (1 µg/ml) before and after transfection. As some of these constructs do not harbour a functional Tat protein, the total amount of viral capsid protein p24 was quantified using an in-house made enzyme-linked immunosorbent assay (ELISA).

Briefly, high Binding ELISA plates (Sarstedt) were coated with 100 µl mouse monoclonal anti-p24 antibody (ExBio) in DPBS overnight at room temperature and in a humid environment. Between the individual incubation steps, plates were washed three times with 350 µl washing buffer. Plates were blocked with blocking buffer for 2 h at 37 °C and sealed

with a sealing tape (Thermo Scientific). A total of 180 µl virus containing cell culture supernatants were lysed in 20 µl lysis buffer and incubated for 1 h at 37 °C prior to their dilution with sample diluent to obtain the desired concentration. 100 µl of a serially 1:2 diluted p24 protein standard (Abcam) and 100 µl of the diluted samples were incubated overnight at room temperature and in a humid environment. ELISA plates were then incubated with 100 µl rabbit anti-p24 serum (Eurogentec) diluted in antibody buffer and afterward with 100 µl goat polyclonal anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Dianova) in antibody buffer, each for 1 h at 37 °C. Lastly, 100 µl SureBlue TMB 1-Component Microwell Peroxidase Substrate (SeraCare Life Sciences) was added and plates were incubated for 20-25 min at room temperature on the Compact Digital MicroPlate Shaker (Thermo Scientific) before interrupting the reaction with 50 µl 0.5 M H_2SO_4. Adsorption was measured at 450 nm with a baseline correction at 650 nm using a Vmax kinetic ELISA microplate reader (Molecular devices). p24 concentrations were calculated using the PRISM 8 Software by interpolating the standard curve using the Sigmoidal, 4PL, X is log(concentration) option.

3.2.8 Stimulation of $CD4^+$ T lymphocytes and MDMs

$CD4^+$ T lymphocytes were isolated as previously described in section 3.2.2. A total of $4x10^6$ cells were seeded in 2 ml supplemented RPMI-1640 medium per well of a 12-well plate and either left unstimulated or treated with IL-2 (Miltenyi Biotec GmbH) (10 ng/ml) alone, IL-2 (10 ng/ml) + IFNα (Pbl assay science) (500 U/ml), IL-2 + IFNγ (Sigma Aldrich) (200 U/ml), IL-2 (10 ng/ml) in combination with anti-CD3/CD28 (Thermo Scientific) beads in a cell:bead ratio of 1:1. Three days post stimulation, cells were harvested, washed once with 1x DPBS, lysed in 100-200 µl Co-IP buffer and processed for Western blot analysis (see section 3.2.10 for further details).

MDMs were isolated from PBMCs as described in section 3.2.2 and $2x10^6$ cells were seeded in 1 ml supplemented DMEM per well of a 12-well Nunc Cell-Culture Treated Multidish (VWR). MDMs were either stimulated with IFNα (500 U/ml) or IFNγ (200 U/ml) on day 7 post differentiation in technical duplicates. Three days post stimulation, cells were washed once with 1x DPBS, lysed directly in the plate and lysates from two identical stimulations were pooled together before processing them for Western blot analysis (see section 3.2.10 for further details).

3.2.9 siRNA transfection and infection of MDMs

Knockdown experiments in MDMs were performed to assess whether endogenous PYHIN proteins restrict HIV-1 replication in primary macrophages. MDMs were isolated from PBMCs as previously described in section 3.2.2 and a total of $2.5x10^6$ cells were seeded in 1 ml supplemented DMEM per well of a 12-well Nunc Cell-Culture Treated Multidish (VWR). At days 7 and 9 post differentiation, MDMs were transfected with either a non-targeting control siRNA or IFI16-, IFIX-, MNDA-, AIM2- or a combination of nuclear PYHIN-specific siRNA pools. siRNA transfections were performed in technical duplicates by mixing 75 µl Opti-MEM with either 1.46 µl siRNA (20 µM) or 3 µl Lipofectamine RNAiMAX Transfection Reagent. Subsequently, the solution of Opti-MEM and Lipofectamine was added to the solution of Opti-MEM and siRNAs, further mixed by pipetting 10 times and incubated at RT for 15 min. Before transfection, MDMs culture medium was replaced with 500 µl fresh supplemented DMEM and the transfection mixes (150 µl/well) were added dropwise to the cells. At 14 h post transfection on day 7 after differentiation, culture medium was replaced with fresh DMEM supplemented with L-glutamine (2 mM), streptomycin (100 µg/ml), penicillin (100 U/ml), human heat-inactivated serum (10 % (v/v)) and M-CSF (15 ng/ml). 14 h post transfection on day 9 after differentiation, MDMs were infected with the macrophage tropic strain AD8. HIV stocks were produced as previously described in section 3.2.3 and stored at 4 °C until the infection. For infection, MDMs culture medium was replaced with a mixture of 500 µl supplemented DMEM and 300 µl virus stock. MDMs culture medium containing the infectious virus was replaced 12 h post infection, cells were washed once with 500 µl 1x DPBS, and 1 ml fresh supplemented DMEM was added afterwards. On day 3 post infection, MDMs culture medium was harvested and replaced with fresh supplemented DMEM. On day 6 post infection, MDMs culture medium was harvested and cells were washed once with 500 µl 1x DPBS. Supernatants harvested on day 3 and 6 post infection were used to determine infectious virus yield via the TZM-bl reporter cell system (see section 3.2.6 for further details). To assess knockdown efficiency, cells were lysed in 100 µl Co-IP lysis buffer directly in the plate and cell lysates were further processed for Western Blot analysis (see section 3.2.13 for further details).

3.2.10 Cas9/gRNA transfection and transduction of $CD4^+$ T lymphocytes

The Clustered Regularly Interspaced Short Palindromic Repeats (CIRSPR)/CRISPR associated protein 9 (Cas9) genome editing technology was employed to knockout IFI16 to investigate its relevance as a retroviral restriction factor in primary $CD4^+$ T lymphocytes.

Cells were isolated as previously described in section 3.2.2. Cells were cultured in RPMI-1640 medium containing 20 % FCS and stimulated with IL-2 (10 ng/ml) and with anti-CD3/CD28 beads. At three days post stimulation, $1x10^6$ cells were nucleofected with the HiFi Cas9 Nuclease V3 (IDT) complexed with either a non-targeting or an IFI16-specific single guide RNA (sgRNA) (IDT) (80 pmol/300 pml), using the Amaxa™ 4D-Nucleofector™ Human Activated T Cell P3 Lonza Kit (Lonza) with the pulse code EO115. At four days post nucleofection, $3x10^5$ cells/sample were transduced with the listed VSV-G pseudotyped HIV-1 primary strains by spinoculation in a Centrifuge 5810R (Eppendorf AG) pre-heated to 37 °C, for 2 h at 1.000 g. The remaining uninfected cells were pelleted and lysed for Western Blot analysis, as further described in section 3.2.12. At three days post transduction, supernatants were harvested, diluted 1:10 in DMEM containing 10 % FCS, L-glutamine (2mM), streptomycin (100 µg/ml) and penicillin (100 U/ml) and used to quantify infectious virus yield, as previously described in section 3.2.6. Cell pellets were frozen at -80 °C and subsequently lysed for RNA extraction and qRT-PCR, as described in section 3.2.4.

3.2.11 Co-immunoprecipitation with benzonase treatment

Co-immunoprecipitation experiments were performed to verify the interaction between PYHIN proteins and Sp1 expressed at endogenous level in HEK293T cells, either in the presence or absence of Benzonase nuclease to investigate the impact of nucleic acids on the interaction. Cells were transfected with an expression construct for C-terminally HA-tagged PYHIN proteins, with one whole 6-well plate per sample (see section 3.2.3 for further details). Two days post transfection, cell culture medium was discarded, cells were washed and harvested in 1 ml of 1x DPBS and pelleted by centrifugation at 1.300 rpm for 5 min. Upon discarding the supernatant, cell pellets were initially frozen for 4 to 16 h at -20 °C and subsequently thawed on ice. Lysis was performed in 1 ml Co-IP lysis buffer (see section for 3.2.10 for further details). Total cell lysates were centrifuged at $4x10^3$ rpm for 20 min at 4 °C and supernatants from the same samples were pooled together. 60 µl of cell lysates were immediately frozen at -20 °C and kept for the input control. Remaining cell lysates were divided into two aliquots of 960 µl, to which 9.6 µl 100 mM $MgCl_2$ were added in addition to either 3.2 µl Benzonase nuclease (750 U/ml) or 3.2 µl Co-IP lysis buffer as a control. Samples were incubated for 2 h at RT on a Stuart tube roller mixer SRT6D (Sigma-Aldrich). Cell lysates were subsequently treated with an HA-tag specific antibody (Abcam) and incubated overnight at 4 °C. To validate efficient removal of nucleic acids, 20 µl of the supernatants were diluted in 6 µl Loading dye (Carl Roth GmbH & Co. KG) and run on a 1

% agarose gel (w/v) (see section 3.2.1.2 for further details). Remaining cell lysates were co-immunoprecipitated using Pierce Protein A/G Magnetic Beads (Thermo Scientific) by incubating the beads-supernatants mixture for 1 h at RT. Beads were washed three times with Nonidet-P40 washing buffer and rinsed once with H_2O HPLC Gradient grade. Proteins were eluted by boiling the beads for 10 min at 95 °C in 60 µl 1x Protein Sample Loading Buffer (LI-COR) and denatured by the addition of 1.75 µl β-mercaptoethanol. Input control lysates were diluted in 20 µl of a 10 % β-mercaptoethanol in 4x Protein Sample Loading Buffer (LI-COR) (v/v) solution and boiled for 10 min at 95 °C. Input and eluted samples were then processed for Western Blot analysis.

3.2.12 Western Blot

Western blot analysis was performed to validate the expression of cellular proteins. Unless stated differently, cells harvested from one well of a 6-well plate were lysed in 200 µl Co-IP lysis buffer. Cell lysates were incubated on ice for 20 min and centrifuged at 14.000 rpm for 20 min at 4 °C. Afterwards, 180 µl of the supernatants were mixed with 60 µl of a 10 % β-mercaptoethanol in 4x Protein Sample Loading Buffer (LI-COR) (v/v) solution and boiled at 95 °C for 5 min. Equal amounts of lysates were run in a 4-12 % NuPAGE Novex Bis-Tris precast polyacrylamide gels (Invitrogen), separated for either 1 or 1.5 h at 130 V in the case of small and big gels respectively. Immobilion-FL Transfer PVDF Membranes (Merck Millipore) were soaked in 100 % methanol for 1 min and then in distilled H_2O for 1 min, before incubating them in transfer buffer for 20 min. Separated proteins were blotted onto activated membranes with the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories GmbH) for 2 hours. Membranes were then subsequently incubated in 50 ml blocking buffer for 1 h at RT. Membranes were stained using primary antibodies directed against IFI16 (Santa Cruz), IFIX (ProSci), MNDA (Santa Cruz), AIM2 (Santa Cruz), HA (Abcam/Cell Signaling), Sp1 (Abcam), FLAG (Sigma Aldrich), GFP/BFP (Abcam) and GAPDH (Santacruz/Biolegend), either by overnight incubation at 4 °C or 2 h at RT. Anti-mouse, anti-goat and anti-rabbit Infrared Dye labeled secondary antibodies (LI-COR) were used for detection of the primary antibodies by incubating the membranes 30 min at RT. Membranes were scanned with an infrared LI-COR Odyssey Imager (LI-COR Biosciences), and band signal intensities were quantified with Image Studio Lite Version 5.0 (LI-COR Biosciences).

3.2.13 Sp1-DNA activity assay

The Sp1-DNA activity assay was carried out to investigate the effects of nuclear PYHIN proteins on binding of Sp1 to its consensus DNA target sequence. For that purpose,

HEK293T cells were transfected with expression vectors encoding either the full length or the N-terminally truncated PYHIN proteins (1 µg). To prepare nuclear extracts, cells were washed with 1 ml of an ice-cold 5 % PIB/PBS (vol/vol) solution 48 h post transfection, detached with 300 µl trypsin/well and harvested in 1 ml of an ice-cold 5 % PIB/PBS (vol/vol) solution. Cells were then pelleted by centrifugation at 1.600 rpm for 5 min at 4 °C, resuspended in 1 ml ice-cold HB and incubated on ice for 15 min to allow the cells to swell. Upon addition of 50 µl of a 10 % Nonidet-P40 (v/v) solution, cells were centrifuged at 14.000 rpm for 1 min at 4 °C, supernatants were discarded and the nuclear pellets were resuspended in 50 µl Complete Lysis Buffer AM1 (ActiveMotif). Tubes were incubated for 30 min at 4 °C with end-over-end mixing and centrifuged afterwards at 14.000 rpm for 10 min at 4 °C. The supernatants containing the nuclear cell extracts were transferred to fresh tubes and quantified using the Pierce Gold BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Nuclear extract volumes were diluted in Complete Lysis Buffer AM1 (ActiveMotif) to reach a final concentration of 40 µg/µl, and the TransAM Sp1 Transcription Factor Assay Kit (ActiveMotif) was used to measure the binding of Sp1 according to the manufacturer's instructions. Absorbance was measured with a VMax kinetic ELISA microplate reader (Molecular Devices, LLC) at 450 nm with a reference wavelength of 655 nm.

3.2.14 Statistical analysis

Statistical analyses were performed with the GraphPad Prism 8 software. p-values (p) were calculated using the one sample t-test or either paired or unpaired Student's t test. Means are to be considered significantly different if $p \leq 0.05$ (*), ≤ 0.01 (**) or ≤ 0.001 (***).

3.2.15 Ethical statement

Experiments involving the isolation and differentiation of $CD4^+$ T lymphocytes and PBMCs from peripheral blood were approved by the Ethics Committee of the Ulm University Medical Center. All donors provided written informed consent.

4 Results

4.1 Nuclear PYHIN proteins inhibit synthesis of viral transcripts

We have previously shown that IFI16 suppresses HIV-1 transcription by reducing the availability of the host transcription factor Sp1 (Hotter et al., 2019). To validate whether other members of the PYHIN family share this antiretroviral activity, we generated expression constructs for IFIX, MNDA and AIM2 carrying an HA-tag at the C-terminus and co-expressing the blue fluorescent protein (BFP) via an internal ribosome entry site (IRES). HEK293T cells were co-transfected with either of these plasmids, together with a proviral construct encoding for the T cell line adapted HIV-1 strain NL4-3 coexpressing an enhanced green fluorescent protein (eGFP) via an IRES (Schindler *et al.*, 2005). Analysis of the eGFP mean fluorescence intensity (MFI) showed that, in line with the IFI16 data (Hotter et al., 2019), also IFIX and MNDA dose-dependently reduce HIV-1-driven eGFP expression (Fig. 7A, left panel). A similar inhibitory effect was observed upon quantification of infectious virus yield from the cotransfected HEK293T cells (Fig. 7A, right panel). In contrast, AIM2 displayed no significant inhibitory effect (Fig. 7A). Real time quantitative PCR (qRT-PCR) confirmed that, similarly to IFI16 (Hotter et al., 2019), IFIX and MNDA dose-dependently reduce the amount of viral mRNAs representing proximal elongation (R-U5/*gag*), distal elongation (*nef*), as well as termination and maturation of transcription (U3-Poly A). In comparison, AIM2 hardly affected viral gene expression (Fig. 7B). Altogether, these results indicate that nuclear PYHIN proteins reduce infectious virus yield by suppressing synthesis of viral transcripts.

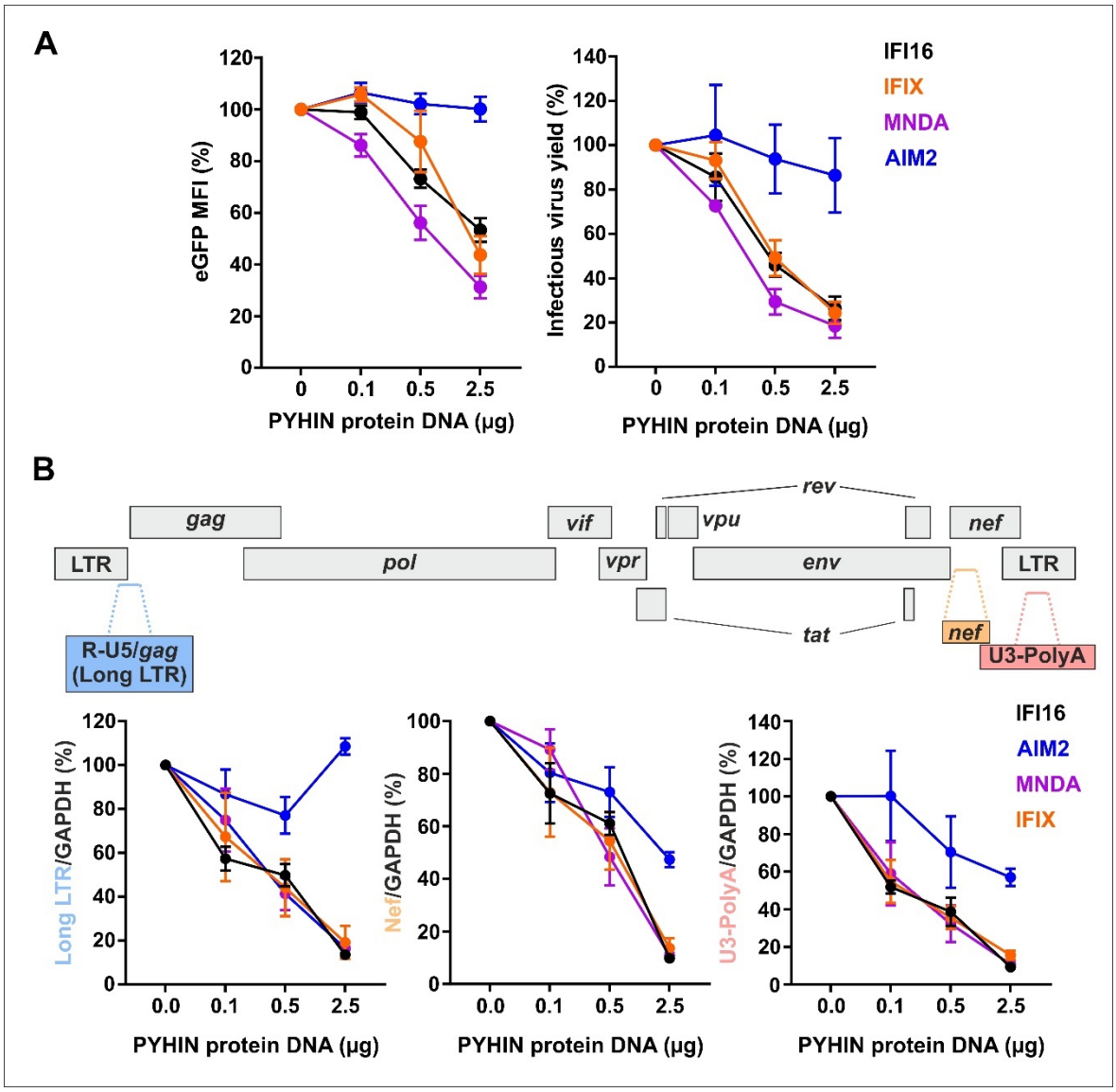

Figure 7. Nuclear PYHIN proteins reduce synthesis of viral transcripts. (A) HEK293T cells were cotransfected with increasing doses of an expression plasmid for different human PYHIN proteins expressing BFP via an IRES and a proviral construct of HIV-1 NL4-3 expressing eGFP via an IRES. LTR-driven eGFP expression in transfected cells was assessed by flow cytometry (left panel), and infectious virus yield was determined by infecting TZM-bl reporter cells (right panel). Results shown are the mean ± SEM, n=5-7. **(B)** Schematic representation of the HIV-1 genome. The regions targeted by primers and probes for qRT-PCR are highlighted by coloured squares (upper panel). HEK293T cells were cotransfected with increasing doses of an expression plasmid for different human PYHIN proteins and a proviral construct of HIV-1 NL4-3. Viral transcripts were quantified via qRT-PCR using previously published primers and probes (Yukl et al., 2018). Results shown are the mean ± SEM, n=3. First published in: Bosso et al., 2020, Fig. 1, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

4.2 PYHIN proteins restrict HIV-1 replication in primary target cells

IFI16 is expressed in primary HIV-1 target cells and moderately upregulated by interferons (IFNs) (Hotter et al., 2019; Søby et al., 2012; Wei et al., 2003). MNDA is constitutively expressed in cells of the myeloid cells (Briggs et al., 1994b; Duhl et al., 1989; Goldberger et al., 1986) and upregulated by IFN-α in monocytes (Briggs et al., 1994a). IFIX and AIM2 are as well expressed in $CD4^+$ T lymphocytes (Monroe et al., 2014) and the latter one in monocyte-derived macrophages (MDMs) as well (Yang et al., 2015). Little is known however about their modulation by interferons. Western blot analysis showed that the three IFI16 isoforms (Johnstone et al., 1998a) are expressed in both $CD4^+$ T lymphocytes and MDMs, and modestly upregulated by IFN-α and both IFN-α and IFN-γ, respectively (Fig. 8A and 8B). At least six isoforms of IFIX are expressed as a result of alternate splicing (α1, α2, β1, β2, γ1 and γ2), of which IFIX α2, β2 and γ2 harbour a deletion of nine conserved amino acids compared to IFIX α1, β1 and γ1 (Ding et al., 2004). Three isoforms were readily detectable in MDMs while only two of them (β and γ) were expressed, at lower levels, in $CD4^+$ T lymphocytes. None of them was however induced by IFN treatment. Stimulation of $CD4^+$ T lymphocytes with anti-CD3/CD28 beads, which mimics physiological T cell activation by antigen presentation (Trickett and Kwan, 2003), led to an average 3.2 fold upregulation of the IFIX β isoform (Fig. 8A and 8B). In agreement with published results (Briggs et al., 1994a; Duhl et al., 1989), MNDA was expressed in primary macrophages, but not $CD4^+$ T cells, and upregulated by IFN-α (Fig. 8A and 8B). AIM2 was detectable in both $CD4^+$ T lymphocytes and MDMs but not induced by IFN stimulation (Fig. 8A and 8B). Overall, IFI16, IFIX, MNDA and AIM2 are expressed in HIV-1 primary target cells and are selectively upregulated by specific stimuli.

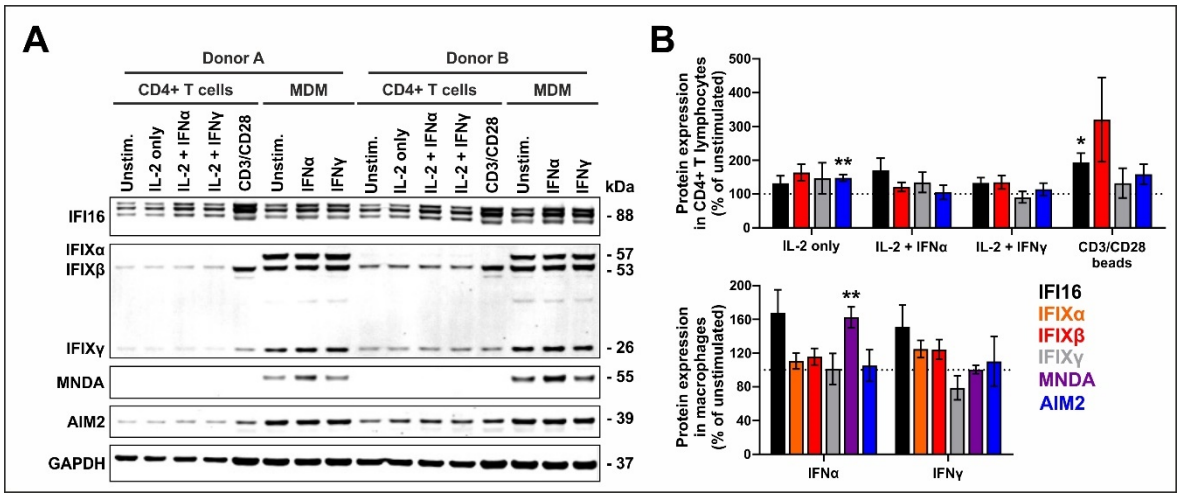

Figure 8. PYHIN proteins are expressed in HIV-1 target cells. (A) $CD4^+$ T cells were stimulated with IL-2 + IFNα, IL-2 + IFNγ, IL-2 + anti-CD3/CD28 beads or IL-2 alone, while monocyte-derived macrophages (MDMs) were stimulated with either IFNα or IFNγ. IL-2 to was added to the $CD4^+$ T lymphocytes to stimulate cell proliferation. PYHIN protein expression was assessed by Western blot analysis. **(B)** Quantification of the protein levels shown in (A). PYHIN expression levels were internally normalized to GAPDH and subsequently normalized to the respective unstimulated (Unstim.) control. Results shown are the mean ± SEM, n=3-5 donors. Asterisks indicate statistically significant differences compared to the unstimulated controls (set as 100%). First published in: Bosso et al., 2020, Fig. 3, S2, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

Endogenous levels of IFI16 in primary MDMs are sufficient to inhibit HIV-1 replication (Hotter et al., 2019). To investigate whether the remaining PYHIN family members also play a relevant role in the antiviral response in primary HIV-1 target cells, siRNA knockdown (KD) assays in MDMs derived from six to nine donors were performed. Western blot analysis confirmed the KD of PYHIN proteins with an average efficiency of approximately 60 % for IFI16, 50 % for IFIX and AIM2 and 80 % for MNDA (Fig. 9A and 9B). In most cases, KD of one PYHIN protein also marginally affected the overall expression levels of the others (Fig. 9B). Individual KD of IFI16 and IFIX resulted in an average 23.1-fold enhancement of HIV-1 replication at 6 dpi while reduced expression of MNDA increased infectious virus yield up to 17.3-fold. In contrast, KD of AIM2 did not enhance infectious virus yield (Fig. 9C). To investigate whether nuclear PYHIN proteins may cooperate to exert antiviral activity, we performed a combined KD by simultaneously transfecting siRNAs directed against IFI16, IFIX and MNDA (nPYHIN). Western blot analysis confirmed reduction of nuclear PYHIN expression levels with an average efficiency of 56 % and this resulted in a 27.6-fold increase in infectious virus yield (Fig. 9B and 9C).

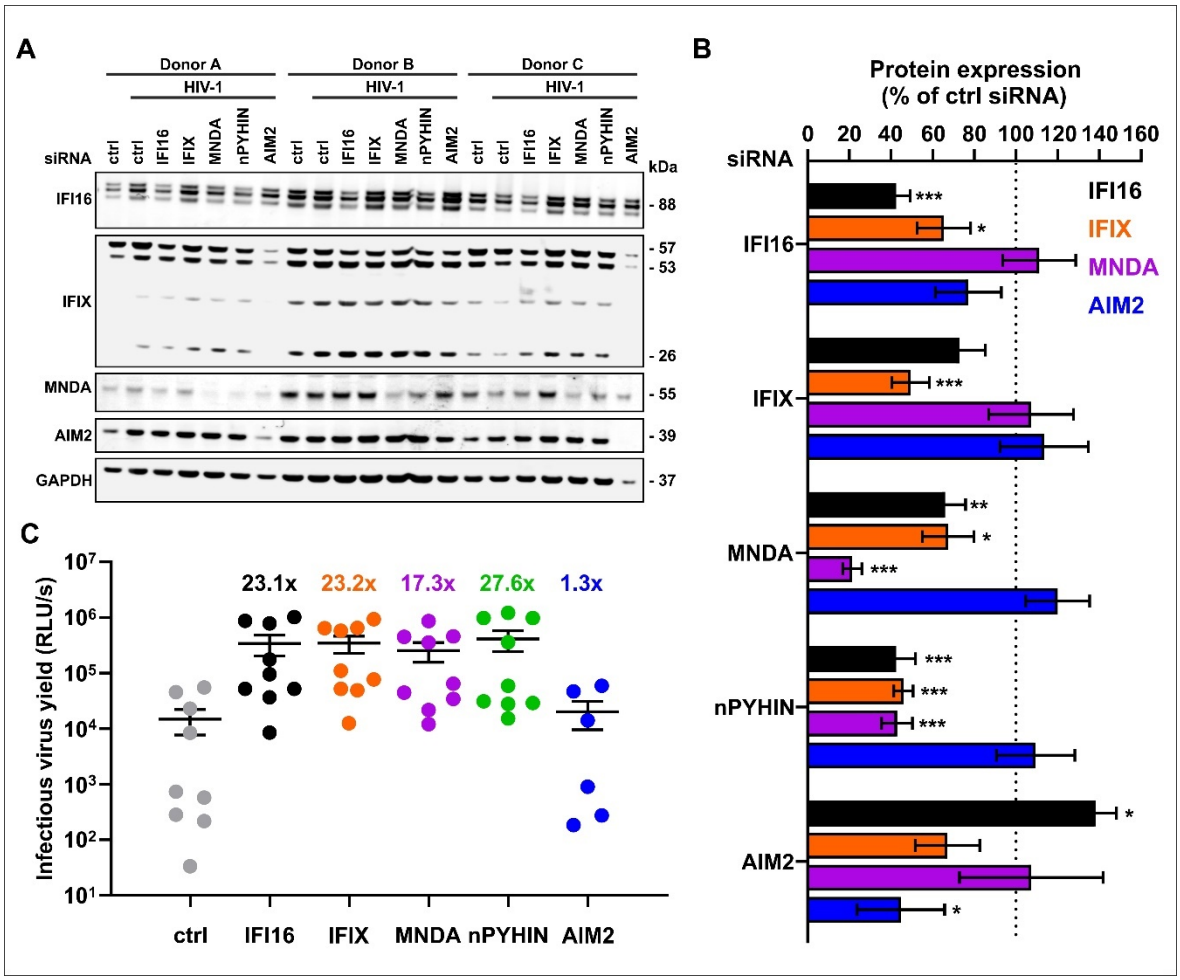

Figure 9. Nuclear PYHIN proteins restricts HIV-1 replication in primary macrophages. (A) Monocyte-derived macrophages were transfected with either a non-targeting (ctrl) or PYHIN-specific siRNAs and infected with the macrophage-tropic HIV-1 AD8 strain. 6 days post infection (d.p.i.), PYHIN expression levels were determined by Western blotting. One representative Western blot with three donors is shown. **(B)** Quantification of PYHIN expression levels analyzed as described in (A). Values were normalized to the respective PYHIN expression levels in the infected control siRNA sample. Results shown are the mean ± SEM, n=6-9 donors. nPYHIN indicate the combined KD of nuclear PYHIN proteins. Asterisks indicate statistically significant differences compared to the infected control siRNA (set as 100%) **(C)** Quantification of infectious virus yield at 6 d.p.i. from siRNA-treated macrophages as described in (A). Mean values obtained from nine donors are indicated by a horizontal line. Numbers indicate n-fold increase compared to the control siRNA. First published in: Bosso et al., 2020, Fig. S2, 3, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

$CD4^+$ T lymphocytes do not express MNDA and relatively low levels of IFIX-α (Fig. 8A). In contrast, IFI16 is detected at comparable levels in macrophages and $CD4^+$ T lymphocytes. To validate the relevance of IFI16 in the latter cell type, we employed three HIV-1 infectious molecular clones (IMCs) that show different sensitivity to this antiviral protein in an

HEK293T-based overexpression assay. HIV-1 clones CH040 and CH058 are inhibited on average 8.1-fold and 5.2-fold, respectively, while the HIV-1 CH042 strain is hardly affected by co-expression of IFI16 (Fig. 10A). Both clones CH040 and CH058 are part of clade B of the pandemic M group of HIV-1 (Ochsenbauer et al., 2012) while CH042 belongs to clade C (Freel et al., 2012). While subtype B is endemic in Europe and North America, subtype C is the most prevalent in India and Central Africa. Notably, subtype C viruses account for nearly 50 % of all HIV-1 infections worldwide (Gartner et al., 2020). Having previously shown that KD of IFI16 in primary macrophages led to higher replication efficiency of subtype B but not subtype C viruses (Hotter et al., 2019), we wanted to validate this result in primary $CD4^+$ T lymphocytes, which represent the main HIV-1 target cell type. To address this question, we did use the recently developed CRISPR-Cas9 genome editing technique (Hultquist et al., 2019) to perform an IFI16 knock-out (KO) in primary $CD4^+$ T lymphocytes. Western blot analysis revealed that nucleofection of the Cas9/IFI16-gRNA complex reduced IFI16 expression levels by approximately 70 % and did not alter total IFIX protein levels (Fig. 10B). Notably, partial depletion of IFI16 enhanced infectious yield of the HIV-1 CH040 and CH058 strains by 18.8-fold and 11.4-fold, respectively, at 3 d.p.i. Reduction of IFI16 protein levels, however, had no enhancing effect on the replication of the HIV-1 CH042 strain (Fig. 10C). Real-time quantitative PCR (qRT-PCR) on infected cells showed a significantly increased synthesis of proximal (R-U5/*gag*), distal (*nef*) and matured viral transcripts (U3-Poly-A) for the HIV-1 CH040 and CH058 IMCs, while no significant change was observed for HIV-1 CH042 (Fig. 10D). Taken together, these results show that nuclear PYHIN proteins restrict HIV-1 replication in primary macrophages and $CD4^+$ T lymphocytes and that HIV-1 strains differ in their sensitivity to IFI16.

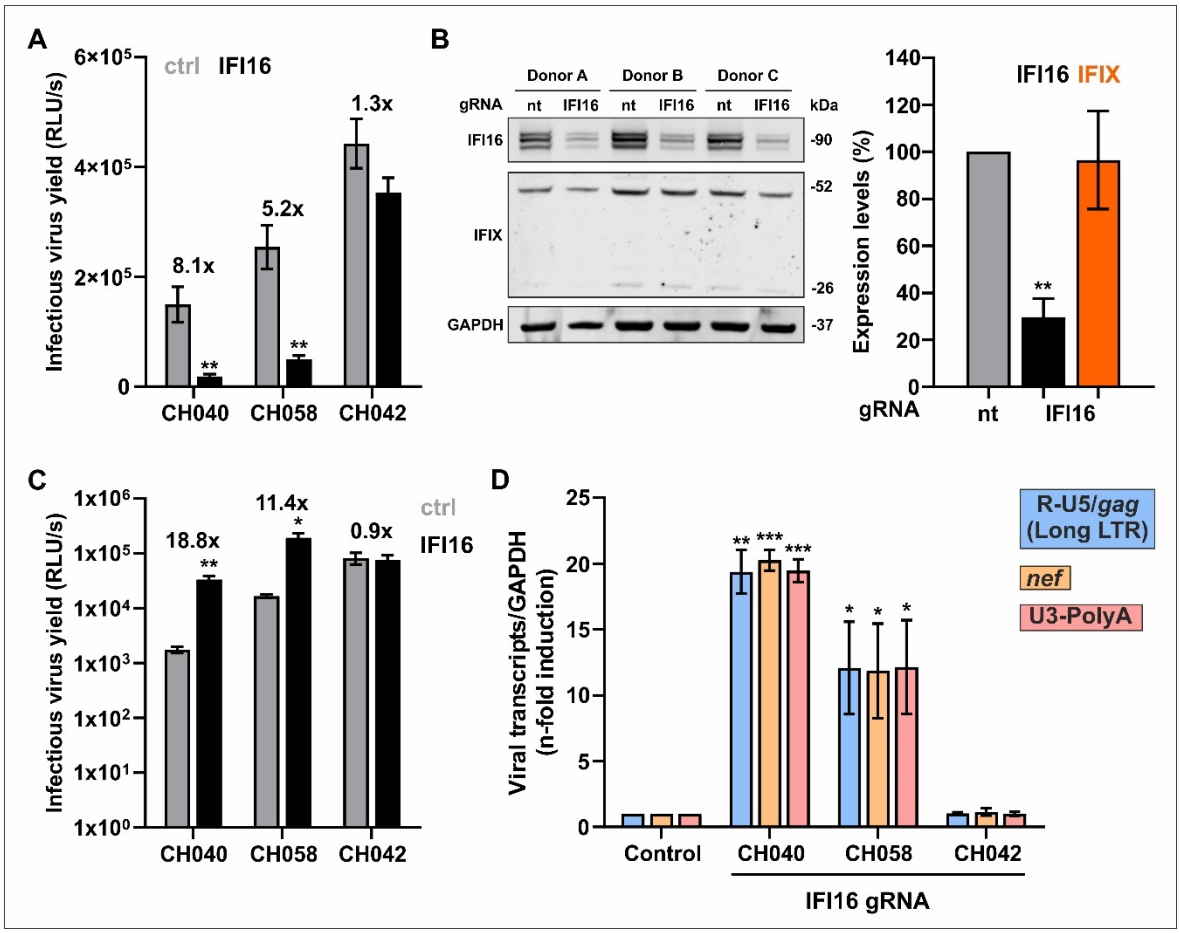

Figure 10. IFI16 suppresses HIV-1 gene expression in CD4⁺ T lymphocytes. (A) HEK293T cells were cotransfected with either an empty vector or an expression plasmid for IFI16 and proviral constructs for the indicated HIV-1 primary strains. Infectious virus yield was measured by infecting TZM-bl reporter cells. Results shown are the mean ± SEM, n=4. Numbers on top indicate n-fold reduction compared to the empty vector. Asterisks indicate statistically significant differences between IFI16 and the empty vector control **(B)** CD4⁺ T lymphocytes were nucleofected with Cas9 complexed with either a non-targeting (nt) or an IFI16-specific gRNA. KO efficiency was assessed via Western blot analysis (left panel) and total protein levels were subsequently quantified (right panel). Results shown are the mean ± SEM, n=3 donors. Asterisks indicate statistically significant differences between the IFI16-specific and nt gRNA treated samples. **(C)** CD4⁺ T lymphocytes were treated as described in (B) and transduced with the indicated VSV-G pseudotyped HIV-1 primary strains. Infectious virus yield was assessed at 3 days post transduction (d.p.t.) by infecting TZM-bl reporter cells. Results shown are the mean ± SEM, n=3 donors. Asterisks indicate statistically significant differences between the IFI16-specific and nt gRNA treated samples. (**D)** CD4⁺ T lymphocytes were treated as described in (B) and (C). Viral transcripts were quantified via qRT-PCR using previously published primers and probes (Yukl et al., 2018). Results shown are the mean ± SD, n=3 donors. First published in: Bosso et al., 2020, Fig. 8, 4, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

4.3 Sp1-binding sites and Tat expression are required for PYHIN-mediated restriction of HIV-1

The available literature illustrating that IFI16, IFIX and MNDA silence transcription of DNA viruses (see Table 1) suggested that the reduction in viral transcript levels (Fig. 7B) might be a consequence of inhibition of the proviral promoter. HIV-1 gene expression is driven by the 5' LTR (Jones et al., 1986) via synergistic transactivation of Sp1 and NF-κB (Perkins et al., 1993) and further enhanced by the viral protein Tat (Garber et al., 1998; Wei et al., 1998). To identify viral elements determining HIV-1 gene expression that are targeted by nuclear PYHIN proteins, we employed a panel of proviral constructs harbouring multiple mutations and deletions in the LTRs, the TAR loop and/or the Tat protein (Das et al., 2007, 2011; Verhoef et al., 2001). To control the production of viral transcripts, the *nef* coding region of these constructs has been replaced by the reverse tetracycline-controlled transactivator (rtTA) gene (Gossen et al., 1995; Urlinger et al., 2000), and two or more *tetO* operator elements have been introduced between the NF-κB and Sp1 binding sites (Das et al., 2007; Verhoef et al., 2001). This setup allows a tight regulation of viral gene expression by the simple addition of doxycycline (Verhoef et al., 2001) (Fig. 11A). The TZM-bl reporter cell system is based on expression of the Tat protein (Wei et al., 2002) and most of these proviral constructs either harbour a non-functional Tat or lack Tat expression. Therefore, to characterize the antiviral effect of IFI16, IFIX and MNDA against these proviral constructs, we quantified the amount of p24 capsid protein released in the cell culture supernatant via enzyme-linked immunosorbent assay (ELISA). Experiments in transfected HEK293T cells revealed that proviral constructs in which the Tat/TAR loop interaction is impaired either by specific point mutations in both elements (Verhoef et al., 1997, 2001) or by substitution of the TAR loop with an unrelated sequence (Das et al., 2007) were inhibited as efficiently as the parental HIV-1 strain by all three nuclear PYHIN proteins. Conversely, proviral constructs either lacking or having scrambled mutations in the Sp1 binding sites, or lacking Tat expression (Das et al., 2011) were hardly susceptible to inhibition by IFI16, IFIX and MNDA (Fig. 11B). These findings indicate that nuclear PYHIN proteins exert their anti-HIV-1 activity via a common mechanism, involving the Sp1 binding sites and Tat expression, but not the Tat/TAR loop interaction.

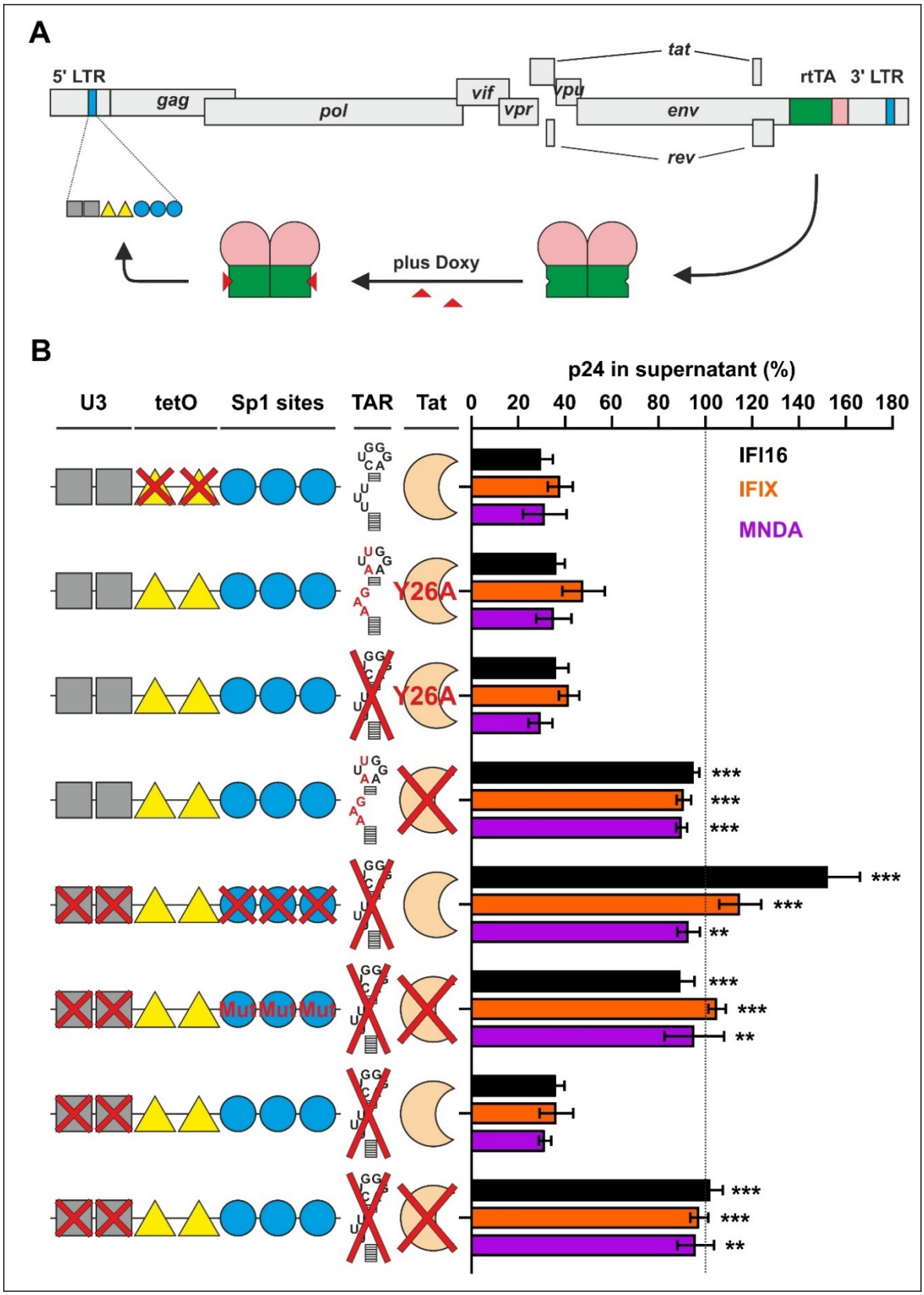

Figure 11. Nuclear PYHIN proteins require Sp1-binding sites and Tat expression to inhibit HIV-1. (A) Schematic representation of the doxycycline-dependent HIV-1 proviral constructs, in which the *nef* reading frame has been replaced by the reverse tetracycline-controlled transactivator (rtTA). Upon addition of doxycycline, the rtTA activates gene expression via two *tetO* operator elements (yellow triangles) introduced between the NF-κB and Sp1 binding sites. **(B)** HEK293T cells were cotransfected with expression plasmids

for human nuclear PYHIN proteins and proviral constructs harbouring the indicated mutations (red letters) or deletions (red crosses) in the LTR, TAR loop and/or Tat. The wild type parental strain is indicated by the lack of the two *tetO* operator elements. Total p24 amounts were quantified in the supernatants via ELISA. Results shown are the mean ± SEM, n=4. Asterisks indicate statistically significant differences between the wild type and the doxycycline-dependent proviruses.

4.4 Nuclear localization in combination with the PYD is sufficient for antiviral activity

In the co-transfection experiments, AIM2 did not show significant antiviral effect (Fig. 7A). Notably, AIM2 is predominantly localized in the cytosol due to the absence of any nuclear localization signal (NLS) (Hornung et al., 2009), and our previous data suggested that artificial delocalization of IFI16 to the cytosolic compartment is sufficient to abrogate its anti-HIV-1 activity (Hotter et al., 2019). To investigate whether the cytoplasmic localization of AIM2 may explain its lack of anti-HIV-1 activity, the region between the PYD and the HIN-A domain of IFI16 containing a bipartite NLS was swapped with that of AIM2. Titration of these chimeras revealed that introduction of the NLS naturally present in IFI16 was sufficient to render AIM2 active against HIV-1. Consistent with published data (Hotter et al., 2019), removal of the NLS from IFI16 abolished its antiviral activity (Fig. 12A). Thus, nuclear localization is required for the antiretroviral activity of human PYHIN proteins.

Accumulating evidence indicated that IFI16 can restrict HIV-1 independently of its sensing ability (Hotter et al., 2019; McLaren et al., 2015). The removal of the two DNA-binding HIN domains did not affect its antiviral activity and its PYD was sufficient to bind Sp1 and competes with DNA for Sp1 binding (Hotter et al., 2019). To investigate whether the PYD of the other PYHIN proteins are sufficient to exert antiviral activity, we generated HA-tagged, C-terminally truncated mutants in which we either removed the HIN domains (PYD+linker) or additionally deleted the region downstream the PYD (PYD only). In line with our previous data on IFI16 (Hotter et al., 2019), the PYD+linker truncation mutants of IFIX and MNDA significantly reduced LTR-driven eGFP expression and infectious HIV-1 yield. Notably, even the PYD only and PYD+linker mutants of AIM2 exhibited antiviral activity, despite lacking any NLS (Fig. 12B). Given their small molecular weight (Bosso et al., 2020), it is possible that they passively translocate in the nucleus and inhibit viral gene expression. In summary, these results highlight the importance of nuclear localization in mediating the antiviral activity of PYHIN proteins and reveal that the N-terminal PYD is sufficient to exert activity against HIV-1.

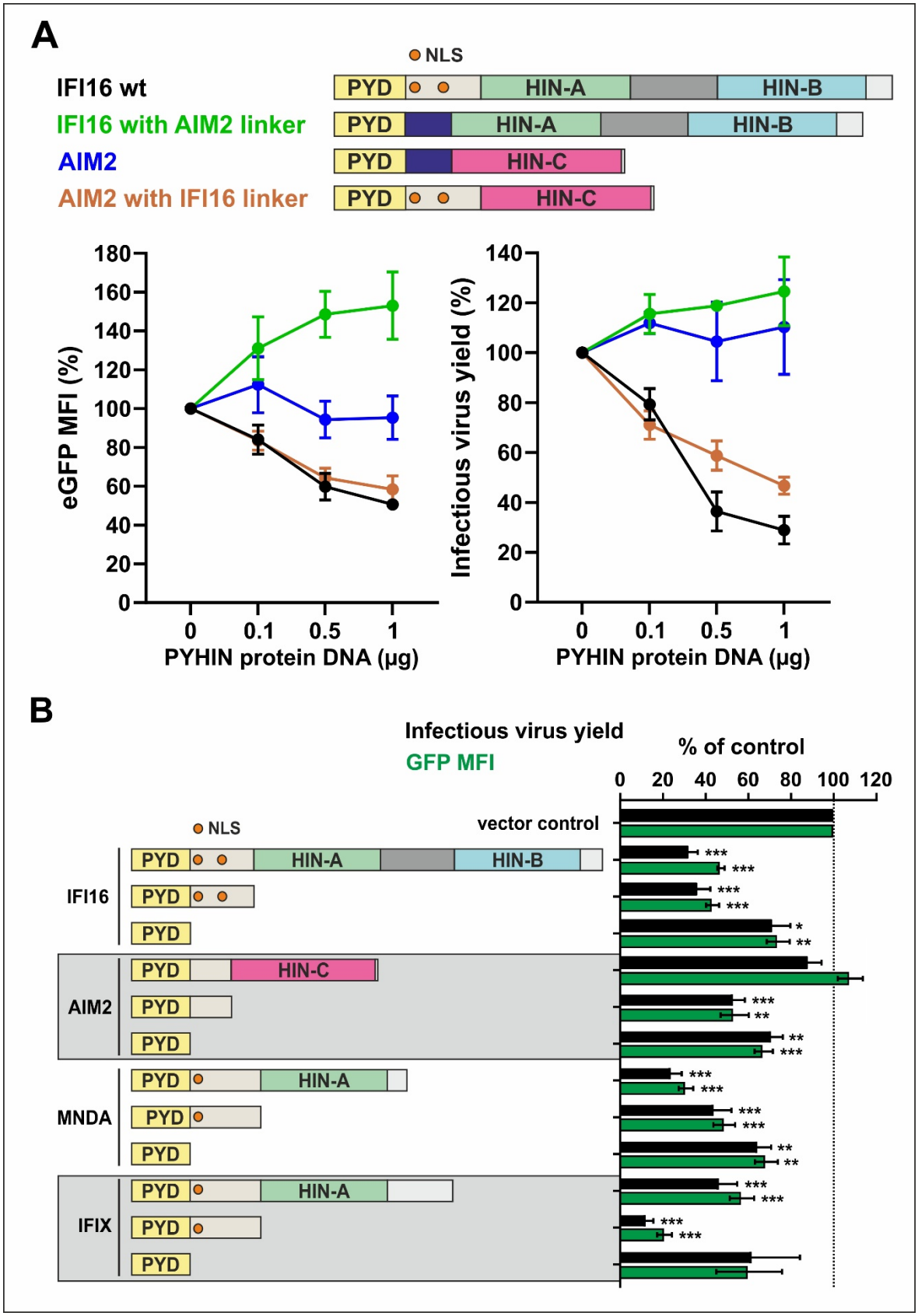

Figure 12. Nuclear localization in combination with the PYD is sufficient to restrict HIV-1. (A) Schematic representation of the IFI16-AIM2 chimeras (upper panel). HEK293T cells were cotransfected with increasing

doses of an expression plasmid for the indicated PYHIN proteins expressing BFP via an IRES and a proviral construct of HIV-1 NL4-3 expressing eGFP via an IRES. LTR-driven eGFP expression in transfected cells was assessed by FACS (left lower panel) and infectious virus yield was measured by infecting TZM-bl reporter cells (right lower panel). Results shown are the mean ± SEM, n=3. **(B)** HEK293T cells were cotransfected with expression plasmids for the indicated wild type and truncated PYHIN proteins expressing BFP via an IRES and a proviral construct of HIV-1 NL4-3 expressing eGFP via an IRES. LTR-driven eGFP expression in transfected cells was assessed by FACS (left lower panel), and infectious virus yield was measured by infecting TZM-bl reporter cells (right lower panel). Results shown are the mean ± SEM, n=5-7. First published in: Bosso et al., 2020, Fig. 5, 6), CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

4.5 Nuclear PYHIN proteins compete with DNA for Sp1 binding

Our findings suggested that all nuclear PYHIN proteins inhibit HIV-1 via a common mechanism and we previously showed that the N-terminal PYD of IFI16 is sufficient to reduce the availability of Sp1 for viral transcription (Hotter et al., 2019). To test whether IFIX and MNDA act the same way, we examined whether HA-tagged variants of these PYHIN proteins co-immunoprecipitated Sp1. To exclude possible crosslinking effects by residual nucleic acids, samples were additionally treated with benzonase. Western blot analysis showed that all HA-tagged PYHIN proteins were efficiently pulled down in either absence of presence of benzonase. Endogenous Sp1 expressed in transfected HEK293T was detectable in the pull down of all PYHIN proteins and signal intensity was stronger in the absence of nucleic acids (Fig. 13A). Quantification of the Sp1-pull down signal from three independent experiments revealed that the enhancement in interaction efficiency was very similar between PYHIN proteins (Fig. 13B). To better understand the functional consequences of the PYHIN protein-Sp1 interaction, we used a commercially available Sp1 binding assay which exclusively relies upon the nuclear fraction of the cell lysate. IFI16, IFIX and MNDA all significantly reduced Sp1 binding to its consensus target sequences, while AIM2 failed to do so. In agreement with the effects on viral gene expression, all PYD+linker truncation mutants prevented Sp1 binding to its consensus target sequence with similar efficiency (Fig. 13C). Sp1 uses its three zinc-fingers to bind DNA (Kadonaga et al., 1987) and our findings suggested that they could have been targeted by the PYD. To validate this hypothesis, we generated C-terminally FLAG-tagged expression constructs of full-length Sp1 or deletion mutants lacking individual functional domains (Fig. 13D). Such constructs were then cotransfected with an expression vector of either an HA-tagged or non-tagged IFI16 and expressing BFP via an IRES, and coimmunoprecipitated with an HA-tag-specific antibody. Western blot analysis confirmed correct expression of the FLAG-tagged

Sp1 constructs (Fig. 13E – Input panel) and efficient HA-mediated IFI16 pull down (Fig 13E – HA-IP panel). Notably, all Sp1 FLAG-tagged but the mutant lacking the three zinc fingers coimmunoprecipitated with HA-tagged IFI16, confirming that PYD competes with DNA for Sp1 binding via the zinc fingers (Fig 13E – HA-IP panel).

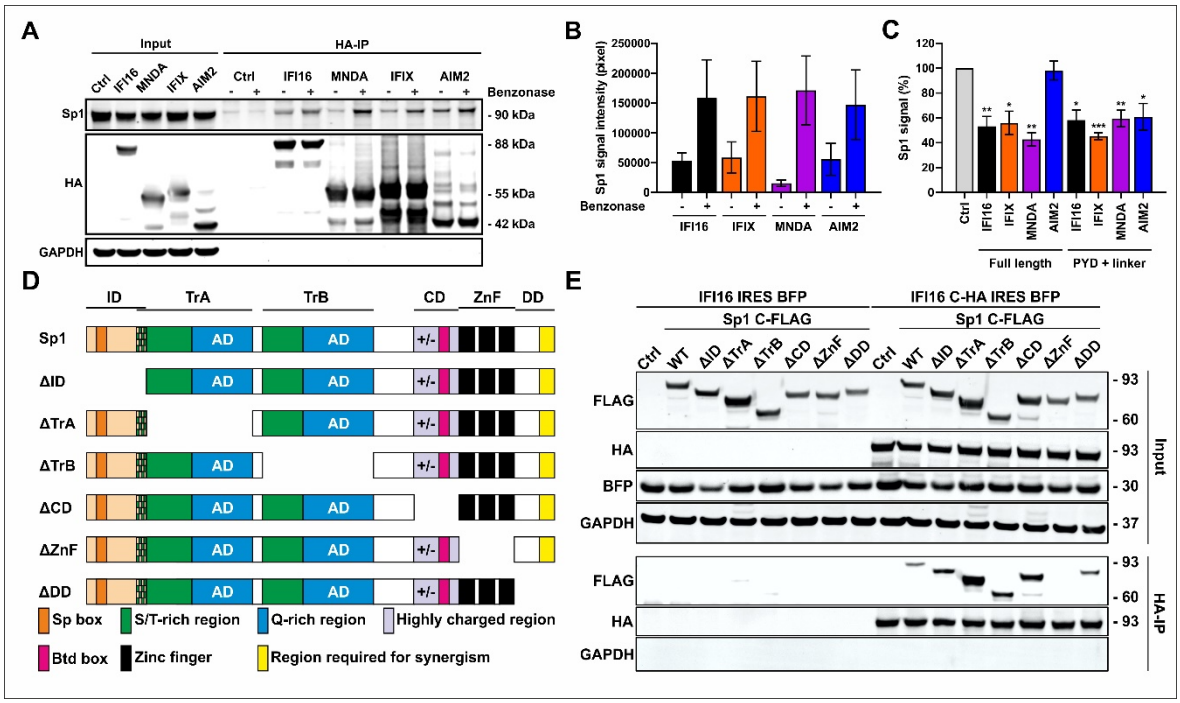

Figure 13. PYHIN proteins compete with DNA for Sp1 binding (A) HEK293T cells were transfected with either an empty vector or expression plasmids for human PYHIN proteins expressing an HA-tag at the C-terminus. HA-tagged proteins were immunoprecipitated, and protein expression as well as co-precipitation of Sp1 were assessed by Western blotting. Where indicated, samples were treated with benzonase. **(B)** Quantification of the Sp1 signal intensity in the HA-tagged pull-down Western blot shown in (A). Results shown are the mean ± SEM, n=3. **(C)** HEK293T cells were transfected with either an empty vector or expression plasmids for wild type or truncated PYHIN proteins. Upon extraction of the nuclear fraction, the amount of Sp1 available for DNA binding was quantified using the TransAM Sp1 Transcription Factor Assay Kit (ActiveMotif). Results shown are the mean ± SEM, n=3. **(D)** Schematic representation of the functional domains of Sp1 and the respective deletion mutants. **(E)** HEK293T cells were cotransfected with an expression construct for IFI16 either harbouring a C-terminally HA-tag or not, and expressing BFP via an IRES, together with either an empty vector or expression constructs for C-terminally FLAG-tagged wild type and deletion mutants of Sp1 represented in (D). HA-tagged IFI16 was coimmunoprecipitated, and protein expression as well as interaction with Sp1 were assessed by Western blotting. First published in: Bosso et al., 2020, Fig. 8, 9, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

5 Discussion

Human Pyrin and HIN domain-containing (PYHIN) proteins are emerging as important components of the host intrinsic immunity against intracellular pathogens (Connolly and Bowie, 2014). While several past studies focused on understanding the relevance of AIM2 and IFI16 in sensing foreign DNA and/or inhibiting viral replication (Dell'Oste et al., 2015; Lugrin and Martinon, 2018; Man et al., 2016), the role of IFIX and MDNA remains poorly characterized. In this study, we demonstrate that human nuclear PYHIN family members share the ability to compete with DNA for Sp1 binding via their N-terminal PYD, an event that limits the availability of this transcription factor and results in the inhibition of LTR-driven gene expression. As Sp1 localizes to the nucleus, this explains why the nuclear paralogues IFI16, IFIX and MNDA suppress HIV-1 replication in primary macrophages and $CD4^+$ T lymphocytes and why artificial nuclear relocalization of AIM2 renders it antivirally active.

The finding that the PYD is sufficient for Sp1 binding raises the questions of what could be the determinant(s) mediating this interaction and their physiological consequences. Phylogenetic analyses suggest that the *PYHIN* locus has undergone extensive differentiation in mammals (Brunette et al., 2012; Cridland et al., 2012). This is reflected in the different number of *PYHIN* members across different species, ranging from 1 in cows to 5 in humans and 14 in mice (Cridland et al., 2012). Additionally, phylogenetic analysis of PYHIN PYDs revealed that all AIM2 orthologues cluster in a separate clade compared to all other PYHINs (Brunette et al., 2012; Cridland et al., 2012). This is further highlighted by the low degree of amino acidic sequence similarity compared to the other human PYHINs (Fig. 14).

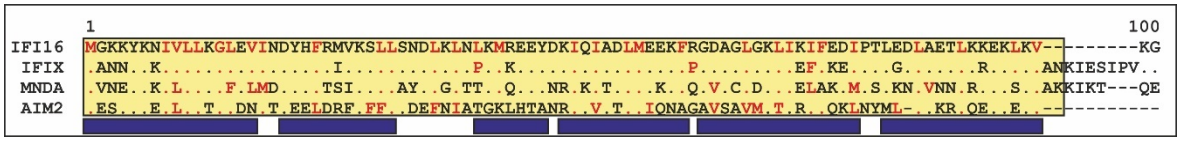

Figure 14. Human PYHIN PYD is evolutionarily poorly conserved. Alignment of the first 100 amino acids of human PYHIN proteins. Dots indicate residues identical to the IFI16 sequence while dashes illustrate gaps in the sequence (indels). The area highlighted in yellow represents the PYD. Amino acids written in red indicate hydrophobic residues potentially involved in the stability and oligomerization of the PYD, while the blue rectangles indicate the position of α-helices determined by NMR or crystal structure (Chu et al., 2015). First published in: Bosso et al., 2020, Fig. S4, CC BY-NC-ND 4.0, https://creativecommons.org/licenses/by-nc-nd/4.0/.

Given the conservation of its unique PYD across evolution, AIM2 has been proposed to be functionally different from other PYHINs. (Cridland et al., 2012). Despite this, our results show that IFI16, IFIX, MNDA and AIM2 interact with Sp1 and that the intensity of this binding is increased upon removal of nucleic acids (Fig. 13A and 13B). Consistently, an altered subcellular localization confers antiviral activity to full length AIM2 and the truncated AIM2 PYD is sufficient to partially reduce LTR-driven gene expression (Fig. 12B) by competing with DNA for Sp1 binding (Fig. 13C). AIM2 is physiologically located in the cytosol where it induces the inflammasome response upon recognition of cytosolic DNA (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). While this was initially thought to be an AIM2-specific pathway (Cridland et al., 2012), additional studies suggest that IFI16 can also trigger the inflammasome pathway in response to viral infections (Kerur *et al.*, 2011; Johnson *et al.*, 2013; Ansari *et al.*, 2013; Monroe *et al.*, 2014). This idea is reinforced by the fact that the inflammasome negative regulator POP3 was shown to interact with the PYD of AIM2 and IFI16 but not with the one of MNDA and IFIX (Khare et al., 2014). Therefore, while divergent evolution of the *PYHIN* genes in humans may have defined specific functions for each family members, our results clearly illustrate that the ability of Sp1 binding is a shared feature of all human PYHIN-PYD.

The PYD is one subfamily member of the death domain fold (DDF) superfamily of protein interaction domains spanning approximately 90 amino acids. Unlike the other DDF subfamilies, it is exclusively located at the terminal ends of proteins (Stehlik, 2007). The structure of the PYD consists of a six α-helical bundle arranged in a globular structure and revolving around a highly conserved hydrophobic core, which stabilizes the whole domain's tertiary folding and exposes highly bipolar charged patches on the surface. A shorter α3 helix and an extended loop linking α2 and α3 as structural features unique to the PYD initially suggested the presence of a subfamily-specific array of interaction partners (Fig. 14) (Kersse et al., 2011). However, this model was challenged by several studies demonstrating the ability of heterotypic interactions between DDF family members and unconventional non-DDF interactions (Stehlik, 2007). Humans encode 23 PYD-containing proteins (Chu et al., 2015) and the findings that the PYHIN-PYD is sufficient to compete with DNA for Sp1 binding (Fig. 13C) makes it tempting to speculate that other PYD-containing proteins share this function. However, this may not be the case, as most of them are either positive or negative modulators of the inflammasome cascade or act as adaptor proteins, and are predominantly localized within the cytosol (de Almeida et al., 2015; Chu et al., 2015; Khare et al., 2014; Le and Harton, 2013; Martinon et al., 2007). The only putative candidate in this

regard is the Pyrin protein, also known as the tripartite motif-containing protein 20 (TRIM20), which is characterized by an N-terminal PYD and two putative NLS situated downstream of it (Centola *et al.*, 1998). The observations that TRIM20 is expressed in primary macrophages and that its total protein levels can be upregulated by IFN-γ (Centola et al., 2000; Kimura et al., 2015) make this protein an interesting candidate for further studies that aim at understanding its role in HIV-1 replication.

Given the low amount of conserved amino acids between different PYDs (Fig. 14), one putative common mechanism which could explain the comparable competition between PYD and DNA for Sp1 binding (Fig. 13B) lies within the ability of PYD to oligomerize. PYD-driven oligomerization is a fundamental event required for the nucleation of multimeric protein complexes that functions as supramolecular platforms crucial to activate the downstream inflammasome response (Chu et al., 2015). The relevance of a tight regulation of the oligomerization is emphasized by the expression of both host Pyrin domain-only proteins (POPs) (Khare et al., 2014; Ratsimandresey et al., 2017; Stehlik et al., 2003) and viral-encoded POP-like proteins (Le and Harton, 2013). Such proteins compete for binding to the PYD of the downstream adaptors, thereby preventing inflammasome assembly. A similar mechanism has been evolved by the human cytomegalovirus (HCMV), which delivers a virion-associated protein that dampens DNA sensing by wrapping around the PYD of IFI16, IFIX, MNDA and AIM2, blocking their oligomerization (Li *et al.*, 2013; Huang *et al.*, 2017). PYD-mediated oligomerization is thought to occur via conserved hydrophobic and charged residues (Chu et al., 2015) that are highly conserved among human PYHIN proteins (Fig. 14). This process has been initially proposed as crucial for AIM2 and IFI16 as a mechanism to distinguish friend or foe and to amplify downstream immune signaling (Morrone et al., 2014, 2015; Stratmann et al., 2015). However, it has been recently reported that the ability of IFI16 to oligomerize is also crucial to mediate its suppression of HSV-1 gene expression and replication, as well as its interaction with other antiviral proteins (Lum et al., 2019). As an oligomerization-deficient mutant of IFI16 is unable to attenuate HIV-1 gene expression and viral infectivity (Bosso et al., 2020) these results indicate that oligomerization could be the *sine qua non* the IFI16-PYD interacts with the zinc fingers of Sp1. IFIX has been shown to form aggregate or “puncta” structures in both mock (Diner et al., 2015) and HSV-1 infected cells (Crow and Cristea, 2017), suggesting that IFIX also exists in an oligomeric state (Stratmann et al., 2015). MNDA represents a potential exception amongst human PYHIN proteins in this regard. It does not form protein aggregates like IFI16, IFIX and AIM2 (Diner et al., 2015), but gives rise to dimers only, via two regions

mapping to the N-terminal PYD (Xie *et al.*, 1997). Nevertheless, this does not exclude that the dimerization of MNDA could be a prerequisite required for its antiviral activity, as the mutant lacking the HIN domain still retains antiviral activity similar to that of the full-length protein (Fig. 12B).

One peculiar finding in regard to the PYDs is that the truncated IFIX-PYD is more active than the full-length protein (Fig. 12B), while both show similar activity in terms of competition with DNA for Sp1 binding (Fig. 13C). One possible reason to explain this observation is that the Sp1 activity assay has been performed in the absence of a proviral construct, suggesting that in the presence of HIV-1 the IFIX-PYD may have a higher affinity towards Sp1. Notably, IFIX interacts with several proteins involved in transcriptional regulation, epigenetic modifications, as well as mRNA maturation and splicing (Crow and Cristea, 2017; Diner et al., 2015), and some of these interactions are more pronounced in the absence of the PYD (Crow and Cristea, 2017). This could well be the opposite case in this situation, in which the removal of the HIN domain may promote or facilitate the interaction between the IFIX-PYD and potentially additional cellular factors which are required for efficient HIV-1 gene expression.

Removal of nucleic acids caused an increase in the interaction between PYHIN proteins and Sp1 (Fig. 13A and 13B) and the truncated PYD was sufficient to reduce binding of the transcription factor to its target sequences (Fig. 13C). This suggested that the PYHIN-PYDs may be targeting the DNA-binding domain of Sp1. Indeed, removal of the zinc fingers region abrogated the Sp1 interaction with IFI16 (Fig. 13D and 13E), and this may also be the case for other PYHIN proteins. It has been reported that the structural arrangement of the first and third zinc finger are essential for a proper nuclear localization of Sp1 (Ito et al., 2009) and that all three are required for efficient binding to importin α (Ito et al., 2010). This suggests that removal of the DNA binding domain may result in a cytoplasmic accumulation of Sp1, rendering it unable to be bound by IFI16 (Fig. 13D and 13E). However, it does not explain the lack of interaction between the ΔZnF mutant and IFI16 in the co-immunoprecipitation experiment, because total cell lysis allows the interaction of proteins that are usually found in different compartments. This is nicely illustrated by cytosolic AIM2, which interacts with endogenous Sp1 in this experimental setup and whose binding is enhanced by the removal of nucleic acids (Fig. 13A and 13B). This raises the question about which residues in the Sp1 DNA binding domain are crucial to mediate interaction with PYHIN proteins. An Sp1 zinc finger mutant carrying cysteine and histidine to alanine

substitutions in the first and third zinc finger shows a reduced nuclear localization and fails to bind the canonical “GC” box (Ito et al., 2009). This effect is probably caused by the loss of the correct tertiary structure or by the unfolding of the exposed α helix responsible for DNA binding (Kuwahara and Coleman, 1990). Consequently, zinc coordination and the correct folding could be required not only for efficient nuclear import but also for a correct interaction with the PYD, given the propensity of α helixes to mediate protein-protein interactions (Moon and Lim, 2015). The gain of interaction in the absence of nucleic acids between PYHIN proteins and Sp1 (Fig. 13A and 13B) also suggests that the PYD could target the nine amino acids (KHA-RER-RHK), which have been predicted to be responsible for directing Sp1 to its consensus DNA target sequence (Fig. 6) (Suske, 1999). Understanding the role of these residues could additionally reveal whether IFI16 and other PYHIN proteins may interact with additional Sp/KLF transcription factors, namely Sp3, Sp4, KLF9, KLF10 and KLF11, whose amino acids involved in DNA binding are highly conserved (Philipsen and Suske, 1999). Naturally, many more variables ranging from basal expression levels to post translational modifications may come into play in regulating the interaction between nuclear PYHIN proteins and Sp transcription factors.

Sp1 is a ubiquitously expressed transcription factor (Saffer *et al.*, 1991; Hagen *et al.*, 1992) which is mostly known for its role as a *trans*-activator of transcription (Kadonaga *et al.*, 1986). Moreover, given the high number of Sp1 binding sites in the human genome (Rouillard et al., 2016) and its crucial role in embryonic development (Marin et al., 1997), it is not surprising that its transcriptional activity has to be tightly regulated. Early findings showing that Sp1 directly interacts with the TATA binding protein (TBP) (Emili et al., 1994) and efficiently drives *in vitro* transcription (Jones and Tijan, 1985) suggested that it could simply regulate expression of TATA-less housekeeping gene promoters (Blake et al., 1990; Jolliff et al., 1991). More recent studies indicate that Sp1 not only is required for basal transcription but it also positively or negatively regulates transcription of cellular genes involved in multiple processes, ranging from metabolism to apoptosis (Black et al., 2001; Oleaga et al., 2012; Wierstra, 2008). In line with the ever-growing role of Sp1 in cancer (Beishline and Azizkhan-Clifford, 2015; Black et al., 2001; Chang and Hung, 2012; Chuang et al., 2012; Fojas de Borja et al., 2001; Li and Davie, 2010; O’Connor et al., 2016; Yang et al., 2013), gene enrichment analysis of all mapped Sp1-binding sites in the human genome (Chen et al., 2013; Kuleshov et al., 2016; Rouillard et al., 2016) shows a pronounced clustering in pathways involved in cancer-related processes (Fig. 15).

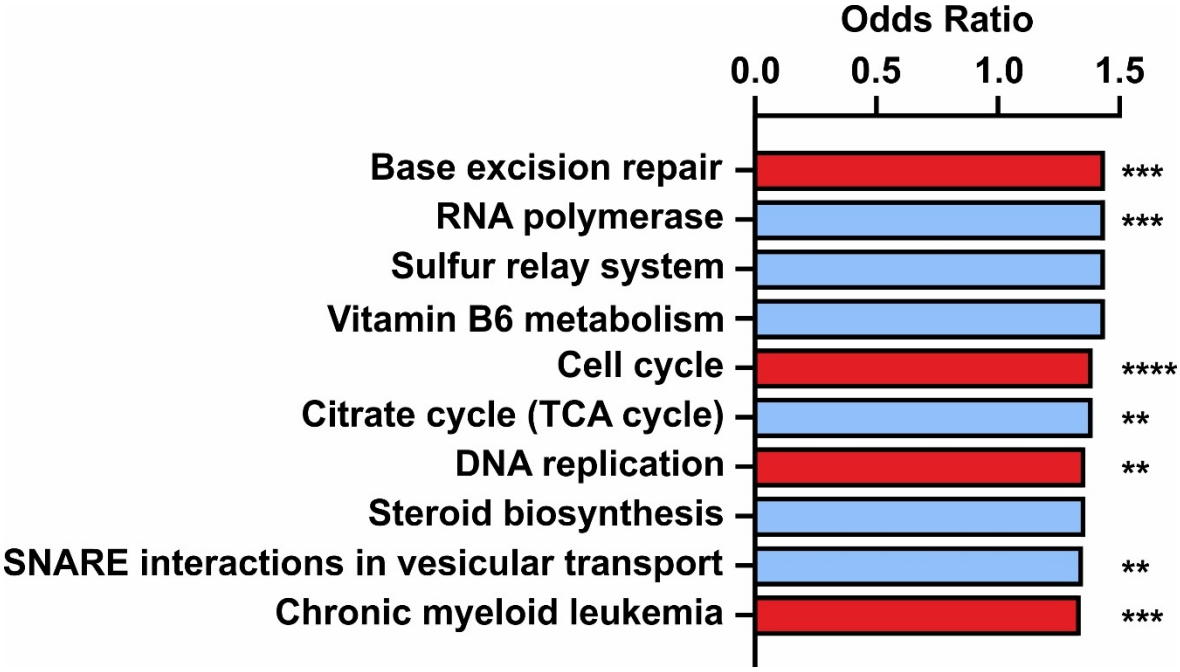

Figure 15. Sp1 regulates expression of cancer-related pathways. The list of 13.904 gene promoters bound by Sp1 derived from the Harmonizome database (Rouillard et al., 2016) was analyzed using Enrichr (Chen et al., 2013; Kuleshov et al., 2016). Top 10 Kyoto encyclopedia of genes and genomes (KEGG) pathways (Kanehisa and Goto, 2000) are shown. Pathways involved in cancer or cancer-related aspects are highlighted in red. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Strikingly, despite the knowledge that IFI16-mediated repression of Sp1-driven transcription has been known since many years (Johnstone et al., 1998b; Luu and Flores, 1997), only a handful of studies investigated the effect of IFI16 on Sp1-driven host gene expression. In this regard, it is known that the inhibitor of kappaB alpha (IκBα) gene promoter basal activity is regulated by one NF-κB and one Sp1 binding sites, which cooperatively bind and induce gene expression (Algarté et al., 1999). In endothelial cells, IFI16 leads to the transcriptional suppression of IκBα by reducing Sp1 recruitment to its promoter and point mutation of the Sp1 binding site relieved repression by IFI16 (Caposio et al., 2007). In a different context, it has been shown that the human telomerase reverse transcriptase (hTERT) gene promoter basal activity is regulated by both Sp1 and Sp3 (Cheng et al., 2015) and that IFI16 downregulates hTERT gene expression (Song et al., 2010). Interestingly however, IFI16 achieves this by reducing expression levels of cellular Myelocystomatosis (c-Myc) transcription factor (Song et al., 2010), probably as a result of a direct binding to the c-Myc promoter (Egistelli et al., 2009). While it cannot be excluded a direct inhibition of hTERT gene expression by IFI16 acting on Sp1 itself, these two brief examples illustrate how

complex the regulatory circuit of Sp1-modulated host gene promoters can be. More studies are clearly needed to better understand the role of IFI16 and other PYHIN proteins in regulating Sp1-driven host gene expression. For instance, it is well-established that IFI16 promotes cell cycle arrest and cellular senescence (Choubey et al., 2008; Clarke et al., 2010; Kim et al., 2005; Kwak et al., 2003; Lin et al., 2017; Raffaella et al., 2004; Song et al., 2008; Xin et al., 2003) but whether these effects are related to its sequestration of Sp1 still remain elusive.

The interplay between IFI16 and Sp1 can be complex as well in the context of viral infections. For instance, HCMV major immediate-early promoter (MIEP) enhancer region harbours three 21-bp repeats containing consensus sequences for Sp1 (Meier and Stinski, 1996). At least one of those is required for efficient viral gene expression at low multiplicity of infection (MOI) (Meier et al., 2002). Additional studies revealed that the Sp1 binding site located between positions -57 and -52 relative to the transcription start site (TSS) was required for minimal promoter activity (Isomura et al., 2004) and that either this one or the Sp1 binding site situated between positions -78 and -70 were required for efficient viral replication (Isomura et al., 2005). Notably, IFI16 does not affect MIEP-driven gene expression (Hotter et al., 2019) nor it impairs immediate early gene expression upon HCMV infection (Gariano et al., 2012), while it restricts HCMV replication by reducing Sp1 binding to the *UL54* viral gene promoter (Gariano et al., 2012; Johnstone et al., 1998b). This clearly shows that the basal activity of a promoter harbouring Sp1 sites is not necessarily negatively modulated by IFI16. Finding that nuclear PYHIN proteins interfere with Sp1 binding (Fig. 13C) and that its C-terminal zinc fingers are necessary to interact with IFI16 (Fig. 13E) further suggest that additional proteins binding to the same domain may reinforce or oppose the interactions with PYHINs by altering Sp1-DNA binding affinity. In this regard, the discovery that histone acetyltransferase p300 preferentially binds to Sp1 zinc fingers and stimulates DNA binding (Suzuki et al., 2000) suggests a potential cellular mechanism to antagonize PYHIN-mediated Sp1 sequestration.

Sp1 transcriptional activity is regulated by interactions with other proteins (Li *et al.*, 2004; Wierstra, 2008; O'Connor *et al.*, 2016), as well as by post-translational modifications (Bouwman and Philipsen, 2002; Chang and Hung, 2012; Tan and Khachigian, 2009). HIV-1 is no exception to the rule, for which Sp1 has been shown to support basal gene expression via the three binding sites in the proviral LTR (Jones et al., 1986). To further enhance its transcriptional *trans*-activation activity, the viral Tat protein promotes Sp1 phosphorylation

on Ser131 by interacting with the DNA-dependent protein kinase (DNA-PK) (Chun et al., 1998). Of note, Chun *et al.* proposed that Tat-mediated phosphorylation of Sp1, rather than the total Sp1 amount, is the limiting factor regulating the activation of the proviral LTR (Chun et al., 1998). The findings that depletion of nuclear PYHIN proteins in primary macrophages and IFI16 in $CD4^+$ T lymphocytes enhances HIV-1 replication and gene expression (Fig. 9A-C and 10B-D) and that IFI16, IFIX and MNDA reduce Sp1 binding to its consensus target sequences (Fig. 13C) argues however against this model (Chun et al., 1998). Lower levels of nuclear PYHIN proteins results in an increased availability of Sp1 for LTR binding, suggesting that in primary cells the availability of this transcription factor could be equally important as its Tat-induced phosphorylation. In the context of HIV-1 infection, nuclear PYHIN proteins may even additionally interfere with Tat itself, as proviral constructs lacking expression of this regulatory protein were hardly inhibited by IFI16, IFIX and MNDA (Fig. 11B). The interplay between Tat and Sp1 is however made more complicated by controversial reports. These either propose a direct interaction between the two (Jeang et al., 1993) requiring a region of Tat spanning between amino acids 30 to 55 (Chun et al., 1998) or claim that the binding between Sp1 and Tat is regulated by additional cellular factors (Loregian et al., 2003). Furthermore, Tat has been shown to *trans*-activate the proviral LTR (Laspia *et al.*, 1989) by acting on the Sp1 binding sites (Berkhout *et al.*, 1990; Das *et al.*, 2011) and to modulate host gene expression by acting on Sp1/3 responsive promoter elements (Howcroft *et al.*, 1995; Marecki *et al.*, 2004; Reeder *et al.*, 2015; Manes *et al.*, 2020). Thus, it is evident that host factors influencing the expression levels or availability of Sp1, Tat or any of their associated cofactors could have a severe impact on viral gene expression and replication. A dysregulation of the fine-tuned mechanisms regulating HIV-1 transcription could however lead to far-reaching consequences, potentially by negatively impacting the development of a definitive cure for AIDS and promoting persistent infection in the infected patient (Donahue et al., 2012; Kamori and Ueno, 2017; Martinez-Picado and Deeks, 2016; Nchioua et al., 2020).

Viral latency is defined as a condition, in which infected cells harbour an integrated provirus but do not release infectious viral particles (Eisele and Siliciano, 2012). It represents the main obstacle towards a complete eradication of HIV-1 (Richman et al., 2009). While it is broadly accepted that establishment of latency revolves around a transcriptional block (Karn, 2011; Siliciano and Greene, 2011), multiple and different mechanisms have been reported to contribute to this status. These include transcriptional interference, epigenetic modifications, sequestration or absence of positive transcription factors (TFs), abundance of

negative TFs (Cary *et al.*, 2016), incorrect functioning of the cellular mRNA export machinery (Lassen et al., 2006) or premature interruption of elongation, maturation and splicing of viral transcripts (Yukl et al., 2018). Regarding the modulation of LTR activity for the establishment and maintenance of HIV-1 latency, the role of Sp1 has been poorly investigated and is controversial. One early study proposed that upon proviral integration downstream of another gene promoter, the binding of Sp1 to the occluded LTR was reduced, potentially contributing to reduced LTR-mediated transcription (Greger et al., 1998). In contrast, another study reported that Sp1 is bound to the viral promoter and promotes latency maintenance by recruiting the cellular transcription factor c-Myc and the histone deacetylase 1 (HDAC1) to inhibit basal and Tat-mediated *trans*-activation of the LTR (Jiang et al., 2007). One study showed that mutation of the Sp1 binding sites in the integrated provirus fully abrogates HIV-1 reactivation from latency (Bosque and Planelles, 2009). These results are further reinforced by our finding that the inhibition of Sp1 binding in latently infected primary $CD4^+$ T lymphocytes reduces HIV-1 reactivation (Hotter et al., 2019). Strikingly, using a cell line carrying a latently integrated provirus, we could also show that Sp1 KO totally abrogated LTR-driven gene expression while the complete loss of IFI16 resulted in ~3.5-fold higher reactivation efficiency compared to the control cell line (Hotter et al., 2019). Globally, these results indicate that Sp1 may play a crucial role in promoting viral reactivation from the latent state and that IFI16 could antagonize it, although more investigations are needed for a better understanding of the mechanism. Given the propensity of HIV-1 to establish a productive infection in activated $CD4^+$ T lymphocytes (Gowda et al., 1989; Mahalingam et al., 1993; Mohagheghpour et al., 1991), the finding that IFI16 potently suppresses transcription and replication of two highly sensitive primary HIV-1 IMCs (Fig. 10A-D) suggests that non-IFI16 sensitive viruses could potentially possess Sp1-independent mechanism to modulate their latency state. Interestingly, additional members of the Sp/KLF family of transcription factors are known to bind the canonical “GC” box (Cook et al., 1998; Hagen et al., 1992; Sogawa et al., 1993), and several members of this family have been shown to modulate LTR-driven gene expression (Imataka et al., 1993; Majello et al., 1994). Notably, Sp3 has been shown to suppress both basal and Tat-mediated LTR transactivation and that its inhibitory effect correlated with its DNA binding ability, probably by competing with the endogenous Sp1 (Majello et al., 1994). As Sp3 has been shown to be expressed in $CD4^+$ T lymphocytes as well (Grekova et al., 2002), Sp3 alone could limit transactivation of the HIV-1 LTR by displacing Sp1 from its binding sites. Alternatively, the sequestration of Sp1 by IFI16 and IFIX could lead to an enhanced binding of Sp3 to the viral promoter.

Multiple strategies have been devised in an attempt to eradicate the latent reservoir. These include bone marrow transplantation, drug-based reactivation and stimulation of the host immune system ('shock and kill' approach), genome editing techniques and 'lock and block' approach (Marsden and Zack, 2019). The latter one consists of inhibiting one or more cellular or viral proteins involved in HIV-1 gene expression, permanently locking the integrated provirus in a silent state (Vansant et al., 2020). Given the crucial role of Sp1 in allowing viral reactivation from latency (Hotter et al., 2019), it may look appealing to target this transcription factor to prevent viral rebound. Attempts to interfere with Sp1-driven gene expression have already been made in the past, upon purification of an antibiotic called mithramycin/plicamycin from *Streptomyces plicatus* (Haskell et al., 1958). Mithramycin binds to minor groove of DNA to the 3'-GGG-5' motif with the highest affinity (Van Dyke and Dervan, 1983), inhibiting Sp1 binding to its target sequences (Sleiman et al., 2011; Snyder et al., 1991). Initially tested as an antineoplastic drug, mithramycin treatment however turned out to cause too many side effects, including thrombocytopenia and fatal internal bleedings (Ahr et al., 1978; Kennedy, 1970; Stewart, 1983). Hence, drugs targeting virus-specific proteins like Tat could represent a better alternative to keep HIV-1 in a permanent latent state (Kessing et al., 2017).

In regard to Sp1 and HIV-1 gene expression, one unexpected finding was the reduced sensitivity of HIV-1 M subtype C to IFI16 (Fig. 10A), IFIX and MNDA compared to other HIV-M subtypes (Bosso et al., 2020; Hotter et al., 2019). In line with our findings, this was mapped to a reduced Sp1-dependency for viral transcription (Hotter et al., 2019). Subtype C is endemic to Southern Africa, Ethiopia and India and represents nearly 50 % of all HIV-1 infections (Gartner et al., 2020). How did subtype C viruses manage to spread so efficiently amongst the population however still remains and open question and multiple aspects could have driven the successful spread of this subtype. These include environmental and socio-economical conditions of the host populations or genetic and viral differences between HIV-1 subtypes (Gartner et al., 2020). In the latter case, the ability of subtype C viruses to efficiently evade PYHIN-mediated restriction in HIV-1 primary target cells (Fig. 10C) (Bosso et al., 2020; Hotter et al., 2019) could represent one of these factors. Thus, my findings identified one selective advantage that distinguishes subtype C viruses from their less prevalent counterparts and may have contributed to their world-wide spread.

6 Summary

Replication of human pathogenic viruses such as HIV-1 is constantly challenged by selective pressures exerted by a large arsenal of host sensors and restriction factors. Consecutively, this drives selection of viruses able to neutralize or evade such host defenses. Human PYHIN proteins (AIM2, IFI16, MNDA and IFIX) are emerging players of the cell-intrinsic immune response. While AIM2 is a well-established cytosolic sensor of exogenous DNA, the role of IFI16 as a nucleic acid sensor is rather controversial. In turn, IFI16 is mostly known to inhibit viral replication by silencing viral gene expression of several DNA viruses. IFIX and MNDA have been shown to be antivirally active against two different herpesviruses, but their precise mechanism remained elusive. As such, the goal of my thesis was to elucidate if and how PYHIN proteins restrict replication of primary HIV-1 isolates. My findings revealed that IFI16, IFIX, MNDA and AIM2 are readily detectable in HIV-1 primary target cells and that all but AIM2 restrict viral transcription. Mechanistic studies showed that nuclear PYHIN proteins inhibit lentiviral gene expression by binding to and reducing the availability of the host transcription factor Sp1. In contrast, AIM2 did not exert antiviral activity. This is due to its physiological cytosolic compartmentalization, as artificial relocalization into the nucleus rendered AIM2 antivirally active. Deletion mutagenesis and functional analyses further revealed that the N-terminal Pyrin domain (PYD) is sufficient for the antiviral function, suggesting that the antiviral mechanism is independent of the DNA interaction and sensing functions of PYHIN proteins. Further experiments in both primary $CD4^+$ T and in a latently infected T cell line further revealed the crucial role of Sp1 in promoting viral reactivation from latency and how this process is antagonized by IFI16. Interestingly, highly prevalent subtype C viruses evade these restriction factors by reducing their dependency on Sp1 for efficient transcription, and this effect seems to be dependent on the proviral LTRs.

In conclusion, my results illustrate how nuclear PYHIN proteins commonly inhibit HIV-1 replication by targeting the host transcription factor Sp1. Finding that a highly prevalent HIV-1 subtype efficiently evades these restriction factors should stimulate further studies aimed at understanding which region in the proviral LTRs is responsible for this effect as well as the underlying mechanism. Notably, Sp1 is required for basal and induced expression of a plethora of cellular genes and it is known to play an important role in cancer. Hence, the PYHIN-Sp1 interaction should be additionally investigated outside of the context of viral infection, to shed more light on the physiological role of PYHIN proteins.

7 References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M.A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. *59*, 284–291.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered Recruitment of Chromatin Modifying and General Transcription Factors to the IFN-β Promoter. Cell *103*, 667–678.

Ahr, D.J., Scialla, S.J., and Kimbali, D.B. (1978). Acquired platelet dysfunction following mithramycin therapy. Cancer *41*, 448–454.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. *4*, 499–511.

Albrecht, M., Choubey, D., and Lengauer, T. (2005). The HIN domain of IFI-200 proteins consists of two OB folds. Biochem. Biophys. Res. Commun. *327*, 679–687.

Alcamí, J., Laín de Lera, T., Folgueira, L., Pedraza, M.A., Jacqué, J.M., Bachelerie, F., Noriega, A.R., Hay, R.T., Harrich, D., and Gaynor, R.B. (1995). Absolute dependence on kappa B responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. EMBO J. *14*, 1552–1560.

Algarté, M., Kwon, H., Génin, P., and Hiscott, J. (1999). Identification by in vivo genomic footprinting of a transcriptional switch containing NF-kappaB and Sp1 that regulates the IkappaBalpha promoter. Mol. Cell. Biol. *19*, 6140–6153.

de Almeida, L., Khare, S., Misharin, A. V., Patel, R., Ratsimandresey, R.A., Wallin, M.C., Perlman, H., Greaves, D.R., Hoffman, H.M., Dorfleutner, A., and Stehlik, C. (2015). The PYRIN Domain-only Protein POP1 Inhibits Inflammasome Assembly and Ameliorates Inflammatory Disease. Immunity.

Almine, J.F., Hare, C.A.J.O., Dunphy, G., Haga, I.R., Naik, R.J., Atrih, A., Connolly, D.J., Taylor, J., Kelsall, I.R., Bowie, A.G., Beard, P.M., and Unterholzner, L. (2017). IFI16 and cGAS cooperate in the activation of STING during DNA sensing in human keratinocytes. Nat. Commun.

Ansari, M.A., Singh, V.V., Dutta, S., Veettil, M.V., Dutta, D., Chikoti, L., Lu, J., Everly, D., and Chandran, B. (2013). Constitutive Interferon-Inducible Protein 16-Inflammasome Activation during Epstein-Barr Virus Latency I, II, and III in B and Epithelial Cells. *87*, 8606–8623.

Ansari, M.A., Dutta, S., Veettil, M.V., Dutta, D., Iqbal, J., Kumar, B., Roy, A., Chikoti, L.,

Singh, V.V., and Chandran, B. (2015). Herpesvirus Genome Recognition Induced Acetylation of Nuclear IFI16 Is Essential for Its Cytoplasmic Translocation, Inflammasome and IFN-β Responses. PLoS Pathog. *11*, e1005019.

Arts, E.J., and Hazuda, D.J. (2012). HIV-1 antiretroviral drug therapy. Cold Spring Harb. Perspect. Med. *2*, a007161.

Athanikar, J.N., Sanchez, H.B., and Osborne, T.F. (1997). Promoter selective transcriptional synergy mediated by sterol regulatory element binding protein and Sp1: a critical role for the Btd domain of Sp1. Mol. Cell. Biol. *17*, 5193–5200.

Ayouba, A., Akoua-Koffi, C., Calvignac-Spencer, S., Esteban, A., Locatelli, S., Li, H., Li, Y., Hahn, B.H., Delaporte, E., Leendertz, F.H., and Peeters, M. (2013). Evidence for continuing cross-species transmission of SIVsmm to humans: characterization of a new HIV-2 lineage in rural Côte d'Ivoire. AIDS *27*, 2488–2491.

Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science *220*, 868–871.

Beishline, K., and Azizkhan-Clifford, J. (2015). Sp1 and the 'hallmarks of cancer.' FEBS J. *282*, 224–258.

Berg, R.K., Rahbek, S.H., Kofod-Olsen, E., Holm, C.K., Melchjorsen, J., Jensen, D.G., Hansen, A.L., Jørgensen, L.B., Ostergaard, L., Tolstrup, M., Larsen, C.S., Paludan, S.R., Jakobsen, M.R., and Mogensen, T.H. (2014). T cells detect intracellular DNA but fail to induce type I IFN responses: Implications for restriction of HIV replication. PLoS One *9*.

Berkhout, B., Silverman, R.H., and Jeang, K.T. (1989). Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell *59*, 273–282.

Berkhout, B., Gatignol, A., Rabson, A.B., and Jeang, K.T. (1990). TAR-independent activation of the HIV-1 LTR: Evidence that Tat requires specific regions of the promoter. Cell *62*, 757–767.

Black, A.R., Black, J.D., and Azizkhan-Clifford, J. (2001). Sp1 and krüppel-like factor family of transcription factors in cell growth regulation and cancer. J. Cell. Physiol. *188*, 143–160.

Blake, M.C., Jambou, R.C., Swick, A.G., Kahn, J.W., and Azizkhan, J.C. (1990). Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. Mol. Cell. Biol. *10*, 6632–6641.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W.,

Crosa, J.H., and Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene *2*, 95–113.

Bosque, A., and Planelles, V. (2009). Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. Blood *113*, 58–65.

Bosso, M., Prelli Bozzo, C., Hotter, D., Volcic, M., Stürzel, C.M., Rammelt, A., Ni, Y., Urban, S., Becker, M., Schelhaas, M., Wittmann, S., Christensen, M.H., Schmidt, F.I., Gramberg, T., Sparrer, K.M.J., Sauter, D., and Kirchhoff, F. (2020). Nuclear PYHIN proteins target the host transcription factor Sp1 thereby restricting HIV-1 in human macrophages and CD4+ T cells. PLoS Pathog. *16*, e1008752.

Bouwman, P., and Philipsen, S. (2002). Regulation of the activity of Sp1-related transcription factors. Mol. Cell. Endocrinol. *195*, 27–38.

Briggs, L.J., Johnstone, R.W., Elliot, R.M., Xiao, C.Y., Dawson, M., Trapani, J.A., and Jans, D.A. (2001). Novel properties of the protein kinase CK2-site-regulated nuclear- localization sequence of the interferon-induced nuclear factor IFI 16. Biochem. J. *353*, 69–77.

Briggs, R., Dworkin, L., Briggs, J., Dessypris, E., Stein, J., Stein, G., and Lian, J. (1994a). Interferon α selectively affects expression of the human myeloid cell nuclear differentiation antigen in late stage cells in the monocytic but not the granulocytic lineage. J. Cell. Biochem. *54*, 198–206.

Briggs, R.C., Kao, W.Y., Dworkin, L.L., Briggs, J.A., Dessypris, E.N., and Clark, J. (1994b). Regulation and Specificity of MNDA Expression in Monocytes, Macrophages, and Leukemia/B Lymphoma Cell Lines. J. Cell. Biochem. *56*, 559–567.

Brunette, R.L., Young, J.M., Whitley, D.G., Brodsky, I.E., Malik, H.S., and Stetson, D.B. (2012). Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. J. Exp. Med. *209*, 1969–1983.

Caposio, P., Gugliesi, F., Zannetti, C., Sponza, S., Mondini, M., Medico, E., Hiscott, J., Young, H.A., Gribaudo, G., Gariglio, M., and Landolfo, S. (2007). A novel role of the interferon-inducible protein IFI16 as inducer of proinflammatory molecules in endothelial cells. J. Biol. Chem.

Cary, D.C., Fujinaga, K., and Peterlin, B.M. (2016). Molecular mechanisms of HIV latency. J. Clin. Invest. *126*, 448–454.

Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J., Wheeler, R., Wong, B., Drenkow, J., Yamanaka, M., Patel, S., Brubaker, S., Tammana, H., Helt, G., Struhl, K., and Gingeras, T.R. (2004). Unbiased mapping of transcription factor binding sites along human

chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell *116*, 499–509.

Centola, M., Aksentijevich, I., and Kastner, D.L. (1998). The hereditary periodic fever syndromes: Molecular analysis of a new family of inflammatory diseases. Hum. Mol. Genet. *7*, 1581–1588.

Centola, M., Wood, G., Frucht, D.M., Galon, J., Aringer, M., Farrell, C., Kingma, D.W., Horwitz, M.E., Mansfield, E., Holland, S.M., O'Shea, J.J., Rosenberg, H.F., Malech, H.L., and Kastner, D.L. (2000). The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. Blood *95*, 3223–3231.

Chang, W.-C., and Hung, J.-J. (2012). Functional role of post-translational modifications of Sp1 in tumorigenesis. J. Biomed. Sci. *19*, 94.

Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., and Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics *14*, 128.

Cheng, D., Zhao, Y., Wang, S., Jia, W., Kang, J., and Zhu, J. (2015). Human Telomerase Reverse Transcriptase (hTERT) Transcription Requires Sp1/Sp3 Binding to the Promoter and a Permissive Chromatin Environment. J. Biol. Chem. *290*, 30193–30203.

Choubey, D., Deka, R., and Ho, S. (2008). Interferon-inducible IFI16 protein in human cancers and autoimmune diseases. Front. Biosci. *13*, 598–608.

Chu, L.H., Gangopadhyay, A., Dorfleutner, A., and Stehlik, C. (2015). An updated view on the structure and function of PYRIN domains. Apoptosis *20*, 157–173.

Chuang, J.-Y., Wang, S.-A., Yang, W.-B., Yang, H.-C., Hung, C.-Y., Su, T.-P., Chang, W.-C., and Hung, J.-J. (2012). Sp1 phosphorylation by cyclin-dependent kinase 1/cyclin B1 represses its DNA-binding activity during mitosis in cancer cells. Oncogene *31*, 4946–4959.

Chun, R.F., Semmes, O.J., Neuveut, C., and Jeang, K.T. (1998). Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. J Virol *72*, 2615–2629.

Lo Cigno, I., De Andrea, M., Borgogna, C., Albertini, S., Landini, M.M., Peretti, A., Johnson, K.E., Chandran, B., Landolfo, S., and Gariglio, M. (2015). The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. J. Virol. *89*, 7506–7520.

Clarke, C.J.P., Hii, L.L., Bolden, J.E., and Johnstone, R.W. (2010). Inducible activation of IFI 16 results in suppression of telomerase activity, growth suppression and induction of cellular senescence. J. Cell. Biochem. *109*, 103–112.

Connolly, D.J., and Bowie, A.G. (2014). The emerging role of human PYHIN proteins in innate immunity: Implications for health and disease. Biochem. Pharmacol.

Cook, T., Gebelein, B., Mesa, K., Mladek, A., and Urrutia, R. (1998). Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-beta-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. J. Biol. Chem. *273*, 25929–25936.

Cosnefroy, O., Murray, P.J., and Bishop, K.N. (2016). HIV-1 capsid uncoating initiates after the first strand transfer of reverse transcription. Retrovirology *13*, 58.

Courey, A.J., and Tjian, R. (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell *55*, 887–898.

Craigie, R., and Bushman, F.D. (2012). HIV DNA integration. Cold Spring Harb. Perspect. Med. *2*, a006890.

Cridland, J.A., Curley, E.Z., Wykes, M.N., Schroder, K., Sweet, M.J., Roberts, T.L., Ragan, M.A., Kassahn, K.S., and Stacey, K.J. (2012). The mammalian PYHIN gene family: Phylogeny, evolution and expression. BMC Evol. Biol.

Crow, M.S., and Cristea, I.M. (2017). Human antiviral protein IFIX suppresses viral gene expression during herpes simplex virus 1 (HSV-1) infection and is counteracted by virus-induced proteasomal degradation. Mol. Cell. Proteomics *16*, S200–S214.

Das, A.T., Harwig, A., Vrolijk, M.M., and Berkhout, B. (2007). The TAR hairpin of human immunodeficiency virus type 1 can be deleted when not required for Tat-mediated activation of transcription. J. Virol. *81*, 7742–7748.

Das, A.T., Harwig, A., and Berkhout, B. (2011). The HIV-1 Tat Protein Has a Versatile Role in Activating Viral Transcription. J. Virol.

Datta, P.K., Kaminski, R., Hu, W., Pirrone, V., Sullivan, N.T., Nonnemacher, M.R., Dampier, W., Wigdahl, B., and Khalili, K. (2016). HIV-1 Latency and Eradication: Past, Present and Future. Curr. HIV Res. *14*, 431–441.

Dell'Oste, V., Gatti, D., Giorgio, A.G., Gariglio, M., Landolfo, S., and De Andrea, M. (2015). The interferon-inducible DNA-sensor protein IFI16: A key player in the antiviral response. New Microbiol.

Dick, M.S., Sborgi, L., Rühl, S., Hiller, S., and Broz, P. (2016). ASC filament formation serves as a signal amplification mechanism for inflammasomes. Nat. Commun. *7*, 11929.

Diner, B.A., Li, T., Greco, T.M., Crow, M.S., Fuesler, J.A., Wang, J., and Cristea, I.M. (2015). The functional interactome of PYHIN immune regulators reveals IFIX is a sensor of viral DNA. Mol. Syst. Biol. *11*, 787.

Diner, B.A., Lum, K.K., Toettcher, J.E., and Cristea, I.M. (2016). Viral DNA Sensors IFI16 and Cyclic GMP-AMP Synthase Possess Distinct Functions in Regulating Viral Gene Expression, Immune Defenses, and Apoptotic Responses during Herpesvirus Infection. MBio *7*, 1–15.

Ding, Y., Wang, L., Su, L.-K., Frey, J.A., Shao, R., Hunt, K.K., and Yan, D.-H. (2004). Antitumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer. Oncogene *23*, 4556–4566.

Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A.D., Singh, M., Skinner, M.A., and Valerio, R. (1989). Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. Proc. Natl. Acad. Sci. U. S. A. *86*, 6925–6929.

Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A.D., Singh, M., and Skinner, M.A. (1990). HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO J. *9*, 4145–4153.

Donahue, D.A., Kuhl, B.D., Sloan, R.D., and Wainberg, M.A. (2012). The Viral Protein Tat Can Inhibit the Establishment of HIV-1 Latency. J. Virol.

DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H., and Calos, M.P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol.

Duhl, D.M., Gaczynski, M., Olinski, R., and Briggs, R.C. (1989). Intranuclear distribution of the human myeloid cell nuclear differentiation antigen in HL-60 cells. J. Cell. Physiol. *141*, 148–153.

Van Dyke, M.W., and Dervan, P.B. (1983). Chromomycin, mithramycin, and olivomycin binding sites on heterogeneous deoxyribonucleic acid. Footprinting with (methidiumpropyl-EDTA)iron(II). Biochemistry *22*, 2373–2377.

Dynan, W.S., and Tjian, R. (1983). The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell *35*, 79–87.

Egistelli, L., Chichiarelli, S., Gaucci, E., Eufemi, M., Schininà, M.E., Giorgi, A., Lascu, I., Turano, C., Giartosio, A., and Cervoni, L. (2009). IFI16 and NM23 bind to a common DNA fragment both in the P53 and the cMYC gene promoters. J. Cell. Biochem. *106*, 666–672.

Eisele, E., and Siliciano, R.F. (2012). Redefining the viral reservoirs that prevent HIV-1 eradication. Immunity *37*, 377–388.

Emili, A., Greenblatt, J., and Ingles, C.J. (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. Mol. Cell.

Biol. *14*, 1582–1593.

Fernandes-Alnemri, T., Yu, J., Datta, P., Wu, J., and Alnemri, E.S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature *458*, 509–513.

Fojas de Borja, P., Collins, N.K., Du, P., Azizkhan-Clifford, J., and Mudryj, M. (2001). Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription. EMBO J. *20*, 5737–5747.

Fouchier, R.A., Meyer, B.E., Simon, J.H., Fischer, U., and Malim, M.H. (1997). HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J. *16*, 4531–4539.

Freed, E.O. (2015). HIV-1 assembly, release and maturation. Nat. Rev. Microbiol. *13*, 484–496.

Freel, S.A., Picking, R.A., Ferrari, G., Ding, H., Ochsenbauer, C., Kappes, J.C., Kirchherr, J.L., Soderberg, K.A., Weinhold, K.J., Cunningham, C.K., Denny, T.N., Crump, J.A., Cohen, M.S., McMichael, A.J., Haynes, B.F., and Tomaras, G.D. (2012). Initial HIV-1 Antigen-Specific CD8+ T Cells in Acute HIV-1 Infection Inhibit Transmitted/Founder Virus Replication. J. Virol. *86*, 6835–6846.

Gao, F., Yue, L., White, A.T., Pappas, P.G., Barchue, J., Hanson, A.P., Greene, B.M., Sharp, P.M., Shaw, G.M., and Hahn, B.H. (1992). Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature *358*, 495–499.

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., Sharp, P.M., and Hahn, B.H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature *397*, 436–441.

Garber, M.E., Wei, P., and Jones, K.A. (1998). HIV-1 Tat interacts with cyclin T1 to direct the P-TEFb CTD kinase complex to TAR RNA. Cold Spring Harb. Symp. Quant. Biol. *63*, 371–380.

Garcia, J.A., Harrich, D., Soultanakis, E., Wu, F., Mitsuyasu, R., and Gaynor, R.B. (1989). Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J. *8*, 765–778.

Gariano, G.R., Dell'Oste, V., Bronzini, M., Gatti, D., Luganini, A., De Andrea, M., Gribaudo, G., Gariglio, M., and Landolfo, S. (2012). The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication. PLoS Pathog. *8*, e1002498.

Gartner, M.J., Roche, M., Churchill, M.J., Gorry, P.R., and Flynn, J.K. (2020).

Understanding the mechanisms driving the spread of subtype C HIV-1. EBioMedicine *53*, 102682.

Goldberger, A., Hnilicatll, L.S., Caseyli, S.B., and Briggssli, R.C. (1986). Properties of a Nuclear Protein Marker of Human Myeloid Cell Differentiation*. J. Biol. Chem. *261*, 4726–4731.

Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766–1769.

Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A., and Saxon, A. (1981). Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. *305*, 1425–1431.

Gowda, S.D., Stein, B.S., Mohagheghpour, N., Benike, C.J., and Engleman, E.G. (1989). Evidence that T cell activation is required for HIV-1 entry in CD4+ lymphocytes. J. Immunol. *142*, 773–780.

Gray, E.E., Winship, D., Snyder, J.M., Child, S.J., Geballe, A.P., and Stetson, D.B. (2016). The AIM2-like Receptors Are Dispensable for the Interferon Response to Intracellular DNA. Immunity *45*, 255–266.

Greger, I.H., Demarchi, F., Giacca, M., and Proudfoot, N.J. (1998). Transcriptional interference perturbs the binding of Sp1 to the HIV-1 promoter. Nucleic Acids Res. *26*, 1294–1301.

Grekova, M.C., Salerno, K., Mikkilineni, R., and Richert, J.R. (2002). Sp3 expression in immune cells: a quantitative study. Lab. Invest. *82*, 1131–1138.

Guntaka, R. V (1993). Transcription termination and polyadenylation in retroviruses. Microbiol. Rev. *57*, 511–521.

Hagen, G., Müller, S., Beato, M., and Suske, G. (1992). Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. Nucleic Acids Res. *20*, 5519–5525.

Harrison, S.M., Houzelstein, D., Dunwoodie, S.L., and Beddington, R.S. (2000). Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. Dev. Biol. *227*, 358–372.

Haskell, T.H., Ryder, A., Frohardt, R.P., Fusari, S.A., Jakubowski, Z.L., and Bartz, Q.R. (1958). The Isolation and Characterization of Three Crystalline Antibiotics from Streptomyces plicatus. J. Am. Chem. Soc. *80*, 743–747.

Van Heuverswyn, F., Li, Y., Neel, C., Bailes, E., Keele, B.F., Liu, W., Loul, S., Butel, C.,

Liegeois, F., Bienvenue, Y., Ngolle, E.M., Sharp, P.M., Shaw, G.M., Delaporte, E., Hahn, B.H., and Peeters, M. (2006). Human immunodeficiency viruses: SIV infection in wild gorillas. Nature *444*, 164.

Hirsch, V.M., Olmsted, R.A., Murphey-Corb, M., Purcell, R.H., and Johnson, P.R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. Nature *339*, 389–392.

Hochrein, H., Schlatter, B., O'Keeffe, M., Wagner, C., Schmitz, F., Schiemann, M., Bauer, S., Suter, M., and Wagner, H. (2004). Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. Proc. Natl. Acad. Sci. U. S. A. *101*, 11416–11421.

Horan, K.A., Hansen, K., Jakobsen, M.R., Holm, C.K., Søby, S., Unterholzner, L., Thompson, M., West, J.A., Iversen, M.B., Rasmussen, S.B., Ellermann-Eriksen, S., Kurt-Jones, E., Landolfo, S., Damania, B., Melchjorsen, J., Bowie, A.G., Fitzgerald, K.A., and Paludan, S.R. (2013). Proteasomal Degradation of Herpes Simplex Virus Capsids in Macrophages Releases DNA to the Cytosol for Recognition by DNA Sensors. J. Immunol. *190*, 2311–2319.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514–518.

Hotter, D., Bosso, M., Jønsson, K.L.K.L., Krapp, C., Stürzel, C.M.C.M., Das, A., Littwitz-Salomon, E., Berkhout, B., Russ, A., Wittmann, S., Gramberg, T., Zheng, Y., Martins, L.J.L.J., Planelles, V., Jakobsen, M.R., Hahn, B.H.B.H., Dittmer, U., Sauter, D., and Kirchhoff, F. (2019). IFI16 Targets the Transcription Factor Sp1 to Suppress HIV-1 Transcription and Latency Reactivation. Cell Host Microbe *25*.

Hu, W.-S., and Hughes, S.H. (2012). HIV-1 reverse transcription. Cold Spring Harb. Perspect. Med. *2*.

Huang, Y., Ma, D., Huang, H., Lu, Y., Liao, Y., Liu, L., Liu, X., and Fang, F. (2017). Interaction between HCMV pUL83 and human AIM2 disrupts the activation of the AIM2 inflammasome. Virol. J. *14*, 34.

Hultquist, J.F., Hiatt, J., Schumann, K., McGregor, M.J., Roth, T.L., Haas, P., Doudna, J.A., Marson, A., and Krogan, N.J. (2019). CRISPR–Cas9 genome engineering of primary CD4+ T cells for the interrogation of HIV–host factor interactions. Nat. Protoc. *14*, 1–27.

Imataka, H., Mizuno, A., Fujii-Kuriyama, Y., and Hayami, M. (1993). Activation of the human immunodeficiency virus type 1 long terminal repeat by BTEB, a GC box-binding transcription factor. AIDS Res. Hum. Retroviruses *9*, 825–831.

Isaacs, A., Cox, R.A., and Rotem, Z. (1963). FOREIGN NUCLEIC ACIDS AS THE STIMULUS TO MAKE INTERFERON. Lancet *282*, 113–116.

Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., and Akira, S. (2006). A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat. Immunol. *7*, 40–48.

Ishikawa, H., and Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature *455*, 674–678.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature *461*, 788–792.

Isomura, H., Tsurumi, T., and Stinski, M.F. (2004). Role of the proximal enhancer of the major immediate-early promoter in human cytomegalovirus replication. J. Virol. *78*, 12788–12799.

Isomura, H., Stinski, M.F., Kudoh, A., Daikoku, T., and Shirata, N. (2005). Two Sp1 / Sp3 Binding Sites in the Major Immediate-Early Proximal Enhancer of Human Cytomegalovirus Have a Significant Role in Viral Replication. J. Virol. *79*, 9597–9607.

Ito, T., Azumano, M., Uwatoko, C., Itoh, K., and Kuwahara, J. (2009). Role of zinc finger structure in nuclear localization of transcription factor Sp1. Biochem. Biophys. Res. Commun. *380*, 28–32.

Ito, T., Kitamura, H., Uwatoko, C., Azumano, M., Itoh, K., and Kuwahara, J. (2010). Interaction of Sp1 zinc finger with transport factor in the nuclear localization of transcription factor Sp1. Biochem. Biophys. Res. Commun. *403*, 161–166.

Jakobsen, M.R., Bak, R.O., Andersen, A., Berg, R.K., Jensen, S.B., Tengchuan, J., Jin, T., Laustsen, A., Hansen, K., Ostergaard, L., Fitzgerald, K.A., Xiao, T.S., Mikkelsen, J.G., Mogensen, T.H., and Paludan, S.R. (2013). IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. Proc. Natl. Acad. Sci. U. S. A. *110*, E4571-80.

Janeway, C.A., and Medzhitov, R. (2002). Innate immune recognition. Annu. Rev. Immunol. *20*, 197–216.

Jeang, K.T., Chun, R., Lin, N.H., Gatignol, A., Glabe, C.G., and Fan, H. (1993). In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. J Virol *67*, 6224–33.

Jiang, G., Espeseth, A., Hazuda, D.J., and Margolis, D.M. (2007). c-Myc and Sp1 Contribute to Proviral Latency by Recruiting Histone Deacetylase 1 to the Human Immunodeficiency

Virus Type 1 Promoter. J. Virol. *81*, 10914–10923.

Jin, T., Perry, A., Jiang, J., Smith, P., Curry, J.A.A., Unterholzner, L., Jiang, Z., Horvath, G., Rathinam, V.A., Ricky, W., Hornung, V., Latz, E., Bowie, A.G.G., Fitzgerald, K.A.A., Xiao, T.S.S., Johnstone, R.W., Hornung, V., Latz, E., Bowie, A.G.G., Fitzgerald, K.A.A., and Xiao, T.S.S. (2013). Structures of The HIN Domain:DNA Complexes Reveal Ligand Binding and Activation Mechanisms of The AIM2 Inflammasome and IFI16 Receptor. Immunity *36*, 561–571.

Johnson, K.E., Chikoti, L., and Chandran, B. (2013). Herpes simplex virus 1 infection induces activation and subsequent inhibition of the IFI16 and NLRP3 inflammasomes. J. Virol. *87*, 5005–5018.

Johnson, K.E., Bottero, V., Flaherty, S., Dutta, S., Singh, V.V., and Chandran, B. (2014). IFI16 Restricts HSV-1 Replication by Accumulating on the HSV-1 Genome, Repressing HSV-1 Gene Expression, and Directly or Indirectly Modulating Histone Modifications. PLoS Pathog. *10*.

Johnstone, R.W., Kershaw, M.H., and Trapani, J.A. (1998a). Isotypic variants of the interferon-inducible transcriptional repressor IFI 16 arise through differential mRNA splicing. Biochemistry *37*, 11924–11931.

Johnstone, R.W., Kerry, J.A., and Trapani, J.A. (1998b). The human interferon-inducible protein, IFI 16, is a repressor of transcription. J. Biol. Chem. *273*, 17172–17177.

Jolliff, K., Li, Y., and Johnson, L.F. (1991). Multiple protein-DNA interactions in the TATAA-less mouse thymidylate synthase promoter. Nucleic Acids Res. *19*, 2267–2274.

Jones, K.A., and Tijan, R. (1985). Sp1 binds to promoter sequences and activates herpes simplex virus ´immediate-early´ gene transcription in vitro. Nature *317*, 179–182.

Jones, K.A., Yamamoto, K.R., and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell *42*, 559–572.

Jones, K.A., Kadonaga, J.T., Luciw, P.A., and Tjian, R. (1986). Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science *232*, 755–759.

Jønsson, K.L., Laustsen, A., Krapp, C., Skipper, K.A., Thavachelvam, K., Hotter, D., Egedal, J.H., Kjolby, M., Mohammadi, P., Prabakaran, T., Sørensen, L.K., Sun, C., Jensen, S.B., Holm, C.K., Lebbink, R.J., Johannsen, M., Nyegaard, M., Mikkelsen, J.G., Kirchhoff, F., Paludan, S.R., and Jakobsen, M.R. (2017). IFI16 is required for DNA sensing in human macrophages by promoting production and function of cGAMP. Nat. Commun.

Kadonaga, J.T., Jones, K.A., and Tjian, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. Trends Biochem. Sci. *11*, 20–23.

Kadonaga, J.T., Carner, K.R., Masiarz, F.R., and Tjian, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell *51*, 1079–1090.

Kamori, D., and Ueno, T. (2017). HIV-1 Tat and Viral Latency: What We Can Learn from Naturally Occurring Sequence Variations. Front. Microbiol. *8*, 80.

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. *28*, 27–30.

Kao, S.Y., Calman, A.F., Luciw, P.A., and Peterlin, B.M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature *330*, 489–493.

Karn, J. (2011). The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. Curr. Opin. HIV AIDS *6*, 4–11.

Keele, B.F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M.L., Bibollet-Ruche, F., Chen, Y., Wain, L. V, Liegeois, F., Loul, S., Ngole, E.M., Bienvenue, Y., Delaporte, E., Brookfield, J.F.Y., Sharp, P.M., Shaw, G.M., Peeters, M., and Hahn, B.H. (2006). Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science *313*, 523–526.

Kennedy, B.J. (1970). Metabolic and toxic effects of mithramycin during tumor therapy. Am. J. Med. *49*, 494–503.

Kersse, K., Verspurten, J., Berghe, T. Vanden, and Vandenabeele, P. (2011). The death-fold superfamily of homotypic interaction motifs. Trends Biochem. Sci. *36*, 541–552.

Kerur, N., Veettil, M.V., Sharma-Walia, N., Bottero, V., Sadagopan, S., Otageri, P., and Chandran, B. (2011). IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe *9*, 363–375.

Kessing, C.F., Nixon, C.C., Li, C., Tsai, P., Takata, H., Mousseau, G., Ho, P.T., Honeycutt, J.B., Fallahi, M., Trautmann, L., Garcia, J.V., and Valente, S.T. (2017). In Vivo Suppression of HIV Rebound by Didehydro-Cortistatin A, a "Block-and-Lock" Strategy for HIV-1 Treatment. Cell Rep. *21*, 600–611.

Kevin Howcroft, T., Palmer, L.A., Brown, J., Rellahan, B., Kashanchi, F., Brady, J.N., and Singer, D.S. (1995). HIV Tat represses transcription through Spl-Like elements in the basal promoter. Immunity *3*, 127–138.

Khare, S., Ratsimandresy, R.A., de Almeida, L., Cuda, C.M., Rellick, S.L., Misharin, A. V, Wallin, M.C., Gangopadhyay, A., Forte, E., Gottwein, E., Perlman, H., Reed, J.C., Greaves,

D.R., Dorfleutner, A., and Stehlik, C. (2014). The PYRIN domain–only protein POP3 inhibits ALR inflammasomes and regulates responses to infection with DNA viruses. Nat. Immunol. *15*, 343–353.

Kim, B., Arcos, S., Rothamel, K., Jian, J., Rose, K.L., McDonald, W.H., Bian, Y., Reasoner, S., Barrows, N.J., Bradrick, S., Garcia-Blanco, M.A., and Ascano, M. (2020). Discovery of Widespread Host Protein Interactions with the Pre-replicated Genome of CHIKV Using VIR-CLASP. Mol. Cell *78*, 624–640.e7.

Kim, E.-J., Park, J.-I., and Nelkin, B.D. (2005). IFI16 is an essential mediator of growth inhibition, but not differentiation, induced by the leukemia inhibitory factor/JAK/STAT pathway in medullary thyroid carcinoma cells. J. Biol. Chem. *280*, 4913–4920.

Kim, Y.K., Bourgeois, C.F., Isel, C., Churcher, M.J., and Karn, J. (2002). Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. Mol. Cell. Biol. *22*, 4622–4637.

Kimura, T., Jain, A., Choi, S.W., Mandell, M.A., Schroder, K., Johansen, T., and Deretic, V. (2015). TRIM-mediated precision autophagy targets cytoplasmic regulators of innate immunity. J. Cell Biol. *210*, 973–989.

Kluge, S.F., Sauter, D., and Kirchhoff, F. (2015). SnapShot: antiviral restriction factors. Cell *163*, 774–774.e1.

Kuleshov, M. V, Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen, G.W., and Ma'ayan, A. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. *44*, W90-7.

Kuwahara, J., and Coleman, J.E. (1990). Role of the Zinc(II) Ions in the Structure of the Three-Finger DNA Binding Domain of the Sp1 Transcription Factor. Biochemistry *29*, 8627–8631.

Kwak, J.C., Ongusaha, P.P., Ouchi, T., and Lee, S.W. (2003). IFI16 as a negative regulator in the regulation of p53 and p21(Waf1). J. Biol. Chem. *278*, 40899–40904.

Landolfo, S., Gariglio, M., Gribaudo, G., and Lembo, D. (1998). The Ifi 200 genes: An emerging family of IFN-inducible genes. Biochimie *80*, 721–728.

Laspia, M.F., Rice, A.P., and Mathews, M.B. (1989). HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell *59*, 283–292.

Lassen, K.G., Ramyar, K.X., Bailey, J.R., Zhou, Y., and Siliciano, R.F. (2006). Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells. PLoS Pathog. *2*, e68.

Le, H.T., and Harton, J.A. (2013). Pyrin- and CARD-only proteins as regulators of NLR functions. Front. Immunol.

Lemey, P., Pybus, O.G., Wang, B., Saksena, N.K., Salemi, M., and Vandamme, A.-M. (2003). Tracing the origin and history of the HIV-2 epidemic. Proc. Natl. Acad. Sci. U. S. A. *100*, 6588–6592.

Li, L., and Davie, J.R. (2010). The role of Sp1 and Sp3 in normal and cancer cell biology. Ann. Anat. *192*, 275–283.

Li, H., Wang, Z.-X.X., and Wu, J.-W.W. (2014). Purification, characterization and docking studies of the HIN domain of human myeloid nuclear differentiation antigen (MNDA). Biotechnol. Lett. *36*, 899–905.

Li, L., He, S., Sun, J.-M., and Davie, J.R. (2004). Gene regulation by Sp1 and Sp3. Biochem. Cell Biol. *82*, 460–471.

Li, T., Diner, B.A., Chen, J., and Cristea, I.M. (2012). Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. Proc. Natl. Acad. Sci. *109*, 10558–10563.

Li, T., Chen, J., and Cristea, I.M. (2013). Human cytomegalovirus tegument protein pUL83 inhibits IFI16-mediated DNA sensing for immune evasion. Cell Host Microbe *14*, 591–599.

Lin, W., Zhao, Z., Ni, Z., Zhao, Y., Du, W., and Chen, S. (2017). IFI16 restoration in hepatocellular carcinoma induces tumour inhibition via activation of p53 signals and inflammasome. Cell Prolif. *50*.

Liu, S.-Y., Sanchez, D.J., Aliyari, R., Lu, S., and Cheng, G. (2012). Systematic identification of type I and type II interferon-induced antiviral factors. Proc. Natl. Acad. Sci. *109*, 4239–4244.

Loregian, A., Bortolozzo, K., Boso, S., Caputo, A., and Palù, G. (2003). Interaction of Sp1 transcription factor with HIV-1 Tat protein: looking for cellular partners. FEBS Lett. *543*, 61–65.

Ludlow, L.E.A., Johnstone, R.W., and Clarke, C.J.P. (2005). The HIN-200 family: more than interferon-inducible genes? Exp. Cell Res. *308*, 1–17.

Lugrin, J., and Martinon, F. (2018). The AIM2 inflammasome: Sensor of pathogens and cellular perturbations. Immunol. Rev. *281*, 99–114.

Lum, K.K., Howard, T.R., Pan, C., and Cristea, I.M. (2019). Charge-Mediated Pyrin Oligomerization Nucleates Antiviral IFI16 Sensing of Herpesvirus DNA. MBio *10*, 1–16.

Luu, P., and Flores, O. (1997). Binding of SP1 to the immediate-early protein-responsive element of the human cytomegalovirus DNA polymerase promoter. J. Virol. *71*, 6683–6691.

Mahalingam, M., Peakman, M., Davies, E.T., Pozniak, A., McManus, T.J., and Vergani, D. (1993). T cell activation and disease severity in HIV infection. Clin. Exp. Immunol. *93*, 337–343.

Majello, B., De Luca, P., Hagen, G., Suske, G., and Lania, L. (1994). Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1. Nucleic Acids Res. *22*, 4914–4921.

Man, S.M., Karki, R., and Kanneganti, T.-D.D. (2016). AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. Eur. J. Immunol. *46*, 269–280.

Man, S.M., Karki, R., and Kanneganti, T.-D. (2017). Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunol. Rev. *277*, 61–75.

Manes, T.L., Simenauer, A., Geohring, J.L., Flemming, J., Brehm, M., and Cota-Gomez, A. (2020). The HIV-Tat protein interacts with Sp3 transcription factor and inhibits its binding to a distal site of the sod2 promoter in human pulmonary artery endothelial cells. Free Radic. Biol. Med. *147*, 102–113.

Marecki, J.C., Cota-Gomez, A., Vaitaitis, G.M., Honda, J.R., Porntadavity, S., St Clair, D.K., and Flores, S.C. (2004). HIV-1 Tat regulates the SOD2 basal promoter by altering Sp1/Sp3 binding activity. Free Radic. Biol. Med. *37*, 869–880.

Marin, M., Karis, A., Visser, P., Grosveld, F., and Philipsen, S. (1997). Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell *89*, 619–628.

Marsden, M.D., and Zack, J.A. (2019). HIV cure strategies: a complex approach for a complicated viral reservoir? Future Virol. *14*, 5–8.

Marshall, N.F., and Price, D.H. (1995). Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J. Biol. Chem. *270*, 12335–12338.

Marshall, N.F., Peng, J., Xie, Z., and Price, D.H. (1996). Control of RNA Polymerase II Elongation Potential by a Novel Carboxyl-terminal Domain Kinase. J. Biol. Chem. *271*, 27176–27183.

Martinez-Picado, J., and Deeks, S.G. (2016). Persistent HIV-1 replication during antiretroviral therapy. Curr. Opin. HIV AIDS *11*, 417–423.

Martinon, F., Gaide, O., Pétrilli, V., Mayor, A., and Tschopp, J. (2007). NALP Inflammasomes: A central role in innate immunity. Semin. Immunopathol. *29*, 213–229.

Massa, D., Baran, M., Bengoechea, J.A., and Bowie, A.G. (2020). PYHIN1 regulates pro-

inflammatory cytokine induction rather than innate immune DNA sensing in airway epithelial cells. *3*, 1–23.

Masur, H., Michelis, M.A., Greene, J.B., Onorato, I., Stouwe, R.A., Holzman, R.S., Wormser, G., Brettman, L., Lange, M., Murray, H.W., and Cunningham-Rundles, S. (1981). An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. N. Engl. J. Med. *305*, 1431–1438.

McLaren, P.J., Gawanbacht, A., Pyndiah, N., Krapp, C., Hotter, D., Kluge, S.F., Götz, N., Heilmann, J., Mack, K., Sauter, D., Thompson, D., Perreaud, J., Rausell, A., Munoz, M., Ciuffi, A., Kirchhoff, F., and Telenti, A. (2015). Identification of potential HIV restriction factors by combining evolutionary genomic signatures with functional analyses. Retrovirology *12*, 41.

Meier, J.L., and Stinski, M.F. (1996). Regulation of human cytomegalovirus immediate-early gene expression. Intervirology *39*, 331–342.

Meier, J.L., Keller, M.J., and McCoy, J.J. (2002). Requirement of multiple cis-acting elements in the human cytomegalovirus major immediate-early distal enhancer for viral gene expression and replication. J. Virol. *76*, 313–326.

Merkl, P.E., and Knipe, D.M. (2019). Role for a Filamentous Nuclear Assembly of IFI16, DNA, and Host Factors in Restriction of Herpesviral Infection. MBio *10*.

Merkl, P.E., Orzalli, M.H., and Knipe, D.M. (2018). Mechanisms of Host IFI16, PML, and Daxx Protein Restriction of Herpes Simplex Virus 1 Replication. J. Virol. *92*.

Mohagheghpour, N., Chakrabarti, R., Stein, B.S., Gowda, S.D., and Engleman, E.G. (1991). Early activation events render T cells susceptible to HIV-1-induced syncytia formation. Role of protein kinase C. J. Biol. Chem. *266*, 7233–7238.

Monroe, K.M., Yang, Z., Johnson, J.R., Geng, X., Doitsh, G., Krogan, N.J., and Greene, W.C. (2014). IFI16 DNA Sensor Is Required for Death of Lymphoid CD4 T Cells Abortively Infected with HIV. Science (80-.). *343*, 428–432.

Moon, H., and Lim, H.S. (2015). Synthesis and screening of small-molecule α-helix mimetic libraries targeting protein-protein interactions. Curr. Opin. Chem. Biol. *24*, 38–47.

Morrone, S.R., Wang, T., Constantoulakis, L.M., Hooy, R.M., Delannoy, M.J., and Sohn, J. (2014). Cooperative assembly of IFI16 filaments on dsDNA provides insights into host defense strategy. Proc. Natl. Acad. Sci. U. S. A. *111*, E62-71.

Morrone, S.R., Matyszewski, M., Yu, X., Delannoy, M., Egelman, E.H., and Sohn, J. (2015). Assembly-driven activation of the AIM2 foreign-dsDNA sensor provides a polymerization template for downstream ASC. Nat. Commun. *6*, 7827.

Mounts, P., Wu, T.C., and Peden, K. (1989). Method for cloning single-stranded oligonucleotides in a plasmid vector. Biotechniques *7*, 356–359.

Muesing, M.A., Smith, D.H., and Capon, D.J. (1987). Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. Cell *48*, 691–701.

Münch, J., Rajan, D., Rücker, E., Wildum, S., Adam, N., and Kirchhoff, F. (2005). The role of upstream U3 sequences in HIV-1 replication and CD4+ T cell depletion in human lymphoid tissue ex vivo. Virology *341*, 313–320.

Murata, Y., Kim, H.G., Rogers, K.T., Udvadia, A.J., and Horowitz, J.M. (1994). Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. J. Biol. Chem. *269*, 20674–20681.

Myers, G., Foley, B., Korber, B., Mellors, J.W., Jeang, K.T., and Wain-Hobson, S. (1997). Human retroviruses and AIDS 1996. A compilation and analysis of nucleic acid and amino acid sequences (Los Alamos, NM).

Nabel, G., and Baltimore, D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature *326*, 711–713.

Nagaoka, M., Shiraishi, Y., and Sugiura, Y. (2001). Selected base sequence outside the target binding site of zinc finger protein Sp1. Nucleic Acids Res. *29*, 4920–4929.

Narayan, V.A., Kriwacki, R.W., and Caradonna, J.P. (1997). Structures of zinc finger domains from transcription factor Sp1. Insights into sequence-specific protein-DNA recognition. J. Biol. Chem. *272*, 7801–7809.

Nchioua, R., Bosso, M., Kmiec, D., and Kirchhoff, F. (2020). Cellular Factors Targeting HIV-1 Transcription and Viral RNA Transcripts. Viruses *12*, 495.

O'Connor, L., Gilmour, J., and Bonifer, C. (2016). The role of the ubiquitously expressed transcription factor Sp1 in tissue-specific transcriptional regulation and in disease. Yale J. Biol. Med.

Ochsenbauer, C., Edmonds, T.G., Ding, H., Keele, B.F., Decker, J., Salazar, M.G., Salazar-Gonzalez, J.F., Shattock, R., Haynes, B.F., Shaw, G.M., Hahn, B.H., and Kappes, J.C. (2012). Generation of Transmitted/Founder HIV-1 Infectious Molecular Clones and Characterization of Their Replication Capacity in CD4 T Lymphocytes and Monocyte-Derived Macrophages. J. Virol. *86*, 2715–2728.

Oka, S., Shiraishi, Y., Yoshida, T., Ohkubo, T., Sugiura, Y., and Kobayashi, Y. (2004). NMR structure of transcription factor Sp1 DNA binding domain. Biochemistry *43*, 16027–16035.

Okabe, Y., Kawane, K., Akira, S., Taniguchi, T., and Nagata, S. (2005). Toll-like receptor-

independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. J. Exp. Med. *202*, 1333–1339.

Oleaga, C., Welten, S., Belloc, A., Solé, A., Rodriguez, L., Mencia, N., Selga, E., Tapias, A., Noé, V., and Ciudad, C.J. (2012). Identification of novel Sp1 targets involved in proliferation and cancer by functional genomics. Biochem. Pharmacol. *84*, 1581–1591.

Orzalli, M.H., DeLuca, N.A., and Knipe, D.M. (2012). Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. Proc. Natl. Acad. Sci.

Orzalli, M.H., Broekema, N.M., Diner, B.A., Hancks, D.C., Elde, N.C., Cristea, I.M., and Knipe, D.M. (2015). cGAS-mediated stabilization of IFI16 promotes innate signaling during herpes simplex virus infection. Proc. Natl. Acad. Sci. *112*, E1773–E1781.

Pascal, E., and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. *5*, 1646–1656.

Pavletich, N.P., and Pabo, C.O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science *252*, 809–817.

Peden, K., Emerman, M., and Montagnier, L. (1991). Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology *185*, 661–672.

Perkins, N.D., Edwards, N.L., Duckett, C.S., Agranoff, A.B., Schmid, R.M., and Nabel, G.J. (1993). A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. EMBO J. *12*, 3551–3558.

Philipsen, S., and Suske, G. (1999). A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res. *27*, 2991–3000.

Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., and Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol.

Raffaella, R., Gioia, D., De Andrea, M., Cappello, P., Giovarelli, M., Marconi, P., Manservigi, R., Gariglio, M., and Landolfo, S. (2004). The interferon-inducible IFI16 gene inhibits tube morphogenesis and proliferation of primary, but not HPV16 E6/E7-immortalized human endothelial cells. Exp. Cell Res. *293*, 331–345.

Rankovic, S., Varadarajan, J., Ramalho, R., Aiken, C., and Rousso, I. (2017). Reverse Transcription Mechanically Initiates HIV-1 Capsid Disassembly. J. Virol. *91*.

Ratnasabapathy, R., Sheldon, M., Johal, L., and Hernandez, N. (1990). The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAs from

various mRNA and snRNA promoters. Genes Dev. *4*, 2061–2074.

Ratsimandresey, R.A., Chu, L.H., Khare, S., De Almeida, L., Gangopadhyay, A., Indramohan, M., Misharin, A. V., Greaves, D.R., Perlman, H., Dorfleutner, A., and Stehlik, C. (2017). The PYRIN domain-only protein POP2 inhibits inflammasome priming and activation. Nat. Commun. *8*, 1–15.

Reeder, J.E., Kwak, Y.-T., McNamara, R.P., Forst, C. V., and D'Orso, I. (2015). HIV Tat controls RNA Polymerase II and the epigenetic landscape to transcriptionally reprogram target immune cells. Elife *4*, 1–44.

Richman, D.D., Margolis, D.M., Delaney, M., Greene, W.C., Hazuda, D., and Pomerantz, R.J. (2009). The challenge of finding a cure for HIV infection. Science *323*, 1304–1307.

Rittner, K., Churcher, M.J., Gait, M.J., and Karn, J. (1995). The human immunodeficiency virus long terminal repeat includes a specialised initiator element which is required for Tat-responsive transcription. J. Mol. Biol. *248*, 562–580.

Roberts, S.G., Choy, B., Walker, S.S., Lin, Y.S., and Green, M.R. (1995). A role for activator-mediated TFIIB recruitment in diverse aspects of transcriptional regulation. Curr. Biol. *5*, 508–516.

Rouillard, A.D., Gundersen, G.W., Fernandez, N.F., Wang, Z., Monteiro, C.D., McDermott, M.G., and Ma'ayan, A. (2016). The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. Database (Oxford). *2016*.

Roy, A., Ghosh, A., Kumar, B., and Chandran, B. (2019). IFI16, a nuclear innate immune DNA sensor, mediates epigenetic silencing of herpesvirus genomes by its association with H3K9 methyltransferases SUV39H1 and GLP. Elife *8*.

Saffer, J.D., Jackson, S.P., and Annarella, M.B. (1991). Developmental expression of Sp1 in the mouse. Mol. Cell. Biol. *11*, 2189–2199.

Saluja, D., Vassallo, M.F., and Tanese, N. (1998). Distinct subdomains of human TAFII130 are required for interactions with glutamine-rich transcriptional activators. Mol. Cell. Biol. *18*, 5734–5743.

Sauter, D., and Kirchhoff, F. (2016). HIV replication: a game of hide and sense. Curr. Opin. HIV AIDS *11*, 173–181.

Schindler, M., Münch, J., and Kirchhoff, F. (2005). Human immunodeficiency virus type 1 inhibits DNA damage-triggered apoptosis by a Nef-independent mechanism. J. Virol. *79*, 5489–5498.

Schubert, U., Bour, S., Willey, R.L., and Strebel, K. (1999). Regulation of virus release by the macrophage-tropic human immunodeficiency virus type 1 AD8 isolate is redundant and

can be controlled by either Vpu or Env. J. Virol. *73*, 887–896.

Selby, M.J., Bain, E.S., Luciw, P.A., and Peterlin, B.M. (1989). Structure, sequence, and position of the stem-loop in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat. Genes Dev. *3*, 547–558.

Shaw, N., and Liu, Z.-J. (2014). Role of the HIN Domain in Regulation of Innate Immune Responses. Mol. Cell. Biol. *34*, 2–15.

Sheldon, M., Ratnasabapathy, R., and Hernandez, N. (1993). Characterization of the inducer of short transcripts, a human immunodeficiency virus type 1 transcriptional element that activates the synthesis of short RNAs. Mol. Cell. Biol. *13*, 1251–1263.

Siliciano, R.F., and Greene, W.C. (2011). HIV latency. Cold Spring Harb. Perspect. Med. *1*, a007096.

Sleiman, S.F., Langley, B.C., Basso, M., Berlin, J., Xia, L., Payappilly, J.B., Kharel, M.K., Guo, H., Marsh, J.L., Thompson, L.M., Mahishi, L., Ahuja, P., MacLellan, W.R., Geschwind, D.H., Coppola, G., Rohr, J., and Ratan, R.R. (2011). Mithramycin is a gene-selective Sp1 inhibitor that identifies a biological intersection between cancer and neurodegeneration. J. Neurosci. *31*, 6858–6870.

Snyder, R.C., Ray, R., Blume, S., and Miller, D.M. (1991). Mithramycin blocks transcriptional initiation of the c-myc P1 and P2 promoters. Biochemistry *30*, 4290–4297.

Søby, S., Laursen, R.R., Østergaard, L., and Melchjorsen, J. (2012). HSV-1-induced chemokine expression via IFI16-dependent and IFI16-independent pathways in human monocyte-derived macrophages. Herpesviridae *3*, 6.

Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S.Z., Popovic, M., Arya, S., Gallo, R.C., and Haseltine, W.A. (1985). Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science *227*, 171–173.

Sogawa, K., Kikuchi, Y., Imataka, H., and Fujii-Kuriyama, Y. (1993). Comparison of DNA-binding properties between BTEB and Sp1. J. Biochem. *114*, 605–609.

Soneoka, Y., Cannon, P.M., Ramsdale, E.E., Griffiths, J.C., Romano, G., Kingsman, S.M., and Kingsman, A.J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. *23*, 628–633.

Song, L.L., Alimirah, F., Panchanathan, R., Xin, H., and Choubey, D. (2008). Expression of an IFN-inducible cellular senescence gene, IFI16, is up-regulated by p53. Mol. Cancer Res. *6*, 1732–1741.

Song, L.L., Ponomareva, L., Shen, H., Duan, X., Alimirah, F., and Choubey, D. (2010). Interferon-inducible IFI16, a negative regulator of cell growth, down-regulates expression

of human telomerase reverse transcriptase (hTERT) gene. PLoS One *5*, e8569.

Stehlik, C. (2007). The PYRIN domain in signal transduction. Curr. Protein Pept. Sci. *8*, 293–310.

Stehlik, C., Krajewska, M., Welsh, K., Krajewski, S., Godzik, A., and Reed, J.C. (2003). The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-κB and pro-caspase-1 regulation. Biochem. J *373*, 101–113.

Stewart, A.F. (1983). Therapy of malignancy-associated hypercalcemia: 1983. Am. J. Med. *74*, 475–480.

Stratmann, S.A., Morrone, S.R., van Oijen, A.M., and Sohn, J. (2015). The innate immune sensor IFI16 recognizes foreign DNA in the nucleus by scanning along the duplex. Elife *4*, e11721.

Su, K., Roos, M.D., Yang, X., Han, I., Paterson, A.J., and Kudlow, J.E. (1999). An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro. J. Biol. Chem. *274*, 15194–15202.

Suske, G. (1999). The Sp-family of transcription factors. Gene *238*, 291–300.

Suzuki, T., Kimura, A., Nagai, R., and Horikoshi, M. (2000). Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding. Genes Cells *5*, 29–41.

Tahirov, T.H., Babayeva, N.D., Varzavand, K., Cooper, J.J., Sedore, S.C., and Price, D.H. (2010). Crystal structure of HIV-1 Tat complexed with human P-TEFb. Nature *465*, 747–751.

Takehisa, J., Kraus, M.H., Ayouba, A., Bailes, E., Van Heuverswyn, F., Decker, J.M., Li, Y., Rudicell, R.S., Learn, G.H., Neel, C., Ngole, E.M., Shaw, G.M., Peeters, M., Sharp, P.M., and Hahn, B.H. (2009). Origin and Biology of Simian Immunodeficiency Virus in Wild-Living Western Gorillas. J. Virol. *83*, 1635–1648.

Tan, N.Y., and Khachigian, L.M. (2009). Sp1 Phosphorylation and Its Regulation of Gene Transcription. Mol. Cell. Biol. *29*, 2483–2488.

Tanaka, M., and Herr, W. (1990). Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell *60*, 375–386.

Theodore, T.S., Englund, G., Buckler-White, A., Buckler, C.E., Martin, M.A., and Peden, K.W. (1996). Construction and characterization of a stable full-length macrophage-tropic HIV type 1 molecular clone that directs the production of high titers of progeny virions. AIDS Res. Hum. Retroviruses *12*, 191–194.

Trickett, A., and Kwan, Y.L. (2003). T cell stimulation and expansion using anti-CD3/CD28

beads. J. Immunol. Methods *275*, 251–255.

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., Fitzgerald, K.A., Paludan, S.R., and Bowie, A.G. (2010). IFI16 is an innate immune sensor for intracellular DNA. Nat. Immunol.

Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H., and Hillen, W. (2000). Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc. Natl. Acad. Sci. U. S. A. *97*, 7963–7968.

Vansant, G., Bruggemans, A., Janssens, J., and Debyser, Z. (2020). Block-and-lock strategies to cure HIV infection. Viruses *12*.

Verhoef, K., Koper, M., and Berkhout, B. (1997). Determination of the minimal amount of Tat activity required for human immunodeficiency virus type 1 replication. Virology *237*, 228–236.

Verhoef, K., Marzio, G., Hillen, W., Bujard, H., and Berkhout, B. (2001). Strict Control of Human Immunodeficiency Virus Type 1 Replication by a Genetic Switch: Tet for Tat. J. Virol. *75*, 979–987.

van Vliet, J., Crofts, L.A., Quinlan, K.G.R., Czolij, R., Perkins, A.C., and Crossley, M. (2006). Human KLF17 is a new member of the Sp/KLF family of transcription factors. Genomics *87*, 474–482.

Watson, N. (1988). A new revision of the sequence of plasmid pBR322. Gene *70*, 399–403.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., and Jones, K.A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell *92*, 451–462.

Wei, W., Clarke, C.J.P., Somers, G.R., Cresswell, K.S., Loveland, K.A., Trapani, J.A., and Johnstone, R.W. (2003). Expression of IFI 16 in epithelial cells and lymphoid tissues. Histochem. Cell Biol. *119*, 45–54.

Wei, X., Decker, J.M., Liu, H., Zhang, Z., Arani, R.B., Kilby, J.M., Saag, M.S., Wu, X., Shaw, G.M., and Kappes, J.C. (2002). Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy. Antimicrob. Agents Chemother. *46*, 1896–1905.

Wichit, S., Hamel, R., Yainoy, S., Gumpangseth, N., Panich, S., Phuadraksa, T., Saetear, P., Monteil, A., Morales Vargas, R., and Missé, D. (2019). Interferon-inducible protein (IFI) 16 regulates Chikungunya and Zika virus infection in human skin fibroblasts. EXCLI J. *18*, 467–476.

Wierstra, I. (2008). Sp1: Emerging roles-Beyond constitutive activation of TATA-less housekeeping genes. Biochem. Biophys. Res. Commun. *372*, 1–13.

Wilen, C.B., Tilton, J.C., and Doms, R.W. (2012). HIV: cell binding and entry. Cold Spring Harb. Perspect. Med. *2*.

Wimmer, E.A., Jäckle, H., Pfeifle, C., and Cohen, S.M. (1993). A Drosophila homologue of human Sp1 is a head-specific segmentation gene. Nature *366*, 690–694.

Xie, J., Briggs, J.A., and Briggs, R.C. (1997). MNDA dimerizes through a complex motif involving the N-terminal basic region. FEBS Lett. *408*, 151–155.

Xin, H., Curry, J., Johnstone, R.W., Nickoloff, B.J., and Choubey, D. (2003). Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence. Oncogene *22*, 4831–4840.

Yang, C.-A., Huang, S.-T., and Chiang, B.-L. (2015). Sex-dependent differential activation of NLRP3 and AIM2 inflammasomes in SLE macrophages. Rheumatology (Oxford). *54*, 324–331.

Yang, W.-J., Song, M.-J., Park, E.Y., Lee, J.-J., Park, J.-H., Park, K., Park, J.H., and Kim, H.-P. (2013). Transcription factors Sp1 and Sp3 regulate expression of human ABCG2 gene and chemoresistance phenotype. Mol. Cells *36*, 368–375.

Yang, Y., Zhao, X., Wang, Z., Shu, W., Li, L., Li, Y., Guo, Z., Gao, B., and Xiong, S. (2019). Nuclear Sensor Interferon-Inducible Protein 16 Inhibits the Function of Hepatitis B Virus Covalently Closed Circular DNA by Integrating Innate Immune Activation and Epigenetic Suppression. Hepatology hep.30897.

Yieh, L., Sanchez, H.B., and Osborne, T.F. (1995). Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter. Proc. Natl. Acad. Sci. U. S. A. *92*, 6102–6106.

Yu, C.Y., Motamed, K., Chen, J., Bailey, A.D., and Shen, C.K. (1991). The CACC box upstream of human embryonic epsilon globin gene binds Sp1 and is a functional promoter element in vitro and in vivo. J. Biol. Chem. *266*, 8907–8915.

Yukl, S.A., Kaiser, P., Kim, P., Telwatte, S., Joshi, S.K., Vu, M., Lampiris, H., and Wong, J.K. (2018). HIV latency in isolated patient CD4+ T cells may be due to blocks in HIV transcriptional elongation, completion, and splicing. Sci. Transl. Med. *10*, eaap9927.

Zenzie-Gregory, B., Sheridan, P., Jones, K.A., and Smale, S.T. (1993). HIV-1 core promoter lacks a simple initiator element but contains a bipartite activator at the transcription start site. J. Biol. Chem. *268*, 15823–15832.

Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B.,

Mathews, M.B., and Price, D.H. (1997). Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev. *11*, 2622–2632.

Zimmerman, E.S., Sherman, M.P., Blackett, J.L., Neidleman, J.A., Kreis, C., Mundt, P., Williams, S.A., Warmerdam, M., Kahn, J., Hecht, F.M., Grant, R.M., de Noronha, C.M.C., Weyrich, A.S., Greene, W.C., and Planelles, V. (2006). Human Immunodeficiency Virus Type 1 Vpr Induces DNA Replication Stress In Vitro and In Vivo. J. Virol.

8 Statutory declaration

I hereby declare that I wrote the present dissertation with the title

“Human nuclear PYHIN proteins inhibit lentiviral gene expression by targeting the host transcription factor Sp1”

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“ (Rules of the University of Ulm for Assuring Good Scientific Practice).

Ulm,

Matteo Bosso

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