

Regulation of membrane scission in yeast endocytosis

Deepikaa Menon¹ and Marko Kaksonen^{1*}

*For correspondence:

Marko.Kaksonen@unige.ch

¹Department of Biochemistry, University of Geneva, Geneva, Switzerland

Abstract

Introduction

In Clathrin-mediated endocytosis, a flat plasma membrane is pulled into a tubular invagination that eventually forms a vesicle. Forces that drive the transition from invagination to spherical vesicle in mammalian cells are provided by constriction of the GTPase Dynamin. Dynamin is now known to act in concert with the crescent-shaped N-BAR proteins Endophilin and Amphiphysin (ref. Dynamin papers). Proline-rich motifs on the Dynamin. In yeast cells, what causes membrane scission is unclear, although the yeast N-BAR protein complex Rvs has been identified as an important component of the scission module. In yeast, the Amphiphysin and Endophilin homologue Rvs is a heterodimeric complex composed of Rvs161 and Rvs167 (Friesen et al., 2006). Deletion of Rvs reduces scission efficiency by nearly 30% and reduces the invagination lengths at which scission occurs (ref Marko, wanda). Apart from a canonical N-BAR domain which forms a crescent-shaped structure, Rvs167 has a Glycine-Proline-Alanine rich (GPA) region and a C-terminal SH3 domain. Rvs161 and Rvs167 N-BAR domains are 42% similar, and 21% identical, but are not interchangeable (Sivadon, Crouzet and Aigle, 1997). The GPA region is thought to act as a linker with no known other function, while loss of the SH3 domain affects budding pattern and actin morphology. Most Rvs deletion phenotypes can however, be rescued by expression of the BAR domain alone (Sivadon, Crouzet and Aigle, 1997), suggesting that the BAR domains are the main functional unit of the Rvs complex. Homology modelling has shown that the BAR domain of Rvs167 is similar to Amphiphysin and Endophilin (Youn et al., 2010), and is therefore likely to function similarly to the mammalian homologues. In keeping with this theory, Rvs has been shown to tubulate liposomes in vitro (Youn et al., 2010). The Rvs complex arrives at endocytic sites in the last stage of the endocytosis, and disassembles rapidly at the time of membrane scission (Picco et al., 2015), consistent with a role in membrane scission. While it is known to be involved in the last stages of endocytosis, a mechanistic understanding of the influence of Rvs on scission however, remains incomplete. u89

We used quantitative live-cell imaging and genetic manipulation in *S.cereviciae* to investigate the influence of Rvs and several Rvs interacting proteins that have been suggested to have a role in scission. We found that arrival of Rvs to endocytic sites is timed by interaction of its BAR domain with a specific membrane curvature. The Rvs167 SH3 domain affects localization efficiency of the Rvs complex and also influences invagination dynamics. This indicates that both BAR and SH3 domains are important for the role of Rvs as a regulator of scission. We tested current models of membrane scission, and find that deleting yeast synaptojanins or dynamin does not change scission dynamics. Interfacial forces at lipid boundaries are therefore unlikely to be sufficient for scission, and forces exerted by dynamin are not required. Furthermore, invagination length is insensitive to overexpression of Rvs, suggesting that the recently proposed mechanism of BAR-induced protein friction on the membrane is not likely to drive scission. We propose that recruitment of Rvs BAR

43 domains prevents scission and allows invaginations to grow by stabilizing them. We also propose
 44 that vesicle formation is dependent on forces exerted by a different module of the endocytic
 45 pathway, the actin network. Preventing premature membrane scission via BAR interaction could
 46 allow invaginations to grow to a particular length and accumulate enough forces within the actin
 47 network to reliably cut the membrane.

48 Results

49 Removal of Rvs167, not Vps1, results in reduced coat movement

50 In yeast, the Dynamin-like protein Vps1 does not contain a Proline Rich Domain, which in mammalian
 51 cells is required for recruitment to endocytic sites. It is however, reportedly recruited to and interacts
 52 with endocytic proteins (refAyscough, Yu, 2004; Nannapaneni et al., 2010; Goud Gadila et al., 2017).
 53 Vps1 tagged both N- and C-terminally with GFP constructs failed to co-localize with endocytic
 54 proteins in our hands (Fig.1 supplement).

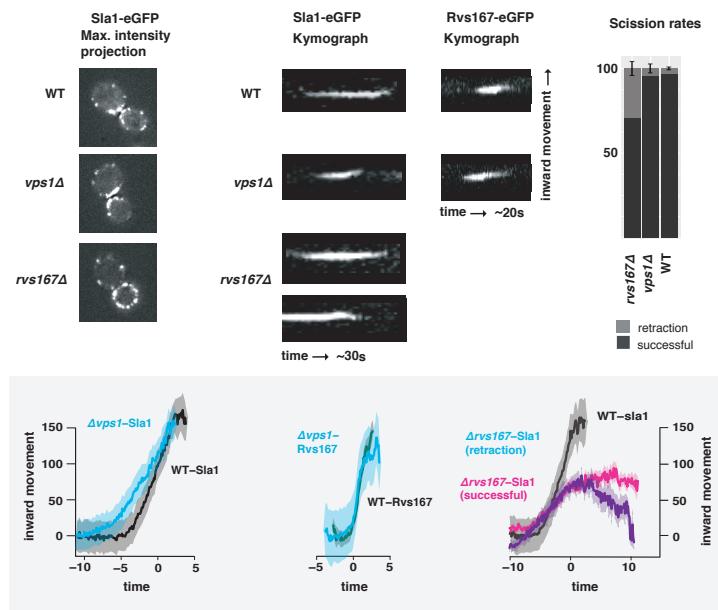


Figure 1. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrap figure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

55 To test whether absence of Vps1 influences scission, endocytic dynamics are observed in cells
 56 lacking Vps1 and compared against wild-type (WT) cells (Fig1a-c). Vps1 deletion is confirmed by
 57 sequencing the open reading frame, and these cells show the growth phenotype at 37°C (Fig.1,
 58 supplement) recorded in other work (ref. ayscough). In Fig.1c, retraction of the membrane in
 59 *vps1Δ* and WT cells is quantified. Membrane retraction, that is, inward movement and consequent
 60 retraction of the invaginated membrane back towards the cell wall is a scission-specific phenotype
 61 (ref.Marko). Sla1 is an endocytic coat protein that acts as a marker for membrane movement.
 62 Upon actin polymerization, the endocytic coat is pulled along with the membrane as it invaginates
 63 (ref.Skuzny?), and thus Sla1 acts as a proxy for the behaviour of the plasma membrane. We
 64 endogenously tagged Sla1 at the N-terminus with eGFP in WT and *rvs167Δ* cells (Fig.1a), and tracked
 65 the dynamics. Retraction rates do not increase in *vps1Δ* cells compared to the WT (Fig.1c).

66

67

68 In order to study the total inward movement of the coat, and therefore the depth of the
69 endocytic invagination, the averaged centroid trajectory of Sla1-eGFP (ref. Andrea) is tracked in 50
70 endocytic sites in *vps1* Δ and WT cells (Fig.1d). In brief, yeast cells expressing fluorescently-tagged
71 endocytic proteins are imaged at the equatorial plane. Since membrane invagination progresses
72 perpendicularly to the plane of the plasma membrane, proteins that move into the cytoplasm
73 during invagination do so in the imaging plane. Centroids of 40-50 Sla1 patches- each patch being
74 an endocytic site- are tracked in time and averaged. This provides an average centroid that can
75 be followed with high spatial and temporal resolution. When different endocytic proteins are
76 simultaneously imaged with Actin Binding Protein Abp1, Abp1 provides a frame of reference to
77 which all the other proteins can be aligned. Abp1 is used because it is abundant at endocytic sites
78 and therefore easily imaged. Time=0 is established as the peak of the Abp1 fluorescence intensity
79 in respective co-tagged strains strains. Abp1 fluorescent intensity maxima in wild-type cells is
80 concomitant with the peak of Rvs167 fluorescent intensity and is time window in which scission
81 occurs (ref2andrea, refwanda). Centroid movement of Sla1-eGFP in WT cells is linear to about
82 140nm. Sla1 movement in *vps1* Δ cells has the same magnitude of movement. In spite of slight
83 differences in the rates of movement, the total inward movement- and so the depth of endocytic
84 invagination- does not change.

85

86

87 Centroid tracking has shown that the number of molecules of Rvs167 peaks at the time of
88 scission, and is followed by a rapid loss of fluorescent intensity, simultaneous with a sharp jump
89 of the centroid into the cytoplasm (ref.Andrea). This jump, also seen in Rvs167-GFP kymographs
90 (Fig.1b), is interpreted as loss of protein on the membrane tube, causing an apparent spatial jump
91 to the protein localized at the base of the newly formed vesicle. Kymographs of Rvs167-GFP (Fig.1b),
92 as well as Rvs167 centroid tracking (Fig.1e) in *Vps1* deleted cells show the same jump.

93

94 Since removal of the Rvs complex is known to increase the retraction rate at endocytic sites,
95 involvement of these proteins in the scission process was investigated further. Rvs161 and Rvs167
96 form dimers (ref.Dominik), so deletion of Rvs167 effectively removes both proteins from endocytic
97 sites. We quantified the effect of deletion of Rvs167 on membrane invagination (Fig.1a-c). 27% of
98 Sla1 patches that begin to form invaginations move inward and then retract in *rvs167* Δ cells (Fig.1c),
99 consistent with retraction rates measured in other experiments (Kaksonen, Toret and Drubin, 2005),
100 and suggesting failed scission in these 27% of endocytic events. Coat movement of the retractions
101 and of successful endocytic events were quantified (Fig.1f) as described in Picco et. al, 2015. Sla1
102 centroid movement in both successful and retracting endocytic events in *rvs167* Δ cells and WT look
103 similar up to about 60nm (Fig.1f). In successful endocytic events, Sla1 is disassembled, and Abp1
104 intensity drops (Fig.1supplement), indicating that scission occurs at invagination lengths between 60
105 -80 nm. That membrane scission occurs at shorter invagination lengths than in WT is corroborated
106 by the smaller vesicles formed in *rvs167* Δ cells by Correlative light and electron microscopy (CLEM)
107 (Kukulski et al., 2012). In retraction events, the Sla1 centroid moves back towards its original position.
108 CLEM has also shown that Rvs167 localizes to endocytic sites after the invaginations are about
109 60nm long (Kukulski et al., 2012). Sla1 movement in *rvs167* Δ indicates therefore that membrane
110 invagination is unaffected till Rvs is supposed to arrive.

111 **Synaptojanins likely influence vesicle uncoating, but not scission dynamics.**

112 There are three Synaptojanin-like proteins in budding yeast: Inp51, Inp52, and Inp53. Inp51-eGFP
113 exhibits a diffuse cytoplasmic signal, and Inp53 localizes to patches within the cytoplasm- cellular
114 localization that is consistent with involvement in trans-Golgi signalling (refGolgi)- Inp53 was not
115 investigated further. Inp52-eGFP localizes to cortical actin patches that are endocytic sites (Fig.2,
116 Fig2 supplement). Spatial and temporal alignment with Sla1 and Rvs167 shows that Inp52 protein
117 molecules arrive in the late scission stage, and localizes to the bud tip, consistent with a role in

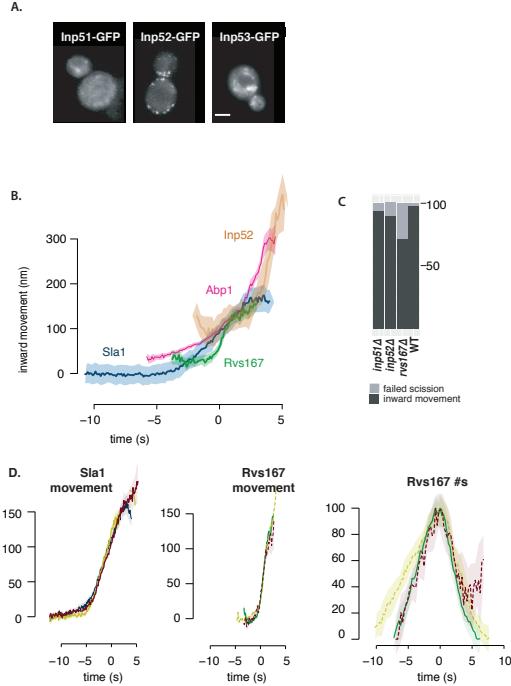


Figure 2. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrap figure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

118 membrane scission (Fig.2c).

119 Inp51 and Inp52 were tested as potential candidates for scission regulators. Sla1-eGFP and
 120 Rvs167-eGFP in cells with either Inp51 or Inp52 deleted were studied. Retraction events do not
 121 significantly increase compared to the WT in either *inp51* Δ or *inp52* Δ cells (Fig2c). Magnitude and
 122 speed of coat movement in *inp51* Δ is the same as the WT (Fig2.d). In *inp52* Δ cells, coat movement
 123 also has the same magnitude and speed, but Sla1-eGFP signal is persistent after membrane scission
 124 (Fig.2d). Similarly, Rvs167 disassembly has a delay, while the assembly is similar to WT (Fig2e).
 125 Assembly of Rvs167 has a delay in *inp51* Δ cells. The magnitude of the inward movement of both
 126 Sla1 and Rvs167 in cells containing either deletion are the same as in WT.

127 **Rvs BAR domains recognize membrane curvature in-vivo**

128 The interaction between Rvs167 and membrane curvature in vivo has not so far been tested. In
 129 order to do so, we deleted the SH3 domain of Rvs167 (henceforth BAR-GPA) and observed the
 130 localization of full-length Rvs167 and BAR-GPA. The GPA region is a disordered region that has no
 131 previously reported function and was retained to ensure proper folding and function of the BAR
 132 domain. Endogenously tagged Rvs167-eGFP and BAR-GPA-eGFP colocalization with Abp1-mCherry
 133 in WT and *sla2* Δ cells are compared (Fig3b). Sla2 acts as the molecular linker between forces exerted
 134 by the actin network and the plasma membrane (ref. Skruzny). *sla2* Δ cells therefore contain a
 135 polymerizing actin network at endocytic patches, but the membrane remains flat and endocytosis
 136 fails. In these cells, the full-length Rvs167 protein co-localizes with Abp1-mCherry, indicating that
 137 it is recruited to endocytic sites (Fig3b). BAR-GPA-eGFP localization is removed, except for rare
 138 transient patches that do not co-localize with Abp1-mCherry, indicating that in the absence of
 139 membrane curvature, the BAR domains cannot localize to endocytic sites (Fig3b, *sla2* Δ).

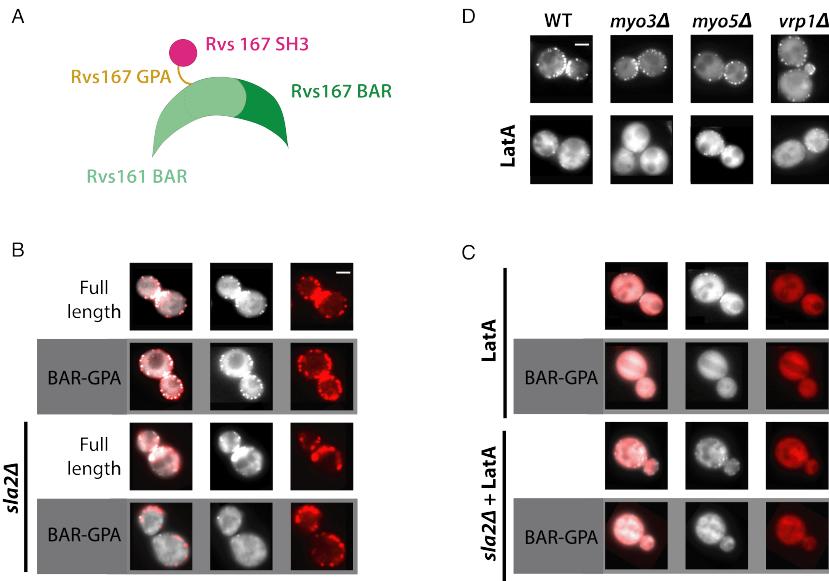


Figure 3. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrap figure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

140 **SH3 domains are likely recruited by Myosin 3**

141 SH3 domains have been shown to interact with several proteins in the actin module of endocytosis.
 142 Type I myosins Myo3 and Myo5, and Vrp1 have genetic or physical interactions with Rvs167 SH3
 143 domains (Lila and Drubin, 1997; Colwill et al., 1999; Madania et al., 1999; Liu et al., 2009). We tested
 144 the interaction between these proteins and the Rvs167-SH3 region by studying the localization of
 145 full-length Rvs167 in cells with one of these proteins deleted, and treated with LatA to reproduce
 146 the situation in which BAR-curvature interaction is removed, and SH3 interaction remains. Deletion
 147 of neither Vrp1 nor Myo5 in combination with LatA treatment removes the localization of Rvs167.
 148 Deletion of Myo3 with LatA treatment removes localization of Rvs167.

149 what about the differences in myo5 and myo3 number...

150 **Loss of Rvs167 SH3 domain affects coat and actin dynamics**

151 In order to further probe the contribution of the Rvs167 SH3 domain to endocytosis, we compared
 152 dynamics of Sla1, as well as Rvs167 and BAR-GPA centroids (Fig4a). Movement of the coat protein
 153 Sla1 is reduced in BAR-GPA cells (Fig4a). Sla1 centroid movement into the cell is remarkably reduced.
 154 Both full length Rvs167 and BAR-GPA however, arrive at endocytic coats when Sla1 centroid is about
 155 30nm away from the initial position (Fig1a). Tubular invaginations are formed in BAR cells, and
 156 qualitatively resemble that in WT cells, as seen by CLEM (Fig.4 supplement). The inward jump of
 157 BAR-GPA is less than that of full-length Rvs167 (Fig4a). Recruitment of BAR is reduced to half that
 158 of Rvs167 (Fig4c), although cytoplasmic concentration of Rvs167 and BAR are not different (Fig4
 159 supplement). We also quantified the number of Abp1 and Rvs molecules recruited to endocytic
 160 sites (Fig4b). Abp1 disassembly is slowed down in BAR-GPA cells compared to WT (Fig4b), and
 161 recruitment is reduced to 50% of WT recruitment (Fig4c).

162 **N-helix and GPA domains do not contribute to recruitment of Rvs or membrane 163 movement**

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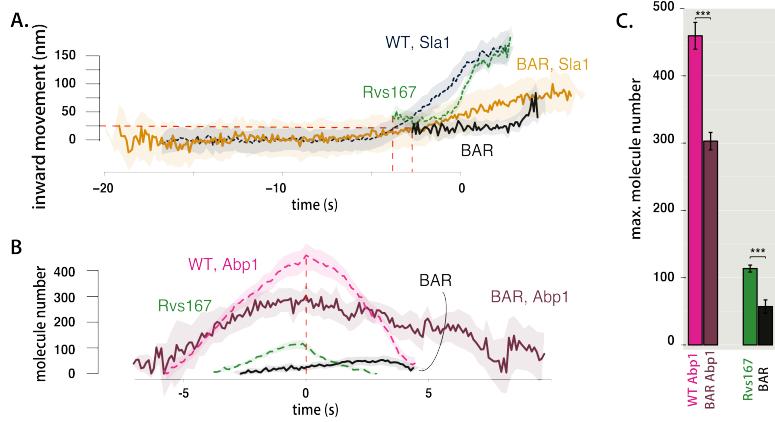


Figure 4. A half-columnwidth image using wrapfigure, to be used sparingly. Note that using a wrapfigure before a sectional heading, near other floats or page boundaries is not recommended, as it may cause interesting layout issues. Use the optional argument to wrapfigure to control how many lines of text should be set half-width alongside it.

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170 **Increased BAR domain recruitment corresponds to increased membrane movement**

171 Decreased Sla1 movement in BAR-GPA cells (Fig3a) can be explained by loss of some interaction
 172 mediated by the SH3 domain, or because BAR-GPA is recruited in smaller numbers to endocytic
 173 sites. To check whether increasing the recruitment of the Rvs complex alone can rescue reduced
 174 Sla1 movement, the Rvs167 and Rvs161 genes were duplicated endogenously (ref Huber) in diploid
 175 and haploid yeast cells. Diploid cells are generated containing either 4 copies of both Rvs genes
 176 by gene duplication, 2 copies (WT diploid) of each gene, or 1 copy, in which one gene of Rvs167
 177 and Rvs161 are deleted. Amount of Rvs167 recruited to sites increases linearly with gene copy
 178 number (Fig5c) without changing either the rate of movement, or significantly modifying total
 179 movement of Sla1. Similarly, in haploid cells, increasing the number of Rvs167 and Rvs161 genes
 180 results in increased recruitment of Rvs167 to nearly twice the WT amount (Fig5f). Sla1 dynamics
 181 however, remains the same as in the WT. Expressing two instead of one copy of the Rvs167 BAR-GPA
 182 domain alone rescues the loss of Sla1 movement in the 1x copy of BAR-GPA, as well the inward
 183 jump of BAR-GPA itself. The loss of inward movement in 1xBAR suggests that smaller vesicles are
 184 produced in these cells, confirmed by CLEM (Fig4 supplement). This would in corollary indicate that
 185 the increased inward movement in 2xBAR produces WT-sized vesicles. We measured similar total
 186 number of Abp1 molecules at endocytic sites for similar Sla1 movements. Total Abp1 numbers
 187 recruited are reduced for 1xBAR and rvsdeletion strains, suggesting a correlation between the
 188 maximum number of Abp1 recruited and total inward endocytic movement.
 189

190 **Discussion**

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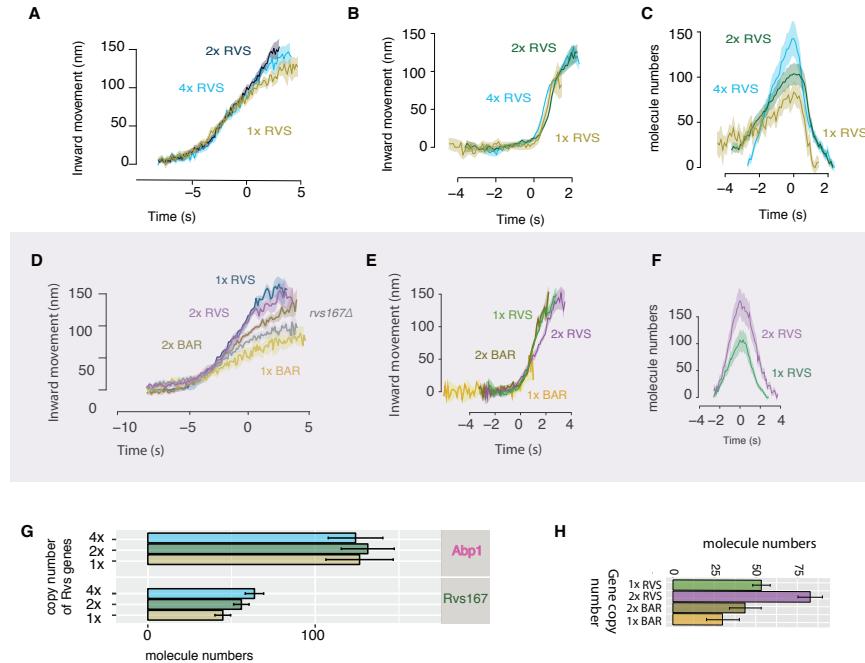


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199 tellus scelerisque quam, pellentesque hendrerit ipsum dolor sed augue. Nulla nec lacus.

200 Methods and Materials

201 Guidelines can be included for standard research article sections, such as this one.

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203 libero. Vivamus viverra fermentum felis. Donec nonummy pellentesque ante. Phasellus adipiscing
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²¹⁹ **Acknowledgments**

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