1 **Atypical chemokine receptor 3 (ACKR3) induces the perturbation of rRNA**

2 **biogenesis: a novel mechanism of colorectal tumorigenesis**

3 Juan Yang¹, Ya-Nan Li¹, Ting Pan¹, Rong-Rong Miao², Yue-Ying Zhang³, Shu-Hua
4 Wu⁴, Xian-Jun Qu², Shu-Xiang Cui^{1*}

5 ¹Toxicology and Sanitary Chemistry, School of Public Health, Capital Medical
6 University, Beijing, 100069, China

7 ²Department of Pharmacology, School of Basic Medical Sciences, Capital Medical
8 University, Beijing, 100069, China

9 ³Department of Experimental Pathology, College of Basic Medical Sciences,
10 Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan,
11 271016, Shandong, China

12 ⁴Department of Pathology, Hospital of Binzhou Medical College, 256603, Shandong,
13 China

14

15 ***Correspondence.** Shu-Xiang Cui. E-mail addresses: cuisx@ccmu.edu.cn

16 **Title:** ACKR3 induces perturbation of rRNA biogenesis in colorectal tumorigenesis

17

18 **Abstract**

19 Atypical chemokine receptor 3 (ACKR3), previously known as C-X-C chemokine
20 receptor type 7 (CXCR7), has emerged as a key player in several biologic processes.
21 Its atypical “intercepting receptor” signaling properties have established ACKR3 as
22 the main regulator in pathophysiological processes in many diseases. However, much
23 less is known the underlying mechanisms of ACKR3 in promoting tumorigenesis. We
24 found, in both human and animal model, that activation of ACKR3 promotes
25 colorectal tumorigenesis through the NOLC1-induced perturbations of rRNA
26 biogenesis. As compared with adjacent non-neoplastic tissue, human colonic cancer
27 tissues demonstrated higher expression of ACKR3, and high ACKR3 expression was
28 associated with increased severity of colonic cancer. Villin-ACKR3 transgenic mice
29 demonstrated the characteristics of ACKR3-induced colorectal cancer, showing the
30 nuclear β -arrestin-1-activated perturbation of rRNA biogenesis. Activation of ACKR3
31 induced nuclear translocation of β -arrestin-1 (β -arr1), leading to the interaction of
32 β -arr1 with nucleolar and coiled-body phosphoprotein 1 (NOLC1). As the highly
33 phosphorylated protein in the nucleolus, NOLC1 further interacted with Fibrillarin, a
34 highly conserved nucleolar methyltransferase responsible for ribosomal RNA
35 methylation, leading to the increase of methylation in Histone H2A, resulting in the
36 promotion of rRNA transcription of ribosome biogenesis. Conclusion: ACKR3
37 promotes colorectal tumorigenesis through the perturbation of rRNA biogenesis by
38 nuclear β -arr1-induced interaction of NOLC1 with Fibrillarin.

39

40 **KEYWORDS:** Colorectal cancer; ACKR3; NOLC1; Fibrillarin; ribosome biogenesis

41

42 **HIGH LIGHTS**

- 43 • ACKR3 is an atypical G protein-coupled receptor (GPCR)
- 44 • ACKR3 promotes colorectal tumorigenesis
- 45 • ACKR3 induces nuclear translocation of β -arr1
- 46 • Nuclear β -arr1 interacts with NOLC1 to activate Fibrillarin
- 47 • Interaction of NOLC1 to Fibrillarin leads to perturbation of rRNA biogenesis
- 48

49 **Introduction**

50 Chemokine receptor ACKR3, formerly named as CXCR7/RDC1, is an atypical G
51 protein-coupled receptor (GPCR) (Bachelier et al., 2014; Balabanian et al., 2005;
52 Burns et al., 2006). Unlike classical GPCRs, ACKR3 usually fails to activate
53 canonical Gi protein-mediated signaling due to the lack of a conserved DRYLAIV
54 structure (Cancellieri et al., 2013). ACKR3 is once regarded as a scavenger receptor
55 and decoy receptor (Luker et al., 2012; Meyrath et al., 2020). During the
56 pathophysiological processes, ACKR3 could be activated by endogenous ligands
57 CXCL11/I-TAC and CXCL12/SDF-1, resulting in the development of numerous
58 diseases, such as cancers, chronic inflammation, and cardiovascular disorders
59 (Sanchez-Martin et al., 2013). ACKR3 preferentially triggers the β -arrestin-dependent
60 signaling, leading to the internalization *via* the AKT or ERK1/2 signaling pathways
61 (Becker et al., 2019; Li et al., 2019; Rajagopal et al., 2010). At present, still little is
62 known about mechanism of the ACKR3-induced β -arrestin-dependent signaling in
63 tumorigenesis. ACKR3 was found overexpression in many cancers. Activation of
64 ACKR3 has been considered to promote cancer growth through the key processes,
65 such as proliferation, anti-apoptosis, angiogenesis, etc (Miao et al., 2007; Sun et al.,
66 2010; Wang et al., 2008). ACKR3 has thus considered as a potential therapeutic target
67 for the clinical management of cancers. However, these ongoing efforts have been less
68 encouraging in animal models because ACKR3 could participate in a complex
69 signaling network, interacting with additional targets and signaling pathways through
70 various crosstalk and compensatory signaling mechanisms. Further investigating the

71 mechanisms of ACKR3 could help us to fully understand the role of ACKR3 in
72 colorectal tumorigenesis (Salanga et al., 2009).

73 As a “crosslinker” receptor, ACKR3 is likely to bind with CXCR4, a classic
74 chemokine receptor for CXCL12, to form the ACKR3/CXCR4 heterodimer. In the
75 ACKR3/CXCR4 heterodimer, ACKR3 might function as modulating the CXCR4
76 signaling to an active and independent signaling receptor (Koch and Engele, 2020).
77 Previously, we revealed a mechanism of ACKR3/CXCR4 heterodimer in promoting
78 colorectal tumorigenesis. ACKR3 was found to modulate or just assist the CXCR4
79 signaling through inducing β -arr1 recruitment to the nucleus, leading to the increase
80 of histone demethylase JMJD2A (Song et al., 2019). Villin-ACKR3 mice developed
81 more exacerbated colorectal cancer than Villin-CXCR4 mice under the same
82 AOM/DSS condition (Song *et al.*, 2019). Accordingly, ACKR3 might function in
83 tumorigenesis independent of CXCR4 signaling. As yet the mechanism of ACKR3,
84 only known an atypical G protein-coupled receptor, has not been fully investigated.

85 In the present study, we uncovered a new mechanism of ACKR3 in the promotion
86 of colorectal tumorigenesis through the NOLC1-induced perturbations of rRNA
87 biogenesis. Since β -arr2 has a strong nuclear export signal (NES) in its C terminus,
88 this specific NES excludes it from sustained presence in the nucleus (Meier, 1996;
89 Scott et al., 2002). Thus, in the present study, we identified β -arr1 as a downstream
90 signaling of activated ACKR3 in the pathophysiological processes of colorectal
91 tumorigenesis. The ACKR3-induced nuclear β -arr1 interacted with NOLC1 to form
92 the β -arr1-NOLC1 complex. NOLC1 is a highly phosphorylated nucleolus coiled

93 protein and a binding protein for RNA polymerase I. NOLC1 is essential for small
94 nucleolar riboprotein synthesis and a critical transcription factor for an array of gene
95 transcriptions (Kim et al., 2003; Meier, 1996). In our model, the phosphorylated
96 NOLC1 was further interacted with Fibrillarin, the rRNA methyl-transferase, leading
97 to the promotion of nucleolus Fibrillarin, resulting in the increase of rRNA
98 transcription of ribosome biogenesis through upregulating the methylation in Histone
99 H2A. We envision that future approaches to treat colorectal cancer should use
100 ACKR3 inhibitors for preventing the ACKR3-activated NOLC1 and Fibrillarin in the
101 nucleolus.

102

103 **Results**

104

105 **High expression of ACKR3 in human CRC specimens**

106 To investigate the role of ACKR3 in colorectal cancer (CRC), we firstly analyzed the
107 data of ACKR3 gene issued in the Oncomine database. An analysis of a Hong
108 Colorectal dataset indicated that the mRNA levels of ACKR3 were significantly
109 higher in colorectal cancers than in normal tissues ($P = 7.50E-5$, Fold Change = 2.427)
110 (Fig 1A). Importantly, the increased ACKR3 was associated with the progression of
111 clinical stages of colorectal cancer as indicated in the GEPIA database (Fig 1B). F
112 value = 2.23, $Pr(>F) = 0.084$. We then performed the immunohistochemistry assay
113 (IHC) to determine ACKR3 levels in human CRC tissues and their paracancerous
114 tissues (Fig 1C). Of the 60 CRC specimens, 57 cases were identified higher ACKR3

115 expression than their paracancerous tissues (Supplementary, Table S4). Western
116 blotting assay further identified higher levels of ACKR3 in human fresh colonic
117 cancer tissues than their paracancerous tissues (Fig 1D). In vitro cultured cells, the
118 cancer cell lines but not human normal colonic cell line demonstrated higher levels of
119 ACKR3 (Fig 1E).

120

121 **Increased ACKR3 in intestinal epithelium cells conferred colorectal** 122 **tumorigenesis in Villin-ACKR3 mice**

123 Villin-ACKR3 mice demonstrated the increased severity of colorectal tumorigenesis
124 as compared to their WT littermates under AOM/DSS induction, showing a
125 significant weight loss (Fig 2A) and an high disease activity index (DAI index) (Fig
126 2B). AOM/DSS induced shorter colorectal length in Villin-ACKR3 mice showed than
127 WT mice (Fig 2C, D). Importantly, Villin-ACKR3 mice developed more colorectal
128 tumors than WT mice (Fig 2E). These colorectal tumors in Villin-ACKR3 mice
129 demonstrated higher levels of ACKR3 (Fig 2F), with significantly increased CXCL12
130 (Fig 2G) than those in their WT littermates. Microscopic analysis of colonic tissues
131 showed the exacerbated colonic damage with higher dysplasia in Villin-ACKR3 mice
132 than WT mice (Fig 2H). These symptoms of colorectal tumorigenesis in
133 Villin-ACKR3 mice might associate with the upregulation of signaling of pluripotent
134 potential and the ERK signaling in the intestinal epithelium cells (Supplementary
135 Figure S1 and Figure S2).

136

137 **ACKR3 induced β -arr1 translocation into the nucleus to interact with NOLC1**

138 As the down-stream signal of GPCR, β -arr1 might respond to the activated ACKR3.
 139 Human colon cancer cells HCT116 exposed to CXCL12 to activation of ACKR3
 140 demonstrated an increase of β -arr1 in the nucleus with time dependent manner ($P <$
 141 0.05 vs. 0 h) (Fig 3A). Since CXCL12 is the ligand of both ACKR3 and CXCR4, we
 142 thus used AMD3100, the specific CXCR4 inhibitor, to prevent action of the
 143 CXCL12-activated CXCR4 on ACKR3, and then analyzed the expression of β -arr1 in
 144 the nucleus. An increase of β -arr1 had remained in the nucleus in the absence of
 145 CXCR4 (Fig 3B). Nuclear β -arr1 was clearly seen in the immunofluorescence staining
 146 cells (Figure 3C). Nuclear β -arr1 was also seen in colorectal cancer of Villin-ACKR3
 147 mice (Fig 3D). Reversely, silencing of ACKR3 significantly prevented the
 148 translocation of β -arr1 into the nucleus (Fig 3E).

149 As β -arr1 was translocated into the nucleus, we next investigated the functions of
 150 nuclear β -arr1. We had searched any information regarding the interaction of β -arr1
 151 with nuclear proteins issued in the HitPredict database. We observed a specific
 152 indication that nuclear β -arr1 might interact with NOLC1. Herein, a strong interaction
 153 of nuclear β -arr1 with NOLC1 was identified in HCT116 cells with activated ACKR3
 154 by CXCL12 (Fig 3F, i and ii). Since NOLC1 levels varied during the cell cycles (Pai
 155 et al., 1995), we thus identified the interaction of nuclear β -arr1 with NOLC1 more
 156 obviously in the interphase and the telophase than in the prophase and the metaphase
 157 (Fig 3G). Knockdown of ACKR3 significantly prevented the interaction of nuclear
 158 β -arr1 with NOLC1 (Fig 3H).

159

160 **The interaction of nuclear β -arr1 with NOLC1 resulted in the promotion of**
 161 **NOLC1 in the nucleolus**

162 NOLC1 is a highly phosphorylated nucleolus protein functions as a regulator of RNA
 163 polymerase I (Kim *et al.*, 2003; Meier, 1996). Still little has been known its roles in
 164 tumorigenesis. The Cancer Genome Atlas (TCGA) reported high levels of NOLC1 in
 165 colorectal cancers (Fig 4A). Our results showed the increased NOLC1 levels in
 166 colorectal cancer grown in Villin-ACKR3 mice (Fig 4B) as well as human colonic
 167 cancer tissues (Fig 4C). We performed the Immunofluorescence assay to mark the
 168 colocalization of NOLC1 with Fibrillarin, the rRNA methyl-transferase, and UBF1,
 169 the nucleolar transcription factor 1. NOLC1 was exactly identified in the nucleolus
 170 (Fig 4D). Reversely, NOLC1 was not presented in the nucleolus of cancer cells
 171 knockdown of β -arr1 (Fig 4E) or ACKR3 (Fig 4F). These results indicated that
 172 activation of ACKR3 resulted in the increase of NOLC1 in the nucleolus through the
 173 induction of nuclear translocation of β -arr1.

174

175 **The increased nucleolus NOLC1 promoted the synthesis of rRNA of ribosome**
 176 **biogenesis**

177 To investigate the nucleolus NOLC1-promoted in tumorigenesis, we analyzed the
 178 level of nuclear AgNOR protein, a marker of cell proliferation, in colorectal cancer
 179 tissues of Villin-ACKR3 mice. More deeply and globally staining of AgNOR proteins
 180 was measured in colorectal cancer of Villin-ACKR3 than that of WT mice (Fig 5A).

181 To illustrate the role of NOLC1 in the nucleolus, we employed cell model silenced
 182 NOLC1 to analyze the levels of synthesis of rRNA of ribosome biogenesis. We
 183 designed 3 types of siRNAs to target NOLC1. The levels of NOLC1 were
 184 significantly reduced by 51.2%, 49.7%, 65.1%, respectively, in si1-, si2-, and
 185 si3-treated HCT116 cells (Fig 5B). Silence of NOLC1 by si3 resulted in a significant
 186 reduction of nucleoli (Fig 5C). We then analyzed the levels of precursor rRNA 45S,
 187 36S, and 32S rRNA by RT-qPCR assay. As shown in Fig 5D, the expression levels of
 188 pre-rRNA 45S, 36S, and 32S were down-regulated in cancer cells with silenced
 189 NOLC1 (siNOLC1 cells) as compared with control WT cells. These results indicated
 190 that silencing gene of NOLC1 resulted in the reduction of rRNA synthesis. We further
 191 analyzed the nucleolar size of cancer cells with silenced NOLC1 under the
 192 transmission electron microscopy. Silencing of NOLC1 gene resulted in the nucleolus
 193 smaller than control cells (Fig 5E). Western blotting analysis demonstrated a higher
 194 level of nucleolar NOLC1 in colonic cancer tissues in Villin-ACKR3-AD than
 195 WT-AD mice. These colonic cancer tissues with elevated NOLC1 exhibited higher
 196 level of POLR1A (RPA194), the largest subunit of RNA Pol I, and higher level of
 197 UBF1, the transcription initiation factor of rRNA transcription, in Villin-ACKR3-AD
 198 than WT mice (Fig 5F). These results suggest that the ACKR3-activated nucleolar
 199 NOCL1 is associated with the synthesis of rRNA of ribosome biogenesis.

200

201 **The interaction of nucleolar NOLC1 with Fibrillarin led to the increase of**
 202 **Fibrillarin and resulted in the promotion of rRNA transcription**

203 Fibrillarin plays an important role in tumorigenesis through inducing rRNA
204 transcription (Shubina et al., 2018). As we identified the function of nucleolar NOLC1
205 in promoting synthesis of rRNA of ribosome biogenesis, we wanted to know whether
206 Fibrillarin was involved in the NOCL1-activated synthesis of rRNA. Analysis of
207 correlation data issued by GEPIA database indicated a correlation of nucleolar
208 NOLC1 to Fibrillarin in CRC tissues (correlation coefficient $R = 0.4$, Fig 6A). Further
209 immunofluorescence analysis indicated a strong colocalization of NOLC1 with
210 Fibrillarin in nucleolus of HCT116 cells (Fig 6B). Western blotting analysis indicated
211 a significant higher Fibrillarin in Villin-ACKR3 mice than WT mice (Fig 6C).
212 Conversely, knockdown of nucleolar NOLC1 significantly reduced Fibrillarin levels
213 (Fig 6D). The interaction of nucleolar NOLC1 with Fibrillarin was clearly seen in
214 colorectal cancer cells of Villin-ACKR3 mice but not obviously in colorectal cancer
215 cells of WT mice (Fig 6E). These results indicated that the interaction of NOLC1 with
216 Fibrillarin induced the upregulation of Fibrillarin and further promoted rRNA
217 transcription in colorectal cancer cells of Villin-ACKR3 mice. We conclude that
218 activation of ACKR3 promotes colorectal tumorigenesis through the increase of
219 ribosome biogenesis by the interaction of nuclear NOLC1 with Fibrillarin (Fig 6F).

220

221 Discussion

222

223 It is known that, as an atypical GPCR, targeting ACKR3 does not lead to typical
224 G-protein-coupled receptor-mediated calcium mobilization and chemotaxis, but rather

225 the recruitment of β -arrestins and the internalization of GPCR (Nguyen et al., 2020).
 226 ACKR3 has long been considered as a scavenger receptor and decoy receptor (Luker
 227 *et al.*, 2012; Meyrath *et al.*, 2020). ACKR3 is upregulated in the inflammatory cells
 228 and the malignant cells. Even though ACKR3 was found to function in the
 229 ACKR3/CXCR4 heterodimer in tumorigenesis (Koch and Engele, 2020), much less is
 230 known about the mechanism of ACKR3. Since Villin-ACKR3 mice developed more
 231 exacerbated colorectal cancer than Villin-CXCR4 mice, thus we hypothesize that
 232 ACKR3 play a crucial role independent of CXCR4. In the present study, our clinical
 233 data showed that high ACKR3 was associated with increased severity of clinical
 234 stages. ACKR3 has been received attention in diagnostics, targeting drug design, and
 235 management of patients.

236 To unveil the mechanism of ACKR3 in colorectal tumorigenesis, we firstly
 237 answered the question that how the activated ACKR3 signaling be translocated into
 238 the cytoplasm and then to the nucleus. We have understood that GPCRs could interact
 239 with β -arrestins (β -arr1 and β -arr2) to function as scaffolds for a multiple kinases that
 240 connect GPCRs to the effector pathways. These complex signaling network and
 241 interactions are facilitated by a conformational change in the β -arrestins that is thought to
 242 occur upon binding to a phosphorylated activated GPCR (Charest et al., 2005).
 243 Previous reports indicated that activation of ACKR3 could recruit the β -arr2 into the
 244 nucleus through the AKT and ERK1/2 signaling pathways (Li et al., 2014; Lin et al.,
 245 2014; Min et al., 2020). Since β -arr2 has a nuclear export signal (NES) in its C
 246 terminus, however, this specific NES excludes it from sustained presence in the

nucleus (Scott *et al.*, 2002). In the present study, we revealed that activation of ACKR3 induced nuclear translocation of β -arr1 but not β -arr2 into the nucleus. Importantly, the ACKR3-induced β -arr1 interacted with NOLC1, a most highly nuclear phosphoprotein, to form the β -arr1-NOLC1 complex.

How NOLC1 was identified as the downstream effector of nuclear β -arr1? We firstly searched for the clues about the interaction related to the nuclear β -arr1 in the Hitpredict database. An interesting link of β -arr1 to NOLC1 was found in the database. Previously, NOLC1 was already identified as a nuclear localization signal-binding protein and functions as a chaperone for shuttling between the nucleolus and the cytoplasm (Meier and Blobel, 1992). However, the underlying mechanisms and biological functions remain largely unknown, and its roles in tumorigenesis are contradictory among the reports (Duan *et al.*, 2013; Huang *et al.*, 2018; Hwang *et al.*, 2009; Yuan *et al.*, 2017b). Since NOLC1 was upregulated in the interphase and the telophase, it is thus suggested that the interaction of nuclear β -arr1 with NOLC1 might play the function of over-proliferation in cancer cells (Pai *et al.*, 1995). However, there were some confused reports that nuclear NOLC1 increased the percentage of cells in their G0/G1 phase (Yuan *et al.*, 2017a). Our further research revealed that the ACKR3-induced interaction of β -arr1 with NOLC1 exactly occurred in the interphase and the telophase but not in the prophase and the metaphase. These results indicated that the interaction of β -arr1 with NOLC1 resulted in the upregulation of NOLC1 in the nucleolus.

We next identified downstream effector of nuclear NOLC1. High ribosome

269 biogenesis has long been considered to associate with key process of
270 over-proliferation and tumorigenesis in many types of cells (Penzo et al., 2019).
271 Ribosome biogenesis is a universal, complex and well-orchestrated cellular process,
272 such as production of ribosomal RNA, synthesis of ribosomal proteins and ribosome
273 assembly (Pelletier et al., 2018). Regulation of ribosome biogenesis, particularly the
274 production of rRNA, is a critical aspect of cell growth control (Mayer and Grummt,
275 2006). Nucleolus, a nuclear subcompartment where ribosomal RNA is synthesized
276 and assembled into the ribosomal subunits, is the main site of ribosome biogenesis
277 (Grummt, 2013). It is a dynamic organelle subject to inputs from growth signaling
278 pathways, nutrients, and stress, whose size correlates with the rRNA synthesis
279 (Boulon et al., 2010). NOLC1 interacts with RNA polymerase I, leading to the
280 regulation of rRNA transcription, perhaps through linking RNA Pol I transcription
281 with pre-rRNA processing. NOLC1 also plays an essential role in rDNA transcription
282 and further inducing rRNA in ribosome biogenesis (Chen et al., 1999; Meier, 2005).
283 Thus, ribosome biogenesis is identified as the downstream effector of nuclear NOLC1.
284 In the present study, we found that colorectal cancer grown in Villin-ACKR3 mice
285 demonstrated increased ribosome biogenesis, and knockdown of NOLC1 resulted in a
286 decrease of pre-rRNA and smaller nucleolar size in cancer cells. It is thus that
287 increased NOLC1 functions a key process in synthesis of rRNA. Increased NOLC1
288 was found to further interact with Fibrillarin in the process of tumorigenesis.
289 Colorectal cancer developed in Villin-ACKR3 mice demonstrated a strong interaction
290 of NOLC1 to Fibrillarin in the nucleolus. Conversely, knockdown of NOLC1

291 downregulated the expression levels of Fibrillarin in the nucleolus. Fibrillarin is a
292 highly conserved nucleolus protein. Fibrillarin plays a crucial role in the regulation of
293 ribosome biogenesis through promoting the methylation of ribosomal RNAs and
294 rDNA histones (El Hassouni et al., 2019). Accordingly, Fibrillarin might be a
295 downstream signaling of NOLC1. However, the mechanism of NOLC1-activated
296 Fibrillarin in the ribosome biogenesis has not been investigated. A report indicated
297 that enhanced Fibrillarin might increase rRNA transcription through activating RNA
298 Pol I methylation in H2AQ104 (Tessarz et al., 2014). In colorectal cancer of
299 Villin-ACKR3 mice, ACKR3-activated NOLC1 promoted nuclear Fibrillarin
300 expression, accordingly, enhanced Fibrillarin functions the upregulation of ribosome
301 biogenesis through promoting methylation of ribosomal RNAs and rDNA histones.

302 In conclusion, ACKR3 promotes colorectal tumorigenesis through the perturbation
303 of rRNA biogenesis by nuclear β -arr1-induced interaction of NOLC1 with Fibrillarin.
304 We envision that future approaches to treat colorectal cancer should use ACKR3
305 inhibitors for preventing the ACKR3-activated NOLC1 and Fibrillarin in the
306 nucleolus.

307

308 **Materials and Methods**

309

310 **TCGA dataset**

311 We referenced the microarray databases of Oncomine (<http://www.oncomine.org>)
312 (Rhodes et al., 2004) and GEPIA (<http://gepia.cancer-pku.cn>) (Tang et al., 2017) for

313 analyzing the expression of ACKR3 and NOLC1 in cancer tissues and normal tissues.
314 To analyze the dataset, thresholds were set as follows: p-value: 0.01; fold change: 2;
315 gene rank: 20%; analysis type: cancer vs normal; data type: mRNA.

316

317 **Human colonic cancer specimens**

318 Human CRC specimens and their paired adjacent non-neoplastic tissues were
319 collected from resected specimens between February and May of 2018 in Binzhou
320 Medical College Hospital (Binzhou, China) (n = 20). The use of human clinical
321 samples was approved by the Ethics Committee of Binzhou Medical College.

322

323 **Human colon cell lines, cell culture and treatment**

324 Human cancer cell lines SW480, SW620, HCT116, HT29 and human normal colonic
325 cell line NCM460 were purchased from China Cell Bank authorized by American
326 Type Culture Collection (ATCC). Cells were cultured in medium of RPMI1640 or
327 DMEM (Gibco) containing 10% FBS (Gibco) at 37°C in a humid atmosphere (5%
328 CO₂). Cells were exposed to 40 ng/ml SDF-1 α (CXCL12) (PeproTech) and
329 AMD3100 10 μ M (Selleck) for analysis of ACKR3 expression and its downstream
330 signaling pathways.

331

332 **Villin-ACKR3 mice and establishment of colorectal cancer**

333 Villin-ACKR3-IREF mice specifically expressed high ACKR3 in intestinal epithelial
334 cells (IECs) were generated by Cyagen Biosciences Inc (Guangzhou, China).

Villin-ACKR3-IREF mice and their WT littermates were maintained under controlled room temperature and allowed unrestricted access to the standard mouse feed (Meidenbauer et al., 2014). Animal experiments were approved by Animal Welfare Committee of Capital Medical University (permit no. AEEI-2016043). Mice model of colorectal cancer was established as previously described (Song *et al.*, 2019).

Histopathology and Immunohistochemical staining assay

Routine hematoxylin-eosin staining (H&E staining) of 5 µm thick sections were used for histological analysis of cancer tissues. Immunohistochemical staining assay (IHC) was performed using antibodies against ACKR3 (Abcam), PCNA (Proteintech Group), NOLC1 (Proteintech Group). IHC scoring was obtained based on the staining intensity and percentage of stained cancer cells as described elsewhere.

RNA interference assay

Small interfering RNA (siRNA) was designed by GenePharma (Suzhou, China). Human siACKR3, siβ-arr1, siNOLC1 were co-transfected into cells using Lipofectamine 2000 (Invitrogen) (Sequences of siRNAs were detailed in Supplementary Table S1). Cells were plated in the 6-well plates for 24 h before interfering. The diluted Lipofectamine 2000 was lightly mixed with an equal volume siRNA suspension. Each sample was incubated at room temperature for 20 min. The siRNA-lipid complex was added and incubated at 37°C for 6 h. Finally, new medium was replaced and cells were incubated for 48 h before treatment.

357

358 **RNA extraction and quantitative PCR (real-time PCR)**

359 Total RNA was extracted using TRIzol reagent according to manufacturer's protocols
360 (Invitrogen). Reverse transcription of total RNA, the first-strand cDNA was
361 synthesized with ReverTra Ace[®] qPCR RT Kit (TOYOBO, Japan). Quantitative PCR
362 was performed in triplicate using SYBR[®] Green Realtime PCR Master Mix
363 (TOYOBO, Japan) on an ABI 7500 Real Time PCR System. β -actin (ATCB) was used
364 as an endogenous control, and fold changes were calculated by means of relative
365 quantification ($2^{-\Delta\Delta CT}$). The primers used for quantitative PCR were listed in
366 Supplementary Table S2.

367

368 **Total protein and nuclear protein extraction**

369 Total protein was extracted using a strong version of RIPA lysate (Beyotime
370 Biotechnology, China), containing 1 mM PMSF. Nuclear protein was isolated using
371 NE-PER nuclear and cytoplasmic extraction reagent (Beyotime Biotechnology, China)
372 according to manufacturer's protocols. Protein concentrations were quantitatively
373 determined using BCA protein assay kit (Beyotime Biotechnology, China).

374

375 **Western blotting assay**

376 Western blotting assay was performed as described previously (Zhang et al., 2020).
377 Primary antibodies were listed in Supplementary Table S3. Appropriate horseradish
378 peroxidase-conjugated secondary antibodies were purchased from ZSGB-BIO, China.

379 Images were obtained by FluorChem FC3 image analyzer (Molecular Devices). Band
380 intensity was analyzed with ImageJ analysis program.

381

382 **Co-immunoprecipitation (CO-IP)**

383 Samples of cultured cells and cancer tissues were obtained by NP-40 lysis buffer
384 (Beyotime Biotechnology, China), containing 1 mM PMSF. Complexes of proteins,
385 protein A/G beads and specific antibodies used Beaver BeadsTM Protein A/G
386 Immunoprecipitation kit (Beaver Biosciences Inc, China) according to manufacturer's
387 protocols. Immunoprecipitates were resolved by 10% SDS-PAGE membranes
388 (Millipore). Membranes were incubated in blocking buffer [1% (w/v) BSA, 5% (w/v)
389 non-fat dry milk, and 0.1% (v/v) Tween-20 in TBS (pH 7.0)] overnight at room
390 temperature. Membranes were subsequently probed with the corresponding antibodies
391 in blocking buffer for 12-16 h at 4°C. These antibodies included NOLC1 polyclonal
392 antibody (Proteintech Group), β -arrestin1 monoclonal antibody (Invitrogen) and
393 Fibrillarin antibody (Santa Cruz). Membranes were incubated with HRP-conjugated
394 anti-rabbit IgG or HRP-conjugated anti-mouse IgG for 1 h at room temperature.
395 Antibody-reactive proteins were detected by western chemiluminescent HRP
396 substrate (ECL) (Millipore). Images were acquired by FluorChem FC3 image
397 analyzer (Molecular Devices).

398

399 **Immunofluorescence assay**

400 Cells ($3-5 \times 10^4$) cultured onto glass were fixed in 100% cold methanol for 10 min,

401 washed in PBS, permeabilized with 0.5% Triton (Sigma-Aldrich) for 30 min, blocked
402 for nonspecific antibody reactions by incubating in solution containing 5% bovine
403 serum albumin for 30 min, incubated with primary antibodies of β -arrestin1
404 anti-rabbit IgG (Abcam), Fibrillarin (Abcam), NOLC1 (Proteintech Group),
405 β -arrestin1 anti-mouse IgG (Invitrogen), UBF1 (Santa Cruz) at 4°C overnight,
406 followed by staining with secondary antibodies of Alexa FluorTM 488 donkey
407 anti-rabbit IgG (Invitrogen) or Alexa Fluor[®] 647 goat anti-mouse IgG (Cell Signaling
408 Technology) for 1 h at room temperature. Cell nuclei were counterstained with
409 4'6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China). Images were
410 acquired under confocal laser imaging system (NIS-Elements Confocal, Japan).

411

412 **AgNOR staining**

413 The method of AgNOR staining was performed as described previously (Trerè, 2000).
414 Paraffin sections deparaffinized were washed for 10 min and then stained in AgNOR
415 Stain solution (Solarbio, China) at room temperature for 40-60 min. After rinsing in
416 distilled water for 1-2 min, slides were counterstained for 1-3 min in methyl green
417 staining solution, washed and finally air dried. AgNOR staining was analyzed by area
418 quantification module of HALO v3.0.311.407 (Indica Labs) software.

419

420 **Transmission electron microscopy (TEM)**

421 Sample preparation for TEM, cancer cells were aspirated into the centrifuge tube and
422 centrifuged at 800 rpm for 5 min. The cell pellets were fixed by adding 2.5%

423 glutaraldehyde (Servicebio, China) for 2 h at 4°C. Cell pellets were rinsed three times
424 and then fixed with 1% osmium tetroxide for 1 h. After dehydration through a graded
425 ethanol series, samples were embedded in propylene oxide and resin (1:1). Sections
426 were examined with a transmission electron microscope (JEM-2100, Japan).

427

428 **Statistical analysis**

429 Data were described as mean $\pm$ SEM. Statistical analysis was done with GraphPad
430 Prism 8. Student's t test was used to compare differences between two groups.
431 Statistical differences among multiple groups were analyzed by one-way analysis of
432 variance (ANOVA) followed by post hoc test with Dunnett (multiple comparisons to
433 the same control). *P* values < 0.05 were considered significant.

434

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438 **Author contributions**

439 S-X Cui conceived and designed the study. J Yang, Y-N Li, T Pan, R-R Miao and Y-Y
440 Zhang performed the experiments. S-H Wu provided human colonic cancer specimens.
441 J Yang wrote the manuscript and performed statistical analysis. X-J Qu provided
442 intellectual inputs and edited the manuscript.

443 **Declaration of interest:**

444 The authors declare no potential conflicts of interest.

445

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576

577 **Figure Legends**

578 **Figure 1. Human CRC specimens were determined high ACKR3 levels.**

579 (A) Level of ACKR3 mRNA copy number in colorectal carcinoma and normal tissue
580 in Hong Colorectal dataset. (B) The expression levels of ACKR3 is varied in different
581 clinical stages of colorectal cancers. (C) The comparison of ACKR3 levels in human
582 CRC tissues and paired adjacent non-cancer tissues. Upper images 10x, lower images
583 40x. The boxed region in each top panel is magnified and shown in the corresponding
584 bottom panel. (10x)Scale bar: 200 μ m, (40x)Scale bar: 50 μ m. $n = 20$. (D) Western
585 blotting analysis of ACKR3 in the representative ($n = 20$) human CRC tissues
586 (marked by T) and adjacent non-cancer tissues (marked by N). (E) The expression
587 levels of ACKR3 in human colonic cancer cell lines and normal colonic epithelial cell
588 line. Protein level was determined by densitometry and normalized to β -actin (right).
589 $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

590

591 **Figure 2. Increased severity of colorectal cancer in Villin-ACKR3 mice.**

592 (A) Body weight changes in mice. $n = 8$. (B). Disease activity index score (DAI)
593 during third DSS cycle: WT-AD (Red), Villin-ACKR3-AD (Blue). $n = 6$. (C) Images
594 of colorectal cancer and (D) colorectal length in mice. $n = 6$. (E) Colorectal tumor
595 number in WT-AD, Villin-ACKR3-AD. $n = 6$. (F) Expression levels of ACKR3 in
596 colorectal cancer tissues of WT, Villin-ACKR3, WT-AD, Villin-ACKR3-AD mice; n
597 = 3. (G) Western blotting analysis of CXCL12 in CRC tissues of WT-AD and
598 Villin-ACKR3-AD mice. $n = 3$. 1, 2, 3 represented the samples from three mice. (H)

599 H&E-stained colorectal cancer tissues in mice. Upper images 10x, lower images 40x.
600 The boxed region in each top panel is magnified and shown in the corresponding
601 bottom panel. (10x) Scale bar: 100 μ m, (40x) Scale bar: 20 μ m. $n = 3$. $*P < 0.05$, $**P$
602 < 0.01 , $***P < 0.001$. WT-AD: wild mice exposed to AOM/DSS. Villin-ACKR3-AD:
603 Villin-ACKR3 mice exposed to AOM/DSS.

604

605 **Figure 3. Activation of ACKR3 induced β -arr1 recruitment to the nucleus,**
606 **interacting with NOLC1.**

607 (A) HCT116 cells exposed to CXCL12 (40 ng/ml) for the indicated times,
608 cytoplasmic and nuclear extracts were subjected to Western blotting assay for analysis
609 of β -arr1. The average of nuclear β -arr1 in the graphics corresponds to the
610 quantification of protein bands normalized to H3. $n = 3$. (B) HCT116 cells were
611 exposed to CXCL12 (40 ng/ml) and CXCL12 (40 ng/ml) + AMD3100 (10 μ M) for 24
612 h, nuclear extracts were subjected to Western blotting assay for analysis of β -arr1. $n =$
613 3. (C) Immunofluorescence staining analyzed the localization of β -arr1 in HCT116
614 cells exposed to CXCL12 and AMD3100. Scale bar: 10 μ m. (D) Western blotting
615 analysis showed the expression of β -arr1 in the nucleus in colorectal cancer tissues of
616 Villin-ACKR3-AD and WT-AD mice. 1, 2, 3 represented the samples from three mice.
617 $n = 3$. (E) Knockdown of ACKR3 reduced the expression levels of β -arr1 and
618 NOLC1. $n = 3$. (F) Co-IP analysis indicated the interaction between β -arr1 and
619 NOLC1. (G) Immunofluorescence analysis showed the variation of NOLC1 and
620 β -arr1 colocalization during cell cycles in HCT116 cells exposed to CXCL12 (40

621 ng/ml). Scale bar: 5 μ m. (H) Knockdown of ACKR3 reduced the colocalization of
622 NOLC1 and β -arr1. Scale bar: 5 μ m. Arrows showed the colocalization between
623 NOLC1 and β -arr1. * P < 0.05. WT -AD: wild mice were exposed to AOM/DSS.
624 Villin-ACKR3-AD: Villin-ACKR3 mice were exposed to AOM/DSS.

625

626 **Figure 4. The interaction of nuclear β -arr1 with NOLC1 resulted in the**
627 **promotion of NOLC1 in the nucleolus.**

628 (A) The expression levels of NOLC1 in CRC in TCGA datasets. Differences were
629 seen in NOLC1 levels between colonic tumor (Orange) and corresponding
630 paracancerous tissues (Grey). COAD: Colon adenocarcinoma, READ: Rectum
631 adenocarcinoma. Log₂(TPM + 1) was used for log-scale. Calculated means $\pm$ SEM
632 were represented by bars and whiskers. (B) Western blotting analysis of NOLC1
633 levels in Villin-ACKR3-AD and WT-AD mice. 1, 2, 3 represented the samples from
634 three mice. n = 3. (C) Expression of NOLC1 in human CRC tissues (marked by T)
635 and paired adjacent non-cancer tissues (marked by N). Upper images 20x, lower
636 images 40x. The boxed region in each top panel is magnified and shown in
637 corresponding bottom panel. (20x) Scale bar: 50 μ m, (40x) Scale bar: 20 μ m. n = 5. (D)
638 NOLC1 was localized to the nucleolus. Co-staining of NOLC1 (Alexa Fluor 488),
639 Fibrillarin and UBF1 (Alexa Fluor 647) in HCT116 cells. Scale bar: 25 μ m. n = 3. (E)
640 Knockdown of β -arr1 reduced the expression of NOLC1 in CRC cells. n = 3. (F)
641 Western blotting analysis shows the expression levels of NOLC1 in cancer cells with
642 knockdown ACKR3. * P < 0.05, ** P < 0.01, *** P < 0.001. WT-AD: wild mice

643 exposed to AOM/DSS. Villin-ACKR3-AD: Villin-ACKR3 mice exposed to
644 AOM/DSS.

645

646 **Figure 5. Nuclear β -arr1-activated NOLC1 promoted the synthesis of rRNA of**
647 **ribosome biogenesis.**

648 (A) AgNOR staining of colonic tissues in WT-AD and Villin-ACKR3-AD mice.

649 Scale bar: 10 μ m. $n = 6$ (B) Western blotting analysis of NOLC1 levels in HCT116

650 cells with knockdown ACKR3. $n = 3$ (C) Immunofluorescence staining of NOLC1 in

651 cancer cells. Scale bar: 10 μ m. $n = 3$. (D) Real-time PCR analyzed the relative levels

652 of pre-RNA 45S, 36S, and 32S in HCT116 cells. $n = 3$ (E) Transmission electron

653 microscopy (TEM) showed the nucleolus in HCT116 cells of control group (Con),

654 siNC group cells (siNC), silenced NOLC1 group cell (siNOLC1). $n = 3$. (F) Western

655 blotting analysis of ACKR3, NOLC1, POLR1A and UBF1 in colonic tissues of

656 WT-AD and Villin-ACKR3-AD mice. 1, 2, 3 represented the samples from three mice.

657 $n = 3$. DFC, FC and nucleolus were indicated with arrows. $**P < 0.01$, $***P < 0.001$.

658 DFC: Dense Fibrillar Component. FC: Fibrillar Center. Nu: Nucleolus. WT-AD: wild

659 mice exposed to AOM/DSS. Villin-ACKR3-AD: Villin-ACKR3 mice exposed to

660 AOM/DSS.

661

662 **Figure 6. The interaction of NOLC1 with Fibrillarin led to the increase of**

663 **Fibrillarin and resulted in the promotion of rRNA transcription.**

664 (A) The correlation of NOLC1 to Fibrillarin in human colorectal cancer tissues in
665 GEPIA database. (B) Immunofluorescence staining of NOLC1 interacted with
666 Fibrillarin in the nucleolus. Scale bar: 10 μ m. $n = 3$. (C) The expression of Fibrillarin
667 in colorectal cancer tissues of Villin-ACKR3-AD and WT-AD mice. 1, 2, 3
668 represented the samples from three mice. $n = 3$. (D) Knockdown of NOLC1 reduced
669 Fibrillarin level in HCT116 cells. $n = 3$. (E) Co-IP analysis indicated an interaction
670 between NOLC1 and Fibrillarin. (F) Proposed mechanism of activated ACKR3 in
671 colorectal tumorigenesis. Activation of ACKR3 induces nuclear translocation of
672 β -arr1, leading to the interaction of NOLC1, resulting in the Fibrillarin-induced rRNA
673 transcription of ribosome biogenesis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. WT-AD:
674 wild mice exposed to AOM/DSS. Villin-ACKR3-AD: Villin-ACKR3 mice exposed
675 to AOM/DSS.
676

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6