MEDICAL UNIVERSITY OF VIENNA

Host-virus protein interactome of L protein of lymphocytic choriomeningitis virus

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Vienna, January 2017

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Declaration

The following thesis has been written in a cumulative format and includes one first author publication. I performed *in vitro* experiments at CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, and *in vivo* experiments at the Department for Biomedical Research of the Medical University of Vienna.

The contribution of other people is described as follows:

I designed and conducted most of the experiments, analyzed and interpreted all obtained data, wrote the manuscript and this thesis. Christopher Schliehe, Anannya Bhattacharya, Bojan Vilagos, Lindsay Kosack, Alexander Lercher, Mehmet Sahin, Daniel D. Pinschewer and Leo C. James helped in conduction of *in vitro* and *in vivo* experiments and/or provided technical assistance and/or provided expert advice. Keiryn L. Bennett and Roberto Sacco performed mass spectrometry analyses. Michael Caldera, Peter Májek, Alexey Stukalov and Jörg Menche performed bioinformatic analysis. Andreas Bergthaler supervised the study (designed, analyzed the experiments and interpreted the obtained data) and wrote the manuscript.

All chapters of this thesis were written by the author. Dr. A. Bergthaler provided input and feedback during the thesis preparation.

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Abstract

Chronic human viral infections represent one of the most prominent global health problems. The molecular mechanisms underlying host-virus interactions in chronic infections remains elusive. Viral RNA-dependent RNA polymerase (RdRp) is a central enzyme responsible for viral genome replication and transcription. It recruits multiple host proteins to perform these functions. A single point mutation at the position 1079 of the RdRp-containing L protein of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a major molecular determinant for the immunobiological differences between the closely related acute Armstrong (ARM) and chronic Clone 13 (Cl13) strains.

We proposed that the L1079 mutation affects L protein interactions with host protein complexes. In order to test this hypothesis, we reverse genetically engineered L protein-tagged LCMV and performed mass spectrometry-based analyses of host-virus interactors in the context of a natural viral infection in human cells. The obtained data on the common as well as strain-specific host interactors of the L protein was used for further validation of the functional role of selected candidates in LCMV life cycle. Furthermore, we integrated our LCMV L protein interactomes with known host-virus interactors from other RdRps of negative and positive RNA viruses. These network analyses demonstrated that viral RdRps target highly interconnected functional modules of human proteins in common as well as virus-specific ways.

Zusammenfassung

Chronische Virusinfektionen stellen ein bedeutendes globales Gesundheitsproblem dar. Dennoch sind die der Wirt-Virus-Interaktion zugrundeliegenden molekularen Mechanismen noch ungenügend untersucht. Die virale RNA-abhängige RNA Polymerase (RdRp) ist das zentrale Enzym, welches für die Transkription und Replikation des viralen Genoms zuständig ist. Um diese Funktionen auszuführen, rekrutiert die Polymerase verschiedene Wirtsproteine. Eine einzige Punktmutation an der Position 1079 im RdRp-enthaltenden L-Protein des prototypischen Lymphozytären-Choriomeningitis Virus (LCMV) aus der Familie der Arenaviridae stellt das wichtigste molekulare Merkmal für die Unterschiede im Infektionsverlauf zwischen den eng verwandten akuten Armstrong (ARM) und persistierenden Clone 13 (Cl13) Stämmen dar.

Unsere Hypothese ist, dass die L1079 Mutation die Interaktionen des L Protein mit Wirt-Proteinkomplexen beeinflusst. Um diese Hypothese zu testen, fügten wir ein Protein-Tag an das L Protein mittels reverser Genetik und bestimmten dessen Interaktion mit Wirtsproteinen durch eine massenspektrometrischer Analyse. Anhand der Daten für allgemeine wie Stamm-spezifische Wirt-Interaktionen des L Protein selektierten wir Kandidaten-Proteine, deren funktionelle Rolle wir im LCMV Lebenszyklus wir genauer untersuchten. Des Weiteren verglichen wir die erhaltenen LCMV L-Protein Interaktionsdaten mit bekannten Wirt-Virus Interaktoren anderer RNA-Polymerasen von Plus- und Minus-RNA Viren. Diese Netzwerkanalyse zeigt, dass virale RNA Polymerasen präferenziell mit hochvernetzten funktionalen Modulen menschlicher Proteine interagieren in allgemeiner und Virus-spezifischer Weise interagieren.

Publications arising from this thesis

Kseniya Khamina, Alexander Lercher*, Michael Caldera*, Christopher Schliehe, Bojan Vilagos, Mehmet Sahin, Lindsay Kosack, Anannya Bhattacharya, Peter Májek, Alexey Stukalov, Roberto Sacco, Leo C. James, Daniel D. Pinschewer, Keiryn L. Bennett, Jörg Menche, Andreas Bergthaler. Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein, December 20, 2017, PLOS Pathogens. https://doi.org/10.1371/journal.ppat.1006758

Abbreviations

α-DG	α-dystroglycan
ALT	alanine aminotransferase
APC	antigen-presenting cells
AP-MS	affinity purification mass spectrometry
ARHGEF2	Rho guanine nucleotide exchange factor 2
ARM	Armstrong strain LCMV
AST	aspartate aminotransferase
Cl13	Clone 13 strain LCMV
CTL	cytotoxic T lymphocytes
FFU	focus forming unit
GP	glycoprotein
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IRF-3	Interferon regulatory factor 3
IFN I	type I interferon
IFNγ	interferon gamma
IGR	intergenic region
IL	interleukin
IRFs	interferon regulatory factors
ISG	interferon-inducible genes
LAG3	lymphocyte-activation gene 3
LCMV	lymphocytic choriomeningitis virus
MAVS	mitochondrial antiviral-signalling protein
MDA5	melanoma differentiation-associated gene 5
MS	mass spectrometry
NK cells	natural killers cells
NKRF	NF-κB repressing factor
NP	nucleoprotein
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PD-1	programmed cell death protein 1
PPI	protein-protein interactions
PRRs	pattern-recognition receptor

PTMs	posttranslational modifications
RdRp	RNA-dependent RNA-polymerase
RIG-I	Retinoic acid Inducible Gene I
RLRs	retinoic-inducible gene 1-like receptors
RNP	ribonucleoprotein
TGF-β	transforming growth factor beta
TLR	Toll-like receptor
Tregs	regulatory T cells
TRIM21	Tripartite motif containing 21
UTR	untranslated region
VSV	vesicular stomatitis virus

Acknowledgements

I deeply appreciate the possibility to carry out my PhD research work in the creative environment of CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, in the lab of Dr. Andreas Bergthaler. Therefore, I would like to thank my scientific supervisor Dr. Andreas Bergthaler for providing me with the opportunity to conduct my doctoral studies in his laboratory and for his support. These years were extremely productive for my scientific and personal development. Furthermore, many people contributed to my research and helped me on this long way.

I would like to particularly express my thankfulness and appreciation to Prof. Giulio Superti-Furga and Prof. Sylvia Knapp for scientific guidance and personal support. I thank Anita Ender and all members of the administrative team of CeMM for helping me to navigate through all essential documentation processes.

I would also like to thank my colleagues from CeMM for the wonderful collaborative atmosphere. Particularly, members of the Dr. Bergthaler lab: Christopher Schliehe for sharing his knowledge and wise attitude to life, Anannya Bhattacharya for support and optimism and Bojan Vilagos for valuable scientific advisce and practical vision of reality.

I would like to thank the DOC Fellowship Program of the Austrian Academy of Sciences for providing me with 2 years of funding. Furthermore, I highly appreciate the help of my PhD committee, including Prof. Franz X. Heinz and Prof. Giulio Superti-Furga, in shaping my research project.

Last but not the least, I want to acknowledge Harald and my family, whose love and support inspired me for this long journey. I would like particularly mention my parents and grandfather, who have always encouraged my dream to become a scientist.

INTRODUCTION

1.General introduction

Chronic human viral infections represent one of the key problems for global health. Over 500 million people around the world are infected with human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus (HCV) (Virgin, Wherry et al., 2009). Despite the rapid development and significant progress in antiviral therapy, our understanding of the molecular processes underlying viral chronicity remains incomplete. This knowledge is of particular importance to combat not only currently clinically relevant viruses but to enable timely response to emerging and re-emerging infections.

The current definition of chronic infection highlights certain features of a pathogen, such as the ability to evade immune response and to establish prolonged (even life-long) infection. This requires from the virus accurate manipulations of the host immunity not only to escape from antiviral response, but also to avoid overwhelming inflammatory reaction that leads to the tissue pathology and may even cause host death. The host-pathogen system in chronic infection aims to achieve an equilibrium stage with maximal viral propagation and minimal host immunopathology (Virgin et al., 2009).

The molecular basis of host-pathogen interactions lies in the direct protein-protein interactions (PPI). Viruses as obligate intracellular parasites recruit various host proteins to perform all essential vital functions, including replication of the viral genome. Cellular factors are indispensable for the functional activity of viral polymerases and may serve as a promising drug target for the development of antivirals (Watashi, Ishii et al., 2005). Furthermore, viruses interfere with immune signaling through direct PPI. Therefore, a comprehensive mapping of host-virus PPI on the global landscape of the human interactome may provide an overview of cellular functions and processes hijacked or targeted by viruses. Additionally, comparative analyses of host-virus interactions of different viruses and/or viral proteins could point out the distinct requirement for various infections.

In this research we focused on the comparative analyses of PPI between host factors and the virus replication machinery of acute Armstrong (ARM) and chronic Clone 13 (Cl13) strains of the lymphocytic choriomeningitis virus (LCMV). LCMV is a benchmark model in virology and immunology, that allows studying basic immunological reactions in the context of animal model (Zinkernagel, 2002). Previous research demonstrated that the prominent differences in immunobiology between the genetically closely related strains of ARM and Cl13 is determined predominantly by a

single amino acid exchange of lysine to glutamine at the position 1079 of the viral L protein that perform RdRps functions (Bergthaler, Flatz et al., 2010, Sullivan, Emonet et al., 2011). We developed a system for proteomic analyses of the LCMV L protein interactome in the context of viral infection and investigated how this single point mutation affects host-virus PPI. Then we performed functional analyses of the identified interactors in the context of LCMV infection using a loss-of-function approach. This allowed to identify novel proviral (DDX3X) as well as antiviral (NKRF, TRIM21) host factors. We infected *Trim21*$^{-/-}$ mice with LCMV and demonstrated impaired virus control in chronic infection with Cl13 strain. Additionally, we characterized the NKRF-deficient mice infected with the LCMV strain Cl13. Furthermore, we integrated the obtained dataset with publicly available PPI of viral RdRps and generated a global overview of cellular processes and pathways targeted by polymerases of different positive and negative RNA viruses.

In order to provide an appropriate context for our research and further discussion, I would like to review in the introduction the following topics:

1.1 Lymphocytic choriomeningitis virus as a model system to study chronic viral infections
1.2 Molecular and cellular biology of the lymphocytic choriomeningitis virus
1.3 Structure and function of viral RNA-dependent RNA polymerases
1.4 Viral evolution and RNA-dependent RNA polymerases
1.5 Immune response to lymphocytic choriomeningitis virus infection
1.6 Host-virus protein-protein interactions and the technological approaches to study them

1.1 Lymphocytic choriomeningitis virus as a model system to study chronic viral infections

LCMV is a prototypic virus from the family of *Arenaviridae*. This family contains various clinically relevant pathogens causing hemorrhagic fever in humans, such as Junin, Lassa, Machupo and others. Although LCMV is a rodent pathogen in the natural settings, it could cause neurological disorders in organ transplantation patients (Fischer, Graham et al., 2006).

Over the last 80 years, LCMV served as an indispensable model system in virology and immunology. Mouse infection with LCMV recapitulates some of the key features of human chronic viral infections, such as HCV and HIV (Rouse & Sehrawat, 2010, Virgin et al., 2009). Infection with LCMV Cl13 strain causes impaired T cell response, generalized immunosuppression, T cell exhaustion and delayed viral clearance. LCMV was used in pioneering works that established key principles of

immunosuppression and T cell exhaustion mechanisms, such as the critical role of programmed cell death protein 1 (PD-1) receptor and lymphocyte-activation gene 3 (LAG-3) (Barber, Wherry et al., 2006, Blackburn, Shin et al., 2009, Sakthivel, Gereke et al., 2012) as well as long-term exposure to transforming growth factor β (TGF-β) (Tinoco, Alcalde et al., 2009) and interleukin 10 (IL-10) (Brooks, Trifilo et al., 2006, Ejrnaes, Filippi et al., 2006) for sustaining suppression of $CD8^+$ T cells during viral persistence and a stage-dependent role of type I interferon signaling in virus control (Teijaro, Ng et al., 2013, Wilson, Yamada et al., 2013). Overall, LCMV provides a unique opportunity to study a naturally co-adapted and co-evolved host-virus pair and explore multiple aspects of innate and adaptive immune responses.

Another important feature of the LCMV model system is the extensive strain diversity with various immunobiological characteristics (Ahmed, Salmi et al., 1984, Pfau, Valenti et al., 1982). ARM leads to acute infection with the efficient viral clearance (undetectable in the blood after one week post infection), whereas Cl13 causes chronic infection with impaired immune response and viremia up to 8 weeks post infection with the same dose of viruses. ARM and Cl13 are genetically closely related strains of LCMV. Only three coding mutations are different between ARM and Cl13: two in the viral gene encoding glycoprotein ($GP260_{F\rightarrow L}$ and $GP176_{N\rightarrow D}$) and one in the viral L protein ($L1079_{K\rightarrow Q}$). Reverse genetic approach allowed to identify, that only a single point mutation that leads to the amino acid exchange lysine (ARM) to glutamine (Cl13) at the position 1079 of L protein determines the differences in the immunobiology of these two LCMV strains (Bergthaler et al., 2010). This mutation is also associated with higher replication rates of Cl13 in myeloid cells *in vitro* and *in vivo* (Bergthaler et al., 2010, Matloubian, Kolhekar et al., 1993). Although the GP260 mutation determines the differences in the affinity to the viral entry receptor α-dystroglycan (α-DG), it only synergistically contributes to the differences in immunopathology (Bergthaler et al., 2010).

LCMV is a noncytopathic virus *in vivo*, although infection with some strains leads to the severe immunopathology. For example, infection with Cl13 or WE, but not with ARM, strains causes CTL-mediated hepatitis (Zinkernagel, Haenseler et al., 1986). The severity of tissue damage could be evaluated both by histology and by measurement of the serum level of enzymes released upon rupture of hepatocytes, such as alanine (ALT) and aspartate (AST) aminotransferases. Similar readouts are used as clinical hallmarks of hepatitis.

Another interesting feature of the LCMV model is a broad cellular tropism, although it can be modulated by glycosylation of viral glycoprotein (GP) (Bonhomme, Knopp et al., 2013).

The route of infection in the LCMV model has a prominent impact on the immunobiological outcome. For example, intravenous infection with $2x10^6$ focus forming units (FFU) of Cl13 causes chronic infection, whereas the same dose of virus injected intracranially would lead to the choriomeningitis with lethal outcome (McGavern, Homann et al., 2002).

Overall, LCMV is an extremely useful and widely applicable model system for virology and immunology. This virus can establish systemic infection in mice, entering multiple organs and body compartments, that provides researchers with a broad range of tools for studies.

1.2 Molecular and cellular biology of the lymphocytic choriomeningitis virus

LCMV is a negative single strand pleomorphic enveloped RNA virus with a two-segmented genome. Long (L) and short (S) segments encode two open reading frames (ORFs) each in ambisense direction (Fields, 2007). The L segment (7.2 Kb) contains two genes, glycoprotein (GP) and nucleoprotein (NP), and the S segment (3.4 Kb) encodes the viral Z and L proteins. The genes are separated from each other by intergenic regions (IGRs) and flanked on the 5′ and 3′ ends with untranslated regions (UTRs) (**Figure 1**). The UTRs are highly structured and involved in long-range interactions (5´UTR - 3´UTR), that leads to genome circularization. This conformation is important for the efficient transcription and replication processes performed by the viral polymerase (Ferron, Weber et al., 2017).

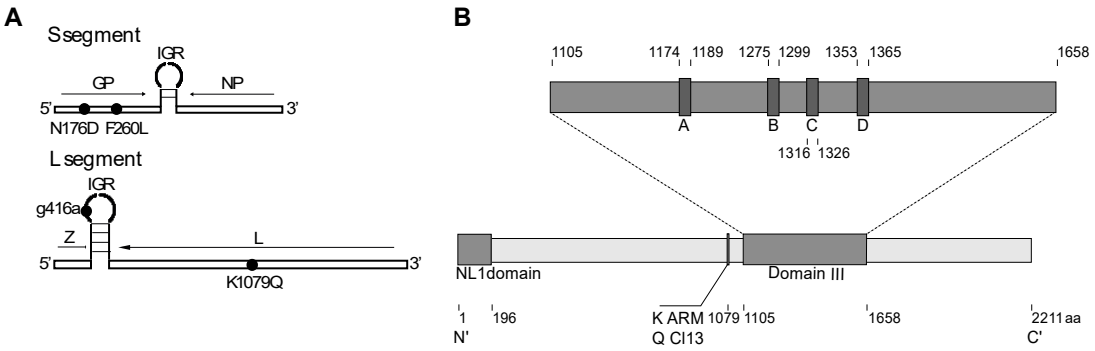

Figure 1. LCMV genome and L protein functional domains (A) Scheme of the LCMV genome (not drawn to scale). Each segment encodes two ORFs: S – GP and NP, L – Z and L. Genes are separated by IGR. Mutations that differ ARM from Cl13 is depicted as black dots (three coding: N176D and F260L in GP ORF, K1079Q in L ORF and one non-coding: g416a) **(B)** Scheme of the L protein LCMV (not drawn to scale) depicting NL1 and domain III (with conservative amino acid residues within domain III).

Viral RNA remains bound to the different viral proteins during all stages of the LCMV life cycle. The most abundant structural protein of LCMV, NP, interacts with viral

RNA to form the ribonucleoprotein complex (RNP). This RNP complex binds to the L protein, which possesses a domain with RdRp activity, to form a minimal viral RNA synthesis complex (Lee, Novella et al., 2000).

The L protein is the largest LCMV protein (around 250 kDa) and contains NL1 domain (type II endonuclease) as well as a palm structure-like domain III with RNA-dependent RNA polymerase activity (**Figure 1**). The NL1 domain consists of 196 amino acid residues and its available crystal structure for it demonstrates the similarity to the N-terminal part of PA protein of influenza. It plays an important role in the "cap-snatching" mechanism by binding to cellular mRNA and cleaving it. This provides viral mRNA with short-capped primers for RNA synthesis initiation from the host transcript (Morin, Coutard et al., 2010). Many negative sense RNA viruses, including *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae*, possess a structurally similar motif (Reguera, Weber et al., 2010). Additionally, the analyses of various negative strand RdRps structures revealed the presence of six conserved domains (I-VI) (Poch, Sauvaget et al., 1989). Since these domains are highly conserved between different families of viruses, they are considered to be important for the functional activity of the L proteins. Particularly, conserved motifs A, B, C and D within domain III play an essential role in viral RNA synthesis (Poch, Blumberg et al., 1990) (**Figure 1B**). The L1079 mutation, that determines immunobiological differences between the LCMV strains ARM and Cl13, lies outside of the known domains of the LCMV L protein (**Figure 1B**). Interestingly, the LCMV L protein forms oligomers and L-L interaction is required for the enzymatic activity of the L protein (Sanchez & de la Torre, 2005). For the Lassa virus L protein the sites mediating oligomerisation were identified both on N- and C-terminus of the protein (Brunotte, Kerber et al., 2011, Brunotte, Lelke et al., 2011). The oligomerisation of RdRps is documented for a range of viruses, e.g. poliovirus, HCV and foot-and-mouth disease virus (FMDV) (Bentham, Holmes et al., 2012, Spagnolo, Rossignol et al., 2010). The oligomerisation results in the cooperative template binding and plays an important role in the functional activity of the viral RdRps.

There is no available full crystal structure neither for the LCMV L protein nor for any other arenaviruses, which hampers the study of their replication machinery. The first RdRp structure of the negative single strand virus was solved for the influenza A and B polymerase complexes, consisting of three polyproteins – PA, PB1 and PB2 (Pflug, Guilligay et al., 2014, Reich, Guilligay et al., 2014) only in 2014. This discovery was followed by obtaining the crystal structures for influenza C polymerase (Hengrung, El Omari et al., 2015). Since influenza is a negative-strand RNA virus as well, these achievements could facilitate the generation of the crystal structure for the LCMV L

protein. The general structural features as well as functions of viral RdRps have been discussed in more details in the chapter 1.3.

The L protein forms RNP together with the most abundant LCMV protein – NP, that is a structural protein that binds to viral RNA as well. To form the RNP complex, which is essential for the functional activity of viral polymerase (transcription and replication of viral genome), NP interacts with other NP molecules through its N terminal domain (Levingston Macleod, D'Antuono et al., 2011, Ortiz-Riano, Cheng et al., 2012b). Recently, a specific amino acid residue (NP_{471}) that is critical for self-association has been reported (Ortiz-Riano, Cheng et al., 2012a). Nevertheless, NP serves not only as a structural protein but it is also involved in the active counteraction of the host immune response. Two regions in the C terminal part of NP, $NP_{370-400}$ and $NP_{500-553}$, are critical both for IFN I and NF-κB response inhibition (Martinez-Sobrido, Emonet et al., 2009, Rodrigo, Ortiz-Riano et al., 2012). The molecular mechanism of this counteraction is not entirely clear, although NP is reported to suppress IFN I signaling through IRF-3 inhibition (Martinez-Sobrido, Giannakas et al., 2007).

Another structural protein of LCMV is a small matrix-like protein with RING finger domain – the Z protein (Salvato, Schweighofer et al., 1992). It is associated with cellular membranes and is considered to be a key player during the LCMV budding process. Interestingly, several posttranslational modifications (PTMs) are required for the functional activity of the LCMV Z protein. For example, myristoylation of glycine at the position 2 is essential for budding of LCMV virions (Perez, Greenwald et al., 2004) and phosphorylation of serine at the position 41 might be involved in defective particle release (Ziegler, Eisenhauer et al., 2016). Similar to the NP of LCMV, the Z protein is involved in counteracting host immune response. The Z protein inhibits RIG-I-like receptors (RLRs) through its N terminal domain and therefore decrease IFN I response (Xing, Ly et al., 2015). There are a few known cellular interactors of the LCMV Z protein: eukaryotic translation initiation factor eIF4E (Campbell Dwyer, Lai et al., 2000), ribosomal protein P0 (Borden, Campbelldwyer et al., 1998) and, promyelocytic leukemia protein PML (Borden, Campbell Dwyer et al., 1998). The functional consequences of these host-virus PPI requires further analyses. Importantly, due to ambisense coding strategy, mRNA from Z ORF is synthesized from antigenomic RNA **(Figure 2)**. This is an additional level of gene expression regulation by LCMV. During the early stage of infection expression level of the Z ORF is relatively low, which may reflect it's role in the regulation of transcription and replication of viral RNA by direct PPI with the L protein (Kranzusch & Whelan, 2011), whereas high expression of the Z gene in the late stages of infection facilitates assembly of viral particles and budding (Perez & de la Torre, 2003). Z protein serves as a regulator of the viral RNA

transcription and replication process, but is not an essential component for efficient RNA synthesis (NP and L are the minimal factors required) (Lee et al., 2000).

NP interacts with the Z protein by direct binding. The binding site is located in the same C terminal domain that is also involved in counteracting IFN I response, although different amino acid residues have been reported to be responsible for this interaction (Ortiz-Riano, Cheng et al., 2011). Probably, NP-Z interaction mediates the correct virion assembly (Shtanko, Imai et al., 2010).

The GP ORF encodes a precursor protein that is processed by cellular protease SKI-1 (subtilisin-kexin-isozyme-1) into the membrane-associated stable signal peptide (SSP) and two proteins: GP1 and GP2 (Beyer, Popplau et al., 2003). These proteins are located on the surface of the virion and form a protein complex that serves multiple functions. GP1 interacts with the cellular surface receptor α-DG (Cao, Henry et al., 1998) and initiates viral entry into the cell (Borrow & Oldstone, 1994), whereas GP2 drives the membrane fusion. It's worthwhile to note, that SSP is involved in posttranscriptional processing of GP precursor as well as in pH-dependent viral entry and budding of the virions (Saunders, Ting et al., 2007). The GPs bear a PTM (glycosylation) that is indispensable for their functional activity and help to escape from antiviral immunity. It is known that the GP has 11 N-glycosylation sites (Bonhomme et al., 2013, Wright, Spiro et al., 1990) that shield the virus from neutralizing antibody response (Wright, Salvato et al., 1989) and affect GP functions such as virion assembly and budding (Bonhomme, Capul et al., 2011). Furthermore, GP1 recognizes the glycosylated sites of the α-DG receptor introduced by the cellular glycosyltransferase LARGE (Kunz, Rojek et al., 2005).

It is important to emphasize that not only LCMV, but also Lassa virus and several other arenaviruses, use α-DG as their main receptor (Cao et al., 1998, Kunz, Borrow et al., 2002). α-DG is a highly conserved and ubiquitously expressed cell surface receptor for extracellular matrix proteins, that is in agreement with wide cellular tropism and broad host range of arenaviruses. Further, LCMV is likely to use also alternative receptors to enter the cell next to α-DG (Kunz, Sevilla et al., 2004).

Altogether, LCMV possesses a rather small genome (10.6 Kb for both segments) with only four ORFs, resulting in five viral proteins and one signaling peptide sufficient for the viral life cycle within the host cell. The miniature size of the viral genome complicates the introduction of any additional genetic material. Nevertheless, a reverse genetic system developed for LCMV provides the opportunity to introduce genetic modification into the viral genome. This approach employs a RNA polymerase I/II-driven reverse genetic system for the recovery of infectious LCMV from cDNA (Flatz, Bergthaler et al., 2006).

A better understanding of the molecular environment required for the functional activity of various viral proteins as well as a comprehensive mapping of the cellular processes perturbed by viruses may shed light on the different steps of the viral life cycle and identify novel targets for antiviral treatment.

1.3 Structure and function of viral RNA-dependent RNA polymerases

Viral RdRp is an enzyme that catalyzes phosphodiester bond formation between ribonucleotides using RNA as a template. Therefore, RdRps from the different viral species are responsible for a similar function: transcription of the viral genome to mRNA and its replication. Despite displaying low homology at the protein primary structure level (some regions demonstrate less than 10% similarity), most of the viral RdRps possess several evolutionary conserved regions (Bruenn, 1991, Bruenn, 2003). Furthermore, the tertiary structure of viral RdRps remains very similar throughout different systematic groups of RNA viruses. This could be explained by the fact that all viral RdRps perform very similar functional steps such as recognition of the appropriate template, binding to it as well as coordination of different steps of RNA synthesis while protecting the viral RNA from degradation.

The RNA-dependent polymerase domain typically consists of a 400 amino acids sequence (Bruenn, 1991). This domain contains seven structural motifs arranged in the following order from N- to C-terminus: G, F, A, B, C, D and E (Bruenn, 1991, Hansen, Long et al., 1997, O'Reilly & Kao, 1998). This specific order remains preserved in all groups of RNA viruses, apart from Birnaviridae and Permutotetraviridae (Gorbalenya, Pringle et al., 2002, te Velthuis, 2014, Zeddam, Gordon et al., 2010). These seven motifs form the right hand-like structure with three subdomains that are called fingers, palm and thumb. Six of the aforementioned motifs (A-E) are located in the palm subdomain and the F motif is located within the finger subdomain. These regions of the polymerase complex remain conserved between positive and negative RNA viruses and are essential for the functional activity of the protein.

The “right-hand” conformation is unique for RdRps and each of the three subdomains involved in the different stages of viral polymerase activity. The thumb subdomain binds RNA and forms the NTP tunnel made of positively charged amino acid residues (Butcher, Grimes et al., 2001). This subdomain can form protrusions in some viral RdRps, that stabilizes NTPs on the single stranded RNA substrate during the initiation stage (Butcher et al., 2001).

The fingers subdomain interacts with the major groove of the RNA template. Some of the structural elements of this domain are involved in RNA binding (Butcher

et al., 2001) and oligomerisation (Pathak, Ghosh et al., 2002). The fingers and the thumb subdomains are variable between different RdRps and can be fully aligned only among positive RNA viruses (Ferrer-Orta, Arias et al., 2006, Monttinen, Ravantti et al., 2014).

The palm subdomain, consisting of A, C and D motifs, is the part of the RdRp complex where phosphodiester bonds between NTPs are formed. The conformational reorganization of the palm domain plays a key role in the catalytic activity. This subdomain contains highly evolutionary conserved aspartate residues within the structural motifs A and C, that are bound to two divalent metal ions (Flanegan & Baltimore, 1977) and play an important role in the positioning of the NTPs and their incorporation into the synthesized molecule (Butcher et al., 2001, Jablonski & Morrow, 1995). These sequences are preserved not only throughout different RNA, but DNA polymerases as well. Motif D participates in the evacuation of the phosphate group from the active catalytic site and initiates translocation of the RNA (te Velthuis, 2014).

The polymerase domain is usually a part of the large L protein (around 250 kDa), that possesses some additional domains responsible for its different functions. As we discussed in Chapter 1.2, LCMV L protein contains an N-terminal type II endonuclease (NL1 domain) (**Figure 1B**). NL1 is essential for cap-snatching activity of Arenaviridae and Bunyaviridae families (Morin et al., 2010, Reguera et al., 2010). VSV and Flaviviral RdRps possess domains with 2′-O-methyltransferase activity, that are important for cap synthesis (Egloff, Benarroch et al., 2002, Li, Fontaine-Rodriguez et al., 2005). These additional enzymatic domains are connected with each other through linker sequences, that allow to perform their functions independently (Lai, Kao et al., 1999).

The structural information about RdRps is available for some of the negative RNA viruses, such as vesicular stomatitis virus (VSV) (Liang, Li et al., 2015), La Crosse orthobunyavirus (Gerlach, Malet et al., 2015) as well as *Orthomyxoviridae* family viruses -influenza A, influenza B (Pflug et al., 2014, Reich et al., 2014) and influenza C (Hengrung et al., 2015). There is no available full crystal structure for LCMV. RdRps of orthobunyavirus and VSV are large multienzymatic polypeptides, whereas the polymerase complex of *Orthomyxaviridae* consists of three different polypeptides (PA, PB1 and PB2) organized in a heterotrimer. Despite these differences, the molecular structure and organization of these polymerase complexes remains similar and reveals well characterized structural motifs. The RdRp motifs F-E are located on PB1, while PB2 possesses a cap-binding domain and PA contains an endonuclease. This remarkable similarity of the RdRps functional domains across different families of

viruses makes polymerases a valuable tool to reconstruct viral evolution (see Chapter 1.4).

Although RdRps are enzymes responsible for transcription and replication of viral genomes, they also control several additional processes, such as template recognition, initiation of RNA synthesis, elongation, modification of the synthesized RNAs with cap structures and others (Ahlquist, 2002).

All polymerases synthesize nucleic acid in the 5´-3´ direction and, therefore, initiate the reaction on 3´ end of the template. In the case of viral initiation, the task is getting more complex due to the necessity to distinguish viral RNA from cellular RNA. Moreover, in case of arenaviruses this is further complicated by the presence of several species of viral RNA in the infected cells due to the ambisense coding strategy: genomic, antigenomic and mRNA (**Figure 2**) (Shao, Liang et al., 2015). Different viruses solve this problem in different ways. Brome mosaic virus requires an additional viral protein, 1a, that bind to both viral RNA and RdRp, acting as a mediator during complex-formation (Chen, Noueiry et al., 2003). Flaviviral and arenaviral RdRps bind only to circularized genomic RNA, that is facilitated by the highly conserved elements on its 5´ and 3´ ends (Filomatori, Lodeiro et al., 2006, Salvato & Shimomaye, 1989). For Picornaviruses circularization is mediated through some viral or cellular proteins and represents an important step in template recognition and initiation (Herold & Andino, 2001).

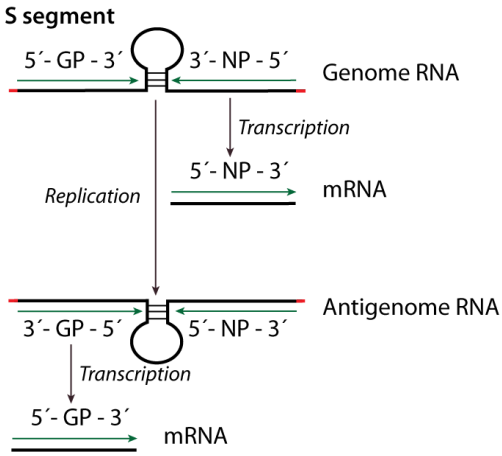

Figure 2. Scheme of the LCMV genome transcription and replication (S segment). Scheme of the LCMV genome replication and transcription for the S segment (not drawn to scale). The S segment encodes GP and NP ORFs. Genes are separated by IGR. Genome RNA is replicated through the step of antigenome RNA. The NP ORF is transcribed from the genome, while GP mRNA is synthesized from the antigenome.

Viral RdRps employ two approaches to initiate the synthesis of RNA: *de novo* and primer-dependent initiation. *De novo* initiation requires interaction of two NTPs,

where first of the NTPs use a 3´-hydroxyl group to form phosphodiester bond with the second NTP. Primer-dependent initiation requires presence of some primers to start polymerization. Viral RdRps use as primers not only oligonucleotides (including fragments of RNA obtained through the cap-snatching mechanism), but also proteins and the 3´terminus of the template (so called back-primed initiation) (te Velthuis, 2014).

The next step after initiation is elongation of viral genome. Transition to this stage is usually reflected in a kinetics shift (Klumpp, Ford et al., 1998) and accumulation of the not yet complete RNA products (Kao, Singh et al., 2001).

Viral RdRps regulate the balance between replication and transcription by the multiple approaches. One of the best studied examples is the life cycle of Sindbis virus. The viral protease shifts the transcription/replication ratio by performing incomplete processing of viral non-structural polyproteins, that support only replication (Lemm, Rumenapf et al., 1994). An alternative approach to regulate the switch between transcription and replication is to use different protein complexes for these processes. The replication complex of VSV contain viral proteins L, P and N, whereas the transcription complex lacks N and requires host proteins: translation elongation factor 1 alpha and Hsp60 (Qanungo, Shaji et al., 2004). LCMV employs the Z protein as a central regulator of the balance between transcription and replication of the viral genome. The Z protein interacts directly with L and locks the viral polymerase in a catalytically inactive state (Kranzusch & Whelan, 2011). This downregulates the viral RNA production and promotes virus assembly.

Termination is the next step in the functional cycle of all RdRps. Transcription termination for some viruses, such as VSV, can be linked to the mRNA polyadenylation process (Hwang, Englund et al., 1998), whereas for others, including LCMV, it is associated with a 3´terminal hairpin structure formed by IGR (Meyer & Southern, 1993).

Altogether, multiple factors affect the efficiency of RdRp, including complex regulatory loops between viral RNA and protein as well as host factors at the different stages of viral replication and transcription.

Various host factors are involved at the different stages of the functional activity of RdRps. Heat shock protein Hsp90 stabilizes and ensures proper folding of several RdRps of negative RNA viruses (Connor, McKenzie et al., 2007). Moreover, a number of host factors are directly involved in the polymerization process. The influenza virus polymerase complex interacts with the cellular polymerase II (Engelhardt, Smith et al., 2005) and ATP-dependent RNA helicase UAP56 (Momose, Basler et al., 2001) during RNA synthesis. Interestingly, many host factors interact both with viral proteins and with viral RNA. Elongation factor 1-alpha (eEF1A) is a protein

required for the enzymatic delivery of tRNAs to the ribosomes in eukaryotes (Mateyak & Kinzy, 2010). eEF1A binds to viral proteins and viral RNA of West Nile virus (Blackwell & Brinton, 1997), tombusvirus (Li, Pogany et al., 2010) and tobacco mosaic virus (Zeenko, Ryabova et al., 2002). These data altogether indicate that host proteins play an important role in the functional activities of viral RdRps. Thus, characterizing the interactomes of RdRps allows to better understand the molecular functions of these enzymes. The development of the various bioinformatics approaches to predict protein-ligand binding sites helps to gain insights into the molecular mechanisms underlying PPI (Roche, Brackenridge et al., 2015, Xie & Hwang, 2015). Furthermore, it may allow to identify potential targets for antiviral pharmaceuticals (Watashi et al., 2005).

As described in the previous example with VSV, that regulates the switch between transcription and translation by changing the composition of the replication complex (Qanungo et al., 2004), host-virus interactions can be very dynamic in the course of infection. Furthermore, the physiological context of infection, the cell type and many other parameters might influence the interactome of viral polymerases. These factors should be taken into the consideration for proteomic analyses of viral RdRps.

1.4 Viral evolution and RNA-dependent RNA polymerases

RdRps are unique enzymes with very distinct characteristics. They display extremely high mutation rates (10^{-3}-10^{-4} mis-incorporations per nucleotide per generation), which coupled together with immense population size and small genomes, leads to the fast evolution and adaptation of RNA viruses to various environmental challenges (Andino & Domingo, 2015, Domingo, Sheldon et al., 2012). These factors determine that each viral genome is likely to differ from the parental molecule by at least one nucleotide exchange. Therefore, individual genomes in viral populations form constellations of closely related mutants, so called quasispecies, that cooperatively respond to the environmental pressure (Domingo & Holland, 1997). Thus, the properties of RdRps define the basic characteristics of viral evolution.

The low fidelity of viral RdRps is not a sign of inefficient functional activity of this enzyme, but is an important property essential for survival of the viruses in a complex environment. One of the classical examples strengthening this hypothesis is the identification and characterization of a single point mutation in the 3D polymerase of poliovirus (3D-G64S), which increases its fidelity (Pfeiffer & Kirkegaard, 2003, Pfeiffer & Kirkegaard, 2005). This mutation was originally identified as a driver of poliovirus resistance to ribavirin (Pfeiffer & Kirkegaard, 2003). Ribavirin is a commonly

used antiviral drug that is considered to act as a nucleotide analog. It increases the error rate during viral RNA synthesis through incorporation into the viral genome pairing with cytosine and uracil (Sidwell, Huffman et al., 1972, Tam, Lau et al., 2001). The 3D-G64S mutant with increased fidelity demonstrated a reduced fitness and virulence *in vivo* compared to the wild type virus (Pfeiffer & Kirkegaard, 2003). This supported the hypothesis that a high error rate is required for viral adaptation upon entering mouse tissues *in vivo*.

Although viral RdRp apparently needs to possess a high mutation rate to enable viral evolution, it should also preserve the genetic information to ensure that a significant proportion of the synthesized RNA molecules remain functional. Thus, the maximal error rate compatible with the maintenance of the viral genetic information serves as an error threshold (Swetina & Schuster, 1982). This creates a certain range of the error rates for different viral RdRps and enables them, on one hand, to maintain the genome integrity, and, on the other hand, to provide a high level of diversity required for viral evolution.

The specific properties of viral RdRps define the basic characteristics of viral evolution; thus a better understanding of viral polymerase activity may allow to artificially shape viral quasispecies and interfere with viral infections.

1.5 Immune response to lymphocytic choriomeningitis virus infection

Infection with LCMV activates both the innate and adaptive immune responses of the host organism. Many important immunological phenomena were originally discovered in the LCMV system. For example, the first idea of the MHC restriction in the context of viral infection came from experiments by Rolf Zinkernagel and Peter Doherty which demonstrated that LCMV-specific $CD8^+$ T cells (or cytotoxic T cells - CTL) require the recognition of both specific viral epitopes and matching MHC molecules (Zinkernagel & Doherty, 1974). This finding elaborates an important characteristic of the immune system in self/nonself-discrimination that remains one of the key ideas of immunity till now.

Another valuable immunological concept that was extensively studied in the LCMV model is T cell exhaustion. This phenomenon is associated with non-responsiveness of T cells to a specific antigen in chronic infections that leads to the impaired control of the infecting agent. This T cell status is often characterized by the loss of effector function accompanied by the expression of certain cell surface markers, such as CTLA4 (cytotoxic T lymphocyte antigen 4) and PD1 (programmed cell death protein 1) (Barber et al., 2006, Blackburn et al., 2009). T cell exhaustion occurs not only in the setting of chronic viral infections but also in cancer since in both cases T

cells constantly deal with persistent antigens and inflammatory reaction (Pauken & Wherry, 2015). The improvement of T cell response by inhibition of the PD1 receptor was initially described in the LCMV system and it is currently being used as a promising therapeutic approach for cancer treatment (Planchard, Yokoi et al., 2016, Robert, Ribas et al., 2014). Overall, the precise mechanistic dissection of the molecular pathways involved in exhaustion is important both for basic immunology and for further development of therapeutic approaches to treat chronic viral infection and cancer.

Chronic LCMV infection induces innate and adaptive immune responses. Various signaling pathways, as well as immune and non-immune cell subsets are involved at every step of the antiviral response. Therefore, I would like to discuss in the current chapter innate (**1.5.1**) and adaptive (**1.5.2**) immune responses to LCMV and highlight the major players involved in host-virus interactions during LCMV infection.

1.5.1. Innate immune response to lymphocytic choriomeningitis virus infection

Innate immune response is a primary immune response against pathogens and is driven by germ-line encoded molecules. Innate immunity includes complex interactions between various signaling molecules and pathways, including:

- activation of intracellular signaling cascades upon recognition of the virus by sensors such as pattern-recognition receptor (PRRs) and retinoic-inducible gene 1-like receptors (RLRs));
- cellular response (macrophages, natural killer (NK) cells and dendritic cells (DCs));
- induction of defense mechanisms within the cell (e.g. IFN I, NF-κB signaling).

The multiple host immune response sensors that activate innate immune response pathways recognize conserved pathogen structures – pathogen-associated molecular patterns (PAMPs).

RIG-like receptors (RLRs) are located in the cytosol and required to sense very common PAMPs during infection: double-stranded and single-stranded viral RNA. Two of the best characterized sensors from the RLRs family - melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I) play a key role in LCMV recognition. MDA5 and RIG-I sense double-stranded RNA and transduce a signal through the adaptor protein mitochondrial antiviral-signaling protein (MAVS) to induce IFN I. Genetic ablation of MAVS signaling leads to the impairment of IFN I production, $CD8^+$ virus-specific T cell response and viral load control in LCMV infection (Clingan, Ostrow et al., 2012). LCMV evolved to actively counteract RIG-I signaling pathway through viral NP protein binding to I-kappa-B

kinase epsilon (IKKε), thereby, preventing IRF3 phosphorylation (Pythoud, Rodrigo et al., 2012).

The toll-like receptors (TLR) family is an evolutionary highly conserved group of pattern-recognition receptors (PRRs) that is associated with cellular and intracellular membranes. The first identified TLR in mammals was TLR4 (Medzhitov, Preston-Hurlburt et al., 1997). Although TLRs include 13 transmembrane receptors, only few of them were implicated in immune signaling induced by LCMV. The most important receptors for LCMV sensing and immune response are TLR2 (Zhou, Halle et al., 2008) and TLR7 (particularly in pDC recognition of LCMV) (Macal, Lewis et al., 2012). Although there are other members of the TLR family involved in viral RNA sensing such as TLR3 (double stranded RNA) and TLR8 (single stranded RNA), the genetic ablation of these receptors doesn't affect the antiviral immune response against LCMV (Edelmann, Richardson-Burns et al., 2004, Jung, Kato et al., 2008). Some of the key molecules downstream from TLRs were shown to be Important in anti-LCMV immunity, such as Myeloid differentiation primary response 88 (MyD88) that serves as an adaptor protein essential for the signal transduction of various TLRs and IL1 (Medzhitov, Preston-Hurlburt et al., 1998). MyD88 is indispensable for fully functional $CD8^+$ T cell response and efficient LCMV clearance (Rahman, Cui et al., 2008, Zhou, Kurt-Jones et al., 2005).

Another important part of the antiviral response are cytokines. These small secreted proteins play a crucial role in orchestrating the immune response (Turner, Nedjai et al., 2014). LCMV infection triggers production of multiple pro-inflammatory cytokines, such as type I interferon (IFN I), IL-1β, IL-6, IL-10, IL-15, IL-21 and IL-33 (Zuniga, Macal et al., 2015), that significantly shapes both innate and adaptive immune responses.

IFN I signaling is considered to be one of the first lines of host defense against viruses. Originally, it was identified as a soluble factor that "interferes" with the spread of viruses between cells in tissue culture conditions (Nagano & Kojima, 1958). This cytokine reprograms gene expression of the host cell and cause up-regulation of multiple interferon-stimulated genes (ISGs). Nevertheless, the role of IFN I response goes far beyond intracellular signaling to hunt the virus and prevent its spread to the neighboring cells. This cytokine may modulate the function of natural killer (NK) cells and CTL response. IFN I was used for decades as an important component of common treatment in the pegylated form for multiple human chronic viral infections, including HCV (Palumbo, 2009). The recent research based on LCMV system demonstrated that activated IFN I signaling facilitates T cell exhaustion in chronic viral infection (Teijaro et al., 2013, Wilson et al., 2013). This surprising finding led to the change of

paradigm not only in infection biology but also in medical application. These studies elucidated the stage-dependent role of IFN I and expanded our idea about so called “cytokine storm” accompanying various infectious and non-infectious disease. The concept of cytokine storm reflects the overproduction and uncontrolled release of multiple proinflammatory cytokines. Similar to many other viruses LCMV developed molecular mechanisms to inhibit IFN I production (Fan, Briese et al., 2010, Rodrigo et al., 2012). Altogether, LCMV research significantly advanced our understanding of IFN I response in the settings of chronic inflammation.

Multiple cytokines act as regulators of the adaptive immune response (see Chapter 1.5.2) as well.

The “IL-1” term not only refers to a single cytokine, but to a cytokine family. These proteins are involved in various signaling pathways highly relevant in LCMV infection. They induce cytokine production (IL-6), apoptosis (Joeckel, Wallich et al., 2012) as well as MAP kinase and NF-κB signaling. Additionally, IL-1 was implicated in the weight loss in the intracranial LCMV infection model (Hildeman & Muller, 2000).

Altogether various signaling molecules, induced by LCMV infection, shape the cellular immune response and have a great impact on the immunobiological outcome.

Innate immune cells play a crucial role in the antiviral response. Dendritic cells (DCs) are important antigen-presenting cells (APCs), required for the successful activation of adaptive immune response. There are two major subsets of DCs in mice defined as plasmacytoid DCs (pDCs) ($CD11b^{-}$ $CD11c^{low}B220^{+}$) and conventional DC (cDCs) ($CD11b^{high}$ $CD11c^{high}B220^{-}$) (Shortman & Liu, 2002). cDCs are professional APCs, whereas pDCs are one of the most important IFN I producer during the early stages of LCMV infection (Reizis, Bunin et al., 2011). LCMV infects different groups of DCs at the beginning of an infection leading to the activation, up-regulation of MHCI and MHCII surface molecule and cytokine production, including IFN I (Lee, Burke et al., 2009). LCMV Cl13 preferentially infects pDCs (Sevilla, Kunz et al., 2003). Cl13 infects DCs more efficiently than ARM (Bergthaler et al., 2010, Sevilla, Kunz et al., 2000). These differences are mainly attributed to the mutation GP260 in the viral glycoprotein that affects the binding efficiency to α-DG. Furthermore, Cl13 inhibits the production of IFN I by pDCs more efficiently and for longer than ARM. During chronic LCMV infection DCs are both rapidly decreasing in number and losing functional activity (Cunningham, Champhekar et al., 2016, Zuniga, Liou et al., 2008). The impaired functionality of DCs might be involved in the T cell exhaustion phenotype observed in Cl13 LCMV infection. Both IFNγ and IFN I are responsible for the generation of suppressive DCs during chronic infection: IFNγ drives the differentiation

of DCs from monocytes and IFNI upregulates PDL-1 (programmed cell death ligand) and IL-10 production (Cunningham et al., 2016).

NK cells are another subset of innate immune cells that play a crucial role in virus clearance both by lysing the infected cells directly and producing cytokines (Biron, Nguyen et al., 2002).

In general, LCMV infection induces multiple innate immune signaling pathways and cellular subsets that facilitate adaptive immune response.

1.5.2 Adaptive immune response to lymphocytic choriomeningitis virus infection

Although adaptive and innate immune responses represent different systems and stages of the host defense in LCMV infection, they are closely connected and mutually dependent (Zuniga et al., 2015). It was demonstrated that adoptive transfer of LCMV-specific $CD4^+$ lymphocytes in chronic infection facilitates virus control and restores CTL and antibody responses (Penaloza-MacMaster, Barber et al., 2015).

Differentiation and specialization of naïve immune cells during LCMV infection are accompanied by transcriptional, epigenetic and metabolic reprograming. Multiple cytokines and other signaling molecules orchestrate a complex process of the adaptive immune response.

$CD4^+$ T cells are required for effective virus control in LCMV infection, for most through their effect on other cell subsets (B cells and $CD8^+$ T cells). LCMV induces both Th1 and Th2 response with long-lasting memory (Whitmire, Asano et al., 1998). Several cytokines contribute to effective $CD4^+$ T cells response. IL-12 is not only one of the key players in the differentiation of naïve T cells in the Th1 subset upon antigen stimulation (Cooper, Magram et al., 1997), it also contributes to the maintenance of the balance between Th1 and Th2. Despite the fact that IL-12 is considered to have a rather minor effect on the $CD8^+$ T cell response upon LCMV infection (Oxenius, Karrer et al., 1999), it synergizes with other cytokines (TNFα, IL-12, IL-18 and IL-33) to induce IFNγ production in effector cells (Freeman, Hammarlund et al., 2012).

Another important regulator for production, differentiation and activation of T cells is IL-10 cytokine. A recent study provided a mechanistic link between constant exposure to the antigen and BLIMP-1-driven IL-10 expression in Th1 cells (Parish, Marshall et al., 2014). During chronic LCMV infection (e.g. with Cl13 strain) it contributes to the T cell exhaustion process (Richter, Perriard et al., 2013). IL-2 has a stage-dependent role in chronic LCMV infection. This cytokine negatively regulates T cell differentiation and expansion during the early phase of infection and promotes

these processes in the late stage of infection as well as memory T cells proliferation (Blattman, Grayson et al., 2003).

In the late stages of infection, another cytokine, IL-6, plays a prominent role. Particularly, it is required for follicular T helper response that, in turn, is important for effective antibody response (Harker, Lewis et al., 2011). Furthermore, IL-6 is a pleiotropic cytokine that, on one hand, promotes differentiation of B and T cells and, on the other hand, has functions similar to hormones that affect metabolism and the neurological system (Hunter & Jones, 2015).

The $CD8^+$ T cell or cytotoxic T lymphocyte (CTL) response plays a critical role in LCMV clearance from the organism. The main function of $CD8^+$ T cells is the elimination of infected cells through the secretion of cytolytic proteins (e.g. granzyme B) and cytokines. Cytokine production is an important feature of functionally active cells. Competent virus-specific $CD8^+$ lymphocytes produce TNF-alpha (TNFα) and IFN gamma (IFNγ) cytokines. The significant decrease in the number of virus-specific $CD8^+$ T cell double producers of TNFα and IFNγ is one of the important characteristics of the exhausted phenotype in chronic LCMV infection (Wherry, 2011). The $CD8^+$ T cell response is significantly shaped by multiple cytokines, such as IL-1 (Joeckel et al., 2012), IL-4 (Renkema, Lee et al., 2016) and others. Infection with several LCMV strains, such as Cl13 and WE, lead to hepatitis that is caused by cell death induced by $CD8^+$ T cells (Kagi, Ledermann et al., 1996). LCMV infection leads to the memory CD8 T cell response, that maintained in the absence of the virus and plays an important role in the immune response to recurrent infections. IL-2, IL-7 and IL-15 contributes to the development of the memory responses against LCMV infection (Blattman et al., 2003, Kieper, Tan et al., 2002, Richer, Pewe et al., 2015).

Differences in immunopathology of various LCMV strains reflects the variations in innate and adaptive immune responses caused by them. For example, infection with the chronic strain Cl13, but not the acute strain ARM leads to the early activation of DCs followed by sustained expansion of myeloid cells (Norris, Uebelhoer et al., 2013). This provides researchers with indispensable tools to dissect the impact of adaptive and innate immune responses on the infection.

Overall, LCMV infection induces multiple innate and adaptive immune response pathways and employs various mechanisms to modify and counteract host immunity.

1.6 Host-virus protein-protein interactions and the technological approaches to study them

Viruses went through a long process of co-evolution and co-adaptation with their host. This is a regular process for most parasites, but it is of a particular importance for viruses since they are obligate intracellular parasites and they rely on host factors in most of the vital functions. Viruses hijack and exploit multiple cellular pathways during their life cycle. Therefore, host-virus interactions on the molecular level play a key role in the determination of infection outcome. This molecular level of host-virus interactions can be elucidated through identification of PPI between viral and cellular proteins.

LCMV provides us with a few relevant examples of how virus interference with cellular immune response is mediated through direct PPI (Martinez-Sobrido et al., 2007, Xing et al., 2015). Nevertheless, the numbers of cellular interaction partners as well as molecular consequences of these interactions for LCMV infection remain elusive and require further investigation.

Identification of host-virus PPI pinpoints cellular pathways and functions targeted by viruses. Two of the most common technological approaches to map these interactions are affinity purification followed by mass spectrometry (AP-MS) analyses and yeast two-hybrid (Y2H) screening.

The AP-MS approach includes two important steps: 1) purification of the protein-of-interest together with associated protein complexes and 2) identification of isolated proteins by MS analysis. The first step of the AP-MS protocol requires specific enrichment of the “bait” (protein-of-interest). This can be achieved either by introducing a specific epitope tag suitable for purification to the protein-of-interest or by application of an antibody specific for the viral protein. The viral protein can be either endogenously tagged or its tagged version can be ectopically overexpressed (the most common approach).

The choice of an appropriate system to study host-virus interactions may significantly affect the results. For instance, ectopic cellular overexpression of the tagged viral protein may impair its function and sub-cellular localization. Additionally, qualitative and quantitative composition of the cellular proteome is significantly different between infected and uninfected cells. Therefore, interactome analyses of endogenously tagged protein from the replication-competent virus may provide more comprehensive results. Nevertheless, this approach requires a very careful choice of the tag size and location. The decision shall be taken based on the domain structure of the given protein and several recombinant viruses shall be tested.

The AP-MS experiments require a thoughtful choice of negative control samples in order to distinguish specific interactors from background contaminants (non-specific interactors). The most common approach is either to employ green fluorescent protein (GFP) tagged with the same epitope and overexpressed in the same cell line as protein-of-interest or to perform purification with the same resin from the same cell line without any epitope-tagged protein. Furthermore, some of the publicly available databases provide an overview of the common contaminators from AP-MS experiments in mammalian cells (Mellacheruvu, Wright et al., 2013). Some groups of proteins are significantly enriched in a contaminant repository, such as elongation factors, ribonucleoproteins and ribosomal proteins. Nevertheless, these proteins might be true interactors for some viral proteins, such as viral polymerases. Overall, the filtration of AP-MS results from each experiment shall be performed using several approaches.

A commonly used strategy to confirm and validate novel host-virus PPI identified by MS analyses is protein co-immunoprecipitation followed by western blot analyses and co-localization by fluorescent microscopy. Still none of these methods allows to distinguish direct from indirect PPI.

The AP-MS approach provides an overview of both direct and indirect protein interactions, whereas Y2H screens identify binary protein interactions (Bartel, Roecklein et al., 1996). Therefore, these two approaches are not only technologically different but also describe complimentary although not identical PPI networks (Yu, Braun et al., 2008).

A rapid development of host-virus interactomes over the last decades is reflected in the growth of virus-host protein interaction databases (Calderone, Licata et al., 2015, Guirimand, Delmotte et al., 2015). By 2016 these public databases contain over 5000 host-virus PPI (Lum & Cristea, 2016). Nevertheless, the main research in this field was concentrated around few clinically relevant viruses such as influenza, HIV, HCV and HBV. This bias restricts our understanding of host-virus PPI landscape to a few particular species and prevents the comprehensive understanding of virus-induced perturbations of the host proteome.

One of the key problems of the current virus-host interactomics is the lack of a comprehensive overview of protein complexes reorganization depending on the stage of infection and tissue/cell type. Most of the publicly available data are produced in cell lines therefore these data do not reflect neither the complexity of the host immune response *in vivo* nor tissue-specific expression of multiple genes. A better understanding of how these factors affect host-virus protein-protein interactions could

highlight novel therapeutic opportunities, provide new insights in the molecular mechanisms of host-virus interactions and, potentially, in some cellular processes.

Multiple innovative therapeutic approaches for the treatment of chronic human viral infections involve direct targeting of viral proteins. This led to significant progress in the treatment of many clinically relevant infections (Keating, 2016). Nevertheless, this approach might be challenged by various complications, such as the emergence of drug resistant mutants. Some efforts on antiviral drug development were concentrated around the identification of critical cellular components of host-virus complexes that could serve as a therapeutic target as well (Flisiak, Horban et al., 2008, Watashi et al., 2005). Moreover, the global overview of molecular pathways targeted by viral proteins could be used to chart the requirements for the life cycle of the respective virus.

Host factors interacting with viral proteins may be subdivided into two groups: antiviral factors (e.g. proteins involved in antiviral immune responses) and proviral factors (cellular proteins required to perform essential functions for the viral life cycle). Both groups have a profound impact on the virus life cycle. A better understanding of host-virus interactions may highlight novel targets for therapeutics.

2. AIMS OF THE THESIS

Although infection with either ARM or Cl13 strains of LCMV leads to a significantly different immunobiological outcome (see Chapter 1.1), the molecular determinant for these immunological differences lies in only a single amino acid exchange of lysine (ARM) to glutamine (Cl13) at the position L1079 (Bergthaler et al., 2010). This research aimed to reveal the host-virus protein interactome of the LCMV L protein and to identify the effect of the L1079 mutation on it. Additionally, we aimed to integrate the obtained LCMV L protein interactome dataset with host-virus PPI network of other viral RdRps to define the general polymerase strategies to target the host proteome. Then, we planned to perform functional validation experiments *in vitro* and *in vivo* to identify host factors, that play a role in the LCMV life cycle.

In order to achieve this, we set the following aims:

1. Develop a strategy to endogenously tag the LCMV L protein using reverse genetic approach, generate recombinant viruses $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ and characterize the obtained viruses *in vivo*.
2. Obtain LCMV L protein interactomes for $Cl13_{L1079K}$ and $Cl13_{L1079Q}$.
3. Integrate the obtained dataset with data on host-virus interaction from public datasets and dissect the approaches of viral RdRps to target the host proteome.
4. Characterize the role of selected interactors in the LCMV life cycle using a loss-of-function approach *in vitro* and *in vivo*.

3. RESULTS

3.1 Prelude

The molecular mechanism underlying host-virus interactions in chronic viral infection remains one of the most puzzling questions in immunology. In the following research article “Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein” by Khamina et al. and Chapter 3.3 “Investigating the effect of the L1079 mutations of LCMV L protein interactomes and functional validation” we addressed all main aims of this thesis, described in Chapter 2. We engineered L protein-tagged infectious LCMV recombinant viruses $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ and characterized them *in vivo*. Since these viruses were generated on a Cl13 backbone, the only difference between $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ lies in the single point mutation L1079. Therefore, all identified strain-specific interactors can be attributed to this mutation. The development of L protein-tagged LCMV allowed us to study host-virus protein interactions in the context of natural viral infection. Then, we integrated the obtained interactome of LCMV L-host protein interactome with public datasets on host-virus interactions of other viral RdRps. This network analyses provided a global overview that highlighted common and virus-specific approaches of RdRps to target host protein modules.

As a follow up on the interactome study, we selected 5 candidates for loss-of-function experiments based on a CRISPR-Cas9 approach to identify their impact on LCMV life cycle. Then we perform characterization of TRIM21, one of the validated L protein interactors, *in vitro* and *in vivo*. Furthermore, we present data on the characterization of NKRF, a $Cl13_{L1079Q}$-specific interactor, upon LCMV infection.

3.2 Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein

PLOS | PATHOGENS

RESEARCH ARTICLE

Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein

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OPEN ACCESS

Citation: Khamina K, Lercher A, Caldera M, Schliehe C, Vilagos B, Sahin M, et al. (2017) Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein. PLoS Pathog 13(12): e1006758. https://doi.org/10.1371/journal.ppat.1006758

Editor: Jens H. Kuhn, Division of Clinical Research, UNITED STATES

Received: July 14, 2017

Accepted: November 17, 2017

Published: December 20, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This project was supported by a grant of the Hochschuljubiläumsstiftung of the City of Vienna (Andreas Bergthaler). Anannya Bhattacharya and Kseniya Khamina were supported by DOC Fellowships from the Austrian Academy of Sciences. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

RNA-dependent RNA polymerases (RdRps) play a key role in the life cycle of RNA viruses and impact their immunobiology. The arenavirus lymphocytic choriomeningitis virus (LCMV) strain Clone 13 provides a benchmark model for studying chronic infection. A major genetic determinant for its ability to persist maps to a single amino acid exchange in the viral L protein, which exhibits RdRp activity, yet its functional consequences remain elusive. To unravel the L protein interactions with the host proteome, we engineered infectious L protein-tagged LCMV virions by reverse genetics. A subsequent mass-spectrometric analysis of L protein pulldowns from infected human cells revealed a comprehensive network of interacting host proteins. The obtained LCMV L protein interactome was bioinformatically integrated with known host protein interactors of RdRps from other RNA viruses, emphasizing interconnected modules of human proteins. Functional characterization of selected interactors highlighted proviral (DDX3X) as well as antiviral (NKRF, TRIM21) host factors. To corroborate these findings, we infected *Trim21*$^{-/-}$ mice with LCMV and found impaired virus control in chronic infection. These results provide insights into the complex interactions of the arenavirus LCMV and other viral RdRps with the host proteome and contribute to a better molecular understanding of how chronic viruses interact with their host.

Competing interests: The authors have declared that no competing interests exist.

Author summary

RNA-dependent RNA-polymerases (RdRps) play a key role in the life cycle of RNA viruses. They interact with cellular proteins during replication and transcription processes and impact the immunobiology of viral infections. This study characterized the host protein interactome of the RdRp-containing L protein of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV). Several L protein interactors with proviral and antiviral effects were identified *in vitro*, and mice lacking the identified L protein interactor TRIM21 exhibited impaired control of chronic LCMV infection. Integration of the L protein interactomes with known RdRp interactomes from other RNA viruses highlighted common and virus-specific strategies to interact with the host proteome, which may indicate novel avenues for antiviral interventions.

Introduction

RNA viruses hijack and utilize host factors at all steps of their life cycle. The viral RNA-dependent RNA polymerase (RdRp) is a key enzyme responsible for transcription and replication of viral genomes. Three-dimensional complete protein structures are available for RdRps from different RNA viruses and feature a similar "right handed" structure containing six conserved motifs [1–3]. The high similarity of RdRps between different viruses suggests that they might interact with similar host factors in the viral life cycle. Global host–virus protein interaction networks provided invaluable insights into infection biology [4–8], and similar approaches hold promise for an in-depth understanding of how viral RdRps interact with the host.

Lymphocytic choriomeningitis virus (LCMV) is a (-) RNA virus of the *Arenaviridae* and represents a well-established model system that led to seminal findings in immunology and host-pathogen research [9–12]. Particularly, it serves as a benchmark model of chronic viral infections [9, 13] whereby the strain Clone 13 (Cl13) causes immunosuppression and persists in mice for several months [14, 15]. The genetically closely related LCMV strain Armstrong (ARM) leads to acute infection, which is efficiently cleared from the blood of infected mice within a week [14, 15]. A single amino acid exchange at the position 1079 of the L protein (K1079Q) was found to be required for the ability of Cl13 to establish persistence with additional contribution of the mutation F260L in the viral glycoprotein [14–16]. The L gene encodes the largest open reading frame (250 kDa) in the LCMV genome, which together with the viral nucleoprotein (NP) forms the ribonucleoprotein complex (RNP) required for viral transcription and replication [17, 18]. The L protein contains several domains of which the NL1 domain serves as a type II endonuclease and domain III possesses RdRp activity [19, 20]. Yet, a better molecular understanding of the L protein and its role in the viral biology is hampered by lack of complete protein structure and unknown host protein interactors.

In this study we set out to determine the host interactome of the LCMV Cl13 L protein to gain insights into the interactions of the LCMV replication complex with host factors and to investigate the molecular environment that modulates viral immunobiology. We developed infectious LCMV virions with a protein tag fused to the L protein, enabling us to identify interacting host proteins by immunoprecipitation followed by mass spectrometry. Functional characterization of selected interaction partners *in vitro* revealed the impact of several interactors on LCMV replication. This was corroborated in mice lacking one of these interactors–TRIM21, which exhibited impaired control of chronic LCMV infection. Finally, we integrated previously reported interactomes from other viruses by a global network analysis and

identified common and unique highly interconnected cellular protein functional modules targeted by different viral RdRps.

Results

Generation and characterization of LCMV strains expressing a tagged L protein

To elucidate the host protein interactome of the L protein of LCMV, we developed a strategy of endogenous protein tagging that enabled us to analyze the host cell interaction partners in the context of the natural infectious life cycle. Arenaviruses have a compact genome and even minor modifications in their genome can severely affect their viability. We, thus, selected four different affinity purification tags with a short size of 8 to 14 amino acid residues (HA, Strep II, FLAG and V5) and introduced the tags either on the N- or C- terminus of the L ORF by insertional cDNA mutagenesis (S1 Table). Reverse genetic rescue of the corresponding viruses revealed that all viruses bearing an N terminally tagged L protein viruses were efficiently generated on a Cl13 LCMV backbone while none of the C terminally tagged L protein constructs resulted in infectious virions (Fig 1A). This demonstrated that it was feasible to generate L protein tagged LCMV variants with retained infectivity in cell culture. Based on the viral titers recovered, we continued to work with a LCMV Cl13 virus whose L protein was N terminally fused with a HA tag ($Cl13_{L\text{-}HA}$). To control for the expression of the tagged L protein, we prepared lysates from cells infected with $Cl13_{L\text{-}HA}$ and detected the L protein at the expected size by anti-HA antibody (S1A Fig). To assess the effect of the introduced tag on viral propagation, we compared the growth kinetics of $Cl13_{L\text{-}HA}$ to an untagged Cl13 virus upon infection of HEK293T cells. This experiment revealed exponential growth of $Cl13_{L\text{-}HA}$ albeit with slight attenuation compared to the untagged Cl13 virus (Fig 1B).

To assess the fitness of the LCMV variant with tagged L protein *in vivo*, we infected mice with $Cl13_{L\text{-}HA}$ or the corresponding untagged Cl13 virus and assessed viral loads in blood (Fig 1C), spleen and liver (Fig 1D). The virus encoding the HA-tagged L protein showed a similar kinetics of viremia compared to the untagged virus, although peak titers were lower when mice were infected with $Cl13_{L\text{-}HA}$ virus. Quantification of virus-specific $CD8^+$ T cell responses showed no significant differences between mice infected with either $Cl13_{L\text{-}HA}$ or untagged Cl13 (Fig 1E). To assess the stability of the HA tag in the LCMV genome, we sequenced virus from the spleen lysates of mice which had been infected with either $Cl13_{L\text{-}HA}$ or untagged Cl13 50 days previously. These results confirmed the presence of the HA tag and did not provide any evidence for acquired mutations in the corresponding region at this late time point (S1B Fig).

Together, these data indicated that $Cl13_{L\text{-}HA}$ has similar immunobiological characteristics as the respective untagged Cl13 strain. These novel tools enabled us to proceed with immunoprecipitation of the L protein in the context of a natural viral infection.

Identification of the LCMV L protein interactome

For the identification of the LCMV L protein interactome, we relied on a well-established cell culture system [21]. HEK293T FlpIn cells infected with $Cl13_{L\text{-}HA}$ were used for mass spectrometry analyses. In addition, we infected HA-GFP overexpressing as well as wild type HEK293T FlpIn cells with untagged Cl13 virus which served as negative controls for the interactome analysis. Subsequently, we performed an one-step anti-HA immunoprecipitation from infected cell lysates (S1C Fig), and analyzed interaction partners of the LCMV L protein by one-dimensional gel-free liquid chromatography tandem mass spectrometry (LC–MS/MS). In

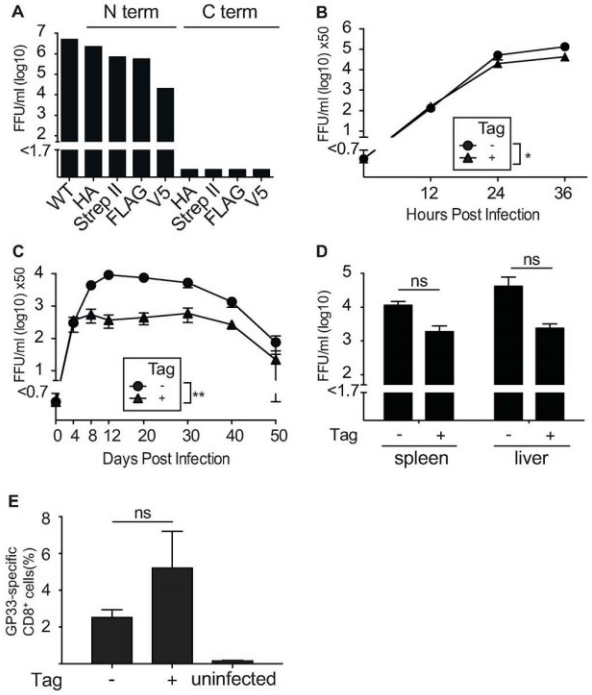

Fig 1. Generation and characterization of LCMV strains expressing a tagged L protein. (A) Viral titer of N- and C-terminal L protein-tagged Cl13 LCMV and WT Cl13 LCMV measured by focus forming assay at 72 hours post infection after reverse genetic rescue on BHK21 cells. (**B**) HEK293T cells were infected at a MOI of 0.01 with either $Cl13_{L\text{-}HA}$ or with untagged Cl13. Supernatant was harvested and viral loads were measured at the indicated time points by focus forming assay. **(C** and **D)** C57BL/6J mice were infected with $2x10^6$ FFU of the indicated viruses. Viral titers were determined in **(C)** blood at indicated time points and in **(D)** organs 20 days post infection. **(E)** C57BL/6J mice were infected with $2x10^6$ FFU of the indicated viruses and the percentage of GP33-tetramer$^+$ CD8$^+$ T cells was quantified in the spleen at 8 days post infection. Each symbol and bar represents the mean ± SEM of three to five mice. Statistical significance was calculated by Two-way ANOVA (B-**C**) or unpaired t-test (**D-E**). Significant p values were indicated as follows: ns—non significant, * $p \leq 0.05$,: ** $p \leq 0.01$.

https://doi.org/10.1371/journal.ppat.1006758.g001

total we identified 555 proteins in the L protein pulldowns. Upon stringent filtration against the background of our control samples (**Materials and Methods**), we obtained 231 interactors for our bait (Fig 2, S2 Table). Our bioinformatic analysis, which was based on global gene set enrichment and functional classification, revealed that the major components of the LCMV L protein interactome are RNA biology-associated proteins (e.g. splicing, ribonucleoproteins, ribosomal and RNA-binding proteins) and proteins related to translation and transcription (Fig 2A and 2B). Importantly, the LCMV nucleoprotein (NP), a known interactor of the L protein [17], was detected in all L protein pulldowns (2 biological replicates and 2 technical replicates each) but not in our control samples infected with untagged Cl13 virus, serving as a valuable internal control (Fig 2B, S1C Fig). In addition to the identified global clusters depicted in Fig 2A, we found a number of interactors with reported roles in signal transduction and innate immunity (Fig 2B). These included DEAD-box helicase 3 (DDX3X), insulin-like growth factor 2 (IGF2BP1), NF-kB repressing factor (NKRF) and Tripartite Motif Containing 21 (TRIM21). To confirm selected host protein interactions for the L protein, we used available antibodies specific to the endogenous interaction partners DDX3X, polyadenylate-binding protein 1 (PABPC1) and TRIM21 and performed Western blots on anti-HA

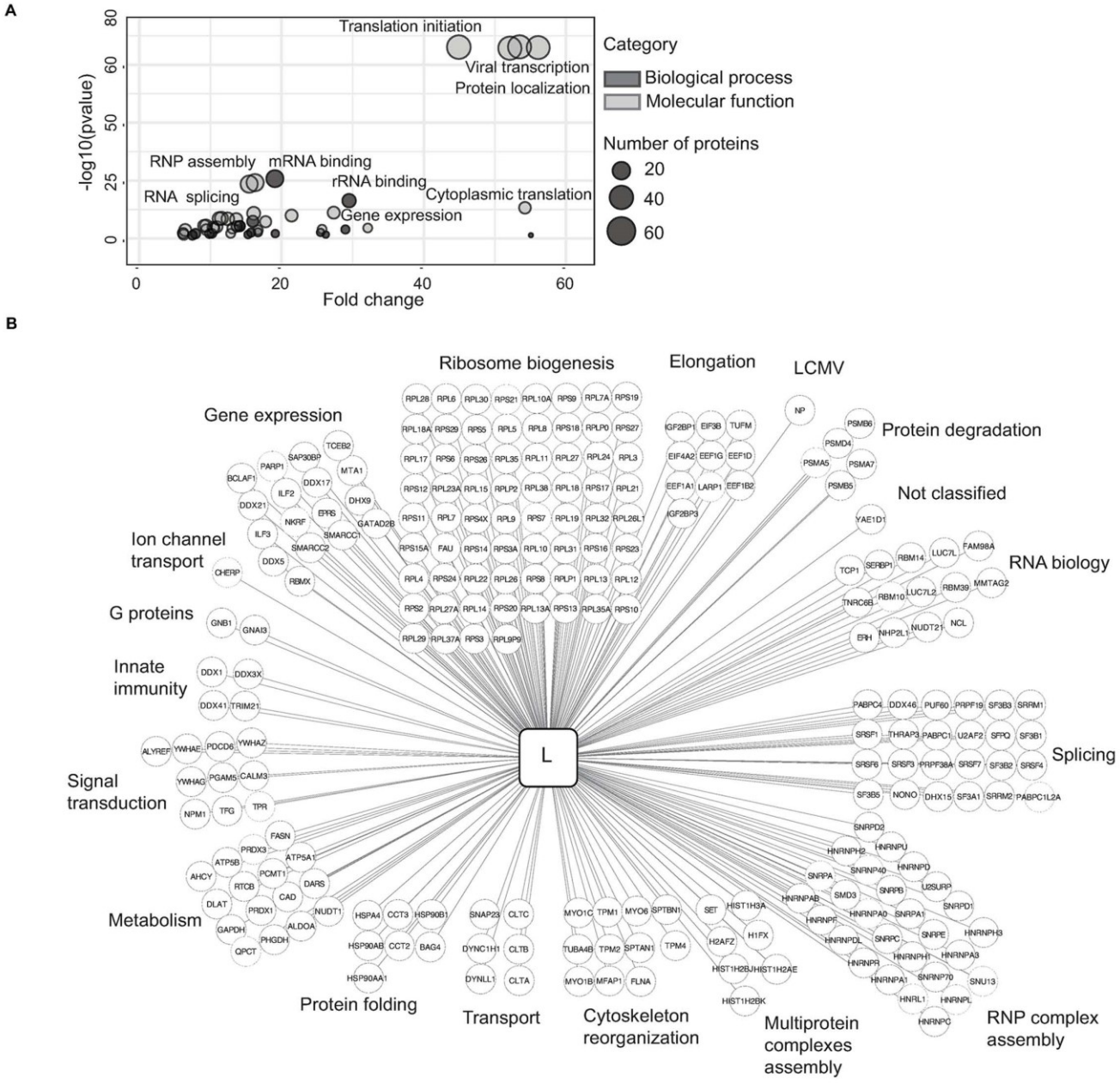

Fig 2. Identification of L protein interactome. (A) GO enrichment analyses for the L protein interactome based on the molecular functions (light grey) and biological processes (dark grey) of interactors followed by visualization with ReviGO. **(B)** Overview of L protein interactomes classified based on the protein functions and visualized in Cytoscape. The data is based on the mass-spectrometry derived list of proteins identified in L protein pulldowns after filtration using Top3 quantitation and SAINTexpress software as detailed in Materials and Methods.

https://doi.org/10.1371/journal.ppat.1006758.g002

immunoprecipitates of $Cl13_{L\text{-}HA}$-infected HEK293T cell lysates (S1D Fig). The L protein of the acute LCMV strain ARM differs in the single amino acid exchange K1079L and plays a key role in viral immunobiology [14–16]. We, thus, generated also an LCMV variant expressing

the HA-tagged L protein of ARM to perform co-immunoprecipitation experiments. These revealed that pulldown of the ARM L protein enriched for DDX3X, PABPC1 and TRIM21 as well, indicating that the interaction is not affected by the mutation K1079L (S1D Fig). To provide additional evidence for direct binding of these host proteins to the L protein in the absence of viral RNA or other viral proteins, we transiently transfected cells with a plasmid encoding the HA-tagged L protein of Cl13. In line with our previous findings HA-specific immunoprecipitation confirmed DDX3X, PABPC1 and TRIM21 as L protein interactors (S1E Fig). We also performed a reciprocal co-immunoprecipitation experiment by transfecting HEK293T cells with plasmids encoding HA-tagged L protein and/or V5-tagged TRIM21 (S1F Fig), corroborating the interaction between the viral L protein and TRIM21.

Together, our experiments with endogenously-tagged infectious LCMV characterized the host protein interactome of the LCMV L protein.

Viral RNA-dependent RNA-polymerases target the host proteome by common and virus-specific strategies

To better understand the global landscape of interactions between viral replication machineries and the host, we integrated the LCMV L protein interactome obtained in this study with publicly available interactome datasets on other RNA viruses (S3 Table) [22], resulting in a comprehensive network of all hitherto known interactomes of viral RdRps (Fig 3A). We then mapped this host-RdRp network onto the global human interactome consisting of 141,296 experimentally verified physical interactions between 13,460 proteins [23], thus allowing us to locate all human proteins targeted by various RdRps. Proteins associated with the same disease have been found to aggregate in the same local neighborhood of the human interactome [23].

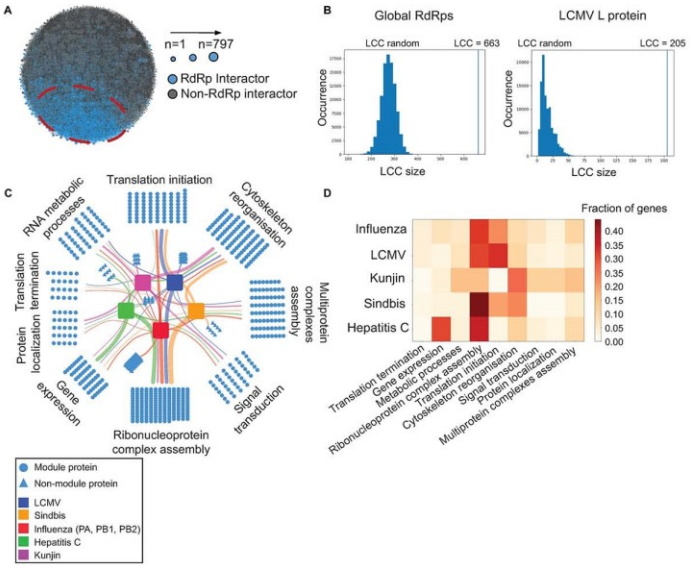

Fig 3. Viral RNA-dependent RNA-polymerases target host proteome by common and virus-specific strategies. (A) Integrated interactome of viral RdRp targets. Host proteins interacting with viral RdRps are highlighted in blue, the rest of the human proteome—in grey. **(B)** Largest connected component (LCC) analyses for global RdRps and LCMV only datasets. **(C)** Functional protein modules targeted by RdRps based on the community detection method. **(D)** Heat map representing virus-specific targeting of protein functional modules.

https://doi.org/10.1371/journal.ppat.1006758.g003

To test whether proteins targeted by RNA viruses display a similar tendency we analyzed the size of the largest connected component (LCC) [24], i.e. the number of directly connected proteins (see Methods and **Fig 3A**). Out of 797 host proteins targeted by various RdRps, 663 proteins are directly connected to each other by protein-protein interactions, suggesting that indeed viruses affect a specific neighborhood of the human interactome. The obtained LCC values were significantly higher than the random expectation both for global RdRps (p-value = 1.47e-36) and for LCMV Cl13 L protein only (p value = 9.77e-84) datasets (**Fig 3B**), indicating that viral RdRps specifically target highly interconnected host protein interactome modules.

To further elucidate the biological function of the targeted interactome neighborhood we performed a gene ontology (GO) enrichment analyses and clustered the targets based on their functional similarity using a network-based community detection method [25]. We identified 9 basic functional protein modules in the human proteome that are targeted by various viral RdRps (**Fig 3C–3D**). While most of them are associated with RNA biology-related processes such as RNA biosynthesis, RNA metabolic processes and ribonucleoprotein complex assembly, we also identified other cellular processes including cytoskeleton reorganization and signal transduction. Interestingly, certain protein modules are commonly targeted by all viruses (e.g. ribonucleoprotein complex assembly) whereas other modules seem to be targeted in a virus-specific manner (**Fig 3D**). These analyses pinpointed that viral RdRps target the host proteome by common and virus-specific strategies.

Functional screening for L protein interactors involved in LCMV infection

In order to elucidate a potential functional role of the identified host factors in LCMV infection, we selected the five proteins DDX3X, FLII, IGF2BP1, NKRF and TRIM21 from the total list of interactors. TRIM21 and DDX3X were among the proteins that are targeted by the polymerase complexes of several viruses [8, 21], which suggested that these host factors may play an important role in the replication of RNA viruses. The other host proteins were selected based on their known immune functions or their role in the life cycle of other viruses. We designed CRISPR-Cas9 assays for the selected candidates and created two independent cell pools by individual single guide RNA per gene. These targeted cell pools and non-target control cells were generated in HeLa S3 cells and pools of early passage 1 or 2 were used. This human cell line supports LCMV replication and mounts an antiviral innate immune response. Specific gene editing was confirmed by T7EI cleavage assay (**S2A Fig**) and TIDE approach (**S2B Fig**) for the targeted cells. Further, we confirmed the decreased protein expression of DDX3X and TRIM21 in the respective targeted cells by Western blot (**S2C Fig**). We also attempted to generate cells targeting the validated L interactor PABPC1, but were unsuccessful, possibly because PABPC1 is an essential gene *[26]*.

The obtained targeted cells were subsequently infected with Cl13 WT and viral propagation was measured by focus forming assay (FFA) (**Fig 4A**). These experiments revealed that Cl13 replicated to higher titers in TRIM21-targeted and NKRF-targeted cells. Conversely, we found reduced viral loads in DDX3X-targeted cells, which is in line with its proviral effect in HCV infection [27]. Other targeted cells did not reveal consistent differences in viral loads, which could reflect functional redundancies or cell type-specific properties. We also determined the effect of LCMV infection on the expression levels of TRIM21 in infected HEK293T cells and found, that it is not significantly changed upon infection (**S3 Fig**).

To further validate the findings of our functional screen, we performed reconstitution experiments with TRIM21. TRIM21-targeted cells were transfected with either a plasmid expressing TRIM21 or with an empty vector (EV) control plasmid and subsequently infected with Cl13 WT. Importantly, reconstitution with TRIM21 reversed the phenotype previously

observed in TRIM21-targeted cells and led to reduced viral titers (Fig 4B). Similar antiviral effects of TRIM21 were observed upon infection with ARM WT (S4A Fig).

Finally, we infected *Trim21*$^{-/-}$ and WT mice with Cl13 WT to assess viral propagation *in vivo* [28]. Interestingly, *Trim21*$^{-/-}$ and WT mice showed comparable levels of viremia in the early stage of infection but approximately two weeks after infection *Trim21*$^{-/-}$ mice exhibited impaired control of Cl13 in the blood compared to WT mice (Fig 4C, p-value <0.0001). Similarly, we found higher viral loads in spleen, liver and kidney of infected *Trim21*$^{-/-}$ mice compared to WT mice (Fig 4D). We also infected *Trim21*$^{-/-}$ and WT mice with the acute strain ARM but were unable to detect differences in viral loads in blood and other organs (S4B and S4C Fig). This was in line with comparable viremia kinetics of Cl13 in *Trim21*$^{-/-}$ and WT mice during the early phase of infection.

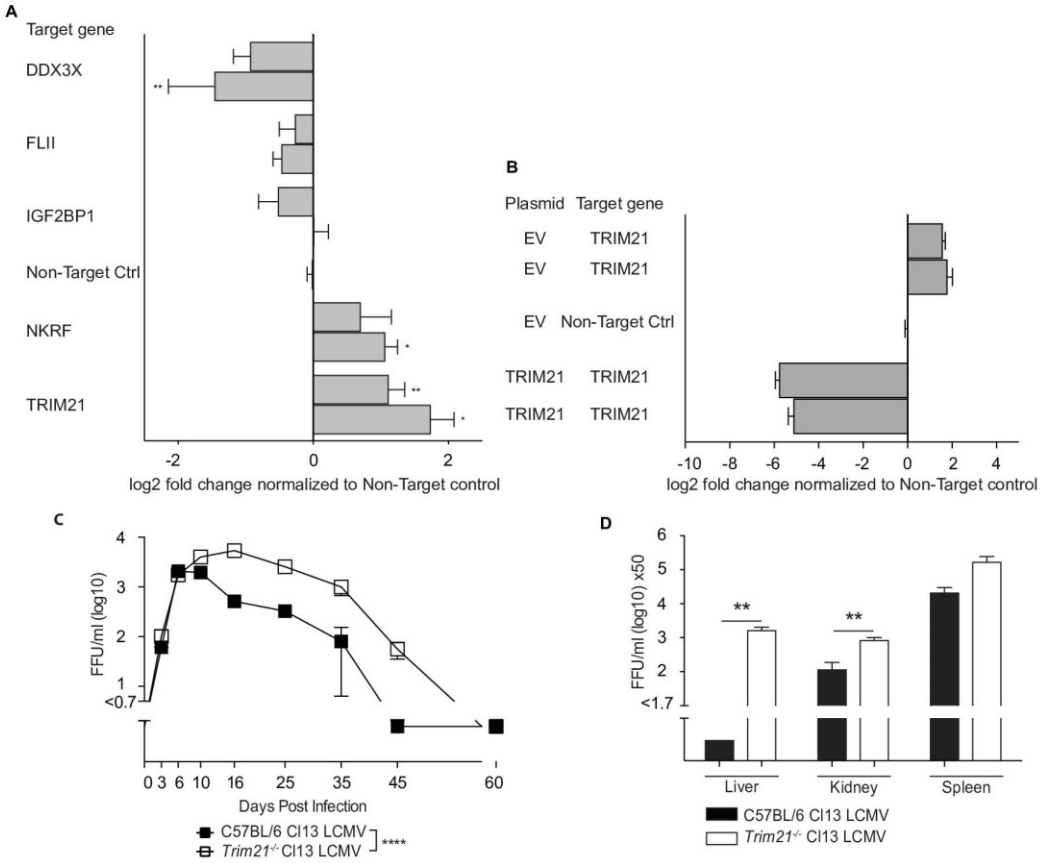

Fig 4. Functional screening for L protein interactors involved in LCMV life cycle. (**A**) Two independently generated HeLa S3 CRISPR-Cas9 targeted cell pools per gene of interest for 5 genes were infected in triplicate wells with LCMV Cl13 WT at a MOI of 0.01 and viral loads were measured at 36 hours post infection by focus forming assay. The obtained data were normalized to the non-target control and log2 transformed. (**B**) Two HeLa S3 CRISPR-Cas9 TRIM21-targeted cell pools were reconstituted either with TRIM21-expressing plasmid or with non-target control and 36 hour post transfection were infected in triplicate wells with LCMV Cl13 WT at a MOI of 0.01. Viral loads were measured at 36 hours post infection by focus forming assay. The obtained data were normalized to the non-target control and log2 transformed. (**C-D**) C57BL/6 and *Trim21*$^{-/-}$ mice were infected with 2x10^6 FFU of the indicated viruses. Viral titers were determined in (**C**) blood at indicated time points and in (**D**) organs at 21 days post infection. The data shown in (**C**) is representative of two similar experiments. Each symbol and bar represents the mean ± SEM of three to five mice. Statistical significance was calculated by unpaired t-test (**A, B, D**) or by Two-way ANOVA (**C**). Significant p values were indicated as follows: ns—non significant, * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

https://doi.org/10.1371/journal.ppat.1006758.g004

In summary, these experiments provided novel insights into the impact of identified L protein interactors on the life cycle of LCMV and uncovered a non-redundant antiviral role of TRIM21 in the control of chronic LCMV infection.

Discussion

This study gives novel insights into how the replication machinery of LCMV interacts with the host on a protein-protein level by the use of reverse genetically engineered endogenously tagged viruses. Similar to recent approaches for endogenous tagging of viral proteins [29–31], this avoids potential artifacts generated by conventional ectopic overexpression of tagged viral proteins due to non-physiological protein abundance and/or localization and may be applicable to the study of other viral protein interactomes. Moreover, it enables the proteomic characterization of viral RdRps and other viral proteins with cellular partners not only in cell lines but also in more complex systems such as primary cells and tissues *ex vivo*. We acknowledge that affinity purification mass-spectrometry approaches may also yield interactors which are not directly binding to the protein-of-interest but rather to complex partners. In the case of the L protein this may include interactions with other components of the arenaviral ribonucleoprotein complex or the viral Z protein [32, 33].

Arenaviruses have compact genomes and any minor modification may severely affect infectivity. Interestingly, N-terminally L-tagged LCMV constructs resulted in infectious virions, whereas tags fused to the C terminus of the L protein prevented viral reverse genetic rescue (**Fig 1A**). This may reflect impaired protein structure-function of the L protein or possibly indicate reduced fitness due to noncoding structural effects on the viral RNA genome [34].

Our proteomic analyses demonstrate that the LCMV L protein targets multiple cellular pathways such as RNA biology-associated modules (e.g. RNA metabolic processes and ribonucleoprotein complex assembly), cytoskeleton reorganization, protein localization and translation. Interestingly, the L protein also binds to numerous host proteins associated with signal transduction and innate immune signaling. This is of interest due to the known intricate and multi-faceted relation of LCMV with the antiviral type I interferon system [35, 36].

By combining our proteomic results with loss-of-function experiments we implicated several interactors in the course of LCMV infection. As examples, the E3 ligase TRIM21 and the RNA helicase DDX3X were identified as interactors of the LCMV L protein and loss of either protein had an effect on viral propagation. TRIM21 is known to regulate the type I interferon response via ubiquitination of multiple interferon regulatory factors (IRFs) and acts as an intracellular Fc receptor [37, 38], whereas DDX3X is involved in innate immune signaling cascades [39, 40]. Of note, both TRIM21 and DDX3X are targeted by the polymerase complexes of influenza virus and HCV [8, 21]. These two proteins provide an example of host factors located at the interface of several viral RdRp interactomes. Bioinformatic analyses of integrated host-viral interactomes can highlight such "hot spots" in the human proteome for further investigations. Moreover, DDX3X was recently also described as interactor of LCMV NP [41], indicating that individual host factors may bind to several viral proteins.

Based on our mass spectrometric analysis and the loss-of-function experiments *in vitro*, we infected *Trim21*$^{-/-}$ mice with LCMV strain Cl13 and found impaired virus control in the late phase of infection (**Fig 3C and 3D**). These results revealed a novel role for TRIM21 in the replication of LCMV both *in vitro* and *in vivo*. Of note, LCMV is an enveloped arenavirus whereas the antibody-mediated antiviral effects of TRIM21 have been predominantly linked to non-enveloped viruses such as adenoviruses [42], caliciviruses [38] and picornaviruses [43]. Further investigations will be required to dissect the TRIM21-dependent mechanisms for the effective control of chronic LCMV infection.

Our proteomic results provide a data-rich resource for the study of arenaviruses in general. Further, the LCMV L protein interactome may bring novel impetus to unravel the functional implications of the L1079 mutation and the associated immunobiological differences between different strains of LCMV and their abilities to persist in the mouse. Finally, our integration of the LCMV L protein interactome with publicly available data of other RdRps emphasizes the impact of mass spectrometry-based proteomics on virological research [44], and provides a global overview of the host factors targeted by viral RNA polymerases. Of note, these available datasets were derived from overexpressed viral RdRps while the interactome data of this study was obtained in the context of endogenously tagged replicating viruses and may thus also contain proteins binding to the polymerase complex. Yet, the high interconnectivity within the cluster of viral targets suggests that RdRps target functionally important protein modules. This could be associated with the conserved structure and similar functional host-dependent requirement of viral RdRps. Further, our network analyses emphasize the fact that viral RdRps from different taxonomy groups target functional host protein modules in common and virus-specific ways. This may highlight RdRp-host protein interactions that are required for the majority of RNA viruses as well as reveal unique features of distinct virus species. This expanded view on the interaction of viral replication machineries with their host proteomes may contribute to the development of novel antiviral therapeutic avenues.

Materials and methods

Animal experiments

C57BL/6J mice were originally obtained from The Jackson Laboratory. *Trim21*$^{-/-}$ mice were generated by gene disruption via insertion of EGFP into exons 3–5, as described [28]. Mice were subsequently rederived by transferring embryos from the original knockout C57BL/6 strain into pathogen-free recipient C57BL/6 females. Mouse experiments were performed with sex- and age-matched animals under specific pathogen-free conditions.

Cells

All cell lines were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FCS (Invitrogen) and Penicillin-Streptomycin-Glutamine (Thermo Fisher Scientific, 10378016). HEK293 Flp-In TREx cells (Invitrogen) with doxycycline-dependent transgene expression were used to generate the cell line for Strep-HA-GFP overexpression according to the manufacture protocol. This cell line was treated for 48 h with doxycycline (1μg/mL) to induce overexpression of the tagged protein.

Viruses and infections

L protein-tagged LCMV was generated using a reverse genetic system approach [45]. A detailed description for the generation of the N-terminal HA-tagged L protein Cl13 virus ($Cl13_{L\text{-}HA}$) is provided below. The sequence encoding the HA tag (TATCCGTATGATGTGC CGGATTATGCG) followed by the sequence encoding a short linker GGS (GGTGGTTCT) was used to design primers for an insertional mutagenesis approach. Additionally, a single nucleotide exchange (non-coding mutation) in the second codon of L ORF (A to G) was introduced to distinguish viruses that may have lost the HA tag from wild type viruses. All primer sequences for this reverse genetic approach are described in **S1 Table**. pl-L-Cl13(-) plasmids were employed for insertional mutagenesis by PCR with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, F530S). The obtained plasmid was further used to generate HA-tagged L1079K virus using insertional mutagenesis. These plasmids encoding the L-tagged

segment were used to rescue corresponding viruses according to the standard reverse genetics protocol [45]. LCMV titers were determined by NP-specific focus forming assay (FFA) as described previously using Vero cells (ATCC-CCL-81) [46].

Reverse genetically rescued WT (untagged) viruses were used throughout all experiments with the exception of Fig 4C and 4D and S4B and S4C Fig. For the latter experiments with WT and *Trim21*$^{-/-}$ mice we used passaged WT ARM and Cl13 virus stocks whose origins are described in [15].

For mass spectrometry (MS) analyses HEK293 FlpIn TREx cells (Invitrogen, R78007) were infected with $Cl13_{L\text{-}HA,}$ negative control cells Strep-HA-GFP expressing HEK293 Flp-In TREx and empty HEK293 FlpIn TREx cells were infected with untagged Cl13 at a multiplicity of infection (MOI) of 0.01, washed and harvested with ice-cold phosphate-buffered saline (PBS) after 36 hours post infection.

Mice were infected intravenously with $2x10^6$ focus forming units (FFU) of the corresponding virus stock. To confirm the presence of the HA tag at 50 days post infection, total cellular RNA was extracted from the spleen of mice infected with either $Cl13_{L\text{-}HA}$ or untagged Cl13 using QIAzol lysis reagent (QIAGEN, 79306) according to the standard protocol to confirm the presence of the genetic tag. cDNA was obtained with random hexamers by Superscript II RT PCR and used as an input for amplification with LCMV-specific primers (5'gtgctgtgaaagctaccagcctatc'3 and 5'tatccgtatgatgtgccggattatg'3) by Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F530S). This PCR product was analyzed by agarose (0.8%) gel electrophoresis, the specific band was extracted from the gel with QIAquick Gel Extraction Kit (QIAGEN, 28706) and analyzed by Sanger sequencing. Furthermore, the presence of the HA-tagged L protein in infected HEK293T (ATCC- CRL-3216) cells was confirmed by western blot analyses with anti-HA antibody.

Affinity purification mass spectrometry

Ten subconfluent 15-cm dishes of HEK293 FlpIn TREx cells were infected with $Cl13_{L\text{-}HA}$ at a MOI of 0.01. Additionally, ten sub-confluent 15 cm dishes of HEK293 FlpIn TREx cells (no bait control) or Strep-HA-GFP expressing HEK293 TREx Flp-In (GFP control) were infected with untagged Cl13 LCMV at a MOI of 0.01. Expression of Strep-HA-tagged GFP in HEK293 TREx Flp-In cell line was induced with doxycycline (1 μg/mL) 24 hours before infection. Cells were harvested 36 hours post infection and lysed for 30 min on ice in the IP buffer (HEPES 50 mM, pH 8.0; NaCl 150 mM, EDTA 5 mM, NP-40 0.5%, NaF 50 mM, Na_3VO_4 1 mM, PMSF 1 mM, protease inhibitors (P8849, Sigma)) and cleared by centrifugation. Affinity purification was performed with anti-HA Clone 7 agarose beads (A2095, Sigma) on Biospin column (732–6008, BioRad) using 50mg of total protein lysate. The flow-through was removed by gravity flow and anti-HA agarose beads were washed 6 times with washing buffer (HEPES 50 mM, pH 8.0; NaCl 150 mM, EDTA 5 mM), the protein complexes were eluted with 100 mM formic acid, neutralized with triethylammonium bicarbonate (TEAB) and digested with trypsin as described previously [47]. The input was normalized based on western blot signal and samples were analyzed by Liquid Chromatography Mass Spectrometry (LC–MS/MS) on a linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (ThermoFisher Scientific) coupled to an Agilent 1200 HPLC nanoflow system (Agilent Biotechnologies). Details of the instrument configuration and methodology are described elsewhere [48].

Co-immunoprecipitation

HEK293T cells were either infected with either $Cl13_{L\text{-}HA}$ or untagged Cl13 or transfected either with L-HA encoding plasmid or empty vector control with Effectene Transfection

reagent (QIAGEN, 301425) in 6 well plates. 36 hours post infection or transfection cells were harvested, lysed for 30 min on ice in the IP buffer, and then the supernatant was cleared by centrifugation. Co-immunoprecipitation of HA-tagged L protein and PABPC1 and DDX3X was performed using anti-HA agarose beads, Clone 7 (A2095, Sigma). Total cell lysate was incubated with anti-HA agarose overnight at 4˚C. Beads were washed 6 times with IP buffer, proteins were eluted with 4% SDS Laemmli sample buffer at 95˚C for 10 min and analyzed by western blot.

For reverse co-immunoprecipitation experiments HEK293T cells were transfected with L-HA and/or TRIM21-V5 encoding plasmids with Effectene Transfection reagent (QIAGEN, 301425) in 6 well plates. TRIM21 cDNA was synthesized using the following primers: 5'caccatggcttcagcagc'3 and 5'atagtcagtggatccttgtgatcca'3 and subcloned with pENTR/D-TOPO cloning kit into a plasmid backbone originating from the pTRACER-V5 plasmid (Invitrogen) according to the manufacturer's protocol (Thermo Fisher Scientific, 450218). L Cl13 LCMV ORF was obtained from the plasmid described previously [45] using the following primers: 5'caccgccatggatgaaatcatctcagaattgagag'3 and 5'gtcgatgtcctcggccacc'3 subcloned by Gateway cloning (Thermo Fisher Scientific) into a plasmid pTO-SII-HA according to the manufacturer's protocol (Thermo Fisher Scientific, 450218).

36 hours post transfection cells were harvested, lysed for 30 min on ice in the IP buffer, and then the supernatant was cleared by centrifugation. Co-immunoprecipitation of HA-tagged L protein and V5-tagged TRIM21 was performed using anti-V5 agarose affinity gel, Clone V5-10 (A7345, Sigma). Total cell lysate was incubated with anti-V5 agarose affinity gel overnight at 4˚C. Beads were washed 2 times with IP buffer, proteins were eluted with 4% SDS Laemmli sample buffer at 95˚C for 10 min and analyzed by western blot.

Western blot

Protein concentration of cell lysates was determined with Pierce Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific, 23200). Proteins were analyzed by SDS-Page (Thermo Fisher Scientific, EA0375BOX) using Westran Clear signal PVDF membranes (Sigma Aldrich, 0485289) and the following antibodies: anti-HA.11 epitope tag antibody (Covance, MMS-101P), anti-DDX3X (Bethyl Laboratories, A300-474A), anti-PABPC1 (Cell Signaling, 4992), anti-TRIM21 (New England BioLabs, 92043) and anti-V5 Clone 5C5 (gift from M. Busslinger). The protein size was determined with a Spectra Multicolor High Range Protein Ladder (Thermo Fisher Scientific, 26625). Signal was detected with Pierce ECL Western blotting substrate (Thermo Fisher Scientific, 32209), or Amersham ECL select Western blotting detection reagent (GE Healthcare Life Sciences, RPN2235). Visualization was performed with the chemiluminescent gel documentation system MF-chemi 3.2 Pro (DNR Bio-Imaging Systems) respectively with the Bio-Rad ChemiDoc XRS Gel Documentation system (Bio-Rad Laboratories).

CRISPR-Cas9 engineering of targeted cells and functional screen

CRISPR-Cas9 single guide RNAs (sgRNAs) for selected target genes were designed using the CRISPR Design Tool [49], sgRNA Designer [50] and E-CRISPR v4.2 [51] online tools based on the target genome sequences obtained from Ensembl genome browser or merged Ensembl/Havana transcripts. Complementary oligo sgRNAs (**S4 Table**) were cloned into *Bsm*BI restriction site of LentiCRISPRv2 [52] (Addgene ID 49535) that was used for lentivirus particle production. To validate the genome editing in the targeted cells, we performed T7EI assays. PCR products were denatured, then temperature was reduced to 25˚C. Hybridized PCR products were digested with T7 endonuclease 1 (NEB) for 20 min at 37˚C in NEBuffer 2 (NEB, USA)

and analyzed by agarose gel electrophoresis (2%). ImageJ 2.0 software was used to quantify the band intensities. PCR products were analyzed by Sanger sequencing and insertion or deletion (Indel) rates were assessed by the TIDE approach [53] using DNA of non-target control cells as a reference control. The non-target control cells were transfected with the empty plasmid, that could not lead to genome editing.

CRISPR-Cas9 engineered HeLa S3 cells (ATCC CLL-2.2) were infected with 0.01 of MOI of LCMV Cl13 WT and supernatant was harvested at 36 hours post infection followed by viral load analyses by FFA. All values were normalized to the non-target control and Log2 transformed.

Reconstitution of TRIM21-targeted cells

CRISPR-Cas9 TRIM21-targeted HeLa S3 cells were transfected with either TRIM21-encoding plasmid or with mock control (empty vector—EV) with Effectene Transfection reagent (QIAGEN, 301425). 36 hours after transfection cells were infected with either wild type LCMV strain ARM or Cl13 at a MOI of 0.01. Supernatants were harvested 36 hours after infection and viral titers were quantified by focus forming assay. Obtained values were normalized to the non-target control transfected with EV plasmid and Log2 transformed.

Real-time PCR

Total RNA was extracted using QIAzol lysis reagent (QIAGEN, 79306) and reverse-transcribed with First Strand cDNA synthesis kit (Thermo Fisher Scientific, K1622) according standard protocol. Gene expression by real-time PCR was analyzed using Taqman Fast Universal Master Mix (Thermo Fisher Scientific, 4352042) and Taqman Gene Expression assays for Trim21 (Thermo Fisher Scientific, Hs00989229_g1) and for LCMV NP as described previously [54]. Gene expression data was normalized to the housekeeping gene HPRT1 (Thermo Fisher Scientific, Hs99999909_m1).

Proteomic analysis

For protein identification the RAW MS data files were converted into Mascot generic format (.mgf) using Proteowizard software v2.1.2708 [55] and searched against the human SwissProt protein database (v. 2013.01) using the two search engines, Mascot (v2.3.02, MatrixScience, London, UK) [56] and Phenyx (v2.6, GeneBio, Geneva, Switzerland) [57]. The search parameters were set with carbamidomethyl cysteine and oxidized methionine as fixed and variable modifications, respectively. One missed tryptic cleavage site was allowed. The Mascot and Phenyx identifications were combined and filtered using the previously described procedure [21] at a $<1\%$ protein false discovery rate (FDR).

Known MS contaminants such as trypsin and keratin were discarded from the results and further filtering of proteins specifically binding to LCMV L protein was achieved by comparing L protein pulldowns with the negative controls (GFP and no HA-tagged bait pulldowns) using Top3 quantitation [58] calculated with Skyline v3.1 [59] and SAINTexpress AP-MS filtering software [60]. Based on Top3 quantitation and an average of SAINT probabilities (AvgP) >0.95, all potential interactors with >120 fold increase in abundance in L pulldowns were considered to be high-confidence interactors. The visualization of the obtained networks was performed in Cytoscape v.3.4.0 [61].

Gene ontology and pathway enrichment analysis

The obtained datasets for the L protein LCMV interactome were integrated with publicly available data on host-virus interactions for other viral RdRps [22] (**S3 Table**). Functional gene

annotations were obtained from the Gene Ontology (GO) database [62]. Gene set enrichment analyses were performed using Fisher's exact test with all approved protein coding genes from the HUGO Gene Nomenclature Committee (HGNC) [63] as background set. The resulting *p*-values were corrected for multiple hypotheses testing according to the Bonferroni procedure using a cut-off of p-value<0.05. For the visualization of the resulting GO terms we used ReviGO [64].

Functional protein modules clustering

In order to identify groups of proteins with similar function we used the following network-based approach: First, we quantified the functional similarity of all protein pairs from the overlap of their respective GO annotations using the Tanimoto coefficient: $T = \frac{c}{a+b-c}$, where "c" gives the number of shared annotations and "a" and "b" the number of annotations of the two respective proteins. Next, we connected all protein pairs whose functional similarity exceeded a threshold of $T \geq 0.1$, resulting in a network of 797 human proteins, 5 viral RdRps and 1033 links. We finally used a community detection algorithm from network theory [65] to identify groups of highly interconnected proteins, i.e. proteins with a high degree of functional coherence. Each community was manually assigned a functional module name based on the most prevalent GO-terms (**S5 Table**). The visualization of the network was performed in Cytoscape v.3.4.0 [61].

Network analysis

The human host proteome we used was manually curated in [23] and contains 13,460 proteins connected by 141,296 physical interactions. To quantify the extent to which a set of viral targets is localized within a certain neighborhood of the host proteome, we analyzed their largest connected component (LCC), i.e., the highest number of viral targets that are directly connected to one another [23]. To test the statistical significance of a measured LCC size, we compared it to the control distribution obtained from 100,000 random simulations in which gene sets of the same size were chosen completely at random from the network.

Statistical analyses and data visualization

Two-tailed Student's t-tests were used for comparisons between two groups, one-way ANOVA with Bonferroni correction were used for comparisons between multiple groups. The values shown in the line and bar graphs indicate the mean +/- standard error of the mean. Significant p-values are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Statistical analyses and graph preparation were performed in GraphPad Prism (version 6.0b).

Ethics statement

Experiments conducted at the animal facility of the Medical University of Vienna were approved by the ethical committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research with the animal protocol number 66.009/0318-II/3b/2012 in accordance with the Austrian law for animal experiments (TVG, BGBl. Nr. 501/1989 i. d.F. BGBI. I Nr. 162/2005). Mouse experiments performed at the University Basel were approved by the Veterinary Office of the Canton on Basel (#2665/28404) and were performed in accordance with the Swiss law for animal protection (TSchG 455).

Supporting information

S1 Fig. Functional validation of $Cl13_{L\text{-}HA}$ virus and HA-immunoprecipitation. (**A**) HEK293T cells were infected at a MOI of 3 with either $Cl13_{L\text{-}HA}$ or with untagged Cl13. Cells were harvested and lysed at 36 hours post infection for western blot analyses with anti-HA antibodies. (**B**) C57BL/6J mice were infected with $2x10^6$ FFU either $Cl13_{L\text{-}HA}$ or untagged Cl13 and spleen samples were analyzed 50 days post infection by Sanger sequencing. (**C**) Fractions from one-step purification of $Cl13_{L\text{-}HA}$ protein were collected during the mass spectrometry sample preparation and analyzed by western blot with antibodies specific to HA and NP LCMV. NP, as a known L interactor, was used as a positive control to confirm the successful immunoprecipitation of L-HA. Percentage indicates the amount of each fraction collected during AP-MS pulldown preparation loaded on the gel. (**D**) HEK293T cells were infected with MOI 3 either with $Cl13_{L\text{-}HA}$ containing either L1079K or L1079Q, or untagged virus. Cells were harvested and lysed 36 hours post infection and co-immunoprecipitation was performed with anti-HA followed by western blot analyses with antibodies specific to the endogenous proteins DDX3X, PABPC1 and TRIM21 as well as HA. IP–immunoprecipitation. **(E)** HEK293T cells were transfected with plasmid encoding HA-tagged L protein or empty vector control. Cells were harvested and lysed 36 hours post transfection and co-immunoprecipitation was performed with anti-HA followed by western blot analyses with antibodies specific to the endogenous proteins DDX3X, PABPC1 and TRIM21. **(F)** HEK293T cells were transfected with plasmid encoding HA-tagged L protein and/or V5-tagged TRIM21. Cells were harvested and lysed 36 hours post transfection and co-immunoprecipitation was performed with anti-V5 followed by western blot analyses with antibodies specific to the HA and V5. * marks a non-specific protein band.
(TIF)

S2 Fig. Confirmation of the genome editing for CRISPR-Cas9 targeted cells. Confirmation of the genome editing for CRISPR-Cas9 targeted cells using (**A**) T7EI cleavage assay followed by the band intensity quantification with ImageJ software and (**B**) Sanger sequencing followed by tracking of indels by decomposition (TIDE) quantification. For TIDE analyses primers were designed covering the respected targeted region using Ensembl genome browser or merged Ensembl/Havana transcripts to PCR-amplify the selected region. To evaluate indel frequencies we used non-target control treated sample (transfected with an empty plasmid that) as a reference control. Bars represent indel frequencies for each cell line. (**C**) TRIM21 and DDX3X CRISPR-Cas9 targeted cells were lysed and analyzed by western blot with antibodies specific to the endogenous TRIM21 or DDX3X and actin.
(TIF)

S3 Fig. *TRIM21* expression in HEK293T cells infected with Cl13 LCMV. HEK293T cells were infected with LCMV Cl13 WT at a MOI of 3 and harvested at the indicated time points. The gene expression for nucleoprotein Cl13 LCMV and *TRIM21* was measured by RT-PCR. The arbitrary units were calculated using HPRT1 as a housekeeping gene, then fold change for each gene was calculated using 0 hpi as a reference point for *TRIM21* and 2 hpi for nucleoprotein Cl13 LCMV.
(TIF)

S4 Fig. Analyses of the L protein of LCMV strain ARM *in vitro* and *in vivo*. (**A**) Two independently generated HeLa S3 CRISPR-Cas9 targeted cell pools per gene of interest for 5 genes were infected in triplicate wells with LCMV ARM WT at a MOI of 0.01 and viral loads were measured at 36 hours post infection by focus forming assay. The obtained data were normalized to the non-target control and log2 transformed. (**B-C**) C57BL/6 and *Trim21*$^{-/-}$ mice were

infected with $2x10^6$ FFU of the indicated viruses. Viral titers were determined in (**B**) blood at indicated time points and in (**C**) organs at 21 days post infection. Each symbol and bar represents the mean ± SEM of three to five mice. Statistical significance was calculated by unpaired t-test (**B**) or by two-way ANOVA (**C**). Significant p values were indicated as follows: ns—non significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
(TIF)

S1 Table. Primers used for reverse genetic engineering of L protein-tagged LCMV.
(XLSX)

S2 Table. Mass spectrometry data.
(XLSX)

S3 Table. Host protein interactomes of viral RdRps from public databases.
(XLSX)

S4 Table. sgRNA oligonucleotide sequences.
(XLSX)

S5 Table. Protein functional modules targeted by L protein and other RdRps.
(XLSX)

Acknowledgments

We like to thank Jacques Colinge, Franz X. Heinz and Giulio Superti-Furga for helpful advice and feedback during the development of the project. We acknowledge Roberto Giambruno for advice on mass spectrometry as well as Alexandra Mariela Popa for critical discussions.

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Characterization of host proteins interacting with the lymphocytic choriomeningitis virus L protein

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Supporting Information

S1 Fig. Functional validation of $Cl13_{L\text{-}HA}$ virus and HA-immunoprecipitation

(A) HEK293T cells were infected at a MOI of 3 with either $Cl13_{L\text{-}HA}$ or with untagged Cl13. Cells were harvested and lysed at 36 hours post infection for western blot analyses with anti-HA antibodies. (**B**) C57BL/6J mice were infected with $2x10^6$ FFU either $Cl13_{L\text{-}HA}$ or untagged Cl13 and spleen samples were analyzed 50 days post infection by Sanger sequencing. (**C**) Fractions from one-step purification of $Cl13_{L\text{-}HA}$ protein were collected during the mass spectrometry sample preparation and analyzed by western blot with antibodies specific to HA and NP LCMV. NP, as a known L interactor, was used as a positive control to confirm the successful immunoprecipitation of L-HA. Percentage indicates the amount of each fraction collected during AP-MS pulldown preparation loaded on the gel. (**D**) HEK293T cells were infected with MOI 3 either with $Cl13_{L\text{-}HA}$ containing either L1079K or L1079Q, or untagged virus. Cells were harvested and lysed 36 hours post infection and co-immunoprecipitation was performed with anti-HA followed by western blot analyses with antibodies specific to the endogenous proteins DDX3X, PABPC1 and TRIM21 as well as HA. IP – immunoprecipitation. **(E)** HEK293T cells were transfected with plasmid encoding HA-tagged L protein or empty vector control. Cells were harvested and lysed 36 hours post transfection and co-immunoprecipitation was performed with anti-HA followed by western blot analyses with antibodies specific to the endogenous proteins DDX3X, PABPC1 and TRIM21. **(F)** HEK293T cells were transfected with plasmid encoding HA-tagged L protein and/or V5-tagged TRIM21. Cells were harvested and lysed 36 hours post transfection and co-immunoprecipitation was performed with anti-V5 followed by western blot analyses with antibodies specific to the HA and V5. * marks a non-specific protein band.

S2 Fig. Confirmation of the genome editing for CRISPR-Cas9 targeted cells

Confirmation of the genome editing for CRISPR-Cas9 targeted cells using (**A**) T7EI cleavage assay followed by the band intensity quantification with ImageJ software and (**B**) Sanger sequencing followed by tracking of indels by decomposition (TIDE) quantification. For TIDE analyses primers were designed covering the respected

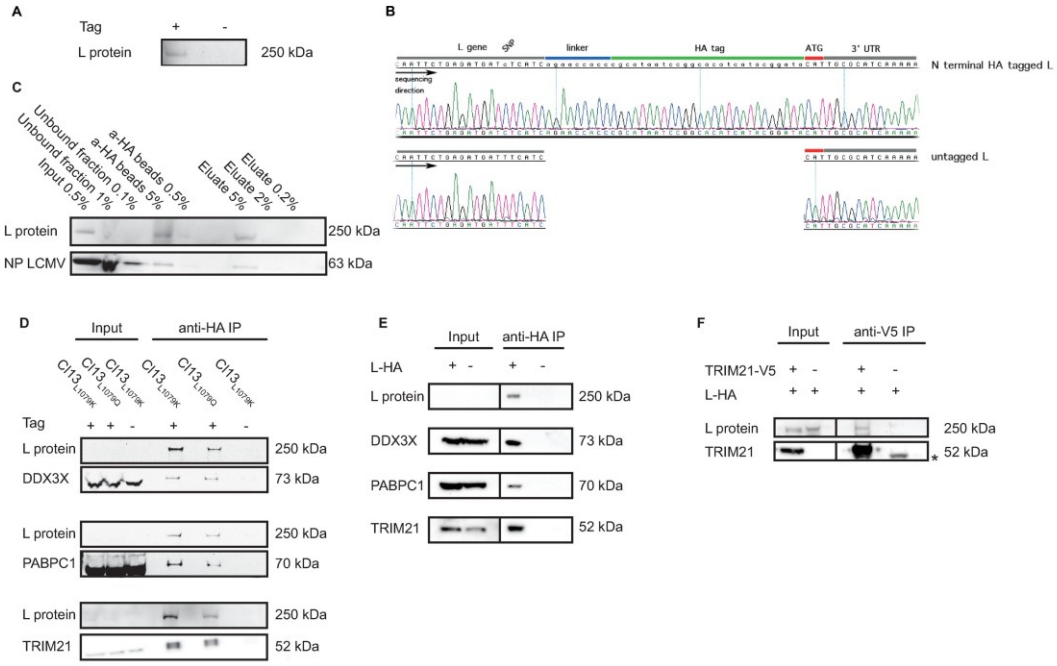
targeted region using Ensembl genome browser or merged Ensembl/Havana transcripts to PCR-amplify the selected region. To evaluate indel frequencies we used non-target control treated sample (transfected with an empty plasmid that) as a reference control. Bars represent indel frequencies for each cell line. (**C**) TRIM21 and DDX3X CRISPR-Cas9 targeted cells were lysed and analyzed by western blot with antibodies specific to the endogenous TRIM21 or DDX3X and actin.

S3 Fig. *TRIM21* expression in HEK293T cells infected with Cl13 LCMV
HEK293T cells were infected with LCMV Cl13 WT at a MOI of 3 and harvested at the indicated time points. The gene expression for nucleoprotein Cl13 LCMV and *TRIM21* was measured by RT-PCR. The arbitrary units were calculated using HPRT1 as a housekeeping gene, then fold change for each gene was calculated using 0 hpi as a reference point for *TRIM21* and 2 hpi for nucleoprotein Cl13 LCMV.

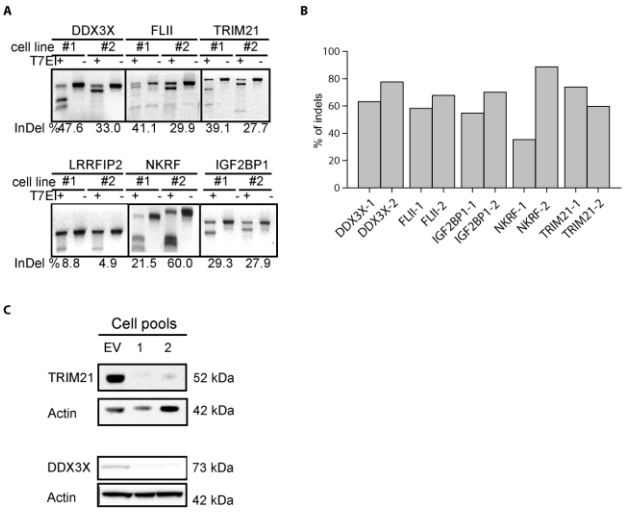
S4 Fig. Analyses of the L protein of LCMV strain ARM *in vitro* and *in vivo*.
(A) Two independently generated HeLa S3 CRISPR-Cas9 targeted cell pools per gene of interest for 5 genes were infected in triplicate wells with LCMV ARM WT at a MOI of 0.01 and viral loads were measured at 36 hours post infection by focus forming assay. The obtained data were normalized to the non-target control and log2 transformed. (**B**-**C**) C57BL/6 and *Trim21*$^{-/-}$ mice were infected with 2x10^{6} FFU of the indicated viruses. Viral titers were determined in (**B**) blood at indicated time points and in (**C**) organs at 21 days post infection. Each symbol and bar represents the mean ± SEM of three to five mice. Statistical significance was calculated by unpaired t-test (**B**) or by two-way ANOVA (**C**). Significant p values were indicated as follows: ns - non significant, * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

S1 Table. Primers used for reverse genetic engineering of L protein-tagged LCMV
S2 Table. Mass spectrometry data
S3 Table. Host protein interactomes of viral RdRps from public databases
S4 Table. sgRNA oligos sequences
S5 Table. Protein functional modules targeted by L protein and other RdRps

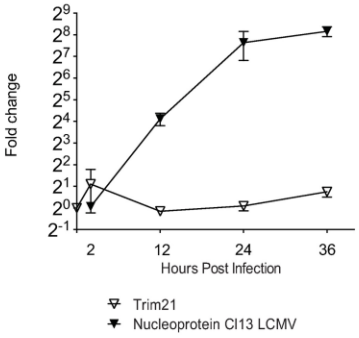
Supplementary Figure 1



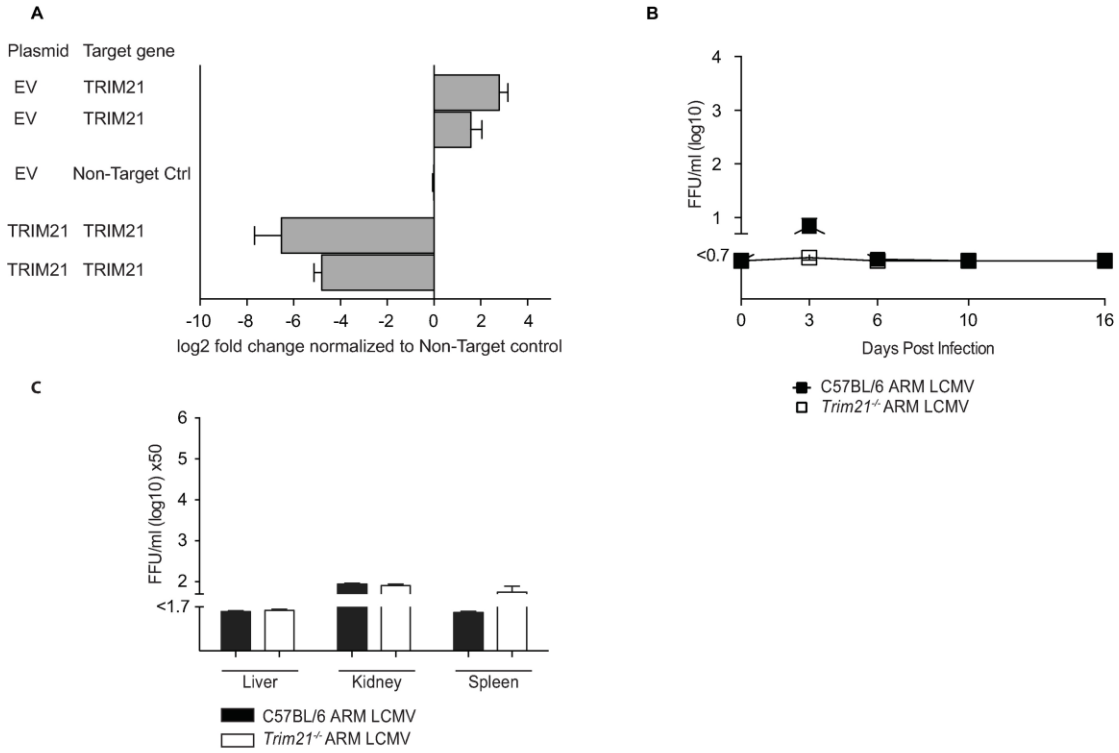
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



3.3 Investigating the effect of the L1079 mutations of LCMV L protein interactomes and functional validation

In order to perform a comparative analysis of interactomes of ARM and Cl13 polymerases and to identify how a single amino acid exchange at the position L1079 affects host-virus interactions, we analyzed the $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ L protein interactome using an MS approach (**Figure 3** and **Figure 2 from the Manuscript**).

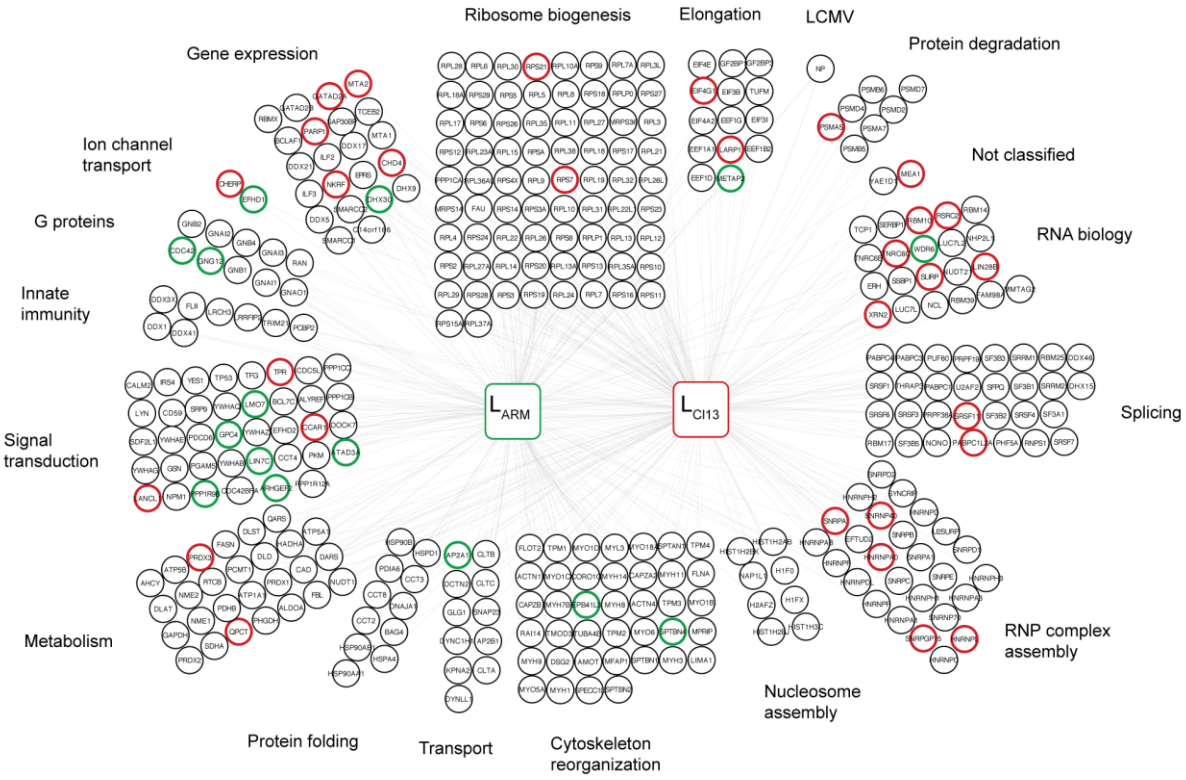


Figure 3. Interactome of $Cl13_{L1079K}$ (L_{ARM}) and $Cl13_{L1079Q}$ (L_{Cl13}) LCMV L protein. Overview of L protein interactomes grouped based on the protein functions and visualized in Cytoscape. Strain-specific interactors are highlighted in green ($Cl13_{L1079K}$) and in red ($Cl13_{L1079Q}$).

Most of the host factors were identified as interactors of both $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ proteins. The LCMV NP, a known L protein interactor (Kerber, Rieger et al., 2011), was detected in the pulldowns of both polymerases. Nevertheless, the experiment revealed strain-specific proteins as well. These factors belong to the different functional groups of host proteins (**Figure 3)**.

One of the host factors identified as a $Cl13_{L1079Q}$-specific interactor is the NKRF protein, a transcription factor regulating NF-kB response as well as an important node of the innate immunity (Feng, Guo et al., 2002, Nourbakhsh, Kalble et al., 2001). Nevertheless, there is no available data on the characterization of the NKRF-deficient mice upon LCMV infection. Therefore, we decided to infect these mice with Cl13 LCMV and follow the course of infection (**Figure 4**).

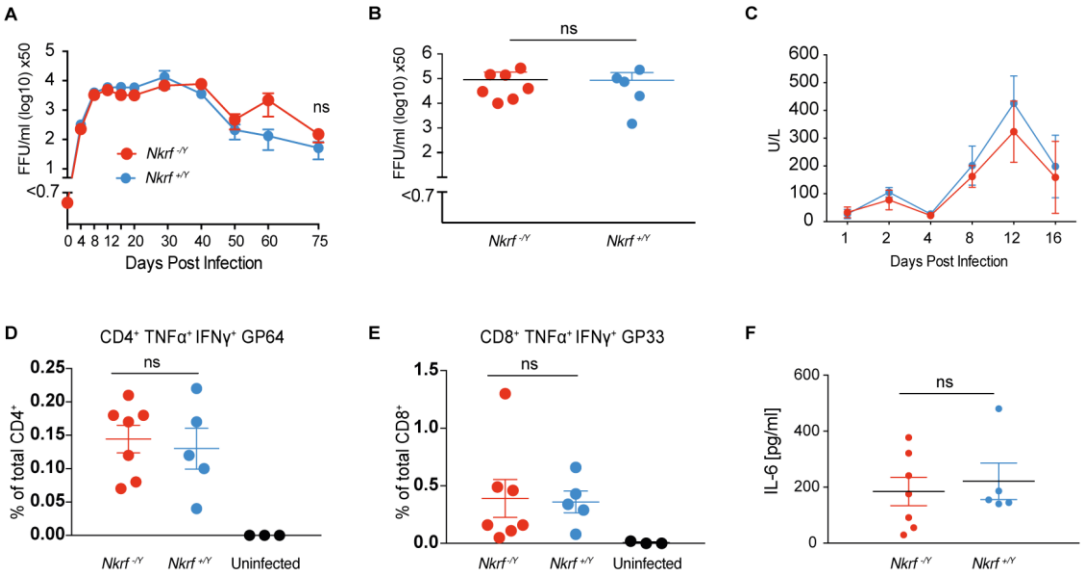


Figure 4. Characterization of *Nkrf* $^{-/Y}$ mice upon LCMV Cl13 infection. *Nkrf* $^{-/Y}$ and littermates control mice were infected with 2x10^{6} FFU of the LCMV strain Cl13. Viral titers were determined in (**A**) blood at indicated time points and in (**B**) kidney at 75 day post infection. Level alanine aminotransferase (ALT) was measured in serum at indicated time points (**C**). Single cell suspension of spleen was stimulated with GP64 peptide and CD4^{+} T cells producing both TNFα and IFNγ were measured at 75 day post infection (**D**). Single cell suspension of spleen was stimulated with GP33 peptide and CD8^{+} T cells producing both TNFα and IFNγ were measured at 75 day post infection (**E**). IL-6 was measured by ELISA in serum of the infected mice at 4 dpi (**F**). The data is representative of two similar experiments. Each symbol represents the mean ± SEM of five to seven mice. Statistical significance was calculated by unpaired t-test (**B, D-F**) or by Two-way ANOVA (**A** and **C**). Significant p values were indicated as follows: ns - non significant.

To investigate whether NKRF contributes to the control of LCMV infection, we infected *Nkrf* $^{-/Y}$ mice with LCMV strain Cl13 and monitored the course of infection. NKRF-deficient mice demonstrate the viremia levels over the course of infection (**Figure 4A**) as well as viral load in kidney (**Figure 4B**) similar to the littermates. Then, we measured serum concentration of ALT, routinely used clinical parameter of liver damage (**Figure 4C**). ALT level is not significantly different between NKRF-deficient and control groups up to 16 day post infection.

Furthermore we did not identify any differences between *Nkrf* $^{-/Y}$ mice and the control group in the number of GP64^{+} CD4^{+} (**Figure 4D**) as well as in the number of GP33^{+} CD8^{+} cells (**Figure 4E**).

Next, we measured serum levels of IL-6 (Kamperschroer & Quinn, 2002), in the infected mice at the day 4 post infection, that were comparable between NKRF-deficient mice and littermate control.

To summarize, we developed a novel approach to study host-virus protein interactions and identified the common and unique interactomes of Cl13$_{L1079K}$ and

$Cl13_{L1079Q}$ L proteins. Then we integrated the obtained data with the publicly available datasets for various RdRps of positive and negative RNA viruses. Next, we performed functional studies to validate the role of the selected host factors in the LCMV life cycle *in vitro* and in *vivo*. This approach allowed us to identify proviral (DDX3X) and antiviral (NKRF, TRIM21) cellular proteins. Furthermore, we demonstrated impaired virus control in *Trim21*$^{-/-}$ mice in chronic LCMV infection and performed characterization of NKRF-deficient mice upon the LCMV strain Cl13 infection.

4. DISCUSSION

4.1 General discussion

This research demonstrates a technological approach to introduce a genetic tag in the two-segmented LCMV genome without loss of viral replication (**Figure 1 from the Manuscript**) and provides an overview of the arenaviral polymerase interactome in the context of natural viral infection (**Figure 2 from the Manuscript**). The presented strategy allowed to obtain L protein-tagged LCMV that sustain the main immunobiological features of the wild type viruses (**Figure 1B-E from the Manuscript**). Potentially, the described workflow can be applied to other viral polymerases and various viral proteins *in vitro* and *in vivo*. Additionally, this approach can be used for *ex vivo* and *in vitro* characterization of viral protein interactomes in a tissue- and cell type-specific context. This can improve our understanding how host-virus interactions changes at different stages of infection and point out novel targets for antivirals.

Our proteomic analyses revealed that the LCMV L proteins of both $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ viruses target multiple cellular pathways such as RNA biology-associated modules (e.g. splicing and ribonucleoprotein complex assembly), cytoskeleton reorganization, protein folding and transport. The annotation of the LCMV L protein interactomes provides a comprehensive overview of the protein functional groups identified in the pulldowns (**Figure 2B from the Manuscript**). The majority of these groups reflects basic biological processes recruited by viral polymerases: splicing, ribosome biogenesis, RNP assembly and elongation.

Interestingly, the L protein also binds to numerous host proteins associated with signal transduction that include players of innate immune signaling such as the dsRNA-sensing helicase DDX1 (Zhang, Kim et al., 2011) and the MyD88 modulators LRRFIP2. This may be of relevance for the intricate and multi-faceted relation of LCMV with the antiviral type I interferon system (McNab, Mayer-Barber et al., 2015, Zuniga, Hahm et al., 2007). In addition, we found several different G protein-coupled receptors in our L protein interactomes (**Figure 3 and Figure 2 from the Manuscript**), indicating viral interference with these central signaling networks (Sodhi, Montaner et al., 2004).

Furthermore, we selected some of the signal transduction-associated proteins (DDX3X, FLII and IGF2BP1) for follow up loss-of-function experiments (**Figure 4 from the Manuscript**). Interestingly, FLII serves as a negative regulator of TLR signaling by direct interaction with the intracellular adaptor protein MyD88 (Dai, Jeong et al., 2009). Interactions between FLII and L protein can indicate that LCMV actively interferes with TLR signaling.

DDX3X is a member of the RNA helicases family, that is involved in many pathways of RNA metabolism (Franca, Belfiore et al., 2007) and innate immune response (Soulat, Burckstummer et al., 2008). It serves as a proviral factor for human cytomegalovirus (Cavignac, Lieber et al., 2015), HCV (Pene, Li et al., 2015), HIV-1 (Frohlich, Rojas-Araya et al., 2016) and as an antiviral factor for influenza A virus (Thulasi Raman, Liu et al., 2016) and dengue virus (Li, Feng et al., 2015). *In vitro* experiments with DDX3X-targeted cell lines revealed a proviral role of this helicase in the LCMV life cycle (**Figure 4A from the Manuscript**).

Several other members of the RNA helicases group were identified as common LCMV L protein host interactors, such as DDX1, DDX4, DDX5, DHX9 and DHX30. These proteins are involved in ATP-dependent remodeling of RNA as well as RNP complexes (Linder & Jankowsky, 2011). Many RNA helicases are implicated in signal transduction and innate immune processes. Altogether this makes RNA helicases attractive targets for antivirals development (Kwong, Rao et al., 2005). Direct protein-protein interactions between the LCMV L protein and multiple RNA helicases suggest that the virus recruits them for RNP remodeling and/or interfere with the cellular signaling.

G protein-coupled receptors (GPCRs) represent another class of signaling proteins identified as LCMV L proteins interactors. Different viruses hijack and modulate functions of G proteins according to their requirements, using them as co-receptors for viral entry, perturbing MAPK signaling and regulating apoptosis (Sodhi et al., 2004). Our data suggest that LCMV-G proteins interactions may have some functional consequences which could provide interesting directions for further studies.

Considering the L1079 mutation in the Cl13 polymerase as a primary determinant of viral persistence, strain specific interactors may play an important role in the determination of the infection course. The differential interactomes of $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ L protein (**Figure 3**) highlight potential candidates. Interestingly, the strain-specific interactors belong to the different host protein modules. The $Cl13_{L1079K}$-specific proteins are part of G proteins and cytoskeleton reorganization proteins groups, whereas the $Cl13_{L1079Q}$-specific interactors belong to the metabolism, splicing, RNP complex assembly, protein degradation and ribosome biogenesis functional modules.

One of the identified strain-specific interactors is NKRF, which was found only in $Cl13_{L1079Q}$ pulldown (**Figure 3**). This protein plays an important role in the innate immune response.

NKRF is a transcription factor repressing the expression of interferon β (IFNβ) (Nourbakhsh, Oumard et al., 2000), IL-8 (Nourbakhsh et al., 2001) and inducible nitric-oxide synthase (iNOS) (Feng et al., 2002) under steady-state conditions. The functional role of NKRF might be significantly changed upon stimulation as it may promote the expression of IL-8 expression in the presence of IL-1 (Nourbakhsh et al., 2001). This context-dependent function of NKRF on the IL-8 promoter suggests that other signaling molecules may affect its functional role. Additionally, NKRF serves as a negative regulator of NF-κB activation through direct binding to p65 and preventing it from translocating to the nucleus (Reboll, Schweda et al., 2011). Furthermore, NKRF was implicated in the inhibition of HIV-1 transcription (Dreikhausen, Hiebenthal-Millow et al., 2005). We identified NKRF as a strain-specific interactor for $Cl13_{L1079Q}$ which suggests a possible role of NKRF in the immunobiology of persistence. The diverse functions of NKRF, both as a transcription factor as well as a NF-κB repressor, might shape the immune response during LCMV infection. Surprisingly, innate immune responses in NKRF-deficient mice are undistinguishable from those elicited in the littermates upon challenges with various pathogens (Froese, Schwarzer et al., 2006).

Since NKRF contributes to innate immunity both as a transcription factor and as a NF-κB activation regulator, and NKRF-targeted cell lines demonstrated increased viral load (**Figure 4A from the Manuscript**), we infected NKRF-deficient mice with the LCMV strain Cl13 and followed the infection course (**Figure 4**). We demonstrated that viral loads in blood and in kidney (**Figure 4A-B**), as well as liver damage (**Figure 4C**), are similar between NKRF-deficient mice and the control group. Furthermore, *Nkrf* $^{-/Y}$ mice showed similar numbers of $GP64^{+}$ $CD4^{+}$ (**Figure 4D**) and $GP33^{+}$ $CD8^{+}$ cells (**Figure 4E**) as well as similar concentration of the cytokine IL-6 in the serum of infected mice (**Figure 4F**).

The performed comprehensive characterization did not reveal any statistically significant differences between NKRF-deficient mice and the control group upon infection with the LCMV strain Cl13. Despite the observed significant differences in viral load between NKRF-targeted cells and the control cells upon the Cl13 strain LCMV infection (**Figure 4A from the Manuscript**), we did not identify a similar effect in the mouse model (**Figure 4A-B**). There are multiple factors that could determine these differences between *in vitro* and *in vivo* models. Various cell types and organs contribute to the antiviral host response during the different stages of the LCMV infection. The L protein interactome could vary between different cell types in the organism and the observed antiviral effect of NKRF could be not present in all cell types. Moreover, some of the host proteins expressed *in vivo*, but not *in vitro*, may potentially compensate the effect from the genetic ablation of NKRF.

To summarize, we demonstrated the antiviral effect of NKRF *in vitro*, but not *in vivo*. Nevertheless, the obtained data may guide future investigations towards better understanding of the NKRF function in immune responses.

It is important to emphasize, that strain-specific factors may play an important role in a context-dependent manner and potentially still affect infection outcome, that can't be detected in the HEK293T cell line. Therefore, our study opens a novel technological and methodological avenue for future studies of host-virus interaction in the context of natural viral infection.

Furthermore, the immunobiological outcome of viral infection could be affected by multiple factors apart from host-protein PPI. Lysine at the position of L1079 in the ARM L protein could serve as a site for various post-translational modifications (PTMs) including methylation, ubiquitination, SUMOylation and N-acetylation (Walsh, Garneau-Tsodikova et al., 2005). PTMs of L protein may affect the protein structure and/or the rate of protein degradation. Additionally, the L1079 mutation could alter the transcription vs replication rate as well as polymerase fidelity. Thus, further investigations should reveal factor(s) responsible for the dramatic changes in the immunobiology caused by L1079 mutations.

The combination of proteomic (**Figure 3** and **Figure 2 from the Manuscript**) and functional screening (**Figure 3 from the Manuscript**) approaches applied here highlighted several interesting candidates that play a role in the LCMV life cycle. We identified TRIM21 and NKRF as antiviral host factors, and DDX3X – as a proviral factor *in vitro* (**Figure 4A from the Manuscript**). The infection experiment with NKRF-deficient mice did not reveal any impact on the LCMV viral load or on the investigated parameters of the host immune response (**Figure 4**).

TRIM21 was identified as an interactor for both $Cl13_{L1079K}$ and $Cl13_{L1079Q}$ strains L proteins. TRIM21-targeted cell lines demonstrated higher viral loads, than EV control cells (**Figure 4A from the Manuscript**). This protein contributes to the innate immunity within the cell. The E3 ubiquitin-protein ligase TRIM21 (Espinosa, Zhou et al., 2006), regulates type I interferon response via ubiquitination of interferon regulatory factors (IRFs), such as IRF3, IRF5, IRF7 and IRF8 (Higgs, Lazzari et al., 2010, Higgs, Ni Gabhann et al., 2008, Kong, Anderson et al., 2007). Furthermore, TRIM21 is implicated in the antibody-mediated immune response acting as a cytosolic Fc receptor (McEwan, Tam et al., 2013). Ubiquitous expression of TRIM21 in the various tissues as well as broad antibody isotype specificity (IgA, IgG, IgM) may suggest wide functional activity in different body compartments (Foss, Watkinson et al., 2015). *Trim21*$^{-/-}$ mice demonstrated impaired virus control after infection with the LCMV strain Cl13 (**Figure 4C-D from the Manuscript**). Interestingly, there are no significant

differences in the viral load between *Trim21*$^{-/-}$ mice and the control group upon infection with the LCMV strain ARM (**Supplementary Figure 4B-C from the Manuscript**). Although TRIM21 was identified as an interactor for both L proteins, the genetic ablation affects only the chronic LCMV infection, but not the acute one. This can be explained by the differences in time course of ARM and Cl13 infections. The LCMV strain ARM is cleared from the blood of infected mice within a week after infection, whereas the Cl13 strain causes a long-lasting viremia (around 8 weeks). The differences in viral load appear in the LCMV strain Cl13 infection only after 2 weeks after infection (**Figure 4C from the Manuscript),** when the ARM strain is already cleared by the immune system. Future investigations shall reveal the molecular mechanism behind this intriguing phenotype.

We integrated the obtained dataset on the LCMV L protein with the available datasets for the RdRps of positive and negative RNA viruses. Network analyses of RdRp interactions provide us with a powerful tool to indicate the main approaches of viral RdRps to target the host protein-protein interaction network. We have demonstrated that RdRps from the different viruses have very similar targets in the host proteome. Moreover, they target a concrete module of proteins, highly connected to each other. This reflects the fact that the viral replication machineries recruit various RNA biology-associated host factors (**Figure 3 from the Manuscript**).

The strategy to obtain interactome data on viral polymerase developed here might be applied for many other viral proteins. Potentially, that allows to dissect protein-protein interactions *in vivo*, that would be of particular importance for both better understanding of host proteome perturbations by viruses in the tissue-specific context as well as for the identification of novel therapeutic targets.

Our results of N- and C-terminus L protein-tagged LCMV highlighted the importance of appropriate site selection to introduce the genetic tag into the viral protein: all viruses containing N-terminally tagged L protein were viable, but none of the C-terminally tagged ones. This could be due to disruption of protein structure by the tag, impaired functional activity or disturbance of interactions with cellular proteins required for transcription and replication of the viral genome.

It is important to take into the consideration not only domain localization within the viral protein, but also available data on the secondary structure of viral RNA. The careful choice of the tag position and testing multiple options are very important to overcome possible technical obstacles.

To summarize, identification of cellular pathways and processes targeted by the LCMV L protein revealed both general viral strategies to perturb the host proteome as well as demonstrate the differences in host-virus interactions between acute and

persistent infections. Functional validation *in vitro* and *in vivo* allowed to pinpoint the impact of TRIM21 on the course of infection.

4.2 Conclusions and future prospects

The developed technology of endogenous tagging of LCMV L protein allows to obtain recombinant viruses that retain important immunobiological characteristics similar to untagged viruses. This methodology could be applicable to other proteins and viruses and, thus, empower host-virus interactomics with the opportunity to analyze stage- and tissue-specific interactions *in vivo* and *ex vivo*. The obtained datasets on the LCMV L protein interactome can significantly change our understanding of molecular mechanisms underlying host-virus interactions during infection and lead to the development of novel therapeutic approaches.

This study provides a comprehensive overview of the host-virus interactome for the LCMV L protein in the context of natural viral infection.

The analysis of host-virus interactions coupled with loss-of-function experiments allowed to pinpoint critical host partners involved in the LCMV life cycle. We identified both proviral (DDX3X) and antiviral (TRIM21, NKRF) host factors. Furthermore, we confirmed that genetic ablation of TRIM21 in the LCMV strain Cl13 infection results in the impaired virus control. Future efforts in dissecting the molecular mechanism underlying the role of TRIM21, NKRF and DDX3X in the LCMV life cycle shall provide a better understanding of host-virus interactions.

Integration of the stage- and tissue-specific data on host-virus PPI with patterns of gene expression changes from the same samples coupled with loss-of-function experiments can provide novel insights in the viral life cycle and virus-induced perturbations.

Furthermore, the obtained global overview of host protein complexes and pathways that are targeted by various RdRps resulting from the integration of the generated datasets with publicly available data highlighted common and virus-specific cellular pathways.

5. MATERIALS & METHODS

Measurements of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured from the serum of mice using a Cobas C311 Analyzer (Roche) or a 747 Automatic-Analyzer (Hitachi).

ELISA

IL-6 concentrations in serum were measured with the Mouse IL-6 DuoSet (#DY406) (R&D Systems) according the manufacturer's protocol.

Mouse experiments

NKRF-deficient mice were kindly provided by Prof. Hansjörg Hauser (Froese et al., 2006). Mouse experiments were performed with sex- and age-matched animals under specific pathogen-free conditions.

Proteomic analysis

Protein identification and subtraction of the non-specific interactors were performed as described in the manuscript. DHX30 and TRIM21 passed the Top3 quantitation (Silva, Gorenstein et al., 2006) filtering step but not the SAINT subtraction (Choi, Larsen et al., 2011). Nevertheless, these proteins were also included in the following analyses due to the highly enriched spectral counts in L protein pulldowns compared to controls (**Supplementary Table 3 from the Manuscript**) and due to their known roles in immune responses and in the life cycles of other viruses. The visualization of the obtained networks was performed in Cytoscape v.3.4.0 (Shannon, Markiel et al., 2003).

For other experimental procedures please refer to the section "Materials and Methods" in the manuscript.

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Curriculum Vitae

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Education

• PhD program, 2012-Present time
Medical University of Vienna, Vienna, Austria
Thesis title: “Host-virus protein interactome of L protein of lymphocytic choriomeningitis virus”

• MSc, Genetics, 2011 (Summa Cum Laude),
Tomsk State University, Tomsk, Russia
Thesis title: “Phylogeny and phylogeography of haplogroups of N clade of human Y-chromosome in Volga-Ural region and Siberian populations” Grade: 5.0 (out of 5.0)

• BSc, Biology, 2009 (Summa Cum Laude)
Tomsk State University, Tomsk, Russia
Thesis title: “Dissection of the Khakass gene pool structure using Y-chromosome markers” Grade: 5.0 (out of 5.0)

Career History

2012 – Present time
PhD Student, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

September 2011 – September 2012
Junior Researcher, Institute of Cytology and Genetics the Siberian Division of the Russian Academy of Science, Novosibirsk, Russia

February 2011 – August 2011
Researcher, State Research Center of Virology and Biotechnology VECTOR, Koltsovo, Russia

August 2009 – July 2011
Student (MSc program) of the Tomsk State University, Tomsk, Russia

February 2006 – January 2011
Junior Researcher, Laboratory of Evolutionary Genetics, Research Institute of Medical Genetics, Tomsk, Russia

August 2005 – June 2009
Student (BSc program) of the Tomsk State University, Tomsk, Russia

Conference participation (selected)

- "Viral Immunity", 20-21 October 2016, San Raffaele Hospital, Milan, Italy (poster presentation)
- "Virus Genomics and Evolution", 8-10 June 2016, Wellcome Genome Campus, Hinxton, Cambridge, UK (poster presentation)
- Cambridge Immunology Forum „Co-Infection: A War on Two Fronts ", 24th September 2015, Cambridge, UK (poster presentation)
- 25th Annual Meeting of the Society for Virology, 18-21 March 2015, Bochum, Germany (poster presentation)
- 4th International Retreat: Viral Infection, Immunity and Autoimmunity, 11-14 August 2015, Koblenz, Germany (oral presentation)
- 17th International Summer School on Immunology FEBS Advanced Lecture Course «Immune System: Genes, Receptors and Regulation», September 14-21, 2013; Rabac, Croatia (poster presentation)
- Student Science Conference, Tomsk State University, 2010 and 2011 (oral presentation)
- 46th International Student Science Conference "Students and Progress in Science and Technology", Novosibirsk State University, 2008 (oral presentation)

Honors and Awards

- Doctoral Fellowship Program (DOC) of the Austrian Academy of Sciences, 2015;
- Medal of the Russian Academy of Sciences for the students (19 awards out of 3500 applicants), 2012;
- Award of the Governor of Tomsk region for students (20 awards out of 850 applicants), 2011;
- Award for the best report at the Student Science Conference, Tomsk State University (1 award out of 15 participants), 2011;
- Medal for the best scientific student work of the Ministry of Education and Science, Russia Federation (12 awards out of 2500 applicants), 2010;
- Award for the best report at the Student Science Conference, Tomsk State University (1 award out of 36 participants), 2010;
- 1st prize of the students State Olympiad of the Russia Federation (command test – 1 award out of 12 commands), 2010;
- 3rd prize of the students State Olympiad of the Russia Federation (personal test – 1 award out of 46 participants), 2010;
- Award for the best report at the International Science Conference "Ecology of Southern Siberia", Khakas State University (1 award out of 43 participants), 2010;
- Award for the best report at the Student Science Conference, Tomsk State University (1 award out of the 35 participants), 2009;
- Award for the best report at the 46th International Student Science Conference "Students and Progress in Science and Technology", Novosibirsk State University (1 award out of 56 participants), 2008;

- Prizewinner of the regional Biological Olympiad for students (1 award out of the 47 participants), 2005;
- Silver medal for academic achievements in the high school, 2005

Publications:

1. **Khamina K.,** Lercher A.*, Caldera M.*, Schliehe C., Vilagos B., Sahin M., Kosack L., Bhattacharya A., Májek P., Stukalov A., Sacco R., James L.C., Pinschewer D.D., Bennett K., Menche J., Bergthaler A. Comparative analyses of L protein interactomes of lymphocytic choriomeningitis virus. *PLOS Pathogens*, 2017 Dec 20; https://doi.org/10.1371/journal.ppat.1006758.

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3. Bhattacharya A, Hegazy AN, Deigendesch N, Kosack L, Cupovic J, Kandasamy RK, Hildebrandt A, Merkler D, Kühl AA, Vilagos B, Schliehe C, Panse I, **Khamina K**, Baazim H, Arnold I, Flatz L, Xu HC, Lang PA, Aderem A, Takaoka A, Superti-Furga G, Colinge J, Ludewig B, Löhning M, Bergthaler A. Superoxide Dismutase 1 Protects Hepatocytes from Type I Interferon-Driven Oxidative Damage *Immunity*. 2015 Nov 17; 43(5):974-86.

4. Schliehe C, Flynn EK, Vilagos B, Richson U, Swaminathan S, Bosnjak B, Bauer L, Kandasamy RK, Griesshammer IM, Kosack L, Schmitz F, Litvak V, Sissons J, Lercher A, Bhattacharya A, **Khamina K**, Trivett AL, Tessarollo L, Mesteri I, Hladik A, Merkler D, Kubicek S, Knapp S, Epstein MM, Symer DE, Aderem A, Bergthaler A. The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection. *Nat Immunol.* 2015 Jan;16(1):67-74.

5. Huber ML, Sacco R, Parapatics K, Skucha A, **Khamina K**, Müller AC, Rudashevskaya EL, Bennett KL. abFASP-MS: affinity-based filter-aided sample preparation mass spectrometry for quantitative analysis of chemically labeled protein complexes. *J Proteome Res.* 2014 Feb 7; 13(2):1147-55.

6. Kharkov VN, **Khamina KV**, Medvedeva OF, Simonova KV, Khitrinskaya IY, Stepanov VA. Gene-pool structure of Tuvinians inferred from Y-chromosome marker data *Genetika.* 2013 Dec;49(12):1416-25 (in Russian).

7. Khar'kov VN, **Khamina KV**, Medvedeva OF, Shtygasheva OV, Stepanov VA. Genetic diversity of Khakassian gene pool: subethnic differensiation and the structure of Y-chromosome haplogroups. *Mol Biol (Mosk),* 2011 May-Jun; 45(3): 446-58 (in Russian).

Teaching experience:

July 2016
Supervisor of High School Summer Student Malak Khan (Vienna International School)

March 2014 – June 2015
Co-supervisor of the Master thesis “Dissection of Virus-Host Interaction in LCMV Infection by Employing CRISPR/Cas9 Technology” Alexander Lercher (University of Vienna).

2008 - 2012
Personal Tutor on Biology and Chemistry for students and pupils