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Cytoskeletal remodeling by C3G to induce neurite-like extensions and inhibit motility in highly invasive breast carcinoma cells

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ABSTRACT

Cytoskeletal remodeling is responsible for cell plasticity and facilitates differentiation, motility and adherence related functions. C3G (RAPGEF1), an exchange factor for Ras family of small GTPases, regulates cytoskeletal reorganization to induce filopodia in epithelial cells and neurite growth in neuroblastoma cells. Here we show that C3G overexpression induces neurite-like extensions (NLE) in MDA-MB-231 and BT549 breast carcinoma cells and not in a variety of other cancer cell lines examined. These processes were actin-rich with nodes, branches and microspikes. C3G associates with the cytoskeleton and its expression enabled stabilization of microtubules. NLE formation was dependent on Rap, Rac and Cdc42. C3G expression was associated with a decrease in cellular β-catenin levels specifically in MDA-MB-231 and BT549 cells. β-Catenin stabilization induced by GSK-3β inhibition, or coexpression of β-catenin, reduced C3G induced NLE formation. Time lapse analysis showed reduced motility of C3G expressing cells compared to GFP expressing cells. Our results suggest that C3G overexpression can induce phenotypic characteristics of neuronal cells in highly invasive breast cancer cells and inhibit their motility.

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1. Introduction

Cytoskeletal remodeling is responsible for maintenance of cell shape, adhesion, motility and vesicular transport and its regulation is important for cellular differentiation and tissue morphogenesis. The metastatic potential of cancer cells is dependent on dynamic rearrangements of the cytoskeleton and regulators of cytoskeleton remodeling contribute to the aetiopathogenesis of malignancy [1]. In response to extracellular stimuli a variety of regulatory molecules signal to changes in the cytoskeleton [2]. Extensive reorganization of the cytoskeleton occurs during neuronal differentiation and therefore it has been important to elucidate the molecular effectors that bring about these morphological changes that precede acquisition of biochemical characteristics by mature neurons [3]. Small GTPases of the Rho and Ras family have generally been implicated in signaling to the cytoskeleton and their activity is triggered by guanine nucleotide exchange factors (GEFs) that respond to stimuli received through transmembrane receptors [4].

The GEF, C3G that activates Rap, R-Ras and TC10 GTPases has been implicated in signaling pathways stimulated by growth factors, cytokines, adhesion receptors and mechanical stress [5–12]. C3G has a catalytic domain in its extreme C-terminus and a central region containing multiple proline-rich sequences responsible for protein interaction [13,14]. It interacts with Crk, p130 Cas, Hck and c-Abl and its activity is known to be regulated through membrane recruitment and tyrosine phosphorylation [13–19]. Tyrosine phosphorylated C3G localizes to the Golgi and sub-cortical cytoskeleton [20]. It interacts with E-cadherin through sequences in the N-terminal domain [21]. In signaling pathways it appears to serve functions dependent on its catalytic activity and/or through protein interactions. Regulation of cell adhesion and migration, cell proliferation and cell death are some of the functions attributed to C3G [11,16,22–24]. Cell and tissue specific differences have been seen in the motility phenotype exhibited upon C3G deregulation. While in some instances it enables cell migration, in other cases it functions to inhibit motility [25–28]. It has been shown to suppress malignant transformation independent of its catalytic activity [29]. Transformation suppression is mediated through actin binding and PP2A activation at the sub-cortical actin cytoskeleton [30]. Inactivation of C3G has been shown to be associated with cervical squamous cell carcinomas [31].

We have recently demonstrated a role for C3G in signaling to actin reorganization during filopodia formation in epithelial cells and neurite growth in neuroblastoma cells [17,32]. C3G plays an essential role in embryonic development as C3G knockout mice show embryonic lethality [33]. Fibroblasts from embryos lacking C3G show defective adhesion and enhanced motility. Mice compromised for C3G expression due to insertion of a hypomorphic allele have defects in multiple systems such as vascular maturation and brain cortical development [34,35]. Our finding that C3G overexpression can induce morphological changes and neurite growth in neuroblastoma cells lead us to investigate the effect of

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C3G overexpression in inducing morphological changes in non-neuronal cells. Here we show that C3G expression can induce the formation of neurite-like extensions (NLEs) specifically in aggressive breast cancer cells. Some molecular events involved downstream of C3G to mediate these morphological changes have also been investigated. C3G associates with cytoskeletal fraction of cells and its expression induces stabilization of microtubules and inhibits cell motility.

2. Materials and methods

2.1. Cell culture, transfections and treatments

MDA-MB-231, HEK 293T, MCF-7, MDA-MB-453, BT549 and HeLa cells were obtained from ATCC and grown in DMEM containing 10% FCS (CDMEM) in a humidified incubator maintaining 5% CO2. MDA-MB-231 cells were also obtained from an alternate source (UCLA) to test whether observations made were specific to a particular stock. Transfections were performed using lipofectamine-Plus reagent from Invitrogen as per manufacturer's instructions. LiCl (50 mM) and BIO (5 μM) (Calbiochem) treatment was given by adding to cells in CDMEM 6 h after transfection for a period of 24 h. Cells were subjected to cytochalasin D (Cyto D) (0.2 μg/ml) or nocodazole (NOC) (1 μg/ml) for a period of 4 h prior to fixation to disrupt microfilaments and microtubules, respectively. Microtubule stability assay was performed as described in Bosc et al. with slight modification [36]. Cells were treated with 5 μg/ml nocodazole for 30 min prior to fixation. Nocodazole, cytochalasin D and the MAPK inhibitors were from Calbiochem.

Cell fractionation for preparation of cytoskeletal fractions was carried out essentially as described by Nam et al. [37]. Cells were lysed by suspending in ice cold buffer (50 mM Tris pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM Na_2VO_4, 1 mM PMSF, protease inhibitors and 0.5% triton X-100) and incubated on ice for 10 min followed by centrifugation at 15,000g for 30 min at 4 °C. The supernatant was collected as triton soluble fraction. The pellet, containing the triton insoluble fraction, was washed with buffer without detergent. The fractions were suspended in SDS sample buffer.

2.2. Plasmids and antibodies

C3G and C3G deletion constructs have been described earlier [16,17]. TC10 T31N-HA has been described [32]. DnRac1N17myc, DnRhoAN19myc, DnCdc42N17myc and constitutively active Rap1 (CA-Rap1), HA-Rap1A-Q63E, were kindly provided by Dr Jean de Gunzburg. GFP-RapGAP was kindly provided by Dr Patrick Casey. GFP construct was from Clontech. GFP-C3G construct was made by excising out C3G form PCDNA3 using Hind III and Xho1 and cloning it into the Hind III and Sal1 sites of GFPN3 vector. GFP-Optineurin has been described [38]. Myc tagged full length β-catenin expression vector was kindly provided by Prof. Marian Waterman. DnR-Ras T43N-GFP was gifted by Dr. John Peranen. CDK2, C3G, β-catenin, α-tubulin, HA, calnexin, vimentin and myc antibodies were from Santa Cruz. Acetylated tubulin (Ac-tub) and GAPDH antibodies were from Sigma and Millipore, respectively. Rhodamine phalloidin and Oregon Green phalloidin were from Molecular Probes. C9 antibody, which detects over expressed C3G, was raised in our laboratory [17].

2.3. Western blotting, immunofluorescence, imaging and quantification of NLE

In all experiments cells were processed for lysate preparation or immunofluorescence after 30 h of transfection. Western blotting and immunofluorescence were performed as described earlier [17]. Immunofluorescence co-staining for two antigens was performed by sequential incubation of primary and corresponding secondary antibodies. Cy3 or Alexa 488 coupled secondary antibodies were used to detect expression. For methanol fixation, cells were washed once with PBS and incubated with pre-cooled methanol for 7 min at −20 °C. After the incubation, cells were washed 3 times with PBS before blocking for performing immunofluorescence. Images were taken on Carl Zeiss Apotome with Axioimager Z1 upright microscope or Leica TCS SP-5 AOBS confocal microscope and analyzed by Axiovision 4.4 or Leica Application Suite (LAS), respectively. All images were captured from a single experiment using similar parameters. Quantitation of NLEs was performed by examining the cells using a 40× objective of an Olympus fluorescent microscope. Cells positive for expression of various antigens were examined for the presence of cellular extensions greater than 2 cell diameters. Cells stained for F-actin were used to quantitate process formation in non-expressing cells. A minimum of 200 expressing cells were counted on each coverslip. Data was averaged from 3 independent experiments carried out on duplicate coverslips and represented as percentage of cells showing extensions among antigen expressing cells. Statistical significance was determined using Student's t test.

2.4. Live cell imaging and tracking of cells

For live cell imaging MDA-MB-231 cells were transfected with GFP-C3G and after 12–16 h of transfection were subjected to imaging using Carl Zeiss LSM 5 live. Images were taken after every 20 min for a period of 24 h and analyzed using LSM 5 image examiner and Meta Morph Version 6.1r0 software was used for tracking the movement of individual cells in the time frame images. For calculating average speed, cells were tracked for either a period of 24 h or till the time they divided or left the field of view. For showing the paths traced, cells were tracked for a period of 6 h and only those cells were taken into consideration which neither divided nor moved out of the field of view. Student's t test was performed to test the statistical significance of the difference in the average speeds of two cell populations.

3. Results

3.1. C3G expression induces neurite-like extensions in aggressive breast cancer cells

The ability of C3G to induce morphological changes in non-neuronal human cell lines was examined by transient overexpression for 30 h. Upon examination of C3G expression by indirect immunofluorescence it was observed that C3G expressing MDA-MB-231 and BT549 cells showed dramatic changes in cell morphology with two or more cytoplasmic extensions that resembled neurite outgrowths (Fig. 1A). C3G was distributed into the cellular processes and therefore these extensions could be easily observed. These extensions very often ranged from 50 to 200 μm. The cell body appeared rounded and smaller, compared to cells that did not express C3G. Other cell types like MCF7, HeLa, HEK293T, or MDA-MB-453 did not show NLEs upon C3G expression, even though C3G could be expressed at high levels in these cells (Fig. 1A and B). In some of these cells, C3G expression has been shown to induce filopodia formation [17]. Expression of GFP did not result in formation of long processes in either MDA-MB-231 or BT549 cells, while about 40% of C3G expressing cells showed dramatic morphological changes at 30 h of expression (Fig. 1C and D and Supplementary Fig. 1). Supplementary Fig. 1 shows representative low magnification images indicating the extent of NLE formation in C3G expressing MDA-MB-231 and BT549 cells. Short extensions of 5–10 μm length were seen in 2–3% of MDA-MB-231 cells that did not express C3G. MDA-MB-231 cells procured from a totally different source also showed similar phenotypic changes upon C3G expression (Supplementary Fig. 1). Expression of optineurin, a protein known to induce NLEs in human fibrosarcoma cells [39], also did not cause any morphological changes in MDA-MB-231 cells (Fig. 1C and D). Co-staining for F-actin showed that C3G induced processes were rich in
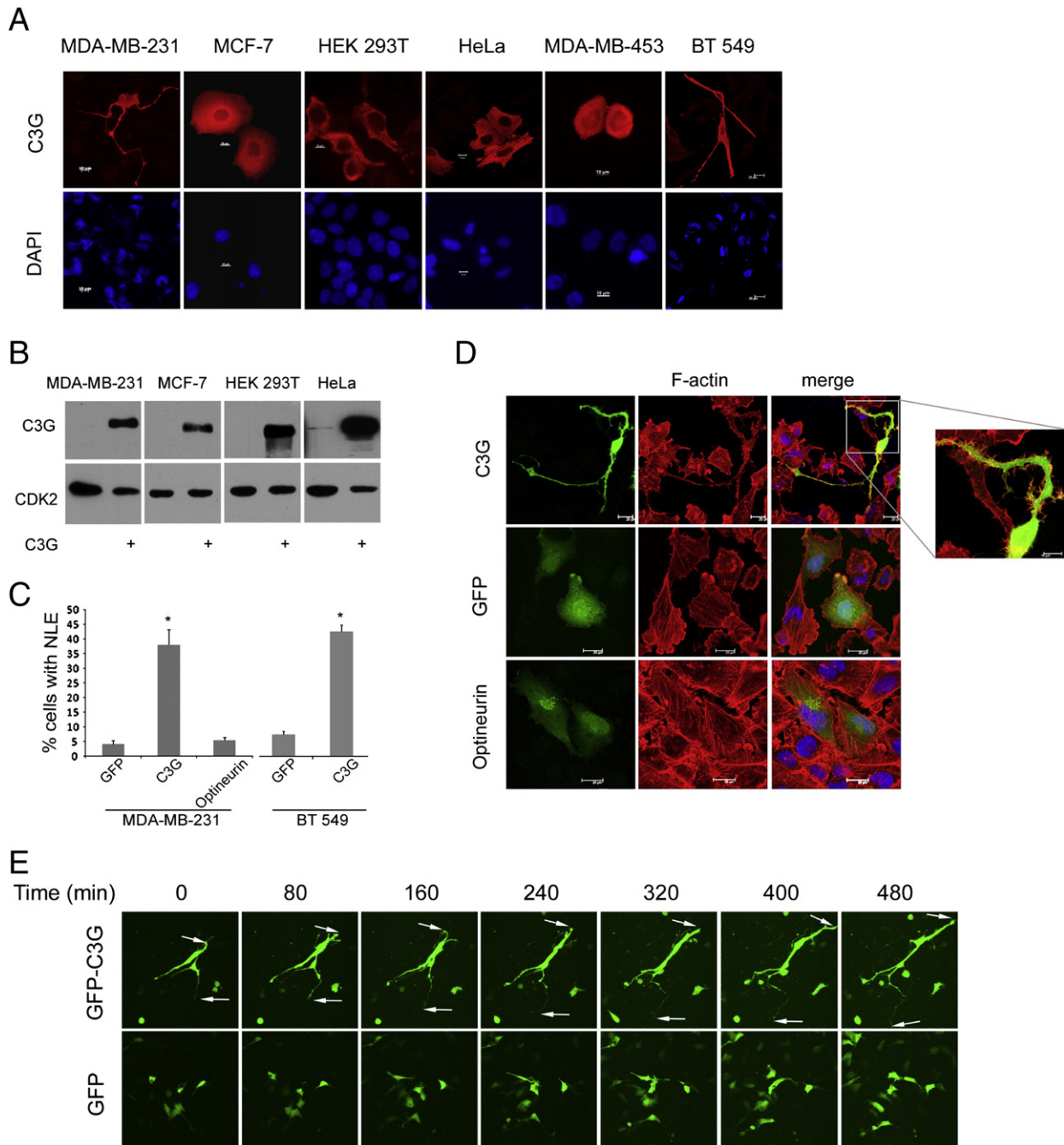
Fig. 1. C3G expression induces neurite-like extensions in MDA-MB-231 and BT549 cells. (A) Various human cell lines as indicated were transiently transfected with C3G expression plasmid for 30 h and stained with anti-C3G antibodies to visualize expressing cells. Panels show morphology of C3G expressing cells captured using fluorescence microscope fitted with a CCD camera. Bar, 10 μm. (B) Immunoblot showing expression of C3G in cell lysates upon transfection for 30 h. (C) Quantitation of cells showing NLEs among MDA-MB-231 and BT549 cells expressing C3G, GFP or optineurin. Data shows proportion of antigen positive cells showing NLEs depicted as mean ± SD obtained by quantitation of cells on duplicate coverslips from 3 independent experiments.*$p < 0.001$. (D) C3G colocalizes with F-actin in extensions. MDA-MB-231 cells expressing C3G, GFP or GFP-OPTN were costained with Rhodamine phalloidin to detect F-actin. Panels show morphology of expressing cells captured using a confocal microscope. Bar, 20 μm. Magnified region shows presence of distinct lateral processes rich in F-actin. Bar, 10 μm. (E) Time lapse images of cells expressing GFP-C3G or GFP captured using a 20× objective at 80 min intervals for a period of 6 h. Imaging was begun 12 h after transfection. Arrows show the position of the growth cone of the NLEs.

F-actin and showed the presence of nodes, branches and microspikes or lateral extensions. Though C3G showed a predominantly cytoplasmic staining, it showed colocalization with F-actin only in the cortical regions, in the extensions, and in microspikes (Fig. 1D, Supplementary Fig. 1C). Cells expressing C3G lacked stress fibers as shown earlier in HeLa cells [17]. These processes resembled extending neurites upon live cell imaging using expression of a GFP tagged C3G construct. They showed the presence of a growth cone like end with filopodia and an increase in length with time (Fig. 1E).

We also determined which domains of C3G were required for the induction of NLE by overexpressing various deletion constructs of C3G, ΔC, ΔN and CBR (depicted schematically in Fig. 2A). ΔC lacks the catalytic domain, ΔN lacks the protein interaction domain and CBR has the protein interaction domain only. ΔN C3G is known to show constitutive catalytic activity [19]. It was observed that all three deletion constructs were partially compromised in their ability to induce NLEs indicating that functions dependent on both the catalytic domain and protein interaction domain contribute to inducing
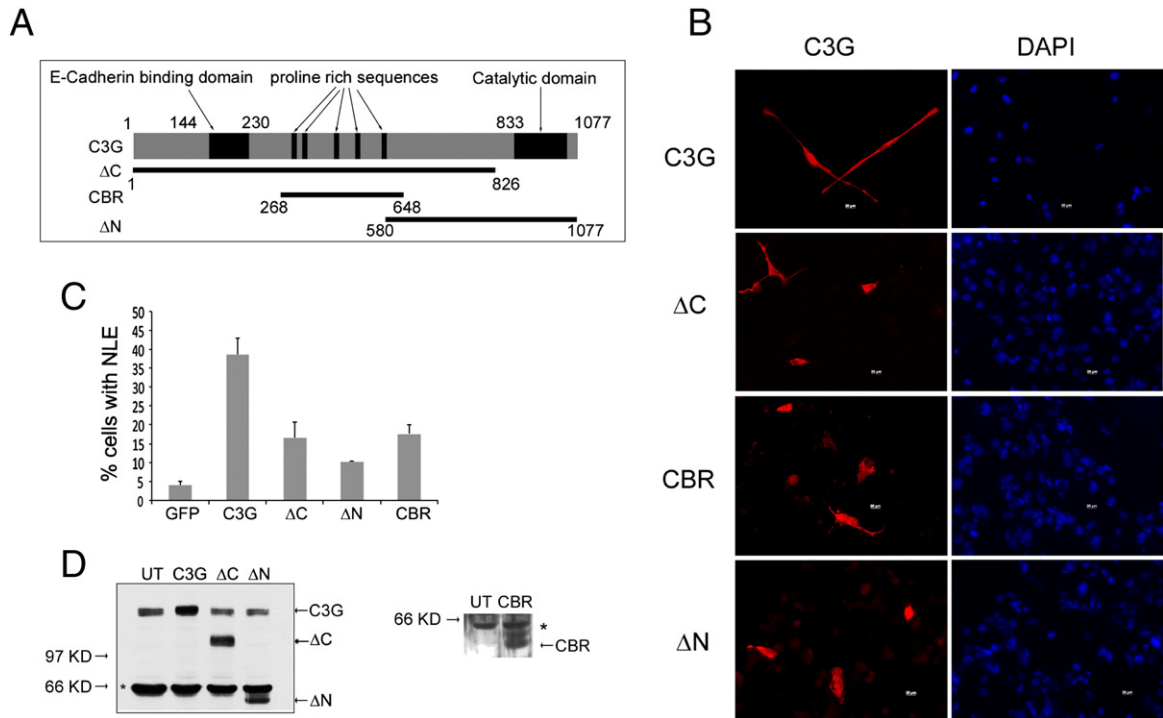
Fig. 2. C3G induces NLEs dependent on both catalytic and protein interaction domains. (A) Schematic diagram showing the various domains in full length C3G and the deletion constructs. (B) MDA-MB-231 cells were transfected with the various C3G constructs, fixed after 30 h and stained to detect expressing cells. Panels show representative fields of cells. Bar, 20 μm. (C) Quantitation of NLE formation induced by the various C3G constructs depicted as mean ± SD. (D) Western blots showing expression of C3G and its deletion constructs. UT, untransfected. *indicates a nonspecific band.

morphological changes (Fig. 2B and C). Differences in their ability to induce NLEs was not due to differences in level of expression (Fig. 2D). Earlier, it has been shown that expression of CBR represses oncogene mediated transformation just as does full length C3G [29]. This domain is known to function dominantly to inhibit catalytic function of endogenous C3G [14]. In our study, coexpression of CBR with C3G did not alter extent of NLE formation induced by C3G alone (data not shown).
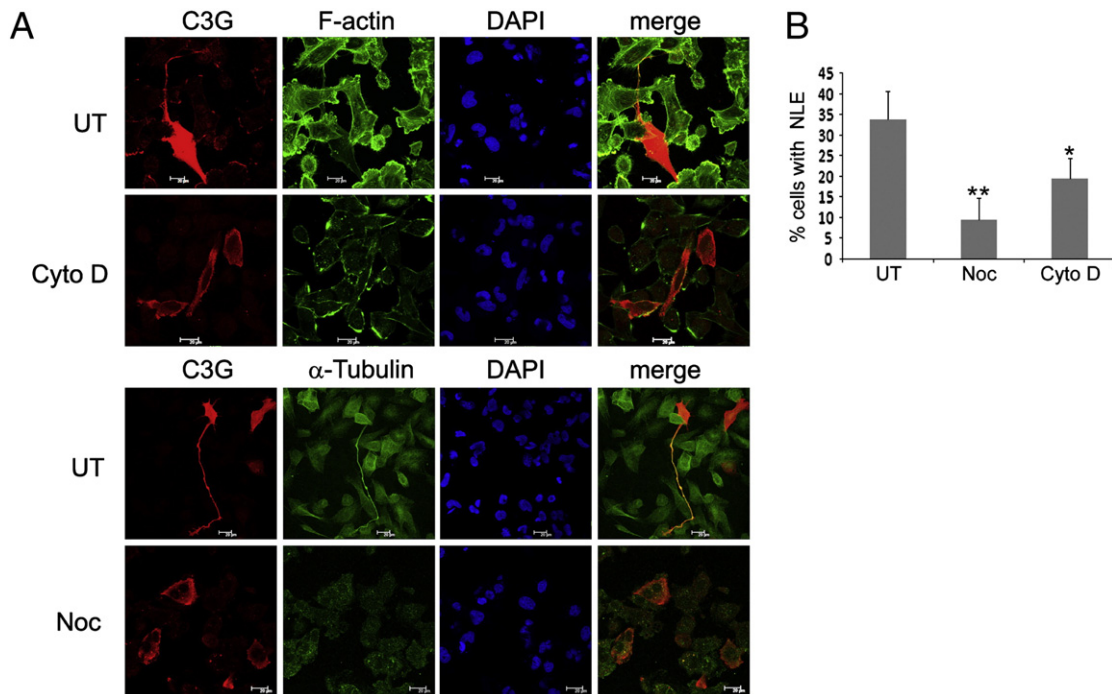

Fig. 3. NLE formation by C3G is dependent on an intact cytoskeleton. (A) C3G expressing MDA-MB-231 cells were treated with either 0.2 μg/ml cytochalasin D (Cyto D) or 1 μg/ml nocodazole (NOC) for 4 h prior to fixation. Cells were stained for C3G expression and F-actin (upper panels) or α-tubulin (lower panels). Images show morphology and staining pattern in representative fields. Bar, 20 μm. (B) Quantitation of NLE formation in C3G expressing cells treated with Cyto D or NOC. *$p < 0.001$, **$p < 0.0001$.
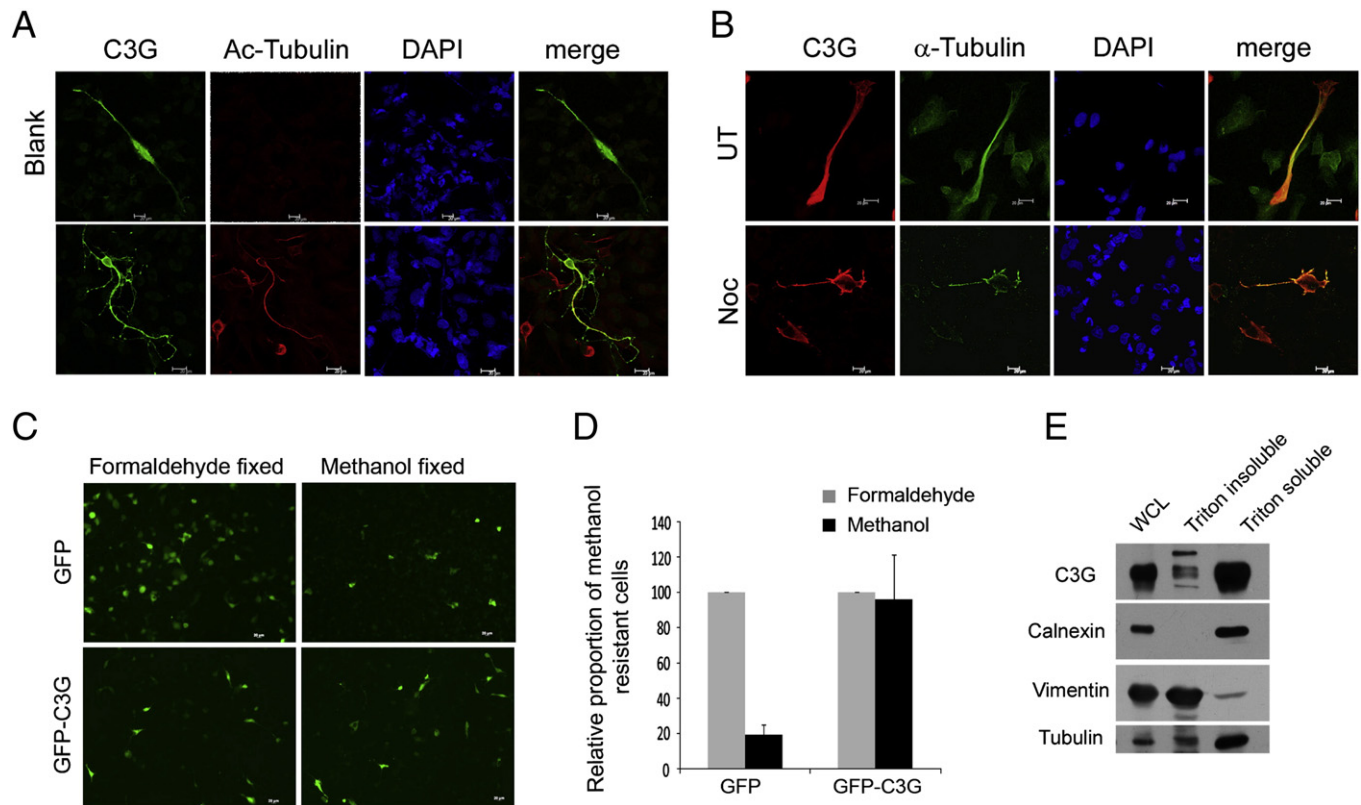
Fig. 4. C3G expression stabilizes microtubules. (A) C3G transfected cells were stained for C3G expression and Ac-tubulin. Blank panels show cells which were not incubated with Ac-tubulin primary antibody. (B) C3G transfected cells were either left untreated (UT) or subjected to treatment with NOC (5 μg/ml for 30 min) and stained for C3G and α-tubulin. Bar, 20 μm. (C) MDA-MB-231 cells were transfected with GFP or GFP-C3G and parallel coverslips fixed using either formaldehyde or methanol. Images show representative fields of cells with GFP expression. Bar, 20 μm. (D) Number of GFP expressing cells were quantitated under conditions of formaldehyde and methanol fixation. Proportion of GFP positive cells seen upon methanol fixation was represented relative to number of GFP positive cells seen upon formaldehyde fixation as 100%. (E) Presence of C3G in cytoskeletal fraction. Triton insoluble and soluble fractions were made from MDA-MB-231 cells expressing C3G for 30 h followed by Western blotting to detect the presence of C3G in these fractions. Calnexin and vimentin were used to determine the purity of triton soluble and cytoskeletal fractions respectively. WCL, whole cell lysate.

3.2. C3G expression induces changes in microtubule organization

Polymerization of microfilaments and microtubules regulates morphology of most cells. But to what extent each of these cytoskeletal elements contribute to process formation often varies. The dependence of C3G to induce processes on intact microfilaments and microtubules was tested by treatment of transfected cells with either cytochalasin D (Cyto D) or nocodazole (NOC), agents that disrupt actin filaments and microtubules (MTs), respectively. Cells expressing C3G were treated with Cyto D or NOC for 4 h prior to fixation (conditions known to disrupt actin and tubulin structures) and stained for C3G and F-actin or C3G and α-tubulin, respectively. C3G induced extensions showed distinct α-tubulin bundles in the shafts of untreated cells. Treatment resulted in loss of organized microfilaments and microtubules as shown in Fig. 3A. It was observed that both agents caused a decrease in number of C3G expressing cells with NLEs (Fig. 3A and B).

Long NLEs are known to be associated with MT rearrangement. C3G transfected MDA-MB-231 cells were stained for C3G expression and acetylated-tubulin (Ac-tubulin), a marker for stabilized microtubules [40]. It was observed that C3G induced extensions showed prominent staining for Ac-tubulin, whereas weak staining was seen in untransfected cells (Fig. 4A). The ability of C3G to affect microtubule dynamics was also examined in a stability assay which monitors nocodazole resistance as an indicator of stable microtubules [36]. C3G transfected cells were treated with 5 μg/ml NOC for 30 min before being processed for indirect immunofluorescence using antibodies against C3G and α-tubulin. It was observed that while MTs were totally disrupted with total loss of α-tubulin staining in untransfected cells, those expressing C3G retained staining for MT (Fig. 4B). α-Tubulin staining was seen even in C3G expressing cells that lacked NLEs, though not equally prominent. These results indicated that C3G expression has a stabilizing effect on microtubules.

Colocalization of C3G with actin and tubulin in cellular extensions and its ability to alter their dynamics indicated that C3G may be binding to cytoskeletal elements in the cell. This was examined by testing for resistance to methanol fixation, which is known to permeabilize cells and release cytosolic proteins while formaldehyde fixation preserves cytosolic proteins [41,42]. MDA-MB-231 cells were transfected with either GFP or GFP-C3G on parallel coverslips and fixed using formaldehyde or methanol. Cells were scored for proportion of GFP positive cells retained upon methanol fixation relative to proportion seen upon formaldehyde fixation. It was observed that only about 20% of GFP expressing cells were retained upon methanol fixation, whereas majority of GFP-C3G expressing cells were retained (Fig. 4C and D). These results indicated that C3G tagged GFP is associated with cellular structures while GFP is primarily cytosolic.

Association of C3G with cytoskeletal elements was also determined by fractionation of MDA-MB-231 cells expressing C3G. A fraction of C3G was found in the triton insoluble fraction, which essentially comprises cytoskeletal components like vimentin (Fig. 4E). The absence of

Fig. 5. C3G induced NLEs are dependent on Rap, Rac and Cdc42 function. MDA-MB-231 cells were transfected with C3G along with control plasmid, RAPGAP or Dn GTPases as indicated. Bar, 20 μm. Representative fields of cells are shown in A and B and quantitation of NLEs in C. *$p < 0.0001$.
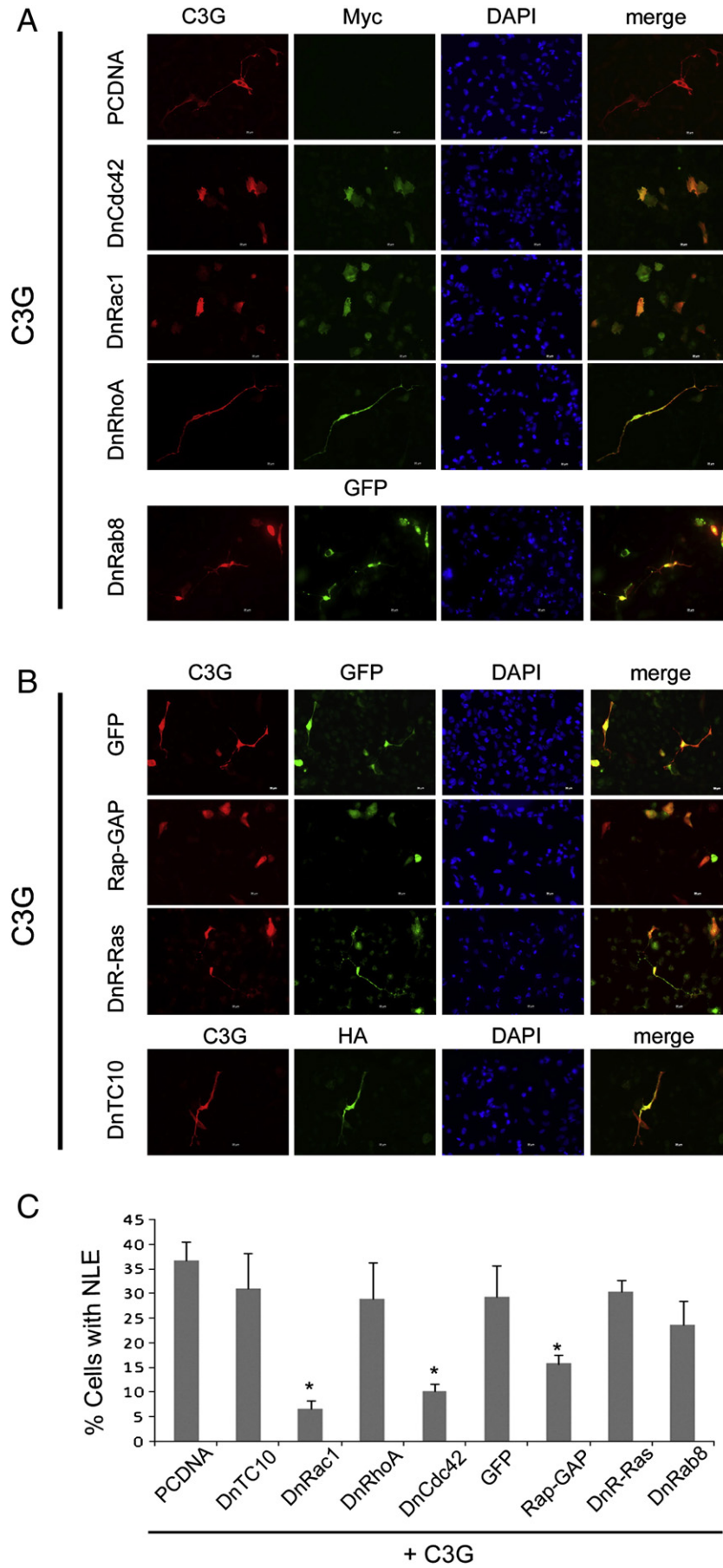
contaminating cytosolic proteins was shown by probing for calnexin, which was present exclusively in the soluble fraction.

3.3. C3G induced NLEs dependent on Rap, Rac and Cdc42

To determine the mechanism by which C3G induces NLEs, the involvement of various signaling effectors known to play a role in cytoskeletal reorganization and neurite growth was examined. Rho family GTPases RhoA, Rac1 and Cdc42 have been primarily implicated in actin reorganization responsible for morphological changes in cells [43]. Their role in C3G induced NLE formation was examined by coexpressing dominant negative (Dn) myc-tagged constructs, which have earlier been used to disrupt the function of endogenous RhoA, Rac1 and Cdc42 [44]. Quantitation of NLEs in cells coexpressing C3G with dominant negative forms of Rho, Rac or Cdc42 showed that C3G induced processes were significantly reduced in cells expressing Dn Rac and Dn Cdc42, but not Dn RhoA (Fig. 5A and C). Expression of dominant negative constructs of Rac and Cdc42 alone (in the absence of C3G) did not have an effect on cell morphology, whereas expression of Dn RhoA alone showed a significant overall increase in NLE formation compared to GFP (data not shown), suggesting that inhibition of endogenous RhoA activity enables actin reorganization for NLE formation. NLE formation induced by ΔC-C3G and CBR was inhibited significantly by Dn Cdc42 and Dn Rac, indicating that the protein interaction domain of C3G signals to cytoskeletal changes dependent on Cdc42 and Rac (Supplementary Fig 2A, and data not shown). Rap1, TC10 and R-Ras are known targets/effectors of C3G [5–7]. Since C3G catalytic activity appeared to contribute to its ability to induce extensions, we determined which of these molecules were involved in signaling downstream of C3G by coexpressing Dn TC10, Dn R-Ras and Rap-GAP, a molecule that inhibits Rap activation. It was seen that only Rap-GAP coexpression inhibited NLE formation partially indicating that Rap function contributed to C3G induced NLE formation (Fig. 5B and C). Lack of significant effect upon coexpressing Dn R-Ras or Dn TC10 suggested that they are not effective in inhibiting C3G mediated downstream signaling to cytoskeletal reorganization. Rap-GAP coexpression did not inhibit NLE formation induced by ΔC-C3G or CBR (data not shown), indicating that the protein interaction domain of C3G does not trigger Rap activation (Supplementary Fig. 2A). Expression of CA-Rap1 alone did not induce NLE formation, nor did it enhance NLEs formed by ΔC-C3G (Supplementary Fig. 2B).

Rab8, a small GTPase involved in vesicle traffic has been shown to induce NLEs upon expression of a constitutively active isoform in HT-1080 cells [39]. To test whether Rab8 activation was involved downstream of C3G in causing cellular extensions, a dominant negative variant was coexpressed. It was observed that Dn Rab8 did not repress C3G induced NLE formation; (Fig. 5A and C) nor did the expression of constitutively active Rab8 induce processes in MDA-MB-231 cells (data not shown). MAP kinases have been implicated in signaling to MT dynamics via phosphorylation of MT associated proteins. To investigate their involvement as mediators in C3G induced NLEs, C3G transfected cells were treated with inhibitors specifically for MAPK (PD 98059) or p38 (SB 203580) for 24 h and their effect on cell morphology was examined. MAPK inhibition had no effect on C3G induced process formation (Supplementary Fig. 3).

3.4. C3G expression suppresses cellular β-catenin levels in MDA-MB-231

Signaling involving APC and β-catenin are known effectors of cytoskeletal remodeling [45]. The consequence of C3G expression on cellular β-catenin levels was examined in MCF-7, HEK 293, MDA-MB-231 and BT549 cells. It was observed that though C3G overexpression was comparable, cellular β-catenin levels were significantly reduced only in MDA-MB-231 and BT549 cells upon C3G expression (Fig. 6A). Down-regulation of β-catenin levels by C3G appears to be mediated through

Rap1 activation, since expression of constitutively active Rap1 also resulted in repression of β-catenin levels (Supplementary Fig. 4). Expression of the protein interaction domain of C3G(CBR) did not alter cellular β-catenin levels. To test whether reduced β-catenin levels were responsible for the morphological changes seen, C3G expressing cells were treated with LiCl or BIO, inhibitors of GSK3-β. This treatment results in stabilization of β-catenin and increase in cellular levels. It was observed that C3G induced NLEs were significantly reduced under these conditions (Fig. 6B and C). Treatment with LiCl did not affect C3G levels, but showed an increase in β-catenin as expected (Fig. 6D). C3G-induced repression of cellular β-catenin was not seen upon LiCl treatment, suggesting that C3G may be affecting β-catenin levels through regulation of GSK-3β. To confirm that high β-catenin levels repress NLE formation, β-catenin expression vector was co-transfected with C3G and NLE formation quantitated among coexpressing cells. Fig. 6E shows reduction in NLE formation due to β-catenin coexpression.

3.5. C3G expression inhibits motility of MDA-MB-231 cells

Since formation of NLEs involves cytoskeletal changes and C3G expression results in loss of stress fibers, the consequence of C3G expression on cell motility was examined by digital time-lapse photomicrography. Cells transfected with GFP or GFP-C3G for 12 h were examined for migration for a 24 h period and total distance compared between GFP positive and negative cells. Cells at sparse density were filmed at 20 min intervals and cell trajectories traced using Meta Morph version 6.10 software (Fig. 7A). While GFP expressing cells or untransfected cells showed an average speed of 17.8 and 18.2 μm/h respectively, GFP-C3G expressing cells moved at an average speed of 8.7 μm/h indicating that C3G expression reduces cell motility (Fig. 7B). C3G expressing cells, with or without processes, showed similar motility, suggesting that reduced motility is not due to extension formation.

4. Discussion

This study demonstrates the formation of long processes specifically in highly invasive breast cancer cell lines upon enhancing C3G levels through exogenous expression. These structures are formed through rearrangement of actin cytoskeleton and microtubule network, enabling bundle formation required for elongation of long cytoplasmic extensions. Accumulation of Ac-tubulin in C3G induced extensions of MDA-MB-231 cells is similar to that seen in neurites formed in IMR-32 neuroblastoma cells upon C3G expression except that long extensions formed by 24 h in MDA-MB-231 cells unlike in the case of extensions in neuronal cells which were evident only 48–72 h after transfection [32]. Morphological similarity complemented by similarity in MT and microfilament bundling suggested that they could be called NLEs [46]. The molecular mechanisms involved in neural process initiation have not been well elucidated. Our results show that C3G associates with cytoskeletal components in MDA-MB-231 cells and therefore suggest that C3G can function to cause actin and microtubule reorganization and morphological changes required during the complex process of neuronal differentiation, in certain non-neuronal cells.

Outgrowth of neurite-like processes in response to certain extracellular stimuli has been shown in other non-neuronal cells [47]. Expression of neuronal proteins like drebrin or Neuro-p24 results in formation of NLE in fibroblasts [48,49]. C3G signaling has been implicated earlier in cell morphology as expression of Myr-CHAT induced cell protrusions with branched morphology in NIH3T3 cells dependent on Crk-C3G-Rap1 pathway [50]. p120 catenin expression resulted in Rac1 dependent formation of dendritic extensions in MDA-MB-231 and NIH 3T3 cells [51]. The Cas family docking protein, HEF1, induced NLEs in MCF-7 cells dependent on Rac and Cdc 42 [52]. ZNRF1 expression causes neurite-like morphological changes in non-neuronal cells [53]. These studies have not described cell type
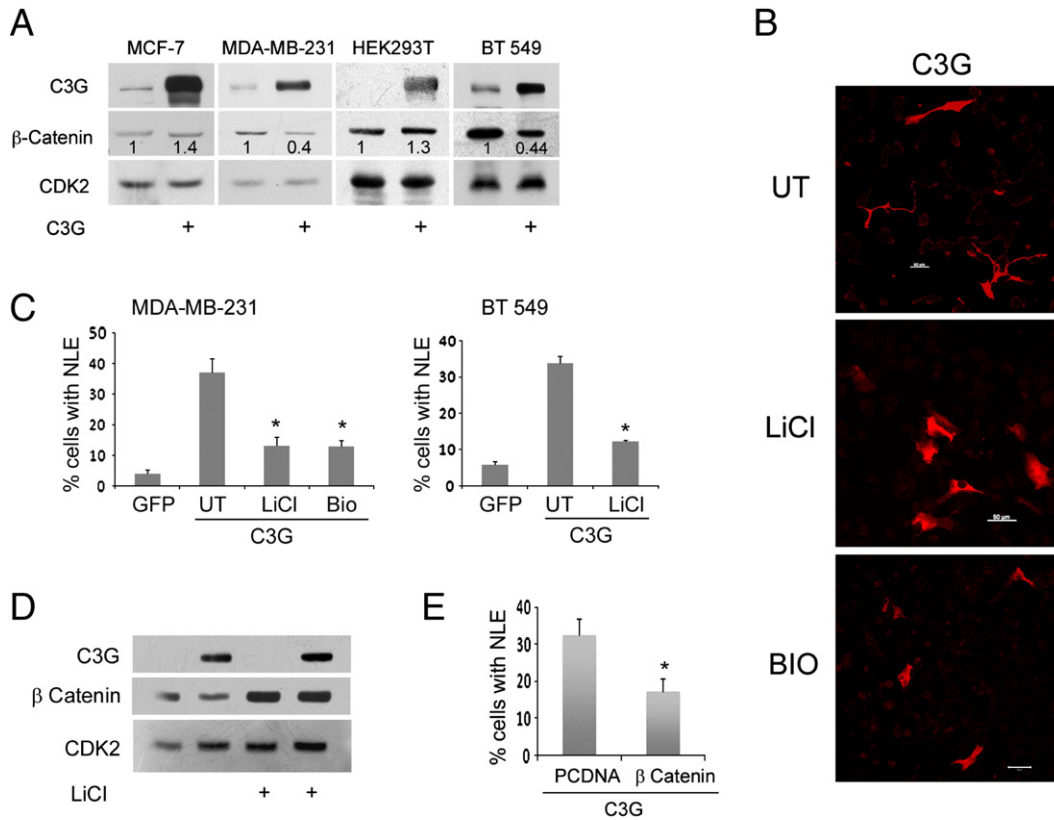
Fig. 6. C3G expression downregulates cellular β-catenin in MDA-MB-231 and BT549 cells. (A) MCF-7, MDA-MB-231, HEK293T and BT549 cells were transiently transfected with C3G expression plasmid (for 30 h) and cell lysates subjected to Western blotting for C3G, β-catenin and CDK2 (loading control). Values indicate relative levels of β-catenin in C3G expressing cells compared to non-expressing cells. (B) MDA-MB-231 cells expressing C3G were left untreated (UT) or treated with LiCl or BIO and examined for effect on cell morphology. (C) Effect of LiCl on extent of NLE formation by C3G in MDA-MB-231 and BT549 cells. $*p<0.01$. (D) Lysates of MDA-MB-231 cells treated as in (C) were subjected to Western blotting for C3G, β-catenin and CDK2. (E) β-catenin coexpression inhibits C3G induced NLE formation. Quantitation of NLE formation in MDA-MB-231 cells expressing C3G along with control or β-catenin expressing plasmids. $*p<0.001$.
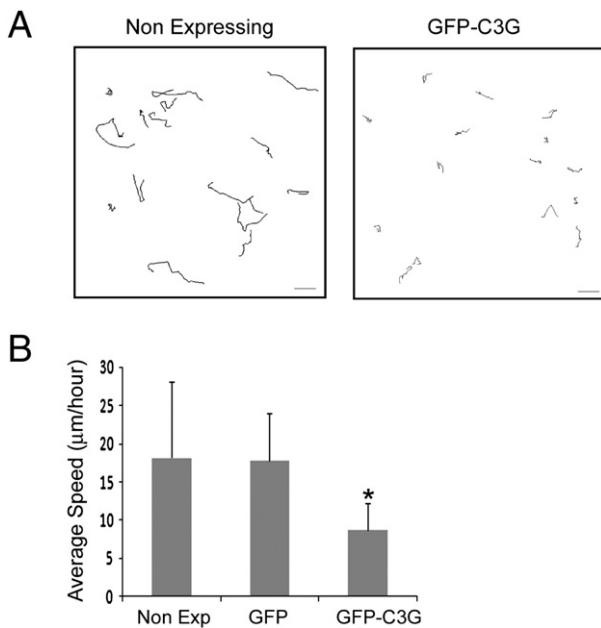

Fig. 7. C3G expression inhibits cell motility. (A) MDA-MB-231 cells transfected with GFP-C3G were subjected to live cell imaging after 16 h and imaged every 20 min for a total period of 24 h. Panels show paths of non-expressing cells and GFP-C3G expressing cells captured for a period of 6 h. Bar, 50 μm. (B) Average speed of non-expressing cells, GFP or GFP-C3G expressing cells tracked for a period of 24 h. Data was averaged using multiple cells from two independent experiments. $*p<0.0001$.

specificity and C3G seems unusual in that it is able to induce these morphological changes only in aggressive breast cancer cells.

Rac and Cdc42 are known mediators of actin based changes at the cell membrane such as lamellipodia and filopodia formation. Our results show that C3G induces NLE by engaging the functions of Rac and Cdc42. This function is mediated by the protein interaction domain of C3G. RhoA activity, which functions in organizing intracellular stress fibers, did not contribute to signaling downstream of C3G. Rho has been described as a neurite inhibitory GTPase and Rac, a neurite promoting GTPase [51]. As shown earlier [46] in our experiments too, RhoA inhibition by a dominant negative construct in the absence of C3G caused NLE formation, indicating that C3G may also function by inhibiting RhoA to cause cytoskeletal reorganization. C3G expression causes loss of stress fibers, which are required for formation of mature focal contacts, required for normal motility of cells [54]. Therefore defective motility in C3G expressing cells may be due to defects in formation of cell substratum adhesions. These results are also in agreement with findings showing enhanced motility of fibroblasts from C3G deficient mouse embryos [33]. Deregulated expression of C3G therefore has implications in suppressing migration in this cell line derived from an invasive tumor.

CA-Rap1 did not result in NLE formation in these cells, though it could signal to reduction in β-catenin levels, indicating that multiple effectors may be engaged by C3G to bring about these morphological changes. Rap1 has recently been described as a negative regulator of β-catenin in endothelial cells [55]. Our results using the deletion constructs showed that protein interaction domain, as well as catalytic domain, is compromised for NLE formation, indicating that various C3G domains have the ability of signaling to cytoskeletal changes. The fact that no additive effect was seen upon coexpression

of CBR with C3G may be due to insufficiency of endogenous molecules engaged by C3G and CBR in signaling to cytoskeletal remodeling. Expression of CA-Rap1 along with protein interaction domain of C3G did not enhance NLE formation to the same extent seen upon expressing full length C3G, indicating that Rap activation state turnover is important for affecting NLE formation. Activation of Rap1 at distinct subcellular locales may also be important for NLE formation, and difference in location of CA-Rap1 compared to that of C3G may be responsible for the inability of CA-Rap1 to induce NLE formation. The adaptor protein CrkII is known to constitutively associate with C3G and aid in its membrane targeting [18]. But expression of Dn CrkII constructs did not affect C3G induced morphological changes, indicating that CrkII does not contribute to causing NLEs formed by C3G.

C3G induced neurite-like process formation correlated with a reduction in cellular β-catenin levels, with forced stabilization of endogenous β-catenin by GSK-3β inhibition or exogenous expression repressing these morphological changes. β-Catenin functions in regulating cell proliferation and metastasis by signaling to actin cytoskeleton changes [54] and β-catenin mediated signaling has been shown to negatively regulate retinal neurite extension [56]. Reduced β-catenin has also been shown to increase extensions in HeLa cells [57]. Reduction in β-catenin protein expression is associated with reduced growth in MDA-MB-231 cells [58]. C3G deficient neuroepithelial cells accumulate β-catenin and fail to exit cell cycle *in vivo* [35]. Effect of C3G on β-catenin levels is cell type specific and correlated with NLE formation, since destabilization was not seen in either MCF-7 or HEK-293 cells. Our results are consistent with the suggestion that C3G induces NLE in cells through multiple mechanisms involving properties such as (1) association with cytoskeleton, (2) signaling to activate Rho family GTPases, Cdc42 and Rac mediated by its protein interaction domain, (3) activation of Rap, and (4) repression of β-catenin levels. Ability of C3G to signal to both actin microfilaments and microtubules in the cell by engaging multiple effectors like Rac, Cdc42, Rap and β-catenin is indicative of its role as an upstream regulator modulating multiple signaling pathways required for cell shape changes.

MDA-MB-231, breast cancer cells derived from a metastatic pleural effusion, have been described as a highly dedifferentiated mesenchymal like cell line. BT549 cells derived from a breast and invasive ductal tumor has been described to have mesenchymal properties. These two cell lines are models for more aggressive metastatic disease [59]. It is not totally evident as to why C3G induces dramatic changes in morphology only in highly metastatic cells. Invasive and aggressive breast cancer cells are known to have unique molecular signatures [60] and our results are indicative of unique properties possessed by cell lines derived from highly invasive breast cancer cells. Recent studies have shown that MDA-MB-231 cells express neuronal and vascular markers [61,62]. One recent hypothesis suggests that tumors arise from cells which retain pluripotency and can therefore be induced to differentiate into distinct lineages [63]. Some breast cancer cells also show lineage infidelity [64]. These cells may therefore serve as a model to study neuronal differentiation of mesenchymal cells.

5. Conclusions

Findings from the present study show that C3G deregulation through overexpression remodels microfilaments and microtubules to induce a neuronal phenotype, and inhibit motility of highly invasive breast cancer cells. MT stabilization and NLE formation represent previously undescribed functions of C3G. C3G induced NLE formation was dependent on Rho family GTPases and destabilization of β-catenin. This study advances our understanding of how signaling components regulate the cytoskeleton for causing the extensive plasticity involved in formation of NLEs. It raises the possibility of trans-differentiating easily accessible breast cancer cells to adopt neuronal morphology for enabling an understanding of complex signaling events required for acquiring a neuronal phenotype. Our findings also suggest that through suppression of cell motility, C3G overexpression could help in inhibiting metastatic properties of invasive breast cancer cells.

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