HUMAN PICORNAVIRUSES: UNCOATING, ASSEMBLY AND INTERACTION WITH CELLULAR RECEPTORS

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ACADEMIC DISSERTATION

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Author’s contribution in each publication:
I. SS performed homology modelling and flexible fitting, analysed and interpreted data from 3-D reconstructions and flexible fitting. This work was presented as a manuscript in JSS’s PhD thesis (2011, University of Helsinki). JSS performed cryo electron microscopy and image processing. SS, JSS and SJB wrote the paper.

II. SS planned experiments and performed electron cryo-microscopy, image processing, 3-D reconstructions, tomography, flexible fitting, analysed and interpreted data. SS and SJB wrote the paper.

III. SS planned experiments; setup a generalised processing pathway for complex macromolecular structure fitting, performed homology modelling, flexible fitting, validation of the models, analysed and interpreted data. All authors wrote the paper.

IV. SS planned experiments, cultured and purified virus, prepared and labelled pentamers, performed electron cryo-microscopy, assisted in processing next generation sequencing data and sequence alignments, analyzed and interpreted data. SS, ECD, SW, RT, PGS and SJB wrote the manuscript.

*These authors contributed equally.
*Patent application filed based on this study.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>3-D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>Å</td>
<td>Ångstrom</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxackie adeno receptor</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CPS</td>
<td>Component placement score</td>
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<tr>
<td>cryo-EM</td>
<td>Cryo transmission electron microscopy</td>
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<td>cryo-ET</td>
<td>Cryo electron tomography</td>
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<tr>
<td>CTF</td>
<td>Contrast transfer function</td>
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<tr>
<td>CVA16</td>
<td>Coxackievirus A16</td>
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<td>CVA21</td>
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<td>CVA6</td>
<td>Coxackievirus A6</td>
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<td>CVA7</td>
<td>Coxackievirus A7</td>
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<tr>
<td>CVA9</td>
<td>Coxackievirus A9</td>
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<tr>
<td>DAF</td>
<td>Death accelerating factor</td>
</tr>
<tr>
<td>DED</td>
<td>Direct electron detector</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DQE</td>
<td>Dynamic quantum efficiency</td>
</tr>
<tr>
<td>EV71</td>
<td>Enterovirus 71</td>
</tr>
<tr>
<td>EV-A</td>
<td>Enterovirus group A</td>
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<tr>
<td>FMDV</td>
<td>Foot-and-mouth disease virus</td>
</tr>
<tr>
<td>GA</td>
<td>Bacteriophage GA</td>
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<tr>
<td>HPeV1</td>
<td>Human parechovirus 1</td>
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<tr>
<td>HPeV3</td>
<td>Human parechovirus 3</td>
</tr>
<tr>
<td>HRV2</td>
<td>Human rhinovirus 2</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>MP</td>
<td>Maturation protein</td>
</tr>
<tr>
<td>MS2</td>
<td>Bacteriophage MS2</td>
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<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PS</td>
<td>Packaging signal</td>
</tr>
<tr>
<td>PV1</td>
<td>Poliovirus 1</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SCARB2</td>
<td>Human scavenger receptor class B, member 2</td>
</tr>
<tr>
<td>SCCC</td>
<td>Segment cross correlation coefficient</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>smFCS</td>
<td>Single molecule fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>STNV</td>
<td>Satellite tobacco necrosis virus</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
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<tr>
<td>TR</td>
<td>Translation repressor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VP</td>
<td>Viral protein</td>
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ABSTRACT

Pathogenic human picornaviruses are known to cause a wide variety of diseases ranging from mild colds to severe paralysis. In addition to their importance in causing disease, they also serve as models for understanding the basic mechanisms of host-pathogen interactions, virus entry, viral genome release, viral synthesis and viral assembly. In picornaviruses, the majority of the structural and host-cell interaction studies have been conducted on polioviruses and human rhinoviruses. Picornaviruses like coxsackievirus A 7, coxsackievirus A 9 and human parechovirus 1 have not been so well studied because of difficulties in culturing them. Recently, the number of cases reported for infection by these viruses has increased dramatically due to better detection methods, thus making structural studies of these viruses and their interactions with their host cells important in order to understand their mode of infection so that better therapeutics can be designed against them.

I have studied coxsackievirus A 7, coxsackievirus A 9 and human parechovirus 1, which are all pathogenic picornaviruses, in order to understand the mechanism of pathogenesis, tropism, viral entry and assembly for these viruses in particular and for picornaviruses in general. Two studies dealt with determining the structure of coxsackievirus A 7, a Human Enterovirus A species for which there was no structural information available at the time when this study was conducted. The genome-filled and empty structure of coxsackievirus A 7 were determined using cryo electron microscopy to sub-nanometer resolution which helped in building pseudo-atomic models for them using homology modelling and flexible fitting. With the help of these models, the majority of the strain variations in the capsid proteins were identified on the
surface of VP1. Such variations are the likely cause of differences in pathogenesis and tropism between strains. Furthermore, superimposition of these models showed that the capsid underwent a conformational change on RNA release. In the process, generalised methods for optimising and comparing results from flexible fitting were developed. The next structural study elucidated the interaction of coxsackievirus A 9, a *Human Enterovirus B* species, with a cellular receptor. Integrins were found to bind sub-stoichiometrically to the capsid using electron cryo-tomography (cryo-ET). Asymmetric reconstruction indicated that this was probably due to steric hindrance. The affinity of this interaction was calculated to be 1nM using surface plasmon resonance. Additionally, the conformational changes which occur on its RNA release were quantified. The fourth study explained the importance of viral RNA in picornavirus assembly. Pentameric intermediates of human parechovirus 1 were isolated and used to identify packaging signals in the viral RNA required for capsid assembly using aptamer library screening and next generation sequencing analysis. Poly-U was identified as the common motif for these packaging signals present on the stem or the loop of the RNA secondary structure.

Overall, this thesis gives an insight into many important aspects of host-virus interactions especially the events occurring on viral RNA exit and during its encapsidation. The work in this thesis could be utilized to identify potential targets for antiviral synthesis and also to define general virus assembly principles.
1. INTRODUCTION

1.1 Picornaviruses

There are a large number of icosahedrally-symmetric small, single stranded (ss) ribonucleic acid (RNA) containing animal viruses that belong to the family Picornaviridae. The family consists of seventeen genera which have about 37 species (Adams et al. 2013) (http://www.picornaviridae.com/, as on 15.11.2013). They are known pathogens of both human and animals and are associated with several important diseases. The majority of the infections by picornaviruses are transmitted by the oral-fecal route (Yun et al. 2008, Baba et al. 2012), are asymptomatic or cause mild respiratory infections like the common cold caused by rhinoviruses (Arnold and Rossmann 1990). However, some enterovirus infections e.g. by poliovirus and enterovirus 71 lead to severe diseases like paralysis and meningitis (Chumakov et al. 1979, Rastogi et al. 1983, Verboon-Maciolek et al. 2002). In cloven-hoofed animals, foot and mouth disease is a highly infectious disease caused by foot and mouth disease virus (FMDV). There have been numerous outbreaks of this disease causing great economic loss to the farming industry (Salmon 1903, Henderson 1969, Knight-Jones and Rushton 2013). Picornaviruses are studied not only because of their infections in human and animals but also because they serve as model system for studying various biological processes like replication of RNA (Tuschall et al. 1982, Van Dyke et al. 1982), apoptosis (Tolkskaya et al. 1995, Schwarz et al. 1998), necrosis (Bozym et al. 2011), immune responses (Bedke et al. 2012, Howe et al. 2012a, Howe et al. 2012b), and self-assembly of macromolecules (Chung et al. 2010, Li et al. 2012, Porta et al. 2013). Picornaviruses are even being tested for biopharmaceutical applications to

1.1.1 Capsid structure

Picornaviruses are composed of the protective protein shells called capsids which keep the encapsidated nucleic acid safe from degradation by nucleases. Among the different common shapes of virus capsids like isometric, rod-shaped helices and pleomorphic, are the small, compact and icosahedrally-symmetric picornavirus capsids (Hogle et al. 1985, Rossmann et al. 1985, Semler 2002). The picornavirus capsids are also responsible for host cell recognition, viral attachment and capsid entry into the host cells. In addition, capsid proteins aid in the release of viral RNA into the host cells. Picornviruses have small genomes ranging from ~7000-8500 nucleotides in size (Kitamura et al. 1981, Racaniello and Baltimore 1981, Carroll et al. 1984, Callahan et al. 1985). Thus, they prudently use several copies of the same or similar proteins capable of assembling into a symmetric structure where all subunits occupy the same environment (e.g. icosahedral or helical capsid) as proposed initially by Watson and Crick for viruses with small genomes (Crick and Watson 1956). The proposed theory of Crick and Watson was later improved upon by Caspar and Klug for icosahedrally-symmetric viral capsids with more than 60 subunits (Caspar and Klug 1962). Their ‘quasi-equivalence theory’ stated that for capsids with more than 60 subunits, each subunit occupies a similar but not identical environment in the capsid. (Caspar and Klug 1962). The triangulation number (T number) describes the number of structural subunits per asymmetric unit (Caspar and Klug 1962). The icosahedral capsid of picornavirus have T=1 (pseudoT=3) symmetry which
means that one asymmetric unit of the capsid is made up of 3 similar but not identical proteins (Caspar and Klug 1962, Hogle et al. 1985, Rossmann et al. 1985, Semler 2002).

![Poliovirus capsid](image)

**Figure 1. Poliovirus capsid**

Adapted from Bubeck D. et al. Journal of Virology 2005 with permission from American Society of Microbiology. The left panel shows three-dimensional surface representation of a poliovirus capsid. The right panel shows an asymmetric unit of the capsid made of VP1 (blue), VP2 (yellow), VP3 (red) and VP4 (green).

Picornavirus capsids are composed of viral proteins VP1, VP2, VP3 and VP4 (Figure 1 and Figure 2) (Hogle et al. 1985, Rossmann et al. 1985, Semler 2002) except viruses in the genera *Parechovirus* and *Kobuvirus* where VP0 does not undergo cleavage into VP2 and VP4 (Yamashita et al. 1998, Stanway and Hyypiä 1999). These four viral proteins compose one asymmetric unit and five such units make up a pentamer (Semler 2002). Twelve pentamers together
make up a T=1 capsid (Semler 2002). There are five-fold, three-fold and two-fold rotational symmetries found in these capsids (Figure 1) (Hogle et al. 1985, Rossmann et al. 1985, Semler 2002). VP1 is found around the five-fold vertices whereas VP2 and VP3 are found around the two-fold and three-fold axes of symmetry (Figure 1) (Hogle et al. 1985, Rossmann et al. 1985, Semler 2002). The small mostly unstructured VP4 is found underneath VP1, VP2 and VP3 (Hogle et al. 1985, Rossmann et al. 1985, Semler 2002). VP1 protrudes from the capsid surface at the five-fold vertices making a ‘mesa’ whereas the VP2 and VP3 extend beyond the capsid surface at the three fold symmetry forming a ‘propeller’ (Figure 1) (Hogle et al. 1985, Bubeck et al. 2005b). In entero- and rhinoviruses, these features give an impression of a ‘canyon’ between them (Figure 1) (Hogle et al. 1985, Rossmann et al. 1985). The depth of the canyon varies among the entero- and rhinoviruses (Semler 2002). The VP3 N-terminus makes an annulus around the five-fold vertices whereas the N-termini of VP1 and VP2 along with VP4 make contact with the RNA (Rossmann et al. 1985, Hadfield et al. 1997, Hendry et al. 1999).

The amino acid sequence identity between VP1, VP2 and VP3 is low e.g. in Poliovirus 1 mahoney strain (UniProt ID: P03300), the sequence identity between VP1 and VP2 is 13.6%, between VP1 and VP3 is 17.4% and between VP2 and VP3 is 15.7% calculated with ClustalW (Larkin et al. 2007) (http://www.ebi.ac.uk/Tools/msa/clustalw2/, accessed on 16.11.2013). However, these capsid proteins have a common conserved β-barrel (jelly-roll) structure with four antiparallel β strands on each side (Figure 1) (Rossmann et al. 1985). Atomic models of many entero- and rhinoviruses have revealed the presence of a lipid molecule called a pocket factor within the hydrophobic region of the VP1 β-barrel (Hogle et al. 1985, Rossmann et al. 1985, Filman et

1.1.2 Host recognition and interaction

Although the overall structure of picornaviruses is similar (see section 1.1.1) (Hogle et al. 1985, Rossmann et al. 1985, Acharya et al. 1989), they utilize a wide variety of cellular receptors in order to gain entry into the host cells (Greve et al. 1989, Mendelsohn et al. 1989, Berinstein et al. 1995, Jackson et al. 2000). The cellular receptors may be proteins, for example, integrin αvβ6 for FMDV and coxsackievirus A9 (CVA9) (Jackson et al. 2000, Heikkilä et al. 2009), sugar moieties (e.g. heparin sulfate for FMDV) (Jackson et al. 1996) or glycoproteins (e.g. CD155 for poliovirus) (Mendelsohn et al. 1989). The protein-based receptors used by picornaviruses mostly belong to the immunoglobulin superfamily (Greve et al. 1989, Mendelsohn et al. 1989, Bergelson et al. 1997) or integrin superfamily (Roväinen et al. 1994, Jackson et al. 2000, Williams et al. 2004, Xing et al. 2004). Some examples of proteinaceous receptors used by picornaviruses are αvβ3 integrin, αvβ6 integrin, intercellular attachment molecule-1 (ICAM-1), polio virus receptor (CD155/Pvr), decay accelerating factor (DAF) and coxsackie adeno receptor (CAR) (Greve et al. 1989, Mendelsohn et al. 1989, Shafren et al. 1995, Bergelson et al. 1997, Seitsonen et al. 2010). These receptors can merely act as an attachment site for the virus (Shafren et al. 1997, Karnauchow et al. 1998) or they can cause instability in the virus capsid and initiate capsid uncoating (He et al. 2000, Ohka et al. 2004).
The canyon on the capsid surface (see section 1.1.1 and Figure 1) is widely used as the site for capsid binding to cellular receptors (Olson et al. 1993, Kolatkar et al. 1999, Belnap et al. 2000a, He et al. 2000, He et al. 2001, Xiao et al. 2001) but it is not the only site, as both sides of the canyon rim and the five-fold vertices are also exploited by picornaviruses to bind to cellular receptors (Hewat et al. 2000, Rossmann et al. 2002).

The same virus can have different receptor binding sites on its capsid surface for infecting different cell types as in the case of Coxsackievirus A9 where it uses the ‘arginine-glycine-aspartic acid’ (RGD) motif on the capsid surface in order to gain entry into many integrin-bearing cell types like African green monkey kidney cells (Roivainen et al. 1991), but it can still infect human rhabdomyosarcoma cells after deletion of the ‘RGD’ motif, indicating the use of a second receptor-binding site (Hughes et al. 1995). The virus can also use different receptors on the same cell at different stages of its entry as in the case of coxsackievirus B3 (CVB3) and coxsackievirus A21 (CVA21) where they uses DAF as a binding receptor but for uncoating they require CAR and ICAM1, respectively (Shafren et al. 1997, Milstone et al. 2005).

1.1.3 Entry and RNA release

From the structural perspective, the virus must have a dynamic but stable capsid so that while escaping the harsh conditions that the virus encounters upon first contact with the human body, it can still interact with its target cell and release the RNA in the host cell cytoplasm. Picornavirus capsids can “breathe”, leading to transient expulsion of the VP1 N-terminus and VP4 at a physiological temperature (Li et al. 1994, Lewis et al. 1998, Katpally et al. 2009, Lin et al. 2012). Subsequently, it has been suggested that once a cellular
receptor comes in contact with a picornavirus, it displaces the pocket factor from the β-barrel of VP1, thereby, destabilizing the virus, at least in the case of entero- and rhino-viruses which have a pocket factor. This results in capsid expansion and embedding of externalized VP1 and VP4 into the host membrane to make a channel into the cell interior so that the RNA gets released into the cytoplasm (Fricks and Hogle 1990, Danthi et al. 2003, Tuthill et al. 2006, Davis et al. 2008).

Although the molecular events associated with RNA release are well described in the literature, there has been controversy in the field about the exit site for RNA on the capsid. Early reports suggested the capsid vertices as the site for RNA release based on the fact that there is a plugged channel present on the five-fold vertices in virion which gets opened by externalization of VP1 N-terminus and VP4 (Hadfield et al. 1997, Belnap et al. 2000b, Belnap et al. 2000a, Hewat et al. 2002, Hewat and Blaas 2004, Bubeck et al. 2005b, Bubeck et al. 2005a). Recently, work on poliovirus has challenged the earlier RNA exit model by identifying holes near the two-fold and quasi three-fold axes in icosahedral reconstructions of early and late empty capsids suspected to be RNA exit sites (Levy et al. 2010) and later confirmed to be near the two-fold symmetry axis by asymmetric reconstructions where the virus was imaged at the stage of RNA release (Bostina et al. 2011). Owing to the low resolution of these asymmetric reconstructions from single particle work and subtomogram averaging and the fact that these particles were not true derivatives of virion-cellular interactions but were prepared by heating the virions at 56°C (Bostina et al. 2011), these experiments gave only indicative evidence of the RNA release site on the capsid surface. Finding the general applicability of this model to other picornaviruses was one of the motivations for the current study.
Even if the RNA comes out of a two-fold, how one site is chosen out of the 30 possible sites on the capsid and how an RNA exit site facing the cellular membrane is chosen remain unanswered questions.

### 1.1.4 Genome organization

![Poliovirus genome organization](image)

**Figure 2.** Poliovirus genome organization, post-translational processing and protein function.


The picornaviruses have positive-sense, single-stranded, non-segmented RNA genomes capable of infection on its own without additional accessory proteins (Colter *et al.* 1957, Alexander *et al.* 1958, Brown *et al.* 1958). The genome can be divided into the 5’ untranslated region (UTR), the coding region and the 3’ UTR (Figure 2) (Kitamura *et al.* 1981). The long 5’ UTR contains the viral-encoded viral protein genome-linked protein (VPg) covalently linked at its 5’ end (Pettersson *et al.* 1978) and an internal ribosome entry site (IRES) (Jang *et al.* 1988, Pelletier *et al.* 1988) (Figure 2). The VPg
acts as a primer for the viral-encoded, RNA-dependent, RNA polymerase (Nomoto et al. 1977, Paul et al. 1998) and the IRES is required for RNA translation (Jang et al. 1988, Pelletier et al. 1988). Since the picornavirus RNA does not carry any accessory protein with it except VPg, the first viral action after release of the RNA in the host cell is viral RNA translation (Semler 2002). The coding region is translated as a single polyprotein which is cleaved into P1, P2 and P3 proteins by viral-encoded proteases (Figure 2) (Holland and Kiehn 1968, Jacobson and Baltimore 1968, Summers and Maizel 1968, Rueckert and Wimmer 1984). Further proteolysis cleaves P3 into 3AB and the 3CD protease. 3CD cleaves P1 into 1ABC and 1D/VP1, and P2 into 2A and 2BC (Figure 2) (Semler 2002, Bedard and Semler 2004). Viral proteins formed at this stage either start functioning for instance by shutting off the host transcription-translation machinery or undergo further cleavage whereby 1ABC cleaves into VP0 and VP3, 2BC cleaves into 2B and 2C, 3AB cleaves to form 3A and 3B, and 3CD cleaves to form 3C and 3D (Figure 2) (Semler 2002, Bedard and Semler 2004). The 3D protein is the RNA-dependent RNA polymerase and once it is made, it starts synthesizing negative-sense RNA which is later used as the template for making more of the positive-sense RNA, thereby, increasing the concentration of the viral RNA and proteins in the host cell (Semler 2002, Bedard and Semler 2004). The 3’ UTR is short, with a poly-A tail at its end (Kitamura et al. 1981). The 3’ UTR is required for the synthesis of negative-sense RNA strand from the positive RNA strand (Pilipenko et al. 1996).

1.1.5 Viral assembly

The double stranded, deoxyribonucleic acid (DNA) viruses (e.g. phi29 bacteriophage) or double stranded RNA viruses (e.g. phi6 bacteriophage) use
packaging motor proteins to package the viral genome into a preassembled procapsid (Gottlieb et al. 1991, Gottlieb et al. 1992, Paatero et al. 1998, Smith et al. 2001). The specificity for packaging the viral genome in these viruses comes from specific sequences on the viral genome as in the case of phi6 where these so called ‘pac sequences’ present on the 5’end, fold into secondary structures (Pirttimaa and Bamford 2000) and are recognized by the viral protein P1 (Qiao et al. 2003). Interestingly, phi6 packages its genome in the form of 3 segmented single-stranded RNAs sequentially by the help of the P4 packaging motor protein (Qiao et al. 1995, Paatero et al. 1998, Mindich 2004). The single-stranded non-segmented RNA viruses like picornaviruses lack a packaging motor protein, therefore assembly of capsid and packaging of the genome should occur simultaneously (Hohn 1976).

Picornavirus assembly is poorly understood at least at the structural level. The capsid proteins are known to assemble into pentamers which either by direct interaction with RNA form genome-filled capsids (Nugent and Kirkegaard 1995) or make empty capsids (Putnak and Phillips 1981, Corrias et al. 1987, Ansardi et al. 1991, Jore et al. 1991). It is still unclear whether the naturally-occurring empty capsids formed are on-pathway intermediates for RNA encapsidation (Basavappa et al. 1994, Curry et al. 1997) or dead-end products which are infection incompetent (Marongiu et al. 1981). In addition, which assembly intermediate is associated with RNA packaging is unclear. Only one study in a cell free system on poliovirus has directly demonstrated that it is the pentamer which is likely to be associated with packaging (Verlinden et al. 2000). This observation is further validated by in vitro assembly work on bovine enterovirus where it has been demonstrated that the dissociation of in vitro formed empty capsids is an energetically unfavorable

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process, therefore, they are not likely to be the packing unit for assembly (Li et al. 2012).

The picornavirus capsid encapsidates only its own viral genome in vivo but where that specificity comes from is debatable. Nevertheless, it has been argued that the specificity for packaging viral RNA into the capsid comes from the viral proteins of the RNA replication complex and the viral RNA itself does not play any role in packaging or assembly (Nugent et al. 1999, Liu et al. 2010b). This argument comes from experiments where successful and efficient encapsidation of chimeric genome was observed when the poliovirus 5’ UTR regions were replaced with regions from other picornaviruses such as coxsackievirus B3 (Johnson and Semler 1988) and human rhinovirus 2 (HRV2) (Gromeier et al. 1996, Plevka et al. 2012) and even with a flavivirus, hepatitis C virus (Lu and Wimmer 1996). These observations were further supported when similar experiments were conducted by removing or replacing P1, 3’ UTR, P2 or VPg encoding regions of poliovirus with other viruses (Reuer et al. 1990, Porter et al. 1995, Porter et al. 1998). In contrast, there have been a few studies which indirectly gave evidence that there could be regions on the viral RNA genome called packaging signals (PSs) which make contact with capsid proteins and ensure that the viral genome gets packaged and not the cellular RNA (Barclay et al. 1998, Jia et al. 1998, Johansen and Morrow 2000b, a). These studies monitored the trans-encapsidation efficiency, in co-transfection of a poliovirus replicon carrying sub-genomic regions like 5’ UTR regions, IRES elements or 3’ UTR regions for poliovirus with the capsid proteins from poliovirus or other picornaviruses expressed on other vector (Barclay et al. 1998, Jia et al. 1998, Johansen and Morrow 2000b, a). These studies observed that the trans-encapsidation efficiency of polioviral sub-genomic regions was
significantly lower for capsid protein of other picornaviruses as compared to poliovirus. The most convincing evidence of PSs in picornaviruses perhaps comes from Aichi virus where mutations in 5’ UTR stem-loop region A keep the Aichi virus mutant replication and translation efficient but instead of genome-filled virions, empty capsids were formed (Sasaki and Taniguchi 2003).

For other single stranded non-segmented RNA viruses like bacteriophage MS2 (MS2) and the plant virus, Satellite Tobacco Necrosis Virus (STNV), it has been demonstrated that specific stem-loops on the viral RNA act as PSs for the assembly of the capsid into an infectious virion (Stockley et al. 2007, Toropova et al. 2008, Basnak et al. 2010, Bunka et al. 2011, Dykeman et al. 2011). In fact, PSs have been identified for other RNA viruses as well such as tobacco mosaic virus (Butler and Klug 1971, Zimmern and Butler 1977), turnip crinkle virus (Qu and Morris 1997), brome mosaic virus (Choi and Rao 2003), human immunodeficiency virus 1 with the well-studied packaging signal \( \Psi \) (Lever et al. 1989), mouse hepatitis virus (Abrescia et al. 2004), severe acute respiratory syndrome coronavirus (Hsieh et al. 2005), transmissible gastroenteritis coronavirus (Morales et al. 2013), rift valley fever virus (Morales et al. 2013) and alphaviruses (Jäälinoja et al. 2008). However, the most comprehensive evidence for RNA-centric assembly in icosahedrally-symmetric viruses comes from MS2.

The crystal structure of the MS2 capsid showed 30 copies of symmetrical capsid protein dimers (C/C) and 60 copies of asymmetrical capsid protein dimers (A/B) arranged in a T=3 capsid (Valegard et al. 1990, Golmohammadi et al. 1993). The capsid protein dimers were shown to initiate assembly by binding to a 19 nucleotide long RNA stem-loop structure called
the translation repressor (TR) which occurs only once in the whole genome sequence (Beckett and Uhlenbeck 1988, Beckett et al. 1988). The co-crystallization of capsid protein dimers not only with TR stem-loop but also with other sequences showed the plausibility of non-TR PSs for MS2 (Valegard et al. 1994, Valegard et al. 1997, Convery et al. 1998, Rowsell et al. 1998, van den Worm et al. 1998, Horn et al. 2004). Later on, several studies showed the conformational switching of the symmetric C/C dimer form to the asymmetric dimer A/B upon its binding to TR stem-loop during assembly and that the assembly requires both dimer types (Stockley et al. 2007, Basnak et al. 2010, Dykeman et al. 2010, Morton et al. 2010). Electron cryo-microscopy (Cryo-EM) in conjunction with icosahedral averaging showed 2 distinct layers of RNA density inside the wild type MS2 capsid in contact with each other (Toropova et al. 2008). Subsequently, a C5 symmetric reconstruction revealed a distinct density below the vertex attributed to a single copy of the maturation protein in complex with RNA termini (Toropova et al. 2011). This observation was later invalidated by symmetry-free subtomogram averaging of the MS2 capsid in complex with its receptor F-pilus showing the point of asymmetry at the two-fold symmetry axis occupied by a single copy of the maturation protein (MP) by restricting the angular search range on the basis of the direction and polarity of the F-pilus (Dent et al. 2013). The MP is responsible for two distinct roles: first is the circularization of the 5’ and 3’ ends of the viral RNA (Nathans et al. 1966, Shiba and Suzuki 1981) which require it to be inside the capsid; and second is the recognition and attachment of the F-pilus to the bacteriophage (Roberts and Steitz 1967, Krahn et al. 1972) which require the MP to be exposed on the capsid surface. Therefore, the presence of MP on the capsid surface partially exposed and partially in contact with the viral genome.
as identified by the subtomogram-based asymmetric reconstruction gives credibility to its dual role at the structural level.

Figure 3. Two-stage model for MS2 assembly.

Recently, reassembly assays for MS2 and STNV, monitored by single molecule fluorescence correlation spectroscopy (smFCS), provided a mechanistic model for capsid-RNA coassembly (Figure 3) (Borodavka et al. 2012). The smFCS assays were carried out by fluorophore tagging of either the capsid proteins or the RNA to monitor capsid assembly or RNA folding, respectively. The main feature of smFCS is that these studies can be carried out at a very low concentration (<1μM) of the components under investigation mimicking the expected scenario in the physiological state (Borodavka et al. 2012). MS2 and STNV assembly were shown to occur in two stages. In the first stage, capsid proteins in sub-stoichiometric concentrations bound to sequence- and structure-specific regions on free-form viral RNA resulting in a quick viral RNA collapse instead of the slow compaction expected if the capsid-RNA interactions were merely electrostatic (Borodavka et al. 2012). This was followed by a rate-limiting second stage, where recruitment of more
capsid proteins either individually or as decamers and hexamers onto the collapsed RNA resulted in the gradual assembly of the whole capsid (Borodavka et al. 2012). Although assembly in the presence of equally-long, non-viral RNAs was also shown to be initiated, it was far more inefficient and no RNA collapse was seen (Borodavka et al. 2012). So, capsid-RNA co-assembly is a function of protein-protein and protein-RNA interactions where capsid proteins bind to PSs distributed throughout the viral RNA genome instead of a single high affinity PS. In fact, multiple PSs have been predicted for MS2 and bacteriophage GA using Hamiltonian paths (see below for description of Hamiltonian paths) (Dykeman et al. 2013) and for STNV using systematic evolution of ligands by exponential enrichment (SELEX) (see section 4.2 for a description of SELEX) (Bunka et al. 2011).

These observations about the capsid assembly pathway using multiple packaging signals along with the distribution of the RNA in the first asymmetric reconstruction of MS2 (Toropova et al. 2011) were described mathematically using Hamiltonian paths (Dykeman et al. 2011). In graph theory, a Hamiltonian path describes the route taken from one graph vertex to another using each vertex only once. Hamiltonian path descriptions for MS2 assembly were established by making assumptions on the binding of MP to the 5’- and 3’-ends of the RNA to circularize the genome and that capsid assembly follows an energetically-favored pathway. Without the constraints there were about 40,500 ways in which RNA could have been packaged in the capsid but with the knowledge from the C5 reconstruction of MS2 (Toropova et al. 2011) about the binding of both end of the RNA to a single copy of maturation protein beneath the vertex, reduced the number of probable hamiltonian paths dramatically to 66. These Hamiltonian pathways also emphasized the spacing
of the stem-loops on the genome to fit within the capsid. Combining the Hamiltonian paths with biochemical and structural studies can potentially be extended to other ssRNA viruses to identify packaging signals on viral genomes as has been shown for MS2 and GA bacteriophage and to describe viral assembly in general (Dykeman et al. 2013).

The above mentioned approaches of identifying assembly and packaging responsible protein epitopes and RNA motifs could be utilized for those picornaviruses which have partially or completely ordered viral RNA as in the atomic models of CVA9 (see section 1.3.2) (Hendry et al. 1999), CVB3 (Muckelbauer et al. 1995), coxsackievirus A6 (CVA6) (Filman et al. 1989), human rhinovirus 14 (Arnold and Rossmann 1990), human rhinovirus 16 (Hadfield et al. 1997), CVA21 (Xiao et al. 2005) in the HPeV1 cryo-EM density map (see section 1.3.3). However, knowledge of the capsid atomic model e.g. from X-ray crystallography and the RNA distribution from cryo-EM is a prerequisite for applying the Hamiltonian paths-based approach.

1.2. Electron cryo-microscopy

Biological science is about studying how things work within and around the whole organism and at the cellular and molecular level. It is well known that the function of a molecule is related to its structure, so it is imperative to understand molecular structure in detail. Over the years, the major workhorse for structure determination has been X-ray crystallography, the method that has contributed about 85000 out of 96000 deposited structures in the Protein Data Bank (http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=explMethod-xray&seqid=100, accessed on 15.11.2013). Recently, nuclear magnetic
resonance (NMR) spectroscopy and cryo-EM are also making in-roads at similar (Bjorndahl et al. 2007) or lower resolution (Seitsonen et al. 2010). X-ray crystallography can be used to solve structures for homogenous samples ranging from small molecules (Mu et al. 2013) to viruses (MDa) (Abrescia et al. 2004, Cockburn et al. 2004) with the prerequisite that diffracting crystals form for which the phases can be solved. NMR spectroscopy based structure determination is mostly employed for smaller-sized homogenous proteins in a hydrated solution state and is useful especially for proteins which have flexible/unstructured regions (Felli and Pierattelli 2012). Big macromolecular assemblies like viruses or metastable ribosome complexes which are not amenable to crystallization and too big for NMR can be studied using cryo-EM in a near native state (Fischer et al. 2010, Zhang et al. 2013). The samples do not necessarily have to be homogenous for cryo-EM as they can be purified digitally from the micrographs (Fischer et al. 2010, Ramrath et al. 2012). Other information which can be pulled out using cryo-EM is different conformational states of a macromolecule or an assembly (Fischer et al. 2010, Ramrath et al. 2012). The majority of my thesis work utilizes cryo-EM (study I, II, III and IV), so I will give a brief introduction to the technique in the following sections.
1.2.1 Transmission electron microscope

A transmission electron microscope (TEM) is analogous to an inverted light compound microscope (Figure 4) and is composed of an electron emission source, an electromagnetic lens system, objective and condenser apertures and image detection systems like a phosphor viewing screen, photographic film or

Figure 4. Comparison of light and transmission electron microscopes.

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CCD camera (Orlova and Saibil 2011). The microscope is maintained at high vacuum to avoid interaction of high energy electrons with anything except the sample (Orlova and Saibil 2011). The sample is inserted into the microscope either using a side entry holder or a top entry holder. The electrons are extracted from a heated emission source which could be a tungsten filament, lanthanum hexaboride crystal, tungsten crystal based field emission gun (Orlova and Saibil 2011). The magnification and focusing of electron beams is controlled by a series of magnetic lenses, comprising condenser, objective and projection lenses (Orlova and Saibil 2011). These lenses are imperfect in shape and suffer from spherical aberrations. The spherical aberration is constant for a particular microscope and can be corrected during image processing. Actually, it is this aberration which is one of the factors that give contrast to unstained vitrified samples imaged in a TEM (Baker et al. 1999). The electrons can pass through the specimen without any interaction (unscattered) or on interaction with the specimen can be either elastically scattered where the travelling electrons do not lose energy or inelastically scattered where the travelling electrons loose energy (Baker et al. 1999, Orlova and Saibil 2011). The interaction of the electrons with the specimen gives rise to amplitude/scattering contrast and phase/interference contrast. If we consider the wave nature of the electron, then the amplitude contrast arises from the difference in the amplitude of two travelling elastic and inelastic scattering waves and is a major source of contrast for thick or stained samples. The phase contrast comes from the phase difference between elastically scattered and unscattered waves and is a major source of contrast in thin unstained samples (Baker et al. 1999, Orlova and Saibil 2011). Phase contrast can be enhanced by underfocussing the image which changes the path lengths of the scattered electrons more compared to the unscattered electrons without a significant loss in resolution because of the
large depth of focus of the magnetic lenses compared to the specimen thickness (Orlova and Saibil 2011).

**1.2.2 Sample preparation and imaging**

Biological samples, especially proteins, made of low atomic weight elements do not scattered electrons appreciably. With the aid of heavy metals stains such as uranyl acetate or tungstate high amplitude contrast can be gained but the samples may suffer from dehydration which can give rise to artifacts due to collapse of the structure and are mainly useful for low resolution work. After pioneering work on the vitrification of water (Dubochet 1981), it has been possible to image samples in a near native state by rapidly cooling the samples in liquid ethane kept in a bath of liquid nitrogen so that the samples become suspended in a glass-like state (vitrified) of water. This metastable state can be maintained in the vacuum of the microscope by keeping the temperature below ~ -160°C so as to prevent conversion of vitrified water into crystalline ice which occurs at temperatures of ~ -140°C (Baker *et al.* 1999). Cooling of samples also helps prevent radiation damage but the unstained samples inherently suffer from low phase contrast (Taylor and Glaeser 1976). So, in order to increase the ratio of signal to noise, many particles from the images are averaged together.
It is important to remember that images formed in the transmission electron microscope are projections of their three-dimensional (3-D) objects (Orlova and Saibil 2011). The relationship between the projection image and the sample itself is defined by the contrast transfer function (CTF) (Baker et al. 1999). The contrast transfer function (CTF) comprises of amplitude and phase scattering components and is given by the following equation:

$$\text{CTF}(v) = -\left(1 - F_{amp}^2\right)^{1/2} \sin \chi(v) + F_{amp} \cos \chi(v) * E(v)$$  \hspace{1cm} \text{Equation (1)}$$

where $\chi(v) = \pi * \lambda * v^2 \left(\Delta f - 0.5 * C_s * \lambda^2 * v^2\right)$  \hspace{1cm} \text{Equation (2)}$$
is the phase shift function with $\Delta f$ as the defocus value ($\mu$m), $\sin \chi(v)$ is the phase contrast, $\cos \chi(v)$ is the amplitude contrast, $F_{amp}$ is the fractional amplitude contrast, $\lambda$ is the electron wavelength ($\AA$) and $v$ is the spatial frequency ($\AA^{-1}$) and $C_s$ is the spherical aberration (mm) of the objective lens (Baker et al. 1999). $E(v)$ is the envelope function given by

$$E(v) = e^{-(\delta v)^2}$$

-Equation (3)

and dependent on beam coherence and specimen drift. The $F_{amp}$ for an unstained cryo-EM sample is close to 0.07 (Toyoshima and Unwin 1988) which makes the phase contrast the dominant component in the CTF (Equation 1). The phase scattering itself depends on the amount of underfocus, the spherical aberration of the objective lens and the electron wavelength (Equation 1 and 2). Due to the spherical aberration in lenses, we see black and white rings called Thon rings appearing in a Fourier transform of the projection image when images are taken underfocus (Figure 5) (Baker et al. 1999). The rings indicate that the image details alternate among black features (negative), featureless (no information or zero) and white features (positive) at different spatial frequencies, this is called phase inversion (Baker et al. 1999). So, this means that some details cannot be resolved as we do not have the information at that spatial frequency; therefore, we need to record images at different underfocii to get a 3-D reconstruction. In addition, many such phase inversions are needed to be corrected in order to get to high resolution.

Imaging under cryo conditions is done at a low electron dose typically ~5-20 electrons/$\AA^2$ so as not to damage the unstained hydrated sample which is extremely sensitive to radiation damage (Baker et al. 1999). The images are
recorded on photographic film or charged coupled device (CCD) (Faruqi and Henderson 2007) and more recently on direct electron detectors (DED) (McMullan et al. 2009). The images are recorded on films by interaction of electrons with silver halide crystals present in photographic emulsion, these electron-exposed films are later on dipped into developer solution followed by fixer solution so that silver halide crystals transform into visible silver grains (Orlova and Saibil 2011). Film is the recording medium of choice for the collection of high resolution data owing to the high dynamic quantum efficiency (DQE) which is a function of contrast and resolution, larger imaging area and finer pixel size (Grigorieff and Harrison 2011). There are several examples where film has been used to record data resulting in high resolution structures of icosahedral viruses including rotavirus double-layered particle (Zhang et al. 2008b), rotavirus VP7 recoated particle (Chen et al. 2009), rotavirus triple-layered particle (Settembre et al. 2011), bovine papillomavirus (Wolf et al. 2010), aquareovirus infectious sub-virion particle (Zhang et al. 2010), adenovirus (Liu et al. 2010a) and dengue virus (Zhang et al. 2013).

Images on CCD are recorded by converting the electrons into photons using a scintillator which passes the photons to photosensitive elements of the CCD chip to convert them into an electric signal (Downing 1995). The advantage of using CCD is that a digitized image is immediately available for image processing compared to photographic films which need to be developed and digitized first. However, in comparison to film, CCD has a lower DQE at higher spatial frequencies, larger pixel size and smaller imaging area. Recent advances in DED technology, start to make them a real alternative to films and they are already far superior to CCD at 300 kV (Campbell et al. 2012). The image is recorded in DED by direct exposure of pixel detectors to the incident electrons (Faruqi and Henderson 2007). These pixel detectors are coupled to a
complementary metal oxide semiconductor (CMOS) to convert the signal into voltage which makes the image recording faster. Two main advantages of DED have been reported, firstly, beam-induced specimen drift and beam damage can both be minimized by careful processing of the image series acquired of each region, and secondly, the DQE is similar or may be even higher than that of photographic film, depending on the operating voltage (Campbell et al. 2012). This helps to increase the signal to noise ratio and contrast in the images which has resulted in more accurate determination of orientations and origins improving resolution (Campbell et al. 2012). Already, DED data have been processed to high resolution reconstructions for various macromolecular assemblies such as rotavirus (Campbell et al. 2012), eukaryotic ribosome (Bai et al. 2013), proteasome (Li et al. 2013), *Sulfolobulus* turreted icosahedral virus (Veesler et al. 2013) and mammalian transient receptor potential channel 1 (Liao et al. 2013).

### 1.2.3 Three dimensional image reconstruction

To combat the low signal-to-noise ratio in low dose cryo-EM images of viruses many different images can be averaged together. The viruses tend to be randomly oriented in the vitreous water, so each micrograph can contain many different views of the same virus if that virus is structurally congruent. Thus the spatial orientations and position of the images with respect to each other or to a reference image (Baker et al. 1999) need to be determined. Inherent symmetry present within the specimen can be used to improve the resolution of the map (Baker et al. 1999).
Figure 6. General workflow of image processing.


A general workflow for 3-D icosahedral image reconstruction is shown in Figure 6. The CTF (see section 1.2.2) is determined for each micrograph and the micrographs showing drift or astigmatism are discarded. There are many programs for determining the CTF of a micrograph, one such popular program is CTFFIND3 (Mindell and Grigorieff 2003) where the defocus and astigmatism determination for a micrograph is fully automated and the power spectrum of the micrograph is fitted to a calculated one. The virus particles are boxed out from the selected micrographs and are normalized to have similar
grey scale. The main goal of image reconstruction is to determine as accurately as possible spatial orientations and origin values for the boxed virus particles. The particle orientations is commonly determined by the common lines method (Crowther 1971) or projection matching (Baker and Cheng 1996, Fuller et al. 1996) for icosahedral viruses distributed randomly in vitrified ice. The common line approach works on the principle that in Fourier space different two-dimensional projections of a 3-D object have at least one common line (DeRosier and Klug 1968). Therefore relative orientation between different particles can be determined in Fourier space from three or more common lines between them. For high symmetry objects the number of these common lines is rather high as in the case of an icosahedron where 6 five-fold axes contribute 12 common lines, 10 three-fold axes contribute 10 common lines and 15 two-fold axes contribute 15 common lines which together have 37 pairs of common line per particle and 60 pairs of cross-common lines between two particles (Crowther 1971). Instead of Fourier space, the common line method can be alternatively applied in real space using angular reconstitution (Van Heel 1987).

An initial model is a prerequisite in projection matching approach. The initial model can be a known related 3-D structure, an object of similar shape and size (Baker and Cheng 1996, Fuller et al. 1996), a starting model from tomography (Sperling et al. 1997), or a series of random models generated from the boxed particles (Yan et al. 2007). The initial model is back projected in Fourier space in all possible orientations with predefined angular sampling and compared to 2-D projections of the boxed particles. The highest correlating projections are taken as the orientations of the boxed particles. These orientations are further refined with finer angular sampling.
In case of random model generation, a subset of particles are assigned random orientations with the assumption that the virus particle center is same as the center of the box in which it is extracted. A 3-D model is calculated and the orientations are further refined by projection matching the boxed particles against the initial model (Yan et al. 2007). The results from a number of such random models are compared, typically 10, and the one with the highest resolution is picked to be used as a model to determine the orientations of the full data set. The advantage of this approach is that it avoids model bias in the initial stages of the reconstruction. Irrespective of the choice of the initial model, the further refinements of orientation and origin require iterative processing where the model reconstructed in the last iteration is used as a reference model for determination of orientation and origins in the next iteration.

Many icosahedrally-symmetric virus capsids have components that do not follow that symmetry but have one or many asymmetric features in them e.g. tailed bacteriophages (Parent et al. 2012), sub-stoichometrically saturated soluble receptors on icosahedral viruses (Hafenstein et al. 2007, Seitsonen et al. 2010), genome density in an icosahedral capsid (Toropova et al. 2011, Dent et al. 2013), single-copy protein in the capsid (Dent et al. 2013), mismatches in symmetry of the capsid proteins (Briggs et al. 2005, Huiskonen et al. 2007, Jäälinoja et al. 2008). Imposing icosahedral symmetry during 3-D reconstruction averages out such features. There are different approaches to obtain an asymmetric reconstruction (Tao et al. 1998, Morais et al. 2001, Briggs et al. 2005, Lander et al. 2006, Hafenstein et al. 2007, Bostina et al. 2011, Toropova et al. 2011, Parent et al. 2012), one such approach is to mask the asymmetric parts initially and impose icosahedral symmetry until optimal
origins and orientations of the virus particles are determined (Tao et al. 1998, Morais et al. 2001, Lander et al. 2006, Parent et al. 2012). Next, the icosahedral symmetry can be relaxed and the asymmetric features are unmasked from the virus particles. A new set of orientations are determined for these particles by aligning the asymmetric features in each particle to a common reference which have a single copy of the asymmetric feature at a predefined position in its structure (Tao et al. 1998, Morais et al. 2001, Lander et al. 2006, Parent et al. 2012).

The information from these 3-D density maps can be extended by fitting atomic models into its 3-D density map in order to obtain ‘pseudo-atomic models (Stewart et al. 1991, Olson et al. 1993, Grimes et al. 1997, San Martin et al. 2001, Zhang et al. 2008a, Shingler et al. 2013). These atomic models for the whole virus, its individual components or structural homologs are derived from X-ray crystallography, NMR (Fabiola and Chapman 2005, Rossmann et al. 2005) or in silico homology modelling (Topf and Sali 2005, Roy et al. 2010) and are fitted either by rigid or flexible fitting (Topf et al. 2008, Ahmed et al. 2012, Lopez-Blanco and Chacon 2013). Even for rigid fitting, the resolution of the reconstruction, the completeness of the atomic model, the predominant secondary structure elements and the shape of both the atomic model and the reconstruction are key factors when trying to find the best fitting solutions. But in addition, the atomic models and the 3-D density maps may have conformational differences which could be due to flexible loop regions of the structure or major changes in the conformation states of the atomic model and the density map or there could be protein structure prediction errors arising from homology modelling (Topf et al. 2008, Ahmed et al. 2012). In order to do fitting for such cases, perturbation of the atomic model is required to find the
optimal conformation of the model with respect to the 3-D density map in conjunction with search for best fits of origins and orientations (Topf et al. 2008, Ahmed et al. 2012, Lopez-Blanco and Chacon 2013). Still there is a need to generalize and test these methods especially for homology models.

1.3. Viruses in this study

1.3.1 Coxsackievirus A7

Coxsackievirus A7 (CVA7) is a member of the Enterovirus A (EV-A) species whose other clinically important members are enterovirus 71 (EV71) and coxsackievirus A16 (CVA 16). EV71 and CVA16 are the main causative agents for hand, foot and mouth disease (Hagiwara et al. 1978, Cabrerizo et al. 2013) whereas CVA7 is similar to poliovirus in pathogenesis (Ranzenhofer et al. 1958) and is associated with acute flaccid paralysis (Habel and Loomis 1957, Grist 1962) and aseptic meningitis (Richter et al. 1971).

Strain-based variations in pathogenesis and tropism are seen for CVA7 in EV-A group. CVA7 Parker and USSR strains are associated with flaccid paralysis (Grist 1962) whereas 275/58 strain is associated with aseptic meningitis (Richter et al. 1971); even the infection in animal models differ among these strains (Habel and Loomis 1957, Richter et al. 1971).

Earlier, unlike EV71, human scavenger receptor class B, member 2 (SCARB2) was not considered a cellular receptor for CVA7 (Yamayoshi et al. 2009) but a recent study showed it to be a likely receptor for CVA7 (Yamayoshi et al. 2012). This discrepancy was due to the successful infection of CVA7 Parker strain in SCARB2 expressing cells as well as SCARB2 deficient cells (Yamayoshi et al. 2009), however, SCARB2 knockdown by
RNA interference in a SCARB2 expressing cell line showed loss of CVA7 Parker strain infection which was regained in rescue experiments (Yamayoshi et al. 2012). So, CVA7 infection in SCARB2 expressing cells is SCARB2 dependent but it is likely to utilize other receptors in non-SCARB2 expressing cells.

Clearly, CVA7 and other members of the EV-A group are clinically a significant group of viruses but there was no structural information available on these viruses when study I was initiated.

1.3.2 Coxsackievirus A9

Coxsackievirus A9 (CVA9) is a member of the Enterovirus B species whose other important members are CVA6, CVB3 and echovirus 1. These viruses are associated with various diseases such as respiratory infection (Whitton et al. 2005), aseptic meningitis (Michos et al. 2007, Cui et al. 2010), myocarditis (Moschovi et al. 2007), encephalitis (Moschovi et al. 2007) and childhood diabetes (Clements et al. 1995, Roivainen et al. 1998). CVA9 is known to utilize integrins ą3 and ą6 for cellular entry by attaching through its VP1 C-terminus ‘RGD’ motif (Roivainen et al. 1994, Williams et al. 2004), although, successful infection with an ‘RGD’ less mutant has also been reported (Roivainen et al. 1996).

These integrins are essential cellular receptors which facilitate cell adhesion (Ross et al. 2013), cell migration (Huttenlocher and Horwitz 2011) and cell differentiation (Streuli 2009). These heterodimers of α-and β-type chains are exploited for their ‘RGD’ motif recognition capabilities by many viruses for cell attachment and internalization (Stewart and Nemerow 2007). Cryo-EM in combination with other techniques revealed that viruses can bind
to different conformation state integrins as shown for echovirus 1 binding to bent state integrin (Xing et al. 2004, Jokinen et al. 2010) or the adenovirus binding to an extended state integrin (Lindert et al. 2009). Similar to natural integrin ligands, the virus binding can induce transition in the integrin state from one conformation to another (Lindert et al. 2009) and also cause clustering of integrins in the cell membrane (Jokinen et al. 2010).

Although there is an atomic model available for CVA9 (Hendry et al. 1999) (PDB: 1D4M), unlike echovirus 1 (Xing et al. 2004, Jokinen et al. 2010), the structural basis for its binding to the integrins was not established prior to this study. The CVA9 atomic model possesses the common characteristics of an enterovirus such as a T=1 (pseudoT=3) capsid, the presence of a canyon, hydrophobic pocket factor and VP4 inside the capsid shell (see section 1.1.1) (Hogle et al. 1985, Hendry et al. 1999). An RNA nucleotide density in contact with a highly conserved tryptophan residue of capsid protein VP2 was also seen in this structure (Hendry et al. 1999). However, RNA expulsion from the capsid could not be elicited from this model.

1.3.3 Human Parechovirus 1

Human parechoviruses (HPeV) 1-16 are members of the Parechovirus genus, although HPeV1 and HPeV2 were originally named as echovirus 22 and 23, respectively, under the genus, Enterovirus (Wigand and Sabin 1961). However, the genome sequencing and N-terminal sequencing of VP1 and VP3 showed HPeV1 to be distinct from other picornaviruses (Hyypiä et al. 1992) and was later categorized into separate parechovirus group. HPeV1 infections usually occur in early childhood with mild respiratory (Russell and Bell 1970,
Abed and Boivin 2006) and gastrointestinal infections (Grist et al. 1978, Ehrnst and Eriksson 1993). However, in some cases these infections can lead to severe diseases of the central nervous system like aseptic meningitis (Ehrnst and Eriksson 1993), encephalitis (Koskiniemi et al. 1989) and encephalomyelitis (Legay et al. 2002) or myocarditis (Maller et al. 1967, Russell and Bell 1970).

Figure 7. HPeV1 capsid-RNA finger-like densities.

A) Central cross section of HPeV1 EM density map (EMDB: 1690) (Seitsonen et al. 2010) with capsid-RNA densities marked with arrow-head. The view is along the two-fold axis. Two-fold, five-fold and three-fold symmetry axes are marked as 2f, 5f and 3f, respectively. B) Slabbed-section of isosurface, radially depth-cued HPeV1 EM density map (EMDB: 1690) (Seitsonen et al. 2010) rendered at 3 standard deviation above mean showing the capsid-RNA density marked by arrow. Arrow-head indicates the density for capsid shell. Color key based on distance from the center of the particle is shown.

Cell culture studies have shown that HPeV1 can grow in a wide variety of cell lines and is most efficient in HT29 cells (Westerhuis et al. 2013), indicative of broad cellular tropism with utilization of many different cellular
receptors. As in the case of CVA9 and echovirus 9, HPeV1, 2, 4, 5 and 6 also have an ‘RGD’ motif on the VP1 C-terminus and binding to ‘RGD’ recognizing integrins such as αvβ3 and αvβ6 have been shown for HPeV1 (Boonyakiat et al. 2001, Joki-Korpela et al. 2001). The structural basis for this binding was established in a cryo-EM study on integrins αvβ3 and αvβ6 with the virus (Seitsonen et al. 2010). A surprising observation which came out of this work was the identification of highly ordered capsid-RNA densities below the vertices with the signal intensity as high as that for the capsid proteins (Figure 7) (Seitsonen et al. 2010). These densities seemed unique to the parechoviruses as none of the earlier reported cryo-EM or X-ray structures for picornaviruses have reported them. We are still debating reason behind this strong RNA-capsid association.
2. AIMS OF THE STUDY

The general aims of these studies were to understand the mechanism(s) underpinning picornavirus-host cell interactions at the structural level. More specifically, we wanted to understand how picornaviruses interact with the cellular receptors; what happens to them upon RNA release; how do they assemble; where does the specificity of assembly comes from? I studied CVA7, CVA9 and HPeV1 with the following specific aims in mind:

A. CVA7: To understand this virus structure as there was no structural information available for this virus group at the beginning of the study. To understand why different strains of CVA7 differ in pathogenesis and tropism; and what happens to the capsid when RNA is released?

B. To improve methods of fitting and validation of homology models and their fits into complex structures that undergo conformational changes.

C. CVA9: To understand the virus-receptor interaction in greater detail using cryo-EM utilizing the known atomic structure of the capsid. Also, to understand the conformational changes in the capsid on RNA egress upon binding to cellular receptors.

D. HPeV1: To understand RNA-based assembly of picornaviruses using HPeV1 as a model system because this virus shows strong interactions at the interface of the capsid proteins and the viral genome.
3. MATERIALS AND METHODS

The majority of the thesis is about the determination of the virus structures either in isolation or in complex with soluble cellular receptors. Although the virus structure can be determined either by X-ray or using electron cryo-microscopy, the choice depends on the sample properties. When the sample is structurally homogenous, of reasonable size and crystallizes then X-ray is the method of choice but in this work, the samples were a mix of genome-filled and empty capsids and when they were in complex with receptors, there was variability in terms of the number of receptors bound to the capsid. So for these reasons, electron cryo-microscopy was employed. In addition, the inherent icosahedral symmetry present in these viruses was imposed in the 3-D image reconstruction which gives a reasonable virus reconstruction with a lower number of particles as described in details in ‘material and method’ section of study I and II (also see section 1.2.3). Besides, icosahedral reconstruction, asymmetric reconstruction was also carried out for receptor-virus complexes in study II because receptor binding to the virus was suboptimal causing its density to smear in the icosahedral reconstruction. In order to investigate the reason behind this sub-stoichiometric binding of the receptor to the capsid, we adopted the asymmetric reconstruction approach used for tailed Sf6 bacteriophages (Parent et al. 2012). I modified this asymmetric reconstruction method by manually attaching a receptor density on one of the 60 equivalent binding positions on a capsid and using it as the reference map for aligning one of the 60 possible positions of receptor-bound virus capsid particles in the asymmetric mode of AUTO3DEM. This asymmetric method helped us to describe the steric hindrance caused by receptor binding to the virus.
The combination of icosahedral reconstruction with flexible fitting (see section 2.3) was maximally utilized in study I and II. Flexible fitting of poor homology models into sub-nanometer resolution 3-D density maps (study I) was particularly challenging in terms of segmentation of the predominantly \(\beta\)-sheet density map, fitting at the interfaces of the capsid proteins, fitting of flexible regions and identification of unreliable regions due to errors in the homology modelling. The lessons learnt from flexible fitting using two different approaches in study I and II led us to develop a general protocol for fitting homology models in low resolution virus 3-D density maps (study III). Study III also demonstrated methods of modelling remote homologs into density maps, modelling of expansion states of macromolecules using known X-ray structures into EM density maps. Finally, study III combined the results for homology models of two different conformational states especially looking at the validation and comparison of different approaches.

**Table 1** Methods used in the study

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in the study</th>
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<tr>
<td>Virus culture and purification</td>
<td>I, II, IV</td>
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<tr>
<td>Electron cryo-microscopy</td>
<td>I, II, IV</td>
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<tr>
<td>Three dimensional image reconstruction</td>
<td>I, II</td>
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<tr>
<td>Homology modelling</td>
<td>I, III</td>
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<tr>
<td>Rigid and flexible fitting</td>
<td>I, II, III</td>
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<tr>
<td>Reverse transcription- polymerase chain reaction</td>
<td>I, IV</td>
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<tr>
<td>Sequence alignments</td>
<td>I, IV</td>
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<tr>
<td>Viral RNA extraction</td>
<td>I</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>I</td>
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<tr>
<td>Mammalian cell culture</td>
<td>I, II, IV</td>
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<tr>
<td>Recombinant protein purification</td>
<td>II</td>
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<td>Electron cryo-tomography</td>
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<td>Asymmetric reconstruction</td>
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<td>Difference imaging</td>
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<td>Surface plasmon resonance</td>
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<td>Biotin labeling</td>
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<td>Native polyacrylamide gel electrophoresis</td>
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<td>RNA SELEX</td>
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<td>Polymerase chain reaction</td>
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<td>Next generation sequencing</td>
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<td>Thermophoresis</td>
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4. RESULT AND DISCUSSION

4.1. RNA: cause and effect in viral entry

Virus capsids are metastable, undergoing a series of structural changes mainly in response to external factors such as interaction with the cellular receptors with the sole aim of multiplying the viral genome by infecting the host. In return, the genome stores all the information which the virus capsids will require for successful infection. In study I and II, we have looked at different events; all associated with a single goal of viral entry into a suitable host so as to release its RNA.

Conformational changes in capsid upon RNA release

The events occurring in the capsid on RNA release had largely been an unaddressed question in the field until recently. In study I & II, three-dimension reconstruction maps of CVA7 and CVA9 genome-filled and empty capsid were determined to between 6-10 Å, where α-helices were evident, but individual β-strands were difficult to discern. The CVA7 maps were fitted with homology models derived from I-TASSER (Roy et al. 2010) whereas the CVA9 atomic model (PDB: 1D4M) (Hendry et al. 1999) was utilized to fit into the CVA9 maps. By comparing the fitted models into the genome-filled and empty maps, the regions in the capsid undergoing conformational changes were identified. In addition, these changes in translation and rotation were quantified using a component placement score (Topf et al. 2008) for the corresponding capsid proteins and individual secondary structure elements between genome-filled and empty capsid fits. This had the advantage of allowing easy description of the key changes occurring, that were not evident by visual
inspection of the 3-D reconstructions alone. The largest translation observed was for VP1 in both CVA7 and CVA9 whereas most of the rotation was contributed by VP2 and VP3. The homology models of CVA7 were improved by the inclusion of the EV71 atomic models that were published in the interim (Plevka et al. 2012, Wang et al. 2012). This led to reduction in the component placement score compared to the one reported in study I and was actually more in line with concurrent results that came out for EV71 (Wang et al. 2012) and CVA16 (Ren et al. 2013). Taken together, these results for CVA7 and CVA9 indicated that the release of RNA is a concerted interplay between capsid proteins VP1-3 which resulted in expansion of the capsid, through VP2 and VP3 rotation of ~5°and VP1 translation of ~4Å. Recently, it has been shown that this results in externalization of the VP1 N-terminus and expulsion of VP4 and RNA for PV1 (Bostina et al. 2011), EV71 (Wang et al. 2012), HRV2 (Garriga et al. 2012) and CVA16 (Ren et al. 2013). Given the similarity of the conformational changes between these different viruses, it is likely that VP1 and VP4 are critical for RNA release in both CVA7 and CVA9.

**Does RNA exit from two-fold holes?**

The effect of these conformational changes can be readily appreciated at the two-fold axis of symmetry where the symmetry-related helices of VP2 move apart in the empty capsid as compared to the genome-filled capsid making the capsid porous in CVA7 and CVA9. These observations are in agreement with recent studies on expanded intermediate capsids of PV1 (Levy et al. 2010, Bostina et al. 2011), CVA16 (Ren et al. 2013), HRV2 (Pickl-Herk et al. 2013) or empty capsids of EV71 (Wang et al. 2012) and HRV2 (Garriga et al. 2012) where the expansion of the capsid and the rearrangement of the capsid proteins creates holes on the two-fold symmetry axis, which are
proposed to be RNA exit sites. This has been a matter of controversy in the literature, as the initial suggestions were that release happened through the vertices (Hadfield et al. 1997, Belnap et al. 2000b, Belnap et al. 2000a, Hewat et al. 2002, Hewat and Blaas 2004) (see section 1.1.3), so attempts to investigate poliovirus uncoating into liposomes assumed C5 symmetry and used a marker placed on the digitized data (Bubeck et al. 2005a). Just as with MS2 (Toropova et al. 2011, Dent et al. 2013), the assumption was false (see section 1.1.5). Latter attempts with tomography have given lower resolution results where the accurate pinpointing of the release has proven difficult (Levy et al. 2010, Bostina et al. 2011). The problem is exacerbated both by the featurelessness of picornavirus capsids, making orientation determination very difficult even at 10 Å resolution, and by the lack of a directional, well-structured internal control to constrain the refinement during subtomogram averaging, such as the F-pilus used in MS2 (Dent et al. 2013) (see section 1.1.5). The recent crystal structure of an expanded intermediate CVA16 capsid where the VP1 N-terminus (residue number 62 onwards, 1-61 residues unresolved) is seen protruding out of the capsid and making the two-fold holes large enough supports the argument that the RNA comes out of the two-fold (Ren et al. 2013) as observed in low resolution cryo electron-tomographs of an expanded poliovirus intermediate (Bostina et al. 2011). Furthermore, in recent work on HRV2 it has been demonstrated that the RNA obtains an ordered conformation prior to its release (Pickl-Herk et al. 2013) and the release is directional with the 3’ end of the RNA egressing first from the capsid (Kumar and Blaas 2013) which could be plausible because the comparatively short 3’ UTR have less secondary structure than the longer 5’UTR.
Is RNA release, a multi-step process?

In study II of this thesis, the structural changes in the CVA9 capsid on binding to its high affinity soluble receptor integrin αvβ6 (Heikkilä et al. 2009) were determined. From surface plasmon resonance, the Kd of the binding of integrin αvβ6 to surface coupled CVA9 was in the subnanomolar range. These integrin binding kinetics could be modelled either as two-state binding or parallel-reaction binding. The two-state binding model assumes change in integrin conformation upon binding to the virus whereas the parallel-reaction assumes that two different conformations of the integrin bind to the capsid either with different affinities or on different binding sites on the capsid. Both the kinetics models could be advantageous to virus infection. In accordance with the two-state model, the virus could bind to only one conformation of the integrin, leaving fewer potential ways for binding on cell surface, however, if the virus binding immediately cause change in integrin conformation that cascade into a series of events leading to internalization of integrin and virus entry then this two-state model could be advantageous for virus entry. Based on the parallel-reaction model, the virus could bind different conformational state integrins which increase the chance of virus binding to the cell surface; hence, this kinetic model could also be helpful in increasing the infection probability of the virus. Irrespective of the chosen kinetic models, the integrins did not occupy all sites on the capsid. This partial occupancy was observed from cryo-ET where different numbers and conformations of integrins were seen bound to the capsid surface. This variation in integrin conformation, high flexibility and low number of bound molecules on the capsid caused smearing of the integrin density on the capsid surface in the icosahedral reconstruction. To test whether or not this weaker density of integrin on capsid surface was due to steric hindrance, I made an asymmetric reconstruction of the CVA9-integrin genome-
filled capsid. To perform the asymmetric reconstruction, I prepared a reference model of integrin αIIbβ3 β-chain (PDB: 3FCS and 3FCU) (Zhu et al. 2008) attached to CVA9 genome-filled capsid at the position determined from CVA9-integrin αvβ6 icosahedral reconstruction. An orientation search was performed for each CVA9-integrin αvβ6 particle where one of the integrin on CVA9 surface was aligned to the reference model. This resulted in alignment of one integrin to the reference model whereas the rest of the integrins could be randomly distributed over the other 59 positions. The signal on the other 59 positions could be equal or stronger at some positions due to preferential distribution caused by either steric hindrance or cooperative binding. The asymmetric reconstruction showed strong density at the position where one of the integrin was aligned to the reference model whereas the rest of the other four equivalent positions around the same five-fold vertex showed weakest integrin signals at the positions closest to where the integrin aligned to the reference model. Using a reference model, where the integrin was bound to an incorrect position on the capsid such as the five-fold vertex showed a comparatively weaker density for the aligned integrins, in addition, density for the other 59 equivalent positions were also weaker in the incorrect model. This implied that the alignment of integrins on the other equivalent 59 positions is much more accurate when the model with integrin attached at the correct position was used. Overall, the asymmetric reconstruction showed that integrin binding to one of the five equivalent sites around the same vertex caused steric hindrance to binding of integrins on either side of it, however, the conformation of the bound integrin could not be discerned from this method.

By iMODfit based flexible fitting (Lopez-Blanco and Chacon 2013) of the CVA9 atomic model (PDB: 1D4M) (Hendry et al. 1999) into the capsid
with and without integrin and performing superimposition of the fits on each other showed no integrin-induced structural changes in the capsid proteins. This observation was seen for both genome-filled and empty capsids. Additionally, the percentage of empty particles in micrographs of capsids with or without integrin was similar. Taken together, these results implies that the integrin \(\alpha_v\beta_6\) acts as an attachment receptor only and further receptors for entry and uncoating are required for destabilizing the capsid and RNA release. Such distinction between attachment and entry receptors has been reported for three other picornaviruses: CVB3, CVA21, and echovirus 1 (Shafren et al. 1997, Xing et al. 2004, Milstone et al. 2005). Such a multi-step process for RNA uncoating by a virus could be useful for escaping the humoral immune response because different receptors may recognize different regions on the capsid so that blocking of an epitope by an antibody may not abort infection. Additionally, such a multi-step process could also be important to ensure that the virus genome is delivered to the correct part of the cell for viral genome replication.

Does RNA encode clues for pathogenesis and tropism differences among viral strains?

For many viruses, such as the CVA7 strains in study I, different strains show difference in tropism and pathogenesis. CVA7 Parker and USSR strains presented with acute flaccid paralysis in human and were pathogenic in suckling mice whereas CVA7 275/58 caused aseptic meningitis in humans but was non-pathogenic in suckling mice (Habel and Loomis 1957, Grist 1962, Richter et al. 1971). We carried out nucleotide sequencing of the capsid protein coding regions of CAV7 USSR and 275/58 strains and compared them to the
CAV7 Parker. This showed that the 275/58 strain was more distant from the Parker strain with ~82% nucleotide sequence similarity and about 96% amino acid sequence similarity. The USSR strains was nearly identical to the Parker strain with about 99.5% nucleotide sequence similarity and about 99% amino acid sequence similarity even though the Parker strain originated in the USA whereas the USSR originated in the former Soviet Union. In fact, these sequence variations held true when the whole genome of Parker, USSR and 275/58 strains were sequenced recently showing Parker and USSR strains to be nearly identical whereas 82.6% nucleotide sequence variation and 96.2% amino acid variation was found between 275/58 and Parker strain (Ylä-Pelto et al. 2013).
Figure 8. CVA7 capsid surface variations.

The CVA7 Parker and 275/58 strains amino acid sequences were aligned to CVA7 USSR strains. The variations in residues (red) were mapped on the pseudo-atomic CVA7 USSR capsid model.
Apparently, these amino acid variations in CVA7 strains were not found in linear regions but were distributed randomly throughout the sequence. Mapping the Parker and 275/58 strain variations on the capsid surface residues of CVA7 USSR pseudo-atomic models showed that variability on VP1’s surface accounted for most of the difference between the strains (Figure 8). These capsid surface variations could be the cause of distinct infections by CVA7 strains and represent hyper-variable regions in the viral RNA genome. Such a difference in pathogenesis and tropism of CVA7 is reminiscent of what is seen in tissue tropism of coxsackievirus subgroup A and B where coxsackievirus subgroup A sites of infection were heart and skeletal muscles in mice whereas subgroup B had broader tissue tropism and were found in the central nervous system, pancreas, brown fat and liver of the mice (Hyypiä et al. 1993).

4.2. Viral RNA guided assembly

Does the picornavirus RNA genome have packaging signals (PSs) for capsid assembly? This is a long-standing question in the field. In order to investigate this issue, HPeV1 was utilized, as a previous 3-D reconstruction (EMDB: 1690) had shown strong ordered density not only for the capsid, but also for the RNA, especially where the two met on the inner surface of the capsid (Seitsonen et al. 2010). No empty capsids were enriched in any fractions from the cesium chloride gradient used for virus purification unlike other picornaviruses for e.g. study I and II of this thesis where the virus band on the gradient contained genome-filled as well as empty capsids. These observations indicate that there is a strong association between the HPeV1 capsid proteins and the viral RNA resulting in very efficient assembly and packaging. In order to understand this association and to identify PSs on viral RNA, the HPeV1
Harris strain capsids were heat-dissociated into pentamers which have been shown to be the building blocks of capsid assembly by *in vitro* assembly assays on bovine enterovirus (Li et al. 2012). These pentamers were panned on to an artificial random library of 40mer RNA aptamers using systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990), in an approach similar to one that had been used for STNV (Bunka et al. 2011). SELEX is a technique developed to enrich functional moieties (aptamers) from a vast variety of DNA or RNA libraries generated randomly with enough diversity that it actually contains at least one of the possible functional moieties that we are hunting for (Joyce 1989, Ellington and Szostak 1990, Tuerk and Gold 1990). The screening for aptamers against HPeV1 pentamers was done by alternating between positive selection with pentamers and negative selection with undisrupted capsids and naked streptavidin beads. The 13th round aptamers were amplified by polymerase chain reaction and sequenced.

By aligning 423,932 unique sequences identified by next generation sequencing (NGS) to the HPeV1 Harris genome (Genbank ID: L02971) (Hyypiä et al. 1992) using an in-house alignment strategy, the five most frequently aligned aptamer sequences were identified. The alignment of the aptamers to the genome was assessed by a Bernoulli score which was generated by calculating the probability of having a number of mismatches between the aptamers and the genome over a length of 40 nucleotides. The alignment of an aptamer was considered significant when the Bernoulli score was 12 or more because a score of less than 12 could occur just by taking a random sequence of 40 nucleotides in length. This alignment of each aptamer sequence to any possible contiguous genomic fragment of 40 nucleotides in length in the Harris genome was done by sliding the aptamer sequence in 1 nucleotide increments.
along the genome resulting in a series of comparison frames. Additionally, alignment of at least 12 nucleotides length of the 5’ end of the aptamer sequence with the 3’ end of the genomic sequence and vice-versa, were also considered so that information from the 5’- and 3’-ends was not missed, in case the genome present itself in a circular form to the pentamers. In particular, we started the alignment procedure by aligning the last nucleotide of the aptamer sequence with the first nucleotide at the 5' end of the genome. The aligned aptamer sequences and the corresponding HPeV1 Harris genome sequences were folded in silico to identify those containing potential stem-loops using the MFOLD program (Zuker 2003). The alignment showed nucleotides 1500-2250 (capsid protein VP1 coding region) and nucleotides 6000-6750 (RNA-dependent RNA-polymerase coding region) on the genome as the regions with the highest Bernoulli score of 17/18. However, the majority of the aptamers were found to align to the 3’ UTR region but with a lower Bernoulli score.

In order to test whether the identified alignment hit regions were conserved across different strains of HPeV1; the 21 fully-sequenced strains of HPeV1 available in Genbank were aligned to each other. This alignment was done by initially dividing the genome sequences into 5’ UTR, coding region and 3’ UTR. The 5’ UTR and 3’ UTR nucleotide sequences and the translated coding region regions were aligned using Clustal-Omega (Sievers et al. 2011). The alignment of translated coding region was further improved by using PAL2NAL (Suyama et al. 2006) which does the nucleotide alignment using the amino acid alignment as the constraint. Finally, the alignment of 5’ UTR, coding region and 3’ UTR were assembled together to obtain the alignment of the full-length genomes. Comparison of these 21 aligned genome sequences to the alignment of aptamers to the Harris genome showed that the identified
alignment hit regions were relatively conserved compared to the rest of the genome. The genome regions with hits of the highest possible Bernoulli scores of 17 or 18 were folded into stem-loop structures by Mfold (Zuker 2003). Those folds which showed strongest similarity with the folds of the five most abundant aptamers returned by SELEX were chosen as the putative PSs. Nucleotide alignment of these PSs using ClustalW (Larkin et al. 2007) indicated a poly-uridine based common sequence motif.

A thermophoresis-based binding study on one of the putative PSs with isolated HPeV1 pentamers gave 12.5 nanomolar affinity. Hence this is preliminary evidence that there are selective, high affinity interactions between the HPeV1 genome and its capsid.

Looking at the geometry of these RNA-capsid interactions in the EM map, 5 PSs per pentamer and maximally 60 PSs for the whole capsid were expected. Our bioinformatics analysis identified 44 occurrences of the poly-uridine based PSs distributed throughout the genome. Comparatively, MS2 and GA bacteriophage (T=3) have multiple PSs distributed at 25 and 23 positions throughout the genome and the STNV (T=1) has 30 occurrence of the PSs motif in its genome (Dykeman et al. 2013). The expectation, given the identification of these PSs, is that the folding of the viral RNA is not a sequential process as with capsid assembly nucleating from one specific position on the genome, rather, RNA folding could be a ‘collapse’ where multiple specific PSs on the genome result in efficient co-assembly of pentamers and RNA into a ‘virion’ as has been seen for MS2 and STNV using smFSC (Borodavka et al. 2012, 2013, Stockley et al. 2013).

One may argue whether or not these PSs need to be specific to a virus type or can they be simple stem-loop structures. The comparison of MS2, GA
and STNV PSs showed that the stem-loop structural motif varies in both length and consensus sequence between the viruses. This variation indicates that viral RNAs have evolved to get specifically packaged into their specific capsids (Bunka et al. 2011, Dykeman et al. 2013). The assembly with non-specific PSs can still occur but will be far more inefficient (Borodavka et al. 2013).

In conclusion, the initial analysis of different HPeV strains indicates conservation of the PSs across different strains. This is not too surprising because these interactions are not under the same selection pressure from the host as the exposed regions of the capsid. There is a need to develop competitive assembly assays in order to test this hypothesis further. The results are encouraging as the basis for developing new antiviral drugs targeting virus assembly.

4.3. Improving model fitting in EM density maps

Study I on CVA7 utilized fitting of homology models derived from remote homologs into the EM density maps of genome-filled and empty capsids at sub-nanometer resolution of 6 and 8Å, respectively. The flexible fitting was performed by Flex-EM by running it in the simulated annealing molecular dynamics mode (Topf et al. 2008). The program treats secondary structure elements as rigid bodies and tries to fit them into the EM density maps. Some regions of the homology models were not fitted into the maps due to low confidence in homology modelling in those regions or ambiguous segmentation of the density maps especially at the subunit interfaces. For study II on CVA9, we fitted atomic models available from X-ray into the EM density maps of genome-filled and empty capsids around the resolution of 9Å using another flexible fitting program, iMODfit due to its higher computational speed.
(Lopez-Blanco and Chacon 2013). It works by normal mode analysis where some dihedral angles are fixed and other are kept flexible.

Experience of working with two different flexible fitting programs led us to develop a generalized protocol for fitting homology models into EM density maps of icosahedral viruses and validating these fits (study III). The protocol begins with generation of homology models and their initial rigid fitting into the target EM density map followed by flexible fitting using Flex-EM and iMODfit programs. Regions of consensus and non-consensus fits between Flex-EM and iMODfit are identified by calculating Ca root mean square deviation (RMSD) between the fits from these two program and by segment cross correlation score (SCCC) estimation between the simulated map of the fit and its corresponding EM density map, followed by SCCC comparison for each segment between Flex-EM and iMODfit. The segment in SCCC can be a secondary structure element (SSE) like α-helices and β-sheets, or an individual protein of an assembly of proteins. Identified regions of non-consensus are further flexibly fitted using Flex-EM to improve the fit. Recently, such a protocol for identifying consensus and non-consensus region using multiple fitting programs has also been described elsewhere (Ahmed et al. 2012). Several important aspects related to fitting homology models into EM density maps are also highlighted in study III such as errors in homology modelling, segmentation difficulties, over-fitting, fitting one conformation of a model into the density map of a different conformation.

To explore the effect of the homology modelling errors that creep into the fitting, we made a homology model of actin using MODELLER (Sali and Blundell 1993) from actin-related protein 3 (Arp3) from an Arp2/3 complex (PDB: 1K8K, chain A) (Robinson et al. 2001) and fitted it into a 9Å resolution
EM density map derived from an actin atomic model (PDB: 2A40) (Chereau et al. 2005) using Flex-EM (Topf et al. 2008) and iMODfit (Lopez-Blanco and Chacon 2013). Comparing these fits against each other and against the crystal structure by Ca RMSD and SCCC showed regions for which the two flexible fitting programs did not have consensus fits. In addition, calculation of local residue error in the actin homology model using the QMEAN server (Benkert et al. 2009) identified six unreliable loop segments when a cut-off >3.5Å was used. The local residue error was calculated based on comparing the absolute quality of the query models to the high-resolution X-rays structures of similar size (Benkert et al. 2009). These loop segments were connected to the SSEs with low consensus fit between Flex-EM and iMODfit. Further refinement of the fit from iMODfit was done using Flex-EM by relaxing all the SSEs having lower SCCC values for iMODfit compared to Flex-EM. This refinement step using Flex-EM improved the fit only for helix 76-78 showing the errors of modelling could not be completely abolished during fitting.

Fitting models into macromolecular assemblies such as virus capsids comes with problems of fitting model of one conformation into a density map of another conformation, segmentation of individual capsid proteins and asymmetric units from the entire density map and issues of overfitting. To demonstrate these issues, we fitted a crystal structure of the EV71 genome filled capsid (PDB: 3VBF) (Wang et al. 2012) into an expanded procapsid EV71 EM density map (EMDB: 5557) (Cifuente et al. 2013) at 9Å resolution for which a corresponding crystal structure (PDB: 4GMP) was also available to serve as the target/reference model (Cifuente et al. 2013). The procapsid EM density map (EMDB: 5557) was segmented into an asymmetric unit by zoning 9Å around the EV71 crystal structure (PDB: 3VBU) asymmetric unit using the
Chimera ‘zone’ tool (Pettersen et al. 2004). The initial rigid fitting was done with the ‘Fit in map’ tool in Chimera followed by flexible fitting in Flex-EM and iMODfit. The SSEs Cα RMSD comparison between Flex-EM and iMODfit showed regions of consensus (<5Å difference in Cα RMSD) and non-consensus. These regions showed direct correlation with the regions in the target map (PDB: 4GMP) where Flex-EM and iMODfit consensus regions have lower Cα RMSD between the target model and fits from Flex-EM and iMODfit compared to the non-consensus regions. This was further validated by generating the whole virus capsid from the fits from Flex-EM and iMODfit where non-consensus regions (VP1 helices: 169-172; VP2 helices 126-128, 159-167) with >10Å difference in Cα RMSD between Flex-EM and iMODfit were found to be involved in clashes at the interfaces of the asymmetric unit with its neighbours. These regions at the interface of asymmetric unit represent the areas where the segmentation is difficult possibly because of intertwining of the proteins from adjacent asymmetric unit. The issue of overfitting was highlighted with the non-consensus fit for VP2 β-hairpin (strands: 83-87, 90-94) where the SCC score was similar for both Flex-EM and iMODfit when compared with the target model but the Cα RMSD was significantly higher for iMODfit (11Å) in comparison to Flex-EM (4Å). The VP2 β-hairpin was possibly fitted incorrectly by iMODfit as this was a region with badly resolved neighbourhood densities. A hybrid final model was generated by refining those SSEs in Flex-EM which had lower SCC values compared to the corresponding iMODfit SSEs.

After doing bench testing on actin and EV71, we performed flexible fitting on CVA7. The CVA7 homology models used in study I were generated from a series of 10 remote homologs where in the best cases, the sequence
identities were only 42% for VP1, 58% for VP2 and 52% for VP3. With the availability of crystal structures from a close homolog, EV71 (Wang et al. 2012) having sequence identity of 60% for VP1, 84% for VP2 and 76% for VP3 we were able to generate improved homology models by giving empty (PDB: 3VBO) and genome-filled EV71 crystal structures (PDB: 3VBF) as the templates in I-TASSER (Roy et al. 2010). On assessing these models by local residue error profile in the QMEAN server (Benkert et al. 2009), we found that the β-barrels and the C-termini of VP1, VP2 and VP3 had lower local residue error compared to the models used in study I. Although in the new models we removed the unreliable 1-73 N-terminus residues and 278-296 C-terminus residues from VP1 and 1-40 N-terminus residues from N-terminus of VP3, we still had 47 more residues in VP1 and 12 more in VP3 compared to the models in study I. The VP1, VP2 and VP3 were assembled into an asymmetric unit by superimposing them on the asymmetric units of EV71 (PDB: 3VBO used for empty capsid models superimposition; PDB: 3VBF used for genome-filled models superimposition) in order to minimize intra-subunit clashes and segmentation errors. This was followed by rigid fitting in the segmented densities of VP1, VP2 and VP3 from study I using the ‘fit in map’ tool in Chimera (Pettersen et al. 2004). The rigidly fitted asymmetric unit was then used to re-segment the CVA7 empty and genome-filled EM density maps by zoning with a radius of 0.9 nm around the asymmetric unit. This was followed by flexible fitting in the segmented maps using Flex-EM and iMODfit.

Fitting in CVA7 empty density map using Flex-EM and iMODfit gave similar fits for β-barrels of VP2 and VP3 based on SCCC values, however for VP1, Flex-EM was slightly better with a SCCC score of 0.62 than iMODfit with a SCCC value of 0.58. Furthermore, there were some helices and sheets in
VP1, VP2 and VP3 which were fitted poorly in Flex-EM compared to iMODfit and vice-versa based on SCCC values. Fitting results in the CVA7 genome-filled density map were comparable using both Flex-EM and iMODfit except for the VP2 β-hairpin as was seen in the EV71 fitting test case. This discrepancy between the two programs for the VP2 β-hairpin was identified from the Ca RMSD which was found to be relatively high at 4.9Å. Interestingly, the Ca RMSD of the VP2 β-hairpin between fits of empty and genome-filled CVA7 using Flex-EM were the same as the corresponding one for EV71 empty (PDB: 3VBO) and genome-filled (PDB: 3VBF) with a value of 4.4Å. This better fit of the VP2 β-hairpin with Flex-EM in case of CVA7 and EV71 fitting could be due to the two-stage refinement process used by it where initially the Flex-EM fitting is done for the models more conservatively by clustering together SSEs with closer contact into a single rigid body using the program RIBFIND (Pandurangan and Topf 2012). This is followed by relaxing all the SSEs as individual rigid bodies in second stage of refinement. This two-stage refinement has been shown to minimize over-fitting during flexible fitting (Pandurangan and Topf 2012). Finally, to arrive at the hybrid final model, all the SSEs which had poor SCCC values in Flex-EM compared to iMODfit were further refined in Flex-EM. The whole capsid was generated for empty and genome-filled capsid models by the ‘oligomer generator’ tool in VIPERdb (Carrillo-Tripp et al. 2009) and any inter subunit clashes were resolved using the Flex-EM conjugant gradient protocol (Topf et al. 2008). In terms of biology, the models of CVA7 from study I and III were still comparable, however, as the new models had fewer truncated residues there was less uncertainty for movement of subunits within densities, hence, the overall conformational changes observed between empty and genome-filled state were more moderate. Such a protocol is not only applicable for fitting
models into virus capsid map but can be extended to other types of density maps as we demonstrated in our actin test case. The protocol presented in this work is similar to the proposal given by Ahmed and Tama (Ahmed and Tama 2013) where they advocated for comparing fitting results from multiple fitting programs so that a consensus fit can be obtained, however, our protocol besides doing fitting from two different programs, also performs an important validation step of these fits based on local fits of models into the density maps.
5. CONCLUSIONS AND FUTURE STUDIES

The studies in this thesis along with recent work of others in the field have built a foundation for understanding the basic mechanisms of picornavirus entry and assembly (Figure 9). This thesis has elaborated the structural events associated with RNA egress by identification and quantification of concerted movements of capsid proteins between genome-filled and empty capsid state. These movements were mainly associated with translation of VP1 and rotation of VP2 and VP3 which resulted in opening of holes at the capsid with the largest ones at the two-fold symmetry axis which could be the sites for RNA release. Identification of these structural events were improved further by

Figure 9. Schematic summary of thesis work.
A) CVA7 and CVA9 attachment, entry and uncoating. B) HPeV1 assembly.
designing of flexible fitting based protocol for fitting models derived from different complementary methods into the EM density maps. This work also elucidated the molecular mechanism of receptor-virus binding where structural evidence was provide for integrin $\alpha_6\beta_6$ functioning merely as an attachment receptor. Moreover, a functional role for RNA in assembly was also shown using HPeV1 as an example. Multiple PSs on RNA responsible for capsid assembly were identified using SELEX. These PSs had a poly-uridine based common motif.

But as is often the case in science, these findings gave rise to more questions than were originally answered. How does the virus choose just one exit point for the RNA out of 30 potential sites on the capsid and which is that site? How does a virus evolve to use cellular receptors as attachment or internalizing receptors? Apart from packaging signals on viral RNA, what other factors affect capsid assembly? In how many ways can viral co-assembly and packaging happen? Does the variation in surface capsid residues really cause the difference in pathogenesis and tropism in vivo? These are a few of the many questions on which we would like to work in the future.

Overall, this thesis provides several important targets such as the symmetry-related helices from two VP2 molecules in CVA7 and CVA9 or the RNA based PSs in HPeV1 which could be exploited for therapeutic potential.
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