

1 Triple A patient cells suffering from mitotic defects fail to localize PGRMC1 to mitotic kinetochore 2 fibers

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15 **Running head:** Cell division in ALADIN mutated cells

16
17 **Abbreviations:** ALADIN, alacrima-achalasia-adrenal insufficiency neurologic disorder; CYP, cytochrome
18 P450; ER, endoplasmic reticulum; GFP, green fluorescent protein; NADPH, Nicotinamide adenine
19 dinucleotide phosphate; NUP, nucleoporin; PGRMC1 and 2, progesterone receptor membrane component 1
20 and 2

21
22 **Keywords:** ALADIN, kinetochore fibers, mitosis, nuclear pore complex, PGRMC1, PGRMC2, triple A
23 syndrome.

24 HIGHLIGHT SUMMARY

- 25 • Investigating cell division in human adrenal cells we show that proliferation is decreased upon over-
26 expression of ALADIN, PGRMC1 or PGRMC2.
- 27 • In immunofluorescence experiments using human adrenal cells and triple A patient fibroblasts we
28 observed that during cell division PGRMC1 localizes to the microtubule kinetochore-fibers in
29 metaphase and to the mid-body in telophase.
- 30 • Depletion of ALADIN results in mis-localization of Aurora A and PGRMC1 in metaphase cells of
31 the human adrenal cell line and fibroblasts derived from patients with triple A syndrome.
- 32 • In real time PCR using RNA of fibroblasts of triple A syndrome patients and healthy controls we
33 measured an increased expression of PGRMC2 in cells with ALADIN mis-function compared to the
34 control cells.
- 35 • We hypothesize that a loss of the regulatory interaction between ALADIN and PGRMC2 leads to an
36 over-regulation and over-expression of PGRMC2 and displaces PGRMC1 at the metaphase spindle.
37 This diminishing of PGRMC1 concentration at kinetochore fibers may lead to mitotic errors and pro-
38 liferation arrest.

40 ABSTRACT

41 Membrane-associated progesterone receptors are restricted to the endoplasmic reticulum and are shown to
42 regulate the activity of cytochrome P450 enzymes which are involved in steroidogenesis or drug
43 detoxification. PGRMC1 and PGRMC2 belong to this group of microsomal receptors and are of interest due
44 to their suspected role during cell cycle. PGRMC1 and PGRMC2 are thought to bind to each other thereby
45 suppressing entry into mitosis. We could previously report that PGRMC2 interacts with the nucleoporin
46 ALADIN which when mutated results in the autosomal recessive disorder triple A syndrome. ALADIN is a
47 novel regulator of mitotic controller Aurora kinase A and depletion of this nucleoporin leads to microtubule
48 instability. In the current study, we present that proliferation is decreased when ALADIN, PGRMC1 or
49 PGRMC2 are over-expressed. Furthermore, we find that depletion of ALADIN results in mis-localization of
50 Aurora kinase A and PGRMC1 in metaphase cells. Additionally, PGRMC2 is over-expressed in triple A
51 patient fibroblasts. Our results emphasize the possibility that loss of the regulatory interaction between
52 ALADIN and PGRMC2 gives rise to a depletion of PGRMC1 at kinetochore fibers and to mitotic errors.
53 This observation may explain part of the symptoms seen in triple A syndrome patients.

54 INTRODUCTION

55 ALADIN is a scaffold nucleoporin (NUP) anchored within the nuclear pore complex by the transmembrane
56 NUP NDC1 (Kind *et al.*, 2009; Yamazumi *et al.*, 2009). ALADIN seems to be involved in building the
57 structural scaffold backbone of the nuclear pore complex (Rabut *et al.*, 2004). Over the last years it has been
58 shown that nuclear pore complexes and its NUPs have fundamental functions beyond nucleo-cytoplasmic
59 transport and control cellular dysfunction in a variety of cellular pathways, especially during mitosis
60 (Fahrenkrog, 2014; Nofrini *et al.*, 2016; Sakuma and D'Angelo, 2017). The first report that ALADIN plays a
61 role during cell division was published in 2015 (Carvalhal *et al.*, 2015). In cooperation with Carvalhal *et al.*
62 we proposed ALADIN as novel regulator of mitotic kinase Aurora kinase A and showed that depletion of the
63 nucleoporin resulted in impaired mitotic spindle assembly and chromosomal alignment at the metaphase
64 plate (Carvalhal *et al.*, 2015). Furthermore, we could recently document that ALADIN is necessary for
65 murine oocyte maturation and for specific stages during meiosis (Carvalhal *et al.*, 2017).

66 Mutations in the human *AAAS* gene, coding for the protein ALADIN, lead to the autosomal recessive
67 disorder named triple A syndrome (Tullio-Pelet *et al.*, 2000; Handschug *et al.*, 2001). Triple A patients
68 present with three distinct symptoms: absent adrenal glucocorticoid and mineralocorticoid synthesis (adrenal
69 insufficiency), impaired movement of the stomach cardia (achalasia) and loss of tear production (alacrima)
70 (Allgrove *et al.*, 1978). These symptoms are highly heterogeneous and are accompanied by progressive
71 impairments of the central, peripheral or autonomous nervous system (Allgrove *et al.*, 1978). Most mutations
72 in *AAAS* result in a mis-localization of ALADIN to the cytoplasm (Cronshaw and Matunis, 2003; Krumbholz
73 *et al.*, 2006).

74 Previously, we identified microsomal PGRMC2 as novel interactor for the nucleoporin ALADIN and
75 provided new insights into the molecular function of the nucleoporin in the pathogenesis of triple A
76 syndrome (Jühlen *et al.*, 2016). PGRMC2 belongs to the group of membrane-associated progesterone
77 receptors. These receptors are restricted to the endoplasmic reticulum (ER) and are thought to regulate the
78 activity of microsomal cytochrome (CYP) P450 enzymes which are involved in steroidogenesis or drug
79 detoxification (Cahill and Medlock, 2017). The first identified membrane-associated progesterone receptor,
80 PGRMC1, gained wide-spread attention due to its several implications in cancerogenesis (Falkenstein *et al.*,
81 1996; Clark *et al.*, 2016; Kabe *et al.*, 2016; Ryu *et al.*, 2017). The mixed-function oxidase system of CYP
82 P450 enzymes requires a donor transferring electrons from NADPH to reduce the prosthetic heme group
83 (Pandey and Flück, 2013). PGRMC1 and PGRMC2 contain a CYP b5-similar heme-binding domain (Ryu *et al.*,
84 2017). PGRMC1 forms stable protein-protein complexes with CYP51A1, CYP7A1, CYP21A2 and
85 CYP3A4 (Hughes *et al.*, 2007). Additionally, PGRMC1 is able to activate CYP19 aromatase (Ahmed *et al.*,
86 2012). PGRMC1 is shown to physiologically affect cholesterol/steroid biosynthesis and metabolism (Ryu *et al.*,
87 2017). It is known that PGRMC2 has similar interaction potential, alters activity of CYP3A4 as possible
88 electron donor, and binds CYP21A2 (Albrecht *et al.*, 2012; Wendler and Wehling, 2013). Most recently,
89 both PGRMC1 and PGRMC2 were identified as putative interacting partners of ferrochelatase, an enzyme
90 catalyzing the terminal step in the heme biosynthetic pathway, thereby possibly controlling heme release as

91 chaperone or sensor (Piel *et al.*, 2016). Interaction of ALADIN with PGRMC2 at the perinuclear ER could
92 influence CYP P450 enzyme activity through electron transfer from NADPH and/or control heme synthesis.
93 In triple A syndrome, altered CYP P450 enzyme activity would consecutively contribute to adrenal atrophy
94 (Jühlen *et al.*, 2016).

95 Human PGRMC1 and PGRMC2 share 67 % of their protein sequence (Cahill, 2017; Cahill and
96 Medlock, 2017). Deficiency of either PGRMC1 or PGRMC2 decreases the anti-apoptotic and anti-mitotic
97 action of progesterone (Peluso *et al.*, 2014). Additionally, increased expression of PGRMC1 or PGRMC2
98 inhibits entry into cell cycle (Griffin *et al.*, 2014; Peluso *et al.*, 2014). On the one hand, PGRMC1 is
99 distributed with β - and γ -tubulin to the mitotic bipolar spindle and spindle poles in metaphase cells and on
100 the other hand, with Aurora kinase B in meiotic cells (Luciano *et al.*, 2010; Lodde and Peluso, 2011).
101 Furthermore, PGRMC1 is thought to regulate microtubule stability (Griffin *et al.*, 2014). PGRMC2 is shown
102 to localize to the mitotic spindles in metaphase and anaphase cells and shall interact with cyclin-dependent
103 kinase 11B (p58) (Griffin *et al.*, 2014). PGRMC1 and PGRMC2 are reported to interact and furthermore, to
104 bind to each other during metaphase, thereby suppressing entry into cell cycle (Peluso *et al.*, 2014).

105 Interestingly, in a large scale interactome mapping of the centrosome-cilium interface the
106 nucleoporin ALADIN and both microsomal PGRMC1 and PGRMC2 have been identified to localize to the
107 cilium transition zone (Hanson *et al.*, 2014; Gupta *et al.*, 2015). The centrosome is an important regulator of
108 cell cycle progression and mitotic spindle assembly (Scholey *et al.*, 2003). Furthermore, ALADIN is strongly
109 dephosphorylated during mitotic exit, whereas PGRMC1 and PGRMC2 are phosphorylated during early
110 mitotic exit (Malik *et al.*, 2009; McCloy *et al.*, 2015). Obviously, ALADIN, PGRMC1 and PGRMC2 seem
111 to have critical roles for the formation and function of the mitotic spindle apparatus in somatic cells.

112 Here, we report that proliferation in human adrenal cells is decreased after increased expression of
113 ALADIN, PGRMC1 or PGRMC2. We show that PGRMC1 localizes to the microtubule kinetochore-fibers
114 in metaphase and to the midbody in telophase of human adrenal cells and fibroblasts. Further, we present that
115 PGRMC1 and Aurora kinase A are mislocalized in metaphase triple A patient fibroblasts. We observed that
116 patient fibroblasts present with increased expression of PGRMC2 and we hypothesize that a depletion of
117 ALADIN in these cells leads to a dysregulation of PGRMC2 and displaces PGRMC1 at the metaphase
118 spindle.

119 RESULTS AND DISCUSSION

120 Adrenal cell proliferation is decreased upon over-expression of ALADIN, PGRMC1 or PGRMC2 and 121 down-regulation of ALADIN

122 In primary skin fibroblasts of triple A patients the cellular proliferation rate is decreased and doubling time
123 of patient cells is significantly increased compared to cells of healthy donors (Kind *et al.*, 2010).
124 Additionally, patient cells show features of senescence even though these cells have not reached replicative
125 senescence as it has been documented for normal skin fibroblasts (Lee *et al.*, 2002; Kind *et al.*, 2010). The
126 nucleoporin ALADIN is ubiquitously expressed with predominant expression in adrenal gland,
127 gastrointestinal and central nervous system structures (Huebner *et al.*, 2002). That may be a reason why
128 patients with triple A syndrome present with characteristic pathogenesis in distinct tissues: adrenal
129 insufficiency, achalasia, alacrima and involvement of the central, peripheral and autonomous nervous system
130 (Allgrove *et al.*, 1978). In order to address the pathogenesis in adrenal tissue in the patients, we reported that
131 loss of ALADIN leads to an impairment of glucocorticoid and mineralocorticoid synthesis in adrenal cells *in*
132 *vitro* (Jühlen *et al.*, 2015).

133 To test whether depletion of ALADIN also results in decreased cellular proliferation in adrenal cells
134 we used inducible adrenocortical NCI-H295R1-TR cells stably expressing *AAAS* shRNA (*AAAS* knock-down)
135 and monitored cellular proliferation using live cell imaging for at least 65 h. *AAAS* knock-down resulted in
136 decreased proliferation in adrenal cells (growth constant k (slope of linear regression line) =0.044) compared
137 to control cells expressing a scrambled shRNA (k =0.062) (wild-type cells: k =0.2) (**Figure 1A**). Surprisingly,
138 in live cell imaging stable over-expression of N-terminal-GFP-tagged ALADIN in adrenocortical NCI-
139 H295R cells also impaired cellular proliferation (k =0.082) compared to over-expression of GFP alone in
140 these cells (k =0.305) (wild-type cells: k =0.16) (**Figure 1B**). It can be assumed that an equilibrated level of
141 ALADIN protein is prerequisite for successful cellular proliferation and that depletion or accumulation of the
142 nucleoporin impairs cellular proliferation. That hypothesis that an equilibrated level of ALADIN is critical
143 for cell division was already postulated by Carvalhal *et al.* (Carvalhal *et al.*, 2015).

144 We firstly investigated, whether cellular proliferation defects are caused by diminished expression of
145 the nucleoporin ALADIN. Secondly, we wanted to know whether an over-expression of PGRMC2, a novel
146 discovered interactor of the nucleoporin ALADIN, impairs the function during proliferation. In ovarian
147 granulosa cells levels of PGRMC2 decrease during G1 phase of cell cycle (Griffin *et al.*, 2014). Increased
148 expression of PGRMC1 or PGRMC2 in these cells is documented to suppress entry into cell cycle (Griffin *et*
149 *al.*, 2014; Peluso *et al.*, 2014). During metaphase both proteins are thought to interact and to impair
150 progression of cell cycle (Peluso *et al.*, 2014). During live cell imaging we observed that transient over-
151 expression of C-terminal-GFP-tagged PGRMC2 in adrenocortical NCI-H295R cells resulted in decreased
152 cellular proliferation (k =0.034) (**Figure 1B**). The same phenotype was seen in adrenocortical cells transiently
153 over-expressing N-terminal-GFP-tagged PGRMC1 (k =0.015) (**Figure 1B**). These results are in accordance
154 with the literature and emphasize a simultaneous role of PGRMC1 and PGRMC2 during cellular

155 proliferation.

156

157 **PGRMC1 is restricted to metaphase spindle during mitosis and mid-body during cytokinesis**

158 Next we determined the localization of PGRMC1 and PGRMC2 during cell division in adrenal cells. So far,
159 we saw that over-expression of ALADIN, PGRMC1 or PGRMC2 leads to an impairment of adrenal cellular
160 proliferation (**Figure 1B**). Thus, we assumed that each of these proteins plays an important role during
161 regulation of cell division. It is known that ALADIN is a novel regulator of mitotic controller Aurora kinase
162 A (Carvalho *et al.*, 2015). Humans have three classes of serine/threonine Aurora kinases: Aurora A, Aurora
163 B and Aurora C (Marumoto *et al.*, 2005). The homologous Aurora kinase A and Aurora kinase B are
164 expressed in most cell types. Despite different localization and activation during cell cycle, they both
165 regulate progression through cell cycle from G2 phase to cytokinesis (Marumoto *et al.*, 2005). In *Drosophila*
166 ALADIN diffuses loosely through the spindle during mitosis (Carvalho *et al.*, 2015). In HeLa cells
167 ALADIN could be found within the mitotic spindle accumulated at spindle poles but the highest amount
168 appeared between centrosome and metaphase plate (Carvalho *et al.*, 2015). This high concentration of
169 ALADIN protein is thought to be an ER-membrane-associated pool of the nucleoporin since it partially co-
170 localizes with calnexin which is an integral microsomal protein (Carvalho *et al.*, 2015). PGRMC1 and
171 PGRMC2 are documented to localize to the mitotic spindle apparatus in metaphase ovarian granulosa cells
172 but their role at the spindle apparatus and during cell cycle is not known (Lodde and Peluso, 2011; Griffin *et al.*, 2014).

174 Firstly, using immunofluorescence in adrenal NCI-H295R cells we show that PGRMC1 throughout
175 prophase and metaphase localized to the centrosome, bipolar spindle and spindle poles (**Figure 2A**). During
176 anaphase PGRMC1 diffuses weakly around chromosomes and in telophase before cytokinesis (cell-cell
177 scission) PGRMC1 localizes to the mid-body (**Figure 2A**). Secondly, during prophase PGRMC1 partially
178 co-localizes with mitotic Aurora kinase A (AURKA) to the centrosome and during metaphase and telophase
179 to spindle poles and mid-body (**Figure 2A**). During telophase and cell-cell scission PGRMC1 could also be
180 found with mitotic Aurora kinase B (AURKB) at the mid-body (**Figure 2B**). PGRMC1 has been documented
181 to associate with Aurora kinase B during meiosis but no such role has been assigned during mitosis (Luciano
182 *et al.*, 2010). Both Aurora kinase A and Aurora kinase B phosphorylate and thereby regulate a variety of
183 mitotic spindle substrates. Dysregulation of these kinases results in fatal mitotic errors (Marumoto *et al.*,
184 2005). Aurora kinase A has distinct roles in centrosome maturation, entry into mitosis, spindle assembly and
185 microtubule (MT) function during anaphase. Aurora kinase B is essential for bi-orientation of chromosomes
186 and cytokinesis (Barr and Gergely, 2007). It is tempting to assume that PGRMC1 is a substrate being
187 phosphorylated by one or both of these mitotic kinases and indeed, PGRMC1 has been found to be
188 phosphorylated at three postulated serines during early mitotic exit (McCloy *et al.*, 2015). Further research
189 should address whether PGRMC1 is phosphorylated by Aurora kinases and should uncover the regulatory
190 effect during mitosis of phosphorylation on one of the three postulated serines in PGRMC1.

191 PGRMC1 efficiently localizes with α - (TUBA) and β -tubulin (TUBB) to metaphase bipolar spindles

in adrenal NCI-H295R cells (**Figure 2B**). It has already been documented that PGRMC1 interacts with β -tubulin that is one of the two main components of MTs which are built from heterodimers of α - and β -tubulin (Scholey *et al.*, 2003; Lodde and Peluso, 2011). Heterodimers are arranged in a head-to-tail fashion into protofilaments whereby in humans 13 of such protofilaments laterally arrange into tubular structures of about 25 nm diameter (Meunier and Vernos, 2012). Within MTs α - and β -tubulin heterodimers reveal a distinct intrinsic polarity in which the minus-end confers to the end where α -tubulin is exposed and the plus-end where β -tubulin is exposed (Meunier and Vernos, 2012). Microtubule polymerization is done at the β -tubulin plus-end and initial nucleation of MTs is facilitated by γ -tubulin including a complex of several proteins (Meunier and Vernos, 2012). Microtubules undergo dynamic cycles of polymerization and depolymerization which is called dynamic instability and is achieved by a variety of regulatory proteins (Meunier and Vernos, 2012). Co-localization of PGRMC1 with β - and γ -tubulin has been proposed before, but a direct interaction of PGRMC1 could only be shown for β -tubulin (Lodde and Peluso, 2011). Here, we show that β -tubulin is a positive target for a novel interaction with PGRMC1 and that association of PGRMC1 with β -tubulin possibly influences polymerization at plus-ends of MTs during mitosis. Furthermore, we show that PGRMC2 was loosely to the mitotic spindle in adrenal metaphase cells and weakly co-localized with PGRMC1 during mitosis (**Figure 2B**). In interphase cells we previously reported that PGRMC2 co-localizes with the nucleoporin ALADIN to the perinuclear space (Jühlen *et al.*, 2016). Here, it appeared that PGRMC2 like ALADIN diffuses loosely in mitotic cells and that only PGRMC1 could be efficiently restricted to the mitotic bipolar spindle (schematically summarized in **Figure 2C**). It is known that PGRMC1 and PGRMC2 interact to suppress entry into cell cycle (Peluso *et al.*, 2014). With immunofluorescence we could however not target PGRMC2 to the same site as PGRMC1 during mitosis. Thus, we hypothesize that PGRMC2 plays a distinct role in regulating the function of PGRMC1 at the mitotic spindle and presumably during cell division.

215

216 **Loss of ALADIN results in mis-localization of PGRMC1 and Aurora kinase A during metaphase**

We have previously shown that the nucleoporin ALADIN interacts with microsomal PGRMC2 and that ALADIN is a new important co-factor of mitotic controller Aurora kinase A (Carvalho *et al.*, 2015; Jühlen *et al.*, 2016). It is thought that PGRMC2 binds its protein homologue PGRMC1 resulting in suppression of cell cycle (Peluso *et al.*, 2014). Here, we demonstrate that over-expression of ALADIN, PGRMC1 or PGRMC2 in adrenal cells leads to impaired cellular proliferation (**Figure 1B**), and that PGRMC1 is restricted to the mitotic bipolar spindle (**Figure 2**). Since both ALADIN and PGRMC2 loosely diffuse in mitotic cells compared to PGRMC1 (**Figure 2C**), we assume that ALADIN and PGRMC2 possibly exploit a regulatory role during cell division. Elucidating this novel role for ALADIN during cell division shall establish new avenues in explaining parts of the pathogenesis in triple A syndrome. We recently showed that a depletion of ALADIN in triple A patient fibroblasts affects the localization of PGRMC2 at the perinuclear ER (Jühlen *et al.*, 2016). Thus, we now tested the effect of over-expression or depletion of ALADIN on the localization of PGRMC1 and Aurora kinase A. We used stable GFP-ALADIN over-expressing and inducible

229 *AAAS* knock-down adrenocortical cells as well as skin fibroblasts from triple A patients. For this purpose, we
230 chose patient cells with three different, and *in vivo* frequently occurring homozygous mutations in the *AAAS*
231 gene: a donor splice mutation IVS14+1G>A (IVS14), a point mutation c.787T>C leading to the missense
232 mutation S263P and a point mutation c.884G>A leading to the nonsense mutation W295X.

233 Using immunofluorescence we revealed that stable GFP-ALADIN over-expressing adrenocortical
234 NCI-H295R cells failed to correctly localize Aurora kinase A to metaphase spindle poles (**Figure 3, A and**
235 **B**, top panels). Aurora kinase A was not restricted to spindle poles in these cells but spread outward onto MT
236 spindles compared to control cells over-expressing GFP (**Figure 3, A and B**, top panels). The same
237 phenotype could be seen in inducible adrenocortical NCI-H295R1-TR cells depleted for ALADIN compared
238 to control cells expressing scrambled shRNA (**Figure 3, A and B**, middle panels). Aurora kinase A normally
239 resides at the centrosome and spindle poles where it regulates centrosome maturation and MT spindle
240 assembly (Barr and Gergely, 2007). Carvalho et al. reported that mitotic HeLa cells depleted for or over-
241 expressing ALADIN have less Aurora kinase A at centrosomes and higher amounts of it at spindle fibers
242 (Carvalho *et al.*, 2015). Our findings further prove that ALADIN partially regulates mitotic Aurora kinase A
243 and raise the question why an equilibrated level of ALADIN protein is of great importance for proper
244 localization of Aurora kinase A. Localization of PGRMC1 was altered in adrenocortical cells depleted for
245 ALADIN (**Figure 3, A and B**, middle panels). PGRMC1 was still correctly targeted to the bipolar MT
246 spindle but distribution onto spindle fibers did not fully extend onto chromosomal kinetochores compared to
247 control scrambled shRNA-expressing cells (**Figure 3, A and B**, middle panels). Only depletion of ALADIN
248 and not over-expression affected localization of PGRMC1 in mitotic cells. We know that ALADIN directly
249 regulates Aurora kinase A and therefore, any change in level of ALADIN protein alters Aurora kinase A
250 localization and function during mitosis (Carvalho *et al.*, 2015). As only depletion of ALADIN alters
251 localization of PGRMC1 in immunofluorescence, it can be assumed that during mitosis ALADIN not
252 directly regulates PGRMC1 but does so rather through secondary mechanisms.

253 Next, we tested our assumptions using fibroblasts from triple A patients. It has been described that
254 triple A patient fibroblasts present with disorganized metaphase plates, shorter MT spindles and less active
255 Aurora kinase A at spindle poles (Carvalho *et al.*, 2015). Here we show in patient fibroblasts that ALADIN
256 depletion results in poleward spread of Aurora kinase A onto metaphase spindles and moreover, in
257 accumulation in the cytoplasm (**Figure 3, A and B**, bottom panels). The highest accumulation of Aurora
258 kinase A in the cytoplasm was seen in patient cells carrying the ALADIN missense mutation S263P and the
259 nonsense mutation W295X (**Figure 3, A and B**, bottom panels). PGRMC1 was correctly restricted to the
260 metaphase bipolar spindle with little decreased spread in direction of kinetochore chromosomes as seen in
261 adrenocortical cells depleted for ALADIN (**Figure 3, A and B**, bottom panels). Nevertheless, PGRMC1 also
262 accumulated in the cytosol of triple A fibroblasts with highest levels in patient cells carrying the missense
263 mutation S263P and the nonsense mutation W295X (**Figure 3, A and B**, bottom panels). Thus,
264 immunofluorescence results in triple A patient fibroblasts verified our previous findings and patient cells
265 even presented with a more profound phenotype regarding Aurora kinase A and PGRMC1 mis-localization

266 than adrenocortical ALADIN knock-down cells.

267 Our recent work presented that depletion of ALADIN alters the localization of PGRMC2 in triple A
268 patient fibroblasts and leads to an increased level of PGRMC2 at the perinuclear ER (Jühlen *et al.*, 2016).
269 Furthermore, we showed that adrenal tissue of female ALADIN knock-out mice exploits higher levels of
270 PGRMC2 protein compared to female wild-type animals (Jühlen *et al.*, 2016). Here we show that fibroblasts
271 from triple A patients carrying the ALADIN missense mutation S263P or the nonsense mutation W295X had
272 about two-fold increased levels of PGRMC2 on mRNA and protein level compared to anonymized healthy
273 control fibroblasts (**Figure 4, A and B**). Cells from the patient carrying the donor splice mutation IVS14
274 presented also with increased protein levels of PGRMC2 but quantitative real-time PCR data was not
275 significant (**Figure 4, A and B**). We hypothesize that increased levels of PGRMC2 in fibroblasts from triple
276 A patients result from the loss of ALADIN in these cells. Furthermore, we assume since ALADIN is a novel
277 interactor of PGRMC2 that ALADIN negatively regulates microsomal PGRMC2. A direct or indirect
278 negative regulation of PGRMC2 results in accumulation of PGRMC2 upon ALADIN depletion. We revealed
279 that ALADIN depleted patient fibroblasts fail to correctly target PGRMC1 fully to the mitotic spindle fibers,
280 instead PGRMC1 accumulates in the cytosol. Levels of PGRMC1 mRNA were not altered in these cells
281 (**Figure S1**) and we already presented that over-expression of either PGRMC1 or PGRMC2 impairs cellular
282 proliferation (**Figure 1B**). Based on the finding that PGRMC1 interacts with PGRMC2, we think that
283 increased levels of PGRMC2 displace PGRMC1 from its correct localization at the mitotic spindle and target
284 PGRMC1 with PGRMC2 to the cytosol impairing cell division and proliferation. Over-expression of
285 PGRMC1 alone would probably also result in the same phenotype while targeting high amount of PGRMC1
286 to the cytoplasm.

287 Adrenocortical ALADIN knock-down cells did not present with alteration of *PGRMC2* expression
288 (Jühlen *et al.*, 2016). Additionally, in immunofluorescence during mitosis PGRMC1 and Aurora kinase A
289 were not targeted to the cytosol in these cells (**Figure 3, A and B**, middle panels). Moreover, fibroblasts
290 from the patient carrying the donor splice mutation IVS14 had less PGRMC1 and Aurora kinase A targeted
291 to the cytosol (**Figure 3, A and B**, bottom panels). Future research has to address in more detail whether the
292 loss of regulation of PGRMC2 and Aurora kinase A is dependent on different levels in ALADIN protein and
293 on different kinds of mutations in the *AAAS* gene. With our previous work we have given evidence, that
294 depletion of ALADIN impairs mitotic cell division and can explain parts of the pathogenesis of triple A
295 syndrome. Our new results shall be the basis for more extended research focusing on mis-regulated
296 PGRMC2 and Aurora kinase A due to loss of ALADIN.

297 298 **PGRMC1 localizes to microtubule (MT) kinetochore fibers**

299 The mature bipolar mitotic spindle is made from different subclasses of MTs depending on their position,
300 functionality and organization: astral MTs, interpolar MTs and kinetochore-fibers (K-fibers) (**Figure 1C**)
301 (Meunier and Vernos, 2012). Astral MTs connect the centrosome with the cell cortex and regulate
302 centrosome separation and spindle positioning (Rosenblatt, 2005). However, it has been shown that mitosis

can occur without astral MTs (Khodjakov *et al.*, 2000; Mahoney *et al.*, 2006). The main body of the mitotic spindle is made up by dynamic interpolar MTs. Interpolar MTs originate from the centrosome towards the center of the spindle and connect in an anti-parallel fashion with interpolar MTs originating from the opposite spindle pole (Meunier and Vernos, 2012). Some interpolar MTs however are shorter and do not emanate have way through the mitotic spindle (Mastronarde *et al.*, 1993). Interpolar MTs are the main component of the central spindle during anaphase and maintain spindle polarity and chromosome segregation (Glotzer, 2009; Vanneste *et al.*, 2011). However, interpolar MTs only indirectly establish chromosome segregation because this function is facilitated by K-fibers (Meunier and Vernos, 2012). K-fibers are big bundles of 20-40 parallel MTs and are responsible of chromosomes attachment to spindle poles and facilitate sister chromatid segregation (McEwen *et al.*, 1997; Meunier and Vernos, 2012). K-fibers are less dynamic and therefore have an average half-life of 4-8 min comparable to that of interphase MTs with 9-10 min (Bakhoun *et al.*, 2009; Meunier and Vernos, 2012). The average half-lives of astral and interpolar MTs are less than 1 min (Zhai *et al.*, 1995; Meunier and Vernos, 2012). Therefore, K-fibers are the most stable MTs when exposed to cold or depolymerizing agents like nocodazole.

From previous work we know that a loss of ALADIN negatively affects K-fiber stability (Carvalho *et al.*, 2015). Moreover, Lodde and Peluso showed that PGRMC1 affects microtubule stability and mitotic progression by the action of progesterone (Lodde and Peluso, 2011). As PGRMC1 localizes to the metaphase bipolar spindle and ALADIN depletion through PGRMC2 up-regulation negatively affects the targeting of PGRMC1 to the spindle, we were eager to find out whether PGRMC1 localizes to a distinct subclass of MTs during mitosis. We exposed cells before immunostaining to cold and observed whether PGRMC1 was still targeted to the spindle in mitotic cells. A positive result would exclude a localization of PGRMC1 to astral or interpolar MTs and would emphasize a role of PGRMC1 at K-fibers. Furthermore, we targeted known K-fiber proteins during immunofluorescence and observed the localization of PGRMC1 compared to these. These K-fiber proteins were firstly, TACC3 (also known as maskin) which is together with clathrin and chTOG (also known as CKAP5) responsible for K-fiber bundling and secondly NDC80 which belongs to the KMN complex (KNL1-MIS12-NDC80 complex) that attaches K-fiber plus-ends to the outer chromosomal kinetochore through polymerization and de-polymerization (Joglekar *et al.*, 2010; Booth *et al.*, 2011). In order to visualize the MT spindle we also stained for α -tubulin (TUBA) and to observe the centromeric region we stained for CENPB (Ando *et al.*, 2002). We used adrenocortical NCI-H295R cells and additionally verified our results in a different cell type using human skin fibroblasts from healthy donors and from earlier mentioned triple A patients.

In **Figure 5, A and B**, we well document that after cold treatment PGRMC1 was still restricted to MT spindles in adrenocortical NCI-H295R cells and human skin fibroblasts. α -tubulin still co-localized with PGRMC1 and thus, targeted PGRMC1 to cold-stable K-fibers (**Figure 5, A and B**). Furthermore, immunofluorescence of CENPB marked the centromeric region of the condensed chromosomes containing the kinetochore. It can be seen that PGRMC1-positive spindle K-fibers reached out to the centromeric region being attached by the KMN complex visualized by immunostaining of NDC80 (**Figure 5, A and B**).

340 Immunofluorescence of PGRMC1 further partly co-localizes with staining of the MT-bundling protein
341 TACC3 in adrenocortical cells and skin fibroblasts (**Figure 5, A and B**). In **Figure 5C** the same
342 immunofluorescence staining pattern can be seen in cold-treated skin fibroblasts from triple A patients
343 carrying the donor splice mutation IVS14, the missense mutation S263P or the nonsense mutation W295X.
344 However, these cells present with shorter and more diffuse K-fibers compared to fibroblasts from healthy
345 donors (**Figure 5, B and C**). We hypothesize that this observation is due to the previously documented
346 decreased stability of K-fibers upon ALADIN depletion (Carvalho *et al.*, 2015).
347 In conclusion we document here that PGRMC1 localizes to cold-stable MT K-fibers in human adrenocortical
348 cells and skin fibroblasts. We verify that K-fibers are less stable upon ALADIN loss of function in triple A
349 patient fibroblasts and we present that these cells fail to target PGRMC1 efficiently to the mitotic spindle.
350 Thus, we assume that part of the phenotype seen in triple A patient cells can be caused by a mis-regulated
351 interaction between ALADIN and PGRMC2 resulting in mis-localization of PGRMC1 during mitosis and
352 less stable K-fibers. Further studies are required to address the exact function of the microsomal protein
353 PGRMC1 at K-fibers and to solve the question whether it is involved in MT bundling and/or MT maturation.

354 MATERIALS AND METHODS

355 *Cell culture*

356 All adrenal carcinoma NCI-H295R cells were cultured in DMEM/F12 medium (Lonza, Cologne, Germany)
357 supplemented with 1 mM L-glutamine (Lonza, Cologne, Germany), 5% Nu-serum (BD Biosciences,
358 Heidelberg, Germany), 1% insulin-transferrin-selenium) (Gibco, Life Technologies, Darmstadt, Germany)
359 and 1% antibiotic-antimycotic solution (PAA, GE Healthcare GmbH, Little Chalfont, United Kingdom).

360 NCI-H295R cells stably expressing GFP-ALADIN fusion protein or GFP were generated as
361 described previously using the gamma-retroviral transfer vectors pcz-CFG5.1-GFP-*AAAS* and pcz-CFG5.1-
362 GFP (Kind *et al.*, 2009).

363 NCI-H295R1-TR cells stably expressing *AAAS* shRNA (*AAAS* knock-down) or scrambled shRNA
364 were generated by our group as previously reported (Jühlen *et al.*, 2015). These cells were cultured with 100
365 µg/ml zeocin (InvivoGen, Toulouse, France) supplemented in culture medium. Doxycycline hydrochloride
366 (MP Biomedicals, Eschwege, Germany) was used at 1 µg/ml for 48 h to turn on the expression of the shRNA
367 sequence.

368 Triple A patient skin fibroblasts and human anonymized control skin fibroblasts were obtained and
369 cultured as described earlier (Kind *et al.*, 2010). All fibroblasts were cultured until passage 20 at the most.
370 Informed consent was obtained from all subjects and experiments were approved by the local ethics review
371 board (Medical Faculty, Technische Universität Dresden, EK820897).

372

373 *Transient adrenal cell transfection*

374 Cells were cultured for proliferation analysis in 24-well culture dishes at a density of 0.4×10^5 cells/well or
375 for immunofluorescence microscopy onto cover slips (Carl Zeiss, Jena, Germany) in 6-well culture dishes at
376 a density of 1.6×10^5 cells/well 24 h before subsequent transfections. Cells were transfected using X-treme
377 GENE HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany). The plasmids for transient
378 transfections pEGFP-C1-PGRMC1 and pCMV6-AC-PGRMC2-GFP vector (RG204682) (OriGene
379 Technologies, Rockville MD, USA) were used at a concentration of 0.01 µg/µl at an optimized transfection
380 ratio of 1:4 diluted in pure DMEM/F12. Proliferation was monitored after 24 h after transfection. Cells for
381 immunofluorescence were fixed after 48 h.

382

383 *Proliferation analysis*

384 Cells were seeded in 24-well culture dishes at a density of 0.4×10^5 cells/well 24 h before proliferation
385 analysis. Confluence measurement was done using live cell imaging on IncuCyte Zoom (Essen BioScience,
386 Ann Arbor MI, USA) over at least 65 h. Measurement was done at least in triplicate and experiments were
387 repeated at least twice.

388

389 *Immunofluorescence microscopy*

390 Cells grown on glass cover slips were fixed with 4% PFA in PBS for 5 min, permeabilized with 0.5% Triton-

391 X-100 in PBS for 5 min and then fixed again. In order to assess K-fiber microtubule stability, cells were put
392 on ice for 10 min prior fixation and permeabilization as reported elsewhere (Carvalho *et al.*, 2015). Blocking
393 was performed with 2% BSA/0.1% Triton-X-100 in PBS for 30 min at room-temperature (RT).

394 All antibodies used for immunofluorescence were diluted in blocking solution. Primary antibodies
395 anti-ALADIN (B-11: sc-374073) (1:25), anti-AURKA (C-1: sc-398814) (1:50), anti-AURKB (A-3: sc-
396 393357) (1:50), anti-CENPB (F-4: sc-376283) (1:50), anti-NDC80 (C-11: sc-515550) (1:50), anti-PGRMC1
397 (C-4: sc-393015) (1:50), anti-PGRMC2 (F-3: sc-374624) (1:50), anti-TACC3 (C-2: sc-376883) (1:50), anti-
398 TUBA (DM1A: sc-32293) (1:50) and anti-TUBB (D-10: sc-5274) (1:50) (Santa Cruz Biotechnology Inc.,
399 Heidelberg, Germany) were incubated at 4°C over-night in a humidified chamber. Secondary antibodies
400 Alexa Fluor 488 goat anti-mouse IgM and Alexa Fluor 568 goat anti-mouse IgG (1:500) (Molecular Probes,
401 Life Technologies) were incubated one hour at RT in the dark. Excess antibodies after primary and
402 secondary antibody staining were removed by three washing steps using 0.1% Triton-X-100 in PBS for 5
403 min. Cover slips were mounted onto microscope slides with VECTASHIELD mounting medium for
404 fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA).

405 Fluorescence was imaged using the confocal laser scanning microscope Zeiss LSM 510 with Zeiss
406 EC Plan-Neofluar 40x objective/ 1.3 Oil and the following lasers: diode 405 nm, Argon 488 nm and DPSS
407 561 nm (Carl Zeiss). Images were acquired and processed using equipment of the Core Facility Cellular
408 Imaging at the Medical Theoretical Centre in Dresden. The experiments were repeated at least three times.

409 *RNA extraction, cDNA synthesis and quantitative real-time PCR*

411 Total RNA from cultured cells was isolated using the NucleoSpin RNA (Macherey-Nagel, Düren, Germany)
412 according to the protocol from the manufacturer. Purity of the RNA was assessed using Nanodrop
413 Spectrophotometer (ND-1000) (NanoDrop Technologies, Wilmington DE, USA). The amount of 500 ng of
414 total RNA was reverse transcribed using the GoScript Reverse Transcription System (Promega, Mannheim,
415 Germany) following the protocols from the manufacturer. Primers for the amplification of the target
416 sequence were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies) and compared to
417 the human genome database for unique binding using BLAST search (National Center for Biotechnology
418 Information, U.S. National Library of Medicine, 2013). Primers for *PGRMC1* (forward, reverse and probe)
419 were used as previously described (Hlavaty *et al.*, 2016). The primer sequences are listed in the
420 supplementary data of this article (**Table S1**).

421 The qPCR amplifications were performed in triplicates using the GoTaq Probe qPCR Master Mix
422 (Promega) according to the manufacturer's reaction parameter on an ABI 7300 Fast Real-Time PCR System
423 (Applied Biosystems, Life Technologies). In all results repeatability was assessed by standard deviation of
424 triplicate C_t s and reproducibility was verified by normalizing all real-time RT-PCR experiments by the C_t of
425 each positive control per run. The experiments were repeated at least five times.

426 *Immunoblots*

428 After SDS-PAGE separation onto 4-12% PAGE (150 V for 1.5 h) and electroblotting (30 V for 1.5 h)
 429 (Invitrogen, Life Technologies) onto Amersham hybond-ECL nitrocellulose membrane (0.45 µm) (GE
 430 Healthcare GmbH, Little Chalfont, United Kingdom) non-specific binding of proteins to the membrane was
 431 blocked by incubation in PBS containing 3% BSA at RT.

432 The membrane was then probed with primary antibodies either anti-ACTB (clone AC-74) (1:40000
 433 in 3% PBS/BSA) (Sigma-Aldrich, Munich, Germany), anti-PGRMC1 (C-4: sc-393015) (1:100 in 3%
 434 PBS/BSA) or anti-PGRMC2 (F-3: sc-374624) (Santa Cruz Biotechnology, Inc.) (1:100 in 3% PBS/BSA)
 435 over-night at 4°C. Secondary antibodies goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000
 436 in 3% PBS/BSA) (Cell Signalling Technology Europe B.V., Leiden, Netherlands) were incubated one hour
 437 at RT. Protein bands were detected using ECL system and visualized on autoradiography film (Hyperfilm
 438 ECL; GE Healthcare, Munich, Germany).

439

440 *Statistics*

441 Statistical analyses were made using the open-source software R version 3.4.2 and R Studio version 1.0.136
 442 (R Core Team, 2017). Unpaired Wilcoxon-Mann-Whitney U-test was performed. During evaluation of the
 443 results a confidence interval alpha of 95% and P values lower than 0.05 were considered as statistically
 444 significant. Results are shown as box plots which give a fast and efficient overview about median, first and
 445 third quartile (25th and 75th percentile, respectively), interquartile range (IQR), minimal and maximal values
 446 and outliers.

447 Growth curve analysis and growth constant k (slope of linear regression line) calculation was done
 448 using multilevel regression technique using R Studio.

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453 (Universität Heidelberg, Mannheim, Germany) kindly provided pEGFP-C1-PGRMC1 plasmid.

454

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460

461 CONFLICT OF INTEREST

462 The authors declare that they have no conflict of interest.

463

464 REFERENCES

Ahmed, I. S. A., Chamberlain, C., and Craven, R. J. (2012). S2R(Pgrmc1): the cytochrome-related sigma-2 receptor that regulates lipid and drug metabolism and hormone signaling. *Expert Opin. Drug Metab. Toxicol.* 8, 361–370.

Albrecht, C., Huck, V., Wehling, M., and Wendler, A. (2012). In vitro inhibition of SKOV-3 cell migration as a distinctive feature of progesterone receptor membrane component type 2 versus type 1. *Steroids* 77, 1543–1550.

Allgrove, J., Clayden, G. S., Grant, D. B., and Macaulay, J. C. (1978). Familial glucocorticoid deficiency with achalasia of the cardia and deficient tear production. *Lancet* 1, 1284–1286.

Ando, S., Yang, H., Nozaki, N., Okazaki, T., and Yoda, K. (2002). CENP-A, -B, and -C Chromatin Complex That Contains the I-Type α -Satellite Array Constitutes the Prekinetochore in HeLa Cells. *Mol. Cell. Biol.* 22, 2229–2241.

Bakhoun, S. F., Thompson, S. L., Manning, A. L., and Compton, D. A. (2009). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nat. Cell Biol.* 11, 27–35.

Barr, A. R., and Gergely, F. (2007). Aurora-A: the maker and breaker of spindle poles. *J. Cell Sci.* 120, 2987–2996.

Booth, D. G., Hood, F. E., Prior, I. A., and Royle, S. J. (2011). A TACC3/ch-TOG/clathrin complex stabilises kinetochore fibres by inter-microtubule bridging. *EMBO J.* 30, 906–919.

Cahill, M. A. (2017). The evolutionary appearance of signaling motifs in PGRMC1. *Biosci. Trends.* 11, 179–192.

Cahill, M. A., and Medlock, A. E. (2017). Thoughts on interactions between PGRMC1 and diverse attested and potential hydrophobic ligands. *J. Steroid Biochem. Mol. Biol.* 171, 11–33.

Carvalho, S., Ribeiro, S. A., Arocena, M., Kasciukovic, T., Temme, A., Koehler, K., Huebner, A., and

- Griffis, E. R. (2015). The nucleoporin ALADIN regulates Aurora A localization to ensure robust mitotic spindle formation. *Mol. Biol. Cell* 26, 3424–3438.
- Carvalho, S., Stevance, M., Koehler, K., Naumann, R., Huebner, A., Jessberger, R., and Griffis, E. R. (2017). ALADIN is required for the production of fertile mouse oocytes. *Mol. Biol. Cell* 28, 2470–2478.
- Clark, N. C., Friel, A. M., Pru, C. A., Zhang, L., Shioda, T., Rueda, B. R., Peluso, J. J., and Pru, J. K. (2016). Progesterone receptor membrane component 1 promotes survival of human breast cancer cells and the growth of xenograft tumors. *Cancer Biol. Ther.* 17, 262–271.
- Cronshaw, J. M., and Matunis, M. J. (2003). The nuclear pore complex protein ALADIN is mislocalized in triple A syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 100, 5823–5827.
- Fahrenkrog, B. (2014). Nucleoporin Gene Fusions and Hematopoietic Malignancies. *New J. Sci.* 2014, Article ID 468306.
- Falkenstein, E., Meyer, C., Eisen, C., Scriba, P. C., and Wehling, M. (1996). Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 229, 86–89.
- Glutzer, M. (2009). The 3Ms of central spindle assembly: microtubules, motors and MAPs. *Nat. Rev. Mol. Cell Biol.* 10, 9–20.
- Griffin, D., Liu, X., Pru, C., Pru, J. K., and Peluso, J. J. (2014). Expression of progesterone receptor membrane component-2 within the immature rat ovary and its role in regulating mitosis and apoptosis of spontaneously immortalized granulosa cells. *Biol. Reprod.* 91, 36.
- Gupta, G. D. *et al.* (2015). A Dynamic Protein Interaction Landscape of the Human Centrosome-Cilium Interface. *Cell* 163, 1484–1499.
- Handschug, K., Sperling, S., Yoon, S. J., Hennig, S., Clark, A. J., and Huebner, A. (2001). Triple A syndrome is caused by mutations in AAAS, a new WD-repeat protein gene. *Hum. Mol. Genet.* 10, 283–290.
- Hanson, D., Stevens, A., Murray, P. G., Black, G. C. M., and Clayton, P. E. (2014). Identifying biological pathways that underlie primordial short stature using network analysis. *J. Mol. Endocrinol.* 52, 333–344.
- Hlavaty, J., Ertl, R., Miller, I., and Gabriel, C. (2016). Expression of Progesterone Receptor Membrane Component 1 (PGRMC1), Progestin and AdipoQ Receptor 7 (PAQPR7), and Plasminogen Activator Inhibitor 1 RNA-Binding Protein (PAIRBP1) in Glioma Spheroids In Vitro. *BioMed Res. Int.* 2016, 8065830.
- Huebner, A., Kaindl, A. M., Braun, R., and Handschug, K. (2002). New insights into the molecular basis of the triple A syndrome. *Endocr. Res.* 28, 733–739.
- Hughes, A. L., Powell, D. W., Bard, M., Eckstein, J., Barbuch, R., Link, A. J., and Espenshade, P. J. (2007). Dap1/PGRMC1 binds and regulates cytochrome P450 enzymes. *Cell Metab.* 5, 143–149.
- Joglekar, A. P., Bloom, K. S., and Salmon, E. D. (2010). Mechanisms of force generation by end-on kinetochore-microtubule attachments. *Curr. Opin. Cell Biol.* 22, 57–67.
- Jühlen, R., Idkowiak, J., Taylor, A. E., Kind, B., Arlt, W., Huebner, A., and Koehler, K. (2015). Role of ALADIN in Human Adrenocortical Cells for Oxidative Stress Response and Steroidogenesis. *PloS One* 10, e0124582.
- Jühlen, R., Landgraf, D., Huebner, A., and Koehler, K. (2016). Identification of a novel putative interaction partner of the nucleoporin ALADIN. *Biol. Open* 5, 1697–1705.

- Kabe, Y. *et al.* (2016). Haem-dependent dimerization of PGRMC1/Sigma-2 receptor facilitates cancer proliferation and chemoresistance. *Nat. Commun.* 7, 11030.
- Khodjakov, A., Cole, R. W., Oakley, B. R., and Rieder, C. L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. *Curr. Biol.* 10, 59–67.
- Kind, B., Koehler, K., Krumbholz, M., Landgraf, D., and Huebner, A. (2010). Intracellular ROS level is increased in fibroblasts of triple A syndrome patients. *J. Mol. Med.* 88, 1233–1242.
- Kind, B., Koehler, K., Lorenz, M., and Huebner, A. (2009). The nuclear pore complex protein ALADIN is anchored via NDC1 but not via POM121 and GP210 in the nuclear envelope. *Biochem. Biophys. Res. Commun.* 390, 205–210.
- Krumbholz, M., Koehler, K., and Huebner, A. (2006). Cellular localization of 17 natural mutant variants of ALADIN protein in triple A syndrome - shedding light on an unexpected splice mutation. *Biochem. Cell Biol.* 84, 243–249.
- Lee, H.-C., Yin, P.-H., Chi, C.-W., and Wei, Y.-H. (2002). Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. *J. Biomed. Sci.* 9, 517–526.
- Lodde, V., and Peluso, J. J. (2011). A novel role for progesterone and progesterone receptor membrane component 1 in regulating spindle microtubule stability during rat and human ovarian cell mitosis. *Biol. Reprod.* 84, 715–722.
- Luciano, A. M., Lodde, V., Franciosi, F., Ceciliani, F., and Peluso, J. J. (2010). Progesterone receptor membrane component 1 expression and putative function in bovine oocyte maturation, fertilization, and early embryonic development. *Reprod. Camb. Engl.* 140, 663–672.
- Mahoney, N. M., Goshima, G., Douglass, A. D., and Vale, R. D. (2006). Making microtubules and mitotic spindles in cells without functional centrosomes. *Curr. Biol.* 16, 564–569.
- Malik, R., Lenobel, R., Santamaria, A., Ries, A., Nigg, E. A., and Körner, R. (2009). Quantitative analysis of the human spindle phosphoproteome at distinct mitotic stages. *J. Proteome Res.* 8, 4553–4563.
- Marumoto, T., Zhang, D., and Saya, H. (2005). Aurora-A - a guardian of poles. *Nat. Rev. Cancer* 5, 42–50.
- Mastronarde, D. N., McDonald, K. L., Ding, R., and McIntosh, J. R. (1993). Interpolar spindle microtubules in PTK cells. *J. Cell Biol.* 123, 1475–1489.
- McCloy, R. A. *et al.* (2015). Global Phosphoproteomic Mapping of Early Mitotic Exit in Human Cells Identifies Novel Substrate Dephosphorylation Motifs. *Mol. Cell. Proteomics* 14, 2194–2212.
- McEwen, B. F., Heagle, A. B., Cassels, G. O., Buttle, K. F., and Rieder, C. L. (1997). Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *J. Cell Biol.* 137, 1567–1580.
- Meunier, S., and Vernos, I. (2012). Microtubule assembly during mitosis – from distinct origins to distinct functions? *J Cell Sci* 125, 2805–2814.
- National Center for Biotechnology Information, U.S. National Library of Medicine (2013). Basic Local Alignment Search Tool, Bethesda MD, USA.
- Nofrini, V., Di Giacomo, D., and Mecucci, C. (2016). Nucleoporin genes in human diseases. *Eur. J. Hum. Genet. EJHG* 24, 1388–1395.
- Pandey, A. V., and Flück, C. E. (2013). NADPH P450 oxidoreductase: structure, function, and pathology of

diseases. *Pharmacol. Ther.* *138*, 229–254.

Peluso, J. J., Griffin, D., Liu, X., and Horne, M. (2014). Progesterone receptor membrane component-1 (PGRMC1) and PGRMC-2 interact to suppress entry into the cell cycle in spontaneously immortalized rat granulosa cells. *Biol. Reprod.* *91*, 1-12.

Piel, R. B., Shiferaw, M. T., Vashisht, A. A., Marcero, J. R., Praissman, J. L., Phillips, J. D., Wohlschlegel, J. A., and Medlock, A. E. (2016). A Novel Role for Progesterone Receptor Membrane Component 1 (PGRMC1): A Partner and Regulator of Ferrochelatase. *Biochemistry (Mosc.)*. *55*, 5204-5217.

R Core Team (2017). R: A language and environment for statistical computing, Vienna, Austria: R Foundation for Statistical Computing.

Rabut, G., Doye, V., and Ellenberg, J. (2004). Mapping the dynamic organization of the nuclear pore complex inside single living cells. *Nat. Cell Biol.* *6*, 1114–1121.

Rosenblatt, J. (2005). Spindle assembly: asters part their separate ways. *Nat. Cell Biol.* *7*, 219–222.

Ryu, C. S., Klein, K., and Zanger, U. M. (2017). Membrane Associated Progesterone Receptors: Promiscuous Proteins with Pleiotropic Functions - Focus on Interactions with Cytochromes P450. *Front. Pharmacol.* *8*, 159.

Sakuma, S., and D'Angelo, M. A. (2017). The roles of the nuclear pore complex in cellular dysfunction, aging and disease. *Semin. Cell Dev. Biol.* *68*, 72–84.

Scholey, J. M., Brust-Mascher, I., and Mogilner, A. (2003). Cell division. *Nature* *422*, 746–752.

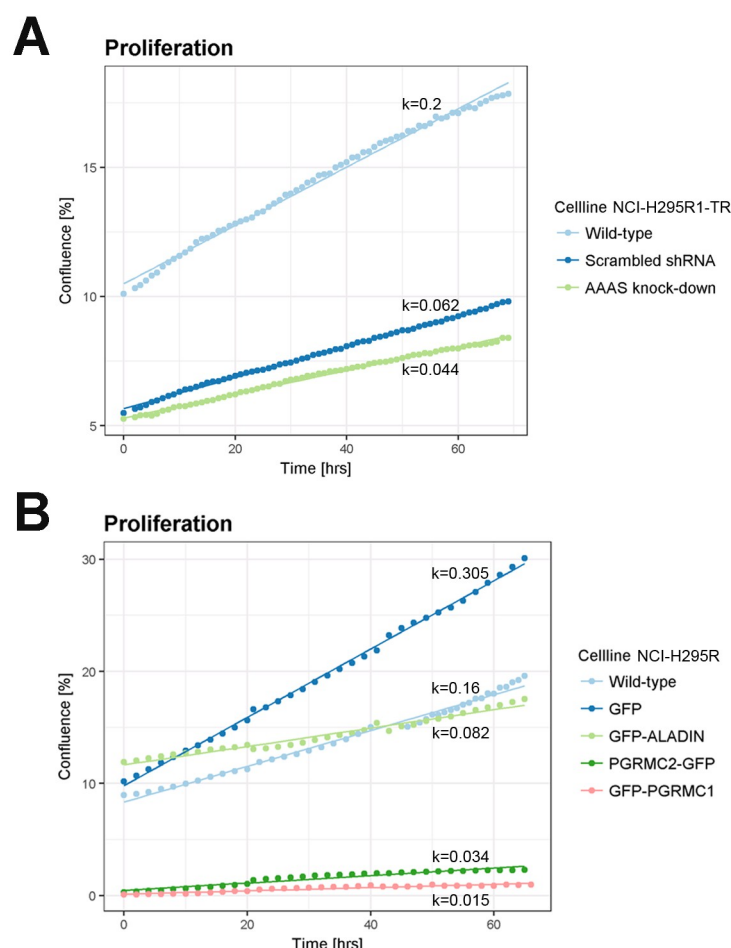
Tullio-Pelet, A. *et al.* (2000). Mutant WD-repeat protein in triple-A syndrome. *Nat. Genet.* *26*, 332–335.

Vanneste, D., Ferreira, V., and Vernos, I. (2011). Chromokinesins: localization-dependent functions and regulation during cell division. *Biochem. Soc. Trans.* *39*, 1154–1160.

Wendler, A., and Wehling, M. (2013). PGRMC2, a yet uncharacterized protein with potential as tumor suppressor, migration inhibitor, and regulator of cytochrome P450 enzyme activity. *Steroids* *78*, 555–558.

Yamazumi, Y., Kamiya, A., Nishida, A., Nishihara, A., Iemura, S., Natsume, T., and Akiyama, T. (2009). The transmembrane nucleoporin NDC1 is required for targeting of ALADIN to nuclear pore complexes. *Biochem. Biophys. Res. Commun.* *389*, 100–104.

Zhai, Y., Kronebusch, P. J., and Borisy, G. G. (1995). Kinetochores microtubule dynamics and the metaphase-anaphase transition. *J. Cell Biol.* *131*, 721–734.



466 **Figure 1. Cellular proliferation is impaired upon over-expression of ALADIN, PGRMC1 or PGRMC2.**
 467 Equal cell numbers were seeded 48 hours before confluence measurement using live cell imaging on
 468 IncuCyte Zoom (Essen BioScience). In case of transient transfection with PGRMC2-GFP and GFP-
 469 PGRMC1 the proliferation of fluorescent cells was measured which starts at cell density of about zero.
 470 Growth curve analysis and growth constant k (slope of regression curve) calculation was done using
 471 multilevel regression technique using R Studio.

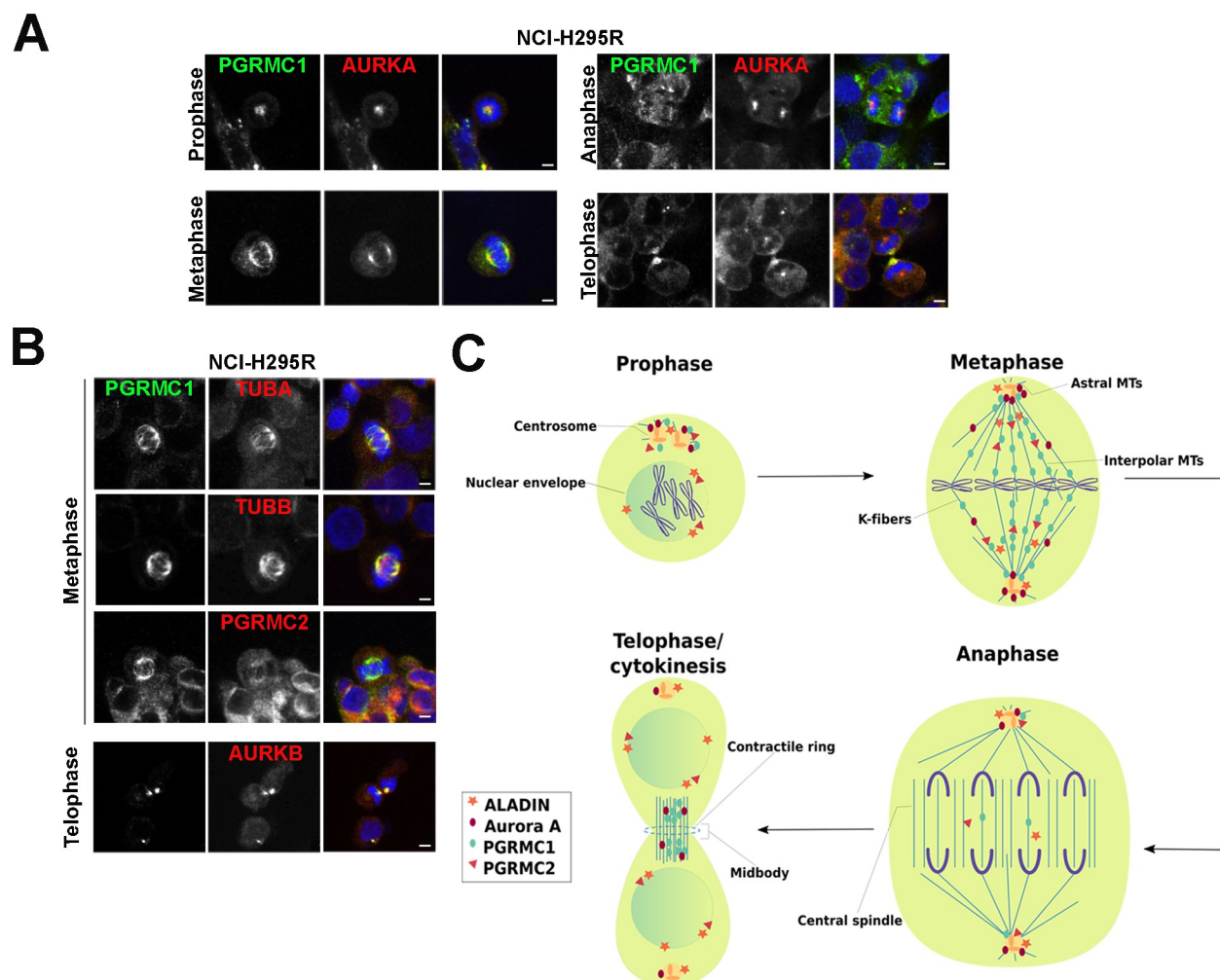


Figure 2. PGRMC1 is restricted to the mitotic spindle and to the mid-body during cytokinesis. (A) Human adrenocortical NCI-H295R cells at different cell division stages were stained with anti-PGRMC1 (green), anti-Aurora kinase A (AURKA) (red) and DAPI (blue). (B) Human adrenocortical NCI-H295R cells at meta- and telophase were stained with anti-PGRMC1 (green), anti- α -tubulin (TUBA) (red), anti- β -tubulin (TUB) (red), anti-PGRMC2 (red), anti-Aurora kinase B (AURKB) (red) and DAPI (blue). Scale bars: 5 μ m. (C) Schematic drawing of cellular localization of ALADIN, Aurora kinase A, PGRMC1 and PGRMC2 during mitosis. MT, microtubule.

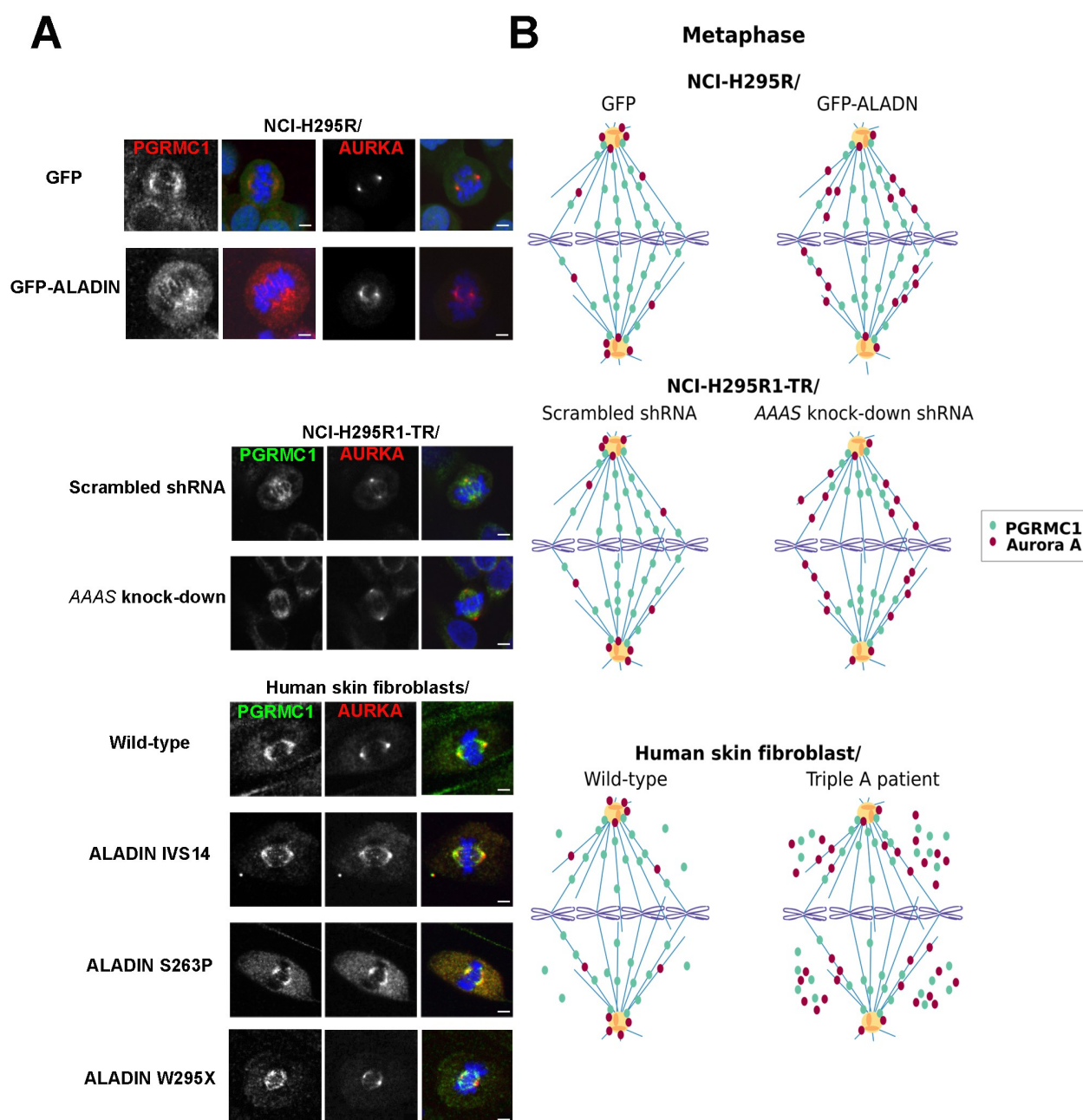


Figure 3. Loss of ALADIN mis-localizes PGRMC1 and Aurora kinase A during mitosis. (A) Human adrenocortical NCI-H295R GFP and GFP-ALADIN over-expressing cells, NCI-H295R1-TR scrambled shRNA and *AAAS* shRNA (*AAAS* knock-down) cells and human skin fibroblasts of healthy wild-type donors and triple A syndrome patients were stained with anti-PGRMC1 (green), anti-Aurora kinase A (AURKA) (red) and DAPI (blue). The different mutations in the human ALADIN protein are denoted as IVS14, S263P and W295X. Scale bars 5 μm, but for NCI-H295R GFP over-expressing cells: 10 μm. (B) Schematic drawing of the immunofluorescence staining of PGRMC1 and Aurora kinase A in (A).

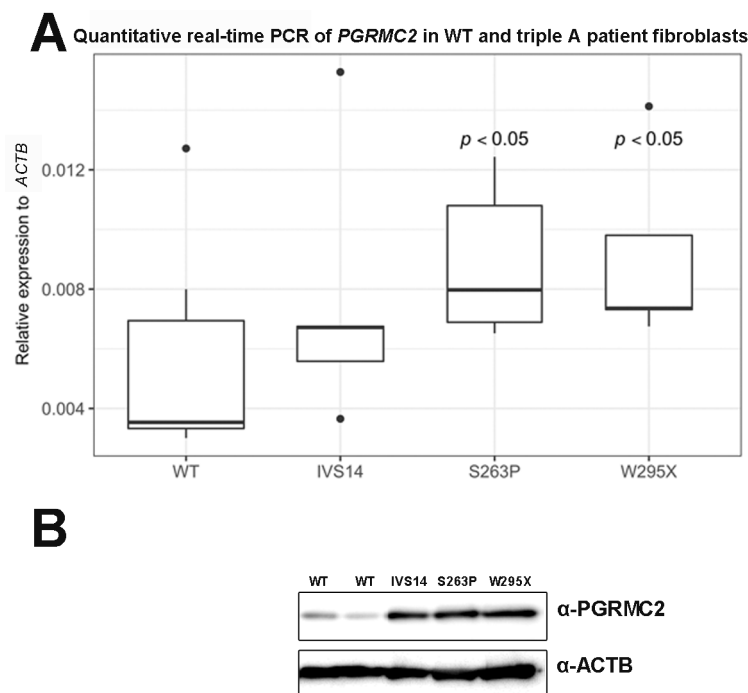
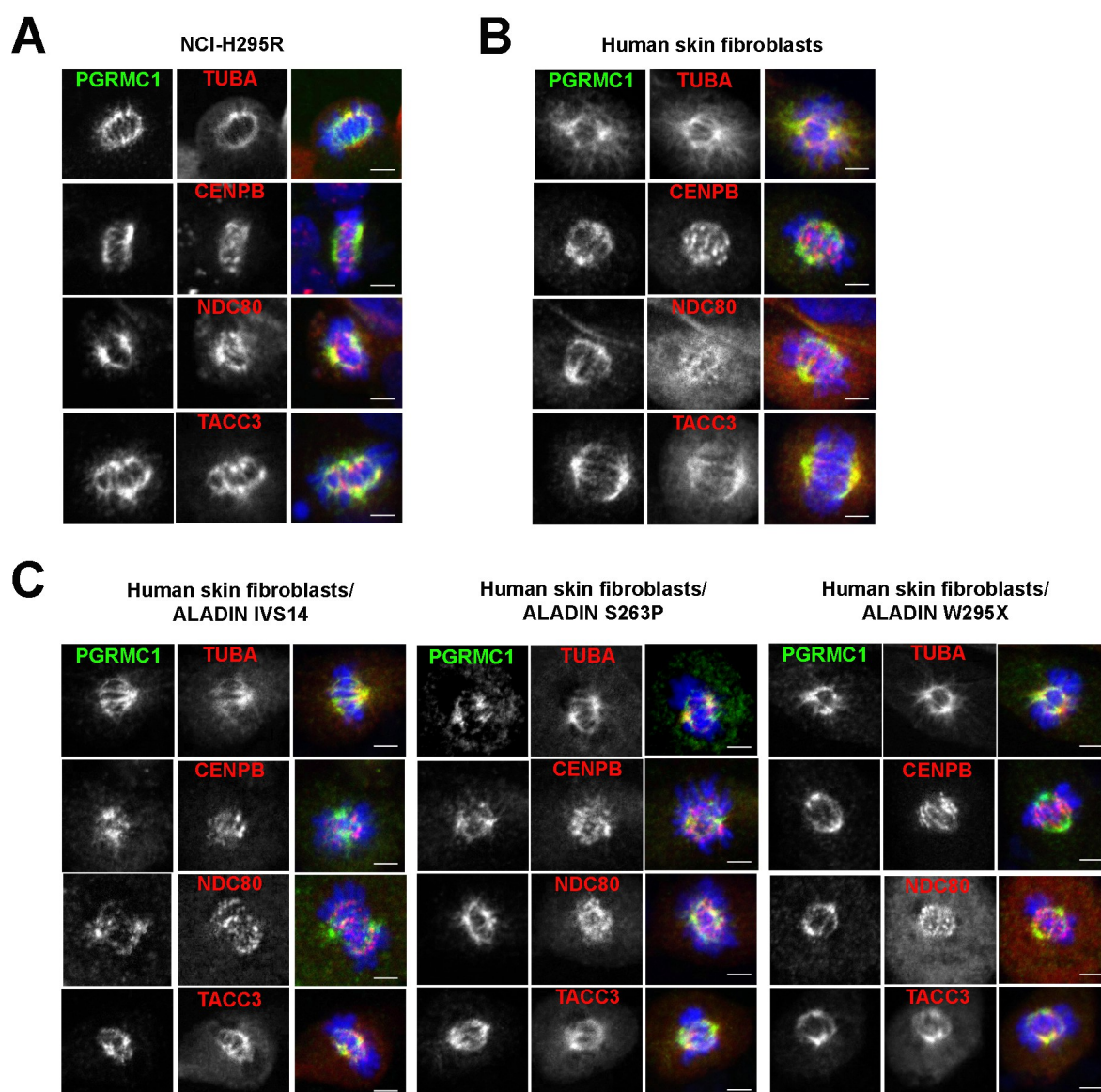


Figure 4. PGRMC2 is over-expressed in triple A patient fibroblasts. (A) Total RNA was isolated from human skin fibroblasts of healthy wild-type donors and patients with triple A syndrome. The different mutations in the human ALADIN protein are denoted on the x-axis of the diagram: IVS14, S263P and W295X. WT, wild-type. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range (IQR) above the upper quartile and below the lower quartile. Outliers were plotted as dots. (B) Total protein was isolated from human skin fibroblasts of healthy wild-type donors and triple A patients followed by western blot with indicated antibodies.



497 **Figure 5. PGRMC1 localizes to cold-stable kinetochore fibers.** Cells were cold-treated and stained with
498 anti-PGRMC1 (green), anti- α -tubulin (TUBA) (red), anti-CENPB (red), anti-NDC80 (red), anti-TACC3
499 (red) and DAPI (blue). (A) Human adrenocortical NCI-H295R cells. (B) Human skin fibroblasts of healthy
500 wild-type donors. (C) Human skin fibroblasts of triple A patients. The different mutations in the human
501 ALADIN protein are denoted as IVS14, S263P and W295X. Scale bars 5 μ m.