TITLE: **C3G dynamically associates with Nuclear speckles and regulates mRNA splicing**

RUNNING HEAD: C3G regulates splicing

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Summary: Nuclear speckles are sites for pre-mRNA splicing. We provide evidence for localization and function of a Ras family GTPase, Rap1 and its exchange factor C3G in nuclear speckles.

Abstract:

C3G (RapGEF1), essential for mammalian embryonic development, is ubiquitously expressed and undergoes regulated nucleo-cytoplasmic exchange. Here we show that C3G localizes to SC35 positive nuclear speckles, and regulates splicing activity. Reversible association of C3G with speckles was seen upon inhibition of transcription and splicing. C3G shows partial colocalization with SC35, and is recruited to a chromatin and RNase sensitive fraction of speckles. Its presence in speckles is dependent on intact cellular actin cytoskeleton, and is lost upon expression of the kinase, Clk1. Rap1, a substrate of C3G, is also present in nuclear speckles and inactivation of Rap signalling by expression of GFP-Rap1GAP, alters speckle morphology and number. Enhanced association of C3G with speckles is seen upon GSK3β inhibition, or differentiation of C2C12 cells to myotubes. CRISPR/Cas9 mediated knockdown of C3G resulted in decreased splicing activity and reduced staining for SC35 in speckles. C3G knockout clones of C2C12 as well as MDA-MB-231 showed reduced protein levels of several splicing factors compared to control cells. Our results identify C3G and Rap1 as novel components of nuclear speckles and a role for C3G in regulating cellular RNA splicing activity.

1 **Introduction:**

2

3 Many molecules function in signalling pathways through dynamic nucleo-cytoplasmic
4 exchange to regulate a variety of nuclear functions like chromatin organization, gene
5 expression and RNA processing. Within the nucleus, proteins may be present in the
6 nucleoplasm, or associated with nuclear sub-structures like chromatin, nuclear matrix,
7 nuclear membrane, nucleoli, Cajal bodies, or nuclear speckles (Handwerger and Gall, 2006).
8 Their localization often provides insights into the functions they perform in the nucleus.
9 Processes like replication, transcription and DNA repair take place in distinct nuclear regions,
10 and are generally defined by dynamics of chromatin remodelling and nuclear architecture
11 (Stein et al., 2003).

12 Nuclear speckles are defined, irregularly shaped structures present in the inter-chromatin
13 regions (Spector and Lamond, 2011). They are complexes of a large number of proteins and
14 snRNA, and are sites of pre-mRNA splicing in cells (Spector, 1993; Zhou et al., 2002).
15 Precise excision of introns from the primary transcript is important to generate spliced
16 translatable mRNAs in eukaryotes. RNA splicing is a regulated process mediated by a
17 multiprotein complex called spliceosome which facilitates the chemical removal of introns
18 (Misteli et al., 1997). Two classes of proteins, the SR family, and hnRNPs, primary localize
19 to speckles and engage in splicing activity. In addition, several other regulatory molecules
20 like kinases and phosphatases also localize to speckles (Spector and Lamond, 2011). Some of
21 these proteins have signature sequences that enable their targeting to speckles (Eilbracht and
22 Schmidt-Zachmann, 2001; Jagiello et al., 2000; Salichs et al., 2009), and phosphorylation
23 plays a major role in regulating localization to speckles (Mermoud et al., 1994; Stamm,
24 2008). While some proteins are constitutive to speckles, and play a role in functional mRNA
25 biogenesis, others show restricted expression and tissue specific alternate splicing events
26 (Castle et al., 2008; Wang et al., 2008). Speckle size and structure are dependent on
27 transcription and splicing activity in cells, and active transcription takes place in the
28 periphery of speckles (Lamond and Spector, 2003; Misteli et al., 1997). RNA transcription
29 and splicing are altered upon transient heat stress, which also affects the localization of
30 various factors to speckles (Biamonti and Caceres, 2009; Lallena and Correas, 1997; Spector
31 et al., 1991; Velichko et al., 2013).

32

3

33 C3G is a ubiquitously expressed guanine nucleotide exchange factor (GEF) that regulates

34 important cellular functions like cytoskeletal remodelling, adhesion, cell proliferation and

35 apoptosis (Radha et al., 2011). It is a 140 kDa protein with a catalytic domain at the C-

36 terminus, and a central protein interaction domain encompassing multiple poly-proline rich

37 tracts. The N-terminus has an E-cadherin binding domain, and the non-catalytic domains

38 serve to negatively regulate its catalytic activity (Hogan et al., 2004; Ichiba et al., 1997). In

39 addition, C3G activity is regulated by tyrosine phosphorylation and membrane localization

40 (Ichiba et al., 1999; Ichiba et al., 1997). Functions of C3G are dependent on its catalytic

41 and/or protein interaction properties. C3G targets GTPases Rap1, Rap2, R-Ras, and TC10

42 (Gotoh et al., 1995; Gotoh et al., 1997; Mochizuki et al., 2000); and interacts with signalling

43 molecules like Hck, Src, Crk, c-Abl, TC-48, and β-Catenin (Dayma et al., 2012; Knudsen et

44 al., 1994; Mitra et al., 2011; Radha et al., 2007; Shivakrupa et al., 2003; Tanaka et al., 1994).

45 We showed that C3G interacts with, and suppresses β-catenin activity, a determinant of cell

46 fate decisions (Dayma et al., 2012). C3G levels increase, and it is required for differentiation

47 of muscle and neuronal cells (Radha et al., 2008; Sasi Kumar et al., 2015). C3G is essential

48 for early embryonic development in mammals, as mice lacking C3G do not survive beyond

49 6-7.5dpc (Ohba et al., 2001).

50 Indirect immunofluorescence experiments have shown that cellular, as well as over-expressed

51 C3G localizes primarily to the cytoplasm (Hogan et al., 2004; Radha et al., 2004). The

52 primary sequence of C3G has functional NLSs, and, fractionation of a variety of cells has

53 shown the presence of C3G in the nuclear compartment (Shakyawar et al., 2017). Inhibition

54 of CRM1 dependent export as well as GSK3β activity, caused an increase in nuclear levels of

55 C3G. In an attempt to understand functions for C3G in the nucleus, we examined its

56 localization to distinct sub-nuclear structures. Our findings demonstrate that C3G and its

57 target Rap1 show dynamic association with nuclear speckles dependent on cellular

58 transcription activity. Differentiation of myocytes to myotubes enhances association of C3G

59 with nuclear speckles and knockdown of C3G causes reduction in cellular splicing activity.

60

61 **Results**

62 **Inhibition of nuclear export causes enrichment of C3G in SC35 positive speckles.**

63

64 The dynamic exchange of C3G between nuclear and cytoplasmic compartments prompted us

65   to study the localization of C3G to distinct sub-nuclear domains, to understand its function in

66   the nucleus. Since C3G localized predominantly to inter chromatin regions upon inhibition of

67   nuclear export, we examined its localization to nuclear speckles by co-staining with antibody

68   for SC35(SRSF2), a spliceosomal protein of SR family, and an established nuclear speckle

69   marker. This antibody recognizes a phospho-epitope of SC35 (Fu and Tom, 1990).   A

70   commercial antibody raised against N-terminal region of C3G that specifically recognizes

71   endogenous C3G, was used (Fig. 1A). This antibody recognizes endogenous C3G in

72   methanol fixed cells when examined by indirect immunofluorescence. Treatment of MDA-

73   MB-231 cells with nuclear export inhibitor Leptomycin B (LMB) showed prominent staining

74   for C3G in nuclear foci positive for SC35, upon analysis by confocal microscope (Fig. 1B).

75   This pattern was seen in a large number (50-60%) of cells. Untreated cells show cytoplasmic

76   and weak nuclear staining.   The efficacy of LMB treatment was validated by observing

77   nuclear localization of NF-κB a protein regulated by CRM1 dependent nuclear export (Fig.

78   S1A). Localization of C3G to speckles in response to LMB, was also seen in other human

79   cell types like MCF-7 (Fig. S2A). Co-localization of endogenous C3G was also seen with

80   overexpressed SC35-GFP upon LMB treatment (Fig. S2B). All cells expressing SC35-GFP

81   showed localization of C3G to splicing factor compartments.

82

83   **Inhibition of transcription results in enhanced localization of C3G to speckles.**

84   The shape & size of speckles is known to change depending on cellular transcription levels

85   (Melčák et al., 2000). Inhibition of transcription causes formation of large intra nuclear foci

86   containing splicing factors in cells (Carmo-Fonseca et al., 1992; Hall et al., 2006; Spector,

87   1993). This is a characteristic feature of nuclear speckles (IGC-inter chromatin granule

88   clusters) which undergo reorganisation and fuse to form large structures which are more

89   rounded and fewer in number. To examine if C3G localization to nuclear speckles responded

90   to inhibition of transcription, α-Amanitin treated MDA-MB cells were dual stained with C3G

91   and SC35antibodies. We observed a redistribution of C3G into enlarged speckles in response

92   to inhibition of transcription, similar to that seen for SC35 (Fig. 1C). No signal was seen in

93   speckles when C3G primary antibody was omitted, and cells were exposed to only secondary

94   antibodies. Quantitation of extent of co-localization between C3G and SC35 showed a

95   significant increase in LMB and amanitin treated cells (Fig. 1D). Inhibition of transcription

96   by amanitin was confirmed by examining RNA pol II using the antibody, H5 which

97   recognises the active Ser2phosphorylated form of RNA Pol II, (Fig. S1B) (Warren et al.,

98   1992). Unlike under condition of inhibition of nuclear export, treatment with amanitin

99   resulted in all the cells showing C3G in speckles. Localization of C3G to nuclear speckles

100   was not specific to a particular cell type, as it was also observed in HeLa, and C2C12 cells

101   (Fig. S3A-B). Endogenous C3G also showed colocalization in nuclear speckles with other

102   ribo-nucleoproteins like U1 snRNP70, U2 snRNP B" and Sm snRNP (Y12) upon inhibition

103   of transcription (Fig. S4A-D).

104   When examined at higher magnification, C3G was distributed irregularly in speckles and

105   appeared to be present only partially co-localized with SC35. Individual speckles were

106   examined by capturing optical z sections at 0.30 microns. 3D reconstruction showed

107   distribution of C3G non-uniformly in the speckle, being prominently seen in the periphery

108   covering the core region where SC35 is primarily located. This was also evident from XY &

109   XZ projections (Fig. 1E, left panel). Quantitative assessment of fluorophore localization

110   using a region of interest (ROI) was carried out to assess the spatial distribution of C3G and

111   SC35 within speckles which showed that intensity peaks do not correspond though the

112   signals overlapped (Fig. 1E). This data indicated that C3G localizes to discrete regions that

113   show only partial overlap with SC35.

114   The dynamics of localization of C3G to speckles was examined using a reversible

115   transcriptional inhibitor DRB (Sehgal et al., 1976). C3G showed reversible association,

116   similar to SC35, as its prominence in speckles was reduced 1 hr after removal of DRB (DRB

117   Rec) (Fig. 2A). Since C3G dynamically exchanges between nuclear & cytoplasmic

118   compartments, we examined if the prominent association with speckles in response to α-

119   amanitin treatment was due to enhanced nuclear translocation or retention. Levels of C3G in

120   nuclear & cytoplasmic fractions were compared in untreated (UT) and amanitin treated cells.

121   Unlike LMB treatment (Shakyawar et al., 2017), amanitin does not increase nuclear C3G

122   levels, indicating that enhanced association with speckles was due to redistribution of nuclear

123   C3G (Fig. 2B).

124   SC35 and snRNPs respond differently to heat shock (HS), which is also known to inhibit

125   transcription (Lallena and Correas, 1997; Spector et al., 1991). We examined the localization

126   of C3G in response to heat shock, and observed that about 40-50% of cells showed the

127   presence of C3G in alternate nuclear structures which were irregular and very few in number

128   per cell. These structures did not colocalize with SC35 positive speckles (Fig. 2C). We also

129   examined the colocalization of C3G and U1 snRNP70 under similar condition and found that

130   unlike SC35, U1 snRNP70 partially colocalized with C3G in these alternate nuclear

131   structures.

132   The rounding up of speckles and clustering of splicing factors upon inhibition of transcription

133   is caused by their accumulation at storage sites, due to reduction in pre-mRNA levels.

134   Reduction in splicing activity also results in similar changes in speckle morphology and

135   number (O'keefe et al., 1994). We examined localization of C3G in cells treated with

136   Isoginkgetin (IGK), a pre-spliceosome complex inhibitor (O'Brien et al., 2008). Reversible

137   association of C3G with speckles was seen upon treatment of cells with IGK (Fig. 3A),

138   suggesting a role for C3G in splicing. Formation of nuclear speckles is dependent on

139   phosphorylation of proteins by the kinase Clk1, and exogenous expression of Clk1 causes

140   redistribution of SR proteins out of speckles (Colwill et al., 1996). GFP-Clk1 and its

141   catalytic mutant (GFP-mClk1) were expressed in MDA-MB cells, and examined for C3G

142   expression in normal and amanitin treated cells. Just as described for other SR/SF proteins,

143   expression of Clk1, but not its kinase inactive variant resulted in loss of speckle pattern of

144   staining for C3G in amanitin treated cells (Fig. 3B). These results suggested that localization

145   of C3G to the speckles is dependent on activity of Clk1 and may be coupled to distribution of

146   SR factors.

147

148   **C3G is associated with nuclear speckles dependent on intact chromatin and RNA.**

149   Molecules generally localize to speckles through association with proteins or RNA (Dye and

150   Patton, 2001; Lallena and Correas, 1997). The association of C3G with speckles was

151   examined by carrying out detergent extraction as well as sensitivity to DNase I & RNase A

152   treatments. When α-Amanitin treated MDA-MB cells were extracted with detergent prior to

153   fixation, C3G as well as SC35 were intact. Treatment with DNase I also did not alter C3G

154   and SC35 staining (data not shown). When treatment with DNase I was followed by 0.4 M

155   NaCl, the SC35 positive intra nuclear foci were intact but C3G staining was greatly reduced

156   as some part of chromatin was lost from the nucleus (Fig. 4A). When digestion was followed

157   by 2M NaCl treatment which removes all chromatin and retains only nuclear matrix, C3G

158   was totally lost from speckles. SC35 foci were intact, indicating tight association with nuclear

159   matrix. Removal of chromatin was confirmed by the loss of DAPI staining.

160   Upon RNase A treatment, speckle localization of C3G was reduced showing diffused nuclear

161   staining, though most of the RNA was lost from the cells (Fig. 4B & S1C). When RNase

162   treatment was followed by 0.4 M and 2M NaCl extraction, the enlarged intra nuclear foci

163   formed by C3G were totally reduced, whereas SC35 foci were nearly intact (Fig. 4B). High

164   salt extraction resulted in retention of SC35 staining only in sharp speckles. The efficacy of

165   RNase A digestion was confirmed by the absence of staining with anti m3G antibody (Fig.

166   S1C) which labels capped snRNAs. These results indicated that localization of C3G to

167   speckles was dependent on the presence of intact chromatin and RNA in cells.

168   We examined the requirement of intact cellular microtubules and microfilaments for

169   localization of C3G to speckles upon amanitin treatment. C3G staining in intranuclear large

170   foci formed after α-amanitin treatment was partially reduced when MDA-MB cells were

171   treated with Nocodazole, an inhibitor of microtubule polymerisation. There was no effect on

172   SC35 foci. When treated with Cytochalasin D (an inhibitor of Actin polymerisation), C3G as

173   well as SC35 showed a weak diffuse pattern, without prominent localization to speckles (Fig.

174   4B). The efficacy of Cytochalasin D and Nocodazole treatments was verified by staining

175   similarly treated parallel coverslips for F-actin and α-tubulin respectively (data not shown).

176

177   **Rap1, a target of C3G localizes to speckles.**

178   Since C3G is a GEF for Ras family GTPases, we examined if Rap1, a target of C3G localizes

179   to nuclear speckles. Localization of Rap1 to the nucleus has been shown earlier (Lafuente et

180   al., 2007). Endogenous Rap1 showed non-uniform staining throughout the cell when

181   methanol fixed cells were examined by immunofluorescence, similar to that shown earlier

182   (Bivona et al., 2004), but in amanitin treated cells, prominent localization was seen at nuclear

183   speckles (Fig. 5A). To determine if activated form of Rap1 is present in speckles, we

184   examined localization of activated (GTP bound) Rap1 by expressing a GFP fusion protein of

185   Ral-GDS-RBD, which binds specifically to activated molecules of Rap1 in cells, and can be

186   observed as enhanced GFP signals (Bivona TG, 2004). We observed prominent GFP

187   fluorescence at nuclear speckles, specifically in α-amanitin treated cells (Fig. 5B), in addition

188   to diffuse signals seen throughout the cell. In untreated cells, intense GFP signals were seen

189   in punctate cytoplasmic structures, which are likely to be endosomes/multi-vesicular bodies

190   as described earlier (Pizon et al., 1994). These results suggest a function for C3G and its

191   target, Rap1 in nuclear speckles.

8

192   This was further validated by examining SC35 speckles in cells expressing GFP-Rap1GAP, a

193   protein known to inhibit Rap activation dependent downstream signalling. We compared

194   structure and number of SC35 speckles in MDA-MB cells expressing GFP-Rap1GAP, (in

195   normally growing and under conditions of transcription inhibition) with those that do not

196   express GFP-Rap1GAP. As shown in Fig. 5C, GFP-Rap1GAP expressing cells show more

197   compacted and significantly fewer speckles compared to non-expressing cells. Difference in

198   speckle morphology and number were not seen under conditions of amanitin treatment, with

199   both expressing and non-expressing cells showing fewer and more rounded speckles.

200

201   <u>C3G is required for pre-mRNA splicing.</u>

202   Earlier work from our laboratory showed that C3G translocates to the nucleus in response to

203   LiCl, an inhibitor of GSK3β (Shakyawar et al., 2017). Upon LiCl treatment which causes

204   increased expression of many genes, we observed that C3G shows enhanced localization to

205   speckles (Fig. 6A). SC35 localization was also altered upon LiCl treatment as shown earlier

206   (Hernández et al., 2004). Efficacy of LiCl treatment was tested by immunostaining of β-

207   catenin, which shows nuclear translocation in response to LiCl treatment (Data not shown).

208   Inhibition of GSK3β promotes myogenic differentiation and SC35 speckles show

209   reorganization during myocyte differentiation, becoming more mis-shapen and larger

210   (Homma et al., 2016; van der Velden et al., 2008). We have earlier shown that C3G is

211   required for C2C12 differentiation, and that C3G translocates to nuclei upon differentiation

212   of myocytes to form myotubes (Sasi Kumar et al., 2015). Co-localization studies showed that

213   C3G localization in SC35 positive speckles of myotubes is higher compared to that seen in

214   undifferentiated cells (Fig. 6B).

215   Splicing activity and structure of nuclear speckles changes during myocyte differentiation

216   (Homma et al., 2016). Based on its dynamic localization to speckles, we hypothesized a role

217   for C3G in mRNA processing. We examined the role of cellular C3G in regulating activity in

218   nuclear speckles by using a clone of C2C12 cells where C3G was knocked down using

219   CRISPR/Cas9 technology (Fig. 6C). Unlike control cells, these cells do not fuse to form

220   myotubes when grown in differentiation medium (Shakyawar et al., 2017). Compared to

221   normal C2C12, cells with reduced cellular C3G showed very poor staining for SC35 speckles

222   (Fig. 6D). In amanitin treated KO cells also, SC35 speckles were weakly stained and less

223   distinct throughout the nucleus compared to WT cells.

9

224   We went on to determine if loss of C3G also impacted cellular splicing activity using a

225   double reporter splicing assay plasmid, pTN24. Ratio of the activities of the two reporter

226   genes, luciferase and β-gal, gives information on the extent of splicing activity in cells. This

227   plasmid was expressed in WT and C3G KO C2C12 cells and splicing efficiency determined.

228   C3G KO cells showed significantly lower splicing activity, suggesting a role for C3G in

229   regulating RNA splicing. (Fig. 6E).

230

231   The reduced levels of SC35 seen in C3G knockout cells could be either a consequence of

232   inefficient assembly into speckles, or due to changes in expression level of SC35. We

233   therefore examined total protein levels of SC35, and a few other splicing factors in WT

234   C2C12 cells and C3G KO cells. As shown in Fig. 7A, expression of many of the tested

235   splicing factors was significantly lower in cells lacking C3G. Similar reduction was also

236   observed in C3G KO clones of MDA-MB-231 cells (generated using CRISPR/Cas9

237   technology) indicating that the effect was not restricted to a particular cell type (Fig. 7B).

238   These results suggested that cellular C3G functions to regulate expression of splicing factors.

239   Presuming that over-expression of C3G would have some consequence on splicing factor

240   levels, we examined levels of these proteins in lysates of MDA-MB-231 and C2C12 cells,

241   overexpressing C3G. It was observed that none of the proteins showed any significant change

242   in their cellular levels upon C3G overexpression (Fig. 7C). We also examined the effect of

243   enhancing cellular levels of C3G on splicing activity and observed that co-expression of C3G

244   with TN24 caused a significant increase in splicing activity (Fig. 7D).

245

Discussion

247   Molecules that exchange between the nuclear & cytoplasmic compartments, regulate

248   important processes in the nucleus. As a follow up of our study identifying nuclear

249   localization of C3G in response to certain physiological stimuli, we carried out a detailed

250   investigation into nuclear functions of C3G. In this study, we identify a novel function for

251   C3G in the nucleus, where it localizes to SC35 positive speckles, and regulates splicing

252   activity. Localization of C3G to speckles is a feature conserved in cells derived from various

253   tissue types, as well as in murine and human cells.

254 Upon inhibition of nuclear export, C3G is retained in the nucleus, with prominent localization
255 seen with SC35 speckles in several cells. Just as in the case of a variety of other molecules
256 involved in RNA splicing activity (Carmo-Fonseca et al., 1992), C3G also shows enhanced
257 localization to speckles upon inhibition of transcription or splicing. Cell fractionation
258 indicated that inhibition of transcription did not particularly increase nuclear levels of C3G,
259 suggesting that nuclear C3G exchanged between nucleoplasm & speckles. The reversibility
260 of release from speckles seen upon removal of transcription or splicing activity inhibition,
261 similar to other splicing factors suggests a common mechanism of dynamic exchange
262 between speckles and nucleoplasm. The fact that C3G is not prominently seen in speckles of
263 exponentially growing cells indicates that it is in constant flux between nucleoplasm and
264 speckles.

265 In response to heat shock, transcriptional reprogramming occurs & general RNA transcription
266 and splicing are also inhibited (Velichko et al., 2013). It therefore came as a surprise that
267 upon heat shock, C3G did not localize to SC35 positive speckles and appears to be present in
268 alternate granular structures in the nucleus which showed partial colocalization with U1
269 snRNP70. It is reported that few splicing factors but not SC35, re-localize to nuclear stress
270 granules which are sites of HSF1 localization and activity upon heat shock (Chiodi et al.,
271 2000; Denegri et al., 2001; Weighardt et al., 1999). It is suggested that splicing factors are
272 sequestered in speckles, and made available in response to specific stimuli (Spector and
273 Lamond, 2011). If C3G is moving to nuclear stress granules in response to heat shock, when
274 enhanced transcription of chaperones takes place, it is tempting to speculate that C3G enables
275 gene specific transcription in response to heat stress.

276 Though C3G localizes to speckles, it does not show superimposition with SC35, and is
277 present in a spatially heterogenous fashion (more in peripheral regions, compared to SC35),
278 suggestive of being involved in distinct functions within the speckle (Mintz and Spector,
279 2000). C3G is released from speckles upon extraction of chromatin, or RNA from nuclei, and
280 differs from SC35 with respect to its extractability from speckles. Removal of C3G from
281 speckles upon mild extraction conditions is in contrast to that seen for several other SR
282 factors, suggesting that C3G is weakly associated with speckles. SC35, and snRNPs differ in
283 their dependence on RNA for retention in speckles (Spector et al., 1991). Proteins in nuclear
284 speckles are known to have specific sequence elements that enable their targeting by binding
285 to RNA and other proteins (Cáceres et al., 1997; Eilbracht and Schmidt-Zachmann, 2001). In
286 the primary sequence of C3G, we could not identify any such features. It is possible that C3G

287   resides in speckles through its ability to interact with other macromolecules present. Since

288   C3G shows properties similar to some snRNPs with respect to extractability, it is possible

289   that localization of C3G to speckles is mediated by snRNPs. Our results show that increased

290   localization to speckles in response to amanitin is dependent on intact cytoskeletal elements.

291   Similar dependence has been shown for other proteins that reside in the speckles (Gieni and

292   Hendzel, 2009). Localization of C3G is also dependent on activity of Clk1 as we observed

293   that enhancing Clk1 activity by its overexpression disrupted the localization of C3G. The

294   possibility of C3G being a substrate of Clk1 is being explored as it is known that Cdk5

295   phosphorylates C3G (Utreras et al., 2013). Since C3G does not possess an RS motif, it will be

296   interesting to identify interacting partners of C3G that enable it to move in & out of speckles

297   in response to Clk1 mediated phosphorylation.

298   Rap1, the small GTPase target of C3G localizes to the nucleus, and regulates gene expression

299   (Lafuente et al., 2007), but its association with nuclear speckles was not known. We show

300   that active Rap1 is a component of nuclear speckles, and inactivation of Rap1 dependent

301   signalling alters speckle number, and morphology. These changes appear similar to the effect

302   of transcriptional and splicing inhibitors, suggesting that RNA transcription and/or splicing

303   may be dependent on Rap1 activity.

304   Rap1 plays a role at cell junctions (Ando et al., 2013) and many junctional proteins and those

305   that regulate actin cytoskeleton have been shown to localize to nuclear speckles (Saitoh et al.,

306   2004). C3G signals to cytoskeleton dependent functions in the cell, and is also known to

307   associate with cytoskeletal elements (Martín-Encabo et al., 2007; Radha et al., 2007). We

308   observed that distribution of C3G, as well as SC35 to speckles is effected upon treatment of

309   cells with Cytochalasin D, a microfilament depolymerizing agent, indicating that enhanced

310   localization of C3G in speckles seen upon inhibition of transcription is dependent on intact

311   cellular microfilaments.

312

313   We have earlier shown that inhibition of GSK3β induces import of C3G into the nucleus,

314   where it localizes to non-heterochromatin domains (Shakyawar et al., 2017). We observed

315   that under these conditions, C3G localizes to speckles, suggesting that localization of nuclear

316   C3G to speckles may be dependent on specific physiological stimuli. C3G shows enhanced

317   localization to speckles in differentiated myotubes, and knockdown resulted in reduced

318   intensity of SC35 speckles. The functional consequence of localization to speckles is a role

319   for C3G in regulating splicing activity. While C3G over-expression enhanced splicing

320   activity, knockdown of C3G resulted in reduced splicing. Alternate splicing is an important

321   mechanism that enables myogenic differentiation (Bland et al., 2010). Our earlier work

322   showed that C3G is required for C2C12 differentiation. Therefore, one of the required

323   properties of C3G to enable myogenic differentiation may be its function in alternate splicing.

324   We also observed that cells lacking C3G show reduced presence of SC35 in speckles. C3G

325   could be playing an adaptor function to enable SR proteins like SC35 to home to speckles,

326   and also playing an independent catalytic function in enabling splicing activity. Surprisingly,

327   examination of cell lysates having low levels of C3G due to CRISPR/Cas9 mediated

328   knockdown, showed dramatic decrease in several splicing factors, which could explain

329   reduced splicing activity in these cells. The splicing factors regulated by C3G are ubiquitous

330   components of speckles and essential for constitutive splicing. One likely explanation for

331   reduction in several of these factors, is proteasomal degradation specific to nuclear speckles

332   (Baldin et al., 2008). If C3G functions to inhibit this activity, reduction of several cellular

333   splicing factor protein levels would be expected. An alternate possibility is the ability of C3G

334   to regulate transcription of splicing factors through some presently unknown mechanism. If

335   C3G affects splicing activity in cells, its loss could result in reduced levels of many proteins

336   that are generated from spliced transcripts. These possibilities are being investigated. Though

337   C3G over expression did not alter levels of these factors, it did show significant, but small

338   increase in splicing activity. This could be because C3G is overexpressed only in 30-40 % of

339   cells.

340   Based on multiple lines of evidence, we describe a novel function for C3G in the nucleus.

341   Localization to splicing factor rich nuclear speckles; dynamic and reversible localization in

342   response to cellular transcription and splicing activity; dependency of localization on intact

343   DNA and RNA; presence of activated Rap1, a target of C3G in speckles; and its requirement

344   for splicing activity enabled us to show that regulation of RNA splicing is an important

345   function of nuclear C3G (Fig. 7E). Ras family GTPases and exchange factors that activate

346   them are involved in multiple signalling pathways regulating cell functions in response to

347   external stimuli. Several of them are known to localize to the nucleus, but their functions in

348   the nucleus are poorly understood. This is the first example of a Ras family GTPase and its

349   exchange factor localizing to, and functioning in nuclear speckles.

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Materials and Methods

Antibodies against C3G(H300), Calnexin, NF-kB, Rap1 and GAPDH were purchased from Santa Cruz biotechnology. Anti-SC35 (S4045-100UL) and Anti-snRNP200 were from Sigma. Anti-SC35 (ab204916), Anti-DHX38 and Anti-Lamin B1 were from Abcam. RNA polymerase-II H5 (Warren et al., 1992) and β-Actin antibodies were from Berkeley Antibody Company and Millipore respectively. U1 snRNP70 (Lerner and Steitz, 1979), U2 snRNP B" (Habets et al., 1985) and Sm snRNP(Y12) (Lerner et al., 1981) antibodies were gifted by David Spector (CSHL, Cold spring Harbour, NY, USA). α-Amanitin, DRB, Cytochalacin D, Nocadazole and LiCl were from Sigma. Leptomycin B was from Santa Cruz Biotechnology. Isoginkgetin was from Merck Millipore. Luciferase reagent was from Promega. Lipofectamine 3000 was from Invitrogen. Horseradish Peroxidase conjugated antibodies were from Amersham GE. Fluorophore conjugated secondary antibodies were from Millipore and Amersham GE.

Plasmid constructs

Expression vector for Flag tagged human C3G was a kind gift from Dr. Tanaka (Tanaka et al., 1994). SC35-GFP construct was a kind gift from Dr. V. Parnaik (Tripathi and Parnaik, 2008), pTN24 plasmid was gifted by Dr. I.C. Eperon. GFP-Clk1 and GFP-mClk1(K190R) plasmids were gifted by David Spector (CSHL, cold spring Harbour, NY, USA). GFP-RalGDS-RBD and GFP-Rap1GAP constructs were gifted by Dr. P.J. Stork (Carey and Stork, 2002) and Dr. Patrick Casey (Meng and Casey, 2002) respectively.

Cell Culture, Transfections and Treatments

MDA-MB-231, MCF-7, HEK293T and HeLa cells were cultured in DMEM with 10% FBS, C2C12 cells were maintained in DMEM with 20% FBS under standard conditions and differentiation was carried out as described earlier (Kumar et al., 2015). Lipofectamine 3000 was used for transfections in cells as per manufacturers protocol. Treatments of α-Amanitin, 50 µg/ml, 5 h; LMB, 37 nM, 6 h; Nocodazole, 1µg/ml, 4 h; Cytochalasin D, 0.2 µg/ml, 4 h; IGK 50 µM, 15 h; LiCl, 50 mM, 24 h; and DRB (5,6-Dichlorobenzimadazole-riboside)

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25µg/ml, 3 h; were given by incubating exponentially growing cells with respective drug(s) in cell culture medium. Transient heat shock treatment of cells was carried out in an incubator at 42°C for 2 h after addition of pre-warmed medium.

Generation of C3G KO clones in C2C12 cells has been described earlier (Shakyawar et al., 2017). Similar strategy was applied to generate C3G KO clones in MDA-MB-231 cells using commercially available CRISPR/Cas9 KO plasmid (sc-401616) and HDR plasmid (sc-401616-HDR).

Immunofluorescence, Image Analysis and Western Blotting

Immunofluorescence was performed as described earlier (Shivakrupa et al., 2003). Co-staining for two antigens was performed by sequential incubation of primary and corresponding secondary antibodies. Parallel coverslips processed without addition of primary antibodies were used as Blank (BL) to show absence of non-specific staining from secondary antibodies. Confocal Z stacks were captured on Leica TCS SP8 Confocal microscope (Leica Microsystems, Germany), and data was analysed using Leica Application Suite and ImageJ software's. Constant image acquisition parameters were used for capturing images of all samples from a given experiment. All confocal images were captured under 63X or 100X objective of the microscope and were digitally processed for presentation using Adobe Photoshop CS6 software. Quantitation of SC35 positive nuclear speckles was carried out with ImageJ software. Images of cells were imported into ImageJ and a digital brightness threshold was applied to each image. A particle analysis tool available in the ImageJ software was applied to thresholded images and number of structures measuring beyond 0.3 µm in size were obtained as output.

Western blotting was performed as described (Radha et al., 1994). Vilber-Lourmat Chemiluminescence System (Germany) or Carestream XBT autoradiogram sheets were used for detection of ECL signal. ImageJ software was used for quantitation of the western blots and values were normalised to loading control.

Cell Fractionation and *In situ* extraction

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Whole cell (W), Cytoplasmic (C) and Nuclear (N) fractions of cells were prepared as described (Radha et al., 1994). In situ extraction of cells plated on coverslips was performed as described (De Conto et al., 2000) with minor modifications. Cells grown on coverslips were rinsed twice with TM buffer (50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$), followed by 10 min incubation on ice with TM buffer containing 0.4% Triton X-100, 0.5 mM $CuCl_2$, 0.2 mM PMSF and protease inhibitor. Cells were washed and treated with DNase I (40 U/ml) or RNase A (20 μg/ml) for 30 min at 37^oC. Cells were subjected to 0.4 M and 2 M NaCl treatment sequentially for 5 min on ice to remove chromatin and RNA. Coverslips were fixed with methanol at each stage and immunostained.

Splicing activity assay

In vivo splicing activity assays was performed as described (Nasim et al., 2002). pTN24 reporter construct encodes β-gal and luciferase, separated by an intronic sequence (derived from human αs-tropomyosin gene) that contains stop codons. The ratio of luciferase to β-gal reflects the splicing activity in cells. Cells were transfected with the dual reporter plasmid pTN24 in addition to indicated constructs. After 48 hours of transfection, lysates were prepared using reporter lysis buffer and assayed for luciferase activity (Turner designs luminometer TD 20/20), and β-gal assay as per Promega protocols. Luciferase to β-gal ratios were normalised with control to determine relative in vivo splicing activity.

Statistical analysis

All data is represented as the mean±s.d. Unpaired Student's t-Test was carried out to obtain the significance of differences in means.

Acknowledgements

Competing Interest

No competing interest declared.

Figure Legends

Fig. 1. C3G localizes to SC35 nuclear speckles. A. MDA-MB-231, HeLa, and C2C12 cell lysates were subjected to immunoblotting and probed for C3G expression using C3G antibody. **B**. Normally growing MDA-MB-231 cells (UT) on coverslips or those treated with LMB, were fixed and immunostained for C3G and SC35. Panels show optical sections taken using a confocal microscope. **C**. MDA-MB-231 cells were subjected to α-Amanitin treatment; and immunostained for C3G and SC35. BL indicates cells processed similarly without addition of primary antibodies. **D**. Bar Diagram represents the Pearson correlation coefficient of colocalization between C3G and SC35 analysed from multiple images, upon indicated treatments. **E**. Optical sections (z-plane step, 0.30 µm) of nuclei from amanitin treated cells were captured on Leica SP8 confocal microscope, and were reconstructed to form a three-dimensional image. Three-dimensional visualization of speckle regions of cells dually labelled with antibodies against C3G (Red) and SC35 (Green) in XY or XZ plane are shown. Line scans showing local intensity distributions of C3G in red and SC35 in green in the ROI drawn across two speckles are shown to the right of the panels.

Fig. 2. C3G shows reversible association with nuclear speckles. A. Untreated MDA-MB-231 cells (UT) plated on coverslips were treated with DRB and either fixed immediately or after recovery from drug treatment for 1 hr (DRB Rec). Cells were subjected to indirect immunofluorescence to detect C3G and SC35 expression. **B.** MDA-MB-231 cells grown in

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the presence or absence of amanitin were subjected to cell fractionation. Lysates were processed for immunoblotting and probed for expression of indicated proteins. Calnexin and Lamin B1 were used to indicate the purity of the cytoplasmic and nuclear fractions respectively. H5 antibody (RNA pol II pS2) was used to show efficacy of α-amanitin treatment. Number indicate ratio of C3G levels in nuclear vs cytoplasmic fraction determined, based on protein loading. Effect of heat shock on C3G localization. MDA-MB-231 cells plated on coverslips were grown at 37°C (UT) or subjected to heat shock (HS). Panel shows confocal images of cells immunostained with antibodies against C3G and SC35 (**C**) or C3G and U1 snRNP70 (**D**).

Fig. 3. C3G localization to speckles increases upon inhibition of splicing and is lost upon overexpression of Clk1. A. MDA-MB-231 cells were either left untreated (UT) or treated with IGK. Cells were also allowed to recover for 6 h post drug washout (IGK-Rec). Cells were fixed and subjected to immunofluorescence with antibodies against C3G and SC35. Images of a single Z-section through the center of the nucleus acquired on a confocal microscope are shown in the panels. **B.** MDA-MB-231 cells expressing GFP-Clk1 or GFP-mClk1 construct were left untreated, or subjected to α-Amanitin treatment, fixed, and immunostained with antibody against C3G. Panels show confocal images of cells expressing C3G and transfected GFP tagged constructs. Arrows indicate GFP expressing cells in the C3G panels.

Fig. 4. C3G localization to nuclear speckles is dependent on the presence of intact chromatin and RNA. Amanitin treated MDA-MB-231 cells were left untreated or subjected to DNAse (**A**) or RNAse (**B**) treatment followed by NaCl extraction, fixed, and immunostained for C3G and SC35. **C.** MDA-MB-231 cells were treated with α-Amanitin, and also exposed to Nocodazole or Cytochalasin D for 4 h prior to fixation. Immunofluorescence was carried out to detect C3G and SC35.

Fig. 5. Rap1 a target of C3G localizes to nuclear speckles. A. MDA-MB-231 cells were treated with (AMA) or left untreated (UT) and immunostained for Rap1 and SC35. BL indicates secondary antibody control where cells were processed without addition of primary antibodies. **B**. GFP-RalGDS-RBD transfected MDA-MB-231 cells were treated with or without α-Amanitin, fixed with formaldehyde and immunostained with SC35. **C.** MDA-MB-231 cells transfected with GFP-Rap1GAP were treated with or without amanitin, fixed with

methanol and immunostained for expression of SC35. Arrows in SC35 panel show GFP-Rap1GAP expressing cells. Bar diagram shows quantitation of number of speckles per nucleus in expressing and non-expressing cells using data obtained from large number of cells from three independent experiments. ***p < 0.001.

Fig. 6. C3G associates with nuclear speckles upon myocyte differentiation and is required for splicing activity. A. MDA-MB-231 cells grown on glass coverslips were subjected to LiCl treatment prior to fixation, and immunostained for expression of C3G and SC35. Images show confocal sections of immunostained cells. **B.** C2C12 cells were grown in the presence of growth medium (GM) or differentiation medium (DM) for 72 h, fixed and immunostained for expression of C3G and SC35. The colocalization coefficient between C3G and SC35 in myoblasts and myotubes was quantitated using large number of cells from 3 independent experiments is shown in bar diagram. ***p < 0.001. **C.** Immunoblot of lysates from control (Con) and C3G KO clone (KO), probed for the expression of C3G; actin was used as loading control. Numbers indicate relative amount of C3G in KO cells compared to control cells. **D**. SC35 staining is altered in C3G KO cells. Control and C3G KO clones of C2C12 were treated with or without α-Amanitin, fixed and immunostained to detect C3G and SC35. Panels show images acquired on a confocal microscope. **E.** Effect of C3G KO on cellular splicing activity. Control and C3G KO clones transiently transfected with pTN24 vector for 48 h and lysates were assayed for luciferase and β-gal activity. Bar diagram shows relative luciferase to β-gal ratio averaged from three independent experiments. ***p < 0.001.

Fig. 7. Altered expression of splicing factors in C3G knockout cells. A. Lysates of C2C12 control and C3G KO clones were subjected to Western blotting and probed for C3G, U5 snRNP200, U5 snRNP116 and SC35. Actin was used as loading control. Numbers indicate relative protein levels in KO compared to control. **B.** Whole cell lysates of MDA-MB-231 control and C3G KO clones, probed for the expression of the indicated proteins. Bar diagram shows relative levels of the proteins averaged from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. **C.** Effect of C3G overexpression on splicing factor levels and activity. HEK-293T cells were cotransfected with pTN24 along with C3G-Flag or control vector. After 48 h, lysates were assayed for luciferase and β-gal activity. **D.** MDA-MB-231 and C2C12 cells were transiently transfected with C3G-Flag construct for 30 h. Lysates were subjected to immunoblotting, and blot was probed for indicated proteins. **E.** Schematic figure depicting dynamic localization of C3G and its effector GTPase Rap1 to nuclear speckles. Inhibition of transcription and splicing as well as physiological stimuli enhance localization

of C3G to SC35 positive speckles, whereas expression of active Clk1 disrupts amanitin induced C3G localization to speckles. Active form of Rap1 is present in nuclear speckles and inactivation of Rap1 mediated by expression of Rap1GAP resulted in altered morphology of speckles and significant decrease in number. Cells lacking C3G due to CRISPR/Cas9 mediated knockdown show reduced levels of splicing factors and decrease in splicing activity.

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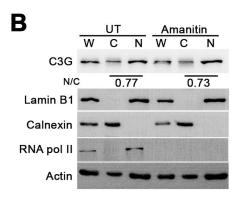
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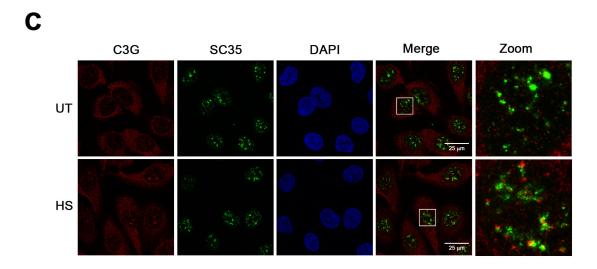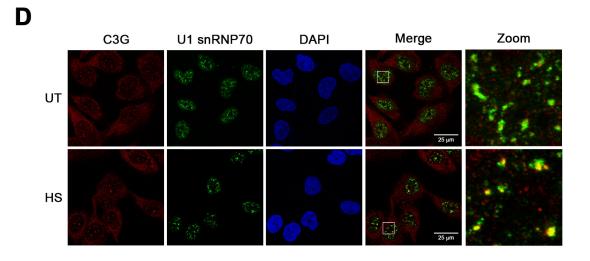
List of abbreviations

RapGEF1: Rap guanine nucleotide exchange factor 1; GSK-3β: Glycogen Synthase Kinase 3 Beta; LMB: Leptomycin B; CRM1: Chromosomal Maintenance 1; IGK: Isoginkgetin; AMA: α-amanitin; DRB: 5, 6-dichloro-1-β-d-ribofuranosylbenzimidazole
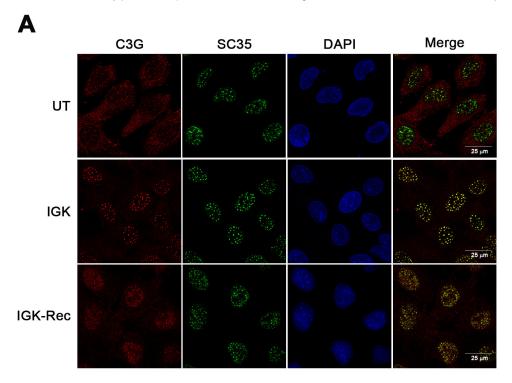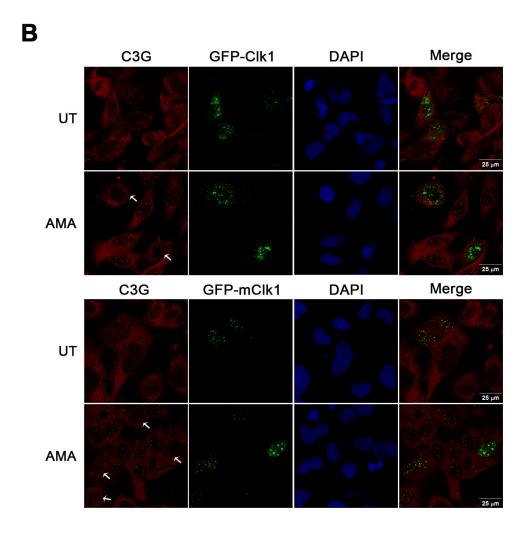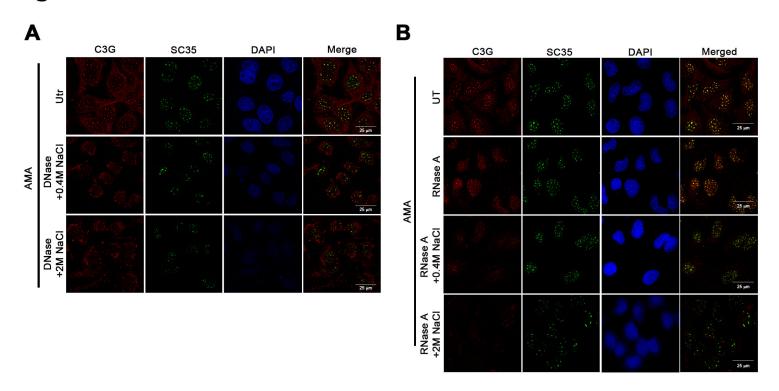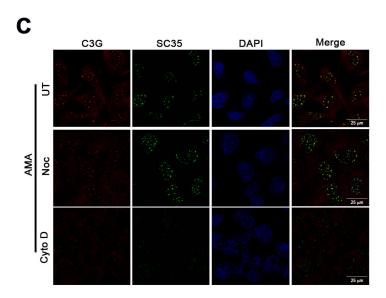
Figure 1

Figure 2

Figure 3

A

B

Figure 4

Figure 5

A

B

C

Figure 6

Figure 7