1   **Caspase-mediated cleavage of Murine Norovirus NS1/2 potentiates apoptosis and is required for**

2   **persistent infection**

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13   **Short title:** Cleavage of MNV NS1/2 potentiates apoptosis and is required for persistence

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16 **Abstract**

17 Human norovirus (HuNoV) is the leading cause of acute gastroenteritis and is spread by fecal shedding that

18 can often persist for weeks to months after infection. Murine norovirus (MNV) is also shed persistently in

19 the feces and provides a tractable model to study molecular mechanisms of enteric persistence. Previous

20 studies have identified non-structural protein 1 (NS1) from the persistent MNV strain CR6 as critical for

21 persistent infection in intestinal epithelial cells (IECs), but its mechanism of action remains unclear. We

22 now find that the function of NS1 in promoting persistence is regulated by apoptosis. Following induction

23 of apoptosis in infected cells, a minority of NS1 is cleaved from the precursor NS1/2 protein, and this

24 cleavage is prevented by mutation of caspase target motifs. MNV strain CR6 with these mutations

25 (CR6$_{\Delta casp}$) is profoundly compromised in infection of IECs and persistence in the intestine. Conversely,

26 replication in tissues outside of the intestine, or in a cultured macrophage cell line, is unchanged, indicating

27 that the requirement of NS1/2 cleavage is intestine-specific. Intriguingly, we also find that cleavage of CR6

28 NS1/2 potentiates apoptosis, suggesting that regulation of cell death is a novel function of this viral protein.

29 Together, these data indicate that the ability of NS1 to promote MNV persistence in IECs is regulated by

30 host caspases, and suggest that potentiation of apoptosis plays a role in viral tropism in the intestine.

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32 **Author Summary**

33 Human Norovirus infection is highly contagious and the most common cause of acute gastroenteritis.

34 Norovirus can persist and be shed for months after infection, leading to continued outbreaks. There are

35 many unanswered questions as to host and viral components of norovirus pathogenesis that can be addressed

36 within the murine norovirus (MNV) model system. We previously identified a critical role for a viral

37 protein, NS1, for intestinal persistence. Herein we describe how the regulation of NS1 is critical for

38 persistent infection in intestinal epithelial cells, but is not required for acute infection of non-epithelial cells,

39 or infection of tissues outside of the gut. Additionally, we demonstrate that NS1 is both regulated by the

40 host cell death machinery, and also reciprocally regulates that machinery to promote cell death during MNV

41 infection, and found that this is specific to persistent strain of MNV. Altogether these data identify a role

42 for how NS1 in a new pathway involved in establishing a persistent norovirus infection in the intestine.

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Introduction

Human norovirus (HuNoV) is the most common cause of epidemic gastroenteritis, and can be particularly dangerous for infants and the elderly [1,2]. Persistent viral shedding often occurs for weeks to months after acute infection, even in asymptomatic individuals [3,4], and is a potential source for continued outbreaks. Despite recent advances in culture systems to study HuNoV [5,6], there is still not an efficient *in vitro* or small animal system for investigating the molecular components of HuNoV infection. Fortunately, HuNoV and murine norovirus (MNV) share genotypic (ssRNA, positive-sense, ~7.5kb genome) and phenotypic (fecal-oral transmission, persistence in intestinal epithelial cells (IECs), and shedding in the feces) similarities, and utilization of the MNV model system has considerably advanced our molecular understanding of norovirus pathogenesis and persistence [7-9].

All noroviruses encode six non-structural proteins (NS1/2, NS3, NS4, NS5, NS6, and NS7) expressed upon cleavage of the open reading frame 1 (ORF 1) polyprotein by the viral NS6 protease [10] and two structural capsid proteins (VP1 and VP2) from ORFs 2 and 3 [11,12], respectively. MNV also has a fourth ORF that encodes a unique virulence factor not present in HuNoV [13]. Although the non-structural protein NS1/2 is not cleaved by the viral protease, it can be cleaved into two additional proteins (NS1 and NS2) [10,14] by the host protease, caspase 3 [10]. However, definitive data regarding the extent and consequences of such caspase cleavage are lacking.

Prior comparison of an acute and a persistent strain of MNV identified two viral proteins that are key components of MNV pathogenesis and persistence [15-17]: the major capsid protein (VP1) of the acute strain CW3 (VP1^{CW3}) drives viral dissemination outside of the intestine, and NS1 of the persistent strain CR6 (NS1^{CR6}) is critical for viral persistence in the intestine. Additional *in vivo* analyses demonstrated NS1^{CR6} is absolutely required for infection of a specialized type of IEC, the tuft cell, identified as the cellular reservoir for MNV persistence [18]. Despite the differences in systemic spread and intestinal persistence, both CW3 and CR6 can infect macrophages, dendritic cells, T-cells, and B-cells *in vitro* and *in vivo* [6,19,20], and utilize the same cell-surface receptor, CD300lf [21,22]. Thus, VP1^{CW3} and NS1^{CR6} are

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71 functioning via mechanisms other than receptor usage and replicative capacity to promote viral

72 dissemination and persistence.

73   In addition to identified roles of viral proteins in norovirus pathogenesis, critical aspects of the host

74 anti-viral response have also been identified. Particularly important is the multi-faceted interferon (IFN)

75 response [23]. Type I (α/β) and type III (λ) IFNs are both key players in the innate anti-viral response,

76 primarily distinguished by the differential expression of their respective receptors [23]. The IFN-α/β

77 receptor is widely expressed on most cell types, whereas the IFN-λ receptor is predominantly expressed on

78 epithelial cells. In regards to MNV, IFN-α/β signaling is critical for control of MNV in myeloid cells,

79 demonstrated by increased titers in macrophages that do not express the IFN-α/β receptor [19], the inability

80 to control systemic MNV infection in mice that specifically lack IFN-α/β receptor expression on myeloid

81 cells [24,25], and increased dissemination and lethality in mice that do not express the downstream signaling

82 molecule, STAT1 [11]. In contrast to systemic spread, intestinal MNV infection is controlled by IFN-

83 λ signaling in IECs, and persistent infection can be cleared and prevented with IFN-λ treatment [26,27].

84 Interestingly, recent data suggests that NS1^{CR6} may have a role in antagonizing the IFN-λ response during

85 intestinal infection of IECs [28], but how NS1^{CR6} may function in this capacity and whether this is its sole

86 function during persistent infection is unknown.

87   In addition to the IFN response, host cells also initiate apoptosis as a means of eliminating virus-

88 infected cells. Correspondingly, viruses have evolved ways to hijack apoptotic machinery to their benefit.

89 Apoptosis is a non-inflammatory, highly-regulated disassembly of the cell mediated by a family of cysteine

90 proteases (caspases), and culminates in the release of the cellular contents within membranous vesicles

91 (apoptotic bodies) that are taken up by professional phagocytes, as well as neighboring cells [29]. Because

92 induction of apoptosis is an innate response to stress or infection, most viruses have developed ways to

93 delay apoptosis in an effort to maximize viral progeny [30], exemplified by a number of large DNA viruses

94 that encode viral homologs of the cellular anti-apoptotic protein, Bcl-2 [31]. Conversely, influenza virus

95 actively induces apoptosis through manipulation of other Bcl-2 family proteins, which increases viral

96   replication and enhances pathogenesis [32,33]. In addition to manipulating apoptosis to prevent cell death

97   or increase viral progeny, a number of viruses, including picornaviruses and flaviviruses, utilize 'apoptotic

98   mimicry' as an alternate mode of egress and viral dissemination [34], and some viruses rely on apoptotic

99   caspases to cleave viral proteins as an additional level of regulation [35-37]. Thus, apoptosis can be both

100   anti-viral and pro-viral, making its regulation critical for viral pathogenesis.

101   HuNoV and MNV infection both induce apoptosis, as indicated by association with markers of

102   apoptosis in infected intestinal tissue [38-40]. Thus, these viruses have likely also developed mechanisms

103   to regulate apoptosis to promote infection. Apoptosis in MNV-infected myeloid cells includes

104   downregulation of an inhibitor of apoptosis protein, survivin, and activation of caspases and other cysteine

105   proteases [41,42]. Furthermore, independent expression of the MNV ORF1 polyprotein, which encodes

106   the non-structural proteins (including NS1/2), is also sufficient to induce apoptosis [43], indicating one or

107   more non-structural proteins could be promoting apoptosis during infection. Additional analyses of the

108   non-structural proteins identified two functional caspase motifs in NS1/2, and demonstrated that the NS1/2

109   protein can be cleaved by recombinant caspase 3 in a cell-free system [10]. Based on these published data,

110   apoptosis during MNV infection may be facilitated by one or more non-structural proteins, and the virus

111   may benefit from apoptotic signaling via utilization of a host caspase to regulate expression of NS1, the

112   critical determinant for intestinal persistence [15].

113   Herein, we further delineate how NS1[CR6] functions to promote viral persistence by characterizing

114   NS1/2 cleavage during MNV infection and its role in intestinal persistence. We demonstrate that at late

115   times post-infection, concurrent with induction of apoptosis, a minority of total NS1/2 protein is cleaved in

116   a caspase-dependent manner. Despite the relatively low proportion of total NS1/2 that is cleaved, our data

117   demonstrate that NS1/2[CR6] cleavage is absolutely required for persistent infection in IECs, and this involves

118   a function separate from IFN antagonism. Moreover, the cleavage of NS1/2[CR6] potentiates apoptotic cell

119   death *in vitro,* suggesting controlled induction of apoptosis is a novel mechanism required for infection of

120   IECs and intestinal persistence.

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Results

Host caspases cleave NS1/2 at late times post-infection. Previous cell-free studies identified two functional caspase motifs (DKAD[121] and DAMD[131]) within NS1/2 that are cleaved by host caspase 3 [10] (Fig 1A), however, the necessity and utility of these sites during viral infection was unknown. Therefore, to assess the role of NS1/2 cleavage during viral persistence, we mutated these sites ($D_{121}G$ and $D_{131}G$) within the persistent MNV strain, CR6, and named this virus CR6$_{\Delta casp}$ (Fig 1B). Analyses of viral protein expression during MNV infection of BV2 cells demonstrates cleavage of a minority of total NS1/2 protein at late times post-infection with both an acute (CW3) and persistent (CR6) strain of MNV (Fig 1C). Cleavage of NS1/2 did not occur in CR6$_{\Delta casp}$-infected cells, demonstrating that it is dependent on the caspase motifs (Fig 1C), and, importantly, these mutations did not disrupt overall expression of full-length NS1/2 protein. To determine whether host caspases are responsible for NS1/2 cleavage, cells were treated at 10h post-infection (PI) with a pan-caspase inhibitor, ZVAD-fmk, and indeed, the ~28kDa NS2 protein was no longer detected in infected cells at late times post-infection (Fig 1D). The ~18kDa NS1 protein was inconsistently detected in cells via western blot and may be a technical result of the small amounts of overall NS1/2 that get cleaved during infection. Together, these data demonstrate that a small portion of total NS1/2 protein is cleaved by host caspases at relatively late times post-infection with MNV.

Cleavage of NS1/2 is not required for viral replication *in vitro*. The abundance of full-length NS1/2 following infection with CR6$_{\Delta casp}$ indicated that viral protein production was not dependent on these cleavage motifs (Fig 1C, D). To further assess whether NS1/2 cleavage is necessary for production of infectious virus, we compared plaque forming units (PFU) produced from cell cultures infected with CR6 vs. CR6$_{\Delta casp}$ over a 48h time course. Inhibition of NS1/2 cleavage did not significantly impair CR6 replication in BV2 cells (Fig 2A), or within wildtype (WT) bone-marrow derived macrophages (BMDCs) (Fig 2C).

147   Our *in vivo* analyses discussed in the following section aimed to assess the role of NS1/2 cleavage

148   on systemic replication as well as intestinal replication of MNV; therefore, we generated CR6 and CR6$_{\Delta casp}$

149   viral strains that could spread systemically due to chimeric expression of VP1^{CW3} (Fig 1B) [15]. We

150   performed growth curves using these capsid chimeras in parallel with growth curves for CR6 and CR6$_{\Delta casp}$

151   described above.   *In vitro* growth kinetics in BV2 cells and WT BMDCs displayed minimal differences

152   between CR6-VP1^{CW3} and CR6$_{\Delta casp}$-VP1^{CW3} (Fig 2B, D). The minor, yet significant, decrease in peak titers

153   of CR6$_{\Delta casp}$-VP1^{CW3} compared to CR6-VP1^{CW3} in WT BMDCs but not in BV2 cells indicated NS1/2

154   cleavage may serve a critical function specific to BMDC cultures. Recently published data suggests a role

155   for NS1^{CR6} in IFN antagonism, therefore, we assessed whether NS1/2 cleavage affected growth in BMDC

156   cultures that are not responsive to IFN (*Stat1$^{-/-}$* BMDCs). All the MNV strains we analyzed demonstrated

157   higher peak titers in *Stat1$^{-/-}$* BMDCs compared to WT BMDCs (Fig 2C-F), confirming the known inhibitory

158   effect of IFNs on MNV replication [19], and the decreased replicative capacity of CR6$_{\Delta casp}$-VP1^{CW3} was no

159   longer evident (compare Fig 2D to 2F). Altogether, these data indicate that NS1/2 cleavage is not necessary

160   for viral replication but may play a role in counteracting the IFN response.

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162   **NS1/2 cleavage is critical for persistence in the intestine**. To analyze whether NS1/2 cleavage impacts

163   MNV infection of systemic and intestinal tissues *in vivo*, we perorally challenged WT B6 mice with 1e6

164   PFU CR6, CR6$_{\Delta casp}$, CR6-VP1^{CW3} or CR6$_{\Delta casp}$-VP1^{CW3}, and analyzed tissues at three days post-infection

165   (dpi) for the presence of infectious virus (Fig 3A-D). As expected, CR6 and CR6-VP1^{CW3} were detected

166   within the mesenteric lymph node (MLN), ileum (devoid of Peyer's patches), and colon, the eventual site

167   of persistence (Fig 3A, B). Consistent with the high titers in intestinal tissue, we also detected infectious

168   virus shed in the feces of mice infected with CR6 and CR6-VP1^{CW3} at 3dpi (Fig 3C, D). However, in mice

169   infected with CR6$_{\Delta casp}$ or CR6$_{\Delta casp}$-VP1^{CW3}, we rarely detected infectious virus in intestinal tissues (Fig 3A,

170   B), nor being shed in the feces (Fig 3C, D), indicating that caspase cleavage of NS1/2 is required for

171   promoting early infection of the intestine.

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172   Although production of infectious virus was severely compromised in intestinal tissues of MNV-

173   infected mice in the absence of NS1/2 cleavage, titers in the spleen were only modestly reduced and not

174   significantly different at 3dpi in the absence of NS1/2 cleavage (Fig 3B), indicating that NS1/2 cleavage is

175   not required for systemic spread. Similarly, we were able to detect MNV RNA within the Peyer's patches

176   of animals infected with $CR6_{\Delta casp}$-$VP1^{CW3}$ using the ultra-sensitive *in situ* RNA hybridization platform,

177   RNAscope (Fig 3F), and this was not visibly different from CR6-$VP1^{CW3}$-infected mice (Fig 3E-G).

178   Together, these data suggest no requirement for NS1/2 cleavage during the initial infection in Peyer's

179   patches or for subsequent systemic spread (Fig 3B, spleen).

180   To further verify that NS1/2 cleavage is not required for systemic MNV infection and pathogenesis,

181   we infected *Stat1*$^{-/-}$ mice with either CR6-$VP1^{CW3}$ or $CR6_{\Delta casp}$-$VP1^{CW3}$ (Fig 3H). Previous work

182   demonstrated that $VP1^{CW3}$ is critical for systemic spread [15,17], and lethality in *Stat1*$^{-/-}$ mice [16,17,24].

183   Accordingly, we found that peroral challenge with CR6-$VP1^{CW3}$ resulted in lethal MNV infection in *Stat1*$^{-}$

184     $^{/-}$ mice, and although NS1/2 cleavage is critical for efficient infection in the intestine, $CR6_{\Delta casp}$-$VP1^{CW3}$

185   infection also resulted in 100% mortality in *Stat1*$^{-/-}$ mice (Fig 3H). As a control, we infected *Stat1*$^{-/-}$ mice

186   with CR6, which persists, but does not result in lethal infection (Fig 3H) [15]. Overall, our characterization

187   of acute infection demonstrates that NS1/2 cleavage is not required for systemic spread of MNV and is not

188   necessary for lethal pathology in *Stat1*$^{-/-}$ mice. However, NS1/2 cleavage is specifically required for

189   efficient MNV replication in the ileum (outside of Peyer's patches) and colon, the source of virus shed in

190   the stool and the site of eventual persistence.

191   Due to limited detection of virus at 3dpi in the intestinal tissues of mice infected with $CR6_{\Delta casp}$ and

192     $CR6_{\Delta casp}$-$VP1^{CW3}$, we predicted that these mutants would also fail to persist. Indeed, whereas CR6 or CR6-

193     $VP1^{CW3}$ persisted in the ileum and colon at 21dpi (Fig 4A, B), and were shed in the stool (Fig 4C, D), we

194   rarely detected persistent virus in any tissues from mice infected with $CR6_{\Delta casp}$ or $CR6_{\Delta casp}$-$VP1^{CW3}$ (Fig 4).

195   Altogether, these findings support a role for NS1/2 cleavage specifically within the intestinal tissue, and

196   temporally link the activity of NS1/2 cleavage to the first 3 days post-infection. Thus, NS1/2 cleavage is

197   required for establishing an infection in the intestine and subsequent persistence.

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199   **NS1/2 cleavage functions independently of IFN.** Due to the dominant role IFN-λ plays in protection

200   against persistent MNV infection of the intestine [26], and the potential role of NS1/2 cleavage in

201   counteracting IFN signaling in BMDCs (Fig 2C, D compared to Fig 2E, F) [28], we sought to more

202   thoroughly characterize CR6$_{\Delta casp}$ infection in the absence of IFN-λ signaling. To this end, we infected WT

203   or *Ifnlr1*$^{-/-}$ mice with CR6 or CR6$_{\Delta casp}$ and monitored shedding in the stool over time (Fig 5A, B), and

204   persistence in the colon at 21dpi (Fig 5C, D). Additionally, to determine whether an early barrier to MNV

205   infection could be overcome with increased infectious dose, we included an experimental group with 10-

206   fold more CR6$_{\Delta casp}$ (1e7 PFU) in the inoculum. MNV was shed in all WT mice infected with CR6 beginning

207   on day 3 and continuing until the experimental endpoint, day 21. Consistent with the protective role of IFN-

208   λ, *Ifnlr1*$^{-/-}$ mice infected with CR6 shed greater than 10-fold more virus relative to WT mice (Fig 5A, B),

209   and CR6 was detected in colon tissue at levels commensurate with fecal shedding at 21dpi (Fig 5C, D). In

210   contrast, we were unable to detect shedding of CR6$_{\Delta casp}$ at any time from WT or *Ifnlr1*$^{-/-}$ mice (Fig 5A, B).

211   These data indicate that NS1/2^{CR6} cleavage promotes persistent infection via a novel mechanism separate

212   from IFN antagonism.

213

214   **Cleavage of NS1/2 is critical for MNV infection of intestinal epithelial cells.** Our preceding data

215   demonstrated that disrupting NS1/2 cleavage leads to an overall reduction of infectious virus produced in

216   intestinal tissues of either WT (Figs 3, 4) or *Ifnlr1*$^{-/-}$ (Fig 5) mice. Therefore, to attain a more thorough

217   understanding of the role of NS1/2 cleavage on cellular tropism, we infected WT or knockout mice with

218   CR6 or CR6$_{\Delta casp}$ and analyzed infection of IECs at 3dpi by flow cytometry and RNAscope (Fig 6). We

219   concurrently quantified MNV infection of Peyer's patches by qPCR to enable contextualization of the IEC

220   infection data. We were able to detect viral RNA in Peyer's patches in the majority of CR6-infected mice

221   and ~50% of CR6$_{\Delta casp}$-infected mice (Fig 6A). Although CR6$_{\Delta casp}$ infection of Peyer's patches was less

222   robust than that of CR6, these data confirm that NS1/2 cleavage is not strictly required for initial infection

223   of non-epithelial cells during acute infection (Figs 3E-F, 6A). This detectable but reduced infection by

224   CR6$_{\Delta casp}$ was observed in mice deficient in IFN-λ receptor (*Ifnlr1$^{-/-}$;* LRKO), doubly-deficient in IFN-λ and

225   IFN-α/β receptors (*Ifnlr1$^{-/-}$/Ifnar1$^{-/-}$*; DKO), as well as receptor-sufficient controls (WT) (Fig 6A),

226   suggesting NS1/2 cleavage amplifies infection in Peyer's Patches.

227   To assess the requirement of NS1/2 cleavage in IEC infection, intestinal tissue devoid of Peyer's

228   patches was mechanically and enzymatically stripped to obtain a single cell suspension and stained for

229   CD45 and EpCAM as markers of immune cells and epithelial cells, respectively. The presence of MNV

230   infection was detected via dual intracellular staining using two distinct antibodies to NS1/2 in mice infected

231   with CR6 or CR6$_{\Delta casp}$ (Fig 6B, C). We analyzed IEC infection in the same mice as in Fig 6A, including

232   knockouts and littermate controls. Regardless of genotype, we only detected IEC infection when mice were

233   infected with CR6, but not CR6$_{\Delta casp}$ (Fig 6B, C). Additionally, tissue sections were examined via

234   RNAscope, and we reproducibly detected CR6 infection of IECs (Fig 6D, E), as well as non-epithelial cells

235   in the lamina propria (Fig 6E) but were unable to find evidence for CR6$_{\Delta casp}$ infection of IECs at 3dpi (Fig

236   6F). These data demonstrate the necessity of NS1/2 cleavage for CR6 infection of IECs, the cellular

237   reservoir of MNV persistence. Furthermore, the profound absence of CR6$_{\Delta casp}$ in IECs would translate to

238   a loss of IEC-dependent viral amplification and may explain the reduced titers in Peyer's patches (Fig 6A)

239   and MLN (Fig 3A, B) during acute infection. Altogether, our data demonstrate that eliminating IFN

240   signaling is not sufficient to rescue CR6$_{\Delta casp}$ infection of IECs (Figs 5 and 6), indicating that cleavage of

241   NS1/2 functions to promote persistent infection of IECs by a mechanism independent of the IFN-λ

242   response.

243

244 **NS1/2 cleavage promotes MNV-induced apoptosis.** We next sought to identify an IFN-λ-independent

245 cellular response altered by NS1/2 cleavage. Because apoptotic-associated caspases have been implicated

246 in cleavage of NS1/2 (Fig 1) [10], and NS1/2 cleavage is critical for developing a persistent MNV infection

247 (Figs. 4, 5), we postulated that apoptosis may be altered by NS1/2 cleavage to promote persistent infection

248 in the gut. Consistent with this hypothesis, we found that CR6 induced significantly more apoptosis in BV2

249 cells compared to $CR6_{\Delta casp}$ (Fig 7). This included an increase in the active form of caspase 3 (Fig 7A, B)

250 and an increase in PARP cleavage (Fig 7A), a known target of activated caspase 3 [44]. The increase in

251 caspase 3 activity at 18hpi (Fig 7B) was not simply due to increased infection in CR6-infected cultures, as

252 there were similar levels of ProPol detected in all infection conditions (Fig 7C). We also measured a

253 significant increase in cells expressing phosphatidylserine on the external plasma membrane via Annexin

254 V staining in CR6-infected cells (Fig 7E-G). Similarly, there was also an increase in these apoptotic

255 markers following infection with CR6 -VP1^{CW3} compared to $CR6_{\Delta casp}$-VP1^{CW3} (Fig 7D, E-H). Furthermore,

256 in support of the hypothesis that NS1/2^{CR6} cleavage promotes apoptosis in a manner that is specific for

257 persistence, the acute strain of MNV, CW3, induced less apoptosis in all our analyses (Fig 7), despite

258 demonstrating NS1/2 cleavage (Fig 1C). Interestingly, CR6-VP1^{CW3} infection resulted in increased cell

259 permeability and death relative to CR6 at 17hpi (Fig 7E, H), consistent with our separate findings that

260 VP1^{CW3} drives lytic cell death and inflammatory cytokine production (Jacob VanWinkle, et al, manuscript

261 under review). However, even in the context of increased cell lysis associated with VP1^{CW3}, we still

262 observed a significant decrease in Annexin V-positivity in the absence of NS1/2^{CR6} cleavage (Fig 7F, H),

263 suggesting NS1/2 cleavage is still promoting apoptosis even in the context of lysis and inflammation driven

264 by VP1^{CW3}. Together with the preceding data, these findings suggest that potentiation of apoptotic cell

265 death by caspase-mediated cleavage of NS1/2 is critical for determining IEC tropism and persistence.

266

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Discussion

Herein, we demonstrate that caspase-mediated cleavage of the nonstructural protein, NS1/2, is critical for developing a persistent MNV infection. We engineered and characterized a strain of MNV in which NS1/2 is not cleaved (CR6$_{\Delta casp}$), and our *in vitro* and *in vivo* analyses demonstrate that NS1/2 cleavage is not required for viral replication in myeloid cells but is necessary for infection of IECs, the eventual site of persistence. Furthermore, our data indicate that NS1/2 cleavage potentiates apoptosis, suggesting apoptosis is a critical aspect of establishing a persistent MNV infection.

NS1/2 was not previously known to play a role in cell death during MNV infection, but is required for viral replication beginning at the earliest stages of replication complex assembly. Norovirus replication occurs in the cytoplasm on cellular membranes with a variety of organelle markers [45], and occurs near membranous vesicles [19,45]. MNV NS1/2 targets to the endoplasmic reticulum (ER) [46,47] and promotes replication via membrane re-organization and through an interaction with the vesicle-associated membrane protein, VAPA [48,49]. Similarly, HuNoV NS1/2 also interacts with VAPA, targets to vesicles, and disrupts vesicle trafficking when ectopically expressed [49]. Our data indicate that the functions of NS1/2 during viral replication remain intact when the caspase cleavage sites are mutated, and viral replication occurs normally (Fig 2). NS1/2 cleavage only occurs after the bulk of viral replication has completed indicating that its role in promoting apoptosis, IEC infection, and persistence involves relatively late events in the viral lifecycle.

The last step of the viral replication cycle is egress. Therefore, NS1/2 cleavage and associated apoptotic death may be important for regulating release of viral progeny. How noroviruses are released from infected cells is not well understood, but infection of macrophages and DCs does result in cell lysis [19], and non-enveloped viruses generally rely on cell lysis to release newly assembled virions. Death by apoptosis may alter this process by reducing lytic virion release and favoring retention of virions within cellular membranes. Indeed, a number of non-enveloped viruses utilize vesicle-associated egress as a means of evasion from immune detection, and vesicles with exposed phosphatidylserine allow for

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293 phagocytic uptake of novel target cells that may not express the viral receptor [50,51]. This type of vesicle

294 egress, known as 'apoptotic mimicry', is also utilized by enveloped viruses which can incorporate

295 phosphatidylserine directly into their viral envelope [34]. Viruses, or virus-filled vesicles, decorated with

296 phosphatidylserine can inhibit IFN responses via activation of Tyro3/Axl/Mer (TAM) receptor tyrosine

297 kinases when phagocytosed by dendritic cells (DCs) [52], which would allow more efficient viral

298 replication in those target cells. Thus, vesicular egress has the potential to dramatically change the outcome

299 of infection.

300   Previous work identified CD300lf as the MNV receptor [21], and accordingly, it is expressed on

301 MNV target cells, including myeloid cells and a specialized IEC subtype, tuft cells. Tuft cells are a rare

302 cell type in the intestinal epithelium and are dispersed among non-tuft IECs [53]. Interestingly, our analyses

303 often found intestinal villi with multiple, adjacent epithelial cells infected (Fig 6E) even though MNV

304 infection has not been shown to alter numbers of tuft cells in the intestine [18]. These findings raise the

305 possibility of receptor-independent transfer of MNV to neighboring IECs, which could be mediated by

306 apoptosis or vesicular egress regulated by NS1/2 cleavage. Future work will be necessary to determine

307 whether vesicle-associated egress occurs during norovirus infection and whether it is required for viral

308 persistence.

309   Regular maintenance of the highly proliferative intestinal epithelium means differentiated epithelial

310 cells have a short lifespan, and undergo a specialized form of apoptotic cell death (anoikis) triggered as the

311 cell detaches from the basal membrane and is shed into the lumen [54]. Specific Bcl-2 family proteins are

312 critical players in anoikis [55,56], and display increased expression as IECs transit up the villus [56]. It is

313 possible cleavage of NS1/2 (and perhaps NS1 directly) interferes with Bcl-2 family proteins as a means of

314 altering the kinetics of the highly-regulated pathways that control apoptosis within differentiated IECs.

315 Interestingly, similar to NS1/2 cleavage promoting apoptosis during infection, some Bcl-2 proteins (e.g.

316 Bid) are also regulated via cleavage, in which the truncated version is the active, pro-apoptotic form of the

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317 protein [57]. It will be interesting to examine whether NS1/2 interactions with the apoptotic machinery

318 plays a role in maintaining IEC infection.

319 Death by apoptosis could also function to avoid immune detection and to limit inflammation

320 associated with cell lysis. Virus production that results from cell lysis would promote inflammatory signals

321 to alert the host of an infection. Thus, promotion of an apoptotic cell death in myeloid cells, mediated by

322 NS1/2^{CR6} cleavage (Fig 7), would limit inflammation and benefit persistence. In addition to professional

323 phagocytes, phagocytosis by non-professional phagocytes (e.g. epithelial cells) is critical for efficient

324 clearance of apoptotic cells, and for limiting inflammation in tissues with high cellular turnover [58].

325 Specifically, in a mouse model of colonic injury, phagocytic uptake of apoptotic cells by IECs was sufficient

326 for limiting inflammation in the colon [59], and phagocytosis mediated by epithelial cells is similarly critical

327 for reducing inflammation in lung tissue [60] and mammary glands [61]. Thus, intestinal infection that

328 promotes apoptotic death and the phagocytic uptake by IECs would limit host detection and provide a non-

329 inflammatory environment that would allow more efficient infection of IECs and benefit MNV persistence.

330 Regardless of whether apoptotic death limits inflammation or functions by another mechanism, our studies

331 here have suggested potentiation of apoptosis following cleavage of NS1/2 as a new pathway critical for

332 sustained infection of IECs and expanded our understanding of the requirements for viral persistence in the

333 intestine.

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Materials and Methods

Cells, virus, and plaque assays

BV2 cells (gift from Herbert Virgin IV, Washington University, St. Louis, MO), a mouse microglial cell line [62], were maintained in DMEM (Life Technologies, ThermoFisher, Carslbad, CA) with 5% fetal bovine serum (FBS) (VWR Seradigm, Radnor, PA), 1X Penicillin/Streptomycin/Glutamine (P/S/G) solution, and 10mM HEPES. For bone-marrow derived dendritic cells (BMDCs), bone marrow was isolated from the long bones of each hind leg of either a WT or $Stat1^{-/-}$ mouse and suspended in RPMI (Life Technologies). Red blood cells were lysed using red blood cell lysis solution (Sigma, St Louis, MO) and remaining bone marrow cells were cultured in RPMI with 10% FBS, 1X P/S/G solution, 10mM HEPES, 1X Sodium Pyruvate, 1X non-essential amino acids, and 20ng/mL GM-CSF obtained from supernatants of GM-CSF-secreting cells (J558L, [63]). After seven days of culture (37C, 5% CO_2), non-adherent cells were collected, infected with indicated MNV strains (MOI = 1), and maintained without GM-CSF. For indicated experiments, ZVAD-fmk (Cell Signaling Technology [CST], Danvers, MA) was re-suspended in DMSO and added to cells at 70μM. As a positive control for apoptosis assays, cells were treated with 1uM staurosporine (SS) (CST) for 3.5h, at 37C.

CR6$_{\Delta casp}$ MNV was generated using site directed mutagenesis as previously described [15] with the following primers: CR6 D$_{121}$G, 5'- GCCTAAGGAAGATAAAGCC**GG**TGCGCCCTCCCATGCG and CR6 D$_{131}$G, 5'-TGCGGAGGACGCCATG**GG**TGCAAGGGAGCCCATAATTGG. The targeted residue is underlined with the mutation in bold. Correct mutations were verified via sanger sequence analysis.

MNV stocks were generated from plasmids, as described [17]. Briefly, plasmids encoding viral genomes of parental CW3 (GenBank accession no. EF014462.1), parental CR6 (GenBank accession no. JQ237823), CR6-VP1^{CW3} [15,17] and the two NS1/2$_{\Delta casp}$ viral genomes generated herein, were individually transfected into 293T cells (ATCC #CRL-3216). Forty-eight hours post-transfection, cells were frozen, thawed, vortexed, and spun at 3,000xg to remove large debris. Supernatants were expanded

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360   by two passages at MOI <0.05 in BV2 cells, and p2 virus supernatant was filtered (0.45um), concentrated

361   by ultra-centrifugation through a 30% sucrose cushion, and titered via plaque assay.

362   Plaque assays were performed in BV2 cells, similar to previously described methods [15]. Briefly,

363   BV2 cells were grown in 6well plates, infected with serial dilutions of each sample (500ul per well, 1h, RT,

364   on a rocking platform), after which the inoculum was removed and cells were overlaid with 1%

365   methylcellulose in complete DMEM. At 2-3d post-overlay, cells were fixed and stained with 20% EtOH /

366   0.1% crystal violet.

367

Mice, infections, and tissue collection

369   *Stat1*$^{-/-}$ (*Stat1*^{tm1Dlv}) mice were originally obtained from the Jackson Laboratories (stock #012606).

370   *Ifnlr1*$^{-/-}$ (generated from *Ifnlr1*$^{tm1a(EUCOMM)Wtsi}$) and *Ifnar1*$^{-/-}$ (*Ifnar1*^{tm1Agt}) were originally obtained from

371   Washington University in St. Louis. *Ifnlr1*$^{-/-}$ and *Ifnar1*$^{-/-}$ were bred to generate *Ifnlr1*$^{-/-}$/*Ifnar1*$^{-/-}$ mice (DKO)

372   and *Ifnlr1*$^{+/-}$/*Ifnar1*$^{+/-}$ (Dhet). Dhet and DKO crossbreeding yielded littermate mice deficient in neither

373   (Dhet), both (DKO), or a single (*Ifnlr1*$^{+/-}$/*Ifnar1*$^{-/-}$ or *Ifnlr1*$^{-/-}$/*Ifnar1*$^{+/-}$) IFN receptor gene(s); these

374   littermates were used for experiments to control for variability between litters.

375   All mice (7-9weeks old) were perorally infected with 1e6 PFU in a 25μL volume administered by

376   pipet, with the exception of experiments in Figure 6 where 1e6 or 1e7 PFU (as indicated in figure) was

377   administered by oral gavage in a 100μL volume. An equal proportion of males and females were

378   maintained among experimental groups to mitigate results due to any unidentified sex-dependent variables.

379   Same-sex mice, infected with the same MNV strain, were often co-housed for acute infection analyses (less

380   than three days), but singly-housed for extended experiments to prevent continued transmission between

381   animals.

382   Tissue samples, or a single fecal pellet, were collected in 2ml cryovials with 1mm zirconia/silica

383   beads (Biospec, Bartlesville, OK), frozen in a dry ice/EtOH bath and stored at -80C. For plaque assay or

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384 RT-qPCR analyses, either 1ml DMEM or 1ml RiboZol (VWR) was added, respectively, and tissues were

385 homogenized via bead-beating (1min, RT) prior to continued analysis.

386

387 **Ethics Statement**

388 All mice were bred on C57BL/6 background and maintained in specific-pathogen-free barrier

389 facilities at Oregon Health & Science University under animal protocols approved by the institutional

390 animal care and use committee at Oregon Health & Science University (protocol # IP00000228) according

391 to standards set forth in the *Animal Welfare Act.*

392

393 **RNA, cDNA, and RT-qPCR**

394 RNA from tissues was extracted via RiboZol (VWR) protocols, and RNA from cells was extracted

395 with the ZR Viral RNA kit (Zymoresearch, Irvine, CA). DNA contamination was removed with Turbo

396 DNase (ThermoFisher), and 1ug of RNA was used to generate cDNA with the ImpromII reverse

397 transcriptase (Promega, Madison, WI). Quantitative PCR was performed using PerfeCTa qPCR FastMix

398 II (QuantaBio, Beverly, MA), and the following oligo/probes were used for detection: MNV genomic

399 RNA - Forward primer CACGCCACCGATCTGTTCTG, Reverse primer GCGCTGCGCCATCACTC,

400 and Probe TGCGCTTTGGAACAATGG; RPS29 - Forward primer GCAAATACGGGCTGAACATG,

401 Reverse primer GTCCAACTTAATG AAGCCTATGTC, and Probe CCTTCGCGTACTGCCGGAAGC.

402

403 **Cell Lysates and Western blot**

404 BV2 cells were grown and infected in 12well plates (2 e5 cells/well). Cells were lifted with

405 trypsin/0.25% EDTA (Gibco, ThermoFisher), washed with 1X cold PBS, resuspended in 100ul NP40 lysis

406 buffer (Alfo Aesar, Havervill, MA) with protease inhibitor cocktail (Sigma, St Louis, MO), incubated on

407 ice (15min with intermittent mixing), cleared via centrifugation (1min, 4C, full-speed), and diluted 1:1 with

408 2x SDS loading buffer (0.125 M Tris [pH 6.8], 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β-

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409 mercaptoethanol). Lysates were run on Novex Tris-Glycine gels (ThermoFisher), transferred to 0.2μm

410 PVDF (BioRad, Hercules, CA), blocked with 5% nonfat dry milk, and probed with the following antibodies

411 (Ab): caspase 3 pAb (CST), PARP pAb (CST), β-Actin pAb (Invitrogen), MNV NS1/2 pAb (a gift from

412 Dr. Vernon Ward, U. of Otago, Australia), or MNV ProPol pAb (a gift form Dr. Kim Green, NIH). Signal

413 was detected using goat, α-rabbit IgG-HRP (Jackson ImmunoResearch), developed with ECL substrate

414 (Pierce, ThermoFisher), and imaged with an ImageQuant LAS4000 (GE Healthcare, Little Chalfont, UK).

415

416 **Intestinal epithelial cell collection**

417 Epithelial fractions were prepared by non-enzymatic striping as previously described [24]. Mice

418 were euthanized and distal small intestine, cecum, and colon (regions of highest MNV replication) were

419 isolated. Peyer's patches were removed from small intestine and intestinal tissues were incubated in

420 stripping buffer (10% bovine calf serum, 15 mM HEPES, 5 mM EDTA, 5 mM dithiothreitol [DTT] in PBS)

421 with shaking for 20 min at 37°C. The dissociated cells were collected as the epithelial fraction, consisting

422 predominantly of IECs and directly used for flow cytometry.

423

424 **Surface and intracellular staining and flow cytometry**

425 BV2 cells were trypsinized and washed once with 1X PBS, stained with the amine-reactive viability

426 dye, Zombie Aqua (Biolegend, San Diego, CA) (20min, on ice), washed once with cold 2% FBS/PBS,

427 washed once with cold Annexin V binding buffer (CST), and stained with Annexin V-FITC (CST) (15min,

428 RT). Cells were immediately analyzed via flow cytometry on an LSR Fortessa (Becton Dickinson [BD],

429 Franklin Lakes, NJ) with FACSDiva software (BD), and data was analyzed using FlowJo software (FlowJo

430 LLC, Ashland, OR).

431 IECs isolated as described above were stained with the Zombie Aqua viability dye (Biolegend), Fc

432 receptors were blocked with purified CD16/CD32 pAb (clone93; Biolegend), and cells were probed for

433   surface markers, EpCAM (clone G8.8; Biolegend) and CD45 (clone 30-F11; Biolegend).   Cells were

434   subsequently fixed with 2% paraformaldehyde/PBS (10min, RT), and blocked/permeablized in 0.2% Triton

435   X/PBS with 2% normal goat serum (20min, RT).   Fixed and permeabilized cells were probed for

436   intracellular viral antigen with 2 distinct antibodies: Rabbit polyclonal anti-NS1/2 (a gift from Dr. Vernon

437   Ward, U. of Otago, Australia) and mouse monoclonal anti-NS1 (clone CM79, a gift from Dr. Ian Clark, U.

438   of Southampton, UK). These unconjugated antibodies were detected with anti-Rabbit Alexa Fluor 647

439   (Molecular Probes, Eugene, OR), and anti-mouse Dylight 405 (Jackson ImmunoResearch), respectively.

440   Cells were analyzed via flow cytometry, and viable cells that were EpCAM+, CD45-, NS1/2 pAb+, and

441   NS1 mAb+ were labeled as infected epithelial cells.

442

443   **RNA *in situ* hybridization via RNAscope®**

444   Sections of approximately two cm from the ileum that contained a Peyer's patch (PP) were selected,

445   cut open lengthwise, pinned apical side up in wax trays, and fixed in 10% neutral-buffered formalin (Sigma)

446   (18-24h, RT), then moved to 70% EtOH. Tissues were embedded in 2% agar (ThermoFisher), the PP was

447   vertically transected, flipped 90 degrees, and re-embedded in 2% agar. Agar blocks were maintained in

448   70% EtOH prior to being paraffin-embedded. Tissue sections (5um) were cut and maintained at RT with

449   desiccant until processed. RNA *in situ* hybridization was performed using the RNAscope Multiplex

450   Fluorescent v2 kit (Advanced Cell Diagnostics [ACDBio], Newark, CA) per protocol guidelines. Probes

451   were designed by ACDBio for detection of CW3 genomic RNA (471891-C1), CR6 genomic RNA (502631-

452   C1), and EpCAM mRNA (418151-C2), and signals were amplified and detected via ACDBio protocols

453   using TSA® plus technology (Perkin Elmer, Waltham, MA). Slides were mounted with ProLong Gold

454   antifade reagent (ThermoFisher), and imaged using a Zeiss ApoTome2 on an Axio Imager, with a Zeiss

455   AxioCam 506 (Zeiss, Jena, Germany).

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457   **Statistical Analyses**

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458   Data were analyzed with Prism 7 software (GraphPad Prism Software, La Jolla, CA), with specified

459   tests and significance noted in the figure legend.

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REFERENCES

1. Ahmed SM, Hall AJ, Robinson AE, Verhoef L, Premkumar P, Parashar UD, et al. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. Lancet Infect Dis. 2014;14: 725–730. doi:10.1016/S1473-3099(14)70767-4

2. Glass RI, Parashar UD, Estes MK. Norovirus gastroenteritis. N Engl J Med. 2009;361: 1776–1785. doi:10.1056/NEJMra0804575

3. Saito M, Goel-Apaza S, Espetia S, Velasquez D, Cabrera L, Loli S, et al. Multiple norovirus infections in a birth cohort in a Peruvian Periurban community. Clin Infect Dis. 2014;58: 483–491. doi:10.1093/cid/cit763

4. Teunis PFM, Sukhrie FHA, Vennema H, Bogerman J, Beersma MFC, Koopmans MPG. Shedding of norovirus in symptomatic and asymptomatic infections. Epidemiol Infect. 2015;143: 1710–1717. doi:10.1017/S095026881400274X

5. Ettayebi K, Crawford SE, Murakami K, Broughman JR, Karandikar U, Tenge VR, et al. Replication of human noroviruses in stem cell-derived human enteroids. Science. 2016. doi:10.1126/science.aaf5211

6. Jones MK, Watanabe M, Zhu S, Graves CL, Keyes LR, Grau KR, et al. Enteric bacteria promote human and mouse norovirus infection of B cells. Science. 2014;346: 755–759. doi:10.1126/science.1257147

7. Karst SM, Wobus CE. A working model of how noroviruses infect the intestine. PLoS Pathog. 2015;11: e1004626. doi:10.1371/journal.ppat.1004626

8. Nice TJ, Robinson BA, Van Winkle JA. The Role of Interferon in Persistent Viral Infection: Insights from Murine Norovirus. Trends Microbiol. 2017. doi:10.1016/j.tim.2017.10.010

9. Baldridge MT, Turula H, Wobus CE. Norovirus Regulation by Host and Microbe. Trends Mol Med. 2016;22: 1047–1059. doi:10.1016/j.molmed.2016.10.003

10. Sosnovtsev SV, Belliot G, Chang K-O, Prikhodko VG, Thackray LB, Wobus CE, et al. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. J Virol. 2006;80: 7816–7831. doi:10.1128/JVI.00532-06

11. Karst SM, Wobus CE, Lay M, Davidson J, Virgin HW. STAT1-dependent innate immunity to a Norwalk-like virus. Science. 2003;299: 1575–1578. doi:10.1126/science.1077905

12. Lambden PR, Caul EO, Ashley CR, Clarke IN. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science. 1993;259: 516–519.

13. McFadden N, Bailey D, Carrara G, Benson A, Chaudhry Y, Shortland A, et al. Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. PLoS Pathog. 2011;7: e1002413. doi:10.1371/journal.ppat.1002413

14. Baker ES, Luckner SR, Krause KL, Lambden PR, Clarke IN, Ward VK. Inherent structural disorder and dimerisation of murine norovirus NS1-2 protein. PLoS ONE. 2012;7: e30534. doi:10.1371/journal.pone.0030534

24

504 15. Nice TJ, Strong DW, McCune BT, Pohl CS, Virgin HW. A single-amino-acid change in murine
505   norovirus NS1/2 is sufficient for colonic tropism and persistence. J Virol. 2013;87: 327–334.
506   doi:10.1128/JVI.01864-12

507 16. Bailey D, Thackray LB, Goodfellow IG. A single amino acid substitution in the murine norovirus
508   capsid protein is sufficient for attenuation in vivo. J Virol. 2008;82: 7725–7728.
509   doi:10.1128/JVI.00237-08

510 17. Strong DW, Thackray LB, Smith TJ, Virgin HW. Protruding domain of capsid protein is necessary
511   and sufficient to determine murine norovirus replication and pathogenesis in vivo. J Virol. 2012;86:
512   2950–2958. doi:10.1128/JVI.07038-11

513 18. Wilen CB, Lee S, Hsieh LL, Orchard RC, Desai C, Hykes BL, et al. Tropism for tuft cells
514   determines immune promotion of norovirus pathogenesis. Science. 2018;360: 204–208.
515   doi:10.1126/science.aar3799

516 19. Wobus CE, Karst SM, Thackray LB, Chang K-O, Sosnovtsev SV, Belliot G, et al. Replication of
517   Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol. 2004;2:
518   e432. doi:10.1371/journal.pbio.0020432

519 20. Grau KR, Roth AN, Zhu S, Hernandez A, Colliou N, DiVita BB, et al. The major targets of acute
520   norovirus infection are immune cells in the gut-associated lymphoid tissue. Nat Microbiol. 2017;2:
521   1586–1591. doi:10.1038/s41564-017-0057-7

522 21. Orchard RC, Wilen CB, Doench JG, Baldridge MT, McCune BT, Lee Y-CJ, et al. Discovery of a
523   proteinaceous cellular receptor for a norovirus. Science. 2016. doi:10.1126/science.aaf1220

524 22. Haga K, Fujimoto A, Takai-Todaka R, Miki M, Doan YH, Murakami K, et al. Functional receptor
525   molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect
526   cells. Proc Natl Acad Sci USA. 2016;113: E6248–E6255. doi:10.1073/pnas.1605575113

527 23. Hoffmann H-H, Schneider WM, Rice CM. Interferons and viruses: an evolutionary arms race of
528   molecular interactions. Trends Immunol. 2015;36: 124–138. doi:10.1016/j.it.2015.01.004

529 24. Nice TJ, Osborne LC, Tomov VT, Artis D, Wherry EJ, Virgin HW. Correction: Type I Interferon
530   Receptor Deficiency in Dendritic Cells Facilitates Systemic Murine Norovirus Persistence Despite
531   Enhanced Adaptive Immunity. PLoS Pathog. 2016;12: e1005684.
532   doi:10.1371/journal.ppat.1005684

533 25. Thackray LB, Duan E, Lazear HM, Kambal A, Schreiber RD, Diamond MS, et al. Critical role for
534   interferon regulatory factor 3 (IRF-3) and IRF-7 in type I interferon-mediated control of murine
535   norovirus replication. J Virol. 2012;86: 13515–13523. doi:10.1128/JVI.01824-12

536 26. Nice TJ, Baldridge MT, McCune BT, Norman JM, Lazear HM, Artyomov M, et al. Interferon-λ
537   cures persistent murine norovirus infection in the absence of adaptive immunity. Science.
538   2015;347: 269–273. doi:10.1126/science.1258100

539 27. Baldridge MT, Lee S, Brown JJ, McAllister N, Urbanek K, Dermody TS, et al. Expression of Ifnlr1
540   on Intestinal Epithelial Cells Is Critical to the Antiviral Effects of Interferon Lambda against
541   Norovirus and Reovirus. J Virol. 2017;91. doi:10.1128/JVI.02079-16

28. Lee S, Wilen CB, Orvedahl A, McCune BT, Kim K-W, Orchard RC, et al. Norovirus Cell Tropism Is Determined by Combinatorial Action of a Viral Non-structural Protein and Host Cytokine. Cell Host Microbe. 2017;22: 449–459.e4. doi:10.1016/j.chom.2017.08.021

29. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008;9: 231–241. doi:10.1038/nrm2312

30. Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of mitochondrial apoptosis. PLoS Pathog. 2008;4: e1000018. doi:10.1371/journal.ppat.1000018

31. Polster BM, Pevsner J, Hardwick JM. Viral Bcl-2 homologs and their role in virus replication and associated diseases. Biochim Biophys Acta. 2004;1644: 211–227. doi:10.1016/j.bbamcr.2003.11.001

32. Tran AT, Cortens JP, Du Q, Wilkins JA, Coombs KM. Influenza virus induces apoptosis via BAD-mediated mitochondrial dysregulation. J Virol. 2013;87: 1049–1060. doi:10.1128/JVI.02017-12

33. Tripathi S, Batra J, Cao W, Sharma K, Patel JR, Ranjan P, et al. Influenza A virus nucleoprotein induces apoptosis in human airway epithelial cells: implications of a novel interaction between nucleoprotein and host protein Clusterin. Cell Death Dis. 2013;4: e562. doi:10.1038/cddis.2013.89

34. Amara A, Mercer J. Viral apoptotic mimicry. Nat Rev Microbiol. 2015;13: 461–469. doi:10.1038/nrmicro3469

35. Richard A, Tulasne D. Caspase cleavage of viral proteins, another way for viruses to make the best of apoptosis. Cell Death Dis. 2012;3: e277. doi:10.1038/cddis.2012.18

36. Mebratu YA, Tipper J, Chand HS, Walton S, Harrod KS, Tesfaigzi Y. Bik Mediates Caspase-Dependent Cleavage of Viral Proteins to Promote Influenza A Virus Infection. Am J Respir Cell Mol Biol. 2016;54: 664–673. doi:10.1165/rcmb.2015-0133OC

37. Al-Molawi N, Beardmore VA, Carter MJ, Kass GEN, Roberts LO. Caspase-mediated cleavage of the feline calicivirus capsid protein. J Gen Virol. 2003;84: 1237–1244. doi:10.1099/vir.0.18840-0

38. Cheetham S, Souza M, Meulia T, Grimes S, Han MG, Saif LJ. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. J Virol. 2006;80: 10372–10381. doi:10.1128/JVI.00809-06

39. Troeger H, Loddenkemper C, Schneider T, Schreier E, Epple H-J, Zeitz M, et al. Structural and functional changes of the duodenum in human norovirus infection. Gut. 2009;58: 1070–1077. doi:10.1136/gut.2008.160150

40. Mumphrey SM, Changotra H, Moore TN, Heimann-Nichols ER, Wobus CE, Reilly MJ, et al. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. J Virol. 2007;81: 3251–3263. doi:10.1128/JVI.02096-06

41. Bok K, Prikhodko VG, Green KY, Sosnovtsev SV. Apoptosis in murine norovirus-infected RAW264.7 cells is associated with downregulation of survivin. J Virol. 2009;83: 3647–3656. doi:10.1128/JVI.02028-08

42. Furman LM, Maaty WS, Petersen LK, Ettayebi K, Hardy ME, Bothner B. Cysteine protease

26

579   activation and apoptosis in Murine norovirus infection. Virol J. 2009;6: 139. doi:10.1186/1743-
580   422X-6-139

581   43.   Herod MR, Salim O, Skilton RJ, Prince CA, Ward VK, Lambden PR, et al. Expression of the
582   murine norovirus (MNV) ORF1 polyprotein is sufficient to induce apoptosis in a virus-free cell
583   model. PLoS ONE. 2014;9: e90679. doi:10.1371/journal.pone.0090679

584   44.   Chang HY, Yang X. Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol
585   Biol Rev. 2000;64: 821–846.

586   45.   Hyde JL, Sosnovtsev SV, Green KY, Wobus C, Virgin HW, Mackenzie JM. Mouse norovirus
587   replication is associated with virus-induced vesicle clusters originating from membranes derived
588   from the secretory pathway. J Virol. 2009;83: 9709–9719. doi:10.1128/JVI.00600-09

589   46.   Hyde JL, Mackenzie JM. Subcellular localization of the MNV-1 ORF1 proteins and their potential
590   roles in the formation of the MNV-1 replication complex. Virology. 2010;406: 138–148.
591   doi:10.1016/j.virol.2010.06.047

592   47.   Doerflinger SY, Cortese M, Romero-Brey I, Menne Z, Tubiana T, Schenk C, et al. Membrane
593   alterations induced by nonstructural proteins of human norovirus. PLoS Pathog. 2017;13:
594   e1006705. doi:10.1371/journal.ppat.1006705

595   48.   McCune BT, Tang W, Lu J, Eaglesham JB, Thorne L, Mayer AE, et al. Noroviruses Co-opt the
596   Function of Host Proteins VAPA and VAPB for Replication via a Phenylalanine-Phenylalanine-
597   Acidic-Tract-Motif Mimic in Nonstructural Viral Protein NS1/2. MBio. 2017;8.
598   doi:10.1128/mBio.00668-17

599   49.   Ettayebi K, Hardy ME. Norwalk virus nonstructural protein p48 forms a complex with the SNARE
600   regulator VAP-A and prevents cell surface expression of vesicular stomatitis virus G protein. J
601   Virol. 2003;77: 11790–11797.

602   50.   Feng Z, Hensley L, McKnight KL, Hu F, Madden V, Ping L, et al. A pathogenic picornavirus
603   acquires an envelope by hijacking cellular membranes. Nature. 2013;496: 367–371.
604   doi:10.1038/nature12029

605   51.   Chen Y-H, Du W, Hagemeijer MC, Takvorian PM, Pau C, Cali A, et al. Phosphatidylserine
606   vesicles enable efficient en bloc transmission of enteroviruses. Cell. 2015;160: 619–630.
607   doi:10.1016/j.cell.2015.01.032

608   52.   Bhattacharyya S, Zagórska A, Lew ED, Shrestha B, Rothlin CV, Naughton J, et al. Enveloped
609   viruses disable innate immune responses in dendritic cells by direct activation of TAM receptors.
610   Cell Host Microbe. 2013;14: 136–147. doi:10.1016/j.chom.2013.07.005

611   53.   Gerbe F, Legraverend C, Jay P. The intestinal epithelium tuft cells: specification and function. Cell
612   Mol Life Sci. 2012;69: 2907–2917. doi:10.1007/s00018-012-0984-7

613   54.   Delgado ME, Grabinger T, Brunner T. Cell death at the intestinal epithelial front line. FEBS J.
614   2016;283: 2701–2719. doi:10.1111/febs.13575

615   55.   Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, et al. Integrins and EGFR
616   coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol. 2003;5: 733–

27

617   740. doi:10.1038/ncb1026

618   56.   Hausmann M, Leucht K, Ploner C, Kiessling S, Villunger A, Becker H, et al. BCL-2 modifying
619   factor (BMF) is a central regulator of anoikis in human intestinal epithelial cells. J Biol Chem.
620   2011;286: 26533–26540. doi:10.1074/jbc.M111.265322

621   57.   Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat
622   Rev Mol Cell Biol. 2008;9: 47–59. doi:10.1038/nrm2308

623   58.   Poon IKH, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and
624   therapeutic potential. Nat Rev Immunol. 2014;14: 166–180. doi:10.1038/nri3607

625   59.   Lee CS, Penberthy KK, Wheeler KM, Juncadella IJ, Vandenabeele P, Lysiak JJ, et al. Boosting
626   Apoptotic Cell Clearance by Colonic Epithelial Cells Attenuates Inflammation In Vivo. Immunity.
627   2016;44: 807–820. doi:10.1016/j.immuni.2016.02.005

628   60.   Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, et al. Apoptotic
629   cell clearance by bronchial epithelial cells critically influences airway inflammation. Nature.
630   2013;493: 547–551. doi:10.1038/nature11714

631   61.   Monks J, Rosner D, Geske FJ, Lehman L, Hanson L, Neville MC, et al. Epithelial cells as
632   phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress
633   inflammatory mediator release. Cell Death Differ. 2005;12: 107–114. doi:10.1038/sj.cdd.4401517

634   62.   Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F. Immortalization of murine microglial cells
635   by a v-raf/v-myc carrying retrovirus. J Neuroimmunol. 1990;27: 229–237.

636   63.   Qin Z, Noffz G, Mohaupt M, Blankenstein T. Interleukin-10 prevents dendritic cell accumulation
637   and vaccination with granulocyte-macrophage colony-stimulating factor gene-modified tumor
638   cells. J Immunol. 1997;159: 770–776.

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Figure Legends

Fig 1. MNV NS1/2 is cleaved by host caspases at late times-post-infection. (A) Schematic of MNV genomic organization, illustrating initial NS1/2 expression within the ORF1 polyprotein. Cleavage sites in ORF1 are marked accordingly - sites cleaved by the viral protease (**v**) and sites cleaved by host caspases (*****), the latter at aspartic acid residues 121 and 131 within the 2 functional caspase motifs, DKAD^{121} and DAMD^{131} [10]. (B) Schematic representation of the five MNV clones used in these studies. Clones with $D_{121}G$ and $D_{131}G$ mutations are referred to as Δcasp, and indicated by the X. (C) BV2 cells were infected with indicated strains of MNV (MOI=1), and whole cell extracts (WCE) were analyzed via western blot (WB) at indicated times post-infection. Image is representative of 4 independent experiments. Arrows are pointing to the full-length NS1/2 at ~45kD, and NS2 at ~28kD. * Denotes a non-specific band at ~30kD. (D) BV2 cells were infected as in C, but subsequently treated with 50uM ZVAD-fmk at 10hpi, and WCE were analyzed via WB. Image is representative of 3 independent experiments. SS, staurosporine.

Fig 2. Cleavage of NS1/2 is not required for MNV replication *in vitro*. (A, B) BV2 cells were infected with the indicated strain of MNV (MOI=1) and infectious virus was analyzed at indicated times post-infection via plaque assay. Data are an average of 3 independent experiments. WT (C, D) or *Stat1$^{-/-}$* (E, F) BMDCs were generated as described in methods, and infected and analyzed as in A-B. Data are an average of 2 independent experiments. Dotted lines indicate limit of detection. Statistical significance was determined via two-way ANOVA with Bonferroni's multiple comparisons test. *, $p \le 0.05$. BMDCs, bone marrow-derived dendritic cells.

Fig 3. Cleavage of NS1/2 is specifically required for early replication in intestinal tissues. WT B6 mice were perorally infected with indicated viruses (1e6 PFU). At 3 days post-infection (dpi), tissues (A, B) and stool (C, D) were harvested and analyzed via plaque assay. Data are from 8-9 individual mice within 3 separate experiments, and mean values are indicated. (E-H) Peyer's patches within the ileum were

674   analyzed via RNA *in situ* hybridization, using RNAscope commercial protocols and probes. MNV genomic

675   RNA was probed within ORF2, which encodes the major capsid (VP1), and is labeled in magenta, epithelial

676   cells were probed via EpCAM, and labeled in green, and DAPI is labeling nuclei in blue. Images are

677   representative of 2-3 mice for each group. (I) Mice (5-6 per group) were infected with indicated virus and

678   survival was plotted over the course of 35 days. Statistical significance was determined via Mann-Whitney

679   test for A-D. *, $p \le 0.05$; **, $p \le 0.01$; ns, not significant; MLN, mesenteric lymph node; yellow scale

680   bar, 50μm; white scale bar, 20 μm.

681

682   **Fig 4. Cleavage of NS1/2 is critical for persistent infection in the intestine.** (A, B) WT B6 mice were

683   perorally infected with indicated viruses, sacrificed at 21 days post-infection (dpi), and indicated tissues

684   were analyzed via RT-qPCR for MNV genomic RNA. Data are combined from 2 separate experiments

685   with data points representing individual mice, and mean values are indicated. (C, D) Fecal pellets were

686   also collected at 21 dpi, and similarly analyzed. Statistical significance was determined via Mann-Whitney

687   test. *, $p \le 0.05$; **, $p \le 0.01$; ns, not significant; MLN, mesenteric lymph node.

688

689   **Fig 5. Caspase cleavage of NS1/2 is required independent of the IFN-λ response.** WT (A, C) or *Ifnlr*

690   *-/-* (B, D) mice were infected by oral gavage with either 1e6 PFU or 1e7 PFU, as indicated. Fecal samples

691   (A, B) were collected at 3, 7, 14, and 21 days post-infection (dpi), and colon tissue (C, D) was harvested at

692   21dpi. All samples were analyzed via RT-qPCR, and the mean of each group (N=4) is indicated. Dotted

693   lines indicate limit of detection.

694

695   **Fig 6. NS1/2 cleavage is necessary for infection of intestinal epithelial cells.** (A-C) Littermate mice

696   deficient for the type III IFN receptor (*Ifnlr1*$^{-/-}$ or *Ifnlr1*$^{-/-}$/*Ifnar*$^{+/-}$, "LRKO"), deficient for both type I and

697   III IFN receptors (*Ifnlr1*$^{-/-}$/*Ifnar*$^{-/-}$, "DKO"), and matched receptor-sufficient controls (*Iflr1*$^{+/+}$ / *Ifnar*$^{+/+}$ or

698   *Iflr1*$^{+/-}$/*Ifnar*$^{+/-}$, "WT") were infected with indicated viruses (9-13 mice per group combined from 3 separate

699   experiments), and analyzed at 3 dpi. (A) Peyer's patches were collected and analyzed via RT-qPCR for

700   MNV genomic RNA and normalized to RPS29. (B, C) Intestinal epithelial cells (CD45-, EpCAM+) were

701   collected and doubly stained for NS1/2 as described in methods. Dotted line indicates limit of detection.

702   (C) Flow plots representing data from 5 mice in B (each mouse is a separate color). (D-F) Ileum tissue

703   from CR6-infected or CR6$_{\Delta casp}$-infected WT mice was analyzed via RNAscope at 3 dpi, as in Figure 3.

704   MNV RNA is in magenta, EpCAM is in green, and nuclei are stained with DAPI in blue. Images are

705   representative of 5 mice infected with each virus. white scale bar, 20μm. Legend refers to data in A and

706   B. Statistical significance was determined by Mann-Whitney test. *, p ≤ 0.05.

707

708   **Fig 7. Cleavage of NS1/2 potentiates apoptotic cell death.** BV2 cells were infected (MOI=1) with

709   indicated MNV strains, and (A-D) whole cell extracts were collected at indicated times and analyzed via

710   WB for caspase 3, PARP, actin, and MNV ProPol. (A, D) Arrows denote cleaved fragments of caspase 3

711   and PARP, indicating activation. Images are representative of 3 independent experiments. Cleaved caspase

712   3 (B), and ProPol (normalized to actin) (C) were analyzed via densitometry, with average and SD shown.

713   (E-H) Cells were harvested at 14h and 17hpi, stained with Annexin V and zombie LIVE/DEAD viability

714   stain, and analyzed via flow cytometry. (E) Representative flow plots are shown from one of six

715   independent experiments. (F) Total Annexin V+ cells were averaged at 17hpi, and individual and mean

716   values are indicated, and (G) live, Annexin V+ (bottom right quadrant of flow plots in E), and (H) dead,

717   Annexin V+ (top right quadrant of flow plots in E) populations were also analyzed separately. Statistical

718   significance was determined by repeated measures one-way ANOVA, with Bonferroni's multiple

719   comparison test. *, p ≤ 0.05; **, p ≤ 0.01; ns, not significant. SS, staurosporine.