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1 **Cancer stem cell marker DCLK1 reprograms small extracellular vesicles toward migratory**

2 **phenotype in gastric cancer cells**

3

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33 **Keywords**

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35

Abstract

Doublecortin-like kinase 1 (DCLK1) is a putative cancer stem cell marker, a promising diagnostic and prognostic maker for malignant tumors and a proposed driver gene for gastric cancer. DCLK1 over-expression in a majority of solid cancers correlates with lymph node metastases, advanced disease and overall poor-prognosis. In cancer cells, DCLK1 expression has been shown to promote an epithelial-to-mesenchymal transition (EMT), driving disruption of cell-cell adhesion, cell migration and invasion. Here, we report that DCLK1 influences small extracellular vesicle (sEV/exosome) biogenesis in a kinase-dependent manner. sEVs isolated from DCLK1 overexpressing MKN1 gastric cancer cells ($MKN1^{OE}$-sEVs) promote the migration of parental isogenic MKN1 cells ($MKN1^{PAR}$). Quantitative proteome analysis of $MKN1^{OE}$-sEVs revealed enrichment in migratory and adhesion regulators (STRAP, CORO1B, BCAM, COL3A, CCN1) in comparison to $MKN1^{PAR}$-sEVs. Moreover, using a specific, small molecule inhibitor of DCLK1, we reversed the observed increase in EV size and concentration, as well as kinase dependent cargo selection of proteins involved in EV biogenesis (KTN1, CHMP1A, MYO1G) and migration and adhesion processes (STRAP, CCN1). Our findings highlight a specific role of DCLK1-kinase dependent cargo selection for sEVs and shed new light on its role as a regulator of signaling in gastric tumorigenesis.

53 **Statement of significance of the study**

54 Gastric cancer (GC) is the 3rd leading cause of cancer mortality worldwide, responsible for over 800,000
55 deaths in 2018 and ranks 5th for cancer incidence**.** The importance of understanding the formation and
56 development of gastric cancer is crucial to developing early detection tools and better therapeutic
57 treatments. Although *DCLK1* gene amplification, overexpression and somatic missense mutations are
58 frequently observed in human GC, the mechanisms by which DCLK1 contributes to gastric
59 tumorigenesis remains poorly understood. Here we show that DCLK1 expression in the gastric cancer
60 cell line MKN1 impacts small extracellular vesicle (sEV) biogenesis both quantitatively and qualitatively
61 in a kinase-dependent manner, revealing a hitherto unknown role for this putative oncogenic kinase.
62 By combining functional and protein dissection of human gastric cancer cell-derived sEVs, we show a
63 DCKL1-dependent regulation of sEVs. These findings will enable future studies seeking to characterize
64 the underlying signaling of cancer stem cells and have implications in defining and therapeutically
65 targeting specific pro-tumorigenic signaling drivers, including kinases such as DCKL1.
66

67 # 1 Introduction

68 Doublecortin-like kinase 1 (DCLK1) was first described in 1999 as a close homologue of doublecortin
69 (DCX) protein, encoded by a gene associated with brain development and neuronal migration defects
70 [1-3]. In the last decade, DCLK1 emerged as a murine marker for gastrointestinal (GI) tuft-cells [15-18] and
71 as a marker of cancer stem cells (CSC) and tumor initiating cells in the GI tract [19-21]. Recently, a
72 functional role of DCLK1 has been shown in promoting an epithelial-to-mesenchymal transition (EMT)
73 in cancer cells and a pluripotent/stem cells state under homeostatic and pathological conditions [22-40].
74 A comprehensive genomic and molecular analysis of 100 primary gastric tumors identified DCLK1 as
75 novel potential driver of gastric cancer (GC) [41]. In addition, a meta-analysis of 18 different studies and
76 13 cancer types showed that high DCLK1 expression correlates with the malignancy status and poor
77 patient outcome. Recently, a strong correlation between DCLK1 expression and anti-tumor immune
78 responses as well as stromal components within the tumor micro-environment was found in gastric
79 and colorectal cancers [42, 43]. We hypothesized whether the altered immune responses and stromal
80 components in these DCLK1 high GI-tumors might be an indirect result of the function of DCLK1
81 resulting in perturbed intercellular communication.

82

83 DCLK1 and DCX are part of the microtubule-associated protein (MAP) family, which regulate the
84 dynamic turnover and distribution of microtubules. Microtubules are involved in a range of essential
85 cellular processes like cellular shape, polarity, migration, cell division, and vesicle and organelle kinesin
86 driven transport. Surprisingly, DCLK1 shares no homology to other MAPs, and binds to microtubules
87 via their two in tandem doublecortin domains (DCs) [4, 5]. Unlike classical MAPs, which bind along the
88 ridges of the microtubule protofilament, DCLK1 binds in the valley between the protofilaments,
89 resulting in laterally and longitudinally stabilized microtubules without overlapping binding sites for
90 kinesin driven transport [6-10]. In addition, the DC domains bind to both polymerized and
91 unpolymerized α/β-tubulin facilitating the polymerization process at the fast growing plus-ends of the
92 microtubules. In contrast to DCX, DCLK1 contains a functional serine/threonine kinase domain at the

93 C-terminal tail regulating microtubule-binding affinity through hyper auto-phosphorylation of the DC
94 domains which reduce microtubule binding. Whilst DCX potently stimulates microtubule
95 polymerization *in vitro*, purified full-length DCLK1 does not unless the purified protein is in the
96 presence of a phosphatase or its kinase domain is rendered non-functional by inactivating point
97 mutations or specific kinase inhibitors [13]. This suggests that the kinase domain of DCLK1 is a negative
98 regulator of microtubule polymerization and stabilisation, at least *in vitro.* In addition, DCX and DCLK1
99 have been linked to supporting roles for neuronal kinesin-3 mediated cargo transport to dendrites.
100 Suggesting that DCLK1 might be directly involved in vesicular trafficking and as a result indirectly
101 altering intercellular communications.
102
103 The cellular secretome represents a fundamental means of intercellular communication, comprised
104 of various growth factors, chemokines, cytokines, and extracellular vesicles (EVs) [44-46]. EVs are
105 secreted lipid-encapsulated vesicles that represent an active element of the cell secretome. EVs
106 include plasma membrane–derived large EVs (lEVs, termed microvesicles, 100-1,500 nm size) and
107 endosomal-derived small EVs (sEVs, termed exosomes, 30-200 nm size) and are well-established
108 mediators of cancer pathology. Even though exosomes and microvesicles have different biological
109 origin, there is overlap in their size range, as such we have used the consensus guidelines of MISEV to
110 term the purified isolated EVs as either large or small EVs. EVs have been established as multifaceted
111 paracrine and autocrine regulators of the tumor microenvironment affecting both cancer and non-
112 cancer cells alike in order to create a milieu conducive to cancer cell survival, proliferation, evasion of
113 immune surveillance and to migration, invasion and the spread of cancer cells to local lymph nodes
114 and distant sites [47-54]. DCLK1 is a CSC marker[19-21] and interestingly, sEVs secreted by CSCs have
115 attracted a particular interest due to their potential use as regenerative mediators and targets for
116 clinical anti-cancer therapies. sEVs isolated from renal CSCs were enriched in pro-metastatic miRNAs
117 and were carrying exclusively pro-angiogenic mRNAs. These sEVs were able to render recipient cells
118 resistant to cytotoxic drugs and induced the formation of a pre-metastatic niche in vivo [55]. Another

119 study showed that CSC-sEVs were reprogramming cells in the tumor microenvironment towards a pro-
120 angiogenic and pro-metastatic phenotype [56]. Recently, it has been shown that cancer-derived sEVs
121 induce epigenetic changes in stem cells, influencing their function in the tumor microenvironment [57].
122 These reports support a role for sEVs in mediating cancer stem cell signaling, particularly in tumor
123 progression. Therefore, we hypothesized that DCLK1 could drive critical pathology in gastric cancer
124 mediated by sEVs. Here, we present a specific role of DCLK1-kinase dependent cargo selection for sEVs
125 and shed new light on its role as a regulator of signaling in gastric tumorigenesis.
126

127 ## 2 Materials and methods

128 ### 2.1 Cell culture

129 The human gastric cancer cell line MKN1 cells ($MKN1^{PAR}$), established from primary gastric
130 adenosquamous carcinoma, was obtained from JCRB Cell Bank (JCRB0252). MKN1 cells were cultured
131 in RPMI-1640 + GlutaMax (Gibco, # 61870036), supplemented with 10% (v/v) fetal calf serum (FCS)
132 (Moregate biotech) and maintained at 37 °C with 10% CO_2. The DCLK1 isoform 1 (accession #
133 NM_004734) was PCR amplified from plasmid RC217050 (Origene) using forward primer 5' agc aag ctt
134 gcc acc atg tcc ttc ggc aga gac atg gag 3'and reverse primer 5' acg gga tcc cta cat cct ggt tgc gtc ttc gtc
135 3' and subcloned into pcDNA3 using HindIII and BamHI restriction sites. The construct was verified by
136 Sanger sequencing and transfected into MKN1 using lipofectamine 2000 (Invitrogen). Cells were
137 selected for 4 weeks with 0.4 mg / mL Genetecin selective Antibiotic (Gibco) substituted to the culture
138 medium. DCLK1 protein expression validated by western blot. Cells were imaged with inverted
139 microscope (Zeiss Axio observer 5) and Zen-blue imaging software.
140

141 ### 2.2 DCLK1-IN-1 dose-response assessment

142 To avoid confounding results caused by potential cytotoxicity of the DCLK1 inhibitor on MKN1 cells,
143 we performed a dose-response assessment in order to select a concentration of the inhibitor which
144 was well below the IC_{50}. For the dose-response assessment 7.5 x 10^3 $MKN1^{PAR}$ and $MKN1^{OE}$ cells were

145 seeded in 96-well plate (Gibco) in quadruplicates, and subjected to a concentration range [0.003, 0.01,
146 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM] of DCLK1-IN-1 small molecule inhibitor or DMSO control [58, 59].
147 After 72 hrs, cell toxicity was quantified using MTS-reagent (Promega, #G1112) and absorbance was
148 measured at 490 nm. The IC_{50} values were determined of the Log_{10} transformed concentrations (X)
149 with a non-linear regression curve fit ($Y = Bottom + \frac{Top-Bottom}{1+\frac{X}{IC50}}$) using GraphPad Prism (v.8.4.3).

150

151 **2.3 EV collection, purification and preparation**

152 For the MKN1 cells, one week prior to EV collection, the FCS in the cell culture media was changed to
153 10% (v/v) EV depleted FCS (centrifuged at 100,000 x g for 18 hrs to remove EVs). For EV collection 8 x
154 10^6 cells were seeded in a multilayer flask (Millicell HY cell culture flask T-1000, #PFHYS1008), in 200
155 mL RPMI-1640 + GlutaMax, supplemented with 10% (v/v) EV-depleted FCS and cultured for 48 hrs
156 prior to collection of conditioned media (CM). DCLK1 inhibitor treated $MKN1^{OE}$ cells ($MKN1^{OE+INH}$) were
157 cultured for 48 hrs in the presence of 1 µM DCLK1-IN-1 small molecule inhibitor [58, 59]. Five
158 independent replicates of each $MKN1^{PAR}$, $MKN1^{OE}$, and $MKN1^{OE+INH}$ CM (5 x 200 mL) were subjected to
159 differential ultracentrifugation as previously described [60] (**Figure 1C**). In brief, the CM was centrifuged
160 (Rotina 380R) at 500 x *g* for 5 min 4 °C to remove dead cells, the supernatant subsequently centrifuged
161 (Rotina 380R) at 2000 x *g* for 10 min 4 °C to remove cell debris. Of the supernatant 180 mL was
162 aliquoted in to 6 fractions, each 30 mL and centrifuged at 10,000 x *g* for 30 minutes at 4 °C (SW 28,
163 Beckman Coulter, Optima L-90k Ultracentrifuge). Supernatant was transferred to new tube and
164 centrifuged at 100,000 x *g* for 60 min at 4 °C (SW 32Ti, Beckman Coulter, Optima L-90k
165 Ultracentrifuge). The 10,000 x *g* (10k) pellets contain large EVs (lEVs), while the 100,000 x *g* (100k)
166 pellets contain sEVs. To wash the 10k and 100k pellets, pellets were resuspended in 100 µL DPBS
167 (Gibco) and pooled per condition per replicate (n=5), and centrifuged at either 10,000 x *g* for 30 min
168 at 4 °C (Eppendorf Centrifuge 5430R) or 100,000 x *g* for 60 min at 4 °C (TLA 55, Beckman Coulter,
169 Optima MAX-TL Ultracentrifuge), respectively. The pooled lEVs (10k) and sEV (100k) pellets were

170 resuspended in 50 µL DPBS and aliquoted for immediate use or stored at -80 °C for further
171 downstream use.
172
173 **2.4 Nanoparticle tracking analysis**
174 Vesicle size was determined using NanoSight NS300, Nanoparticle tracking analysis (NTA) (Malvern)
175 fitted with a NS300 flow-cell top plate with a 405 nm laser. lEV and sEV samples (1 µg/µL in filtered
176 (0.2 µm) Milli-Q (1:1,000 dilution) were injected with 1 mL syringes (BD) (detection threshold = 10,
177 flowrate = 50, temperature = 25 ºC). For each sample 5 replicate 60 s video captures were made. To
178 calculate vesicle size and concentration, videos were analysed as described using NTA software 3.0
179 (ATA Scientific) [61].
180
181 **2.5 Protein lysate preparation and Western blot analysis**
182 The sEVs and MVs were solubilized with 2% (w/v) sodium dodecyl sulfate (SDS) and ultrasonicated
183 (Digital Pro ultrasonic cleaner) for 10 min. Protein was quantified using microBCA Protein assay kit
184 (Thermo Fisher, #23235). lEV and sEV relative protein abundances were normalized to their
185 corresponding MKN^{PAR} lEVs or sEVs. SDS-PAGE (Invitrogen, #NP0321PK2) was performed (200 V, 35
186 min) on denatured (70 °C, 10 min) protein lysate (15 µg, 50 mM Dithiothreitol (DTT), 125 mM Tris–
187 HCl, pH 6.8, 12.5 % (v/v) glycerol, 0.02 % (w/v) bromophenol blue). Western blot was performed using
188 iBLOT system (Invitrogen), on PVDF-membranes (Invitrogen, #IB401001). Membranes were blocked
189 in blocking-buffer (5 % (w/v) milk in PBS-0.1 % Tween-20) for 1 hr at RT and subsequently probed with
190 primary antibodies (1:1000 dilution in blocking buffer) over night at 4 °C against ALIX (Cell Signaling
191 Technology, #2172), TSG-101 (BD Biosciences, #612696), DCLK1 (Abnova, #H00009201-A01), and
192 GAPDH (Sigma-Aldrich, #G9545). Secondary HRP-linked antibodies goat anti-mouse (DAKO, #P0447)
193 or goat anti-rabbit (DAKO, #P0448) were diluted (1:7500) in blocking-buffer for 1 hr at RT with orbital

shaking. Protein bands were visualized using ECL-substrate (Pierce, #32106) and Chemidoc^{TM} (Biorad XRS, imagelab^{TM} software).

2.6 Transwell cell migration assay

Transwell migration assay was performed using 8 µm transwell inserts (Falcon, #353097) seeded with 4 x 10^4 MKN1 or MKN1^{OE} cells in 100 µL serum-free RPMI-1640 + Glutamax (Gibco, # 61870036). Cells were supplemented with either 30 µg/mL MKN1^{PAR}-sEVs, 30 µg/mL MKN1^{OE}-sEVs, 30 µg/mL MKN1^{OE+INH}-sEVs or DPBS (no EVs). Inserts were nested onto 24-well plate (Falcon, #353047), as chemoattractant 20% (v/v) EV-depleted FCS was added to RPMI-1640 + Glutamax and incubated for 48 hrs (37 °C). EV-depleted FCS was used to fully attribute altered migration to the added EVs. Non-migrating cells were removed with cotton-swab and inserts were fixed with methanol and serial stained in Diff-Quik staining solution (Millipore). Washed membranes were air-dried prior to mounting to glass slide with dibutylphthalate polystyrene xylene (DPX). Slides were imaged and analyzed using Aperio ImageScope and eSlide Manager (Leica Biosystems).

2.7 Proteomic liquid chromatography–tandem mass spectrometry

sEVs were solubilized in sodium dodecyl sulphate (SDS) 2% (v/v), 50 mM triethylammonium bicarbonate (TEAB), pH 8.0, centrifuged at 16,000*g* for 10 min at 4 °C, and quantified by microBCA. For mass spectrometry-based proteomics, samples (5 µg) were normalized and reduced with 10 mM DTT for 45 min at 50 °C followed by alkylation with 10 mM iodoacetamide for 30 min at 25 °C in the dark. The reaction was quenched to a final concentration of 20 mM DTT. Lysates were precipitated with six volumes of acetone overnight at -20 °C. Protein pellets were centrifuged at 10,000 x *g*, 10 min at 4 °C and resuspended in 50 mM TEAB, pH 8.0. Samples digested with trypsin (Promega, V5111) at a 1:50 enzyme-to-substrate ratio for 16 h at 37 °C. The peptide mixture was acidified to a final concentration of 2% formic acid, 0.1% trifluoroacetic acid (TFA) and centrifuged at 16,000*g* for 5 min, frozen at -20 °C for 30 min, and dried by vacuum centrifugation. For proteomic analysis, peptides were

220 resuspended in 2% acetonitrile, 0.07% TFA, quantified by Fluorometric Peptide Assay and normalized
221 to 1 µg per 3 µl.
222
223 Peptides were analyzed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X
224 hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source in positive
225 mode as described [62]. Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-
226 size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo
227 Technos Co. Ltd) with a gradient of 2–28% acetonitrile containing 0.1% formic acid over 110 minutes
228 at 300 nL min^{-1} at 55°C. An MS1 scan was acquired from 350–1,650 *m/z* (60,000 resolution, 3×10^6
229 automatic gain control (AGC), 128 mseconds injection time) followed by MS/MS data-dependent
230 acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000
231 resolution, 1×10^5 AGC, 60 mseconds injection time, 28% normalized collision energy, 1.3 *m/z*
232 quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species
233 were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for
234 30 seconds. RAW data is available in ProteomeXchange Consortium via the PRIDE (#PXD021371).
235
236 **2.8 Data Processing and Bioinformatics Pipeline**
237 Peptide identification and quantification were performed as described previously [62, 63] using
238 MaxQuant (v1.6.14) with its built-in search engine Andromeda [64]. Tandem mass spectra were
239 searched against Homo sapiens (human) reference proteome (74,811 entries, downloaded 1-2020)
240 supplemented with common contaminants. Search parameters included carbamidomethylated
241 cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as
242 variable modifications. Data was processed using trypsin/P as the proteolytic enzyme with up to 2
243 missed cleavage sites allowed. The search tolerance and fragment ion mass tolerance were set to 7
244 ppm and 0.5 Da, respectively, at less than 1% false discovery rate on peptide spectrum match (PSM)
245 level employing a target-decoy approach at peptide and protein levels. Label free quantification (LFQ)

246 algorithm in MaxQuant was used to obtain quantification intensity values and processed using Perseus
247 as described [65]. LFQ intensities were log2 transformed after removing contaminants and reverse
248 identifications. Proteins with no missing values among all sample groups are subjected to two-tail t-
249 test with p-value adjusted at 5% permutation-based FDR. Missing values between technical replicates
250 imputed using Perseus built-in imputation feature from a normal distribution with 1.8 downshift and
251 0.3 width. Normalized intensities were log2 transformed, with statistical analyses performed using
252 Student's T-test or ANOVA (q-value <0.05 was considered significant). Gene enrichment functional
253 annotation clustering analysis was performed using DAVID bioinformatics recourses[66]. Graphpad
254 Prism and Rstudio were used for visualization of analysis.
255

256 # 3 Results

257 ## 3.1 DCLK1 overexpression increases cell protrusions and secretion of small extracellular vesicles
258 ## (sEVs).

259 In light of the frequent overexpression of DCLK1 in solid tumors and its associated role as a cancer
260 stem cell marker and putative driver of gastric cancer [15, 16, 20, 33, 35, 38, 41, 43, 67], we established a clonal
261 MKN1^{OE} gastric cancer cell line, which stably over-expressed DCLK1 at a level approximately 4.5 fold
262 higher compared to parental MKN1 (MKN1^{PAR}) cells (**Figure S1A**). Consistent with the reported role of
263 DCLK1 as an inducer of EMT in various cancer cells [25, 26, 28, 29], MKN1^{OE} cells display morphologically
264 different to MKN1^{PAR} cells (**Figure 1A**). While the MKN1^{PAR} gastric cancer cells already have a
265 mesenchymal-like morphology, upon DCLK1 overexpression we observe an increased number of
266 cellular protrusions suggesting increased plasma membrane dynamics in MKN1^{OE} cells (**Figure 1A**).
267 In light of the increased membrane dynamics observed in cells overexpressing DCLK1 and the pivotal
268 role that membrane forces play in the shedding of extracellular vesicles, we investigated the impact
269 of DCLK1 and of its catalytic kinase activity on the release and composition of EVs. To avoid potential
270 cytotoxicity a dose-response assessment was performed to select a concentration of the inhibitor well
271 below the IC_{50}. Consistent with previous reports on colorectal and pancreatic cancer cells, DCLK1-IN-

272 1 had little effect on cell viability at concentrations up to 1 µM with an IC_{50} of 14 and 49 µM for
273 MKN1^{PAR} and MKN1^{OE} cells, respectively (**Figure 1B**) [58, 59]. Based on these results and the known IC_{50}
274 of 57 nM for the inhibition of the catalytic activity of the DCLK1 kinase [59], we decided to use DCLK1-
275 IN-1 at a concentration of 1 µM for all treatments in this study.
276
277 We collected conditioned media from MKN1^{PAR} cells and MKN1^{OE} cells grown for 48 hr in presence or
278 absence of DCLK1-IN-1[58, 59] and to gain insight into composition of sEVs, subsequently purified sEVs
279 using differential ultracentrifugation from large EVs and other non-EV components (**Figure 1C**).
280 Marker expression of sEVs was confirmed for TSG101 and ALIX, revealing enrichment of sEVs from
281 lEVs and cell lysate; however, we did not detect DCLK1 in either lEVs or sEVs (**Figure 1D, S1B-D**). The
282 relative protein abundance resulted in a significant (p=0.041) increase in total protein amount in sEVs
283 from MKN1^{OE} cells and a 2.1 fold increase in total protein amount from lEVs purified from MKN1^{OE+INH}
284 cells (**Figure S1E**). This result was further validated by nanoparticle tracking analysis (NTA) confirming
285 increased concentrations of particles detected, which coincide with the increased protein amounts in
286 MKN1^{OE} sEVs and MKN1^{OE+INH} lEVs (**Figure 1E-F, S1E-F**). We observed a significantly increased
287 concentration of particles in the 200-600 nm range for MKN1^{OE} sEVs compared to MKN1^{PAR}, and a
288 concomitant decrease upon DCLK1 inhibition in MKN1^{OE+INH} sEVs (**Figure 1F**). The MKN1^{PAR} lEVs are
289 significantly smaller compared to lEVs from either MKN1^{OE} and MKN1^{OE+INH} (**Figure S1F**). Collectively,
290 we observe DCLK1 overexpression induces cellular protrusions and increase generation of enlarged
291 vesicles released, the latter is reversed upon DCLK1 inhibition.
292
293
294 **3.2 Quality control of sEV proteome replicates**
295 EVs of endosomal origin need to be transported along microtubules to the plasma membrane, in
296 contrast to ectosomal originating EVs. Endosomal EVs, which are smaller (30-200 nm) than ectosomal
297 EVs (100-1,500 nm) and therefore more likely to end up in the sEV fraction, in addition the lEV fraction

298 consists of a very heterogeneous pool of EVs (exosome, microvesicles and apoptotic bodies). Given
299 the importance of sEVs in the tumour microneivornment and transfer of oncogenic cargo in several
300 key studies (REFS!!!), we focused on understanding the composition of sEVs in the context of DCKL1.
301 Therefore, to investigate the capacity of DCLK1 in regulating sEV proteome composition, we
302 performed quantitative proteomics on MKN1^{PAR}, MKN1^{OE}, and MKN1^{OE+INH} sEVs, identifying 1492
303 unique proteins with high stringency (present in 4 or more replicates) across all sample groups, with
304 1290, 1265, and 1362 proteins in each group, respectively (**Figure S1A, Table S1**). Proteomics analysis
305 further identified in sEV proteome several classical EV marker proteins: ALIX, TSG101, CD81, CD82,
306 FLOT1 and FLOT2 (**Figure S1B, Table S1)**. To assess data variance and sample grouping, we performed
307 a correlation matrix (**Figure S1C**) and principal component analysis (**Figure S1D**), demonstrating that
308 MKN1^{OE} and MKN1^{OE+INH} sEV proteomes clustered together and could be distinguished from MKN1^{PAR}
309 sEVs. This revealed that our replicate MKN1^{OE4} (OE4) consistently generated outlier results and
310 therefore was excluded from further downstream analyses (**Figure S1C-D**).

311

312 **3.3 Overexpression of DCLK1 induces reprogramming of sEV composition to support adhesion and**
313 **migration**

314 To investigate the influence of DCLK1 overexpression on sEV proteomes, we initially compared sEV
315 proteomes between MKN1^{OE} and MKN1^{PAR} cells, and observed significantly (students t-test p<0.05)
316 altered abundance of 381 of the identified 1424 proteins, including 96 down- and 283 up-regulated
317 (**Figure 2A, Table S2-3**). We next performed enrichment map analysis of these proteins to gain insight
318 into enriched pathways and functions (Gene Ontology (GO), KEGGs). Subsequent gene enrichment
319 analysis revealed that a third of these proteins are assigned to either cell adhesion (gene ontology GO:
320 0007155, 27.6%) and cell migration (GO: 0016477, 14.5%) (**Figure 2A, S2E, Table S4-5**). The
321 unsupervised cluster analysis shows the distribution of the 381 differentially expressed proteins in
322 MKN1^{OE} in comparison to MKN1^{PAR}, proteins are linked to cell migration or cell adhesion biological
323 processes. (**Figure 2B**). Refining of the two GO-term hierarchies revealed altered protein abundance

324 in more specific clusters in a pro-tumorigenic way, namely the upregulation of epithelial cell migration
325 (GO:0010631, p=5.68E-04) and cell-matrix adhesion (GO:0007160, p=0.010), and the down-regulation
326 of leukocyte migration (GO:0050900, p=4.37E-03) and cell adhesion regulation (GO:0045785,
327 p=0.012) (**Figure 2C**). Major altered proteins are basal cell adhesion molecule (BCAM) and collagen
328 type III α1 (COL3A1) (both involved in extracellular matrix (ECM) reorganization) and serine/threonine
329 Ras-activated protein (STRAP) and coronin 1B (CORO1B) which is involved in cell migration and
330 invasion. Downregulated proteins integrin subunit alpha 2 (ITGA2) and unconventional myosin 1G
331 (MYO1G) (both involved in leukocyte migration), cysteine-rich 61 (CCN1) (ECM-protein regulating cell
332 adhesion), CD59 and CD55 (integrins involved in complement cascade activation) (**Figure 2C**). Most of
333 the aforementioned proteins have been implicated with poor prognosis and metastasis in gastro-
334 intestinal cancers [68-72].
335 We next questioned whether sEV derived from MKN1^{OE} could regulate DCLK1-mediated function,
336 associated with cell migration and adhesion [58, 59] . Indeed, sEVs from MKN1^{OE} increased cell migration
337 of MKN1^{PAR} cells, in comparison to MKN1^{PAR} sEVs (**Figure 2D-E**). Thus, our data suggest that DLCK1
338 reprograms sEVs to support pro-migratory phenotype in gastric cancer cells.
339

340 **3.4 Molecular inhibition of DCLK1 identifies proteins modified in expression in sEVs.**

341 To understand DCLK1 kinase-dependent cargo selection for sEVs, we compared the proteome profiling
342 between MKN1^{OE} and MKN1^{OE+INH} sEVs. Across the 1400 identified proteins, this revealed 61 proteins
343 with altered abundance (students t-test p<0.05), including 16 up-regulated and 45 down-regulated
344 proteins (**Figure 3A, Table S6-7**). Interestingly, 31% of these proteins are associated with cell adhesion
345 (GO:0007155, 21%) and/or cell migration (GO: 0016477, 18%) (**Figure 3A, S2F, Table S8-9**), supporting
346 a functional association of DCKL1 with these cellular processes. Among the proteins displaying a
347 DCLK1-kinase activity dependent abundance (**Figure 3B**), we identified CCN1, KTN1, STRAP, RCC2,
348 SBDS and JAK1 that collectively have been implicated previously with cell migration, EMT or ECM
349 regulation in gastric or other malignancies [73-78].

350

351 **3.5 Identification of 55 DCLK1-kinase dependent sEV cargo proteins**

352 We next questioned the association of DCLK1 activity with the sEV proteome – looking whether the
353 61 altered sEV proteins upon DCLK1 inhibition are also altered upon overexpression. We performed a
354 correlation analysis of these differentially expressed components in sEVs ($MKN1^{OE}/MKN1^{PAR}$ vs
355 $MKN1^{OE+INH}/MKN1^{OE}$) revealing a strong negative correlation (R^2=-0.745, p = 5.37e-12, Pearson
356 correlation) and resulting in 55/61 proteins that are altered in a kinase dependent way (**Figure 3C,**
357 **Table S6**). Of which 13 are downregulated upon overexpression ($MKN1^{OE}/MKN1^{PAR}$) and upregulated
358 upon DCLK1 inhibition ($MKN1^{OE+INH}/MKN1^{OE}$) and vice versa 45 proteins are up and then down
359 regulated, respectively. Two key proteins upregulated upon DCLK1 overexpression and
360 downregulated upon inhibition (up – down) include DEK (oncoprotein associated with chromatin
361 organization) and KTN1 (microtubule-based movement, adhesion and migration), while opposite
362 behaving (down - up) proteins include the extracellular matrix binding protein CCN1 (associated with
363 cell proliferation and cell adhesion) and endosomal sorting protein CHMP1A (**Figure 3C**). The heatmap
364 reveals that the $MKN1^{PAR}$ and $MKN1^{OE+INH}$ replicates cluster together separate of the $MKN1^{OE}$
365 replicates, indicating the inhibition of DCLK1 brings these protein levels down to baseline ($MKN1^{PAR}$)
366 (**Figure 3D**). Interestingly, 15/55 proteins are associated with cell adhesion and/or cell migration. Thus,
367 it appears that DCLK1 can modulate the composition of sEVs in a kinase-dependent manner, resulting
368 in key changes in pro-adhesive and pro-migratory factors, supported by the known functions of DCLK1
369 in cell migration and adhesion as mentioned above.

370

371 **4 Discussion**

372 In this study, we establish a new functional role for the DCLK1 in supporting sEV biogenesis, secretion
373 and reprogramming sEV cargo towards a pro-migratory phenotype, *in vitro*. This is in line with previous
374 reports linking DCLK1 expression to the induction of signaling pathways effecting cancer cell motility,
375 invasion and EMT [22-29, 59, 79]. Our results also align and extend mechanistic models of DCLK1 as a

376 polymerizer and stabilizer of microtubules and therefore facilitator of vesicular trafficking [4, 5, 11-14]
377 (Figure 4).
378
379 The exact mechanism of how DCLK1 can alter EV biogenesis or influence cargo selection is currently
380 unknown. Nevertheless, the reversible nature of sEV size, cargo quantity and composition after DCLK1
381 kinase inhibition is a clear indicator of an important role for the catalytic activity of DCLK1 in all or
382 some of these processes. Whilst our sEV proteome analysis is of limited use in deciphering the
383 intracellular processes directly regulated by DCLK1, it has uncovered several candidates that may
384 explain the promotion of EV biogenesis in DCLK1 over-expressing cells (Figure 4A). Most intriguingly is
385 Kinectin (KTN1), an organelle transmembrane receptor shown to be involved in intracellular organelle
386 motility [80, 81]. KTN1 anchors vesicles and organelles to kinesins, which are transported in a forward
387 movement towards the plus ends of the microtubules [82]. Further, binding of KTN1 to kinesin
388 stimulates kinesin-ATPase activity, releasing kinesin from its inactive compact formation [83]. This
389 observation is consistent with the known localization of DCLK1 at the plus ends of microtubules and
390 doublecortin-stabilized microtubules are substrates for kinesin translocase motors and for
391 depolymerase kinesins [10]. The combination of both increased DCLK1 and KTN1 levels could be a
392 mechanism through which kinesins are facilitated to bind to both the microtubules and the vesicles,
393 increasing vesicular and organelle transport stability and rate. This could explain the increased amount
394 of secreted sEVs *in vitro*. Another explanation for this might be the reduction of MYO1G, which has
395 been shown to be essential for lysosome stability in different human cancer cell types [85]. The
396 downregulation of MYO1G and probable destabilization of the lysosome might influence the decision
397 of MVBs being fused to the plasma membrane rather than with the lysosome [86]. In contrast, charged
398 multivesicular body protein 1A (CHMP1A), is a protein which, in yeast, has been shown to directly
399 interact with vacuolar protein sorting 4 (VPS4) [84], a component of the endosomal sorting complex
400 required for transport III (ESCRT-III), which is mainly responsible for the scission of the intraluminal
401 vesicles (ILVs) into the MVBs [84]. This might suggest that a lack of CHMP1A may cause delays in scission

402 leading to potential defects in the generation of MVBs and may help explain the larger vesicles
403 observed after forced DCLK1 expression and their reversion to normal size after inhibitor treatment.
404
405 Several studies have shown that high expression of DCLK1 induces EMT and increases migration and
406 invasion in several different cancer types through various mechanisms [22-29]. In this study, in addition
407 to identifying upregulated pro-migratory cargo proteins within sEVs from DCLK1 overexpressing MKN1
408 cells, we also show that these sEVs indeed increase transwell cell migration of MKN1 parental cells *in*
409 *vitro*, thus revealing an as of yet unappreciated role of DCLK1 in indirectly reprogramming recipient
410 cells. Two most interesting kinase dependent cargo proteins associated with epithelial cell migration
411 are coronin 1B (CORO1B) and serine/threonine kinase receptor-associated protein (STRAP) which are
412 increased in MKN1OE-sEVs and decreased in MKN1OE+INH-EVs (Figure 4B). CORO1B is a type I
413 cornonin, regulating various actin-dependent cellular processes via its interaction with the Arp2/3
414 complex promoting cell protrusion, migration and scission. Silenced or kinase dead CORO1B has been
415 shown to reduce migration in a multitude of different cancer and non-cancer cells and interestingly,
416 type I cornonins have been associated with poor prognosis and metastasis in GC. In addition, STRAP is
417 significantly upregulated in GCs compared to adjacent normal tissue [71, 100] and STRAP silencing has
418 been shown to reduce cell migration and invasion *in vitro,* and metastasis *in vivo* in CRC and
419 osteosarcoma [71, 101]. Mechanistically, STRAP binds to GSK-3β which reduces the phosphorylation,
420 ubiquitination, and stops the degradation of β-catenin, resulting in activated Wnt/ β-catenin pathway
421 promoting cancer stemness, migration and metastasis [71]. A different study showed that STRAP is
422 tethered to collagen mRNAs and facilitates its translation and thus indirectly regulating ECM stiffness
423 and cell-matrix adhesion [102]. Stiffening of the ECM induces focal adhesion formations within the cells,
424 which are essential for directional cancer cell motility [92-94].
425

426 As well as pro-migratory proteins, the sEVs released by MKN1^{OE} cells also carried more abundant cell-
427 matrix adhesion promoting proteins, of which BCAM and COL3A1 are the top two associated proteins
428 (Figure 4C). BCAM is a member of the immunoglobulin superfamily and a receptor for the extracellular
429 matrix protein laminin. Interestingly, BCAM levels are significantly higher in primary GC tumors of
430 patients with metastasis and predict an overall worse survival [68] and increase cell migration, invasion
431 and metastasis by mediating tumor-ECM interactions [91]. COL3A1 is a type III collagen and part of the
432 interstitital matrix regulating stromal components, and is upregulated in gastric cancer versus normal
433 stomach tissue and is a marker of poor prognosis in many cancer types [24, 89, 90]. Extracellular matrix
434 protein cyCCN1 is a DCLK1 kinase-dependent sEV cargo protein and is downregulated upon
435 overexpression and upregulated upon inhibition. CCN1 is secreted into the ECM and regulates a broad
436 spectrum of cellular activities, including cell adhesion and migration in a cell type and context
437 dependent manner. High CCN1 levels are linked to sites of inflammation and wound healing processes
438 [95], activates NFκB signalling in macrophages polarizing them towards a pro-inflammatory M1
439 phenotype [96] and can induce cell type specific apoptosis of fibroblasts through the activation of FasL,
440 TNFα or integrins [97-99]. The downregulation of CCN1, and upregulation of BCAM and COL3A1 upon
441 DCLK1 overexpression is suggestive of a cell extrinsic role for DCLK1 in the regulation of immune
442 evasive, matrix stiffening, pro-migratory and pro-fibroblastic processes.

443 The limitation of this study is that the work has been done in a single cell line, however the amount of
444 material required to understand sEV composition and function is a key requirement and focused this
445 study in utlising the single model. Subsequent studies could address whether the same effect in EV-
446 biogenesis and pro-migratory cargo selection is observed in other (non)-cancerous cell lines or primary
447 GI-derived cells from healthy patients. Further, to understand the contribution of other factors in the
448 secretome, including other types of EVs (lEVs and exomeres) and soluble factors could also raise
449 further insights into this crosstalk between cancer and non-cancer cells and the role of DCLK1 in this
450 signaling.

451

452 In conclusion, our data has uncovered a novel role for DCLK1 in sEV biogenesis. We found kinase-
453 dependent and independent functions for DCLK1 in sEV biology relating to size, composition and
454 secretion. One of the principal impacts of DCLK1-reprogrammed sEVs is the ability to promote cell
455 migration in recipient cells, *in vitro.* Other altered cargo proteins are associated with GO biological
456 processes that weaken cell-cell adhesion, strengthen cell-matrix adhesion and influence leukocyte
457 migration. These novel insights into DCLK1 function may pave the way for a better understanding of
458 its role as a maker of cancer stem cells and driver of tumorigenesis.
459

460 ## Figure legends

461 **Figure 1. The effect of DCLK1 overexpression and inhibition on cell morphology and viability, and**
462 **isolation and characterization of small extracellular vesicles from MKN1PAR, MKN1OE and**
463 **MKN1OE+INH conditioned media. A)** Morphological images of $MKN1^{PAR}$ and $MNK1^{OE}$ cells, scalebars
464 = 20 µm, cell protrusions are indicated with arrowheads. **B)** DMSO normalized cell viability dose-
465 response assay with DCLK1-IN-1 inhibitor. Data is represented as mean ± SEM of n = 4 technical
466 replicates and are representative of n = 3 independent experiments, horizontal dotted line = IC_{50},
467 vertical dotted line = 1 µM of DCLK1-IN-1 inhibitor. **C)** Flow chart of the sEV isolation procedure by
468 sequential differential centrifugation. **D)** Representative western blot for ALIX, TSG101, DCLK1, and
469 GAPDH for full cell lysate and sEVs. **E)** Relative protein abundance of sEVs normalized to the $MKN1^{PAR}$
470 subset. Data represented are average (n = 5) ± SEM (error bars), with unpaired Student's t-test, * p <
471 0.05. **F)** Histogram of Nanoparticle Tracking Analysis for particle concentration (particles / ml) and size
472 distribution of sEVs of $MKN1^{PAR}$ (blue), $MKN1^{OE}$ (red) and $MKN1^{OE+INH}$ (green), grouped per 100 nm.
473 Data represent average of 5 replicate measurements ± SEM (error bars), * p < 0.001.
474

475 **Figure 2. Overexpression of DCLK1 induces aberrant significant (p<0.05) differentially abundant**
476 **proteins in sEVs isolated from MKN1.** Proteins are present in >75% of replicates in at least one group.
477 **A)** Overview of 55 unique and 381 significant differentially expressed proteins (p < 0.05) proteins in
478 $MKN1^{OE}$ sEVs in comparison to $MKN1^{PAR}$ sEVs, showing the percentage of significant altered proteins
479 associated with GO:0016477~cell migration, GO:0007155~cell adhesion, both or other GO-terms. **B)**
480 Unsupervised clustering analysis of significantly differentially expressed proteins (p<0.05) for each
481 replicate of $MKN1^{PAR}$ (PAR) and $MKN1^{OE}$ (OE) sEVs, values are z-scores of the LFQ intensities (missing
482 values = grey), side columns link proteins are to GO:0016477~cell migration or GO:0007155~cell
483 adhesion (black lines). **C)** Volcano plot showing differentially expressed proteins. The horizontal axis
484 depicts the $\log_2$ fold change, the vertical axis represent the –log(*p*-value, students t-test), with
485 significance threshold at *p*-value = 0.05 (dashed line). Proteins are mapped to their GO-terms:

486 epithelial cell (square) and leukocyte (triangle) migration (orange), positive regulation of cell adhesion
487 (square, purple), or regulation of cell-matrix adhesion (triangle, purple) **D)** Representative images of
488 the trans-well migration membrane of MKN1^{PAR} cell with and without 20% FCS, and sEVs secreted by
489 either MKN1^{PAR} or MKN1^{OE}. Top shows the whole membrane, 1x magnification, scale bar = 1 mm.
490 Bottom row shows 10x magnification, scale bar = 100 um. **E)** Nuclear count of Aperio™ analysis of
491 complete membrane of trans-well migration assay in D (n = 3, error bars = SEM, *p*-value = 0.0053.
492

493 **Figure 3. DCLK1-kinase dependent cargo selection for sEVs.** DCLK1 overexpressing cells (MKN1^{OE})
494 treated with the small molecule inhibitor DCLK1-IN-IN (MKN1^{OE+INH}) resulted in significant differential
495 expression of 61 proteins in sEVs. Proteins are present in >75% of replicates in at least one group. **A)**
496 Overview of 15 unique and 61 significant differentially expressed proteins ($p < 0.05$) proteins in
497 MKN1OE sEVs in comparison to MKN1PAR sEVs, showing the percentage of significant altered proteins
498 associated with GO:0016477~cell migration (red), GO:0007155~cell adhesion (blue), both (checkered,
499 red-blue), and other GO-terms (grey). **B)** Volcano plot showing differentially expressed proteins;
500 showing differential log_2 FC of MKN1^{OE+INH} *versus* MKN1^{OE}, the Y-axis shows the –log(*p*-value, students
501 t-test), with significance threshold at *p*-value 0.05 (dashed line). Proteins are mapped to their GO-
502 terms: GO:0016477~cell migration (orange), GO:0007155~cell adhesion (purple) or both (red-blue
503 halved circles). **C)** Correlation coefficient analysis of log_2 fold change of MKN1^{OE}/MKN1^{PAR} (x-axis)
504 versus log2 fold change of MKN1^{OE+INH}/MKN1^{OE} (y-axis) of significantly differential proteins in response
505 to DCLK1 inhibitor treatment. R-value represents Pearson correlation. **D)** Hierarchical clustering
506 analysis (unsupervised clustering) of 55 kinase dependent proteins in sEVs from MKN1^{PAR} (PAR),
507 MKN1^{OE} (OE) and MKN1^{OE+INH} (INH); values are z-scores of LFQ intensities (missing values = grey), side
508 columns link proteins are to GO:0016477~cell migration or GO:0007155~cell adhesion (black lines).
509

510 **Figure 4. Schematic summary and proposed mechanism of action of DCLK1 on extracellular vesicle**
511 **biogenesis & downstream biological effects. Throughout this figure** sEV cargo proteins that are
512 upregulated are shown in red and downregulated in cargo proteins are in blue. **A)** The effect of DCLK1
513 on extracellular vesicle biogenesis where KTN1 facilitates anchoring of multivesicular bodies (MVBs)
514 to kinesins, therefore facilitating transport along microtubules. Lower CHMP1A levels might explain
515 the larger vesicles found, CHMP1A is a regulator of vesicular scission. Lastly, downregulation of
516 MYO1G results in destabilization of lysosomes favoring the decision of sEVs to be released rather than
517 recycled. **B)** These secreted sEVs can alter intracellular changes upon uptake and main altered cargo
518 proteins involving cell-cell adhesion and cell migration are: STRAP, CORO1B, CD59 and CD55. **C)** The
519 effect of secreted sEVs on extracellular changes and ECM remodeling include altered cargo proteins
520 BCAM, COL3A1 and CCN1 associated with cell-matrix adhesion and cell migration biological processes.
521
522

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649

650 **Conflict of Interests:**

651 F.M.F. and N.S.G. are inventors on a patent application related to the DCLK1 inhibitors described in
652 this manuscript (WO/2018/075608). N.S.G. is a founder, science advisory board member (SAB) and
653 equity holder in Gatekeeper, Syros, Petra, C4, B2S, Aduro, Jengu, and Soltego (board member). The
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656

Supporting information

Figure S1. Supplementary Figure 1.

A) Quantification of DCLK1 overexpression on westernblot relative to GAPDH expression using ImageLab software (data represents the mean (n=4), ± SEM (error bars), with unpaired Student's t-test, * $p = 0.032$. **B)** Relative protein abundance of lEVs normalized to the MKN1PAR subset. Data represented are average (n = 5) ± SEM (error bars), with unpaired Student's t-test, * $p < 0.05$, * $p < 0.01$. **C)** Histogram of Nanoparticle Tracking Analysis for particle concentration (particles / ml) and size distribution of lEVs of MKN1PAR (blue), MKN1OE (red) and MKN1OE+INH (green), grouped per 100 nm. Data represent average of 5 replicate measurements ± SEM (error bars), * $p < 0.001$. **D)** Venn diagram of proteins identified within 80% of replicates for MKN1^{PAR} (blue), MKN1^{OE} (red) and MKN1^{OE+INH} (green) sEVs. **E)** LC-MS/MS LFQ of EV markers ALIX, TSG-101, CD81, FLOT1, and FLOT2. Data represents the mean (n=5) ± SEM (error bars) for MKN1^{PAR} (blue), MKN1^{OE} (red), and MKN1^{OE+INH} (green) sEVs. **F)** Correlation matrix performed using log2 transformed LFQ intensity values of high-confident proteins from each replicate (5 replicates per group). The color scale represent the Pearson correlation values. **G)** Principal component analysis for proteomic profiling of each replicate based on the log2 transformed LFQ values of proteins that have been quantified in minimum 80% of replicates for each group (MKN1^{PAR} (blue), MKN1^{OE} (red) and MKN1^{OE+INH} (green)). **H)** Geneset enrichment analysis for significant biological processes for the 381 significantly altered proteins (MKN1^{OE} / MKN1^{PAR}), with number of proteins found in upregulated (red) and downregulated (blue) subset. **I)** Geneset enrichment analysis for significant biological processes for the 61 significantly altered proteins (MKN1^{OE+INH} / MKN1^{OE}), with number of proteins found in upregulated (red) and downregulated (blue) subset.

680 **Table S1. Quantitative mass spectrometry of sEVs derived from MKN1^{PAR}, MKN1^{OE}, and MKN1^{OE+INH}.**
681 Log_2 LFQ values are displayed for each replicate and validity percentage, indicating in how many
682 replicates the protein has been identified.

683 **Table S2. Significant differentially expressed proteins in sEVs upon DCLK1 overexpression.** With the
684 mean Log2 LFQ values, SD and Log2 fold changes for MKN1^{OE} vs MKN1^{PAR} and significance ($p < 0.05$,
685 students t-test).

686 **Table S3. Differentially expressed proteins in sEVs derived from from MKN1^{OE} and MKN1^{PAR} (master**
687 **table).** Log_2 LFQ values are displayed for all replicates, with the validity percentage of at least 80% in
688 one group and significance ($p < 0.05$, students t-test).

689 **Table S4. Gene ontology enrichment cluster analysis for the 381 significantly altered proteins found**
690 **in MKN1^{OE} vs MKN1^{PAR}**. GO-biological processes are filtered for $p<0.05$ and $FDR<0.02$. Highlighted GO
691 terms are used in Table S5.

692 **Table S5. The up- and downregulated proteins for each highlighted GO-term found in MKN1^{OE} vs**
693 **MKN1^{PAR}**. With their percentage of up- (orange) and downregulated (blue) proteins, used for figure
694 S1H.

695 **Table S6. Significant differentially expressed proteins upon DCLK1 inhibition.** Showing the mean Log2
696 LFQ values and SD values for MKN1^{PAR}, MKN1^{OE}, and MKN1^{OE+INH} and Log2 FCs and significance
697 (students t-test) for [MKN1^{OE} vs MKN1^{PAR}] and [MKN1^{OE+INH} vs MKN1^{OE}].

698 **Table S7. Differentially expressed proteins in sEVs derived from MKN1^{OE} and MKN1^{OE+INH} (master**
699 **table).** Log_2 LFQ values are displayed for all replicates, with the validity percentage of at least 80% in
700 one group and significance ($p < 0.05$, students t-test).

701 **Table S8. Gene ontology enrichment cluster analysis for the 61 significantly altered proteins found**
702 **in MKN1-OE+INH vs MKN1-OE**. GO-biological processes are filtered for $p < 0.05$. Highlighted GO terms
703 are used in Table S9.

704 **Table S9. The up- and downregulated proteins for each highlighted GO-term found in MKN1^{OE+INH} vs**

705 **MKN1^{OE}**. With their percentage of up- (orange) and downregulated (blue) proteins, used for figure

706 S1H.

707