1   **Delta-catenin is required for cell proliferation in virus positive Merkel cell**

2   **carcinoma cell lines but not in human fibroblasts**

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11   **Running Head:** δ-catenin in virus positive Merkel cell carcinoma cells

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Abstract: Merkel cell carcinoma (MCC) is a highly aggressive neuroendocrine skin cancer often driven by the integration of Merkel cell polyomavirus (MCPyV) into the host genome and the persistent expression of its viral oncoproteins, small tumor (ST) antigen and truncated large tumor (t-LT) antigen. While human fibroblasts support MCPyV replication, the cell of origin for MCC remains unknown. We hypothesized that MCPyV initially replicates in fibroblasts but, in rare cases, infects Merkel cell progenitors, contributing to MCC development. Using TurboID mass spectrometry, we identified δ-catenin as a novel ST interactor in fibroblasts. However, while ST binds δ-catenin in fibroblasts, this interaction is absent in virus-positive (VP)-MCC cell lines. Despite this, δ-catenin is essential for VP-MCC, but not for fibroblast, cell proliferation. We found that fibroblasts predominantly express δ-catenin isoform 1, whereas VP-MCC cells mainly express isoform 3. Overexpression of isoform 1 in VP-MCC failed to restore ST binding. δ-catenin promotes VP-MCC proliferation by regulating cell cycle gene expression through its interaction with Kaiso, a transcriptional repressor. Additionally, we found that LSD1 (KDM1A) regulates δ-catenin isoform 3 expression by modulating ESRP1, a δ-catenin splicing factor. Our findings reveal novel host factors involved in MCPyV infection and MCC tumorigenesis, suggesting that the host cell supporting viral replication and the MCC cell of origin may be distinct cell types.

Importance: Merkel cell polyomavirus (MCPyV), the only known human oncogenic polyomavirus, is the primary cause of Merkel cell carcinoma (MCC), a rare and aggressive type of skin cancer. MCC is driven by two viral proteins: small T (ST) and large T (LT). While the virus can replicate in skin fibroblasts, it is still unknown which type of skin cell becomes cancerous. We found that ST binds to a host protein, δ-

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45 catenin in fibroblasts, potentially playing a role in the virus lifecycle, but this interaction

46 is missing in the cancer cells. Our study provides evidence that the cells in which the

47 virus replicates and causes cancer are different.

48

49 **INTRODUCTION**

50 Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin tumor with

51 two distinct etiologies: virus-negative (VN) and virus-positive (VP) MCC. VN-MCC

52 tumors are caused by chronic ultraviolet (UV) exposure and are associated with high

53 mutational burden frequently involving mutations in p53 and RB1 (1, 2). VP-MCC

54 tumors are driven by the clonal integration of Merkel cell polyomavirus (MCPyV)

55 genome into the host genome and the expression of the truncated large T (t-LT) and

56 small T (ST) antigens, which are essential for tumorigenesis (3). Tumor-specific LT

57 truncations inactivate LT's DNA helicase function, disrupt viral replication while

58 preserving the LXCXE motif, which is essential for binding to and inhibiting the tumor

59 suppressor RB1 (4). In contrast, ST remains full length and forms a complex with L-Myc

60 and EP400 that upregulates MDM2 and CK1α to activate MDM4 to suppress p53

61 activation (5). Furthermore, ST in complex with L-Myc and the EP400 complex,

62 activates the expression of LSD1 (lysine-specific histone demethylase 1), also known as

63 KDM1A, and other CoREST complex members including RCOR2 and INSM1. This

64 complex mediates oncogenic transformation and tumorigenesis by repressing the

65 expression of master regulators of the neuronal linage in MCC while promoting tumor

66 cell proliferation (6, 7). In addition, several studies have shown that the expression of

3

67 ST alone is sufficient to transform rodent fibroblast cells (8, 9) and human primary

68 fibroblast (10) in vitro. Surprisingly, ST transformation functions are independent of the

69 conserved interaction with protein phosphatase 2A (PP2A), seen with the oncogenic

70 simian polyomavirus 40 (SV40) (8, 11). Furthermore, ST has shown oncogenic activity

71 in mouse models (12, 13) indicating that ST is the dominant transforming viral protein.

72 The cell of origin of MCC remains unknown. MCC tumors were initially thought to arise

73 from Merkel cells because they share expression of neuroendocrine markers. However,

74 Merkel cells are highly differentiated and non-proliferative (14). As a results, other

75 candidates have been suggested such as keratinocytes (HFK). The co-expression of ST

76 and ATOH1, a Merkel cell linage transcription factor, is sufficient to initiate development

77 of epidermis-derived MCC-like tumors in mice (15). Other candidates such as epidermal

78 and dermal stem cells in addition to skin-derived precursors have been suggested (16)

79 although none has been conclusively linked to MCPyV infection or development of

80 MCC. Recently, MCC-like tumors were observed upon co-expression of MCPyV T

81 antigens and ATOH1, in addition to the knockout of p53 in a keratin 5 (KRT5) promoter

82 driven-mouse model (15). Interestingly, Merkel cells originate from basal epidermal

83 progenitor cells that express both keratin 14 (KRT14) and through ATOH1-dependent

84 transcriptional reprograming (17). However, whether these basal epidermal cells or their

85 descendants, Merkel cells, are the cell of origin of MCC remains unclear.

86 So far, the only cell type that is permissive for the MCPyV lifecycle in vitro is human

87 dermal fibroblasts (human foreskin fibroblasts or HFFs) (18). Typically, permissive cells

88 support viral replication without undergoing transformation as observed in other

4

89   polyomaviruses such as SV40 (19). This prompted us to investigate the cellular

90   interactors of ST in HFFs using proximity labeling mass spectrometry (TurboID MS).

91   Notably, since establishing an MCPyV infection model in cell culture remains technically

92   challenging due to the difficulty of propagating MCPyV, we focused instead on

93   expressing ST in HFFs. Among potential interactors, δ-catenin exhibited the strongest

94   association with ST in HFFs. Additionally, δ-catenin has been shown to be required in

95   the life cycle of human papillomavirus (HPV) (20). However, the role of δ-catenin in the

96   life cycle of MCPyV and oncogenesis was unknown.

97   δ-catenin, also known as p120 catenin and encoded by the CTNND1 gene, is part of the

98   catenin protein family, which also includes β-catenin. Human δ-catenin isoforms,

99   referred to as isoform 1 through 4, differ from each other by the start codon used.

100   Additional isoforms are derived from combinations with alternatively used exons A and

101   B near the C- terminal end of the open reading frame and also with exon C in the middle

102   of the open reading frame (21, 22). The relative expression of each isoform is different

103   depending on the tissue and cell type. Moreover, the specific role of each isoform,

104   especially isoforms 2 and 4, is poorly understood. Each isoform shares the central

105   armadillo domain, which is essential for binding with cadherins on the cell membrane

106   (22, 23) or transcription factors such as Kaiso in the nucleus (24). Therefore, the

107   functional differences between the isoforms may be attributed to the N-terminus. δ-

108   catenin isoform 1 being 101 amino acid residues longer at the N-terminus compared to

109   isoform 3, potentially enables unique protein–protein interactions specific to isoform 1

110   (21, 22, 25). While δ-catenin isoform 1 has been reported to share regulatory

111   mechanisms with β-catenin, the regulatory mechanisms governing δ-catenin isoform 3

112   are unclear. δ-catenin isoform 1 harbors GSK3β and CK1α phosphorylation sites within

113   its unique N-terminal domain, which are absent in isoform 3. These sites facilitate β-

114   TrCP recognition and subsequent degradation (26). It was unknown which δ-catenin

115   isoforms are expressed in HFFs and MCC cell lines as well as how these isoforms are

116   regulated.

117   While δ-catenin isoform 1 is highly expressed in mesenchymal cells, isoform 3

118   predominates in epithelial cells (25). During epithelial-mesenchymal transition (EMT)

119   and cancer progression, transcription factors, such as Slug and Snail, promote isoform

120   1 expression (27), while Epithelial Splicing Regulating Proteins, ESRP1 and ESRP2,

121   favor isoform 3 in epithelial tissues (28, 29). δ-catenin binds to cadherin at the plasma

122   membrane through its armadillo domain supporting cell-cell adhesion (23). Loss,

123   downregulation of or changes in δ-catenin phosphorylation prevents its binding with E-

124   cadherin contributing to cell dissemination and is linked to metastasis and poor

125   prognosis in several human cancers (23, 30). δ-catenin has also been found in the

126   nucleus as it has two putative nuclear localization signals that interact with transcription

127   factors including Kaiso. By directly interacting with Kaiso, δ-catenin indirectly activates

128   the Wnt/β-catenin pathway (24). Kaiso is a BTB/POZ protein that has a bi-modal DNA-

129   binding activity: it binds methylated CpG dinucleotides or sequence-specific consensus

130   Kaiso binding sites within target gene promoters (31, 32) such as the Wnt/β-catenin

131   genes Wnt11, Cyclin D1 and Matrilysin 7, to promote cell proliferation and migration

132   (33-35). However, the role of δ-catenin in MCC was unknown. Interestingly, proteomic

133   analysis identified δ-catenin among the most downregulated proteins following LSD1

134   inhibition, further suggesting its regulation by LSD1 (6). However, the mechanisms still

6

135   are not understood. We hypothesized that while MCPyV initially infects HFFs for

136   production of MCPyV virions, in rare instances, MCPyV may get into Merkel Cell

137   progenitors contributing to the development of MCC. This prompted us to investigate

138   the cellular interactors of ST in HFFs. We found δ-catenin exhibited strong binding to ST

139   in HFFs while this interaction was absent in VP-MCC cells. Nonetheless, δ-catenin is

140   essential for VP-MCC proliferation by regulating expression of genes involved in cell

141   proliferation such as Cyclin B1 via interaction with Kaiso. Furthermore, we found that

142   LSD1 regulates the expression of the δ-catenin isoform 3 by modulating ESRP1

143   expression, thereby promoting cell proliferation in VP-MCC.

144   **RESULTS**

145

146   **Proximity labeling mass spectrometry identifies δ-catenin as a potential**

147   **interactor of ST in fibroblasts.**

148

149   Human dermal fibroblasts are the primary host cell type productively infected by MCPyV

150   (18), prompting us to identify ST interactors in human foreskin fibroblasts (HFFs) using

151   proximity labeling mass spectrometry (36). HFFs were transduced with lentiviral vectors

152   encoding TurboID (TID) biotin ligase fused to the N-terminus of ST (TID-ST).

153   Since ST localizes to the nucleus without a known nuclear localization signal, likely

154   through binding its interactors (9), we tested if TID fusion affected its localization.

155   Subcellular fractionations confirmed that, like ST, TID-ST remains nuclear, suggesting

156   that it retains its ST function and interactome (Fig. 1A). HFFs expressing TID or TID-ST

157   were labeled with biotin, and biotinylated proteins were purified using streptavidin beads

7

158 for mass spectrometry. Of 635 detected proteins, 121 were enriched in HFFs

159 expressing TID-ST, including known ST interactors EP400 and HSP90 (5), validating

160 this approach (Fig. 1B, blue dots). Among these, seven candidates were selected for

161 further investigation for their roles in oncogenic and tumor-suppressive pathways: AIP

162 (37), CACYBP (38), δ-catenin (23, 30), ING3 (39), TNK1(40), YAP, and AMOT (41)

163 (Fig. 1B, red dots).

164

165 To validate these interactions, each candidate was HA-tagged and co-transfected with

166 ST in 293A cells followed by immunoprecipitation with an anti-HA antibody. ST co-

167 immunoprecipitated with PP2AC, our positive control (8) (Fig. 1C, lane 10), and most of

168 the putative interactors (Fig. 1C, lanes 2-9), though AIP and CACYBP showed no

169 binding (lane 2,3), and YAP, TAZ and AMOT bound weakly (lanes 6, 8, 9). The

170 strongest interaction was observed with δ-catenin (lane 4), leading us to focus our

171 attention on δ-catenin.

172

173 To confirm the ST- δ-catenin interaction in HFFs, cells were transduced with a lentiviral

174 vector encoding HA-tagged ST (ST-HA) or red fluorescent protein (RFP) as a negative

175 control, followed by anti-HA immunoprecipitation and immunoblotting for endogenous δ-

176 catenin. Consistent with the proximity labeling experiment, endogenous δ-catenin co-

177 immunoprecipitated with ST-HA, but not RFP (Fig. 1D).

178 We then mapped the domain of δ-catenin interacting with ST. HA- δ-catenin isoforms 1-

179 4 (Fig. S1A) were co-transfected with ST, followed by immunoprecipitation using an

180 anti-HA antibody. ST bound strongly to isoform 1 (Fig. S1B, lane 3), weakly to isoforms

181 2 and 3 (lanes 4 and 5), and not to isoform 4 (lane 6) suggesting ST binds the N-

182 terminal domain. Deletions mutants of isoform 1 (Fig. S1C) further defined the binding

183 region. Isoform 1 served as a positive control, whereas isoform 4 served as a negative

184 control (Fig. S1D, lanes 2 and 3). The Δ55-102 mutant had reduced binding (lane 4),

185 while Δ103-324 mutant completely lost binding to ST (lane 5) suggesting that ST binds

186 within the regulatory domain of δ-catenin. The Δ55–324 and the Δ325-968 mutants also

187 failed to bind to ST, indicating that neither the coiled-coil domain alone (lane 6) nor the

188 coiled-coil and regulatory domain together (lane 7) were sufficient for binding.

189 Given that the regulatory domain is heavily phosphorylated (42), we tested if

190 phosphorylation was required for interacting with ST by generating a phospho-dead

191 isoform 1 mutant (ΔP). Co-transfection of ST with ΔP in 293A cells showed that

192 phosphorylation was not required for ST binding (Fig. S1E, lane 4).

193 Together, these findings suggest that ST binds δ-catenin within the regulatory domain,

194 likely within the region spanning amino acids 102 to 324, and this interaction occurs

195 independently of its phosphorylation. While the coiled-coil domain is not sufficient for

196 binding, it may enhance the binding mediated by the regulatory region.

197 **MCPyV ST does not bind to δ-catenin in VP-MCC cells**

198

199 Given the cell-type specificity of δ-catenin isoforms, we investigated their expression in

200 MCC cells. Mesenchymal cells primarily express isoform 1, whereas epithelial cells

201 predominantly express isoform 3 (25, 27). To determine which isoforms are expressed

202 in MCC, the expression of δ-catenin was analyzed in a panel of 12 MCC cell lines,

9

203   including variant VN-MCC and classical VN- and VP-MCC cell lines. Classical MCC

204   cells grow as spheroids in suspension and express neuroendocrine markers (CHGA,

205   SYP, ENO2) and Merkel cell markers (KRT20, ATOH1, SOX2) while variant MCC cells

206   adhere in culture and show low or no expression of these markers (43)(Fig. S2A, S2B).

207   δ-catenin isoform expression varies across these cell lines. Human foreskin

208   keratinocytes (HFK) and classical VP-MCC cells predominantly expressed δ-catenin

209   isoform 3, whereas HFFs and variant MCC cells expressed primary δ-catenin isoform 1.

210   Most classical VN-MCC cells did not express δ-catenin (Fig. 2A). qRT-PCR analysis

211   using isoform-specific primers (Fig. 2B), as expected, showed that HFFs and variant

212   MCC cells expressed mostly isoform 1 mRNA (Fig. 2C, top) while HFK and classical

213   VP-MCC cells predominantly expressed isoform 3 mRNA (Fig. 2C, bottom). Most

214   classical VN-MCC cells show minimal δ-catenin mRNA, suggesting silencing of

215   CTNND1 gene in these cells.

216

217   To test ST- δ-catenin interaction in VP-MCC cells, co-immunoprecipitation assays were

218   performed using Ab5 antibody to pull down endogenous ST from MKL-2 whole-cell

219   lysates. δ-catenin did not co-immunoprecipitate with Ab5 (Fig. 2D, lane 3), despite

220   successful co-immunoprecipitation of PP2AC, a known ST interactor (lane 3). To

221   confirm these results, a reciprocal co-immunoprecipitation was performed using an anti-

222   δ-catenin antibody. Again, no interaction between endogenous ST and δ-catenin was

223   observed in MKL-2 cells (Fig. 2E, lane 3).

10

224 To rule out the possibility that the lack of interaction was due to the antibodies used, ST

225 was HA-tagged and overexpressed in MKL-2 cells using a doxycycline-inducible

226 lentiviral vector followed by anti-HA immunoprecipitations. Again, endogenous δ-catenin

227 did not co-immunoprecipitate with HA-ST in MKL-2 cells (Fig. 2F, lane 4). Given that

228 VP-MCC cells express low δ-catenin isoform 1 levels compared to HFFs, (Fig. 1D), HA-

229 tagged δ-catenin isoforms 1 and 3 were overexpressed in MKL-2 cells using a

230 doxycycline-inducible lentiviral vector, and each of the isoforms was immunoprecipitated

231 with an anti-HA antibody. ST still failed to bind either isoform (Fig. 2G, lanes 5 and 6)

232 despite successful co-immunoprecipitation of Kaiso, a known interactor of δ-catenin (24)

233 (lane 5 and 6). These findings suggest that ST and δ-catenin interaction requires more

234 than δ-catenin isoform 1 being 101 amino acid residues longer at the N-terminus

235 compared to isoform 3.

236 **δ-catenin is required for cell proliferation in VP-MCC cells**

237 Although ST and δ-catenin do not interact in VP-MCC cells, we hypothesized that δ-

238 catenin might still be necessary for cell proliferation, as it has been shown to promote

239 cell proliferation in other cancers (33, 44, 45). To test this, MKL-1 and MKL-2 cells were

240 transduced with two doxycycline-inducible lentiviral vectors encoding shRNA targeting

241 δ-catenin, (Fig. 3A and 3B), and cell proliferation was assessed using trypan blue

242 exclusion assays. δ-catenin knockdown significantly reduced cell proliferation in both

243 MKL-1 and MKL-2 cells (Fig. 3C and 3D). This effect was specific to VP-MCC cells, as

244 δ-catenin knockdown (Fig. 3E and 3F) did not affect proliferation in MCC14/2, a variant

245 MCC cell line, or in HFFs (Fig. 3G and 3H). Notably, MCC14/2 cells and HFFs

246 predominantly express δ-catenin isoform 1, whereas MKL- 1 and 2 cells primarily

247 express isoform 3. Since t-LT and ST are required for cell proliferation, we investigated

248 whether δ-catenin knockdown affected their expression. However, δ-catenin knockdown

249 had little to no effect on t-LT and ST in MKL-1 and 2 (Fig. 3A and 3B). Our findings

250 indicate that δ-catenin is essential for cell proliferation in VP-MCC cells but not in HFFs

251 and variant MCC and is completely dispensable in 3/4 VN-MCC cell lines.

252 **δ-catenin regulates genes involve in cell proliferation in MKL-2 cells**

253

254 δ-catenin promotes cell proliferation by interacting with Kaiso, a transcriptional

255 repressor of the Wnt/β-catenin pathway, thereby inhibiting its repressive activity (24, 34,

256 35). Consistent with this, endogenous Kaiso was successfully co-immunoprecipitated by

257 δ-catenin isoform 3 in MKL-2 cells (Fig. 2G, lane 6). Kaiso regulates Wnt/β-catenin

258 target genes, including Wnt11**,** Cyclin D1**,** and Matrilysin 7, in various cancers (33-35)

259 but these were absent in classical VN and VP-MCC cell lines, while variant MCC cells

260 expressed only Cyclin D1 (Data not shown). To identify δ-catenin target genes involved

261 in proliferation, bulk RNA sequencing was performed following δ-catenin knockdown in

262 MKL-2 cells. As expected, downregulation of cell cycle genes, including Cyclin B1 (46),

263 Cyclin B3 (47), Cyclin A2 (48) was observed (Fig. 4A). Gene set enrichment analysis

264 using the REACTOME collection showed that silencing δ-catenin in MKL-2 cells was

265 associated with the downregulation of gene signatures related to cell proliferation (Fig.

266 4B).

267

268 To determine if these genes are Kaiso target genes, we analyzed their promoters for

12

269 Kaiso binding sites (KBS). Cyclin B1, B3, A2, and CDC6 contain KBS within their

270 promoter regions (Fig. 4C and Fig. S3), suggesting potential regulation by Kaiso. As a

271 proof of principle, we focused on Cyclin B1, a key regulator of the G2-to-mitosis

272 transition (46). A luciferase reporter plasmid driven by the Cyclin B1 promoter was co-

273 transfected into 293A cells along with Kaiso and/or δ-catenin isoform 3 (Fig. 4D).

274 As predicted, Kaiso overexpression repressed Cyclin B1 luciferase activity, whereas δ-

275 catenin isoform 3 overexpression enhanced Cyclin B1 luciferase activity. Co-

276 transfection of both Kaiso and isoform 3 attenuated Kaiso-mediated repression. To

277 further confirm Cyclin B1 as a Kaiso target, we generated a luciferase reporter under

278 the control of a Cyclin B1 promoter lacking KBS. Co-transfection of Kaiso, δ-catenin

279 isoform 3, or both had negligible effects on luciferase activity (Fig. 4D), confirming that

280 Kaiso binding is required for transcriptional regulation. Of note, we were unable to

281 achieve efficient Kaiso knockdown in MKL-2 cells to assess its impact on cell viability

282 and expression of cell cycle genes such as Cyclin B1 (data not shown).

283

284 Together, these findings suggest that δ-catenin promotes proliferation, at least in part,

285 by alleviating Kaiso-mediated repression of Cyclin B1. The presence of KBS in the

286 promoters of Cyclin B3 and A2 further suggests that these genes may also be potential

287 Kaiso targets.

288

289 **LSD1 regulates the expression of δ-catenin isoforms through ESRP1**

290

291   To investigate how δ-catenin isoforms are regulated, we examined the impact of LSD1

292   inhibition, as it has been shown that LSD1 inhibition alters δ-catenin expression (6).

293   Treating MKL-2 cells with an LSD1-GSK inhibitor reduced cell viability in a dose-

294   dependent manner (Fig. 5A). To confirm specificity, we analyzed PROM1 expression, a

295   known LSD1 target, by western blot (6). As expected, LSD1 inhibition upregulated

296   PROM1, validating LSD1 inhibition. Notably, LSD1 inhibition shifted δ-catenin

297   expression from isoform 3 to isoform 1 (Fig. 5B). To confirm that this switch was specific

298   to LSD1 inhibition, MKL-2 cells were transduced with LSD1-targeting shRNAs.

299   Depletion of LSD1 resulted in a switch from δ-catenin isoform 3 to isoform 1 expression

300   (Fig. 5C).

301   Since ESRP1 regulates δ-catenin isoform expression (29), we hypothesized that LSD1

302   modulates δ-catenin splicing through ESRP1. HFK and classical MCC cell lines, which

303   mostly express isoform 3, express ESRP1 while HFFs and variant MCC cells lines,

304   which mostly express isoform 1, lack ESRP1 (Fig. 2A). Supporting this, LSD1 inhibition

305   and knockdown in MKL-2 cells each decreased ESRP1 expression, leading to a switch

306   from isoform 3 to isoform 1 (Fig. 5B and 5C).

307   To confirm ESRP1's role in δ-catenin splicing, MKL-1 cells were transduced with

308   ESRP1-targeting shRNAs. Knockdown of ESRP1 resulted in a switch from δ-catenin

309   isoform 3 to isoform 1 (Fig. 5D), as confirmed by qRT-PCR analysis (Fig. 5E).

310   Conversely, overexpression of HA-tagged ESRP1 in MCC14/2 and HFF cells, which

311   lack ESRP1 and predominantly express isoform 1, shifted δ-catenin splicing from

312   isoform 1 to isoform 3 (Fig. 5F). Additionally, ESRP1 knockdown (Fig. 5D) reduced

14

313   proliferation in MKL-1 cells (Fig. 5G). ST induces the expression of LSD1 (6, 7) and our

314   findings also add that LSD1 regulates δ-catenin isoform expression in VP-MCC cells

315   through ESRP1, which is both necessary and sufficient for δ-catenin isoform 3

316   expression (Fig. S4) to promote cell proliferation in VP-MCC cells.

317

318   **DISCUSSION**

319   While HFFs have been identified as a cell type permissive for the MCPyV viral lifecycle

320   (18), the MCC cell of origin has yet to be determined. Typically, permissive cells are not

321   transformed by viruses. For example, the related polyomavirus SV40 does not cause

322   tumors in its natural host, rhesus macaque. Indeed, SV40 infection of permissive cells

323   does not result in transformation because the intracellular environment supports viral

324   replication, egress, and eventual cell death (19). In contrast, SV40 infection of non-

325   permissive cells, such as hamster and mouse cells, is abortive, eliminating viral

326   replication functions, and driving cellular transformation (49, 50). Using HFFs, a

327   permissive cell for MCPyV infection, we identified δ-catenin as an ST interactor (Fig. 1B,

328   C). While δ-catenin bound ST in HFFs (Fig. 1D), this interaction was absent in VP-MCC

329   cells (Fig. 3A). Interestingly, while δ-catenin was required for cell proliferation in VP-

330   MCC cells (Fig. 3C, D), at least in part by regulating the expression of cell cycle genes

331   through Kaiso (Fig. 4A, E), δ-catenin was dispensable in HFFs (Fig. 3G, H). Although

332   previous studies have shown that δ-catenin isoform 3, but not isoform 1, binds Kaiso

333   (51, 52), we found that both isoforms bound Kaiso, at least in VP-MCC cells (Fig. 2G).

334   Notably, while our proximity labeling spectrometry identified YAP and TAZ as ST

15

335   interactors in HFFs (Fig. 1B, C), MCC cells do not express YAP or TAZ (53). Our

336   findings plus those of others indicate that HFFs provide a physiologically relevant

337   environment for ST and δ-catenin interaction as well as YAP and TAZ, which may play

338   a role in the MCPyV lifecycle. Moreover, this interaction is absent in VP-MCC cells,

339   suggesting that the MCC cell of origin is distinct from fibroblasts.

340

341   Several skin cell types have been proposed as the MCC cell of origin, including Merkel

342   cells, keratinocytes, or epidermal progenitor cells. However, none of these has been

343   conclusively linked to MCC development. MCC tumors not only express neuroendocrine

344   markers but also epithelial markers (54, 55). Our study reveals that MCC tumors

345   express the epithelial markers ESRP1 (29) and δ-catenin isoform 3 (25) both of which

346   are absent in HFFs and variant MCC cell (Fig. 2A). Loss of ESRP1, as seen during

347   LSD1 inhibition, causes a switch from δ-catenin isoform 3 to isoform 1 in VP-MCC cells

348   (Fig. 5B, C), likely resulting in a loss of epithelial identify. LSD1 repression inhibits MCC

349   differentiation, upregulates expression of pro-neuronal genes such as NEUROD1 and

350   INSM1 and promotes cell death in VP-MCC cells (6, 7). Interestingly, δ-catenin isoform

351   1 has been implicated in neurogenesis (56-58) suggesting that δ-catenin isoform 1 may

352   contribute to the up-regulation to these neuronal genes, as observed previously (6, 7).

353   Of note, LSD1 inhibition in MCC14/2, a variant MCC cell that primarily express isoform

354   1, did not affect its expression (data not shown) suggesting that LSD1 specifically

355   regulates the expression of isoform 3 in VP-MCC cells.

356

357   While the MCC cell of origin remains elusive, our data suggest that MCC may arise from

358   a cell with epithelial characteristics. While HFKs express both ESRP1 and isoform 3

359   (Fig. 2A), they are not considered a likely MCC precursor because viral genes are not

360   expressed following viral infection (18). Most likely, MCC originates from a basal

361   epidermal progenitor cell, precursors of Merkel cells (17). These epidermal progenitor

362   cells probably express ESRP1 and isoform 3, as MCC cells do. Given that MCPyV is

363   unlikely to drive oncogenesis in a permissive cell such as HFF, one possibility is that,

364   while MCPyV replicates in HFFs, it occasionally infects Merkel progenitor cells, a non-

365   permissive cell, in which the transcriptional environment represents a dead-end for viral

366   replication, favoring viral integration and cellular transformation. Our work supports the

367   idea that two distinct cells are involved: HFFs supports the MCPyV lifecycle, while MCC

368   may arise of epidermal progenitor cells.

369   **Acknowledgements**

387   **MATERIALS AND METHODS**

388   **Cell lines:** MCC13 (#10092302), MCC14/2 (#10092303) and MCC26 (#10092304) cells

389   were from Sigma. Variant VN-MCC and VP-MCC cells were culture in RPM1 1640

390   HEPES (Thermo fisher) with 10% tetracycline-negative FBS (GeminiBio), 1X non-

391   essential amino acids (Gibco) and 50 units/mL penicillin and 50 ug/mL streptomycin

392   (Gibco). Classical VN-MCC cells were maintained in media containing 50% DMEM

393   (Gibco), 30% Neurobasal medium (Gibco), 15% chick embryo extract (Thermo fisher),

394   1% N2 (Gibco), 2% B27 (Gibco), 117nM retinoic acid (Sigma), 20ng/ml bFGF (R&D),

395   20ng/ml IGF-1 (R&D), 1% non-essential amino acids (Gibco), 1% penicillin and

396   streptomycin (Gibco) and 50uM 2-βmercaptoethanol (Sigma). 293A cells and 293T cells

397   (Invitrogen) were maintained in DMEM (Gibco) with 10% FBS (Corning), 1x GlutaMAX

398   (Gibco), 1X non-essential amino acids (Gibco), 50 units/mL penicillin and 50ug/mL

399   streptomycin (Gibco). Fibroblasts were cultured in DMEM (Gibco) with 10% FBS

400   (Corning), 1x GlutaMAX (Gibco), 1X non-essential amino acids (Gibco), 50 units/mL

18

401   penicillin and 50ug/mL streptomycin (Gibco). Keratinocytes were cultured with

402   keratinocyte growth medium 2 (PromoCell) per the manufacturer's instructions. All cell

403   lines were maintained at 37°C in 5% CO_2.

404   **Plasmids:** ST interactors, δ-catenin isoforms and δ-catenin mutants as well as ESRP1-

405   HA were codon-optimized, synthesized as dsDNA fragments (IDT) and cloned into

406   pcDNA3 or pTRIPZ using the In-fusion HD Cloning Kit (Takara).

407

408   **Transfections and lentiviral transductions:** For transient transfections, 293A were

409   plated in 10cm plates, at ~80% confluence the next day, transfected with TransIT®-293

410   Mirus following the manufacturer's instructions. Cells were harvested 36 hours post-

411   transfection.

412

413   To generate lentiviral particles, 293T cells were transfected with psPAX2 and pMD2.G

414   (gifts from Didier Trono, Addgene #12260, #12259) and lentiviral vectors (see Appendix,

415   Table S1 or pTRIPZ (Adgene, #206981) expressing δ-catenin isoform 1-HA, isoform 3-

416   HA, ST-HA or ESRP1-HA. After 36h, supernatants were collected, filtered through a

417   0.45μM filter, and supplemented with 6μg/mL polybrene. Target cells were incubated

418   with lentiviral supernatants for 24h, followed by fresh medium for another 24h.

419   Transduced cells were then selected with 0.5μg/mL puromycin for 4 days before

420   expansion in puromycin-free medium.

421   **Cell growth assays:** Cells transduced with doxycycline-inducible lentiviral vectors were

422   treated with 0.5ug/ml doxycycline, refreshed every 3 days. MKL-1 cells transduced with

19

423   shRNA against ESPR1, and MKL-2 cells transduced with shRNA against LSD1 were

424   refresh every 3 days. GSK-LSD1 (Sigma, #SML1072,) was reconstituted in DMSO and

425   added to the media. At each time point, cells were treated with accutase (Stem Cell

426   Technologies) for 10 minutes at room temperature and cell number was assessed by

427   0.2% trypan blue staining.

428   **Co-Immunoprecipitation, immunoblotting and antibodies:** Cell were lysed in 1%

429   NP-40 lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% NP-40) with protease and

430   phosphatase inhibitors. Protein concentrations were determined using BCA protein

431   assays (Thermo Fisher). For co-immunoprecipitations, 500ug of lysates were pre-

432   cleared with 25ul protein A/G magnetic beads (Pierce) for 1h at 4°C with rotation, then

433   incubated with 25ul anti-HA beads (Pierce) for 1h at 4°C. Beads were washed five times

434   with 1%NP-40 buffer and bound . Bound proteins were eluted with 1x LDS sample

435   buffer (Novex) plus 100 mM DTT at 70 °C for 10 min.

436   Twenty μg of cell lysate and IP elute were separated on 8 to 16% denaturing tris-glycine

437   gels (Thermo Fisher), transferred to PVDF membranes (Bio-Rad), blocked in 3% BSA in

438   TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature followed by

439   incubation with primary antibodies overnight at 4°C. Blots were washed with TBST 3

440   times followed by HRP-conjugated secondary antibodies for 1 h at room temperature

441   followed by washes with TBST 3 times. Blots were developed using Clarity Max

442   Western ECL chemiluminescent substrate (Bio-Rad) and imaged on ChemiDoc (Bio-

443   Rad).

444 For immunoblotting alone, cells were lysed in RIPA buffer (25nM Tris-HCL, 150mM

445 NaCl, 1% NP-40 and 1% deoxycholate) supplemented with protease and phosphatase

446 inhibitors and process as above. Antibodies used are listed in Appendix, Table S2.

447 **Microscopy:** Bright-field microscopy images were taken at 10x magnification using the

448 EVOS microscope.

449

450 **Luciferase assay:** Human CCNB1 promoter (Appendix, Table S3) was cloned into the

451 pGL4.11 luciferase vector (Promega). 2×10^4 293A cells in a 96 well plate were

452 transfected with 1ug pGL4.1 CNNB1-luc, 1ug CMV renilla pGL4.75 (Promega) and 1ug

453 pcDNA3 Kaiso and/or 1ug pcDNA3 δ-catenin isoform 3, with pcDNA3 empty vector

454 added to equalized total DNA. Cells were lysed and analyzed using the Dual-Glo

455 luciferase assay kit (Promega) on a Veritas microplate luminometer (Turner

456 BioSystems).

457 **Subcellular fractionations:** Subcellular fractionation was performed using a Thermo

458 Fisher kit (#78840). Lysates were processed as described previously. Antibodies used

459 are listed in Appendix, Table S2.

460 **RNA extraction and qRT-PCR:** Total RNA was isolated using the RNeasy Mini Kit

461 (Qiagen) per manufacturer's protocol and quantified with a NanoDrop spectrometer.

462 cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Thermo

463 Fisher) according to the manufacturer's instructions. qRT-PCR was performed using

464 PowerUp SYBR Green Master Mix (Thermo Fisher) and StepOnePlus Real-Time PCR

465 system (Applied Biosystems). Differential expression of δ-catenin isoform 1 and 3 was

466   calculated using the 2−ΔΔCt method and normalized to housekeeping gene 36B4

467   expression. Primers sequences are listed in Appendix, table S4.

468   **Bulk RNA Sequencing:** Total RNA was isolated using the RNeasy Mini Kit (Qiagen),

469   with RNA integrity assessed by TapeStation (Agilent Technologies) and quantificatify

470   using a Trinean DropSense96 spectrophotometer (Caliper Life Sciences). RNA-seq

471   libraries were prepared with the TruSeq stranded mRNA kit (Illumina) using 500ng total

472   RNA, and library size was validated using a TapeStation. Additional quality control,

473   pooling of indexed libraries, and cluster optimization were performed using the

474   Invitrogen Qubit® 2.0 Fluorometer (Life Technologies-Invitrogen). Libraries were pooled

475   (9-plex) and clustered onto a P2 flow cell before sequencing on an Illumina NextSeq

476   2000 with a paired-end, 50 bp (PE50) strategy.

477   Image analysis and base calling were performed using Illumina's Real Time Analysis

478   v3.4.4 software, followed by demultiplexing and FASTQ generation with bcl2fastq

479   Conversion Software v2.20. Reads were aligned to the GRCm38 reference using STAR

480   v2.7.7a in two-pass mode, and gene quantification was performed with STAR's

481   quantMode using Gencode v38 annotations. Differential expression was analyzed using

482   Bioconductor's edgeR with a significance threshold of $\log_2 FC \geq 1$ or ≤ -1 at 5% FDR.

483   Gene Set Enrichment Analysis (GSEA) was performed on pre-ranked gene lists to

484   identify enriched pathways.

485   **Proximity Labeling Mass Spectrometry:** HFFs TID and ST-TID were cultured with

486   biotin-free DMEM media (Gibco) supplemented with 10% FBS (Corning), 1x GlutaMAX

487   (Gibco), 1X non-essential amino acids (Gibco), 50 units/mL penicillin and 50ug/mL

22

488   streptomycin (Gibco). At 90% confluency, cells were incubated with DMEM containing

489   500 µM biotin for 30 minutes, then placed on ice, washed with ice-cold PBS, and

490   collected. Cells were resuspended in ice-cold 1% NP-40 lysis buffer with benzonase

491   (Sigma) and rotated for 30 minutes at room temperature. Protein concentrations were

492   measured by BCA assay. Six mg of protein was incubated with 125 µL streptavidin

493   magnetic beads (Pierce) that were pre-methylated using a reductive alkylation kit

494   (Hampton Research HR2-434) for 2 hours at room temperature. Beads were washed

495   twice with 1% NP-40 buffer, and proteins were eluted in 8 M urea/100 mM Tris (pH 8.5).

496   Disulfide bonds were reduced with 5 mM TCEP for 15 minutes and alkylated with 10

497   mM 2-chloroacetamide for 30 minutes in the dark at room temperature. Samples were

498   digested with 250 ng rLys-C at 37°C for 4 hours, diluted to 1 M urea with 100 mM Tris

499   (pH 8.5), supplemented with 1 mM $CaCl_2$, and further digested overnight with 1.6 µg

500   trypsin at 37°C. After acidification with 5% formic acid, peptides were desalted using

501   ZipTips (Millipore), eluted with 70% acetonitrile/0.1% TFA, and dried in a Speedvac.

502

503   Peptides were resuspended in 2% acetonitrile in 0.1% formic acid and analyzed by

504   LC/ESI MS/MS on a Thermo Scientific Easy1200 nLC coupled to a tribrid Orbitrap

505   Eclipse with FAIMS Pro. In-line desalting was accomplished using a reversed-phase

506   trap column (100 µm × 20 mm, Magic C18AQ, 5 µm, 200 Å; Michrom), followed by

507   separation on a reversed-phase column (75 µm × 270 mm, ReproSil-Pur C18AQ, 3 µm,

508   120 Å; Dr. Maisch). A 90-minute gradient from 8% to 30% B (80% acetonitrile in 0.1%

509   formic acid) at 300 nL/min was used. Data were acquired in a data-dependent mode

510   with survey scans in the Orbitrap and MS/MS spectra in the linear ion trap (HCD

511   activation) with dynamic exclusion for 60 seconds after one repeat.

512

513   Data analysis was performed using Proteome Discoverer 2.5 (Thermo Scientific). The

514   data were searched against Uniprot Human (UP000005640_Human_052618.fasta) that

515   included common contaminants (cRAPome, cRAP_012915_NP.fasta) and the ST

516   sequence (PC_KD_1622.fasta). Searches were performed with settings for the

517   proteolytic enzyme trypsin. Maximum missed cleavages were set to 2. The precursor

518   ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.6 Da.

519   Dynamic peptide modifications included oxidation (+15.995 Da on M). Dynamic

520   modifications on the protein terminus included acetyl (+42.-11 Da on N-terminus) and

521   static modification carbamidomethyl (+57.0215 on C). Minora was used for peak

522   abundance and retention time alignment. SEQUEST HT was used for database

523   searching. All search results were run through Percolator for scoring. Raw data was

524   normalized at median value across samples. Missing data were imputed with half of

525   global minimum values. P-values for pairwise comparisons were calculated by t-test.

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725   **FIGURE LEGENDS**

726

727   **Figure 1: δ-catenin is a novel interactor of MCPyV ST in human foreskin fibroblast**

728   **(HFFs). A)** Subcellular localization of ST or TID-ST transduced into HFFs was detected

729   with Ab5 antibody. TID-ST was detected with V5 antibody. Cytoplasmic and nuclear

730   fractions were confirmed by GAPDH and histone H3 markers, respectively. W: whole

731   lysate. C: cytoplasmic fraction. N: Nuclear fraction. **B)** Volcano plot of proteins identified

732   by mass spectrometry enriched in HFFs TID- ST vs TID samples. Known interactors of

733   MCPyV ST are highlighted in blue. Putative MCPyV ST interactors are highlighted in

734   red. **C)** Western blots of HEK293A cell lysates transfected with ST and HA-AIP, HA-

735   CACYBP, HA- δ-catenin, HA-ING3, HA-TNK1, HA-YAP, HA-AMOT or HA-TAZ. Lysates

736   were immunoprecipitated with HA antibody and blotted for MCPyV ST. **D)** Western blots

737   of HFFs cell lysates expressing either empty vector (-) or MCPyV ST-HA (+). Lysates

738   were immunoprecipitated with HA antibody and blotted for δ-catenin.

739

Figure 2: Failure of MCPyV ST binding to δ-catenin isoform 3 in VP-MCC Cells. A)

Lysates from normal, variant, and classical virus negative and virus positive cells were

immunoblotted for δ-catenin isoforms, ESRP1, LSD1 and MCPyV T antigens. **B)**

Schematic representation of primers to detect δ-catenin isoform 1 and 3. Primers were

designed to target exon-exon junctions specific to each isoform. **C)** qRT-PCR analyses

of relative expression of δ-catenin isoform 1 and 3 in all 14 cell lines normalized to 36B4

expression. Data are shown as mean of n=3 ±SD. **D and E)** Western blots of MKL-2

lysates and anti-ST and anti-δ-catenin co-immunoprecipitations, respectively. **F)**

Western blots of MKL-2 lysates overexpressing ST-HA and anti-HA co-

immunoprecipitations after 6 days of doxycycline treatment. **G)** Western blots of MKL-2

lysates overexpressing HA-δ-catenin isoform 1 and 3 and anti-HA co-

immunoprecipitations after 6 days of doxycycline treatment.

Figure 3: δ-catenin is required for cell proliferation in VP-MCC cells but not in

MCC14/2 and HFFs cells . A) and B) Knock down of δ-catenin in MKL-1 and MKL-2.

Western blots of cell lysates harvested after 8 days doxycycline treatment. **C) and D)**

Growth curves of MKL-1 and MKL-2 knock down δ-catenin following doxycycline

treatment. Data are shown as mean of n=3 ± SD; 2-way ANOVA, * P<0.05, ***P<0.001,

****P<0.0001. **E and F)** Knock down of δ-catenin in MCC14/2 and and HFFs. Western

blots of cell lysates harvested after 8 days doxycycline treatment. **G and H)** Growth

curves of MCC14/2 and HFFs knock down δ-catenin following doxycycline treatment.

Data are shown as mean of n=3 ± SD; 2-way ANOVA, ns: no significant

30

763 **Figure 4 δ-catenin regulates expression of genes involve in cell cycle. A)** Volcano

764 plot of differentially expressed genes in MKL-2 knock down δ-catenin vs MKL-2 control

765 cells from bulk RNA sequencing. **B)** Dot plot representing the changes in gene set

766 enrichment analysis results illustrating processes associated with loss of δ-catenin in

767 MKL-2. **C)** Nucleotide sequence of the human cyclin B1 promoter region. Consensus

768 Kaiso binding sites are highlighted in yellow. Arrow indicates the transcription initiation

769 site. **D)** Schematic representation of Kaiso and δ-catenin transcription regulation. **E)**

770 Human CCNB1 promoter luciferase reporter in 293A cells co-transfected with Kaiso

771 and/or δ-catenin isoform 3. Luciferase activity was normalized to CMV-driven renilla

772 activity. Relative luciferase activity was determined using Cyclin B1 alone. Data are

773 shown as mean of n=3 ± SD.

774

775 **Figure 5: LSD1 regulates the expression of δ-catenin isoform 3 via ESRP1 in VP-**

776 **MCC cells. A)** Growth curve of MKL-2 after treatment with GSK-LSD1 inhibitor (LSD1-

777 i). Data are shown as mean of n=3 ± SD. **B)** Western blots of δ-catenin, ESRP1 and

778 PROM1 after 10 days treatment with LSD1-i in MKL-2 cells. **C)** Western blot of LSD1,

779 ESRP1 and δ-catenin after 10 days knock down of LSD1 in MKL-2**. D)** Western blots of

780 ESRP1 and δ-catenin after 10 days knock down of ESRP1 in MKL-1. **E)** qRT-PCR

781 analyses of relative expression of δ-catenin isoform 1 and 3 after 10 days knock down

782 of ESRP1 in MKL-1. Relative expression was normalized to 36B4. Data are shown as

783 mean of n=3 ± SD; 2-way ANOVA, ****P<0.0001. **F)** Western blots of overexpressing

784 HA tagged ESRP1 in MCC14/2 and HFFs cells after 10 days of doxycycline treatment.

785   **G)** Growth curve of MKL-1 knock down ESRP1. Data are shown as mean of n=3 ± SD;

786   2-way ANOVA, ****$P<0.0001$.

787

A

MKL-2 shRNA δ-catenin vs control

B

Downregulated
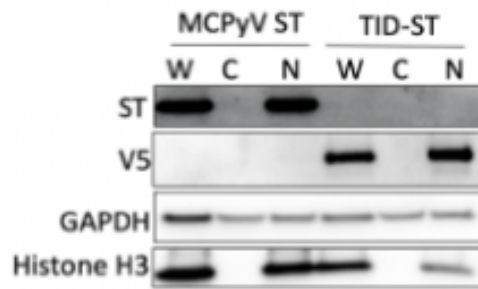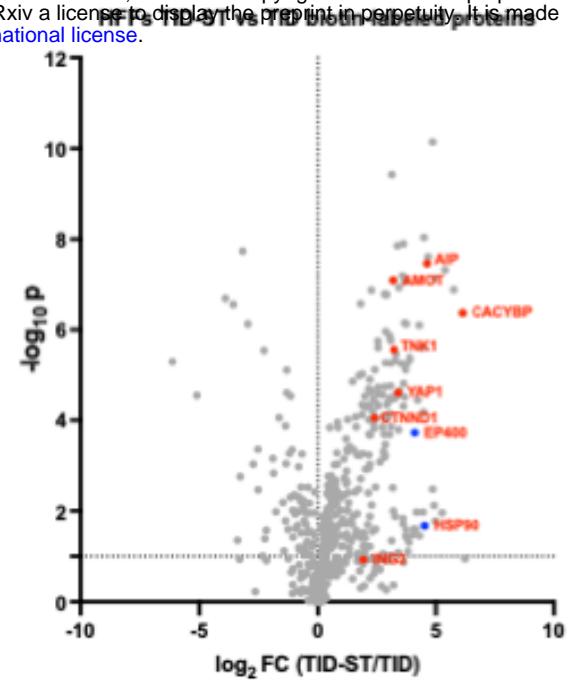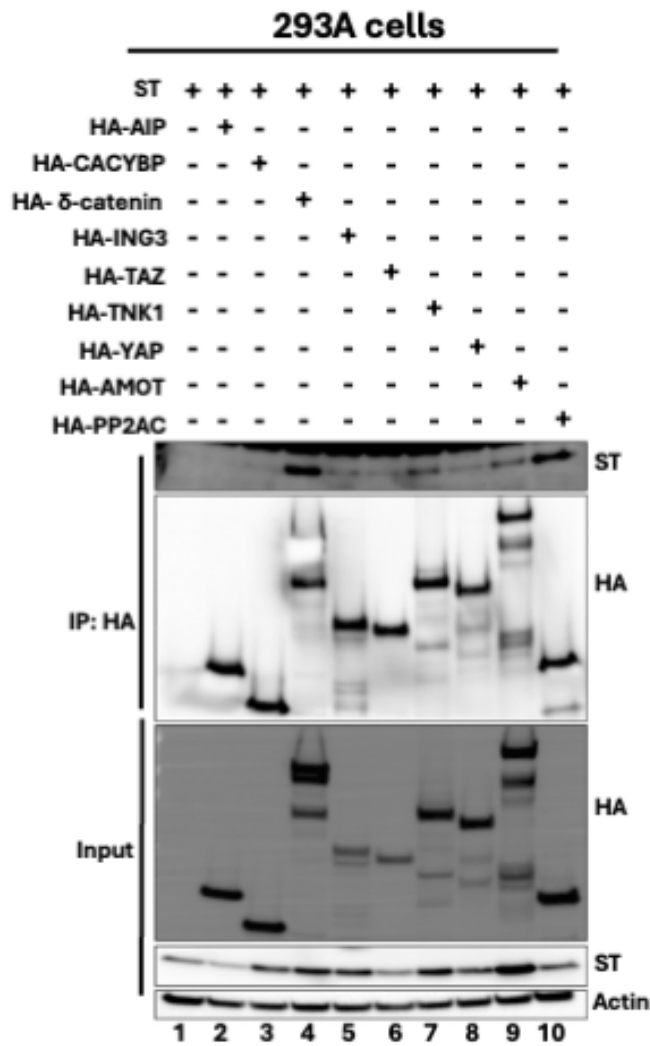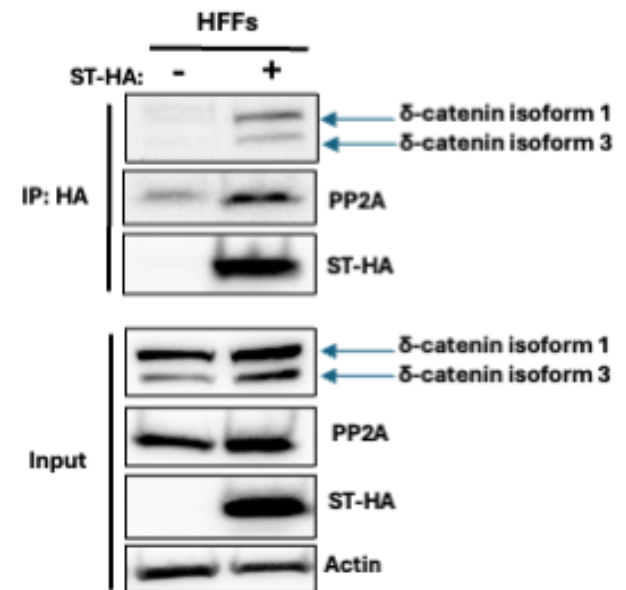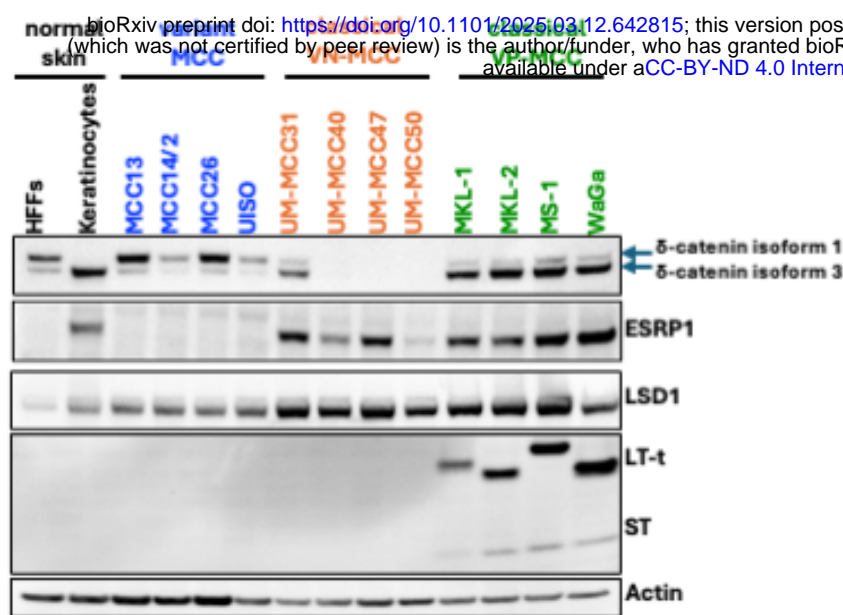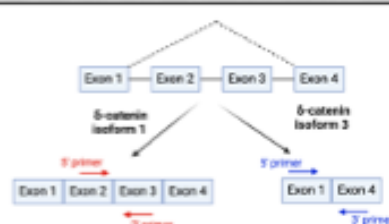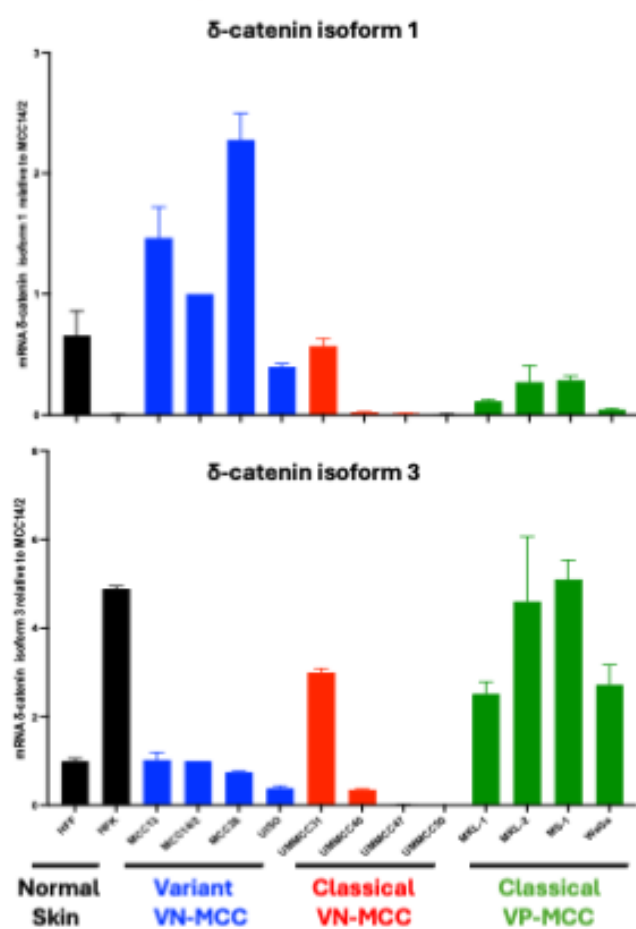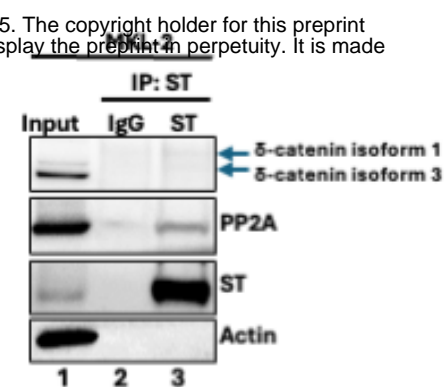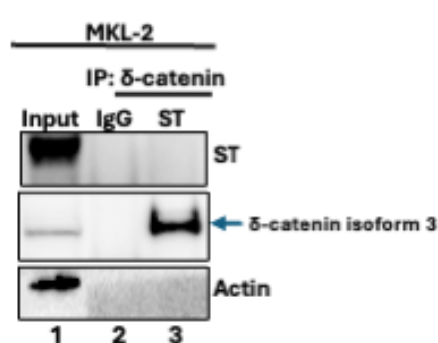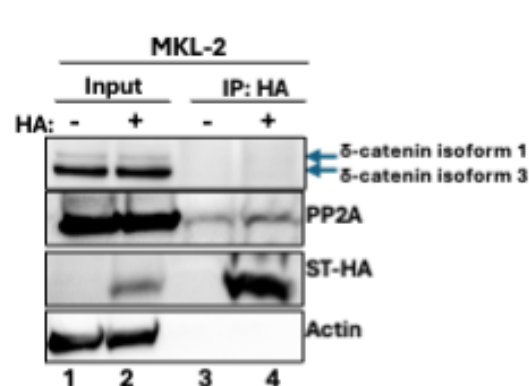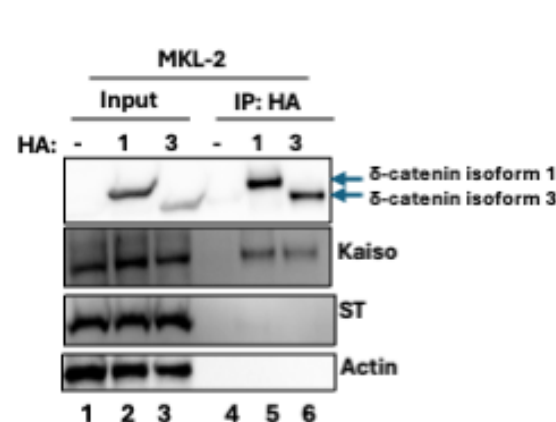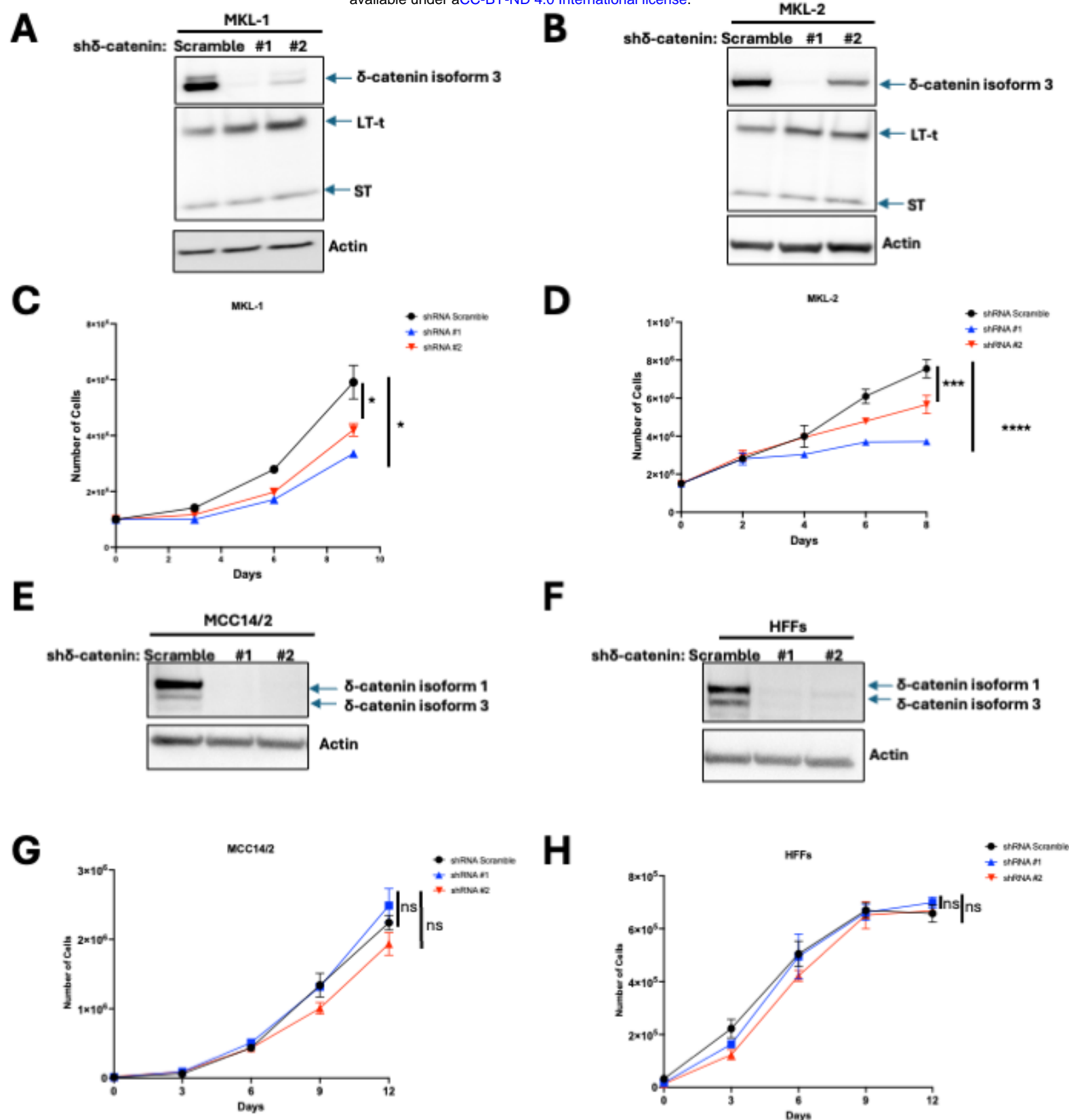
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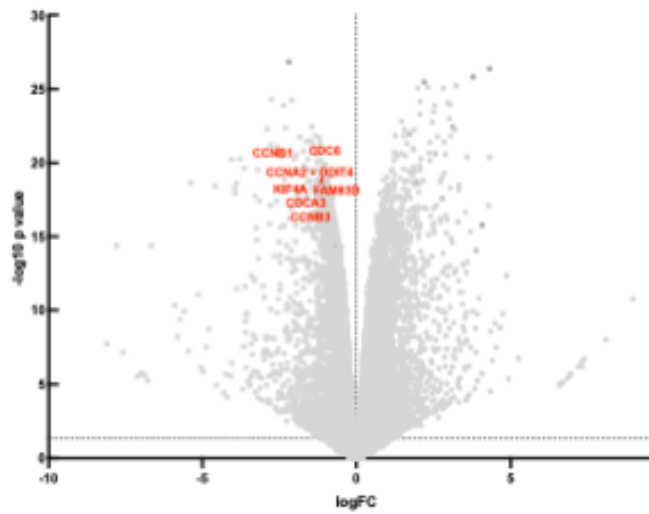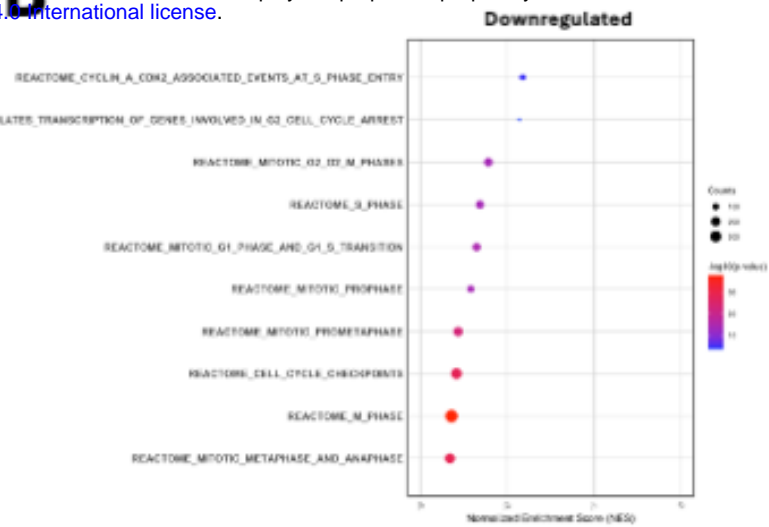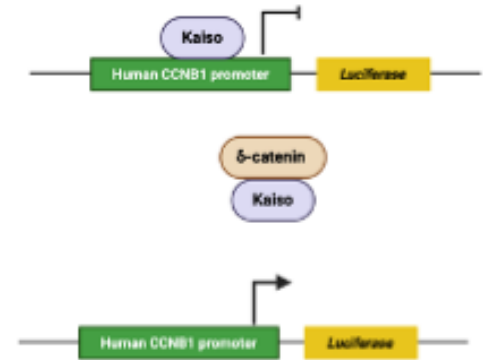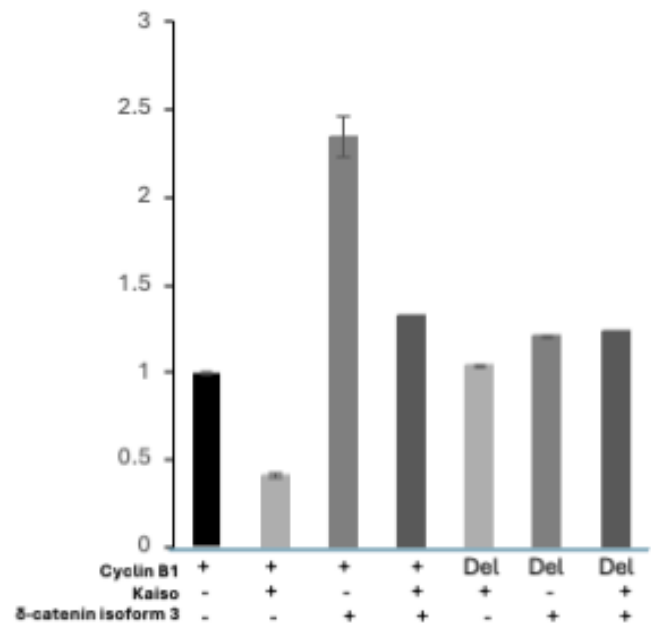## Human Cyclin B1 promoter

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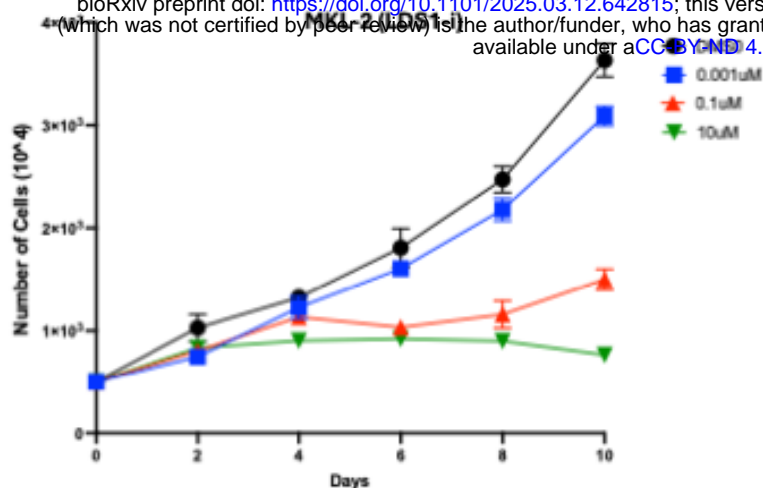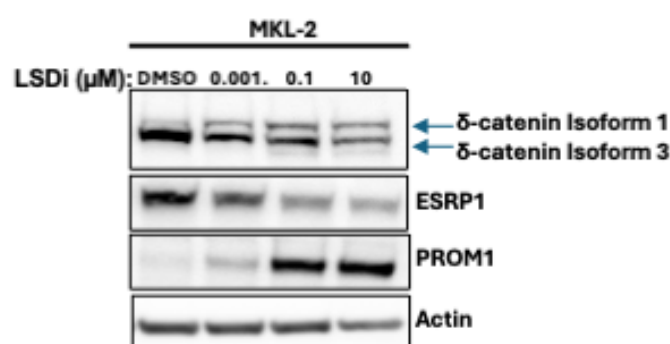TCTGCTTCTCCCCGCTGAGCTGCTGCCTGGTGAAGAGGAAGCC

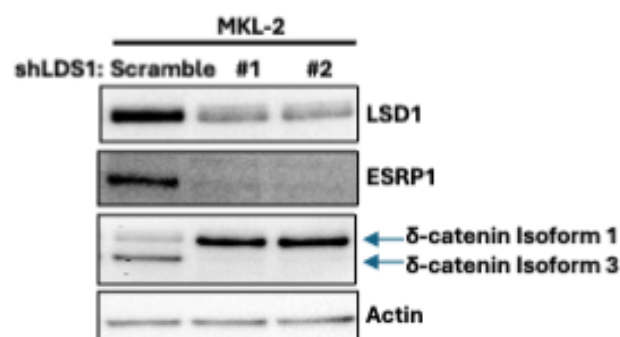**D**
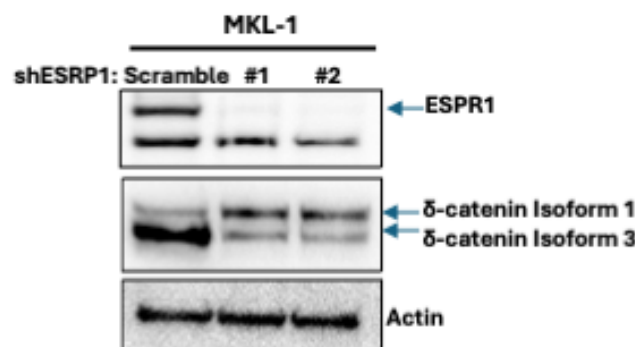

E

Cyclin B1 Luciferase

Cyclin B1	+	+	+	+	Del	Del	Del
Kaiso	-	+	-	+	+	-	+
δ-catenin isoform 3	-	-	+	+	-	+	+