Virion structure and genome delivery mechanism of sacbrood honeybee virus

Michaela Procházková,a Tibor Fúzik,a Karel Škubník,a Jana Moravcová,a Zorica Ubiparip,b Antonín Přidal,b and Pavel Plevkab,1

*Central European Institute of Technology, Masaryk University, 625 00 Brno, Czech Republic; and †Faculty of Agronomy, Mendel University, 613 00 Brno, Czech Republic

Edited by Wolfgang Baumeister, Max Planck Institute of Biochemistry, Martinsried, Germany, and approved June 14, 2018 (received for review December 22, 2017)

Infection by sacbrood virus (SBV) from the family Iflaviridae is lethal to honey bee larvae but only rarely causes the collapse of honey bee colonies. Despite the negative effect of SBV on honey bees, the structure of its particles and mechanism of its genome delivery are unknown. Here we present the crystal structure of SBV virion and show that it contains 60 copies of a minor capsid protein (MiCP) attached to the virion surface. No similar MiCPs have been previously reported in any of the related viruses from the order Picornavirales. The location of the MiCP coding sequence within the SBV genome indicates that the MiCP evolved from a C-terminal extension of a major capsid protein by the introduction of a cleavage site for a virus protease. The exposure of SBV to acidic pH, which the virus likely encounters during cell entry, induces the formation of pores at threefold and fivefold axes of the capsid that are 7 Å and 12 Å in diameter, respectively. This is in contrast to vertebrate picornaviruses, in which the pores along twofold icosahedral symmetry axes are currently considered the most likely sites for genome release. SBV virions lack VP4 subunits that facilitate the genome delivery of many related dicistroviruses and picornaviruses. MiCP subunits induce liposome disruption in vitro, indicating that they are functional analogs of VP4 subunits and enable the virus genome to escape across the endosome membrane into the cell cytoplasm.

Results and Discussion

SBV Virion Structure and Its Comparison with Iflaviruses Containing P-Domains. The virion structure of SBV has been determined to a resolution of 2.1 Å using X-ray crystallography (SI Appendix, Table S1). The maximum diameter of the SBV virion is 312 Å, which is 90 Å less than the maximum diameters of related iflaviruses DWV and SBPV (7, 8, 13). The virions of DWV and SBPV are larger because their VP3 subunits contain 160-residue-long C-terminal extensions, which fold into globular P-domains positioned at the virion surface (Figs. 1 and 2). The capsid of SBV is of spherical shape, with plateaus around icosahedral fivefold symmetry axes and shallow depressions at twofold

Significance

Honey bee pollination is required to sustain the biodiversity of wild flora and for agricultural production; however, honey bee populations in Europe and North America are declining due to virus infections. Sacbrood virus (SBV) infection is lethal to honey bee larvae and decreases the fitness of honey bee colonies. Here we present the structure of the SBV particle and show that it contains 60 copies of a minor capsid protein attached to its surface. No similar minor capsid proteins have been previously observed in any of the related viruses. We also present a structural analysis of the genome release of SBV. The possibility of blocking virus genome delivery may provide a tool to prevent the spread of this honey bee pathogen.

Author contributions: M.P. and P.P. designed research; M.P., T.F., J.M., Z.U., and A.P. performed research; M.P., T.F., K.S., and P.P. analyzed data; and M.P., T.F., and P.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: Cryo-EM maps of the SBV virions from the different conditions have been deposited in the Electron Microscopy Data Bank [accession nos. 3863 (native virion, pH 7.4); 3881 (empty particle, pH 7.4); 3865 (virion, pH 5.8); 3866 (empty particle, pH 5.8, expansion state I); and 3867 (empty particle, pH 5.8, expansion state II), and the corresponding coordinates have been deposited in the Protein Data Bank (PDB ID codes SY7P (native virion, pH 7.4); 6E1W (empty particle, pH 7.4); 6EGV (virion, pH 5.8); 6EGX (empty particle, pH 5.8, expansion state I); and 6EH1 (empty particle, pH 5.8, expansion state II)). The crystal structure of the SBV virion also has been deposited in the Protein Data Bank (PDB ID code 5L5I). The consensus nucleotide sequence of the SBV capsid proteins has been deposited in GenBank (accession no. KY617033).

1To whom correspondence should be addressed. Email: pavel.plevka@ceitec.muni.cz.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1722018115.

Published online July 9, 2018.
Organization of P1 polyproteins of iflaviruses SBV and DWV, dicistrovirus cricket paralysis virus, and picornavirus poliovirus-1. Capsid protein MiCP of SBV and P-domain VP2, and VP3 organized with pseudo-T3 icosahedral symmetry (Fig. 3). The VP1 subunits form pentamers around fivefold axes, whereas the VP2 and VP3 subunits constitute heterohexamers positioned at the icosahedral threefold axes. The major capsid proteins have jelly roll β-sandwich folds with β-strands named according to the virus capsid protein convention B–I (6). The two antiparallel β-sheets forming the core of each of the capsid proteins contain the strands BIDG and CHEF, respectively (Fig. 3A). The N termini of the capsid proteins are located on the inside of the capsid, whereas their C termini are exposed at the virion surface. The crystallographic electron density map of the SBV virion enabled the building of residues 1–243 out of 247 of VP1; residues 3–241 out of 242 residues of VP2; and residues 1–272 out of 280 residues of VP3. SBV encodes a 36-residue-long VP4 subunit (Fig. 2); however, the electron density map of SBV virion does not contain resolved features that could be interpreted as residues of VP4.

In contrast to all other viruses from the order Picornivirales that have been structurally characterized at high resolution so far (32 picornaviruses, 4 dicistroviruses, and 2 iflaviruses), the SBV virion contains a short protein attached to the capsid surface (Fig. 3A). We termed the peptide the MiCP. The structure of the MiCP could be built for residues 22–47 out of 48 residues. The amino acid sequence of the MiCP is located between the C terminus of VP3 and the N terminus of VP1 in the P1 polypeptide of SBV (Fig. 2). LC-MS-MS was used to verify the protein cleavage sites that separate the MiCP from VP3 and VP1 (SI Appendix, Fig. S1). The identified cleavage site, Ser708/Arg709/Arg710, is in agreement with the previously characterized target sequences of picornavirus-like proteases (16, 17).

VP1 subunits of enteroviruses have been shown to form hydrophobic pockets that can be targeted by artificial compounds that prevent virus–receptor interaction or genome release (18–20). Similar to SBPV and DWV (7, 8), the VP1 of SBV does not contain such a pocket and, it is unlikely that the virus could be inhibited by capsid-binding inhibitors targeting the hydrophobic core of VP1.

SBV Virions Do Not Contain VP4 Subunits. The formation of mature infectious virions of most picorna-like viruses requires the cleavage of VP4 subunits from the N terminus of their VP0 precursors that is not performed by the virus proteases (21–23). It has been speculated that the proteolysis of the VP4 subunits of picornaviruses is catalyzed by the RNA genome (24, 25). In dicistroviruses, a conserved motif, Asp-Asp-Phe (DDF), in VP1 has been shown to catalyze the VP4 cleavage (22). Triatoma virus and black queen cell virus, from the family Dicistroviridae, do not encode VP4. VP4 has been shown to be essential for the infectivity of picornaviruses (16, 17).}

Fig. 1. Virion structures of SBV (PDB ID code 5LSF) (A), DWV (PDB ID code SL7Q) (B), SBPV (PDB ID code SL96) (C), and poliovirus 1 (PDB ID code 1ASJ) (D). The molecular surfaces of the respective virions are rainbow-colored according to their distance from the center. The locations of the selected symmetry axes are denoted by pentagons for fivefold, triangles for threefold, and ellipses for twofold.

Fig. 2. Organization of P1 polyproteins of iflaviruses SBV and DWV, dicistrovirus cricket paralysis virus, and picornavirus poliovirus-1. Capsid proteins within P1 are labeled and colored according to the picornavirus convention: VP1 in blue, VP2 in green, VP3 in red, and VP4 in yellow. The MiCP of SBV and P-domain of DWV are highlighted in magenta. Note that translation of the P1 sequence of CrPV is initiated from an independent internal ribosomal entry site located after the coding sequence for the virus polymerase. Arrowheads indicate positions of protease cleavage sites, and the target cleavage sequences are shown. Parts of the proteins resolved in experimentally determined structures are shown in bright colors; the parts highlighted with hatching are not structured.
not contain structured VP4 subunits (26, 27), but the VP4 peptides are present in the virions and likely function in the transport of the genome across the endosome membrane into the cell cytoplasm (27). Some iflaviruses, including SBV and SBPV, also contain the DDF motif in their VP1 subunits (7, 8). The DDF motif of VP1 of SBV is located at the inner face of the SBV virion next to the N terminus of the VP3 subunit of another protomer from the same pentamer (SI Appendix, Fig. S2). Therefore, the DDF motif might function in the cleavage of VP0 subunits of SBV. Nevertheless, VP4 subunits are not detected in the electron density maps of SBV virions and could not be detected in the particles by MS analysis (SI Appendix, Fig. S1). Similarly, VP4 subunits were not resolved in the structures of SBPV and DWV (7, 8), and they likely were not present in the virions. It is possible that the short 38-residue VP4 subunits of SBV diffuse from the virions after the maturation cleavage of VP0. Iflaviruses lacking the VP4 subunits need to use a different mechanism to penetrate the host membrane than that used by the previously studied picornaviruses and dicistroviruses.

**Structure and Putative Receptor-Binding Function of the MiCP.**

The MiCP is a 48-aa-long protein with a molecular weight of 5.4 kDa. Residues 1–21 of the MiCP, which are not resolved in the SBV virion structure, are more varied among the different SBV isolates than residues 22–47, which form the structured part of the peptide (SI Appendix, Fig. S3). The higher tolerance of the flexible part of the MiCP to mutations indicates that its biological function might not depend on a specific structure. In contrast, the structured part is more conserved, as it is required for binding to the virion surface. The structured 26 residues of MiCP form a loop that turns 360° on itself (Fig. 3A). The MiCP is positioned above the core of subunit VP1 almost equidistant from the icosahedral twofold, threefold, and twofold symmetry axes of the capsid (Fig. 3A). The N-terminal arm of the MiCP extends toward the icosahedral fivefold axis and interacts with subunits VP1 and VP3. The MiCP binds to the capsid through an interface area of 1,100 Å² formed by 24 residues of VP2, 7 residues of VP1, and 6 residues of VP3 (Fig. 4A). An occupancy refinement showed that MiCP peptides are present in all 60 positions at the virion surface (Table 1).

The volume at the surface of the SBV virion occupied by the MiCP is taken up by the EF loop known as the “puff” loop from the VP2 subunit in enteroviruses (Fig. 4B and C) (9, 28). In poliovirus 1, the 65-residue-long puff loop forms the outer rim of the canyon (Fig. 4C) (28). In contrast, the puff loop of SBV is only 18 residues long (Fig. 4B). In addition, subunit VP3 of SBV lacks a loop known as a “knob” formed by residues before the β-strand B of enteroviruses (Fig. 4D and E). It has been shown that residues from the puff and knob of many enteroviruses participate in receptor binding (29). Therefore, because of its location at the virion surface, it is possible that the MiCP is involved in SBV receptor recognition.

**Evolutionary Relationships Within the Family Iflaviridae.** There are no structural or sequence similarities between the 48-residue-long MiCP of SBV and the 160-residue-long P-domains of SBPV...
and DWV. Nevertheless, the locations of the sequences coding the MiCP and P-domains in Iflavirus genomes show that both the MiCP and P-domains evolved as C-terminal extensions of VP3 subunits (Fig. 2). The evolution of the MiCP required the introduction of a cleavage site for virus protease 3C. The phylogenetic tree based on a comparison of the P1 sequences of iflaviruses shows that the family can be divided into two groups: viruses that lack P-domains and viruses with P-domains (Fig. 5 A and SI Appendix, Fig. S4). The P-domain appears to be a filial feature. Based on sequence analysis, it is not clear whether all the iflaviruses that lack the P-domains contain MiCPs. A structure-based phylogenetic tree shows branching inside the Iflaviridae family of viruses with and without P-domains (Fig. 5 B), corroborating the sequence-based separation of P-domain–containing iflaviruses into a subgroup within the family Iflaviridae.

### Genome Release Mechanism of SBV

The cell entry mechanism of iflaviruses is unknown, but the process likely involves receptor-mediated

### Table 1. Comparison of SBV structures under various pH and expansion states

| Parameter                                      | Crystal structure | Virion, pH 7.4 | Empty particle, pH 7.4 | Virion, pH 5.8, expansion state I | Empty particle, pH 5.8, expansion state II |
|------------------------------------------------|-------------------|----------------|------------------------|-----------------------------------|------------------------------------------|
| Resolution, Å                                  | 2.10              | 3.22           | 3.87                   | 3.18                              | 4.06                                     | 7.25                                     |
| MiCP occupancy                                 | 1.02              | 0.86           | 0.77                   | 0.94                              | 0.93                                     | 0.79                                     |
| Particle radius, Å                             | 136.4             | 136.1          | 137.4                  | 136.1                             | 137.9                                    | 139.8                                    |
| Pentamer distance, Å                          | 135.8             | 134.4          | 135.7                  | 134.4                             | 136.2                                    | 138.4                                    |
| Pentamer contacts, Å                          | 4,250             | 4,550          | 1,750                  | 4,450                             | 1,750                                    | 700                                      |
| Average B-factor, Å                           | 25                | 39             | 88                     | 43                                | 118                                      | 200                                      |
| Diameter of pore on threefold axis, Å         | 4.4               | 3.6            | 4.4                    | 3.6                               | 5.0                                      | 7.4                                      |
| Average B-factor of residues close to threefold axis, Å² | 23                | 35             | 104                    | 40                                | 143                                     | 200                                      |
| Diameter of pore on fivefold axis, Å          | 5.2               | 5.2            | 5.6                    | 5.6                               | 5.0                                      | 12.0                                     |
| Average B-factor of residues close to fivefold axis, Å² | 19                | 36             | 102                    | 41                                | 137                                     | 200                                      |
| Diameter of pore on twofold axis, Å           | 2.6               | 2.6            | 2.8                    | 2.4                               | 3.0                                      | 2.8                                      |
| Average B-factor of residues close to twofold axis, Å² | 21                | 35             | 55                     | 37                                | 73                                      | 200                                      |

*Particle radius is defined as the distance of the center of mass of the icosahedral asymmetric unit from the particle center.

†Pentamer distance is defined as the distance of the centers of mass of two neighboring pentamers.

**Fig. 5.** Phylogenetic analyses of viruses from the families Iflaviridae, Dicistroviridae, and Picornaviridae. (A) Maximum likelihood tree constructed from whole polyprotein sequences of selected iflaviruses with cricket paralysis virus as an outgroup (highlighted in red). Iflaviruses with P-domain are highlighted in the magenta box. The dashed box indicates viruses with observed MiCP; the asterisk indicates viruses with an experimentally determined virion structure. Values at nodes represent thorough bootstrap support. (B) Structure-based neighbor-joining phylogeny tree with picornaviruses highlighted in blue, dicistroviruses in red, and iflaviruses in green. The subgroup of iflaviruses with P-domains is highlighted in magenta.
The MiCP induces disruption of liposomes at pH 5.5. Liposomes contain pores of 5.0 Å in diameter located at the icosahedral threefold axes, whereas the pores in the more expanded particle are 7.4 Å in diameter (Fig. 6A and B, Table 1, and SI Appendix, Fig. S8). These pores are not of sufficient size to allow passage of the single-stranded SBV RNA genome. However, amino acids that form the loops of capsid proteins adjacent to the pores have higher temperature factors than the rest of the structure (Table 1). This indicates that the loops are flexible. Furthermore, the presence of two expansion intermediates suggests that the empty SBV capsids are dynamic. The possible role of pores at threefold axes of SBV capsids as channels for genome release is consistent with our previous study of the genome release of SBPV (15). In contrast, Organtini et al. (13) speculated that the genome of DWV may escape from particles through a channel in a fivefold vertex of its capsid. In native SBV virions, the N termini of VP3 subunits form a 5.2-Å narrow iris-like constriction of the channel along the fivefold axis (Fig. 6C, Table 1, and SI Appendix, Fig. S8). However, in the more expanded acidic pH empty capsid of SBV, residues 1–48 of VP3 are not structured (Fig. 6D and SI Appendix, Fig. S8). Thus, the more expanded empty SBV particles also contain 12-Å-diameter pores along the fivefold axes (Fig. 6D, Table 1, and SI Appendix, Fig. S8). The putative release of the SBV genome through pores along threefold or fivefold symmetry axes differs from the currently accepted genome release mechanism of picornaviruses, in which the pores along twofold icosahedral symmetry axes have been implicated (35–37). Pores along the twofold axes of SBV particles are not expanded after genome release, as is the case in enteroviruses (Fig. 6E and F, Table 1, and SI Appendix, Fig. S8).

endocytosis, as is the case for related picornaviruses (29). Endosomal entry involves exposure of the virions to an environment with acidic pH (30, 31). In picornaviruses, the acidic pH triggers the formation of activated (A) particles that are expanded, contain pores in capsids, and spontaneously release their genomes (29, 31–33). Freshly purified samples of SBV contain 5% empty particles (SI Appendix, Fig. S5A). We used cryo-EM to determine the structures of the full virions and empty particles to resolutions of 3.2 Å and 3.8 Å, respectively (SI Appendix, Figs. S6 and S7 and Table S1). The structure of the full virion is nearly identical to that determined by X-ray crystallography, with an rmsd of the corresponding Cα atoms of the two structures of 0.44 Å (SI Appendix, Table S2). The structure of the empty virion at pH 7.4 (A, C, and E) and the more expanded empty particle at pH 5.8 (B, D, and F) are shown.

![Comparison of capsid protein conformations close to the threefold, fivefold, and twofold axes of SBV virions and empty particles. The capsid protein loops that form contacts in the vicinity of rotation axes of the capsid are shown in cartoon representation. VP1, VP2, and VP3 are shown in blue, green, and red, respectively. Side chains of residues closest to the rotation axes are shown as sticks; the density map is depicted as a blue mesh. Details of native virion at pH 7.4 (A, C, and E) and the more expanded empty particle at pH 5.8 (B, D, and F) are shown.](image)

Fig. 6. Comparison of capsid protein conformations close to the threefold, fivefold, and twofold axes of SBV virions and empty particles. The capsid protein loops that form contacts in the vicinity of rotation axes of the capsid are shown in cartoon representation. VP1, VP2, and VP3 are shown in blue, green, and red, respectively. Side chains of residues closest to the rotation axes are shown as sticks; the density map is depicted as a blue mesh. Details of native virion at pH 7.4 (A, C, and E) and the more expanded empty particle at pH 5.8 (B, D, and F) are shown.

![Normalized Fluorescence Intensity](image)

Fig. 7. The MiCP induces disruption of liposomes at pH 5.5. Liposomes containing carboxyfluorescein were mixed with MiCP at a final concentration of 2 μM in a solution at pH 7.4 (blue line) at the 50-s time point (MiCP/SBV arrow). Liposomes were mixed with SBV virions at a final concentration of 2 nM, corresponding to an MiCP concentration of 120 nM in a solution at pH 7.3 Å (SI Appendix, Figs. S6 and S7 and Table S1). The smaller particle contains pores 5.0 Å in diameter located at the icosahedral threefold axes, whereas the pores in the more expanded particle are 7.4 Å in diameter (Fig. 6A and B, Table 1, and SI Appendix, Fig. S8). These pores are not of sufficient size to allow passage of the single-stranded SBV RNA genome. However, amino acids that form the loops of capsid proteins adjacent to the pores have higher temperature factors than the rest of the structure (Table 1). This indicates that the loops are flexible. Furthermore, the presence of two expansion intermediates suggests that the empty SBV capsids are dynamic. The possible role of pores at threefold axes of SBV capsids as channels for genome release is consistent with our previous study of the genome release of SBPV (15). In contrast, Organtini et al. (13) speculated that the genome of DWV may escape from particles through a channel in a fivefold vertex of its capsid. In native SBV virions, the N termini of VP3 subunits form a 5.2-Å narrow iris-like constriction of the channel along the fivefold axis (Fig. 6C, Table 1, and SI Appendix, Fig. S8). However, in the more expanded acidic pH empty capsid of SBV, residues 1–48 of VP3 are not structured (Fig. 6D and SI Appendix, Fig. S8). Thus, the more expanded empty SBV particles also contain 12-Å-diameter pores along the fivefold axes (Fig. 6D, Table 1, and SI Appendix, Fig. S8). The putative release of the SBV genome through pores along threefold or fivefold symmetry axes differs from the currently accepted genome release mechanism of picornaviruses, in which the pores along twofold icosahedral symmetry axes have been implicated (35–37). Pores along the twofold axes of SBV particles are not expanded after genome release, as is the case in enteroviruses (Fig. 6E and F, Table 1, and SI Appendix, Fig. S8).
The MiCP May Facilitate Delivery of SBV Genome into Cytoplasm. MiCP subunits are tightly associated with the native virions of SBV (Table 1). In contrast, in empty SBV particles, particularly those exposed to acidic pH, the nucleoprotein density corresponding to the MiCP is visible only at lower contour levels than the rest of the capsid. The occupancy refinement indicates that some of the MiCP subunits detach from empty SBV particles at pH 5.8 (Table 1). The dissociation of MiCPs from the SBV capsid occurs in similar conditions as the release of VP4 subunits from the virions of dicistroviruses and picornaviruses before genome egress (29, 31–33). Furthermore, the hydrophobic profile of the MiCP is similar to that of the VP4 of HRV16 (SI Appendix, Fig. S3B). The MiCP with an N-terminal His6-SUMO tag is expressed as soluble protein in Escherichia coli and remains soluble after cleavage of the tag (SI Appendix, Fig. S9). However, MiCP expression blocks growth of the bacterial culture at 1 h after induction (SI Appendix, Fig. S9). In buffer with pH 5.5, MiCP subunits at micromolar concentrations induce the disruption of liposomes with phospholipid content mimicking that of endosomes (Fig. 7 and SI Appendix, Fig. S10) (38). The effect of SBV virions on liposomes in the same conditions is limited (Fig. 7 and SI Appendix, Fig. S10); however, in native conditions, receptor binding might contribute to membrane fusion of MiCP subunits from virions and thus allow the protein to disrupt the endosome membranes. The biological function of the MiCP may be similar to that of the VP4 of picornaviruses, to facilitate transport of the virus genome across the endosome membrane into the cell cytoplasm (39, 40).

In summary, our results show that SBV virions are structurally distinct from virions of DWV and SBPV. We demonstrate that acidic pH triggers expansion of SBV capsids, genome release, and the partial dissociation of MiCP subunits from the virion surface. Subsequently, the MiCP subunits may disrupt the endosome membrane to allow the virus genome to reach the cell cytoplasm and initiate infection.

Methods

The propagation of SBV in honey bee larvae was performed as described in The COLOSS BeeBOOK (41). Purified SBV was applied onto holey carbon grids and vitrified by plunge-freezing in liquid ethane. Micrographs were recorded with a Falcon II camera in a Titan Krios transmission electron microscope (Thermo Fisher Scientific). Acquired data were processed using the RELION package (42). Crystals of SBV were obtained from the obtained material with the hanging-drop technique in crystallization conditions containing 0.05 M magnesium chloride, 0.1 M MES, 8% isopropanol, and 4% PEG 4000. The MiCP was expressed in E. coli, purified with Ni-NTA chromatography, and diluted in two types of buffer (pH 8.0 and pH 5.5). A suspension of liposomes filled with carboxylfluorescein dye was distributed to a 96-well plate, and the MiCP was added to wells with respective buffer conditions. Resulting changes in fluorescence levels were recorded before and after the addition of 1% Triton X-100. The methodology is described in detail in SI Appendix, Methods.

Acknowledgments. X-ray data were collected at Soleil Synchrotron, beamline Proxima 1. We acknowledge the Central European Institute of Technology (CEITEC) Core Facilities Cryo-Electron Microscopy and Tomography, Proteomics, and Biomolecular Interactions, supported by Czech Infrastructure for Integrative Structural Biology Project LM2015043, funded by the Ministry of Education, University and Research (MEYS) of the Czech Republic. This research was carried out under the project CEITEC 2020 (LQ1601), with financial support from the MEYS under National Sustainability Program II. This work was supported by the IT4I Project (CZ.1.05/1.1.00/02.0070), funded by the European Regional Development Fund, and the national budget of the Czech Republic via the Research and Development for Innovations Operational Program, as well as the MEYS via Grant LM2011033. The research leading to these results received funding from the European Research Council under Grant Agreement 335855 (to P.P.) and EMBO Grant Agreement IG3041 (to P.P.).