1 **Epidermal YAP Activity Drives Canonical WNT16/β-catenin**

2 **Signaling to Promote Keratinocyte Proliferation *in vitro* and in the**

3 **Murine Skin**

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**ABSTRACT**

The skin constantly self-renews throughout adult life. Wnt/β-catenin signaling plays a key role in promoting keratinocyte proliferation in the hair follicles and in the interfollicular epidermis. A recent report demonstrated that epidermal YAP activity drives β-catenin activation to promote keratinocyte proliferation in the murine skin. However, it remains unclear whether this is caused by paracrine activation of canonical Wnt signaling or through other YAP/β-catenin regulatory interactions. In the present study, we found that XAV939-inhibition of canonical WNT signaling in skin of YAP2-5SA-ΔC mice resulted in diminished β-catenin activation, reduced keratinocyte proliferation, and a mitigation of the hyperplastic abnormalities in the interfollicular epidermis, signifying a canonical WNT ligand-dependent mechanism. Our subsequent analyses determined that WNT16 is produced in response to YAP activity in keratinocytes both *in vitro* and *in vivo*, and that WNT16 drives HaCaT keratinocyte proliferation via canonical WNT16/β-catenin signaling. We conclude that under normal physiological conditions WNT16 is the paracrine WNT ligand secreted in response to epidermal YAP activity that promotes cell proliferation in the interfollicular epidermis. This study delineates a fundamental YAP-driven mechanism that controls normal skin regeneration, and that may be perturbed in human regenerative disease displaying increased YAP and WNT signaling activity.

**KEYWORDS**

YAP, WNT signaling, epidermis, regeneration.

## INTRODUCTION

The skin is a unique organ characterized by its continuous regeneration. In order to maintain normal skin homeostasis, epidermal stem/progenitor cell proliferation must be tightly regulated, and any variation of this well-balanced process may result in the development of skin disease. However, the molecular mechanisms involved in epidermal stem/progenitor cell proliferation remain poorly understood.

The Hippo kinase pathway controls organ development, tissue regeneration and stem/progenitor cell self-renewal (Camargo et al., 2007; Dong et al., 2007; Lian et al., 2010). This kinase cascade is classically known to phosphorylate the downstream effectors Yes-Associated Protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ), resulting in their inactivation through cytosolic retention. Unphosphorylated YAP/TAZ translocate to the nucleus and bind the Transcriptional Enhancer Associate Domain (TEAD) transcription factors to activate gene expression and cell proliferation. Recently, mechanical factors and the tissue microenvironment were shown to play a major overarching role over the Hippo kinase pathway in controlling YAP/TAZ activity, which since then are known to act as mechanosensors in control of tissue homeostasis (Aragona et al., 2013; Dupont et al., 2011; Piccolo, Dupont, & Cordenonsi, 2014; Yu, Zhao, & Guan, 2015).

The canonical WNT pathway also has a crucial role during development, cell fate determination and stem/progenitor self-renewal in many tissues (Clevers, 2006). WNT signaling is activated by binding of WNT ligands to the Frizzled transmembrane receptors. This results in the dissociation of β-catenin from the cytosolic destruction complex, and its nuclear translocation and binding to TCF/LEF family transcription factors to activate the expression of target genes and epidermal stem/progenitor cell proliferation (Clevers, Loh, &

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65    Nusse, 2014; Huelsken, Vogel, Erdmann, Cotsarelis, & Birchmeier, 2001; Lo Celso, Prowse,

66    & Watt, 2004). Non-canonical WNT pathways such as the planar cell polarity and

67    WNT/calcium pathways act independently of the cytosolic destruction complex and β-catenin

68    transcriptional activity, and mainly control cellular polarity and motility (Katoh, 2005; Kohn

69    & Moon, 2005).

70    Canonical WNT/β-Catenin signaling has well-established roles in the activation of

71    keratinocyte proliferation during hair follicle cycling (Huelsken et al., 2001). Furthermore,

72    recent reports have resolved a longstanding contentious issue in the field, and unequivocally

73    established that paracrine Wnt signaling and β-Catenin transcriptional activity are required to

74    promote basal keratinocyte proliferation in the interfollicular epidermis (Choi et al., 2013;

75    Lim et al., 2013), and not keratinocyte differentiation, as initially thought (Huelsken et al.,

76    2001). However, the WNT ligands that are responsible for driving β-Catenin transcriptional

77    activity in the interfollicular epidermis remain unknown.

78    The Hippo/YAP pathway interacts with many signaling pathways to control the homeostasis

79    of tissues, including in the skin (Akladios, Mendoza-Reinoso, et al., 2017; Akladios,

80    Mendoza Reinoso, et al., 2017; Piccolo et al., 2014). Cross-talk between the Hippo/YAP and

81    WNT/β-Catenin signaling pathways was reported to be mediated by the cytosolic β-catenin

82    destruction complex, through regulatory interactions taking place in the cell nuclei (Heallen

83    et al., 2011; Rosenbluh et al., 2012; Wang et al., 2014), or through other mechanisms

84    (Azzolin et al., 2014; Cai, Maitra, Anders, Taketo, & Pan, 2015; Imajo, Miyatake, Iimura,

85    Miyamoto, & Nishida, 2012; Oudhoff et al., 2016; Park & Jeong, 2015). Recently,

86    interactions between these two pathways were also shown to occur during epidermal

87    homeostasis, and we and others showed that increased YAP activity in the basal keratinocytes

88    of murine skin results in β-catenin activation (Akladios, Mendoza-Reinoso, et al., 2017), and

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89    in severe epidermal hyperplasia in the interfollicular epidermis and in the hair follicles

90    (Beverdam et al., 2013; Schlegelmilch et al., 2011; Zhang, Pasolli, & Fuchs, 2011). However,

91    the precise underlying molecular mechanism of how epidermal YAP activity promotes β-

92    catenin activity to drive keratinocyte proliferation remains elusive.

93    In the present study, we found that inhibition of canonical Wnt/β-catenin signaling in the skin

94    of YAP2-5SA-ΔC mice results in an amelioration of the epidermal hyperplasia in the

95    interfollicular epidermis. In addition, we found that epidermal YAP promotes WNT16

96    expression in HaCaT keratinocytes and in the mouse interfollicular epidermis *in vivo*.

97    Furthermore, we established that WNT16 promotes keratinocyte proliferation both

98    independently of β-catenin, and through canonical WNT16/β-catenin signaling depending on

99    the levels of WNT16 expression. We conclude that WNT16 is the WNT ligand that under

100    normal physiological conditions promotes canonical WNT/β-catenin signaling to drive

101    proliferation of keratinocytes in response to epidermal YAP activity *in vitro* and in the

102    murine interfollicular epidermis *in vivo*.

103    **MATERIAL AND METHODS**

104    **Animals**

105    All animal experimental procedures performed were conducted under protocols approved by

106    the UNSW Australia's Animal Care and Ethics Committee Unit, and in compliance with the

107    National Health and Medical Research Council 'Australian code of practice (8th edition,

108    2013). Thirty-eight days old YAP2-5SA-ΔC mice were topically treated with 100μl of 5μM

109    XAV-939 (Chem-Supply, X0077-25MG) diluted in DMSO and vehicle solution (DMSO)

110    daily for 13 days.

111    **Tissue processing and Histological and Immunofluorescence staining**

112   Full thickness mice skin tissues were fixed in 4% paraformaldehyde, paraffin embedded,

113   sectioned, and histology-stained following routine procedures. Antigen retrieval was

114   performed using 10mM sodium citrate buffer (pH 6.0) and a Milestone RHS-1 Microwave at

115   110°C for 5 minutes. Tissue sections were immunostained using previously standardized

116   methods, and confocal images were captured using an Olympus FV1200 laser scanning

117   confocal microscope. Epidermal thickness and immuno-signal intensity were quantified using

118   3 mice and 3 skin regions per mouse. Immuno-signal was quantified in a semi-automated

119   fashion using ImageJ software. Antibody information is available in Table S1.

**Western blots and qRT-PCR analysis**

121   Protein and RNA were isolated from full thickness mouse skin biopsies and HaCaT cells

122   using TRIzol® reagent (Thermo Fisher Scientific, 15596026) following standard protocols.

123   Protein lysates were analysed by western blot, intensity of bands was quantified with ImageJ

124   software and normalized to GAPDH, GSK3β or β-catenin. Primary and secondary antibody

125   information is available in Table S1. Quantitative RT–PCR assays were carried out using Fast

126   SYBR® Green Master Mix (Thermo Fisher Scientific, 4385612) and Mx3000P qPCR System

127   (Agilent Technologies), and were analysed by the comparative cycle time method,

128   normalizing to *18S* ribosomal or *GAPDH* RNA levels. Human and mouse primer information

129   is available in Table S2.

**Cell lines and transfections**

131   HaCaT immortalized keratinocytes of passages between 15 and 21 were maintained in

132   DMEM/F-12 (Sigma, D8062), supplemented with 10% FBS (Gibco, 10437-028) and 1X

133   Penicillin-Streptomycin (Gibco, 15140-122) in a 5% CO2 incubator at 37ºC. Transient

134   transfections were performed using Lipofectamine3000 (Thermo Fisher Scientific,

135   L3000015) according to manufacturer's instructions. To overexpress dominant active YAP,

136      pDsRed Monomer C1-YAP2-S127A mutant (Addgene, 19058) and pDsRed Monomer vector

137      control DNA were used. WNT16 overexpression was carried out using pcDNA3.2-WNT16-

138      V5 (Addgene, 35942) and pcDNA3.2 vector control. *YAP* knockdown was performed using

139      MISSION® Universal and *YAP* siRNA (Sigma). *WNT16* knockdown was performed using

140      MISSION® *GFP* and *WNT16* esiRNA (Sigma). MTT assays were performed using Thiazolyl

141      Blue Tetrazolium Bromide (Sigma). Immunostaining assays were performed following

142      standard protocols. Antibody information is available in Table S1. Imaging was performed

143      using an Olympus FluoView™ FV1200 Confocal Microscope.

144      **Statistical analysis**

145      Statistical significance was determined by Student's unpaired t-tests. Error bars represent

146      mean ± SEM. Asterisks indicate statistical significance, where $P < 0.05$ was used as

147      significance cut-off.

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150 **RESULTS**

151 **XAV939 inhibition of canonical WNT signaling ameliorates epidermal hyperplasia of**

152 **YAP2-5SA-ΔC mice.**

153 We recently established that epidermal YAP activity promotes β-catenin activity to drive

154 keratinocyte proliferation in the mouse skin *in vivo* (Akladios, Mendoza-Reinoso, et al.,

155 2017). However, it remains unclear whether these interactions are mediated by paracrine

156 activation of canonical Wnt signaling, or by other YAP/β-catenin regulatory interactions.

157 Firstly, we assessed whether these were caused by activation of canonical Wnt/β-catenin

158 signaling activity. To do so, we used YAP2-5SA-ΔC mice, which display increased

159 epidermal YAP activity, increased β-catenin activity, and severe hyperplasia of the basal

160 layer due to increased keratinocyte proliferation. Furthermore, the skin of these mice also

161 display abnormal hair follicles and hyperkeratosis (Akladios, Mendoza-Reinoso, et al., 2017;

162 Beverdam et al., 2013). We topically treated YAP2-5SA-ΔC mice with XAV-939 or vehicle

163 daily for thirteen days. XAV-939 is a small molecule that selectively inhibits canonical (and

164 not non-canonical) Wnt signaling through stabilizing Axin2 and promoting the destruction of

165 cytosolic β-catenin (Distler et al., 2013).

166 Histological analysis showed that the thickness of the hyperplastic interfollicular epidermis of

167 XAV-939-treated YAP2-5SA-ΔC mouse skin was approximately a third of that of vehicle-

168 treated YAP2-5SA-ΔC mouse skin (Figure 1c-d.1; $P<0.04$, $N=3$). Effective inhibition of

169 Wnt/β-catenin signaling activity by XAV-939 was confirmed by reduced nuclear active β-

170 catenin (Figure 1f, f.1; $P<0.002$, $N=3$), and reduced nuclear localization of β-catenin direct

171 target Cyclin D1 (Figure 1h, h.1; $P<0.0001$, $N=3$) in the interfollicular epidermis.

172 Furthermore, nuclear expression of the basal epidermal stem/progenitor cell marker P63

173 ($P<0.0001$, $N=3$) (Figure 1i *vs*. j & j.1), and of proliferation marker Ki67 ($P<0.008$, $N=3$)

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174   (Figure 1k *vs*. l & l.1) were also significantly reduced in the XAV-939- *vs*. vehicle-treated

175   YAP2-5SA-ΔC interfollicular epidermis. Altogether, these data are in line with our previous

176   findings (Akladios, Mendoza-Reinoso, et al., 2017), and furthermore establish that epidermal

177   YAP activity promotes canonical Wnt signaling to drive β-catenin nuclear activity and basal

178   keratinocyte proliferation in the interfollicular epidermis of the mouse skin *in vivo*.

179   **YAP activity promotes *WNT16* expression in keratinocytes grown *in vitro* and in the**

180   **murine epidermis.**

181   Next, we wanted to identify which WNT ligands are responsible for driving WNT/β-catenin

182   signaling in response to epidermal YAP activity to promote keratinocyte proliferation.

183   Transcriptomics approaches found that *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt6*, *Wnt7A*, *Wnt7B*, *Wnt10A*,

184   *Wnt10B* and *Wnt16* are expressed in the developing or regenerating mouse epidermis (Rezza

185   et al., 2016; Sennett et al., 2015). These and their human orthologues were selected for

186   analyses. Quantitative real time RT-PCR assays revealed significantly increased expression

187   of *Wnt16* expression (Figure 2a. $P<0.0001$, $N=3$), and significantly decreased expression of

188   *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt6* and *Wnt10b* (Figure 2a, $P<0.0025$, $N=3$) in YAP2-5SA-ΔC *vs*.

189   wildtype skin. Then we assessed the expression of these *WNT* ligand genes upon YAPS127A

190   overexpression in immortalized human HaCaT keratinocytes. We chose to overexpress

191   YAPS127A rather than YAP2-5SA-ΔC because previous reports have shown that the latter

192   acts as a dominant negative YAP mutant protein *in vitro* due to loss of its transactivation

193   domain encoded by its C-terminus (Hoshino et al., 2006; Zhao et al., 2007). We found

194   significantly increased gene expression of *YAP* ($P<0.0005$; $N=3$), and of YAP/TEAD direct

195   target genes *CTGF* ($P<0.0002$; $N=3$) and *ANKRD1* ($P<0.04$; $N=3$) in YAPS127A *vs*. control-

196   transfected HaCaT keratinocytes, confirming YAP activation in YAPS127A-transfected

197   keratinocytes (Figure 2b). Furthermore, expression of *WNT16* ($P<0.007$; $N=3$) was also

198 increased, whereas *WNT6* (*P*<0.007; *N*=3) and *WNT10B* (*P*<0.03; *N*=3) expression levels

199 were decreased in YAPS127A *vs*. control-transfected HaCaT keratinocytes (Figure 2b).

200 *WNT3*, *WNT3A*, *WNT4*, *WNT7A*, *WNT7B*, and *WNT10A* expression levels were unchanged

201 (*N*=3, Figure 2b). si*YAP*-transfected HaCaT keratinocytes on the other hand, displayed

202 decreased *YAP* expression levels (*P*=0.0002; *N*=3), confirming effective knock down, and

203 reduced expression of *WNT4* (*P*=0.0007; *N*=3), *WNT7B* (*P*<0.008; *N*=3), *WNT10B* (*P*=0.03;

204 *N*=3), and *WNT16* (*P*<0.007; *N*=3) compared to scramble control-transfected cells (Figure

205 3c). *WNT10A* expression levels were slightly increased (*P*<0.005; *N*=3) (Figure 3c), whereas

206 expression of *WNT3*, *WNT3A*, *WNT6* and *WNT7A* were unchanged (*N*=3; Figure 3c). These

207 data show that YAP activity positively controls the expression levels of *WNT16* in

208 proliferating HaCaT keratinocytes grown *in vitro*.

209 Next, we investigated gene and protein expression in skin biopsies of YAP2-5SA-ΔC mice,

210 and first confirmed significantly higher *hYAP* (*P*<0.003; *N*=3) and *Wnt16* (*P*<0.02; *N*=3)

211 RNA expression levels compared to in skin biopsies of wildtype littermate mice (data not

212 shown). Moreover, immunofluorescence assays displayed increased nuclear YAP and

213 increased activated β-catenin in the dramatically thickened basal Keratin14-expressing layer

214 of the interfollicular epidermis of YAP2-5SA-ΔC mice compared to wildtype (Figure 2d, g,

215 h), which is in line with previously reported (Akladios, Mendoza-Reinoso, et al., 2017;

216 Beverdam et al., 2013). Furthermore, the YAP2-5SA-ΔC basal epidermis displayed increased

217 WNT16 protein levels (Figure 2e, f), and increased levels of phospho-GSK3β relative to

218 wildtype littermate skin (Figure 3i). These results show that YAP activity in basal

219 keratinocytes promotes WNT16 expression and canonical WNT/β-catenin signaling in the

220 regenerating mouse interfollicular epidermis *in vivo*.

221 **YAP activity in proliferating keratinocytes promotes canonical WNT16/β-catenin**

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222 **signaling.**

223 A previous study reported that WNT16B overexpression in normal human keratinocytes

224 (NHEKs) resulted in activation of non-canonical WNT signaling and increased cell

225 proliferation, but not in β-catenin activation (Teh et al., 2007). However, WNT16 is reported

226 to act both as a canonical and as a non-canonical WNT ligand (Christodoulides, Lagathu,

227 Sethi, & Vidal-Puig, 2009; Mazieres et al., 2005; Moverare-Skrtic et al., 2014; Nalesso et al.,

228 2017).

229 To understand if YAP activity promotes canonical WNT16/β-catenin signaling to drive

230 keratinocyte proliferation, immunofluorescence assays were performed in HaCaT

231 keratinocytes grown *in vitro* at low and high density. Predominantly nuclear YAP and

232 activated β-catenin were detected in low-dense proliferating keratinocytes as previously

233 reported (Akladios, Mendoza-Reinoso, et al., 2017). Furthermore, WNT16 protein expression

234 levels were relatively high compared to in high dense and quiescent keratinocytes in which

235 YAP and β-catenin were sequestered in the cytosol and the cell junctions (Figure 4a-d).

236 These data are consistent with the hypothesis that WNT16 drives β-catenin activation and

237 cell proliferation in response to YAP activity in keratinocytes.

238 To further test this hypothesis, we assessed the effect of increased YAP activation on WNT16

239 expression levels, GSK3β phosphorylation, and β-catenin activation in HaCaT keratinocytes.

240 WNT16 protein expression was indeed increased in YAPS127A-transfected cells ($P<0.015$;

241 $N=3$; Figure 4e, e.1), in line with our RNA expression data (Figure 2a). Furthermore, we

242 observed increased phospho-GSK3β ($P<0.04$; $N=3$; Figure 4f, f.1), and increased activated β-

243 catenin ($P<0.004$; $N=3$; Figure 4f, f.2) compared to control-transfected cells. These data show

244 that YAP activity increases WNT16 expression and canonical WNT/β-catenin signaling in

245 proliferating HaCaT keratinocytes *in vitro*.

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**WNT16 promotes keratinocyte proliferation through canonical WNT16/β-catenin signaling.**

Next, we tested the effect of WNT16 overexpression on β-catenin activation and cell proliferation in HaCaT keratinocytes. *WNT16* RNA and WNT16 protein expression levels were increased in V5-WNT16- *vs*. control transfected HaCaT keratinocytes (Figure 4a and b), levels of activated *vs*. pan β-catenin were significantly reduced (Figure 4b and b.1), and expression levels of β-catenin transcriptional targets *CCDN1* and *cMYC* were unchanged (Figure 4a). Furthermore, V5-WNT16-transfected HaCaT keratinocytes proliferated at a mildly but significantly increased rate (Figure 4c). These data confirm that WNT16 overexpression promotes keratinocyte proliferation, but not β-catenin activity, in line with the earlier study (Teh et al., 2007).

A recent publication showed that WNT16, in the presence of excessive levels of WNT ligands, antagonised canonical WNT signaling (Nalesso et al., 2017). So conceivably, the high levels of WNT16 expression in overexpression studies with NHEK (Teh et al., 2007) and HaCaT keratinocytes (this study) may have caused a shutdown of canonical WNT signaling in these cells. To test the effect of reduced *WNT16* expression on canonical WNT signaling and keratinocyte proliferation, we assessed GSK3β phosphorylation, β-catenin activity, and cell proliferation in esi*Wnt16*- *vs*. control transfected HaCaT keratinocytes. Quantitative real time RT-PCR assays confirmed reduced *WNT16* RNA expression levels in esi*WNT16*- *vs*. control-transfected keratinocytes ($P<0.002$; $N=3$; Figure 4d). Furthermore, protein expression levels of phospho-GSK3β ($P<0.05$; $N=3$; Figure 4e and e.1), and of activated β-catenin ($P<0.0005$; $N=3$; Figure 4e and e.2) were reduced in esi*WNT16*-transfected HaCaT keratinocytes, and MTT assays established significantly reduced cell proliferation rates in esi*WNT16*- *vs*. control transfected HaCaT keratinocytes (Figure 4f).

270   These data show that at physiological levels WNT16 promotes proliferation of HaCaT

271   keratinocytes through activation of canonical WNT16/β-catenin signaling.

272   Altogether, these data establish that YAP activity in keratinocytes promotes WNT16

273   expression and keratinocyte proliferation via canonical WNT16/β-catenin signaling.

274   **DISCUSSION**

275   Recent investigations have demonstrated that YAP drives keratinocyte proliferation in the

276   murine epidermis *in vivo* and in HaCaT keratinocytes *in vitro* (Akladios, Mendoza-Reinoso,

277   et al., 2017; Beverdam et al., 2013; Schlegelmilch et al., 2011; Zhang et al., 2011) through

278   promoting β-catenin activity (Akladios, Mendoza-Reinoso, et al., 2017). However, it is still

279   unclear whether YAP drives β-catenin activation and keratinocyte proliferation through

280   paracrine activation of canonical Wnt signaling, or through other regulatory mechanisms.

281   Here, using two independent experimental models, we demonstrate that epidermal YAP

282   activity drives WNT16 expression to promote keratinocyte proliferation through canonical

283   WNT16/β-catenin signaling (Figure 5a). These findings are consistent with reports showing

284   that WNT16 promotes keratinocyte proliferation *in vitro* (Teh et al., 2007), and in the murine

285   hair follicle bulge *in vivo* (Kandyba et al., 2013).

286   Recent reports resolved a contentious issue on the role of β-catenin in the control of

287   keratinocyte proliferation in the interfollicular epidermis, and unequivocally demonstrated

288   that auto/paracrine WNT/β-catenin signaling promotes keratinocyte proliferation, and not

289   differentiation as suggested by earlier studies (Choi et al., 2013; Huelsken et al., 2001; Lim et

290   al., 2013). However, the responsible WNT ligands remained elusive. Our studies are in

291   support of these two recent reports, and furthermore reveal WNT16 as a WNT ligand driving

292   canonical WNT/β-catenin signaling and keratinocyte proliferation *in vitro* and *in vivo*.

293 WNT16 is generally recognized to act both as a canonical and as a non-canonical WNT

294 ligand in osteoblasts (Moverare-Skrtic et al., 2014), chondroblasts (Nalesso et al., 2017),

295 leukemia (Mazieres et al., 2005), and in adipogenesis (Christodoulides et al., 2009). A

296 previous study reported that WNT16B overexpression in normal human keratinocytes

297 (NHEKs) results in increased cell proliferation, but not in increased canonical WNT signaling

298 or β-catenin activation (Teh et al., 2007). Instead, it was reported to activate a non-canonical

299 WNT signaling pathway through the N-Jun-terminal kinase cascade *in vitro* (Teh et al.,

300 2007). Our studies confirm that WNT16 positively regulates keratinocyte proliferation. This

301 was very obvious from our studies in which we repressed WNT16 expression in HaCaT

302 keratinocytes (Figure 4f) relative to those where we overexpressed WNT16 in HaCaT

303 keratinocytes (Figure 4c). The relatively small effect of WNT16 overexpression on

304 promoting keratinocyte proliferation may be explained by the fact that keratinocytes were

305 already in a highly proliferative state and were unable to be induced even further. We also

306 found that WNT16 overexpression did not result in β-catenin activation in HaCaT

307 keratinocytes. Rather, it resulted in reduced β-catenin activation in these cells (Figure 5b).

308 Nevertheless, we also showed that inhibition of endogenous WNT16 expression in HaCaT

309 keratinocytes by siRNAs resulted in decreased canonical Wnt signaling and β-catenin

310 activity, and reduced cell proliferation, demonstrating that WNT16 can also promote

311 canonical Wnt signaling to drive keratinocyte proliferation. These seemingly contradictory

312 observations may be explained by a recent publication, which proposed that WNT16 will

313 counteract canonical WNT signaling in the presence of excessive levels of WNT ligands

314 (Nalesso et al., 2017). Since in our RNA interference studies, we repressed endogenous levels

315 of WNT16 expression, we propose that under normal physiological conditions WNT16 will

316 act as a canonical WNT ligand to promote keratinocyte proliferation (Figure 5a).

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317    In contrast, WNT16 protein expression was mildly elevated due to increased YAP activity in

318    the YAPS127A-transfected HaCaT keratinocytes (Figure 3e-f) and in the YAP2-5SA-ΔC

319    epidermis (Figure 2d-h). Since we also detected concurrent activation of canonical WNT

320    signaling, we conclude that the WNT16 expression levels induced by YAP in these systems

321    drive cell proliferation, but are apparently not high enough to lead to repression of canonical

322    WNT signaling through this recently reported counteracting mechanism (Nalesso et al.,

323    2017). Interestingly, the mildly increased epidermal WNT16 expression levels in skin of

324    YAP2-5SA-ΔC mice still appear to be somewhat compensated for by means of reduced

325    expression levels of other WNTs expressed in the skin biopsies (Figure 2a) through an

326    alternative unknown mechanism.

327    How YAP activates *WNT16* expression remains unclear. We found that *WNT16* expression is

328    tightly regulated by YAP, indicating that it may be a direct YAP/TEAD transcriptional target

329    in basal keratinocytes. However, a previous study in breast cancer cells failed to identify

330    *WNT16* as a direct YAP/TEAD target gene in a different epithelial cell type (Zanconato et al.,

331    2015). Therefore, *WNT16* expression may also be activated indirectly, in response to other

332    effects downstream of YAP/TEAD transcriptional activity in epithelial cells (Figure 5a).

333    There is a heightened interest in the stem cell field with an ever-increasing body of high

334    impact publications demonstrating that the Hippo/YAP and WNT/β-catenin pathways

335    interact to control cell proliferation in tissue homeostasis and in cancer development. A

336    recent study proposed a model where YAP/TAZ are integral components of β-catenin

337    destruction complex, which explained a number of contradictory published observations

338    (Azzolin et al., 2014; Azzolin et al., 2012; Oudhoff et al., 2016). In this model, the nuclear

339    translocation of YAP and β-catenin depends on the presence of WNT ligands, and the

340    activation of canonical WNT signaling. In our study, we established that YAP activity

341     promotes WNT16 expression in epidermal regeneration, revealing that YAP may be able to

342     control its own activity by promoting the dissociation of the β-catenin destruction complex.

343     Our work may therefore reveal yet another level of regulatory interactions between YAP and

344     β-catenin in stem cell control during tissue homeostasis, but does not exclude that these

345     proteins may also undergo additional regulatory interactions to control epidermal

346     stem/progenitor cell proliferation, for instance similar to those that were previously reported

347     to occur in the nucleus (Heallen et al., 2011; Rosenbluh et al., 2012; Wang et al., 2014), in

348     the β-catenin destruction complex (Azzolin et al., 2014; Cai et al., 2015), or otherwise (Imajo

349     et al., 2012; Oudhoff et al., 2016; Park & Jeong, 2015).

350     Uncontrolled proliferation of tissue stem/progenitor cells can result in regenerative disease

351     such as cancer. Interestingly, both YAP and WNT signaling have been implicated in

352     carcinogenesis in various tissues (reviewed in Piccolo et al., 2014), including in the skin

353     (reviewed in Andl, Zhou, Yang, Kadekaro, & Zhang, 2017). Furthermore, YAP/TAZ have

354     been reported to regulate mesenchymal and skeletal stem cells during osteogenesis (Tang,

355     Feinberg, Keller, Li, & Weiss, 2016; Tang & Weiss, 2017), and WNT16 plays a well-

356     recognized role in joint formation and bone homeostasis (Guo et al., 2004; Moverare-Skrtic

357     et al., 2014; Wergedal, Kesavan, Brommage, Das, & Mohan, 2015). Therefore, our findings

358     have important implications for our understanding of the molecular aetiology of regenerative

359     skin disorders, cancer, and cartilage and joint diseases that display increased nuclear YAP

360     and/or WNT16 activity.

361 **CONFLICT OF INTEREST**

362 The authors have no conflicts to declare.

363 **ACKNOWLEDGMENTS**

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566　**FIGURE LEGENDS**

567 **Figure 1. XAV939 inhibition of canonical WNT signaling ameliorates epidermal**

568 **hyperplasia of YAP2-5SA-ΔC mice.**

569 Representative images of YAP2-5SA-ΔC mice treated with vehicle (a, c, e, g, i, k), or XAV-

570 939 (b, d, f, h, j, l). H&E staining of boxed skin areas (c,d). Quantification of epidermal

571 thickness (d.1). Immunofluorescence analysis showing active β-catenin (e,f), Cyclin D1 (g,h),

572 p63 (i, j) and Ki67 (k, l) staining. Quantification of active β-catenin (f.1), Cyclin D1 (h.1),

573 p63 (j.1) and Ki67 (l.1) positive nuclei in the epidermis. H&E, Hematoxylin and eosin;

574 DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 $\mu$m.

575 **Figure 2. YAP activity promotes *WNT16* expression in keratinocytes grown *in vitro* and**

576 **in the murine epidermis.**

577 Quantitative RT-PCR analyses showing *WNT3*, *WNT3A*, *WNT4*, *WNT6*, *WNT7A*, *WNT7B*,

578 *WNT10A*, *WNT10B* and *WNT16* expression levels relative to 18S or *GAPDH* in lysates of

579 YAP2-5SA-ΔC skin (a) and HaCaT keratinocytes transfected with YAPS127A (b) or si*YAP*

580 (c). Immunofluorescence staining of dorsal skin sections of adult wildtype and YAP2-5SA-

581 ΔC littermate mice detecting K14 (e), YAP (f), active β-catenin (g), WNT16 (h), phospho-

582 GSK3β (i). DAPI, 4, 6-diamidino-2-phenylindole. Scale bars = 20 $\mu$m.

583 **Figure 3. YAP activity drives canonical WNT16/β-catenin signalling in proliferating**

584 **keratinocytes.**

585 (a-d) Immunofluorescence staining of proliferating (top) and quiescent (bottom) HaCaT

586 keratinocytes showing Ki67 (a), YAP (b), active β-catenin (c) and WNT16 expression (d).

587 Western blots of (e) YAP, WNT16, GAPDH, and (f) phospho-GSK3β, total GSK3β, active

588 β-catenin, total β-catenin, and GAPDH, in protein lysates of vector and YAPS127A

589  transfected HaCaT keratinocytes (*N*=3). Quantification of WNT16 expression (e.1), GSK3β

590  phosphorylation (f.1), and active β-catenin expression (f.2).

591  **Figure 4. WNT16 promotes cell proliferation independently of β-catenin activation or**

592  **through canonical WNT16/β-catenin signaling.**

593  (a) Fold-change of *WNT16*, *CCND1* and *cMYC* expression normalized to *GAPDH* in HaCaT

594  keratinocytes transfected with V5-WNT16 or vector control. (b) Western blots detecting  V5-

595  WNT16, active β-catenin, total β-catenin, and GAPDH in protein lysates of vector control

596  and V5-WNT16 (right) transfected HaCaT keratinocytes (*N*=3). (b.1) Quantification of fold-

597  change protein expression of active β-catenin normalized to total β-catenin lysates of vector

598  control and V5-WNT16 transfected HaCaT keratinocytes. (c) MTT proliferation assay using

599  HaCaT keratinocytes transfected with vector control and V5-WNT16 (*N*=3).

600  Fold-change of *WNT16* mRNA expression normalized to *GAPDH* in HaCaT keratinocytes

601  transfected with esi*GFP* and esi*WNT16* RNA (d). Western blots showing phospho-GSK3β

602  expression normalized to total GSK3β (e and e.1) and active β-catenin normalized to total β-

603  catenin (e and e.2) in protein lysates of esi*GFP*-  and esi*WNT16*-transfected (right) HaCaT

604  keratinocytes. (f) MTT proliferation assay using HaCaT keratinocytes transfected with

605  esi*GFP* and esi*WNT16* RNA (*N*=3).

606  **Figure 5. YAP promotes WNT16/β-catenin signalling to drive keratinocyte proliferation**

607  (a) Working model of how YAP activity in keratinocytes promotes WNT16 production and

608  canonical WNT/β-catenin signalling to drive cell proliferation in the murine epidermis. (b)

609  Excessive WNT16 signals drive keratinocyte proliferation independently of β-catenin

610  activity.

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612     **Table S1. List of primary and secondary antibodies.**

| Antibody | Company | Catalogue# | Host | Dilution | |
| --- | --- | --- | --- | --- | --- |
| | | | | IF | WB |
| Active-β-catenin (Clone 8E7) | Merck Millipore | 05-665 | Mouse | 1:200 | 1:500 |
| β-catenin | Abcam | ab6302 | Rabbit | 1:200 | 1:500 |
| CyclinD1 | Abcam | ab134175 | Rabbit | 1:200 | - |
| GSK3β | Abcam | ab73173 | Rabbit | - | 1:500 |
| GSK3β (27C10) | Cell Signaling Technology | 9315S | Rabbit | - | 1:500 |
| phospho-GSK3β | Cell Signaling Technology | 9323P | Rabbit | 1:50 | 1:500 |
| K14 | Australian Biosearch | PRB-155P | Rabbit | 1:500 | - |
| Ki67 | Abcam | ab15580 | Rabbit | 1:200 | - |
| P63 | Santa Cruz | sc-8343 | Rabbit | 1:200 | - |
| V5 | ThermoFisher Scientific | R960CUS | Mouse | - | 1:5000 |
| WNT16 | Sigma | HPA027030 | Rabbit | 1:200 | 1:500 |
| YAP | Santa Cruz | 101199 | Mouse | 1:200 | 1:500 |
| HRP-conjugated anti-rabbit | Amersham | NA934 | Donkey | - | 1:5000 |
| HRP-conjugated anti-mouse | Amersham | NA931 | Sheep | - | 1:5000 |
| Alexa 594-anti rabbit | Life Technologies | A21207 | Donkey | 1:200 | - |
| Alexa 488-anti mouse | Life Technologies | A11001 | Goat | 1:200 | - |

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623 **Table S2. List of human and mouse primer sequences.**

| Gene | Sequence | Species |
|---|---|---|
| *ANKRD1*-F<br>*ANKRD1*-R | 5'-AGTAGAGGAACTGGTCACTGG-3'<br>5'-TGGGCTAGAAGTGTCTTCAGAT-3' | Human |
| *CCND1*-F<br>*CCND1*-R | 5'-TCAAATGTGTGCAGAAGGAGGT-3'<br>5'-GACAGGAAGCGGTCCAGGTA-3' | Human |
| *c-MYC*-F<br>*c-MYC*-R | 5'-GCAAACCTCCTCACAGCCCAC-3'<br>5'-AACTTGACCCTCTTGGCAGCA-3' | Human |
| *CTGF*-F<br>*CTGF*-R | 5'-AGGAGTGGGTGTGTGAGGA-3'<br>5'-CCAGGCAGTTGGCTCTAATC-3' | Human |
| *GAPDH*-F<br>*GAPDH*-R | 5'-CTCCTGCACCACCAACTGCT-3'<br>5'-GGGCCATCCACAGTCTTCTG-3' | Human |
| *WNT3*-F<br>*WNT3*-R | 5'-GGAGAGGGACCTGGTCTACTA-3'<br>5'-CTTGTGCCAAAGGAACCCGT-3' | Human |
| *WNT3A*-F<br>*WNT3A*-R | 5'-AACTGCACCACCGTCCAC-3'<br>5'-AAGGCCGACTCCCTGGTA-3' | Human |
| *WNT4*-F<br>*WNT4*-R | 5'-AGGAGGAGACGTGCGAGAAA-3'<br>5'-CGAGTCCATGACTTCCAGGT-3' | Human |
| *WNT6*-F<br>*WNT6*-R | 5'-GGCAGCCCCTTGGTTATGG-3'<br>5'-CTCAGCCTGGCACAACTCG-3' | Human |
| *WNT7A*-F<br>*WNT7A*-R | 5'-CTTCGGGAAGGAGCTCAAA-3'<br>5'-GCAATGATGGCGTAGGTGA-3' | Human |
| *WNT7B*-F<br>*WNT7B*-R | 5'-GTCAGGGATGTTTGTCCC-3'<br>5'-TCTGGTAGGTCCTTGTGC-3' | Human |
| *WNT10A*-F<br>*WNT10A*-R | 5'-CCGCATGTTCTCCATCACT-3'<br>5'-ATCCACGCGAGAATGAGG-3' | Human |
| *WNT10B*-F<br>*WNT10B*-R | 5'-ATGCGAATCCACAACAACAG-3'<br>5'-TCCAGAATGTCTTGAACTGG-3' | Human |
| *WNT16*-F<br>*WNT16*-R | 5'-CAATGAACCTACATAACAATGAAGC-3'<br>5'-CAGCGGCAGTCTACTGACAT-3' | Human |
| *YAP*-F<br>*YAP*-R | 5'-ACAGGCCGCTACTGATGC-3'<br>5'-ATGAGCTCGAACATGCTGTG-3' | Human |
| *18S*-F<br>*18S*-R | 5'-GATCCATTGGAGGGCAAGTCT-3'<br>5'-CCAAGATCCAACTACGAGCTTTTT-3' | Mouse |
| *Wnt16*-F<br>*Wnt16*-R | 5'-TGAATGCTGTCTCCTTGGTG-3'<br>5'-GAGCTGTGCAAGAGGAAACC-3' | Mouse |

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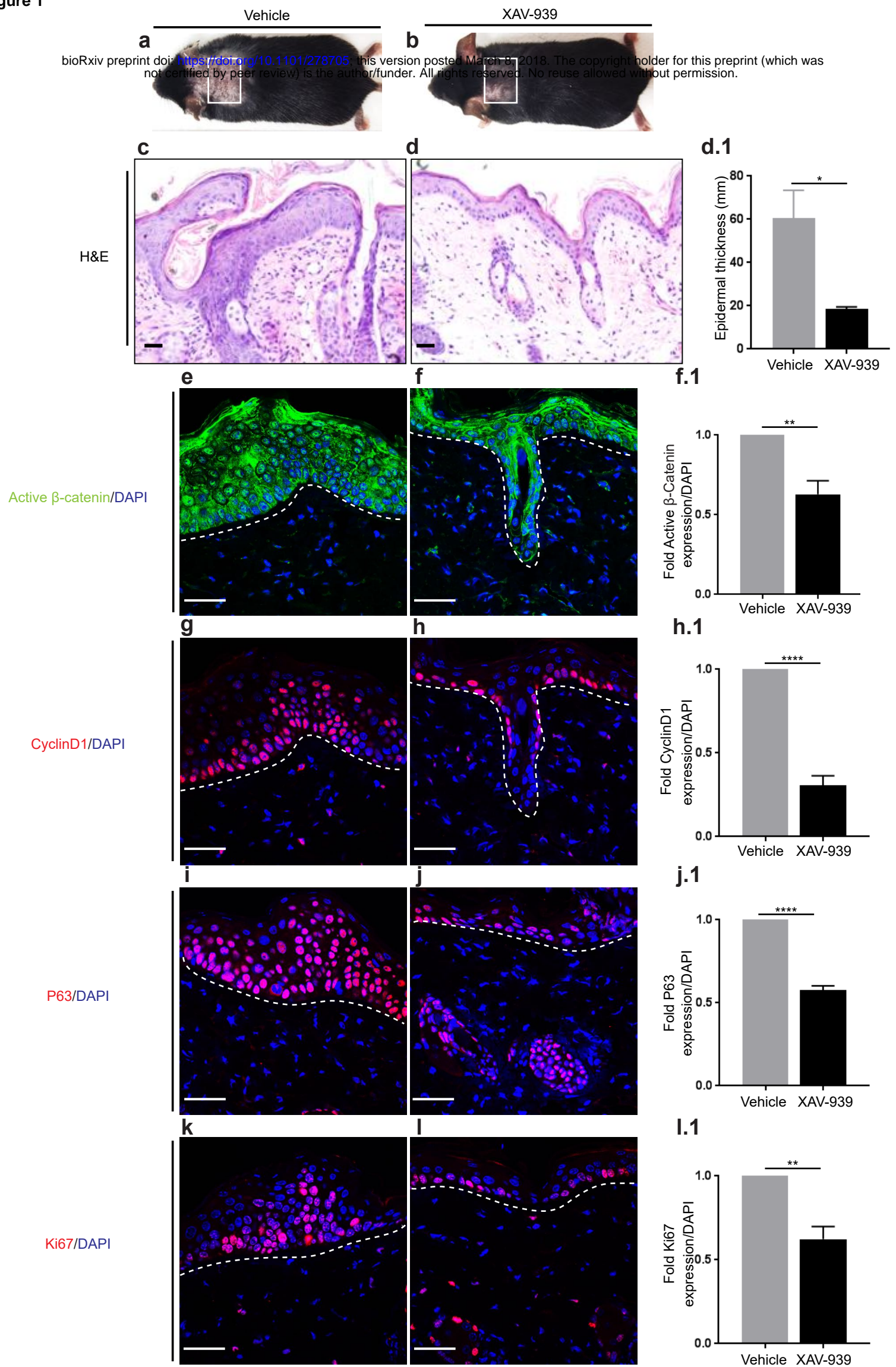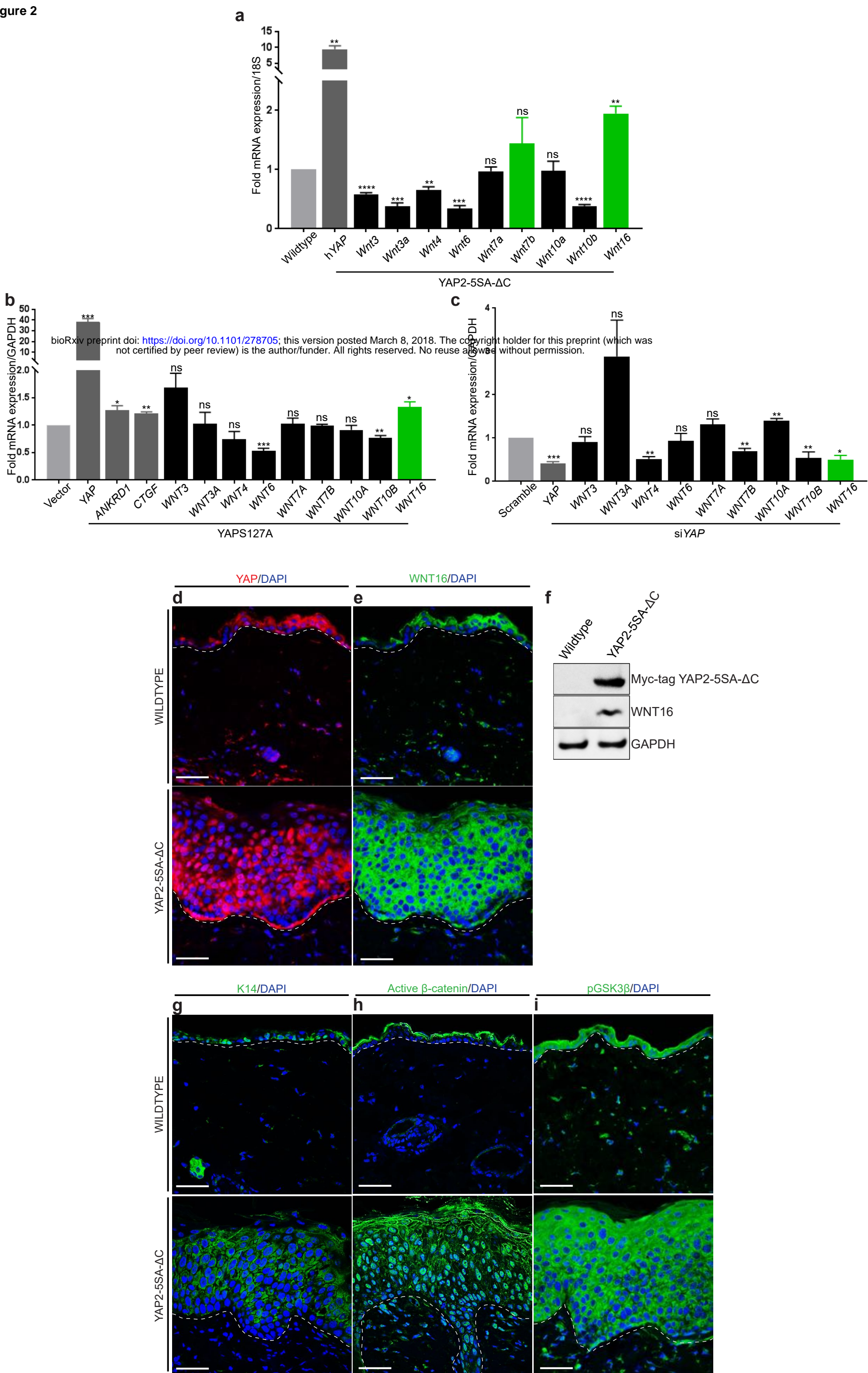
**Figure 1**

**Figure 2**

**Figure 3**



| | Ki67/DAPI | YAP/DAPI | Active β-catenin/DAPI | WNT16/DAPI |
|---|---|---|---|---|
| PROLIFERATING | a | b | c | d |
| QUIESCENT | | | | |

e

Vector    YAPS127A

YAP

WNT16

GAPDH

e.1

WNT16/GAPDH

**

f

Vector    YAPS127A

phospho-GSK3β

GSK3β (Abcam)

Active β-catenin

β-catenin

GAPDH

f.1

Phospho-GSK3β/
total GSK3β

*

f.2
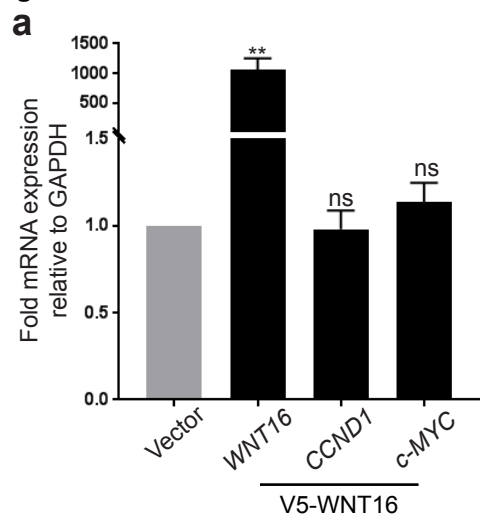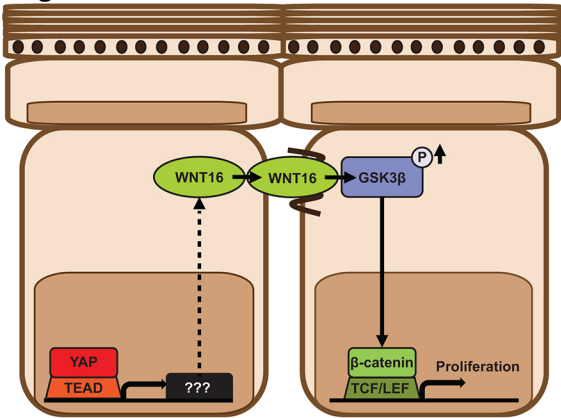
Active β-catenin/
total β-catenin

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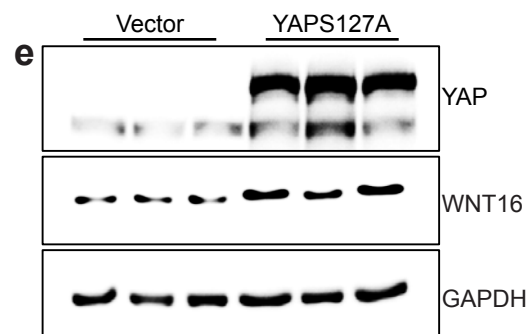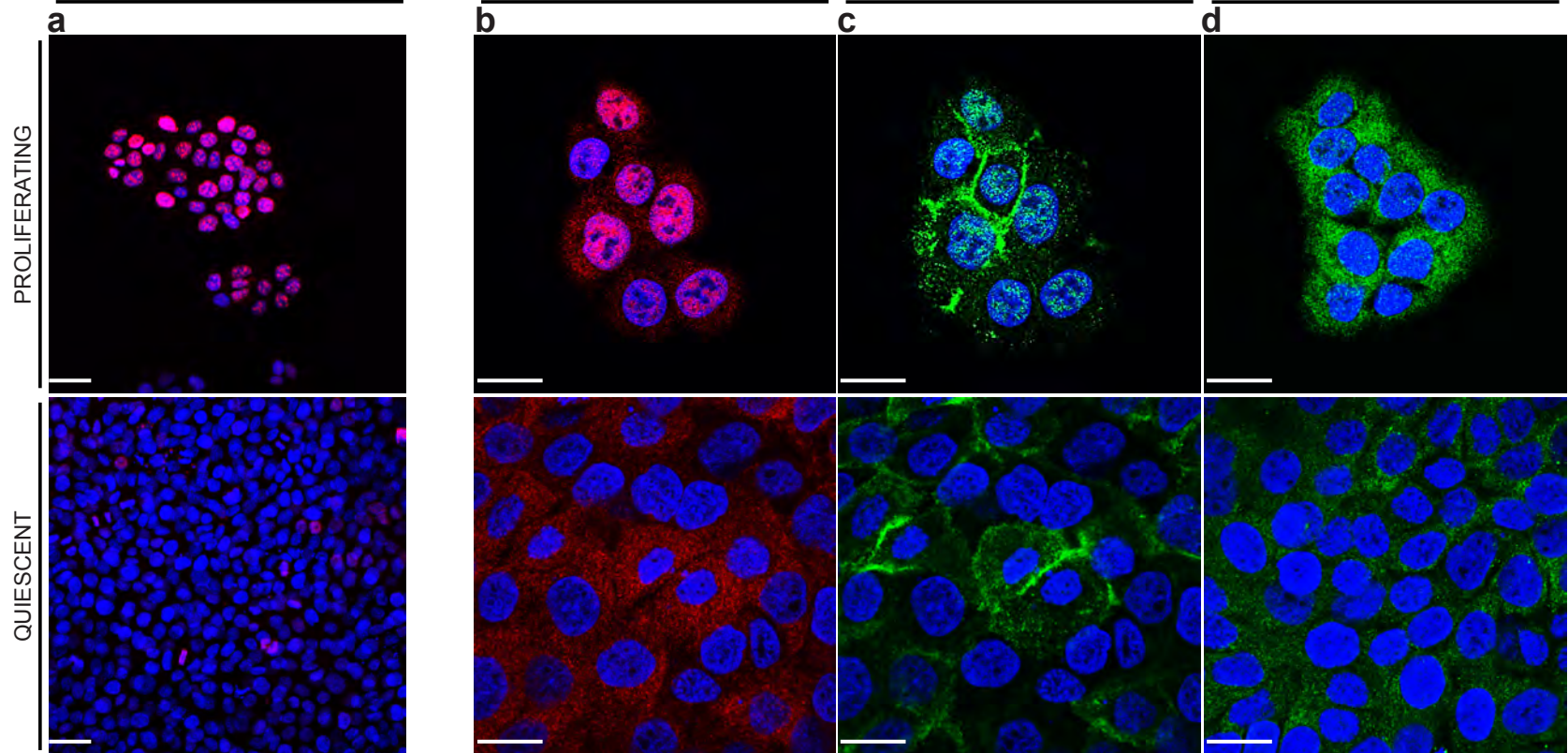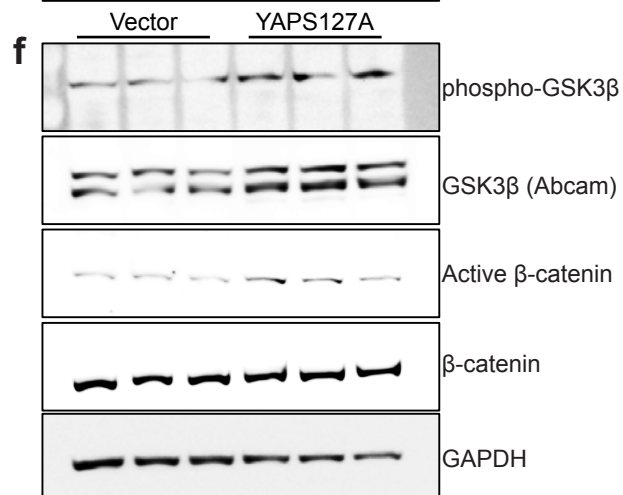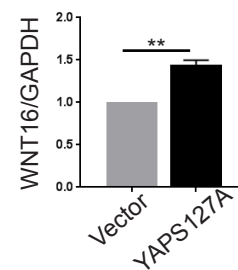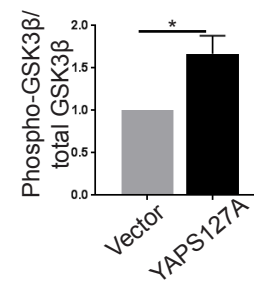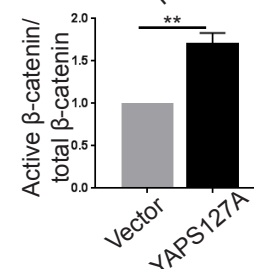
**Figure 4**

**Figure 5**
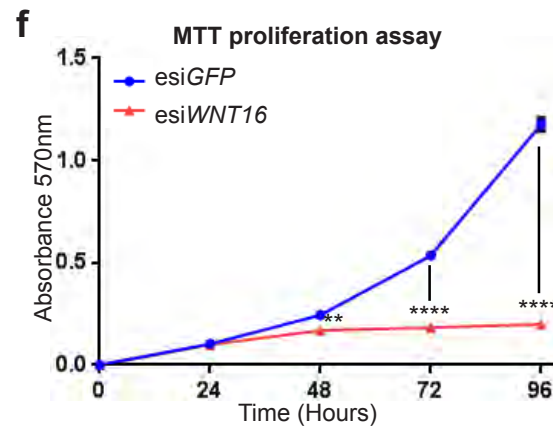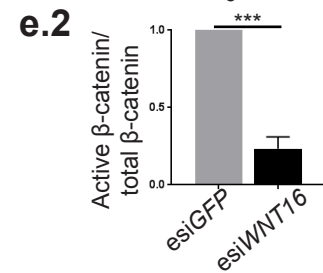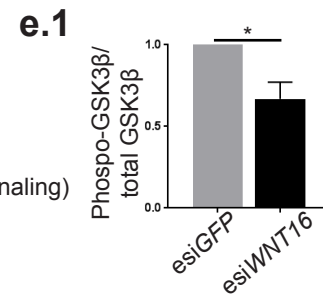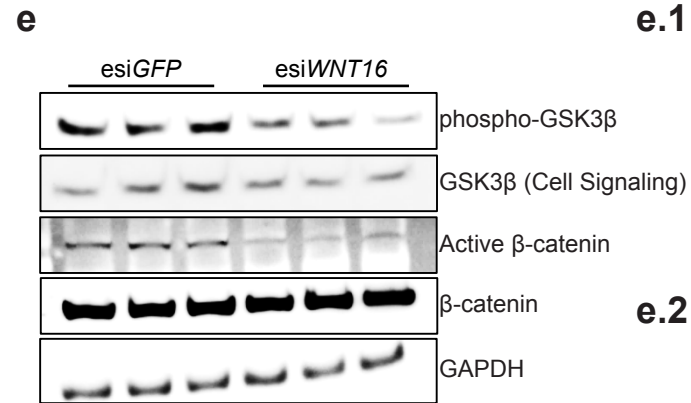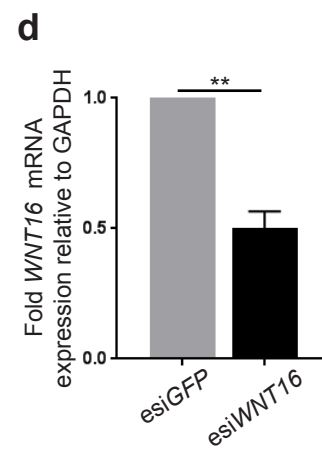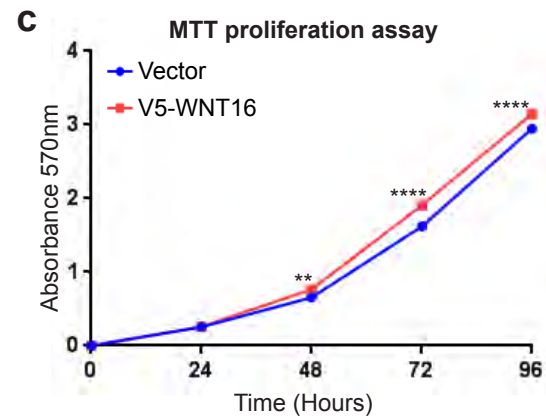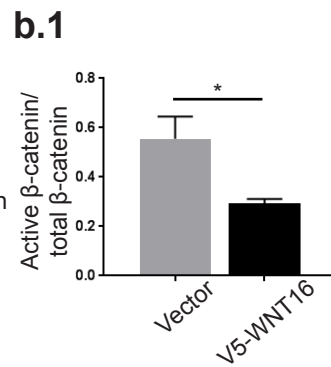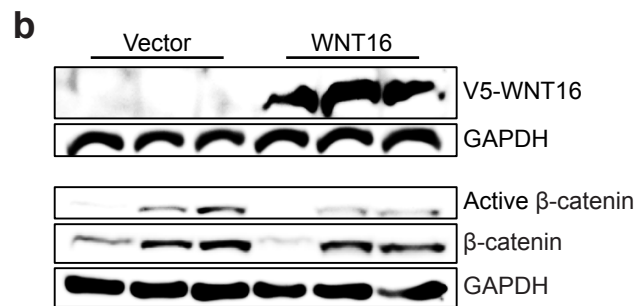
A: Endogenous WNT16 expression levels

B: Excessive WNT16 expression levels