Master's Thesis

The importance of ubiquitome in enteroviral infections

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Enteroviruses are ubiquitous RNA viruses, capable of causing a wide spectrum of diseases in humans. Despite the efforts to develop effective broad-spectrum antivirals, currently there are no clinically approved therapeutics against enteroviral infections, and thus, novel antiviral strategies are needed. Many viruses can interact with the cellular ubiquitome system, which mediates numerous cellular processes and proteasomal degradation of protein substrates. This system can both hinder and promote viral infections, mainly through the activities of ubiquitin ligases and deubiquitinating enzymes. The aim of this thesis was to examine the importance of ubiquitome in enteroviral infections. Previously, nine ubiquitome-related genes were connected to coxsackievirus B3 (CVB3), a type of enterovirus. To validate the previous findings, in this study, the expression of the genes was knocked down with small interfering RNAs (siRNA) and the progression of CVB3 infection was monitored using immunofluorescence labelling and confocal microscopy, as well as western blotting. In addition to CVB3, infections of coxsackievirus B1 (CVB1) and echovirus 30 (EV30) were examined to assess the broad-spectrum effects of silencing ubiquitome-related factors. Knockdown of three genes, namely UCHL1, CHD4 and FBXL14, reduced infections caused by all enteroviruses tested. In addition, the knockdown of CAND2 inhibited CVB1 and EV30 infections. To further inspect the potential of these ubiquitome-related factors in antiviral development, a cytotoxicity assay was conducted for the hit siRNAs. Although UCHL1, FBXL14 and CHD4 knockdown had a slight effect on cell viability, none of the hit siRNA treatments were radically toxic. The anti-enteroviral effects observed in this study may be related to processes such as immune responses or cell cycle regulation. On the other hand, the viruses may also require ubiquitome factors to complete different stages of their life cycle. The exact antiviral mechanisms, however, remain to be determined. In terms of developing broad-spectrum antivirals, the finding that several enteroviral serotypes can utilize same ubiquitome factors is promising.

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Enterovirukset ovat yleisiä RNA-viruksia, jotka aiheuttavat monenlaisia sairauksia ihmisille. Huolimatta pyrkimyksistä kehittää tehokkaita ja laajakirjoisia antiviraaleja, tällä hetkellä enterovirusinfektioiden hoitamiseksi ei ole kliinisesti hyväksyttyjä lääkkeitä, ja uusia antiviraalistrategioita tarvitaan. Monet virukset vuorovaikuttavat isäntäsolun ubiquitomi-järjestelmän kanssa. Soluissa tämä järjestelmä osallistuu useisiin perustavanlaatuisiin prosesseihin sekä substraattien proteosomaaliseen hajotukseen. Koska ubiquitomi-järjestelmä voi sekä estää, että edistää virusinfektioita erilaisten entsyymien välityksellä, tämän tutkielman tarkoituksena oli selventää sen merkitystä enteroviruksille. Aiemmin yhdeksän ubiquitomigeenin havaittiin vaikuttavan enteroviruksiin kuuluvan coxsackievirus B3:n (CVB3) infektioon. Havaintojen vahvistamiseksi tässä tutkielmassa kyseiset geenit hiljennettiin pienillä häiritsevillä RNA-molekyyleillä (siRNA), ja transfektoiduissa soluissa etenevää CVB3-infektiota seurattiin immunofluoresenssileimauksella ja konfokaalimikroskopialla, sekä western blot -menetelmällä. CVB3-infektion lisäksi myös coxsackievirus B1:n (CVB1) ja echovirus 30:n (EV30) infektioita tarkasteltiin mahdollisten laaja-alaisten vaikutusten selvittämiseksi. Yhdeksästä geenistä kolmen havaittiin vaikuttavan kaikkiin tutkittuihin viruksiin. Näiden geenien, UCHL1, CHD4 ja FBXL14, hiljentämisellä oli infektioita alentava vaikutus. CVB1- ja EV30-infektiot alenivat myös, kun CAND2-geeni hiljennettiin. Tulosten perusteella valituille siRNA-molekyyleille tehtiin sytotoksisuuskoe, jotta saataisiin lisätietoa merkittäväksi havaittujen ubiquitomitekijöiden potentiaalista antiviraalikehityksessä. Vaikka UCHL1-, CHD4- ja FBXL14-geenien hiljentämisellä oli pieni vaikutus solujen elinkykyyn, mikään siRNA-käsittelyistä ei ollut äärimmäisen toksinen. Havaitut ubiquitomitekijöiden vaikutukset voivat liittyä niiden rooliin enterovirusten elinsyklissä, tai prosesseihin kuten immuunipuolustus ja solusyklin säätely. Vaikka tarkkoja antiviraalimekanismeja ei vielä tunneta, löydös useille enteroviruksille yhteisistä ubiquitomitekijöistä on lupaava.

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ABBREVIATIONS

BSA	Bovine serum albumin
CV	Coxsackievirus
DUB	Deubiquitinating enzyme
EBV	Epstein-Barr virus
EV	Enterovirus
EV30	Echovirus 30
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPV	Human papillomavirus
IRES	Internal ribosomal entry site
MOI	Multiplicity of infection
NuRD	Nucleosome remodelling histone deacetylase
PBS	Phosphate buffered saline
PV	Poliovirus
RT	Room temperature
SCF	S phase kinase-associated protein 1– cullin 1 – F-box
siRNA	Small interfering RNA
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
VEMU	Vemurafenib
VP	Viral protein

1 INTRODUCTION

Enteroviruses (EVs) are small, highly prevalent RNA viruses that belong to the *picornaviridae* family. These viruses are capable of infecting humans, primates and other mammals and they are responsible for extensive morbidity every year (Knowles et al. 2010). Currently the *enterovirus* genus contains 15 species of viruses, seven of which have human infecting serotypes. The species found in humans include EVs A, B, C and D as well as rhinoviruses (RVs) A, B and C (reviewed in Chen et al. 2020). To this day over one hundred serotypes of human EVs have been identified, including types of polioviruses (PV), coxsackieviruses (CV) A and B, echoviruses and numbered EVs (reviewed in Brouwer et al. 2021).

1.1Enterovirus structure

As is often the case with viruses, the structure of EVs is relatively simple, consisting of viral genome encapsulated within a protein capsid. Unlike some viruses, EVs do not have a lipid membrane surrounding their capsid. The genome of EVs is constructed of a single positive-sense RNA molecule, which is approximately 7.5 – 8.0 kb in length, and contains a non-coding region (NCR) in both of its ends. The NCR of the 5′ end includes a structure called RNA cloverleaf and an internal ribosomal entry site (IRES). In addition, a small viral protein (VPg) is linked to the 5′ NCR (Palmenberg et al. 2010). This region of the genome is necessary in the viral translation and RNA replication of EVs (Rohll et al. 1994). The 3′ NCR, and a short poly(A) tract following it, are also essential for efficient viral replication (Rohll et al. 1995).

The protein capsid of EVs has an icosahedral structure and a diameter of approximately 30 nm. It is constructed of four structural proteins (VP1-VP4) assembled into 12 pentamers. These proteins, as well as some non-structural proteins, are derived from precursors (P1-P3) that are initially cleaved from a single large polyprotein encoded by the viral RNA (Fry and Stuart 2010). Structural proteins VP1, VP2 and VP3 form the external surface of the viral capsid, whereas VP4 is a component of the internal surface (Figure 1). The capsid surface of many EVs and RVs contains a deep depression, termed canyon, surrounding the five-fold axes of symmetry. Beneath the canyon floor lies a hydrophobic pocket, which often contains a stabilizing small molecule known as the pocket factor. For many EVs, such as PV1 and CVB3, the canyon functions as a site for receptor binding (Belnap et al. 2000, He et al. 2001). On the other hand, some EVs have been found to bind their receptors outside the canyon (He et al. 2002). Due to variable surface-exposed loops of the outer

capsid proteins, EVs have high antigenic diversity (reviewed in Baggen et al. 2018).

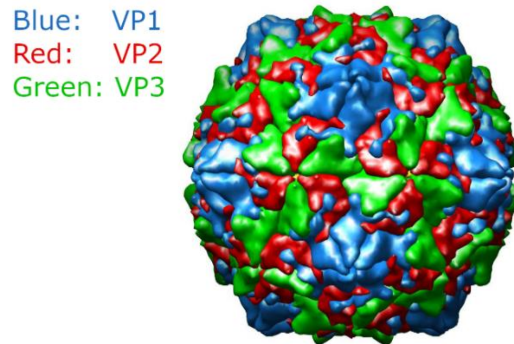

Figure 1. Schematic illustration of coxsackievirus B3 (CVB3) capsid. The icosahedral capsid of enteroviruses is constructed of four structural proteins (VP1-VP4). VP1 (shown in blue), VP2 (shown in red) and VP3 (shown in green) form the outer surface of the virus, while VP4 (not shown) is located in the internal surface. The figure was obtained from VIPERdb (http://viperdb.org) with PDB ID for CVB3 (1COV) (Montiel-Garcia et al. 2020). Structural details of 1COV were originally presented by Muckelbauer et al. (1995).

The non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D and the intermediates 2BC, 3AB and 3CD) have been linked to several processes. Since the genome of EVs is small, its coding capacity is limited. Therefore certain host cell factors are needed to accomplish viral replication. To harness the endogenous mechanisms of their host, EVs modify cellular proteins. This is achieved by using virus-encoded proteases which include the non-structural proteins 2A and 3C (Saeed et al. 2020). These proteases as well as the intermediate 3CD, are also responsible for cleaving the viral polyprotein (reviewed in Baggen et al. 2018). Furthermore, the viral proteases can have a protective function, as they may supress antiviral pathways by altering specific proteins of the host cell (Barral et al. 2009). Most of the non-structural proteins of EVs participate in viral replication either directly by affecting the RNA synthesis, or indirectly by mediating rearrangement of the hosts cytoplasmic membranes (Rozovics and Semler 2010).

1.2 Disease and control

Human infecting EVs spread primarily via fecal-oral route, with the gastrointestinal tract being the primary site of infection. Some members of the enterovirus genus may also use other routes of transmission, or target alternative sites to establish infection. For example, EV-D68 has been primarily isolated from respiratory specimen, indicating that the virus replicates in the respiratory tract (Oberste et al. 2004). Additionally, RVs have commonly been connected to infections of the respiratory tract (El-Sahly et al. 2000).

The human EVs and RVs are common pathogens, capable of causing a wide spectrum of diseases. These diseases include, for example, hand foot and mouth disease (HFMD) caused by certain EVs, and the common cold, which is often caused by RVs (Huang et al. 2018). Enteroviral infections are most often either mild or asymptotic, but these viruses can also cause severe and even fatal diseases if they spread from the primary infection site to other tissues and organs (reviewed in Wells and Coyne 2019). One well-known example of these diseases is poliomyelitis, an infection caused by PV, that may result in acute flaccid paralysis (reviewed in Bitnun and Yeh 2018). Other, non-polio EVs have also been associated with multiple severe conditions, such as encephalitis, aseptic meningitis, acute flaccid myelitis, myocarditis and pericarditis (Gaaloul et al. 2014, Hasbun et al. 2017, Schubert et al. 2019). Furthermore, EVs can also contribute to several chronic disorders, including type I diabetes and asthma (Krogvold et al. 2015, Wang et al. 2018). Especially young children and immunocompromised patients may be at risk of developing severe complications as a result of EV infection (reviewed in Baggen et al. 2018).

Currently PV and EV strain A71 are the only members of the *enterovirus* genus against which vaccines have been produced (reviewed in Laajala et al. 2020). Although vaccination is an effective way to prevent infectious diseases, it is considered unpractical to produce vaccines for every EV, given the high number of serotypes. Since the antigenic variation of EVs constrains vaccine development, additional strategies to control viral infections are needed. One such strategy is the development of broad-spectrum antivirals. Ideally these therapeutics could reduce the pathology and duration of infections caused by several different serotypes. In addition, the antivirals could reduce the excretion and shedding of the viruses (reviewed in Benschop et al. 2015). Although some potential EV inhibitors have been found, at this moment there are no effective and clinically approved antivirals against any of the EVs (reviewed in Laajala et al. 2020).

1.3 Enteroviral life cycle and antivirals

The life cycle of EVs can be divided into several stages, each of which could potentially be targeted with antivirals, either by targeting the virus itself or some host factors the virus depends on (Figure 2). In the beginning of enteroviral life cycle, the virus attaches to specific surface receptors of its host. Attachment to the receptors induces intracellular signals that promote endocytic uptake of the host, facilitating viral entry. EVs can use different types of receptors, many of which are either immunoglobulin-like domains or integrin molecules. For those viruses binding through the canyon, attachment to a receptor releases the pocket factor and causes conformational changes of the virus (Zhang et al. 2008). This can initiate subsequent steps of the life cycle, including uncoating of the virus and genome release (Wang et al. 2012). EVs that bind receptors outside the canyon may require other trigger to initiate these steps. For example, certain viruses in the CVB group need to interact with

two receptors to complete the early steps of the life cycle: decay accelerating factor (DAF) promotes attachment, but the coxsackievirus and adenovirus receptor (CAR) is needed for the conformational changes that initiate uncoating (Milstone et al. 2005).

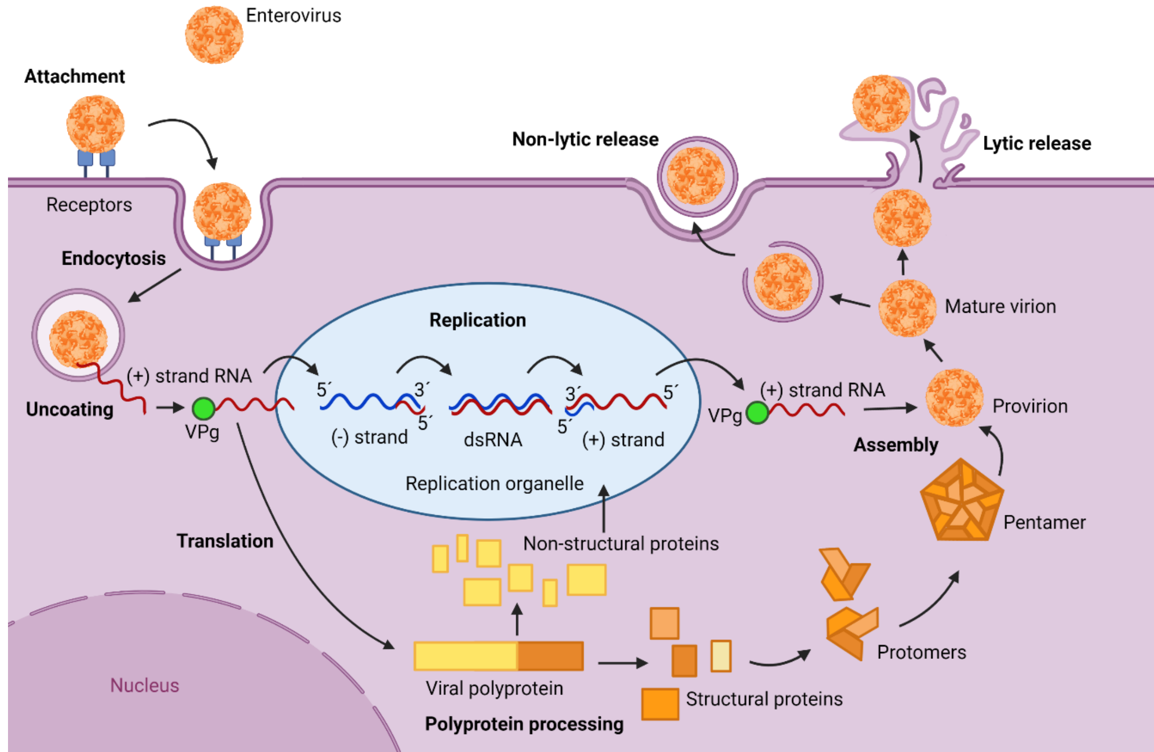

Figure 2. Schematic illustration of enteroviral life cycle. In the beginning, the virus attaches to receptors located on the host cell surface. Following attachment, the virus is transported into host cell cytoplasm through endocytosis. Within the cytoplasm, the virus uncoats and releases its positive-sense (+) RNA genome. The genome is translated to produce viral polyprotein, which is further processed into several structural and non-structural proteins. Genome replication takes place in replication organelles, structures modified from cellular membranes by the virus. During genome replication, a negative-sense (-) RNA strand template is synthesized, to produce new (+) RNA molecules through a double-strand RNA (dsRNA) intermediate. Genome replication requires VPg, a small protein attached to the viral genome. The process is also assisted by the non-structural proteins. The structural proteins, on the other hand, are components of the viral capsid. These proteins self-assemble into multiple protomers and pentamers, and finally, form provirions together with newly synthesized viral RNA. The RNA molecule induces viral maturation, and in the end, mature virions exit the host cell by either lytic, or non-lytic release mechanisms. The image was modified from a review by Baggen et al. (2018) using BioRender.com image creating tool.

Following receptor attachment, EVs are internalized in endocytic vesicles via distinct uptake pathways that are influenced by both virus and host features. Some viruses, such as the EV-A71, require acidic environment to finish

uncoating and use clathrin-mediated acidifying endosomal pathway to enter the host (Hussain et al. 2011). By contrast, several EVB species can be internalized in neutral endosomes in a clathrin-independent manner (Marjomäki et al. 2015).

In the next stage of the viral life cycle, the virus uncoats and releases its genome into the host cell's cytoplasm. Triggers for uncoating are diverse and vary between EV species. These cues may allow the viruses to release their genome in the right cellular compartment at the right time (reviewed in Baggen et al. 2018). The process of enteroviral genome release, however, is not fully elucidated yet. Nevertheless, it is known that the RNA is delivered to the cytoplasm through a pore in the endosomal membrane. This event is likely assisted by cellular adipose-specific phospholipase A2 enzyme (PLA2G16), although the exact mechanism has not been confirmed (Staring et al. 2017). Once in the cytoplasm, viral genome is translated by the hosts cellular machinery to produce viral polyprotein. The mechanisms of this process are conserved among different EVs. The IRES element of the viral genome is important mediator of the translation as it is responsible for ribosome recruitment. The functions of IRES, on the other hand, are dependent on IRES trans-acting factors (ITAFs) of the host (reviewed in Abdullah et al. 2023).

After translation the polyprotein is processed by viral proteases 2A, 3C and the precursor protein 3CD (reviewed in Baggen et al. 2018). A previous study revealed that certain host cell proteases, calpains, may also be involved in the polyprotein processing (Laajala et al. 2019). If the polyprotein is successfully cleaved, the viral life cycle proceeds to genome replication. This process is known to occur on tubulovesicular replicative organelles (ROs) with the aid of the non-structural proteins, such as the 3D polymerase, viral ATPase 2C and membrane-associated 2BC and 3A (reviewed in Baggen et al. 2018). Some cellular factors, such as phosphatidylinositol 4-kinases (PI4Ks), can also be harnessed to support viral replication (Hsu et al. 2010).

Close to the end of the viral life cycle, structural proteins are assembled to form the capsid around replicated RNA. The mechanisms of this process are not fully understood yet, however, the encapsidation specificity has been proposed to be controlled by interactions of the viral 2C and the structural protein VP3 (Liu et al. 2010). A couple of cellular proteins, such as the heat shock protein 90 (HSP90) and glutathione (GSH) have also been linked to the assembly process (Geller et al. 2007, Thibaut et al. 2014). If not disrupted earlier, the life cycle of EVs ends as newly synthesized virions exit the host cell through lytic or non-lytic egress mechanisms. Whether the EVs are released as a result of apoptosis and host lysis, or exit in extracellular vesicles without killing the host, depends on features of both the virus and the infected cell (reviewed in Owusu et al. 2021).

Potential antiviral compounds and their targets in different stages of the enteroviral life cycle have been broadly studied (Table 1). Generally the antiviral treatments can be targeted against any of the viral or host factors the virus requires in its infection. However, both of these approaches have

challenges, as targeting the host may induce cytotoxic responses, whereas targeting viral factors can lead to the emergence of resistant mutants (reviewed in Laajala et al. 2020). Targeting receptor usage, endocytosis or viral egress, is typically not considered as potential antiviral strategy, since the EVs may utilize a broad spectrum of receptors in their attachment, and blocking the intake and release of compounds from the cell would likely disturb cellular homeostasis. Targeting the rest of the stages has yielded some promising results, although, as noted, none of the antiviral compounds studied thus far have passed clinical trials due to, for example, efficacy issues, toxicity and unwanted side effects (reviewed in Laajala et al. 2020).

Table 1. Examples of virus and host-targeted (HT) anti-enteroviral compounds. Abbreviations: IRES, internal ribosome entry site; ITAF, IRES trans-acting factor; pro, protease; pol, polymerase; VPg, viral protein genome-linked; PI4KB, phosphatidylinositol 4-kinase beta; HSP90, heat shock protein 90; GSH, glutathione.

Antiviral group	Affected stage of infection	Example drug	Mechanism of action	References
Capsid binders	Uncoating and other early stages	Pirodavir, Pleconaril	Substitute pocket factor in the hydrophobic pocket to prevent structural changes of the capsid	Andries et al. 1992, Pevear et al. 1999
IRES inhibitors	Translation	Quinacrine	Prevents the activity of IRES by blocking interactions between IRES and cellular ITAFs	Wang et al. 2013
$3C^{pro}/2A^{pro}$ inhibitors	Polyprotein processing and other	Rupintrivir/ Telaprevir	Attach to the active sites of $3C^{pro}/2A^{pro}$ and prevent proteolytic processing of substrates	Binford et al. 2005, Musharrafieh et al. 2019
$3D^{pol}$ inhibitors	Replication	Amiloride	Binds to $3D^{pol}$ and inhibits 3D-dependent VPg uridy-lation and RNA elongation	Gazina et al. 2011
2C inhibitors	Replication	Fluoxetine	Inhibits ATPase activity of viral 2C by binding to the protein	Bauer et al. 2019
PI4KB inhibitors (HT)	Replication	Enviroxime	Inhibits the enzymatic activity of PI4KB and disrupts required lipid micro-environment	van der Schaar et al. 2012
HSP90 inhibitors (HT)	Assembly	Geldanamycin	Inhibits ATPase activity of HSP90, accelerating the degradation of viral capsid proteins	Tsou et al. 2013
GSH inhibitors (HT)	Assembly	TP219	Causes GSH depletion, which disturbs viral morphogenesis	Thibaut et al. 2014

1.4 Ubiquitination and the human ubiquitome

Ubiquitination is an intracellular protein modification process, that can target substrates for proteasomal degradation, or affect substrate activity and localization in the cell (reviewed in Akutsu et al. 2016). This post-translational process is involved in the regulation of almost all fundamental cellular operations, including cell cycle, signalling, endocytosis, apoptosis as well as stress- and immune responses (reviewed in Ciechanover and Schwartz 1998). During ubiquitination, a small, 76 amino acids long protein called ubiquitin (Ub) is linked to a lysine residue of the targeted protein. The substrate proteins can be ubiquitinated at one or many lysine residues at the same time. Additionally, the Ub molecule can be attached to another Ub to form polyubiquitin chains. The Ubs themselves have several lysine residues (K6, K11, K27, K29, K33, K48 and K63) and one methione residue (M1) through which they can be attached to other molecules. This offers countless ways to construct a signal mediating polymer (reviewed in Akutsu et al. 2016).

Substrate ubiquitination is catalysed by enzymes of three distinct classes (Figure 3). In the beginning of the process, ubiquitin-activating enzyme (E1) binds to the Ub and activates the C-terminus of the protein in a reaction that requires ATP. After the Ub has been activated, it is transferred to ubiquitin-conjugating enzyme (E2), which together with ubiquitin ligase (E3) passes the Ub to the substrate. A covalent bond is catalysed between Ub and the substrate protein by E3 (reviewed in Ciechanover and Iwai 2004). When the substrates have been tagged with one or multiple Ubs, proteins with specialized ubiquitin-binding domains (UBD) can recognize them and mediate varying substrate-specific responses (reviewed in Dikic et al. 2009). Whether the substrate is linked to a polyubiquitin chain, a single Ub molecule at one site (monoubiquitination), or several Ubs at distinct sites (multi-monoubiquitination) determines its fate in the cell (reviewed in Woelk et al. 2007)

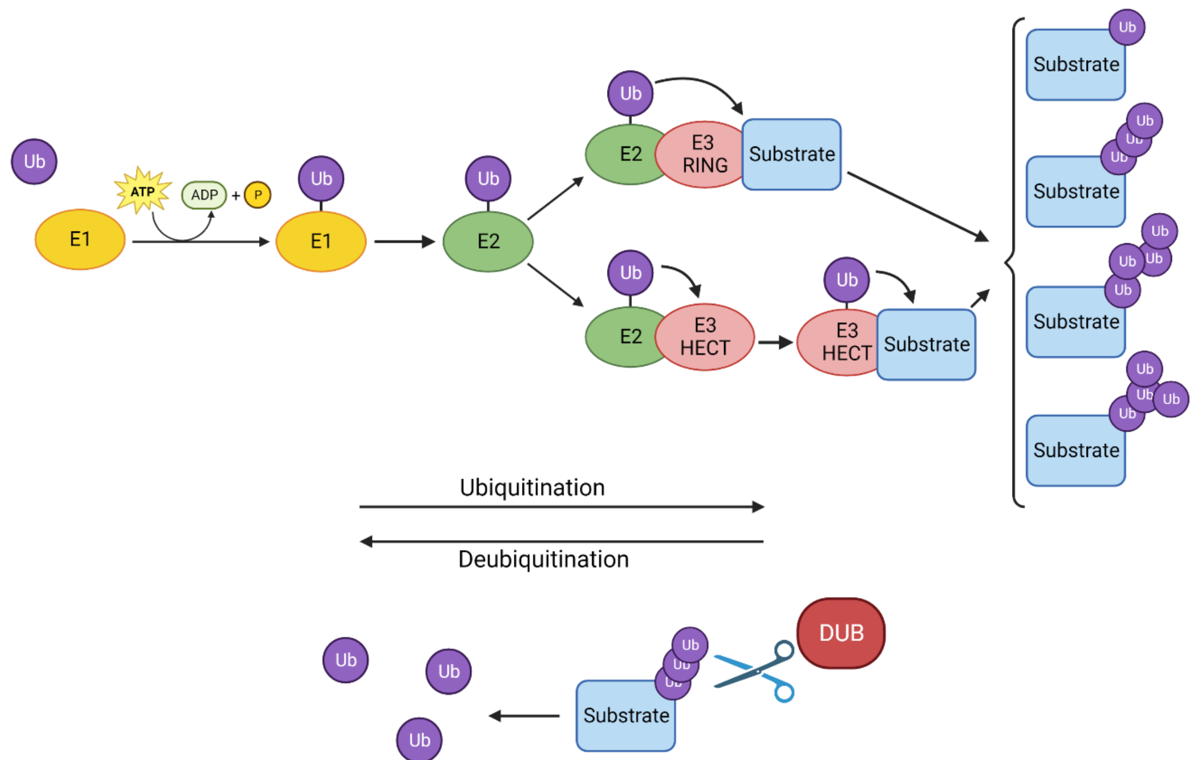

Figure 3. The processes of ubiquitination and deubiquitination. In the beginning of the cycle, ubiquitin activating enzyme (E1) binds to ubiquitin (Ub) and activates the protein in an ATP-dependent manner. From E1, Ub is transferred to ubiquitin conjugating enzyme (E2), which passes the protein to ubiquitin ligating enzyme (E3). Depending on the type of E3, Ub is transferred to the substrate either directly from E2, or by E3-Ub intermediate. From the major types of E3s, those with really interesting new gene (RING) finger domain have the former, while homologous to the E6AP carboxyl terminus (HECT) domain containing E3s have the latter mode of action. During ubiquitination, substrate proteins may be tagged with one or multiple Ubs. The type of polymer constructed determines the fate of the substrate within the cell. In addition to the three enzymes catalysing the process of ubiquitination, the cellular ubiquitome contains deubiquitinating enzymes (DUB), that can remove ubiquitin from the substrates. This releases free ubiquitin for new ubiquitination cycles. The image was modified from a review by Woelk et al. (2007) using BioRender.com image creating tool.

Proteins tagged with compact polyubiquitin chains are often targeted for degradation (reviewed in Ciechanover and Iwai 2004). In eukaryotic cells the degradation is carried out by 26S proteasome, that has a number of active sites with the ability to bind various ubiquitinated proteins. As the substrate is degraded, deubiquitinating enzymes (DUBs) release Ubs for new conjugation cycles. The DUBs can also rescue ubiquitinated proteins targeted for degradation, or edit polyubiquitin chains related to other, non-degradative responses (reviewed in Komander et al. 2009). As whole, the ubiquitin-proteasome system (UPS) has regulative role in a number of cellular processes,

since it can selectively modulate the expression of many regulatory proteins (reviewed in Ciechanover and Schwartz 1998).

The human ubiquitome contains two E1s and approximately 40 E2s. E3s on the other hand, are a diverse group of over 600 enzymes (reviewed in Gu and Fada 2020). E3s can be further divided into several classes, based on their structural features and activities. The most common E3 ligases contain a really interesting new gene (RING) finger domain, and they can transfer the Ub molecule directly from the E2 to the substrate they have bound (reviewed in Ciechanover and Iwai 2004). Other types of E3s include ligases containing homologous to the E6AP carboxyl terminus (HECT) domain, or RING-between-RING (RBR) domain. For both of these, the Ub transfer from the E2 to the substrate is a two-step process that involves the formation of E3-Ub intermediate (reviewed in Gu and Fada 2020). In addition to the E1s, E2s and E3s, approximately 100 DUBs are found in humans. Most of these enzymes are further classified as cysteine proteases, while a small group of metalloprotease DUBs have also been recognized (reviewed in Clague et al. 2019).

1.5 Ubiquitination and viruses

Upon viral infections, various cellular defence mechanisms are activated through complex signalling pathways. Ubiquitination and deubiquitination are both essential regulators of the extent of immune responses (reviewed in Zinngrebe et al. 2014). As noted, linkage type of the Ub chain affects the signalling outcome. K63-linked polyubiquitination, for example, has been connected to the activation of nuclear factor kappa B (NF-κB) and interferon regulatory factors (IRF), two essential effectors of the innate immunity (Zeng et al. 2010). Together with other transcription factors the NF-κB and IRF can induce the production of antiviral compounds, such as type I interferons (reviewed in Budroni and Versteeg 2021). In addition to its immune response enhancing role, ubiquitination may also target viral proteins for degradation (Barajas et al. 2009, Liu et al. 2021). Deubiquitination, on the other hand, is often involved in the negative regulation of immune responses to prevent their harmful overactivation, although, several DUBs may also promote the activities of the immune system (Liu et al. 2018).

Despite its role in the antiviral pathways, the UPS can also promote viral infections, as viruses have adapted to utilize the system for their own purposes. Among other things, viruses can manipulate the UPS to degrade cellular components that are essential for the host's antiviral defences (Querido et al. 2001, Huh et al. 2007). On the other hand, they can also supress immune responses by preventing the degradation of transcription factor inhibitors, such as IκBα that usually inhibits the unnecessary activation NF-κB (Whitmer et al. 2015). In addition to immune evasion, viruses can harness the UPS to support different stages of their life cycle (reviewed in Gu and Fada 2020). As an example, CVB3 has been found to utilize the UPS to process polyprotein fragments in order to complete its replication (Si et al. 2008, Voss et al. 2021).

Along with manipulating the pre-existing cellular ubiquitome factors, viruses may exploit the UPS through E3 ligases and DUBs they have encoded themselves (Coscoy et al. 2001). In conclusion, since ubiquitination and deubiquitination are both important for viral pathogenesis, some of the virus associated E3s, DUBs and their regulatory factors could be potential targets for antiviral treatments.

1.6. Purpose of the study

Given the apparent clinical significance of EVs and the fact that no clinically approved antivirals exist on the market, new antiviral strategies are needed. The purpose of this study was to examine the importance of ubiquitome in enteroviral infections. Previously nine ubiquitome-related genes coding E3s, DUBs, or regulatory factors of these, were found to affect CVB3 infection by the Marjomäki group. When these genes were downregulated by small interfering RNAs (siRNAs), CVB3 infection was either reduced or increased. In this study, the aim was to confirm the preliminary results using new siRNAs from another manufacturer. In addition to CVB3, two other enterovirus strains, namely CVB1 and EV30, were included to the experiments to inspect whether silencing of the ubiquitome-related genes has broad-spectrum effects. It was thought that enzymes coded by ubiquitome genes relevant to multiple strains could be potential targets for antiviral development. The main hypothesis in this study was that the new siRNAs, which were used to downregulate the genes, would validate most of the previously found hits. In addition, common ubiquitome factors were expected to be found between different EV serotypes.

2 MATERIALS AND METHODS

2.1 Cells and viruses

The experiments were carried out using adenocarcinomic human lung (A549) cells (ATCC), which were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 1% glutamax (Gibco) and 1% penicillin and streptomycin (pen/strep, Gibco) in an incubator at +37 °C and 5% CO_2. In the experiments, the cells were grown either on 96-well imaging plates (Greiner Bio-One) for confocal microscopy or on regular 96-well plates (Sarstedt) for western blotting. Three enterovirus strains were used in the screens: CVB3 (Nancy-strain, ATCC), CVB1 (ATCC-strain) and EV30 (Bastianni-strain, kind gift from Michael Lindberg lab, Linnaeus University, Kalmar Sweden).

2.2 siRNA transfection

The cells were reverse transfected with ubiquitome targeting siRNAs (TriFECTa Kit DsiRNA Duplex, Integrated DNA Technologies [IDT]) following instructions of the manufacturer. Nine different genes (CAND2, CHD4, FBXL14, OTUD4, PHF11, RNF216, TRAF3, UCHL1, USP7) were targeted with three different oligos against the same gene (see Appendix 1). Additionally, cells were transfected with non-targeting siRNA (negative control, IDT), Hypoxanthine-guanine phosphoribosyltransferase (HPRT) targeting siRNA (positive control, IDT), or fluorescently labelled siRNA (transfection efficiency control, IDT).

First, Dharmafect transfection reagent (Horizon discovery) was mixed with serum and antibiotic-free medium (-DMEM) after which the mixture was added on top of the siRNAs in separate tubes. The transfection reagent/ siRNA -mixtures were then added on marked wells of a 96-well plate. The final volume of transfection reagent in each well was 0.12 µl in total volume of 125 microliters, and the final amount of siRNA in each well was 50 nM. Plain -DMEM was added to empty wells to prepare virus and Vemurafenib (VEMU) controls with no transfection, and cell control with no transfection or infection. The transfection reagent/siRNA -mixtures were incubated on the plate at RT for 30 min, or at +4 ºC while cells were sub-cultured and counted. Then, 8000 cells were added into each well of the 96-well plate in antibiotic free DMEM. The cells were reverse transfected for 48 h in total at +37 ºC and 5% CO_2. Halfway through the incubation the medium was changed to DMEM with 10% FBS, 1% glutamax and 1% pen/strep.

2.3 Virus infection

After 48 h of transfection, the cells were infected with MOI 25 or 50 of CVB3, CVB1 or EV30. Each virus stock was diluted in DMEM with 1% FBS and 1% glutamax. Virus was added to each sample and control well with the exception of cell control wells. Antiviral drug VEMU (Selleckchem), which was used as a control for infection inhibition, was diluted (final concentration 5 µM) and added into the VEMU control wells. The infection was allowed to proceed for 5.5 h at + 37 ºC and 5% CO_2 after which it was terminated.

2.4 Immunofluorescence labelling and confocal microscopy

For immunofluorescence labelling and confocal microscopy, the infection was terminated by fixing the cells with 4% paraformaldehyde (PFA). The cells were incubated with PFA for 30 min at RT after which PFA was removed, and phosphate buffered saline (PBS) was added to the wells. The cells were permeabilized by incubating them with 0.2% Triton X-100 for 5 min at RT.

Primary antibodies were diluted in 3% bovine serum albumin (BSA) in PBS. Anti-beta-tubulin IgG1 (Santa Cruz, final concentration 0.4 µg/ml) and

Anti-enterovirus clone 5-D8/1 IgG2a (DAKO, 1:1000 dilution) were used to label each siRNA sample, negative control and VEMU control. Positive control and cell control were labelled with HPRT1 rabbit polyclonal antibody (Invitrogen, final concentration 3.25 µg/ml). Primary antibodies were incubated on the cells for 1 h at RT. After incubation, the samples were washed three times for 5 min with PBS. Next, secondary antibodies were prepared. Alexa Fluor 488 conjugated goat anti-mouse IgG1 antibody (Thermo Scientific, final concentration 5 µg/ml), Alexa Fluor 555 conjugated goat anti-mouse IgG2a antibody (Thermo Scientific, final concentration 5 µg/ml) and Alexa Fluor 488 conjugated goat anti-rabbit antibody (life technologies, final concentration 5 µg/ml) were diluted in 3% BSA in PBS and added on samples labelled with corresponding primary antibodies. Secondary antibodies were incubated on the cells for 30 min at RT in the dark, after which the samples were washed three times for 5 min with PBS. The second wash also contained 4′,6-diamidino-2-phenylindole (DAPI, life technologies, dilution 1:40 000) to stain the nuclei.

The cells were imaged with Nikon A1R laser scanning confocal microscope using CFI Plan Apochromat Lambda 40X air objective (Numerical aperture [NA] = 0.95), 405 nm diode laser, 488 nm multiline Argon-laser and 561 nm Sapphire laser. NIS Elements confocal imaging software and JOBS module were used to create automated imaging program. Cells were recognized by the microscope according to DAPI signal from the nuclei and the optimal focal plane was adjusted automatically. The microscope was set to image 500 to 700 cells from randomly selected areas in each well. Resolution of all images acquired was 512 x 512 pixels.

2.5 Western blotting

For western blotting the infection was terminated by collecting the cells into 2X Laemmli buffer. The samples were boiled for 9 min and loaded into 4 – 20% Miniprotean TGX premade gels (BioRad). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was ran through with 160 V. Separated proteins were then electrophoretically blotted with 100 V for 1 h to transfer them from the gels to polyvinylidene difluoride (PVDF) membranes (Merck). Non-specific binding sites were blocked by incubating the blots overnight at +4 ºC in 5% BSA/Tris buffered saline (TBS) with 0.05% tween 20 (Calbiochem).

For immunostaining, antibodies were diluted in 5% BSA/TBS-tween. Blots with the siRNA samples, negative control, VEMU control and virus control were stained with Anti-enterovirus clone 5-D8/1 (1:4000 dilution) and ab6160 rat gamma-tubulin (loading control, Abcam, final concentration 0.1 µg/ml). HPRT1 polyclonal antibody (final concentration 0.65 µg/ml) and GAPDH antibody (loading control, Santa Cruz, final concentration 0.01 µg/ml) were used to stain the blot with positive control and cell control. Primary antibodies were incubated on the blots for 1 h at RT in a rocker, after which three 5 min washes with 0.05% tween in TBS were performed. Secondary antibodies, Anti-

mouse IgG horseradish peroxidase (HRP) -linked antibody (Cell Signaling Technology, dilution 1:3000), Anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, dilution 1:3000) and Anti-rat IgG HRP-linked antibody (Cell Signaling Technology, dilution 1:20 000) were diluted and incubated on the blots stained with corresponding primary antibodies. After incubation, the blots were washed three times with 0.05% tween in TBS and once with 1X TBS. To detect stained proteins, the blots were treated with Supersignal West Pico PLUS Chemiluminescent Substrate (Thermo scientific) for 5 min in the dark, and imaged with ChemiDoc MP imaging system (BioRad).

2.6 Cytotoxicity assay

To assess the cytotoxicity of the hit siRNAs, cells were transfected and grown on a 96-well plate as before. As a positive control for cytotoxicity, three wells with just the cells were treated with 1 µM staurosporine (Sigma) for 24 h. After two days, 100 µl of Cell Titer Glo (Promega) was added to the siRNA and control wells. The samples were incubated for 10 min at RT, after which cell viability was assessed by measuring the cellular luminescence using VICTOR™ X4 multilabel reader (PerkinElmer).

2.7 Data analysis

Imaging data was analysed using CellProfiler version 4.2.1 (Stirling et al. 2021). First, the software was set to identify nuclei as primary objects using global threshold strategy with otsu as thresholding method. Then, using the previously-identified primary objects as a reference, the cytoplasms of infected cells were identified with propagation method and global threshold strategy. Manual thresholding method was chosen for the identification of infected cells. Individual settings were set according to visually detected cell and background intensities. After object identification, the sizes and exact intensities of infected cells were measured, and the data was extracted into excel. Based on visual analysis of the imaging data, a threshold value was determined to distinguish infected cells from uninfected. Infection per cents of different samples and controls were calculated.

Western blot data was analysed using FIJI 2.1.0 image processing software version 1.53o (Schindelin et al. 2012) and excel. To quantify the expression of VP1 and HPRT in the samples, protein bands of the blots were first plotted into histograms using a gel analyser tool in FIJI. Intensities of the bands were then measured with FIJI's tracing tool. Finally, the intensities of sample bands were normalized against corresponding loading bands using excel.

2.8 Statistical testing

GraphPAD PRISM version 6.07 (Dotmatics, San Diego, USA) was used for the statistical analysis of replicated imaging experiments and the cytotoxicity assay. Infection per cent data was first converted with arcsine function to transform binomial distribution data to normal distribution data. Statistical significance of the results was then measured using one-way analysis of variance (ANOVA) with Bonferroni test. A p-value of 0.05 or less was considered to be statistically significant.

3 RESULTS

3.1 Controls and the optimization of experimental conditions

The purpose of this study was to assess the importance of nine cellular, ubiquitome-related genes on different enteroviruses by knocking these genes down with various siRNA molecules. Before the experiments, the infection level and transfection conditions were optimized. Using CVB3, desired level of infection was achieved with MOI 25 (Figure 4A). This multiplicity of infection led to intermediate infection level, which enabled the assessment of both inhibiting and promoting effects of the siRNAs. VEMU, which was used as infection inhibition control, knocked CVB3 infection down fully with 5 µM concentration (Figure 4B).

Transfection efficiency was investigated with a control containing fluorescently tagged siRNAs. In the 96-well plate format, using 0.12 µl of transfection reagent in total volume of 125 µl, and 50 nM siRNA per well, approximately 50 per cent of the cells were transfected (Figure 4C). In addition to the transfection efficiency, RNA interference was studied in more detail using a positive control for functional knockdown. The ability of the siRNAs to knock down targeted structures in the cells was investigated with HPRT targeting siRNA. Targeting HPRT with the positive control siRNA resulted in similar expression of HPRT in comparison to the cell control when studied visually under the microscope (Figure 4D). In order to confirm the result, HPRT level was also studied in whole cell populations using western blotting. The assay revealed approximately 25 per cent knockdown of HPRT in the positive control compared to the non-transfected cells (Figure 4E). Although the knockdown of HPRT in the positive control was not as high as expected, the experiments were carried on, as the transfection efficiency and other experimental conditions were good enough to detect some effects of the siRNAs.

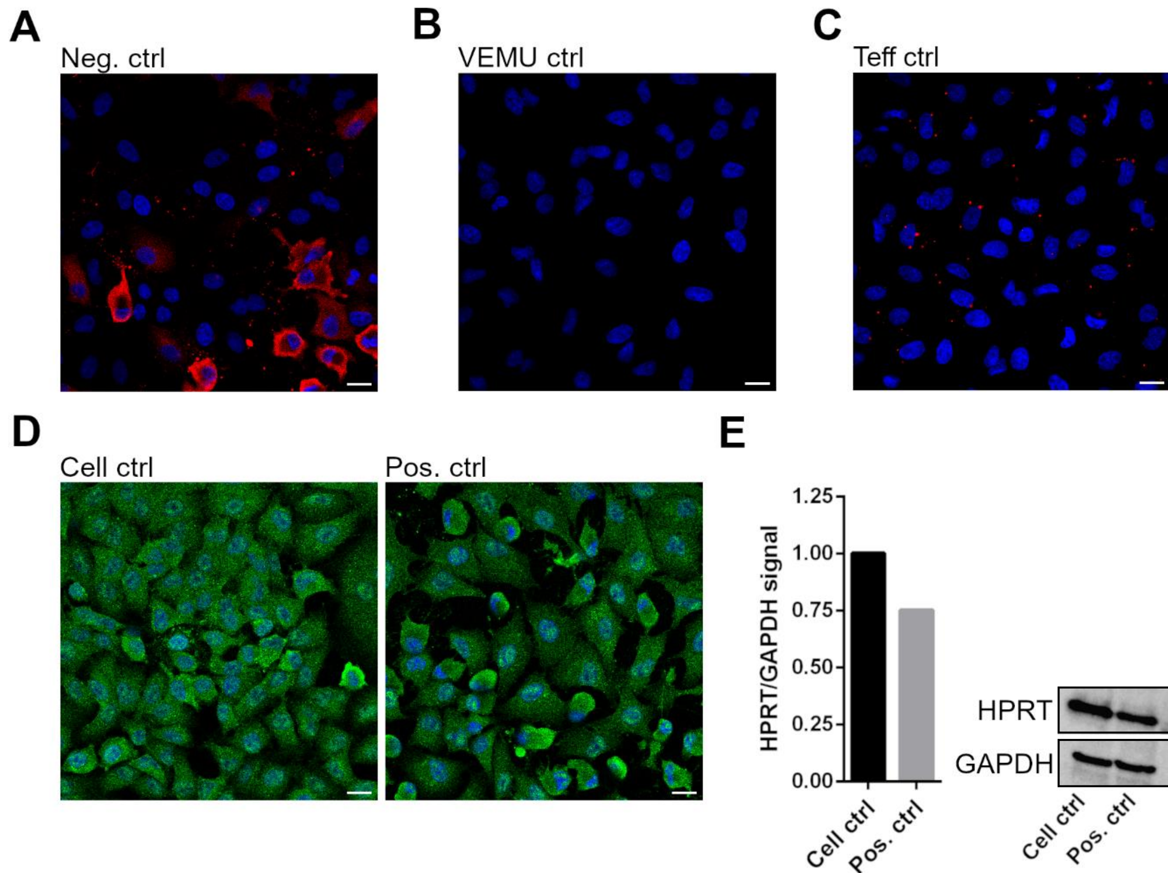

Figure 4. Confocal microscope images and western blot data of screening controls. All experiments were conducted using A549 cells. Blue represents nuclei in each of the microscope images. The scale bars correspond to 20 µm. (A) Negative control showing the natural state of coxsackievirus B3 (CVB3) infection (MOI 25) in cells transfected with a non-targeting siRNA. Red represents viral capsid protein VP1 after 5.5 hours of infection. (B) Infection inhibition control showing complete knockdown of infection. Cells were infected with CVB3 (MOI 25) and treated with 5 µM Vemurafenib (VEMU). Red represents VP1 after 5.5 hours of infection. (C) Transfection efficiency (Teff) control showing fluorescently labelled siRNAs (shown in red) in cells after 48 h of transfection with 50 nM siRNA. (D) Hypoxanthine-guanine phosphoribosyltransferase (HPRT) signal (shown in green) in untreated cells (cell control) and in cells transfected with 50 nM HPRT targeting siRNA (positive control) for 48 h. (E) The expression of HPRT in whole cell populations. Untreated cells and cells transfected with 50 nM HPRT targeting siRNA were collected after 48 h of transfection and analysed with western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The signal of HPRT was normalized against that of GAPDH and finally the positive control was normalized against the cell control.

3.2 Several ubiquitome targeting siRNAs had effects on CVB3

To validate the importance of ubiquitome factors in CVB3 infection, nine ubiquitome-related genes were knocked down by transfecting cells with ubiquitome targeting siRNAs. Each gene was targeted with three different oligos. The cells were then infected and examined by immunofluorescence labelling and confocal microscopy (Figure 5). In untreated control infection, CVB3 was able to infect approximately 50 per cent of the cells transfected with a non-targeting siRNA, whereas treatment with an antiviral (VEMU) blocked the infection fully. Compared to the controls, three siRNA oligos – UCHL1.1, CHD4.1 and FBXL14.2 – inhibited the infection, while two – RNF216.3 and USP7.3 – enhanced it. Other tested siRNAs did not have significant effects on the infection.

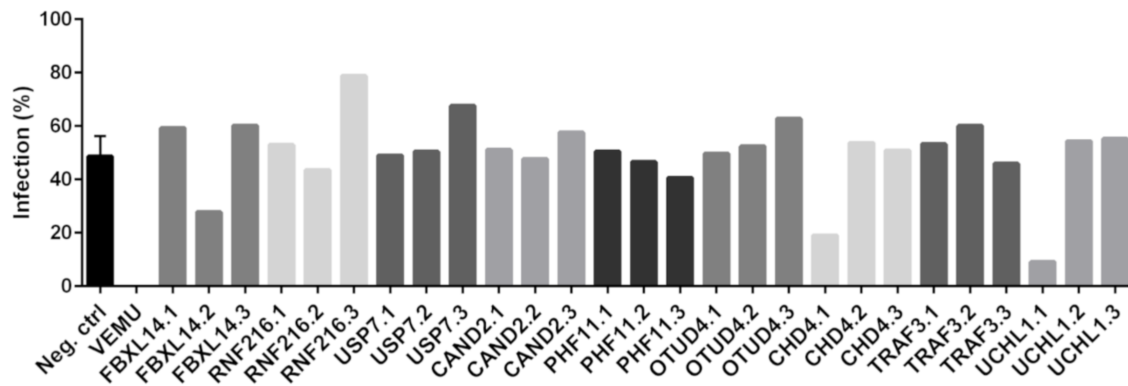

Figure 5. The effects of ubiquitome targeting siRNAs on Coxsackievirus B3 (CVB3) infection. Infectivity of CVB3 was examined in A549 cells treated with various siRNAs. Nine ubiquitome-related genes were targeted with three different oligos (marked with running numbers 1–3). Non-targeting siRNA was used as a control (Neg. ctrl) for normal infection. Antiviral drug Vemurafenib (VEMU) was used as a control for infection inhibition. Cells were infected with MOI 25, and the number of infected cells was determined 5.5 hours post-infection by immunofluorescent labelling of viral capsid protein 1 (VP1) and confocal microscopy. The data is presented from one replicate with the exception of Neg. ctrl which is presented as mean ± standard error of mean (SEM) from three replicates.

3.3 Targeting ubiquitome factors affects CVB1 infection

In order to find cellular factors showing broad-spectrum effects on different enteroviruses, the effects of ubiquitome targeting siRNAs were also studied in CVB1 infection using western blotting (Figure 6). The expression of viral proteins in the samples was compared to the negative control, but not the virus control, as the latter proved to be inconsistent. VEMU treatment was used as a control for infection inhibition, and the drug blocked the infection fully with 5 µM concentration. In comparison to the controls, many of the siRNAs affected

VP1 expression. The siRNAs that most clearly inhibited CVB1 infection included UCHL1.1, CHD4.1 and FBXL14.2. Additionally, several siRNAs – RNF216.1, RNF216.2, USP7.1, CAND2.2 and OTUD4.2 – were also potential inhibitors as they decreased VP1 expression notably. The infection was enhanced in cells transfected with FBXL14.3, OTUD4.3, CHD4.2, TRAF3.1, TRAF3.3, UCHL1.2 and UCHL1.3. However, the loading control indicated slightly uneven loading of the samples, which made the analysis complicated.

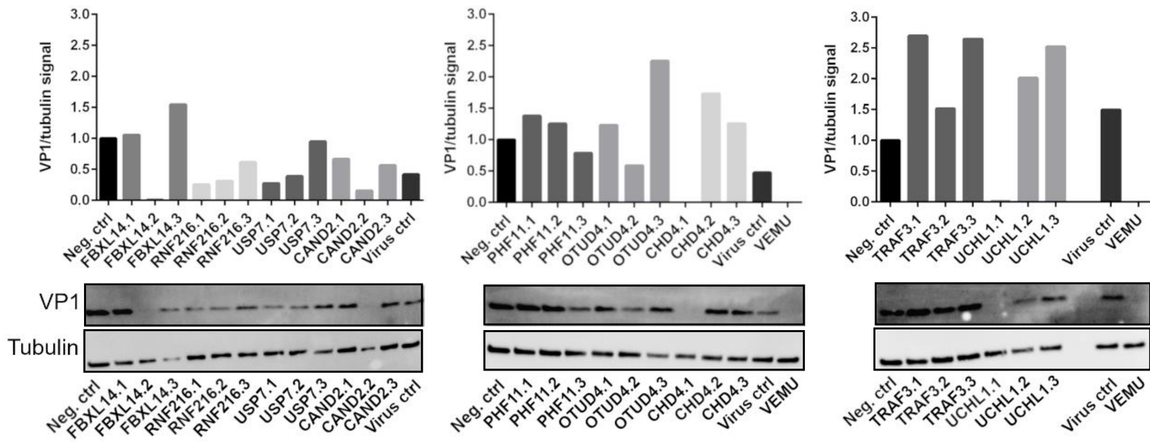

Figure 6. The effects of ubiquitome targeting siRNAs on coxsackievirus B1 infection. A549 cells were transfected with siRNAs targeting nine genes in total. Different oligos targeting the same gene are marked with running numbers 1–3. Non-targeting siRNA was used as a control (Neg. ctrl) for normal infection and antiviral drug Vemurafenib (VEMU) served as a control for infection inhibition. Infection was also tracked in non-transfected cells (Virus ctrl). The cells were infected with MOI 25 and collected 5.5 hours post-infection. The cell extracts were analysed by western blotting using antibodies against viral protein 1 (VP1) and tubulin (loading control). The intensity of VP1 signal was normalized against the intensity of tubulin signal, and finally, all data was normalized against the Neg. ctrl. The data is presented from one replicate.

3.4 Targeting ubiquitome factors affects EV30 infection

In addition to the two coxsackieviruses, the role of different ubiquitome factors in EV30 infection was also studied, given the clinical significance of this enterovirus. The effects of ubiquitome targeting siRNAs on EV30 infection were assessed with immunofluorescence labelling and confocal microscopy. The level of infection was approximately 10 per cent in normal, unaffected infection, where the cells had been transfected with a non-targeting siRNA (Figure 7). Treatment with VEMU reduced the infection to uninfected level. Out of all siRNAs tested, UCHL1.1, CHD4.1, CAND2.2, FBXL14.2 and OTUD4.2 were found to inhibit the infection. By contrast, USP7.3 and OTUD4.3 enhanced the infection, but only by few per cent.

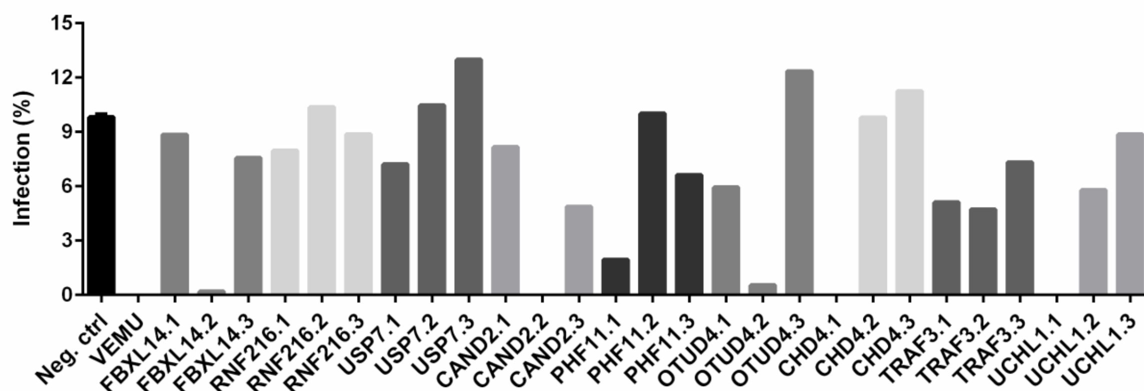

Figure 7. The effects of ubiquitome targeting siRNAs on echovirus 30 infection in A549 cells. Nine ubiquitome-related genes were targeted with three different oligos (marked with running numbers 1–3). Non-targeting siRNA was used as a control (Neg. ctrl) for normal infection, and antiviral drug Vemurafenib (VEMU) was used as a control for inhibited infection. Cells were infected with MOI 25 and the number of infected cells was determined at 5.5 hours post-infection by immunofluorescent labelling of viral capsid protein 1 (VP1) and confocal microscopy. The data is presented from one replicate, with the exception of Neg. ctrl which is presented as mean ± standard error of mean (SEM) from three replicates.

3.5 Each infection was inhibited by at least three siRNAs

Based on the screenings of nine ubiquitome factors with different enteroviruses, the most potential infection altering siRNAs – UCHL1.1, CHD4.1, FBXL14.2, CAND2.2 and OTUD4.2 – were chosen for further studies. All siRNAs chosen were infection inhibitors, as their effects were more consistent throughout the screens, and easier to detect reliably. To confirm the effects observed in the initial screens, three sample replicates were prepared for each chosen siRNA with each virus. All triplicates were studied by immunofluorescence labelling and confocal microscopy.

UCHL1.1, CHD4.1 and FBXL14. were found to have statistically significant effect on CVB3 infection (Figure 8A). Although some cells still got infected, each of the hit siRNAs reduced the number of infected cells at least by half compared to normal infection in negative control. In contrast, CAND2.2 and OTUD4.2 did not have significant effect on CVB3 infection. On a single cell level, the infection had similar appearance in FBXL14.2 treated samples as in the negative control (Figure 8B, blow-ups), but the number of infected cells was clearly reduced. This was also the case for UCHL1.1 and CHD4.1 treated samples (data not shown).

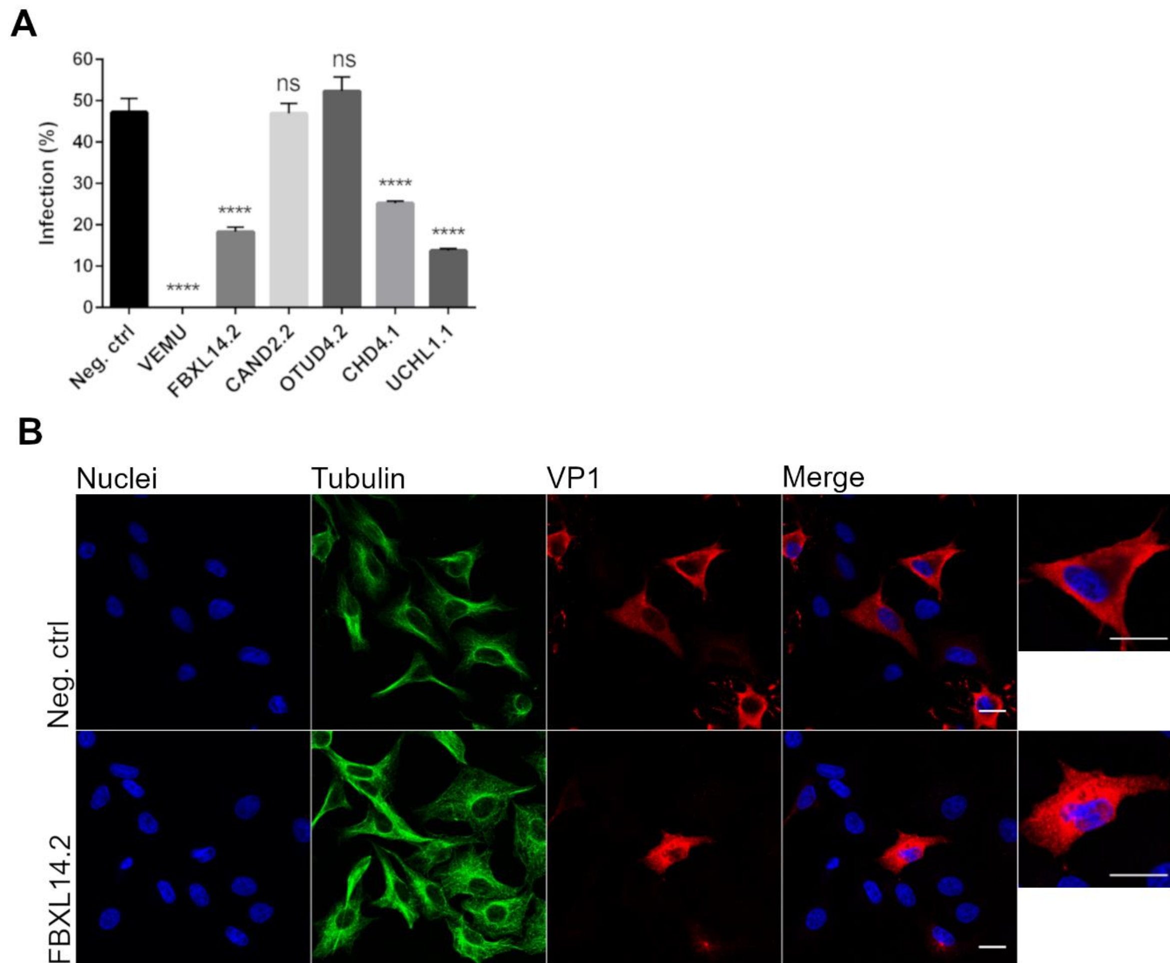

Figure 8. Inhibition of coxsackievirus B3 infection in A549 cells transfected with five ubiquitome targeting siRNAs. Non-targeting siRNA was used as a control for normal infection (Neg.ctrl), while antiviral drug Vemurafenib (VEMU) was used as a control for inhibited infection. The cells were infected with MOI 25, and the infection was detected 5.5 hours post-infection by immunofluorescent labelling of viral capsid protein VP1 and confocal microscopy. (A) A bar chart representing infection per cent data of three replicates. The results are shown as mean of the replicates ± standard error of mean (SEM). Statistical significance of the results compared to the negative control was resolved using one-way ANOVA with Bonferroni test; ns= not significant, ****p-value<0.0001. (B) An image panel representing infection in FBXL14.2 transfected cells in contrast to negative control. Nuclei are shown in blue, tubulin in green and VP1 in red. The scale bars correspond to 20 µm.

The same siRNAs that inhibited CVB3 infection reduced also CVB1 infection (Figure 9A). UCHL1.1 had the most significant effect on the infection, followed by CHD4.1 and FBXL14.2. In addition, CAND2.2 reduced CVB1 infection, although not as significantly, while OTUD4.2 did not affect the infection notably. On a single cell level, the infection had similar appearance in cells transfected with FBXL14.2 as in the negative control (Figure 9B, blow-ups). The number of infected cells, however, was clearly reduced by the siRNA. UCHL1.1

and CHD4.1 had also similar effect on the infection, as well as CAND2.2 on a lower rate (data not shown).

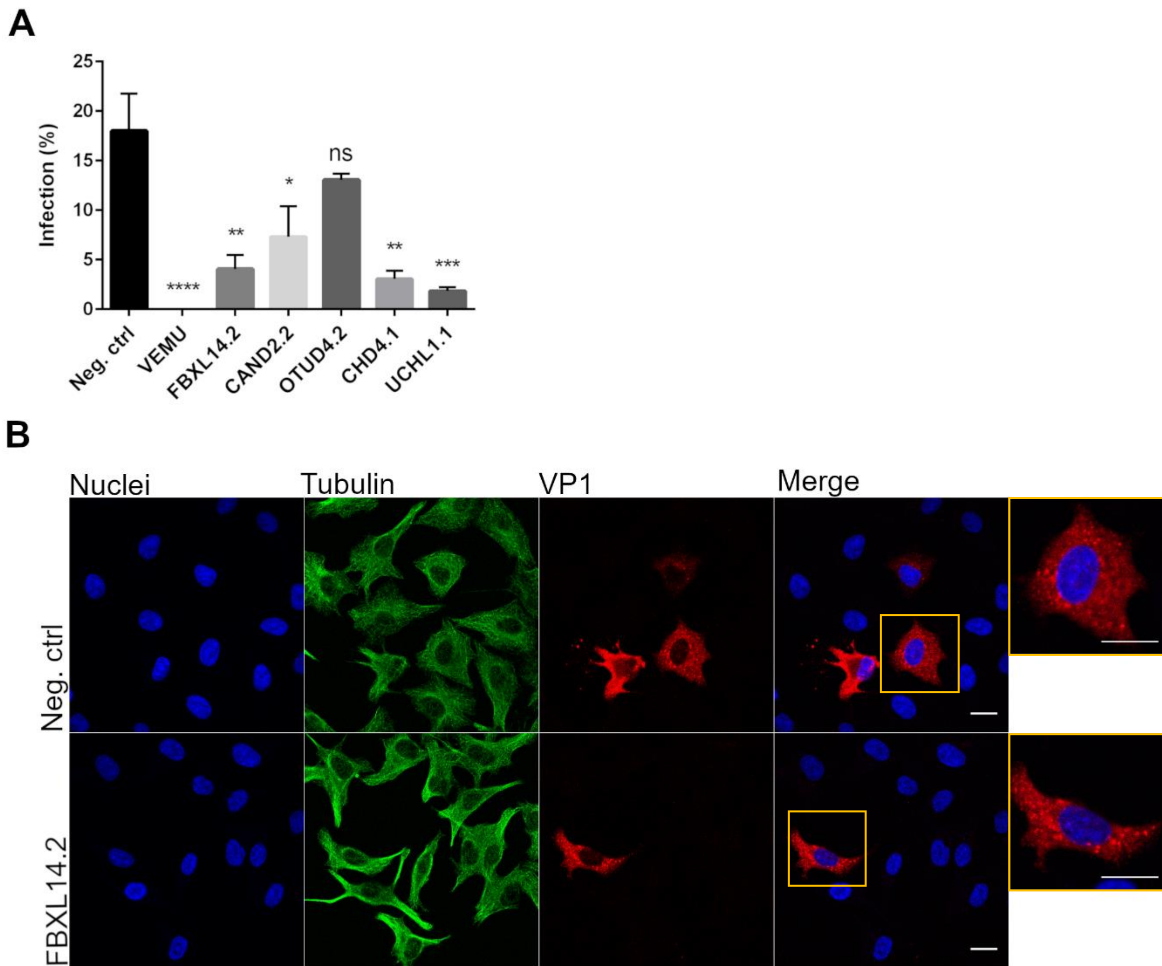

Figure 9. Inhibition of coxsackievirus B1 infection in A549 cells transfected with five ubiquitome targeting siRNAs. Non-targeting siRNA was used as a control for normal infection (Neg. ctrl), while antiviral drug Vemurafenib (VEMU) was used as a control for inhibited infection. The cells were infected with MOI 25, and the infection was detected 5.5 hours post-infection by immunofluorescent labelling of viral capsid protein VP1 and confocal microscopy. (A) A bar chart representing infection per cent data of three replicates. The results are shown as mean of the replicates ± standard error of mean (SEM). Statistical significance of the results in comparison to the Neg. ctrl was resolved using one-way ANOVA with Bonferroni test; ns= not significant, *p-value<0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001. (B) An image panel representing infection in FBXL14.2 transfected cells in contrast to negative control. Nuclei are shown in blue, tubulin in green and VP1 in red. The scale bars correspond to 20 µm.

EV30 infection was also reduced by the same siRNAs that inhibited CVB3 and CVB1 infections (Figure 10A). Statistically, UCHL1.1, CHD4.1 and FBXL14.2 had the most notable effect on the infection. CAND2.2, the siRNA that inhibited CVB1 but not CVB3, had also noticeable inhibiting effect on EV30 infection. As with the other viruses tested, OTUD4.2 did not inhibit infection caused by EV30. The infection was not reduced in the infection inhibition control either, although in the earlier EV30 screen VEMU knocked the infection down. On a single cell level, some VP1 could still be detected in cells treated with FBXL14.2, however, compared to fully progressed infection, the VP1 signal was low and appeared dotted (Figure 10B, blow ups). UCHL1.1 and CHD4.1 had also similar effect on the infection (data not shown). Treatment with CAND2.2, on the other hand, still allowed the infection to progress fully in some cells, while in others the infection had low and dotted appearance (data not shown).

In conclusion, CVB3, CVB1 and EV30 infections were all clearly inhibited by at least three siRNAs: UCHL1.1, CHD4.1 and FBXL14.2. In addition, CVB1 and EV30 infections were inhibited by CAND2.2. None of the infections were inhibited by OTUD4.2. Although some cells were still infected with CVB3 and CVB1 after the hit siRNA treatments, the number of infected cells was notably reduced by the siRNAs. In EV30 infection, by contrast, treatment with the hit siRNAs resulted mostly in low VP1 signal and dotted appearance of the infection within the cells.

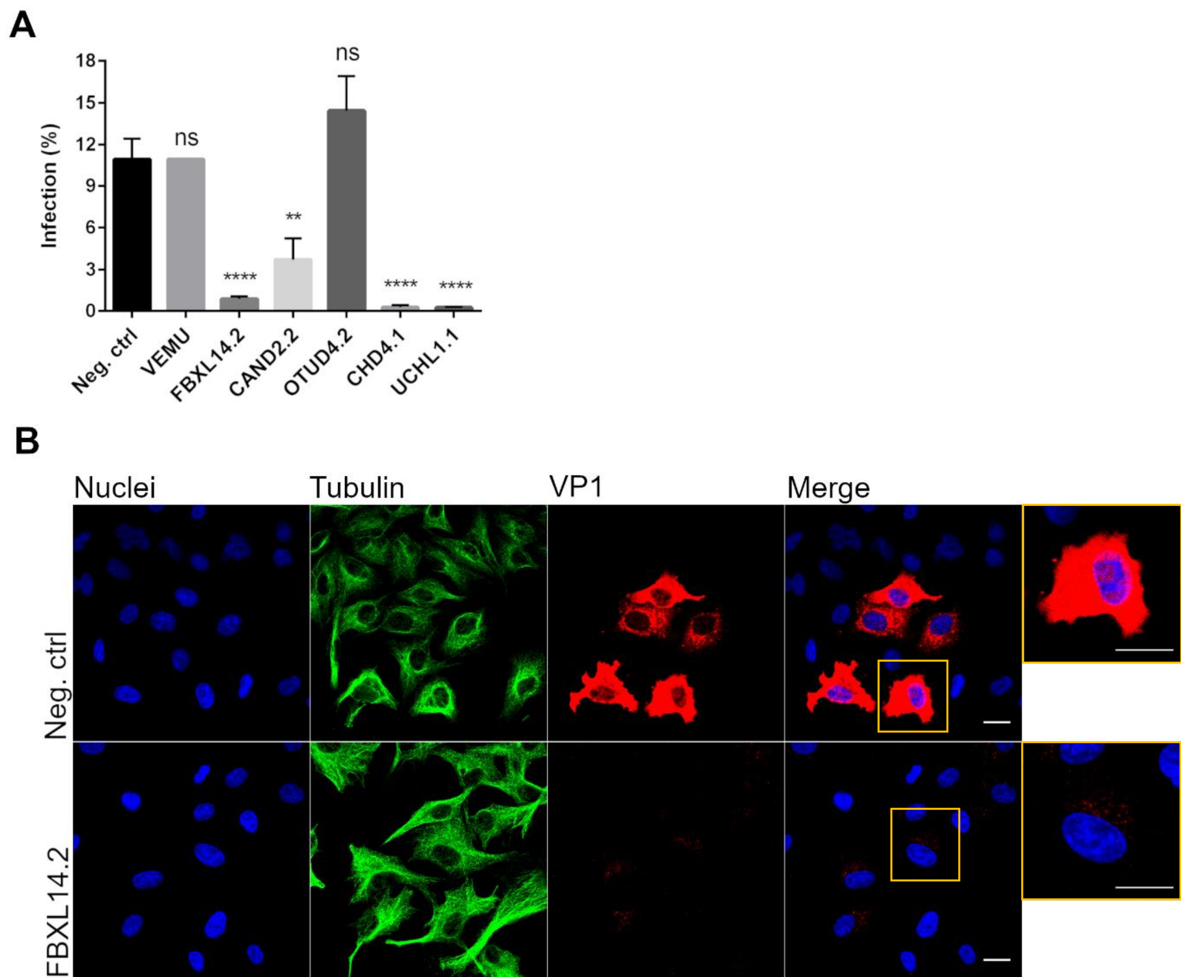

Figure 10. Inhibition of echovirus 30 infection in A549 cells transfected with five ubiquitome targeting siRNAs. Non-targeting siRNA was used as a control for normal infection (Neg. ctrl), and Vemurafenib (VEMU) was used as a control for inhibited infection. The cells were infected with MOI 50, and the infection was detected 5.5 hours post-infection by immunofluorescent labelling of viral capsid protein VP1 and confocal microscopy. (A) A bar chart representing infection per cent data of three replicates. The results are shown as mean of the replicates ± standard error of mean (SEM). Statistical difference of the samples in comparison to Neg. ctrl was resolved using one-way ANOVA with Bonferroni test; ns= not significant, **p-value<0.01, ****p-value<0.0001. (B) An image panel representing infection in FBXL14.2 transfected cells in contrast to negative control. Nuclei are shown in blue, tubulin in green and VP1 in red. The scale bars correspond to 20 µm.

3.6 Cell viability was slightly reduced by the hit siRNAs

Cytotoxicity assay was conducted for UCHL1.1, CHD4.1, FBXL14.2, CAND2.2 and OTUD4.2 to further assess the potential of these ubiquitome factors in antiviral development (Figure 11). Out of the five siRNAs, FBXL14.2, UCHL1.1 and CHD4.1 affected cell viability to some extent while CAND2.2 and OTUD4.2 did not. However, none of the siRNA treatments reduced cell viability as radically as an apoptosis inductor staurosporine, which was used as a positive control for cell cytotoxicity. Although FBXL14.2, UCHL1.1 and CHD4.1 lowered the cell viability in a statistically significant manner, the viabilities were still over 70% in cells treated with these siRNAs. In contrast, staurosporine treatment resulted in only 30% cell viability, showing more biologically relevant cell cytotoxicity. All in all, the results indicate that the knockdown of UCHL1, CHD4, FBXL14, CAND2 and OTUD4 did not affect the cell viability radically, although a small decrease was detected.

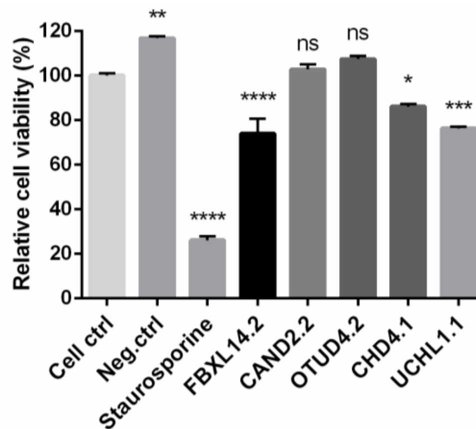

Figure 11. Relative viability of A549 cells transfected with five ubiquitome targeting siRNAs. The viability of cells transfected with non-targeting siRNA (Neg. ctrl) was also measured. Non-transfected cells were used as a control for viable cells (Cell ctrl) and a protein kinase inhibitor staurosporine (1 µM) was used as a positive control for cytotoxicity. Cell viability was assessed by luminescence measurements using Cell Titer Glo (Promega). The results are shown as mean of three sample replicates ± standard error of the mean (SEM). Statistical difference of the samples against the cell control was resolved using one-way ANOVA with Bonferroni test; ns= not significant, *p-value<0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001.

4 DISCUSSION

The ubiquitin-proteasome system has previously been connected to various viral infections, including that of enteroviral strain CVB3. During CVB3 infection, ubiquitination of proteins increases locally at virus-modified cellular membranes, where viral replication and assembly takes place (Voss et al. 2021). If ubiquitination is disturbed by silencing ubiquitin expression, the infection decreases (Si et al. 2008). It is also known that disturbing proteasome-mediated degradation of ubiquitinated proteins by proteasome inhibitors impairs CVB3 replication and protein synthesis (Si et al. 2008, Voss et al. 2021) This was recently attributed to the finding that CVB3 requires the UPS to process viral precursor protein P1, which contains the capsid proteins, and the 3CD fraction of precursor protein P3 (Voss et al. 2021). While the importance of the UPS to CVB3 has been demonstrated earlier, little is known about the roles of different ubiquitome-related enzymes during enteroviral infections. Thus, on the basis of preliminary screenings by the Marjomäki group, this study was set out to examine the importance of nine ubiquitome factors during CVB3, CVB1 and EV30 infections in the hopes of finding potential targets for antiviral development.

Out of the nine ubiquitome-related genes examined in this study, the knockdown of four had consistent effects on enteroviral infections. The products of these genes, UCHL1, CHD4, FBXL14 and CAND2, are likely modified and/or utilized by the viruses, as their knockdown by siRNAs led to inhibition of the infections. Interestingly, while UCHL1, CHD4 and FBXL14 targeting siRNAs affected CVB3, CVB1 and EV30 each, the knockdown of CAND2 was found to affect only CVB1 and EV30 infections. This indicates that different enterovirus strains can interact with different ubiquitome factors.

4.1 UCHL1 and viral infections

The gene UCHL1, also known as PGP 9.5, codes for Ubiquitin C-terminal Hydrolase L1, a deubiquitinating enzyme that hydrolyses small, C-terminal adducts of ubiquitin (Wilkinson et al. 1989, Larsen et al. 1998). In this study, the knockdown of UCHL1 with an siRNA reduced CVB3, CVB1 and EV30 infections, suggesting that the protein is relevant for each of these viruses. It has been found earlier that the inhibition of UCHL1 with an active-site directed inhibitor LDN-57444 adds to the anti-CVB3 effect of proteasomal inhibition (Si et al. 2008). This could imply that the protein is involved in the replicative cycle of CVB3. Interestingly, however, the inhibition of UCHL1 alone with the drug did not have antiviral effect (Si et al. 2008). The contradiction between our result and the previous finding could be related to the differences in the mechanism by which UCHL1 was inhibited (inhibitor vs. siRNA). However, the possibility of UCHL1.1 siRNA having an off-target effect cannot be excluded. Although the

result was consistent, only one out of the three UCHL1 targeting siRNA oligos inhibited the EV infections.

The exact mechanism of the antiviral effect of UCHL1 knockdown remains to be studied in the future. Previously UCHL1 has been recognized as negative regulator of immune responses during human papillomavirus (HPV) infection (Karim et al. 2013). The protein was found to interfere with pattern recognition receptor (PRR) mediated signalling, and thus type I IFN and the NF-κB pathways, during high-risk HPV infection. By contrast, when UCHL1 was knocked down with short hairpin RNA (shRNA), the production of cytokines and chemokines was enhanced in high-risk HPV infected keratinocytes (Karim et al., 2013). If the protein has similar role in enteroviral infections, inhibition of the EV infections following UCHL1 knockdown could be related to the immune responses.

The importance of UCHL1 has also been implicated in Epstein-Barr virus (EBV) infected B cells, where its expression is enhanced by a viral noncoding RNA (EBER2) in order to interfere with the cell cycle (Li et al. 2021). The increased expression of UCHL1 was found to boost the expression of Aurora kinases and cyclin B1, which in turn enabled fast division of the EBV infected cells and was thought to benefit the viruses by offering them reservoirs (Li et al. 2021). Ubiquitination in general has previously been connected to the disturbance of cell cycle during CVB3 infection. However, the process is exploited by the virus to increase the degradation of cellular cyclin D1, and this halts the cell cycle rather than boosts it (Luo et al. 2003). Whether UCHL1 is relevant in this process or not is still unclear.

An aspect that should be noted while interpreting the results and planning future studies is that UCHL1 is not evenly expressed in all tissues. In fact, the protein was initially thought to be neuron-specific, and it is especially abundant in neurons and neuroendocrine cells (Doran et al. 1983, Wilson et al. 1988). However, some UCHL1 expression has also been detected in, for example, gonads and fibroblasts (Wilson et al. 1988, Olerud et al. 1998). Additionally, the expression of UCHL1 has been connected to several malignant tissues and cell lines, including the adenocarsinoma cell line (A549) used in this study (Yao et al. 2022). Keeping this in mind, the expression level of UCHL1 could have been different to begin with in the cells of this study in comparison to the tissues enteroviruses normally target. Several viruses, such as the HPV and EBV can induce the expression of UCHL1 within the tissues they target (Karim et al. 2013, Li et al. 2021). However, the EBER2 of EBVs, for example, has only been shown to induce UCHL1 expression in cells that have initially had some expression of the gene (Li et al. 2021). By contrast, it appears that the expression of UCHL1 does not change during CVB3 infection (Si et al. 2008).

4.2 CHD4 may be utilized for varying purposes by viruses

The gene CHD4 codes for Chromodomain Helicase DNA Binding Protein 4, an essential component of the Nucleosome Remodelling Histone Deacetylase

(NuRD) complex, in which it is responsible for the ATP-dependent remodelling of chromatin (Xue et al. 1998, Wang and Zhang 2001). Either independently, or as part of the NuRD complex, CHD4 has been connected to the regulation of gene transcription, cell cycle control, DNA-damage response (DDR) and the development of, for example, T and B cells (Williams et al. 2004, Polo et al. 2010 Arends et al. 2019). In addition to the NuRD complex, the protein is a component of the ChAHP complex that acts to regulate the expression of lineage-specifying genes (Ostapcuk et al. 2018). CHD4 has been connected to the ubiquitome through its effects on several DDR associated E3s. In this context, CHD4 facilitates the initiation of cellular responses to DNA damage, as the chromatin remodelling activity of CHD4 promotes ubiquitin conjugation at the site of DNA damage (Luijsterburg et al. 2012).

Here, the importance of CHD4 as a factor regulating enteroviral infections was examined, and the knockdown of the protein was found to inhibit CVB3, CVB1 and EV30 infections. Although it is still not known how CHD4 is related to enteroviral infections, there is some evidence, that the protein can be utilized by viruses. Human cytomegalovirus (HCMV) was earlier found to require NuRD components, including CHD4, for efficient replication (Terhune et al. 2010). In line with the current study on enteroviruses, the knockdown of CHD4 with a shRNA disturbed HCMV infection, which was revealed by a dramatic decrease in the accumulation of HCMV immediate-early RNAs and DNA post-infection (Terhune et al. 2010). In contrast to these results, shRNA-mediated knockdown of CHD4 was recently found to enhance the replication of Kaposi sarcoma-associated herpesvirus (KSHV), and the virus was suggested to require CHD4-mediated repression of viral lytic genes to promote latency (Kumar et al. 2022). Thus, it appears that CHD4 may be utilized by different viruses for varying purposes. An interesting question for future studies is whether the effect of CHD4 on enteroviral infections is related to the DNA damage associated ubiquitination process, or some other function of the protein.

4.3 FBXL14 and CAND2 are both connected to SCF complexes

F-Box and Leucine Rich Repeat Protein 14, FBXL14 in short, is one of many F-box proteins that form S phase kinase-associated protein 1 (SKP1) – cullin 1 (CUL1) – F-box protein (SCF) E3 ligation complexes. Within these complexes, the F-box proteins are responsible for binding the substrates (Bai et al. 1996). In this study, all enteroviral infections tested were inhibited upon the silencing of FBXL14, which indicates the importance of the protein for these viruses. Although several F-box proteins have been connected to viral infections, there is little, if any, previous information about FBXL14 in this context (Lan et al. 2007, Baresova et al. 2012, Surjit et al. 2012). The known substrates of FBXL14 include factors associated with, for example, epithelial-mesenchymal transition and neuronal differentiation, and the protein has been shown to target these for proteasomal degradation (Viñas-Castells et al. 2010, Chen et al. 2017). Whether

FBXL14 mediates the degradation of some cellular or viral factors essential for enteroviral infections remains to be determined.

Another ubiquitome-related factor examined in this study, namely Cullin Associated and Neddylation Dissociated protein 2, or CAND2, was found to be important in CVB1 and EV30 infections. Intriguingly, while the knockdown of the protein inhibited both of these EVs, it had no notable effect on CVB3. In terms of function, CAND2 has been predicted to interact with SCF complexes, since its close homolog CAND1 regulates the assembly of these complexes and acts as an exchange factor of F-box proteins (Zheng et al. 2002, Pierce et al. 2013). Indeed, CAND2 was earlier found to attach to the CUL1 component of SCF complexes to inhibit SCF-mediated ubiquitination in the context of muscle cell myogenesis (Shiraishi et al. 2007). While CAND1 acts as an inhibitor of EBV replication, not much is known about the role of CAND2 in viral infections (Gastaldello et al. 2013). The results of this study, however, suggest that at least some enteroviruses may have mechanisms to utilize CAND2 in their infection.

In addition to utilizing different ubiquitome factors, different enteroviruses may be able to utilize same ubiquitome factors in different phases of their infections. In this study, the ubiquitome targeted siRNAs reduced the overall number of CVB3 and CVB1 infected cells, but EV30 infection had distinct phenotype from these two coxsackieviruses. Despite the treatments with the hit siRNAs, some VP1 could still be detected on a single-cell level from EV30 infected cells. This could imply that the virus has managed to initiate, but not go through, its life cycle within the cells. However, more mechanistical studies are still needed to confirm whether infections caused by different enteroviruses are halted in different phases upon the knockdown of ubiquitome-related factors.

4.4 The effect of ubiquitome factor knockdown on cells

One challenge of antiviral development is that targeting cellular factors may always have consequences on the health of the cells. Here, in comparison to untreated cells, the siRNA-mediated knockdown of UCHL1, CHD4 and FBXL14 was found to reduce the viability of A549 lung adenocarsinoma cells modestly. Treatment with CAND2 targeting siRNA, on the other hand, had no apparent effect on the viability of the cells. Many ubiquitome-related factors, including UCHL1, CHD4 and FBXL14, have been connected to a variety of cancerous tissues. Previous studies have shown that silencing UCHL1 raises the probability of apoptosis and reduces migration of A549 cells, while the knockdown of CHD4 also supresses proliferation and migration of these cells (Xu et al. 2020, Yao et al. 2022). Interestingly, the knockdown of FBXL14 in breast cancer cells, on the other hand, increases the abundance of certain cancer promoting factors and facilitates the migration of the cells (Cui et al. 2018). The results of the current study suggest that in addition to UCHL1 and CHD4, FBXL14 may also have some function in A549 cells, since the knockdown of the protein slightly reduced A549 viability.

Most importantly, although a small decrease of viability was detected, none of the hit siRNA treatments reduced cell viability as radically as treatments with apoptosis inducing staurosporine. While this is encouraging, it should be noted that the transfection efficiency with the set experimental conditions was around 50 per cent, and thus, the expression of any of the genes studied was not impaired in all of the cells. This could at least partially explain the relatively low reduction of cell viability upon the knockdown of the ubiquitome-related factors. The transfection efficiency could also explain why some cells still got infected after the siRNA treatments, while others did not.

4.5 Conclusions

In this study, the importance of ubiquitome in enteroviral infections was once again demonstrated, as several ubiquitome-related factors were found to be required in the infection. Although not all results from the preliminary studies were validated, several common ubiquitome factors were recognized for enteroviral serotypes CVB3, CVB1 and EV30, as expected. The relevance of these factors has previously been implicated mainly in infections of different members of the *Herpesviridae* family, or other DNA viruses, such as the HPV. Previous studies on these viruses have suggested various roles for the hit ubiquitome factors in infection, connecting these proteins to processes such as the immune responses, cell cycle regulation, viral replication and lytic-latency switch. Since the DNA viruses have notable structural and functional differences in comparison to small, RNA-genome containing EVs, it is possible that EVs utilize the ubiquitome factors for different purposes, and furthermore, by different mechanisms in comparison to the DNA viruses.

As the exact antiviral mechanisms were not examined in this study, it is still not known how the enteroviral infections were inhibited when the ubiquitome-related factors were silenced. To rule out the possibility of the siRNAs having off-target effects, an important step in the future studies is to check that the expression of the targeted genes is indeed knocked down after the siRNA treatments. Another interesting aspect for the future studies is to examine in which phase the infection is inhibited, and whether infections caused by different enteroviruses are halted in different phases upon the knockdown of ubiquitome factors. In the future, the use of ubiquitome factor targeted inhibitors instead of siRNA-mediated knockdown would allow time of addition experiments which could yield information about the antiviral mechanisms. The use of inhibitors in different concentrations could also offer further information about the cytotoxicity of targeting ubiquitome factors.

All in all, this study has shed light on the potential of ubiquitome-related factors in the development of antivirals against enteroviruses. Since antiviral development is especially focused on creating broadly acting treatments, the finding that different EV serotypes can utilize same ubiquitome factors in their infections is promising. However, more research is still needed on the mechanisms behind the antiviral effects observed in this study.

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REFERENCES

Abdullah S.W., Wu J., Wang X., Guo H. & Sun S. 2023. Advances and Breakthroughs in IRES-Directed Translation and Replication of Picornaviruses. *mBio.* 14, e0035823, doi: 10.1128/mbio.00358-23.

Akutsu M., Dikic I. & Bremm A. 2016. Ubiquitin chain diversity at a glance. *J Cell Sci.* 129: 875 – 880.

Andries K., Dewindt B., Snoeks J., Willebrords R., van Eemeren K., Stokbroekx R. & Janssen P.A. 1992. In vitro activity of pirodavir (R 77975), a substituted phenoxy-pyridazinamine with broad-spectrum antipicornaviral activity. *Antimicrob Agents Chemother.* 36: 100-7.

Arends T., Dege C., Bortnick A., Danhorn T., Knapp J.R., Jia H., Harmacek L., Fleenor C.J., Straign D., Walton K., Leach S.M., Feeney A.J., Murre C., O'Connor B.P. & Hagman J.R. 2019. CHD4 is essential for transcriptional repression and lineage progression in B lymphopoiesis. *Proc Natl Acad Sci U S A*. 116: 10927-10936, doi: 10.1073/pnas.1821301116.

Baggen J., Thibaut H.J., Starting J.R.P.M & van Kuppenveld F.J.M. 2018. The life cycle of non-polio enteroviruses and how to target it. *Nat Rev Microbiol*. 16: 368-381.

Bai C., Sen P., Hofmann K., Ma L., Goebl M., Harper J.W. & Elledge S.J. 1996. SKP1 Connects Cell Cycle Regulators to the Ubiquitin Proteolysis Machinery through a Novel Motif, the F-Box. *Cell* 86: 263-274.

Barajas D., Li Z. & Nagy P.D. 2009. The Nedd4-type Rsp5p ubiquitin ligase inhibits tombusvirus replication by regulating degradation of the p92 replication protein and decreasing the activity of the tombusvirus replicase. *J Virol*. 83: 11751-11764, doi: 10.1128/JVI.00789-09.

Baresova P., Pitha P.M. & Lubyova B. 2012. Kaposi sarcoma-associated herpesvirus vIRF-3 protein binds to F-box of Skp2 protein and acts as a regulator of c-Myc protein function and stability. *J Biol Chem.* 287: 16199-208, doi: 10.1074/jbc.M111.335216.

Barral P.M., Sarkar D., Fisher P.B. & Racaniello V.R. 2009. RIG-I is cleaved during picornavirus infection. *Virology* 391: 171–176.

Bauer L., Manganaro R., Zonsics B., Strating J.R.P.M., El Kazzi P., Lorenzo Lopez M., Ulferts R., van Hoey C., Maté M.J., Langer T., Coutard B., Brancale A. & van Kuppeveld F.J.M. 2019. Fluoxetine Inhibits Enterovirus Replication by Targeting the Viral 2C Protein in a Stereospecific Manner. *ACS Infect Dis*. 5: 1609-1623, doi:10.1021/acsinfecdis.9b00179.

Belnap D.M., McDermott B.M. Jr., Filman D.J., Cheng N., Trus B.L., Zuccola H.J., Racaniello V.R., Hogle J.M. & Steven A.C. 2000. Three-dimensional structure of poliovirus receptor bound to poliovirus. *Proc Natl Acad Sci U S A.* 97: 73-8.

Benschop K.S., Van Der Avoort H.G., Duizer E. & Koopmans M.P. 2015. Antivirals against Enteroviruses: A Critical Review from a Public-Health Perspective. *Antiviral Therapy* 20: 121-130, doi:10.3851/IMP2939.

Binford S.L., Maldonado F., Brothers M.A., Weady P.T., Zalman L.S., Meador J.W., Matthews D.A., & Patick A.K. 2005. Conservation of amino acids in human rhinovirus 3C protease correlates with broad-spectrum antiviral activity of rupintrivir, a novel human rhinovirus 3C protease inhibitor. *Antimicrob Agents Chemother*. 49: 619-626.

Bitnun A. & Yeh E.A. 2018. Acute Flaccid Paralysis and Enteroviral Infections. *Curr Infect Dis Rep*. 20, 34, doi: 10.1007/s11908-018-0641-x.

Brouwer L., Moreni G., Wolthers K.C. & Pajkrt D. 2021. World-Wide Prevalence and Genotype Distribution of Enteroviruses. *Viruses* 13, 434, doi: 10.3390/v13030434.

Budroni V. & Versteeg G.A. 2021. Negative Regulation of the Innate Immune Response through Proteasomal Degradation and Deubiquitination. *Viruses* 13, 584, doi: 10.3390/v13040584.

Chen B-S., Lee H-C., Lee K-M., Gong Y-N. & Shih S-R. 2020. Enterovirus and Encephalis. *Front Microbiol.* 11, 261, doi: 10.3389/fmicb.2020.00261.

Chen F., Zhang C., Wu H., Ma Y., Luo X., Gong X., Jiang F., Gui Y., Zhang H. & Lu F. 2017. The E3 ubiquitin ligase SCFFBXL14 complex stimulates neuronal differentiation by targeting the Notch signaling factor HES1 for proteolysis. *J Biol Chem.* 292: 20100-20112, doi: 10.1074/jbc.M117.815001.

Ciechanover A. & Iwai K. 2004. The Ubiquitin system: From Basic Mechanisms to the Patient Bed. *IUBMB Life* 56: 193 – 201.

Ciechanover A. & Schwartz A.L. 1998. The ubiquitin-proteasome pathway: The complexity and myriad functions of proteins death. *Proc Natl Acad Sci U S A*. 95: 2721-2730.

Clague M.J., Urbé S. & Komander D. 2019. Breaking the chains: deubiquitylating enzyme specificity begets function. *Nat Rev Mol Cell Biol*. 20: 338–352.

Coscoy L., Sanchez D.J. & Ganem D. 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J Cell Biol.* 155: 1265-1273.

Cui Y-H., Kim H., Lee M., Yi J.M., Kim R-K., Uddin N., Yoo K-C., Kang J.H., Choi M-Y., Cha H-J., Kwon O-S., Bae I-H., Kim M-J., Kaushik N. & Lee S-J. 2018. FBXL14 abolishes breast cancer progression by targeting CDCP1 for proteasomal degradation. *Oncogene* 37: 5794–5809.

Dikic I., Wakatsuki S. & Walters K.J. 2009. Ubiquitin-binding domains — from structures to functions. *Nat Rev Mol Cell Biol.* 10: 659–671.

Doran J.F., Jackson P., Kynoch P.A.M., & Thompson R.J. 1983. Isolation of PGP 9.5, a New Human Neurone-Specific Protein Detected by High-Resolution Two-Dimensional Electrophoresis. *J Neurochem*. 40: 1542–1547.

El-Sahly H.M., Atmar R.L., Glezen W.P. & Greenberg S.B. 2000. Spectrum of clinical illness in hospitalized patients with "common cold" virus infections. *Clin Infect Dis.* 31: 96–100.

Fry E.E. & Stuart D.I. 2010. Virion structure. In: Ehrenfeld E., Domingo E. & Roos R.P. (eds.), *The picornaviruses*, ASM Press, Washington, DC, pp. 59–64.

Gaaloul I., Riabi S., Harrath R., Hunter T., Hamda K.B., Ghzala A.B., Huber S. & Aouni M. 2014. Coxsackievirus B detection in cases of myocarditis, myopericarditis, pericarditis and dilated cardiomyopathy in hospitalized patients. *Mol Med Rep.* 10: 2811–2818.

Gastaldello S., Chen X., Callegari S. & Masucci M.G. 2013. Caspase-1 promotes Epstein-Barr virus replication by targeting the large tegument protein deneddylase to the nucleus of productively infected cells. *PLoS Pathog*. 9, e1003664, doi: 10.1371/journal.ppat.1003664.

Gazina E.V., Smidansky E.D., Holien J.K., Harrison D.N., Cromer B.A., Arnold J.J., Parker M.W., Cameron C.E. & Petrou S. 2011. Amiloride is a competitive inhibitor of coxsackievirus B3 RNA polymerase. *J Virol.* 85: 10364-74, doi: 10.1128/JVI.05022-11.

Geller R., Vignuzzi M., Andino R. & Frydman J. 2007. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev.* 21: 195–205.

Gu H. & Fada B.J. 2020. Specificity in Ubiquitination Triggered by Virus Infection. *Int J Mol Sci.* 21, 4088, doi: 10.3390/ijms21114088.

Hasbun R., Rosenthal N., Balada-Llasat J.M., Chung J., Duff S., Bozzette S., Zimmer L. & Ginocchio C.C. 2017. Epidemiology of Meningitis and Encephalitis in the United States, 2011-2014. *Clin Infect Dis.* 65: 359-363.

He Y., Chipman P., Howitt J., Bator C.M., Whitt M.A., Baker T.S., Kuhn R.J., Anderson C.W., Freimuth P. & Rossmann M.G. 2001. Interaction of coxsackievirus B3 with the full length coxsackievirus-adenovirus receptor. *Nat Struct Mol Biol*. 8: 874–878.

He Y., Lin F., Chipman P.R., Bator C.M., Baker T.S., Shoham M., Kuhn R.J., Medof M.E. & Rossmann M.G. 2002. Structure of decay-accelerating factor bound to echovirus 7: a virus-receptor complex. *Proc Natl Acad Sci U S A.* 99: 10325–9.

Hsu N.Y., Ilnytska O., Belov G., Santiana M., Chen Y.H., Takvorian P.M., Pau C., van der Schaar H., Kaushik-Basu N., Balla T., Cameron C.E., Ehrenfeld E., van Kuppeveld F.J., Altan-Bonnet N. 2010. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell* 141: 799–811.

Huang J., Liao Q., Ooi M.H., Cowling B.J., Chang Z., Wu P., Liu F., Li Y., Luo L., Yu S., Yu H., & Wei S. 2018. Epidemiology of Recurrent Hand, Foot and Mouth Disease, China, 2008-2015. *Emerg Infect Dis.* 24: 432–442, doi: 10.3201/eid2403.171303.

Huh K., Zhou X., Hayakawa H., Cho J.Y., Libermann T.A., Jin J., Harper J.W. & Munger K. 2007. Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. *J Virol*. 81: 9737–9747.

Hussain K.M., Leong K.L., Ng M.M. & Chu J.J. 2011. The essential role of clathrin-mediated endocytosis in the infectious entry of human enterovirus 71. *J Biol Chem.* 286: 309–321, doi: 10.1074/jbc.M110.168468.

Karim R., Tummers B., Meyers C., Biryukov J.L., Alam S., Backendorf C., Jha V., Offringa R., van Ommen G.J., Melief C.J., Guardavaccaro D., Boer J.M. & van der Burg S.H. 2013. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. *PLoS Pathog*. 9, e1003384, doi: 10.1371/journal.ppat.1003384.

Knowles N.J., Hovi T., King A.M.Q. & Stanway G. 2010. Overview of Taxonomy. In: Ehrenfeld E., Domingo E. & Roos R.P. (eds.), *The picornaviruses*, ASM Press, Washington, DC, pp. 34–38.

Komander D., Clague M. & Urbé S. 2009. Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol*. 10: 550–563.

Krogvold L., Edwin B., Buanes T., Frisk G., Skog O., Anagandula M., Korsgren O., Undlien D., Eike M.C., Richardson S.J., Leete P., Morgan N.G., Oikarinen S., Oikarinen M., Laiho J.E., Hyöty H., Ludvigsson J., Hanssen K.F., Dahl-Jørgensen K. 2015. Detection of a Low-Grade Enteroviral Infection in the Islets of Langerhans of Living Patients Newly Diagnosed with Type 1 Diabetes. *Diabetes* 64: 1682–1687.

Kumar A., Lyu Y., Yanagihashi Y., Chantarasrivong C., Majerciak V., Salemi M., Wang K-H., Inagaki T., Chuang F., Davis R.R., Tepper C.G., Nakano K., Izumiya C., Shimoda M., Nakajima K., Merleev A., Zheng Z-M., Campbell M. & Izumiya Y. 2022. KSHV episome tethering sites on host chromosomes and regulation of latency-lytic switch by CHD4. *Cell Rep*. 39, 110788, doi: 10.1016/j.celrep.2022.110788.

Laajala M., Hankaniemi M.M., Määttä J.A.E., Hytönen V.P., Laitinen O.H. & Marjomäki V. 2019. Host Cell Calpains Can Cleave Structural Proteins from the Enterovirus Polyprotein. *Viruses* 11, 1106, doi:10.3390/v11121106.

Laajala M., Reshamwala D. & Marjomäki V. 2020. Therapeutic targets for enterovirus infections. *Expert Opin Ther Targets* 24: 745–757.

Lan K., Verma S.C., Murakami M., Bajaj B., Kaul R. & Robertson E.S. 2007. Kaposi's sarcoma herpesvirus-encoded latency-associated nuclear antigen stabilizes intracellular activated Notch by targeting the Sel10 protein. *Proc Natl Acad Sci U S A*. 104: 16287-92, doi: 10.1073/pnas.0703508104.

Larsen C.N., Krantz B.A. & Wilkinson K.D. 1998. Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry* 37: 3358–68.

Li Z., Baccianti F., Delecluse S., Tsai M.H., Shumilov A., Cheng X., Ma S., Hoffmann I., Poirey R. & Delelcuse H.J. 2021. The Epstein-Barr virus noncoding RNA EBER2 transactivates the UCHL1 deubiquitinase to accelerate cell growth. *Proc Natl Acad Sci U S A*. 118, e2115508118, doi: 10.1073/pnas.2115508118.

Liu Q., Wu Y., Qin Y., Hu J., Xie W., Qin F.X. & Cui J. 2018. Broad and diverse mechanisms used by deubiquitinase family members in regulating the type I interferon signaling pathway during antiviral responses. *Sci Adv*. 4, eaar2824, doi: 10.1126/sciadv.aar2824.

Liu X., Xu F., Ren L., Zhao F., Huang Y., Wei L., Wang Y., Wang C., Fan Z., Mei S., Song J., Zhao Z., Cen S., Liang C., Wang J. & Guo F. 2021. MARCH8 inhibits influenza A virus infection by targeting viral M2 protein for ubiquitination-dependent degradation in lysosomes. *Nat Commun*. 12, 4427, doi: 10.1038/s41467-021-24724-2.

Liu Y., Wang C., Mueller S., Paul A.V., Wimmer E. & Jiang P. 2010. Direct Interaction between Two Viral Proteins, the Nonstructural Protein 2CATPase and the Capsid Protein VP3, Is Required for Enterovirus Morphogenesis. *Plos Pathog.* 6, e1001066, doi: 10.1371/journal.ppat.1001066.

Luijsterburg M.S., Acs K., Ackermann L., Wiegant W.W., Bekker-Jensen S., Larsen D.H., Khanna K.K., van Attikum H., Mailand N. & Dantuma N.P. 2012. A new non-catalytic role for ubiquitin ligase RNF8 in unfolding higher-order chromatin structure. *EMBO J*. 31: 2511–27.

Luo H., Zhang J., Dastvan F., Yanagawa B., Reidy M.A., Zhang H.M., Yang D., Wilson J.E. & McManus B.M. 2003. Ubiquitin-Dependent Proteolysis of Cyclin D1 Is Associated with Coxsackievirus-Induced Cell Growth Arrest. *J Virol.* 77: 1–9.

Marjomäki V., Turkki P. & Huttunen M. Infectious Entry Pathway of Enterovirus B Species. 2015. *Viruses* 7: 6387 – 6399.

Milstone A.M., Petrella J., Sanchez M.D., Mahmud M., Whitbeck J.C., Bergelson J.M. 2005. Interaction with coxsackievirus and adenovirus receptor, but not with decay-accelerating factor (DAF), induces A-particle formation in a DAF-binding coxsackievirus B3 isolate. *J Virol.* 79: 655–660.

Montiel-Garcia D., Santoyo-Rivera N., Ho P., Carrillo-Tripp M., Johnson J. E. & Reddy V.S. 2020. VIPERdb v3. 0: a structure-based data analytics platform for viral capsids. *Nucleic Acids Research*, doi: 10.1093/nar/gkn1096.

Muckelbauer J.K., Kremer M., Minor I., Tong L., Zlotnick A., Johnson J.E., Rossmann M.G. 1995. Structure determination of coxsackievirus B3 to 3.5 A resolution. *Acta Crystallogr D Biol Crystallogr*. 51: 871–87.

Musharrafieh R., Ma C., Zhang J., Hu Y., Diesing J.M., Marty M.T. & Wang J. 2019. Validating Enterovirus D68-2A^{pro} as an Antiviral Drug Target and the Discovery of Telaprevir as a Potent D68-2A^{pro} Inhibitor. *J Virol.* 93, e02221-18, doi: 10.1128/JVI.02221-18.

Oberste M.S., Maher K., Schnurr D., Flemister M.R., Lovchik J.C., Peters H., Sessions W., Kirk C., Chatterjee N., Fuller S., Hanauer J.M. & Pallansch M.A. 2004. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. *J Gen Virol.* 85: 2577–2584.

Olerud J.E., Chiu D.S., Usui M.L., Gibran N.S. & Ansel J.C. 1998. Protein gene product 9.5 is expressed by fibroblasts in human cutaneous wounds. *J Investig Dermatol.* 111: 565-572.

Ostapcuk V., Mohn F., Carl S.H., Basters A., Hess D., Lesmantavicius V., Lampersberger L., Flemr M., Pandey A., Thomä N.H., Betschinger J. & Bühler M. 2018. Activity-dependent neuroprotective protein recruits HP1 and CHD4 to control lineage-specifying genes. *Nature* 557: 739–743.

Owusu I.A., Quaye O., Passalacqua K.D. & Wobus C.E. 2021. Egress of non-enveloped enteric RNA viruses. *J Gen Virol.* 102, 001557, doi: 10.1099/jgv.0.001557

Palmenberg A., Neubauer D. & Skern T. 2010. Genome Organization and Encoded Proteins. In: Ehrenfeld E., Domingo E. & Roos R.P. (eds.), *The picornaviruses*, ASM Press, Washington, DC, pp. 3

Pevear D.C., Tull T.M., Seipel M.E. & Groarke J.M. 1999. Activity of pleconaril against enteroviruses. *Antimicrob Agents Chemother*. 43: 2109–15.

Pierce N.W., Lee J.E., Liu X., Sweredoski M.J., Graham R.L., Larimore E.A., Rome M., Zheng N., Clurman B.E., Hess S., Shan S.O. & Deshaies R.J. 2013. Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins. *Cell* 153: 206–15.

Polo S.E., Kaidi A., Baskcomb L., Galanty Y. & Jackson S.P. 2010. Regulation of DNA-damage responses and cell-cycle progression by the chromatin remodelling factor CHD4. *EMBO J*. 29: 3130–9.

Querido E., Blanchette P., Yan Q., Kamura T., Morrison M., Boivin D., Kaelin W.G., Conaway R.C., Conaway J.W. & Branton P.E. 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev.* 15: 3104–3117.

Rohll J.B., Moon D.H., Evans D.J & Almond J.W. 1995. The 3′ Untranslated Region of Picornavirus RNA: Features Required for Efficient Genome Replication. *J Virol.* 69: 7835–44.

Rohll J.B., Percy N., Ley R., Evans D.J., Almond J.W. & Barclay W.S. 1994. The 5'-Untranslated Regions of Picornavirus RNAs Contain Independent Functional Domains Essential for RNA Replication and Translation. *J Virol.* 68: 4384–4391.

Rozovics J.M. & Semler B.L. 2010. Genome Replication I: the Players. In: Ehrenfeld E., Domingo E. & Roos R.P. (eds.), *The picornaviruses*, ASM Press, Washington, DC, pp. 122–125.

Saeed M., Kapell S., Hertz N.T., Wu X., Bell K., Ashbrook A.W., Mark M.T., Zebroski H.A., Neal M.L. Flodström-Tullberg M., MacDonald M.R., Aitchison J.D., Molina H. & Rice C.M. 2020. Defining the proteolytic landscape during enterovirus infection. *PLoS Pathog.* 16, e1008927, doi: 10.1371/journal.ppat.1008927.

Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch T., Preibisch S., Rueden C., Saalfeld S., Schmid B., Tinevez J-Y., White D.J., Hartenstein V., Eliceiri K., Tomancak P. & Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676–682.

Schubert R.D., Hawes I.A., Ramachandran P.S., Ramesh A., Crawford E.D., Pak J.E., Wu W., Cheung C.K., O'Donovan B.D., Tato C.M., Lyden A., Tan M., Sit R., Sowa G.M, Sample H.A., Zorn K.C., Banerji D., Khan L.M., Bove R., Hauser S.L., Gelfand A.A., Johnson-Kerner B.L., Nash K., Krishnamoorthy K.S., Chitnis T., Ding J.Z., McMillan H.J., Chiu C.Y., Briggs B., Glaser C.A., Yen C., Chu V., Wadford D.A., Dominguez S.R., Fan Ng T.F., Marine R.L., Lopez A.S., Nix W.A., Soldatos A., Gorman M.P., Benson L., Messacar K., Konopka-Anstadt J.L., Oberste M.S., DeRisi J.L. & Wilson M.R. 2019. Pan-viral serology implicates enteroviruses in acute flaccid myelitis. *Nat Med.* 25: 1748–1752.

Shiraishi S., Zhou C., Aoki T., Sato N., Chiba T., Tanaka K., Yoshida S., Nabeshima Y., Nabeshima Y. & Tamura T. 2007. TBP-interacting Protein 120B (TIP120B)/Cullin-associated and Neddylation-dissociated 2 (CAND2) Inhibits SCF-dependent Ubiquitination of Myogenin and Accelerates Myogenic Differentiation. *J Biol Chem.* 282: 9017–9028.

Si X., Gao G., Wong J., Wang Y., Zhang J. & Luo H. 2008. Ubiquitination Is Required for Effective Replication of Coxsackievirus B3. *PLoS One* 3, e2585, doi: 10.1371/journal.pone.0002585.

Staring J., von Castelmur E., Blomen V., Blomen V.A., van den Hengel L.G., Brockmann M., Baggen J., Thibaut H.J., Nieuwenhuis J., Janssen H., van Kuppeveld F.J.M., Perrakis A., Carette J.E. & Brummelkamp T.R. 2017. PLA2G16 represents a switch between entry and clearance of Picornaviridae. *Nature* 541**:** 412–416.

Stirling D.R., Swain-Bowden M.J., Lucas A.M., Carpenter A.E., Cimini B.A. & Goodman A. 2021. CellProfiler 4: improvements in speed, utility and usability. *BMC Bioinformatics* 22, 433, doi: 10.1186/s12859-021-04344-9.

Surjit M., Varshney B. & Lal S.K. 2012. The ORF2 glycoprotein of hepatitis E virus inhibits cellular NF-κB activity by blocking ubiquitination mediated proteasomal degradation of IκBα in human hepatoma cells. *BMC Biochem*. 13, 7, doi: 10.1186/1471-2091-13-7.

Terhune S.S., Moorman N.J., Cristea I.M., Savaryn J.P., Cuevas-Bennett C., Rout M.P., Chait B.T. & Shenk T. 2010. Human cytomegalovirus UL29/28 protein interacts with components of the NuRD complex which promote accumulation of immediate-early RNA. *PLoS Pathog*. 6, e1000965, doi: 10.1371/journal.ppat.1000965.

Thibaut H.J., van der Linden L., Jiang P., Thys B., Canela M-D., Aguado L., Rombaut B., Wimmer E., Paul A., Pérez-Pérez M-J., van Kuppeveld F.J.M. & Neyts J. 2014. Binding of Glutathione to Enterovirus Capsids Is Essential for Virion Morphogenesis. *PLoS Pathog.* 10, e1004039, doi: 10.1371/journal.ppat.1004039.

Tsou Y.L., Lin Y.W., Chang H.W., Lin H.Y., Shao H.Y., Yu S.L., Liu C.C., Chitra E., Sia C. & Chow Y.H. 2013. Heat shock protein 90: role in enterovirus 71 entry and assembly and potential target for therapy. *PLoS One* 8, e77133, doi:10.1371/journal.pone.0077133.

van der Schaar H.M., van der Linden L., Lanke K.H., Strating J.R., Pürstinger G., de Vries E., de Haan C.A., Neyts J. & van Kuppeveld F.J. 2012. Coxsackievirus mutants that can bypass host factor PI4KIIIβ and the need for high levels of PI4P lipids for replication. *Cell Res*. 22: 1576-92.

Viñas-Castells R., Beltran M., Valls G., Gómez I., García J.M., Montserrat-Sentís B., Baulida J., Bonilla F., García de Herreros A. & Díaz V.M. 2010. The Hypoxia-controlled FBXL14 Ubiquitin Ligase Targets SNAIL1 for Proteasome Degradation2. *J Biol Chem*. 285: 3794–3805.

Voss M., Braun V., Bredow C., Kloetzel P-M. & Beling, A. 2021. Coxsackievirus B3 Exploits the Ubiquitin-Proteasome System to Facilitate Viral Replication. *Viruses* 13, 1360, doi: 10.3390/v13071360.

Wang H.B. & Zhang Y. 2001. Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. *Nucleic Acids Res*. 29: 2517–21.

Wang J., Du J., Wu Z. & Jin Q. 2013. Quinacrine Impairs Enterovirus 71 RNA Replication by Preventing Binding of Polypyrimidine-Tract Binding Protein with Internal Ribosome Entry Sites. *PLoS One* 8, e52954, doi: 10.1371/journal.pone.0052954.

Wang X., Peng W., Ren J., Hu Z., Xu J., Lou Z., Li X., Yin W., Shen X., Porta C., Walter T.S., Evans G., Axford D., Owen R., Rowlands D.J., Wang J., Stuart D.I., Fry E.E. & Rao Z. 2012. A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. *Nat Struct Mol Biol*. 19: 424–9.

Wang Y.C., Tsai C.S., Yang Y.H., Huang K.Y., Hsieh W.C., Kuo T.Y., Chin-Hung Chen V., Wong J., Ponton L. & Wang T.N. 2018. Association Between Enterovirus Infection and Asthma in Children: A 16-year Nationwide Population-based Cohort Study. *Pediatr Infect Dis J*. 37: 844–849.

Wells A.I. & Coyne C.B. 2019. Enteroviruses: A Gut-Wrenching Game of Entry, Detection, and Evasion. *Viruses* 11, 460, doi: 10.3390/v11050460.

Whitmer T., Malouli D., Uebelhoer L.S., DeFilippis V.R., Früh K. & Verweij M.C. 2015. The ORF61 Protein Encoded by Simian Varicella Virus and Varicella-Zoster Virus Inhibits NF-κB Signaling by Interfering with IκBα Degradation. *J Virol*. 89: 8687–8700.

Wilkinson K.D., Lee K., Deshpande S., Duerksen-Hughes P., Boss J.M. & Pohl J. 1989. The Neuron-Specific Protein PGP 9.5 Is a Ubiquitin Carboxyl-Terminal Hydrolase. *Science* 246: 670–673.

Williams C.J., Naito T., Arco P.G., Seavitt J.R., Cashman S.M., De Souza B., Qi X., Keables P., Von Andrian U.H. & Georgopoulos K. 2004. The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. *Immunity* 20: 719–33.

Wilson P.O., Barber P.C., Hamid Q.A., Power B.F., Dhillon A.P., Rode J., Day I.N., Thompson R.J. & Polak J.M. 1988. The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br J Exp Pathol*. 69: 91–104.

Woelk T., Sigismund S., Penengo L. & Polo S. 2007. The ubiquitination code: a signalling problem. *Cell Div*. 2, 11, doi: 10.1186/1747-1028-2-11.

Xu N., Liu F., Wu S., Ye M., Ge H., Zhang M., Song Y., Tong L., Zhou J. & Bai C. 2020. CHD4 mediates proliferation and migration of non-small cell lung cancer via the RhoA/ROCK pathway by regulating PHF5A. *BMC Cancer* 20, 262, doi: 10.1186/s12885-020-06762-z.

Xue Y., Wong J., Moreno G.T., Young M.K., Côté J. & Wang W. 1998. NURD, a Novel Complex with Both ATP-Dependent Chromatin-Remodeling and Histone Deacetylase Activities. *Mol Cell* 2: 851–861.

Yao J., Reyimu A., Sun A., Duoji Z., Liang S., Hu S., Wang X., Dai J. & Xu X. 2022. UCHL1 acts as a potential oncogene and affects sensitivity of common anti-tumor drugs in lung adenocarcinoma. *World J Surg Oncol.* 20, 153, doi: 10.1186/s12957-022-02620-3.

Zeng W., Sun L., Jiang X., Chen X., Hou F., Adhikari A., Xu M. & Chen Z. J. 2010. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell* 141: 315–330.

Zhang P., Mueller S., Morais M.C., Bator C.M., Bowman V.D., Hafenstein S., Wimmer E. & Rossmann M.G. 2008. Crystal structure of CD155 and electron microscopic studies of its complexes with polioviruses. *Proc Natl Acad Sci U S A.* 105: 18284–18289.

Zheng J., Yang X., Harrell J.M., Ryzhikov S., Shim E-H., Lykke-Andersen K., Wei N., Sun H., Kobayashi R. & Zhang H. 2002. CAND1 Binds to Unneddylated CUL1 and Regulates the Formation of SCF Ubiquitin E3 Ligase Complex. *Mol Cell* 10: 1519–26.

Zinngrebe J., Montinaro A., Peltzer. & Walczak H. 2014. Ubiquitin in the immune system. *EMBO Rep.* 15: 28–45.

APPENDIX 1. siRNA duplex sequences

CAND2.1 5′ – GGCUUGGACCUCAUGUUUCAUUUCT – 3′
3′ – GUCCGAACCUGGAGUACAAAGUAAAGA – 5′

CAND2.2 5′ – GACAGAGGAUAGUGAAUUCAGUGAG – 3′
3′ – CUCUGUCUCCUAUCACUUAAGUCACUC – 5′

CAND2.3 5′ – GUCAACGAGAGCGACAUGCAUGUGG – 3′
3′ – ACCAGUUGCUCUCGCUGUACGUACACC – 5′

CHD4.1 5′ – AGCAUGUCCUUACUAGAAUUGGUGT – 3′
3′ – GGUCGUACAGGAAUGAUCUUAACCACA – 5′

CHD4.2 5′ – UAUCAAUAGCUAUUCUGUUUCUGAT – 3′
3′ – UCAUAGUUAUCGAUAAGACAAAGACUA – 5′

CHD4.3 5′ – AACCACAGCAAAUUUCUUGAUGAAA – 3′
3′ – UUUUGGUGUCGUUUAAAGAACUACUUU – 5′

FBXL14.1 5′ – AGCUUUUUACUUGCUAGGAUGGAAT – 3′
3′ – AGUCGAAAAAUGAACGAUCCUACCUUA – 5′

FBXL14.2 5′ – CAGUUUCCCCAUGAGAUAGAGGAAT – 3′
3′ – UCGUCAAAGGGGUACUCUAUCUCCUUA – 5′

FBXL14.3 5′ – GAUAGAGGAAUGUCUACGUAUUUCA – 3′
3′ – CUCUAUCUCCUUACAGAUGCAUAAAGU – 5′

OTUD4.1 5′ – GUAUUUAAAACUGAUGUUAGUAAAA – 3′
3′ – UCCAUAAAUUUUGACUACAAUCAUUUU – 5′

OTUD4.2 5′ – AAAUGGAAAUCAUUAUGAUAUUGTG – 3′
3′ – AGUUUACCUUUAGUAAUACUAUAACAC – 5′

OTUD4.3 5′ – CUCCUUCACAAGUAACAGAAAAUAA – 3′
3′ – AAGAGGAAGUGUUCAUUGUCUUUUAUU – 5′

PHF11.1 5′ – GCCAAGAGUCAUGUCAAAUUGCAAT – 3′
3′ – UUCGGUUCUCAGUACAGUUUAACGUUA – 5′

PHF11.2 5′ – AAAAACUCAUGGAUGAGACUACUTC – 3′
3′ – UCUUUUUGAGUACCUACUCUGAUGAAG – 5′

PHF11.3 5′ – GAUCUUAUGUCAAGUUCUACAUCAA – 3′
3′ – CUCUAGAAUACAGUUCAAGAUGUAGUU – 5′

RNF216.1 5′ – GGAAUCUCUGAAUUCACUAAGCCAA – 3′
3′ – GACCUUAGAGACUUAAGUGAUUCGGUU – 5′

RNF216.2 5′ – CUGAGGAUGACUACGGUGAAUUUCT – 3′
3′ – AAGACUCCUACUGAUGCCACUUAAAGA – 5′

RNF216.3 5′ – UACCUUCUGGUAGUAAAAAUAGATA – 3′
3′ – AAAUGGAAGACCAUCAUUUUUAUCUAU – 5′

TRAF3.1 5′ – GCAACAUCUUGGUCUAGUAAGAACC – 3′
3′ – UACGUUGUAGAACCAGAUCAUUCUUGG – 5′

TRAF3.2 5′ – GAUAAGGUGUUUAAGGAUAAUUGCT – 3′
3′ – UUCUAUUCCACAAAUUCCUAUUAACGA – 5′

TRAF3.3 5′ – GUCCAAAAUGUACAGCGUGUCAAGA – 3′
3′ – UUCAGGUUUUACAUGUCGCACAGUUCU – 5′

UCHL1.1 5′ – CCAUGCAGUCUAAAAUGCUUCAGTA – 3′
3′ – GGGGUACGUCAGAUUUUACGAAGUCAU – 5′

UCHL1.2 5′ – ACGCAGUGGCCAAUAAUCAAGACAA – 3′
3′ – AGUGCGUCACCGGUUAUUAGUUCUGUU – 5′

UCHL1.3 5′ – GUCGGGUAGAUGACAAGGUGAAUTT – 3′
3′ – UACAGCCCAUCUACUGUUCCACUUAAA – 5′

USP7.1 5′ – AUCAGCAGCUUAAGAUGAAAAUCAC – 3′
3′ – GAUAGUCGUCGAAUUCUACUUUUAGUG – 5′

USP7.2 5′ – GUUUGGCUUCUCUGUAUCUAUUGAC – 3′
3′ – GUCAAACCGAAGAGACAUAGAUAACUG – 5′

USP7.3 5′ – AAGGUACUUUAAGAGAUCUUCUACA – 3′
3′ – ACUUCCAUGAAAUUCUCUAGAAGAUGU – 5′