Multifaceted functions of Rab23 on primary cilium and Hedgehog signaling-mediated granule cell proliferation

CHH Hor[*1,2], WY Leong[2], and ELK Goh[*2,3,4,5]

Affiliations:

[1]Department of Chemistry, Research Cluster on Health and Drug Discovery, Faculty of Science, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR

[2] Neuroscience Academic Clinical Programme, Duke-NUS Medical School, Singapore

[3] Department of Research, National Neuroscience Institute, Singapore

[4]Neuroscience and Mental Health Faculty, Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

[5]KK Research Center, KK Women's and Children's Hospital, Singapore

*Co-corresponding authors

Email: Eyleen_Goh@nni.com.sg; catherinehor@hkbu.edu.hk

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Sonic Hedgehog (Shh) signaling from the primary cilium drives cerebellar granule cell precursor (GCP) proliferation. Mutations of hedgehog (Hh) pathway repressors could cause medulloblastoma, the most prevalent and malignant childhood brain tumor that arises from aberrant GCP proliferation. We demonstrate that brain-specific knockout of a Shh pathway repressor *Rab23* in mice caused mis-patterning of cerebellar folia and elevated GCP proliferation during early development, but with no prevalent occurrence of medulloblastoma at adult stage. Strikingly, *Rab23*-depleted GCPs exhibited up-regulated basal level of Shh pathway activities despite reduced ciliation, and were desensitized against stimulations by Shh and Smoothened (Smo) agonist in primary GCP culture. These results illustrate dual functions of Rab23 in repressing the basal level of Shh signaling, while facilitating Shh signal transduction via Shh/Smo on primary cilium. Collectively, our findings unravel instrumental roles of *Rab23* in GCP proliferation and ciliogenesis. *Rab23's* potentiation of Shh signaling pathway through the primary cilium and Smo, suggests a potential new therapeutic for Smo/primary cilium-driven medulloblastoma.

Author Summary

C.H.H conceived, designed, lead, and performed all *in vitro* and *in vivo* experiments, analyzed data and wrote the manuscript. W.Y performed QPCR experiments and primary GCP cultures and analyzed data. E.L.G conceived and directed the study.

1   **Introduction**

2

3   Cerebellar development in mammals is highly dependent on Shh signaling. In

4   particular, Shh signaling dictates the proliferation of granule cell precursors (GCP)

5   (1,2). GCPs give rise to granule neurons, the most abundant neuronal type in the brain.

6   In the developing cerebellum, GCPs receive mitotic signals from Shh ligands released

7   from the neighboring Purkinje cells to sustain its proliferation (1,2). Besides paracrine

8   Shh signaling, GCPs were also capable of self-regulated autocrine-induced cell

9   proliferation (3). Perturbation of Shh pathway activity during early embryonic or

10   postnatal development results in cerebellar dysplasia, hypoplasia as well as malignant

11   childhood brain tumor medulloblastoma (2,4–7). For example, genetic mutations of

12   Shh signaling components such as *Patched* (*PTCH), Smoothened* (*SMO*), *Gpr161* or

13   *Suppressor of Fused* (*SUFU*) are known to lead to the formation of medulloblastoma

14   (8–11)·(12).

15

16   In the past decade, emergence of primary cilium as an indispensable organelle for Shh

17   signal transduction has facilitated discoveries that recognized the seminal roles of

18   primary cilium in cerebellar development and medulloblastoma formation. The primary

19   cilium is a non-motile cilium found on the surface of nearly every cell. It functions

20   primarily as an "antenna'' on the cell membrane to receive and transduce extracellular

21   signals. In the Shh pathway, Shh ligand binds to the Ptch receptor to release its

22   suppression of Smo on the cell membrane. This subsequently triggers Smo and

23   cytosolic factors such as the Gli transcription factors and SuFu to interact within the

24   primary cilium before translocating into the nucleus to activate Shh downstream target

25   genes (13–15). Although the exact molecular mechanism and trafficking cargoes that

3

26 mediate dynamic ciliary entry and exit of Shh signaling components remain

27 incompletely understood, it has been well established that Shh signal transduction is

28 inevitably deregulated in the absence of a functional primary cilium. For instance,

29 knockout (KO) of genes known to be required for primary cilium formation (i.e. *Kif3a*

30 or *Ift88*) diminished Shh activities in the cerebellum and contributed to the

31 manifestation of cerebellar hypoplasia and distorted foliation due to substantial

32 shrinkage of granule cell precursors pool (16,17).

33

34 Intriguingly, recent findings have revealed that the primary cilium could exert both

35 inducing or suppressing forces on Shh pathway and cancer progression (15,18,19).

36 Depending on the pathogenic origin of the medulloblastoma, primary cilia could

37 potentiate tumor growth driven by Smo, and on the other hand, inhibit tumor growth

38 driven by Gli2 (18). Adding to the complexity of the tumor biology, the same study

39 also showed that there are ciliated and non-ciliated sub-categories of medulloblastoma,

40 with the ones bearing primary cilia often associated with increased Shh and Wnt

41 pathway activities, whereas those without cilia do not exhibit Shh or Wnt pathway

42 activation (18). Given the opposing functions of primary cilium on Shh pathway-

43 mediated tumor progression, as well as the heterogeneity in ciliation capacity among

44 the tumor cells; the multifaceted functions of primary cilium might underlies variable

45 patients' responses to Smo-specific drug, Vismodegib treatment in the clinical trials

46 that targets Shh-subtype medulloblastoma (20,21,22). Therefore, further insights on the

47 interaction between primary cilium and the Shh pathway, and their roles in GCP

48 proliferation would lay critical foundation for further development of effective

49 intervention for medulloblastoma.

50

4

51 Rab23 is a brain-enriched small GTPase (21) known to antagonize the Shh pathway *in*

52 *vivo*, as evidenced by developmental mouse genetic studies. In humans, mutations of

53 *RAB23* cause Carpenter syndrome, an autosomal recessive disorder characterized by

54 aberrant skull fusion, polydactyly and branchydactyly. Other variable developmental

55 abnormalities including heart defect, genu valgum, cornea defect, umbilical hernia,

56 obesity, developmental delay, as well as central nervous system (CNS)-related

57 conditions including cerebral and cerebellar malformations, hydrocephaly, intellectual

58 disability and schizophrenia (22–29). In mouse, the *Rab23*-encoding *open brain* (*opb*)

59 null allele mutant exhibited embryonic lethality at mid-gestation stage, exencephaly

60 and ectopic neural tube ventralization (30,31), which largely recapitulated the

61 phenotypes of other Shh repressor mutants such as *Patched1* (*Ptch1*) and *Suppressor*

62 *of fused (Sufu)* KOs (32–34). Nonetheless, owing to the early embryonic lethality of

63 *Rab23*-null mutant in mouse, true implications of Rab23 in Shh signaling-mediated

64 CNS development beyond the mid-gestation stage are not known.

65

66 Genetic study revealed that *Rab23* represses Hh activities via *Gli2* and promotes the

67 proteolytic cleavage of Gli3 into its cleaved repressor form (31). In addition, *Rab23*

68 also appeared to regulate Hh pathway activity through *Smo*. Concomittant deletion of

69 *Smo* in the *Rab23*-null mutant has partially weakened Shh activation level in the neural

70 tube as compared to that of *Rab23* mutant (31). Besides, a molecular study in

71 mammalian cell line model reported that Rab23 mediates the protein turnover dynamics

72 of Smo in the primary cilium, although it was not clear as to how this may influence

73 Shh pathway activity(35). Another *in vitro* study further revealed that Rab23

74 antagonizes the nuclear translocation of Gli1 transcription activator to impede Shh

75 pathway activation (36). Taken together, these findings suggest that Rab23 casts

5

76   multiple actions in the modulation of Hh signaling cascade. However, how it

77   orcheastrates Shh pathway in the context of GCP proliferation and medulloblastoma

78   formation remains to be determined.

79

80   Although independent studies have implicated the functions of *Rab23* in primary cilium

81   formation and ciliary trafficking, its role in ciliogenesis remains obscure due to

82   inconsistent observations from different cell types. For instance, overexpression of the

83   dominant-negative form, Rab23DN perturbed ciliation in the immortalized retinal

84   pigmented epithelial cells (37). Supporting this observation, a recent study has

85   identified that the GDP-GTP exchange factors (GEF) of Rab23 namely Inturned and

86   Fuzzy, were localized to the primary cilium at proximal end, and played essential role

87   in the primary cilia formation of human and mouse cells(38). The same study

88   demonstrated that depletion of GEF (i.e. Intu and Fuzz), or Rab23 perturbed primary

89   cilium formation in culture IMCD3 cells. On the contrary, $Rab23^{-/-}$ mouse embryo

90   showed unaltered node cilia during early development(39). Taken together, these data

91   suggest that Rab23's action in the primary cilium formation is possibly operating in a

92   context-dependent manner. In the IMCD3 cells that have morphologically normal

93   primary cilium, Rab23 forms protein complex with Kif17 and Dopamine receptor 1

94   (D1R), and it was required for their ciliary localization (40,41). These findings

95   indicated that Rab23 plays crucial roles in ciliary protein targeting. Despite the known

96   function of Rab23 in primary cilium formation and Hh signaling, as well as the long

97   perceived function of primary cilium-dependent Hh signaling in GCP proliferation,

98   whether Rab23 is required for the primary cilium formation in the CNS and cerebellar

99   GCP is not known. Moreover, how Rab23 may mediate primary cilium-dependent Shh

100   signal transduction, and its impact on GCP proliferation and cerebellar development

101   remain to be further characterized.

102

103   In this study, we demonstrate that conditional KO of *Rab23* in the developing mouse

104   brain at E10.5 resulted in abnormal cerebellar foliation, as well as unexpected opposing

105   changes in the cerebellar sizes and Shh activities during embryonic and postnatal

106   cerebellar development. Interestingly, our data suggest that loss of Rab23 did not casue

107   medulloblastoma despite an increase in the basal level of Shh pathway activities and

108   GCP proliferation. We found that KO of *Rab23* affected ciliation in GCP, and rendered

109   the cells less responsive to pathway activation by Shh and Smo agonist. These results

110   suggest that the *Rab23*-KO GCPs have an attenuated response to paracrine Shh stimuli

111   from primary cilium. Taken together, we have uncovered novel functions of Rab23 in

112   GCP proliferation, acting both positively and negatively via Shh signaling. Our results

113   indicate that Rab23 represses basal level of Shh signaling pathway activities, while

114   facilitates Smo-mediated Shh pathway activation in a primary cilium-dependent

115   manner.

116

117

118   **Results**

119

120   **Rab23 dictates proper cerebellar morphogenesis and development**

121   In order to investigate the functions of Rab23 in central nervous system (CNS)

122   development, mouse bearing Nestin-cre (Nes) was crossed with *Rab23-floxed* (42)

123   homozygous mutant to achieve conditional knock-out (CKO) of *Rab23* in the neural

124   progenitor cells at approximately embryonic (E) day 10.5. Gross morphological

125 examination of the whole brain isolated from Nes-CKO mutant revealed noticeable

126 cerebellar enlargement at earlier developmental stages (i.e. postnatal (P) day one and

127 four) but appeared smaller at later adult stage as compared to the control ($Rab23^{f/f}$)

128 counterpart (Fig. 1A-B, E, yellow asterisk). Histological examination of the mid-sagital

129 cerebellar sections by hematoxylin-eosin (H&E) staining revealed cerebellar dysplasia

130 in Nes-CKO brains. This was consistently observed at P1, P4 and adult stages (Fig. 1C-

131 D, F). Disrupted patterning of the cerebellar folia was more prominent at the caudal

132 region. Moreover, the external granular layer (EGL) at the posterior lobules appeared

133 thicker and disorganized as compared to the control group (Fig. 1C-D, red arrows). In

134 the adult mutant, the posterior cerebellar folia were irregularly formed and lack

135 distinctive laminar layering of molecular layer (ML), Purkinje cell layer (PCL) and

136 internal granule layer (IGL) (Fig 1F). Taken together, these data indicate that a loss of

137 *Rab23* resulted in defects in cerebellar folia patterning during postnatal CNS

138 development.

139

140 **Depletion of Rab23 disrupted cerebellar radial glial scaffold and innervations of**

141 **granule cells**

142 The disorganized laminar layering, as well as the cerebellar folia anomaly prompted

143 further examination of the Bergmann glial (Bg) scaffold, which acts as the

144 cytoarchitectural scaffold to aid in neuronal migration and lamination (43–45). We used

145 antibodies against Nestin and glial fibrillary acidic protein (GFAP) to immunolabel

146 radial glia and the Bg scaffold at early postnatal and later adult stages respectively. In

147 the $Rab23^{f/f}$ (control) group, radial fibers of Bg at P1, P4 and P15 appeared

148 perpendicularly aligned and extended from the cell bodies at the lower ML and PCL

149 towards the pial surface of the cerebellum. In contrast, the processes of Nes-CKO

8

150   mutant Bg in the disrupted lobules appeared tangled and misaligned, with some of them

151   unable to extend processes to the pial surface, thus indicating an impairment of the Bg

152   scaffold (Fig. 2A-C). Additionally, hyperplastic lesion-like ectopic nuclei accumulation

153   were detectable at the pial surface in P4 (Fig 2B, asterisk) and adult cerebellum (Fig.

154   2D, white arrows). In line with this defect, the NeuN-positive granule cells in the adult

155   mutant were aberrantly localized to the pial surface and the ML instead of the deeper

156   IGL. In addition, a subpopulation of the granule cells was randomly scattered at the

157   posterior region, concomitant with a loss of laminar structure. The cell soma of GFAP-

158   positive astrocytes/Bg were also found to be ectopically misplaced at the pial surface

159   and ML, indicating a misalignment of radial glial scaffold at the adult stage (Fig. 2D,

160   D', D"). These data indicate that an abnormal glial scaffold in Nes-CKO mutants may

161   hinder proper invagination and migration of granule cells to the deeper IGL during early

162   postnatal cerebellar development.

163

164   Given the defective radial glial scaffold and ectopic accumulation of granule cells at

165   the pial surface and ML, we asked if the inward radial migration of granule cell at the

166   earlier embryonic and postnatal stages was affected. Anti-Pax6 antibody was used to

167   immunolabel both amplifying granule cell precursors (GCP) transiently residing in the

168   EGL (source of granule cells), and the early inwardly migrated post-mitotic granule

169   cells in the granular layer at E15.5 (46). The Pax6-expressing granule cells in the

170   control group were more well-dispersed and scattered further into the deeper granule

171   layer. Conversely, *Rab23*-depleted granule cells appeared less dispersed and are largely

172   confined to the region adjacent to the EGL (Fig. 3A, yellow dotted lines). Because all

173   granule cells arise from the EGL and innervate the inner granular layer as they undergo

174   maturation, we quantified the innervation/migration rate by counting the number of

9

175   Pax6-positive cells that have populated the inner granular layer (innervated) against

176   total Pax6-positive cells. Indeed, the proportion of innervated granule cells in the Nes-

177   CKO mutant appeared markedly reduced compared to the control counterpart (Fig. 3B),

178   suggesting an impaired or delayed innervation. In addition, a two-hour EdU-pulse

179   labeling assay was used to track all early innervated progenitor cells. The proportion of

180   EdU-labelled cells that have innervated (from EGL – magenta arrows, and VZ – yellow

181   arrows) the granular layer was scored against all EdU-labelled cells. Similarly, *Rab23*-

182   depleted cells showed a lower percentage of innervated cells in the granular layer (Fig.

183   3C).

184

185   For the postnatal stage, we examined the migration of granule cells by 48 hours EdU-

186   pulse labelling for P5 to P7. The percentages of cell innervation in different lobules

187   were then analyzed by quantifying the percentages of EdU-labelled cells residing in the

188   EGL, ML and IGL of each lobule. In agreement with the results from the embryonic

189   stage (Fig. 3A-C), the proportions of mutant cells reaching IGL were greatly reduced,

190   concomitant with an increase in the percentage of cells that are accumulated in the EGL

191   (Fig. 3D-E). Taken together, these data suggest that deletion of *Rab23* caused a

192   misalignment of the radial glial scaffold, leading to perturbations in granule cells

193   innervation and lamination. As a result, the cerebellar laminae and folia could not be

194   properly formed during postnatal cerebellar development.

195

196   **Rab23-ablation caused thickened EGL and enhanced GCP proliferation, but no**

197   **discernible tumorigenesis**

198   In view of the thickened EGL observed in H&E staining, as well as the enlarged

199   cerebellum of Nes-CKO at P1 and P4, a more detailed analysis of cell proliferation is

200   warranted. We performed co-immunolabeling of Pax6 and Calbindin to visualized GCP

201   and Purkinje cells respectively. At P1, there was an overall increase in the number of

202   Pax6-expressing GCPs in the Nes-CKO mutant compared to the control. Besides, the

203   Pax6-labelled EGL in Nes-CKO appeared greatly thickened, more so near the posterior

204   folia (Fig. 4A, white asterisks). On the other hand, the Calbindin-expressing Purkinje

205   cells in the mutant PCL appeared to be lower in density and more sparsely distributed

206   as compared to the more densely aligned Purkinje cell layer in the control counterpart

207   (Fig. 4B), implying a perturbed PCL lamination.

208

209   Two hours EdU-pulse labelling was performed on E15.5 and P4 animals to probe GCP

210   proliferation in further details. Compared to the control, the pools of EdU-positive

211   proliferative cells are substantially expanded in the EGL of Nes-CKO mutant at both

212   time points (Fig. 4C-D, red and yellow double heads arrows), indicating aberrantly

213   enhanced GCP proliferation during both embryonic and postnatal cerebellar

214   development. These phenotypes were further confirmed by a quantification of EdU-

215   positive nuclei in the EGL at E15.5, which revealed a significant up-regulation of

216   proliferative cells in the mutant EGL as compared to the control group (Fig. 4E).

217   Accordingly, another cell proliferation marker, *Ki67*, and a GCP-specific marker *Atoh1*,

218   also showed markedly elevated expression levels in the cerebellar tissue of Nes-CKO

219   mutants (Fig. 4F). Taken together, these data suggest that depletion of *Rab23*

220   potentiated GCP proliferation during early cerebellar development. Excessive GCP

221   proliferation often give rise to medulloblastoma (47)'(48,49). Given this, one would

222   expect the development of medulloblastoma at a later postnatal stage. However, we did

223   not find detectable manifestation of medulloblastoma in adult mutant animals, despite

224   the occurrence of hyperplastic lesions-like tissue clumps at P4 (Fig. 1F, 2B-asterisk,

225   D-white arrows).

226

227   **Shh signaling is differentially perturbed in the developing cerebellum**

228   Previous genetic studies have reported that *Rab23* negatively regulates Shh signaling

229   (30,50). As Shh signaling is the key signaling pathway that modulates GCP

230   proliferation (1,2,51,52), we reasoned that it is likely a main factor driving aberrant

231   GCP proliferation in the *Rab23*-deficient cerebellum. To address this possibility, we

232   examined the expressions of *Gli* transcription factors, which are downstream effectors

233   of Shh signaling in CNS development. The Shh signaling activities in cerebellar tissue

234   were examined in both embryonic E15.5 and late postnatal P15. In accordance with the

235   increased CGP proliferation in Nes-CKO at E15.5 and P4, Shh signaling pathway

236   activities were robustly up-regulated at E15.5, as shown by an increase in *Gli1* and *Gli2*

237   expressions compared to the control group (Fig. 5A). Intriguingly, at P15, the

238   expression level of *Gli1* transcripts was significantly down-regulated in the Nes-CKO

239   mutant cerebellum, despite up-regulated levels of *Gli2, Gli3, Ki67* and *Atoh1* (Fig. 5B).

240   Because *Gli1* activates Shh-regulated genes, and its expression is dependent on both

241   *Gli2* and *Gli3*, it could serve as the ultimate readout of Shh signaling pathway activitity

242   (C Brian Bai & Joyner, 2001; C Brian Bai, Stephen, & Joyner, 2004; Lee, Platt,

243   Censullo, & Ruiz i Altaba, 1997). We also compared the expression profile of *Gli1*

244   transcripts at embryonic and postnatal stages. Compared to the control group which

245   exhibited relatively unaltered *Gli1* transcript level between E15.5 and P15, the Nes-

246   CKO mutant showed a significant reduction in *Gli1* transcript level at P15 compared to

247   its embryonic stage (Fig. 5C). Given the perturbed Shh signaling pathway activities, we

248   further examined if the *Shh* transcripts were affected. Interestingly, *Shh* transcripts level

249   in the Nes-CKO appeared largely unchanged at both developmental time points (Fig.

250   5A-B), implying that the mutant cerebellar tissues are not short of Shh stimulants

251   despite the greatly mis-patterned cerebellum. Taken together, these data show that the

252   Shh signaling activities in the Nes-CKO mutants were initially enhanced during

253   embryonic cerebellar patterning, but became down-regulated at later postnatal time

254   point as compared to the control counterpart. Notably, this correlated well with the

255   differential changes in the cerebellar size as aforementioned (Fig. 1A-B, E). Together,

256   these results revealed that Shh signaling pathway activities were differentially

257   perturbed as a result of Rab23 deficiency during embryonic and postnatal stages of

258   cerebellar patterning.

259

260   **Rab23 could regulate Shh signaling in the GCP at basal level as well as in a cilium-**

261   **dependent manner**

262   The alterations of Shh signaling pathway activities on GCP in the whole cerebellar

263   tissues could be a secondary effect resulting from abnormal changes in the

264   cellular/tissue composition. We ruled out this possibility by monitoring Shh signaling

265   in primary GCP culture isolated from P7 cerebellar tissue. Primary culture data show

266   that *Rab23*-KO GCP indeed exhibited elevated expressions of *Gli1* mRNA at the basal

267   level, indicating an over-activation of Shh pathways in the Nes-Cre mutant GCP.

268   Accordingly, primary culture of mutant GCP also displayed potentiated cell

269   proliferation, as illustrated by the up-regulation of *Atoh1* and *Ki67*, as well as an

270   increase in the percentage of EdU-positive proliferative cells (Fig. 6A-C). Furthermore,

271   Shh pathway over-activation and cell proliferation were significantly inhibited by co-

272   expressing *Rab23* wild-type cDNA, or its constitutive active form, *Rab23QL*, in the KO

273   GCP (Fig. 6A-C), suggesting that the effects observed were indeed due to the loss-of

274   *Rab23* gene functions. Together, these results suggest a negative role for *Rab23* in

275   regulating Shh signaling-mediated GCP proliferation.

276

277   Previous findings have demonstrated that primary cilium is required for Shh signaling-

278   mediated CGP proliferation during cerebellar development (16,17). Rab23 has also

279   been reported to be involved in ciliogenesis and ciliary signaling in other cell types

280   (40,41,56). Therefore, it is conceivable that the impact of Rab23 on GCP proliferation

281   was exerted through changes to the primary cilia. We examined primary cilia

282   morphology in the E15.5 and P15 cerebellar GCP by immunolabeling of Arl13b, a

283   primary cilium-specific marker. Interestingly, Arl13b immunostaining showed that the

284   *Rab23*-depleted GCPs exhibited a significantly reduced number of cells bearing

285   primary cilium, whereas nearly all GCPs in the control counterpart showed positive

286   staining of Arl13b (Fig. 6E). This finding is further strengthened by the analysis of

287   primary cilia in primary GCP culture isolated from P7 cerebellar tissues, whereby the

288   *Rab23*-deleted GCPs in culture similarly displayed a significant reduction of ciliation,

289   which could be reversed by co-expressing *Rab23* wild-type cDNA, or the constitutive

290   active *Rab23QL* (Fig 6D). Taken together, our data provide the first indication that

291   Rab23 influences ciliogenesis in the cerebellar GCP *in vivo*. Importantly, these results

292   also hinted at a novel cilium-dependent role of Rab23 in coordinating Shh pathway and

293   GCP proliferation.

294

295   Given the perturbations in primary cilia morphology, we hypothesized that *Rab23*-KO

296   GCP may be compromised in primary cilium-dependent Shh signal transduction. In

297   order to address this hypothesis, primary GCP cultures were subjected to Shh ligand

298   stimulation *in vitro.* Despite its higher basal level of Shh signaling activities as

299   compared to control (Fig. 6A), *Rab23*-KO GCP showed markedly weaker response to

300   Shh ligand stimulation, as illustrated by a lower fold-increase in the expression of *Gli1*

301   mRNA as compared to the control counterpart (Fig. 6F). *Rab23*-KO GCPs also

302   exhibited lower fold-enhancement in cell division as compared to the control group, in

303   which the expression of *Ki67,* as well as *Atoh1* were both significantly lower than

304   control group upon Shh ligand stimulation (Fig. 6F). To check if the Shh signaling

305   occurs through Smo, GCP cultures were treated with a Smo agonist that promotes its

306   localization to the cilium. Control GCPs exhibited robust elevation *Gli1* expression 24

307   hours after SAG treatment (Fig. 6G). Conversely, *Rab23*-KO GCPs' response to SAG

308   induction was significantly compromised, as shown by lower expression level of *Gli1*

309   (Fig. 6G)*, thus implying a desensitization to Shh signaling at the level of primary cilium

310   and Smo. Together, these data demonstrated that silencing *Rab23* impaired ciliation

311   and GCP's response to Shh or Smo stimulations, thereby impeding Shh-mediated GCP

312   proliferation. These suggest a novel positive role of Rab23 in modulating primary

313   cilium-dependent Shh signaling and GCP expansion during early cerebellar

314   development.

315

316   **Discussion**

317

318   Fine tuning Shh signaling during cerebellar development is essential to facilitate a

319   temporally and spatially-defined transit amplification of granule cell precursors (GCP)

320   to ensure proper patterning and growth of the cerebellar folia (2,57–59). We have

321   demonstrated here that *Rab23* has a role in the patterning and growth of cerebellar folia

322   during early cerebellar development. Deletion of *Rab23* resulted in foliation anomalies

323   due to dramatically perturbed radial glial scaffold formation, granule cells lamination

324 and GCP proliferation. This study presents *Rab23* as a novel regulator of GCP

325 proliferation, remarkably, acting both positively and negatively via the Shh signaling

326 pathway. Excitingly, our data showed for the first time that Rab23 has a role in primary

327 cilium-dependent Shh signal transduction during cerebellar development. This

328 demonstration is made possible as the brain-specific KO of *Rab23* in our genetic model

329 did not result in mid-gestation lethality in mice as compared to a global loss of *Rab23*

330 in the *open brain* mutant.

331

332 Previous examination of primary cilia in the node of 2 to 6 somite stage *Rab23*-null

333 embryo reported largely unaltered morphology and similar overall percentage of

334 ciliation as compared to the control (39). Interestingly, unlike the node cilia, our data

335 revealed defective ciliation in the *Rab23*-null GCP during embryonic and early

336 postnatal cerebellar development. siRNA-mediated knockdown studies performed on

337 different cell lines have reported inconsistent conclusions with regards to the role of

338 Rab23 in ciliogenesis (40,41,56,60). These discrepancies suggest that the functions of

339 *Rab23* in primary cilia could vary in a context or cell-type specific manner. Our data

340 supported a GCP-specific role of Rab23 in ciliogenesis *in vivo*. In line with the *in vivo*

341 data, primary culture of *Rab23*-KO GCPs also showed deficiencies in ciliation and

342 compromised response to Shh ligand and SAG-mediated Smo activation, implicating a

343 disrupted primary cilium-dependent Shh signaling.

344

345 Mutations of Shh pathway repressor genes, including *Ptch1, Gpr161* and *Sufu,*

346 commonly lead to the development of medulloblastoma(9,10,61,62) via *Gli1*

347 upregulation (63). We showed that loss of *Rab23*, unlike other Shh repressors, did not

348 promote the development of medulloblastoma despite the basal level up-regulation of

16

349   *Gli1* expression in the GCPs. We further showed that the overall amount of Shh ligands

350   in *Rab23*-deleted cerebellar tissues remained relatively similar to that of control at both

351   embryonic and postnatal stages, suggesting a sufficient source of Shh stimulants in the

352   KO cerebellar tissue environment. Given the above, we deduce that one possible

353   explanation for an absence of tumorigenicity is the defective primary cilium in *Rab23*-

354   KO GCPs. The compromised response to primary cilium-dependent Shh activation may

355   lead to insufficient paracrine pathway stimulations to drive tumor formation in the

356   *Rab23*-KO cerebellum. This is in line with the indispensable role of primary cilium for

357   medulloblastoma formation (18,64).

358

359   Harboring the primary cilium defect in GCP, *Rab23*-KO cerebellum partially

360   phenocopied other ciliopathy mutants, which often exhibit severe cerebellar size

361   shrinkage, abnormal foliation and reduced GCP proliferation due to impaired Shh

362   signaling (16–18,64). In this regard, the postnatal Nes-CKO displayed profoundly mis-

363   patterned folia, and smaller cerebellum at later adult stage, similar to other ciliopathy

364   mutants. Nevertheless, in contrast to most ciliopathy mutants, Shh signaling in the

365   *Rab23*-KO mutant was not completely inhibited. Instead, there was a ligand-

366   independent upregulation of Shh pathway at basal level, which underlies the increase

367   in GCP proliferation and transiently enlarged cerebellum at earlier postnatal stages.

368   *Rab23* was known to influence *Gli2 and Gli3* expression at the transcript level (65) and

369   it could also antagonize Gli1's nuclear translocation and transcriptional activation in

370   cytosolic compartment in the absence of ligand stimulation (36). Given the ligand-

371   independent function of Rab23 in Shh pathway, it is therefore plausible that the Shh

372   pathway in GCP became over-activated due to a basal increase in Gli activations in the

373   absence of Rab23 function. However, limited by the incompetency to respond to Smo

17

374   activation from cell/cilium membrane (Fig 6F-G), Rab23-deficeint GCP could not

375   reach or sustain the full capacity of ectopic Shh pathway activation, causing them less

376   susceptible to tumor formation as compared to other repressors such as *Ptch1* and *Sufu*

377   mutants that are not known to exhibit primary cilium defect.

378

379   Previous work has demonstrated that Rab23 maintains the overexpressed-Smo protein

380   turnover in the primary cilium of MDCK cells upon Shh stimulation (66), however, the

381   underlying mechanism, and how this regulation would affect Shh signaling output

382   remain elusive. Our data show that Rab23-depleted GCPs were less responsive to a

383   Smo agonist (SAG). As SAG activates Shh signaling pathway by facilitating Smo

384   translocation to the cilium axoneme, the compromised response observed in mutant

385   cells could possibly cause by the lack of intact and functional primary cilium for Smo-

386   mediated signaling transduction, and/or impaired maintenance of Smo turnover in the

387   primary cilium of *Rab23*-KO GCP. Our data suggest that the cilium malformation could

388   be the underlying reason. However, the relatively short cilia in GCP cells was

389   technically difficult for detection or quantification of the cilium localization of Smo in

390   primary GCP. We are therefore not able to ascertain if the ciliary turnover/trafficking

391   of Smo protein is affected in the mutant GCP. Nevertheless, given its previous

392   implicated role on Smo protein turnover in MDCK cells (66), Rab23 could potentially

393   mediate the Smo recycling in primary cilium to influence Shh pathway.

394

395   Taken together, our findings suggest that Rab23 confers dual functions in regulating

396   Shh signaling and GCP proliferation; it potentiates primary cilium and Shh/Smo-

397   dependent signaling cascade, while antagonizing basal level Gli transcriptional

398   activation. Our data thus present a previously unappreciated aspect of Rab23 in

399   mediating Shh signaling upstream of Smo. This study sheds new light into the genetic

400   and mechanistic insights underpinning Shh signaling-mediated GCP proliferation and

401   cerebellar development.

402

403   **Materials and Methods**

404

405   ***Animals.*** *Rab23-floxed* animal was generated by Ozgene Pty Ltd. Conditional *Rab23-*

406   *floxed* allele was designed by flanking exon 4 for *Rab23* gene with loxP sites. Nestin-

407   Cre (Jackson Lab cat. no. 003771) was a kind gift from Shawn Je H.S. form Duke-Nus

408   Medical School. All animals were housed in Specific Pathogen Free (SPF) animal

409   facility at Duke-NUS Medical School, Singapore. All animal related procedures were

410   carried out in compliance to animal handling guidelines and protocol approved by

411   IACUC Singhealth, Singapore.

412

413   ***Expression vectors.*** For *in vitro* viral transduction assay, Rab23 over-expression or

414   cDNA were cloned into lentiviral pFUGW backbone. Wild-type (WT) Rab23

415   overexpression construct, previously described full-length Rab23 sequence (67) was

416   sub-cloned into pFUGW vector driven by Ubc promoter. All plasmids were amplified

417   according to the recommended protocol using Endofree® plasmid purification kit

418   (Qiagen, Germany).

419   ***Viral transduction and culturing of mouse primary GCP.*** For viral transduction of

420   primary GCP, self-inactivating murine lentiviruses were prepared according to

421   previously described protocol (42). GCP culture method was modified from standard

422   protocol. Briefly, P7 cerebellar tissues dissected were cut into small pieces and digested

423   in digestion buffer (EBSS / Papain, 1000 times dilution factor (Worthington

424   Biochemical Corporation cat#:3126) / 0.1 mg/ml DNaseI (Roche cat#:11284932001) /

425   5.5 mM cysteine-HCL) for 15 mins at 37 °C prior to dissociation into single cells.

426   Digestion was terminated by resuspension in 10 % FBS/culture medium. Suspension

427   culture was passed through 70 μm cell strainer (Corning cat#352350) to remove

428   undigested tissue clumps. Dissociated single-cell GCPs were plated on poly-D-lysine

429   (Sigma Aldrich cat#: P6407) coated culture plates at the desired cell densities in

430   Neurobasal (Gibco®, Life Technologies, USA) medium containing B27 supplement,

431   200uM GlutaMAX^{TM}-I (Gibco®, Life Technologies, USA), sodium pyruvate (1 mM),

432   penicilin/streptomycin and KCl (250 μM). Half of the culture medium was refreshed

433   every other day. Viral transduction was performed 2 to 3 hours after culture while

434   replacing fresh culture medium. The efficiencies of overexpression were validated by

435   real-time QPCR assay of DIV7 culture. For SAG stimulation, 0.2 μM of SAG (Cayman

436   Chemical, cat#: 11914-1) was added to the DIV 1 culture 24 hours prior to total RNA

437   extraction. Equal volume of DMSO was added as the untreated negative control group.

438   For Shh stimulation, 2 μg/ml of Shh (Stem Cell Technologies, cat#: 78065) was added

439   to the DIV 1 culture 24 hours prior to total RNA extraction.

440

441   ***EdU-pulse labelling assays***. EdU labeling assay was carried out according to the

442   manufacturer's protocol. Click-iT ® EdU Alexa Fluor^{TM} 647 Imaging Kit

443   (ThermoFisher Scientific, cat #: C10340). For GCP culture labeling, 10 μM Edu was

444   added to the culture and incubated for 3 hours before fixation. For E15.5 embryos

445   labelling, 0.25 mg EdU was injected intraperitoneally into the pregnant mice 2 hours

446   before fixing the embryo. For postnatal animals, 25ug EdU was injected

447   subcutaneously 2 hours prior to brain fixation.

448

20

449 ***Cryosectioning, immunohistochemistry and imaging***. Mice were perfused with saline

450 followed by fixative in 4 % paraformaldehyde (Sigma Aldrich cat#:P6148) /

451 HistoChoice (Amresco, cat#: H120) mixture of 1:1 ratio, and whole brains extracted

452 were post-fix at 4 °C for 2 hours, saturated in 30 % sucrose in 0.12 M phosphate buffer

453 and subjected to cryosection at 20 μm thickness. All cerebellar tissues were sectioned

454 at sagital angle and mounted on pre-coated glass slides (Superfrost® Plus,

455 Fisherbrand®). Mid-sagital sections were selected for immunostaining. Antibodies and

456 the dilution factor used were: Pax6 (Covance, 1:1000), Nestin (Sigma, 1:800), NeuN

457 (Milipore, 1:800), GFAP (Milipore, 1:1000), Arl13b (Proteintech, 1:1000). For histo-

458 immunostaining, tissue sections were incubated at 100 °C for 10 mins in pH 6 10 mM

459 sodium citrate buffer with 0.05 % Tween-20 for antigen retrieval, washed twice with

460 phosphate buffer saline (PBS), blocked 1 hour in 1 % BSA/2 % horse serum/0.3 % Tx-

461 100 and incubated 4°C overnight with primary antibodies diluted in blocking buffer.

462 After 3 times of 5 minutes washes with PBS, tissue sections were incubated with

463 secondary antibodies (Alexa Fluor®, Life Technologies, USA) for 1 hour (hr) at room

464 temperature. Tissue sections were mounted in mounting media after 3 times PBS

465 washes. Fluorescence images were taken using Zeiss LSM710 confocal system.

466

467 ***Real-time quantitative PCR.*** Total RNA was extracted using Qiagen's RNeasy Mini

468 Kit. Equal amount of total RNAs from each sample were subjected to reverse

469 transcription to produce cDNA. Equal volume of cDNA was used to perform

470 quantitative PCR assay using SYBR® Select Master Mix (Applied Biosystems^{TM}

471 #4472908). Standard QPCR protocol was carried out according to manufacturer's

472 instruction manual. Primers used were: mouse GAPDH: F-5'-

473 TTCACCACCATGGAGAAGGC-3', R-5'- GGCATGGACTGTGGTCATGA-3';

21

474   mouse   *Rab23*:   F-5'-AGGCCTACTATCGAGGAGCC-3',   R-5'-

475   TTAGCCTTTTGGCCAGTCCC-3';   mouse   *Gli1*:   F-5'-

476   CCCATAGGGTCTCGGGGTCTCAAAC-3',   R-5'-

477   GGAGGACCTGCGGCTGACTGTGTA

478   A-3';   mouse   *Gli2*:   F-5'-CATGGTATCCCTAGCTCCTC-3',   R-5'-

479   GATGGCATCAAAGTCAATCT-3';   mouse   *Gli3*:   F-5'-

480   CATGAACAGCCCTTTAAGAC-3',   R-5'-TGATATGTGAGGTAGCACCA-3';

481   mouse   *Ptch1*:   F-5'-TGCTGTGCCTGTGGTCATCCTGATT-3',   R-5'-

482   CAGAGCGAGCATAGCCCTGTGGTTC-3';   mouse   *Atoh1*:   F-5'-

483   AGTCAATGAAGTTGTTTCCC-3',   R-5'-ACAGATACTCTTATCTGCCC-3';

484   mouse   *Ki67*:   F-5'-CATTGACCGCTCCTTTAGGTATGAAG-3',   R-5'-

485   TTGGTATCTTGACCTTCCCCATCAG-3'.

486

Acknowledgement

496

497

Figure legends

499

Figure 1. Nestin-Cre-driven knock-out of *Rab23* causes expanded cerebellar size and abnormal foliation.

A) Representative whole mount images of control and Nes-CKO mutant brains showing gross morphology of mouse cerebellum at P1, P4 and P15.

B) P4 cerebellar sizes as determined by measuring 2D surface area of cerebellum on images captured at similar angle. Control, n = 6; CKO, n = 4 Statistical significance, unpaired student t-test. P value ** ≤ 0.01. Error bars depict ±SE

C-D) Representative images showing H&E staining of control and Nes-CKO cerebellar sagital sections of P1 (C) and P4 (D) animals. Red arrows highlight morphological changes in the external granule layer of Nes-CKO compared to the control.

E) Representative image showing whole brain of 2 months adult mice. Yellow asterisk shows smaller cerebellum of Nes-CKO mutant compared to the control.

F) Representative images showing H&E staining of sagital cerebellar sections of 10 months adult mice.

514

Figure 2. KO of *Rab23* perturbs radial glial scaffold formation and causes partial loss of cerebellar laminar structure

A-B) Representative images showing immunostaining of Nestin (red) on P1 (A) and P4 (B) sagital cerebellar tissue sections to illustrate radial glial scaffold.

C) Representative images showing immunostaining of GFAP (red) on P15 sagital cerebellar tissue sections to illustrate radial glial scaffold.

D) Representative images showing co-immunostaining of NeuN and GFAP on ten months adult cerebellum to illustrate cerebellar cytoarchitecture, laminar layers and glial cells.

23

524   D'-D") Close up images showing NeuN-positive granule neurons and GFAP-positive

525   glial cells at the internal granule layer and pial surface.

526

527   **Figure 3. Depletion of Rab23 leads to GCP migration defect**

528   A) Representative images showing immunostaining of Pax6 on E15.5 sagital sections

529   of cerebellar primordium to illustrate the GCPs residing in the EGL and early inward

530   migrating GCPs. White arrows show inward migration paths. EGL, external granular

531   layer; RL, rhombic lip

532   B) Quantification of the proportion of innervated Pax6+ GCPs against all Pax6-labelled

533   GCPs. 2 to 3 sections (~50-100 μm apart) of the cerebellar primordium were counted

534   for each animal. Control, n = 3; CKO, n = 3. Statistical significance, unpaired student

535   t-test. P value * ≤ 0.05. Error bars depict ±SE

536   C) Representative image and graph showing two-hours EdU labelled progenitors in the

537   cerebellar primordium. Magenta arrows show migration paths of progenitors from

538   EGL, yellow arrows show migration paths of progenitors from VL. Control, n = 3;

539   CKO, n = 3. 2 to 3 sections (~50-100 μm apart) were counted for each animal. Statistical

540   significance, unpaired student t-test. P value *** ≤ 0.001. Error bars depict ±SE

541   D) Representative images showing cerebellar lobules of 48 hours Edu-labelled cells

542   from P5-P7 to illustrate proportions of cells innervated the IGL after 48 hours of pulse-

543   chase labeling. EGL, external granular layer; ML, molecular layer; ICL, internal

544   granule layer.

545   E) Quantification of the proportion of Edu-labelled cells in each laminar layer as

546   indicated. Control, n = 3; CKO, n = 3. Statistical significance, two-way ANOVA,

547   Bonferroni posttests. P value *** ≤ 0.001, **≤ 0.01, *≤ 0.05. Error bars depict ±SE

548

549   **Figure 4. *Rab23*-deficient cerebellum exhibited thickened EGL and elevated GCP**

550   **proliferation**

551   A-B) Representative images showing co-immunostaining of Pax6 (green) and

552   Calbindin (red) on P1 sagital sections of cerebellum to illustrate the GCPs and Purkinje

553   cells layers. Asterisks show a thickened EGL layer in Nes-CKO cerebellum compared

554   to the control.

555   C-D) Representative images showing two-hours EdU labelled dividing progenitors in

556   the E15.5 (C) and P4 (D) cerebellum. Double headed arrows highlight expanded pools

557   of dividing cells in the Nes-CKO EGL as compared to the control counterparts.

558   E) Quantification of the percentages of 2 hours Edu-labelled proliferative cells in the

559   EGL at E15.5. 2 sections (~100 μm apart) of the cerebellar primordium were counted

560   for each animal. Control, n = 3; CKO n = 3. Statistical significance, unpaired student t-

561   test. P value *** ≤ 0.001. Error bars depict ±SE

562   F) Graphs illustrating the fold change of the gene expression levels of E15.5 cerebellar

563   tissues quantified by real-time quantitative PCR. Control, n = 4; CKO n = 4. Nes-CKO

564   values were normalized to its respective control group. Statistical significance, unpaired

565   student t-test. P value *** ≤ 0.001. Error bars depict ±SE

566

567   **Figure 5. Shh activity is differentially perturbed in the embryonic and postnatal**

568   **cerebellar tissues.**

569   A) Graphs illustrating the fold change of the gene expression levels of E15.5 cerebellar

570   tissues quantified by real-time quantitative PCR. Control, n = 4; CKO n = 4. Nes-CKO

571   values were normalized to its respective control group. Statistical significance, unpaired

572   student t-test. P value ** ≤ 0.01. Error bars depict ±SE. n.s., not significant.

25

573   B) Graphs illustrating the fold change of the gene expression levels of P15 cerebellar

574   tissues quantified by real-time quantitative PCR. Nes-CKO values were normalized to

575   its respective control group. Control, n = 3; CKO n = 4. Statistical significance,

576   unpaired student t-test. P value *** ≤ 0.001. Error bars depict ±SE. n.s., not significant.

577   C) Graphs illustrating the basal level *Gli1* expression profiles of E15.5 and P15

578   cerebellar tissues quantified by real-time quantitative PCR. E15.5 Control, n = 4; CKO

579   n = 4; P15 Control, n = 3; CKO n = 4. Statistical significance, one-way AVONA,

580   Bonferroni's Multiple Comparison Test. P value *** ≤ 0.0001. Error bars depict ±SE.

581   n.s., not significant.

582

583   **Figure 6. *Rab23* regulates ciliogenesis and Shh signaling in the GCPs.**

584   A) Graphs showing gene expression levels of P7 GCPs primary cultures harvested at

585   DIV 7. Lentiviral carrying the over-expression constructs as indicated were transduced

586   into the primary cultures at Day 0, 2 to 3 hours after seeding cells. Quantifications

587   depict 4 independent experiments. Statistical significance, one-way AVONA,

588   Bonferroni's Multiple Comparison Test. P value *** ≤ 0.0001; **≤ 0.01, *≤ 0.05. Error

589   bars depict ±SE.

590   B-C) Representative images (B) and graph (C) showing three-hours EdU labelled

591   (magenta; blue: DAPI) dividing progenitors in P7 GCPs primary cultures of each

592   indicated groups fixed at DIV 7. Cell proliferation was determined by the percentages

593   of Edu-labelled cells out of total number of DAP-positive nuclei in each image taken.

594   For quantification of each batch, 3 fluorescence images were randomly taken from each

595   respective group as indicated. Quantifications depict 3 independent experiments.

596   Statistical significance, one-way AVONA, Bonferroni's Multiple Comparison Test. P

597   value *** ≤ 0.0001; **≤ 0.01. Error bars depict ±SE.

598   D) Quantification of the percentages of ciliation in P7 GCPs primary cultures at DIV7

599   determined by counting the number of cells bearing Arl13B-labelled primary cilium

600   against all DAPI-positive nuclei in each image taken. For quantification of each batch,

601   3 fluorescence images were randomly taken from each respective group as indicated.

602   Quantifications depict 3 independent experiments. Statistical significance, one-way

603   AVONA, Bonferroni's Multiple Comparison Test. P value *** ≤ 0.0001; **≤ 0.01.

604   Error bars depict ±SE.

605   E) Representative images showing immunostaining of Arl13B on E15.5 (top) and P15

606   (bottom) sagital sections to illustrate the primary cilia of GCPs residing in the EGL.

607   EGL, external granular layer; ML, molecular layer.

608   F-G) Graphs showing gene expression levels of P7 GCPs primary cultures treated with

609   Shh (F) and SAG (G) on DIV 1 respectively. Total RNAs were extracted from DIV 2

610   culture, 24 hours after the respective treatments. Quantifications depict double delta Ct

611   values of 3 independent experiments. Delta Ct values of the treated groups were

612   normalized to its respective untreated group, which gave double delta Ct values as

613   plotted. Statistical significance, unpaired student t-test. P value *** ≤ 0.0001; **≤ 0.01.

614   Error bars depict ±SE.

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Figure 1

Figure 2

Figure 3

Figure 4

A Control / Nes-CKO — Pax6 (green), Calbindin (red); lower panels Pax6, Calbindin, DAPI. 100 µm

B Control / Nes-CKO — Calbindin (red), DAPI (blue)

C Control / Nes-CKO — Edu, DAPI; EGL, RL; E15.5; 50 µm

D Control / Nes-CKO — Edu, DAPI; P4; 200 µm

E E15.5 — % of Edu +ve cells in EGL; Control, Nes-CKO; ***

F ki67, Atoh1, Rab23 — Fold change; Control, Nes-CKO; ***

Figure 5

A E15.5

B P15

C

Figure 6

Figure 7