Biochimica et Biophysica Acta 1853 (2015) 2629–2639

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

C3G (RapGEF1), a regulator of actin dynamics promotes survival and myogenic differentiation of mouse mesenchymal cells

K. Sasi Kumar, A. Ramadhas, S.C. Nayak, S. Kaniyappan, K. Dayma, V. Radha *

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 17 June 2015
Accepted 27 June 2015
Available online 30 June 2015

Keywords:
C3G
RapGEF1
C2C12 cells
Myogenic differentiation
Adhesion
Survival

ABSTRACT

RapGEF1 (C3G) is a ubiquitously expressed protein that is essential for mammalian embryonic development. We have shown earlier that C3G regulates cytoskeletal dynamics and is required for neuronal differentiation. To determine if C3G plays a wider role in differentiation of multiple tissue types, we examined its role in skeletal muscle differentiation using the model system of C2C12 cells in culture. C3G protein is highly expressed in mouse skeletal muscle and its transcript and protein levels increase as C2C12 cells are induced to differentiate. Increase in C3G was predominantly seen in the nuclei of myotubes. Ectopic expression of C3G promoted myotube formation when cells were cultured in growth as well as differentiation medium and, enhanced MHC levels were associated with C3G expression. C3G induced differentiation required its catalytic and protein interaction domains and was dependent on the function of cellular R-Ras. Knockdown of cellular C3G using small hairpin RNA reduced expression of muscle specific markers and β-catenin, resulting in impaired differentiation. Disabling C3G function also resulted in enhanced cell death suggesting that cellular C3G is required for cell survival. In cells grown in growth medium, over-expressed C3G increased Akt activity, and C3G knockdown reduced it. C3G expression also suppressed cyclin D1 levels, and induced p27 expression, molecules involved in regulating cell proliferation. Endogenous C3G localizes to focal adhesions in myotubes and C3G expressing cells show distinct stress fibers, elongation and parallel alignment. Expression of a dominant negative construct of C3G, disrupts actin cytoskeleton and formation of focal adhesions resulting in detachment of cells from the substratum and inhibition of differentiation. Our results provide evidence that C3G plays an important role in myogenic differentiation by coordinating cell cycle exit, actin dynamics and survival signaling.

1. Introduction

Early mammalian development involves coordinated regulation of cell renewal and achievement of specialized functions through the process of cell cycle arrest and differentiation. Development of the skeletal muscle involves differentiation of myoblasts into myocytes which fuse to form multinucleated myotubes [1]. These steps are under the control of myogenic transcription factors like MyoD and myogenin [2]. These events are also recapitulated in adult skeletal muscle in response to injury [3]. Myotube formation involves changes in cytoskeletal organization and membrane fusion, in addition to altered gene expression [4]. Molecular determinants that signal to these events are not fully understood.

Guanine nucleotide exchange factors (GEFs) responsible for activation of small GTPases serve as important regulators of signaling pathways leading to diverse cellular functions. Through activation of Rap sub-family of GTPases, RapGEF1 (C3G) is implicated in integrin mediated signaling and cytoskeletal remodeling; thereby controlling processes such as proliferation, differentiation, transformation and apoptosis [5]. C3G is a 140 kDa, multi-domain protein with catalytic sequences at the C-terminal; and multiple proline rich sequences in the central region (known as Crk binding region or CBR) through which it interacts with SH3 domain containing molecules like Crk, Src family kinases (SFKs) and c-Abl [6–8]. A region in the N-terminal is responsible for interaction with E-cadherin [9]. The non-catalytic sequences are known to negatively regulate exchange factor activity of C3G [10]. C3G is activated by tyrosine phosphorylation at Y504, and through interaction with Crk, and membrane association [11–13]. C3G has effector functions dependent on both, or either its catalytic domain and protein interaction domains. C3G is ubiquitously expressed and shows conservation across vertebrate species as well as in *Drosophila* [5]. It is expressed in fetal and adult mouse tissues and in several human cell lines. Adult skeletal muscle and placenta show high expression levels of C3G transcripts [14,15]. Mouse models lacking C3G show early

Abbreviations: GM, growth medium; DM, differentiation medium; GEF, guanine nucleotide exchange factor; MHC, myosin heavy chain; CBR, Crk binding region; ShRNA, small hairpin RNA; FAs, focal adhesions; SFK, Src family kinase; Cl-casp-3, cleaved caspase-3; Dn, dominant negative; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

* Corresponding author.
E-mail address: vradha@ccmb.res.in (V. Radha).

http://dx.doi.org/10.1016/j.bbamcr.2015.06.015
0167-4889/

embryonic lethality [16]. Studies on mice expressing a hypomorphic allele have shown defects in vascular and nervous system development [17]. Null mutants of C3G in *Drosophila* show partial lethality with defects in muscle architecture [18].

C3G is an interacting partner of c-Abl and is required for actin cytoskeletal remodeling and filopodia formation [7]. In neuroblastoma cells, C3G overexpression induces neurite growth and its knockdown compromises NGF induced differentiation [19]. C3G levels are upregulated during differentiation of hematopoietic cells and neuronal cells, suggesting that C3G plays an important role in differentiation of multiple cell types. Our recent studies showed that C3G also interacts with β-catenin and functions as a repressor of β-catenin dependent transcription [20]. β-Catenin is a major effector of Wnt signaling pathway and regulates cell proliferation and differentiation during embryonic development and tissue morphogenesis [21].

We therefore aimed at investigating a role for C3G in skeletal muscle differentiation using cell culture model of mouse mesenchymal C2C12 cells that can be induced to form multinucleate muscle fibers positive for myosin heavy chain (MHC) under conditions of growth in low serum [22]. Molecular events in myogenic differentiation of C2C12 mouse mesenchymal cells are known to mimic events that take place in vivo during muscle development. Increase in C3G RNA as well as protein during C2C12 differentiation suggested that it may play a role in myogenic differentiation. This was examined by determining the consequence of overexpressing or knocking down C3G. C3G overexpressing cells show myogenic differentiation reflected by enhanced myotube formation and expression of muscle specific protein, MHC. Disabling C3G function compromised myogenic differentiation and caused cell detachment and death. Activation of Akt and repression of cyclin D1 are some of the molecular changes regulated by C3G in these cells.

2. Materials and methods

2.1. Cell culture, differentiation, transfections and treatments

Mouse myoblast cell line C2C12, was grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum. H9c2 rat myocardial cells were cultured in DMEM containing 10% fetal calf serum. C2C12 cells at 90% confluency were washed with DMEM and induced to differentiate with differentiation medium (DM) containing 2% horse serum in DMEM for 72–96 h. Differentiation medium was changed every day during the period of differentiation. Mouse embryonic fibroblasts (MEFs) derived from SV129XC57BLXCD1 mice were plated on gelatin (0.01%) coated dishes and grown in differentiation medium for 24–144 h. H9c2 cells were differentiated by culturing sub-confluent cells in DMEM containing 1% fetal calf serum for 5 days. For transfecting C2C12 cells, Lipofectamine-Plus reagent and Lipofectamine LTX from Invitrogen were used as per manufacturer's instructions. Transfection efficiency was generally in the range of 25–30%. PV treatment (inhibits cellular tyrosine phosphatases and enables monitoring tyrosine phosphorylation on proteins), was given as previously described [23]. C2C12 cells were treated with 10 μM wortmannin (inhibitor of PI3K) for 18 h prior to sample preparation. MAP kinase inhibitors, PD 98059 (20 μM), which inhibits ERK, or SB 203580 (10 μM), which inhibits p38 were added to cells along with DM for 72 h.

2.2. Expression vectors and antibodies

Vectors expressing C3G and C3G-GFP, have been described previously [8,24]. pEGFP vector was from Clontech. Control vector pcDNA3.1 was obtained from Invitrogen Life Technologies. N and C terminal C3G deletion constructs lacking N-terminal (ΔN) and C-terminal (ΔC) were gifts from Dr M. Matsuda, Department of Pathology, Research Institute, International Medical Centre of Japan, Tokyo. CBR expressing plasmid which lacks both N and C terminal was a gift from Dr P.J.S. Strok, Oregon Health and Science University. GFP-Rap1GAP was provided by Dr Patrick Casey, Duke University School of Medicine. GFP-R-Ras43N dominant negative construct was gifted by Dr Johan Peranen, Institute of Biotechnology, and University of Helsinki, Finland. Adenoviral vectors were generated using the AdEasy System provided by Dr Bert Vogelstein (Johns Hopkins Kimmel Cancer Center, Baltimore, MD and Johns Hopkins Oncology Center Baltimore, MD, USA). C3G was isolated from C3G-GFP N3 by BamH1, followed by end filling with Klenow and Hind III digestion, and cloned into the pShuttle-CMV plasmid digested with Hind III and EcoR V, resulting in pShuttle-CMV-C3G. CBR was isolated from CBR-pcDNA3 by Hind III and Xba I digestion and cloned into the pShuttle-CMV plasmid digested with Hind III and Xba I under the control of the CMV promoter terminated by SV40 polyadenylation signal, resulting in pShuttle-CMV-CBR. Plasmid pAdTrack-CMV was used as control vector. AdEasier cells were used to generate recombinant plasmids by homologous recombination. These recombinant adenoviral plasmids were used for transfections in HEK 293T cells using Lipofectamine 2000 (Invitrogen). After 18 days, cells were collected with media and subjected to three freeze–thaw cycles, and the supernatant containing virus was used for further rounds of infection to achieve high titer. Infection was done with supernatant for 4 h in C2C12 cells and 60–80% cells showed expression by 48 h of infection.

pmU6, a U6 promoter-based vector was used to clone ShRNA directed against mouse C3G (ShA and ShC). Mouse C3G sequence targeted by ShA was from nucleotides 3324 to 3342 and the sequence targeted by ShC was from 331 to 349 (GenBank™ accession NM_001039087.1). ShRNA having unrelated sequence of same length cloned in pmU6 was used as control (con-Sh). C3G, pY514C3G, β-catenin, α-tubulin, myogenin, and CDK2 antibodies were from Santa Cruz (California, USA). GAPDH, vinculin and actin antibodies were from Millipore (San Diego, CA, USA). Cyclin D1, cleaved caspase 3, Akt and p-Akt (pS473) antibodies were from Cell Signaling (Danvers, Massachusetts, USA). Anti-MHC (A4-1025) mouse monoclonal antibody was derived using a clone gifted by Prof. Blau H.M. (Department of Microbiology and Immunology, Stanford University School of Medicine, CA). C9 antibody, which detects over-expressed C3G, was raised in our laboratory [7].

2.3. Indirect immunofluorescence and western blotting

Cells were fixed either with 4% formaldehyde or cold methanol and immunostaining carried out as described earlier [8]. For visualization of proteins, immunofluorescence was performed using secondary antibodies tagged with either Cy3 (Amersham) or Alexa 488 (Invitrogen). Cells were stained for F-actin using Rhodamine phalloidin (Molecular Probes). Non-specific signals due to secondary antibodies were ruled out by carrying out incubations without adding primary antibodies. This is indicated as blank in the figures. Images of stained cells were captured using Leica TCS SP-5 AOBS confocal microscope from Leica Microsystems (Germany), or Axioimager Z1 upright fluorescence microscope from Carl Zeiss (GmBH, Jena, Germany). Western blotting was done as described earlier [8]. Densitometric analysis of bands normalized with corresponding loading controls was used to quantitate the protein levels.

2.4. Quantitation of myotube formation, myotube length and apoptosis

Transfected cells were differentiated for the indicated time, fixed and stained with MHC antibody. Cells positive for MHC with three or more nuclei were considered as myotubes. Percentage of myotubes was calculated from ratio of expressing cells with three or more nuclei to that of total number of transfected cells. Cell length was measured using Carl Zeiss Axiovision Rel 4.7 software. Apoptotic cells were scored by morphological observation as described earlier [8]. Expressing and non-expressing cells that showed condensed chromatin, cell shrinkage, membrane blebbing, and apoptotic body formation were scored as apoptotic. Data represent mean ± s.d. of percent apoptotic cells after background (percent apoptosis in non-expressing cells) subtraction.

2.5. Quantitative and semiquantitative RT-PCR

RNA isolation was done using Trizol reagent (Sigma). Superscript first strand synthesis kit (Invitrogen) was used to make cDNA from RNA. For Quantitative PCR, the RNA was treated with DNase I. PCR & Q-PCR were performed using the cDNA for amplification of C3G, myogenin, MHC, GAPDH and actin using the same master mix of template. Primers used for C3G were F-5′TCCTCCTTCCGAGCCTA3′, and R-5′CCACCGCTTGGAGAAGTT3′; for myogenin were F-5′TGCCGTGGGC ATGTAAGGT3′, and R-5′TGCGCAGGATCTCCACTTTAG 3′; for actin were F-5′GGCTGTATTCCCCTCCATCG3′ and R-5′CCAGTTGGTAACAATGCCAT GT 3′; for MHC were forward, 5′ CTTCAAGTTTGGACCCACGGT 3′ and reverse 5′AGCCTCGATTCGCTCCTTTT 3′; and for GAPDH were F, 5′ TGAAGTCGCAGGAGACAACCT 3′ and R 5′ ATGGCCTTCCGTGTTCCTA 3′. Densitometric analysis of bands normalized with corresponding loading control was used to quantitate RNA levels.

2.6. Statistical analysis

Student's t test was used to determine the statistical significance of experiments.

3. Results

3.1. C3G expression increases during myocyte differentiation

A role for C3G in myogenic differentiation was investigated by examining C3G expression during the process of differentiation of C2C12 mouse myoblasts. Lysates of C2C12 cells grown in growth medium (GM) or differentiation medium (DM) were prepared and subjected to western blotting. C3G protein levels increased during differentiation concomitant with an increase in myogenin, a muscle specific differentiation marker (Fig. 1a). Results of several experiments show that C3G

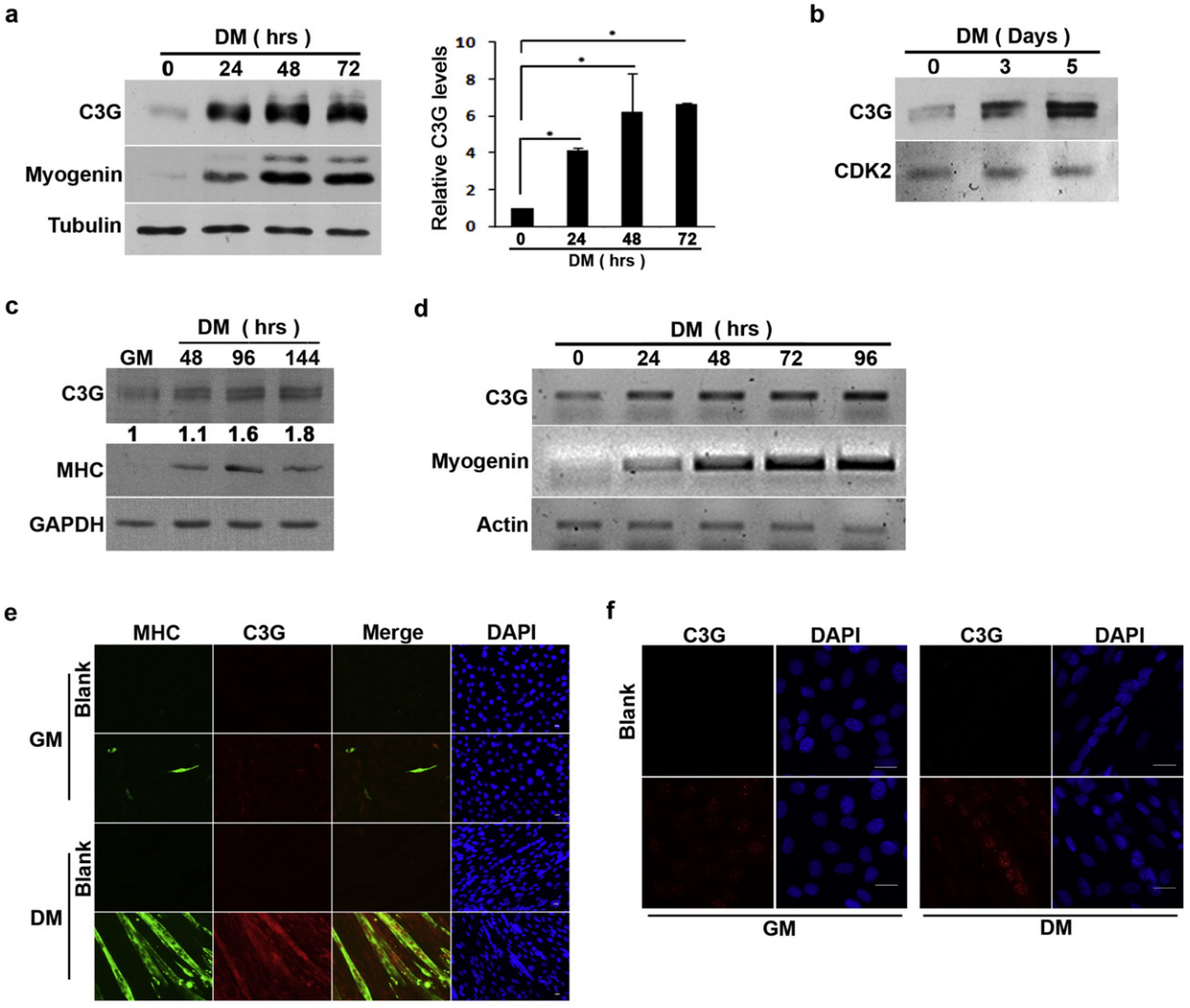

Fig. 1. C3G levels are upregulated during skeletal muscle cell differentiation. (a) C3G protein levels increase during C2C12 differentiation. Undifferentiated (0) and differentiated C2C12 cell lysates were collected at 24 h intervals and subjected to western blotting for detection of C3G, myogenin and tubulin using indicated antibodies. Average change in levels of C3G from 3 independent experiments using tubulin as protein loading control is indicated in the bar diagram. *$p < 0.05$ (b) C3G levels increase during H9c2 cell differentiation. Exponentially growing cells, 3-day and 5-day differentiated H9c2 cell lysates were used for western blotting to detect C3G levels. CDK2 was used as protein loading control. (c) C3G protein increases during myogenic differentiation of MEFs. Lysates of MEFs differentiated for the indicated time were examined for C3G expression. MHC was used as a marker of skeletal muscle differentiation and GAPDH as protein loading control. (d) C3G transcript levels are upregulated upon C2C12 differentiation. Semiquantitative PCR was carried out using cDNA prepared from RNA extracted from undifferentiated (0 h), and differentiated C2C12 cell lysates made at 24 h intervals. (e) C3G expression and localization in C2C12 myoblasts and myotubes. Undifferentiated C2C12 cells (GM) and 96 h differentiated C2C12 cells (DM) were fixed, costained with C3G and MHC antibodies to detect myotubes. Blank images represent cells that were not incubated with C3G and MHC primary antibodies. Images were captured using a fluorescence microscope. Bar, 20 μm. (f) C3G predominantly localizes to myotube nuclei. Exponentially growing (GM) and 96 h differentiated C2C12 cells (DM) were fixed with methanol, and stained with C3G antibody. Blank images represent cells not incubated with C3G antibody. Images were captured using confocal microscope. Bar, 25 μm.

levels on an average increase by 6-fold at 48 h. An increase in C3G levels was also observed upon differentiation of H9c2 rat cardiomyocyte cells (Fig. 1b) and during myogenic differentiation of mouse embryonic fibroblasts (Fig. 1c). The increase in C3G during differentiation was also examined at the transcript level using semi-quantitative RT-PCR of samples prepared at different times during differentiation. C3G transcripts increased coincident with increase in myogenin transcript levels (Fig. 1d). C3G protein is also highly expressed in mouse skeletal muscle tissues (Supplementary Fig. S1a). Treatment of cells with ERK and p38MAPK inhibitors (PD98059 & SB203580 respectively) during differentiation showed that the increase in C3G protein levels was dependent on the activity of ERK, and not on p38MAPK (Supplementary Fig. S1b). ERK is activated in a biphasic manner during C2C12 differentiation [25], and may signal to C3G upregulation.

Increased levels of C3G protein were particularly observed in MHC positive myotubes compared to myocytes which did not fuse (Fig. 1e). Endogenous as well as exogenously expressed C3G was earlier shown to predominantly localize to the cytoplasmic compartment [9,14,23,26]. Since C3G levels increased during differentiation and appeared to localize to the nucleus, we examined sub-cellular localization of C3G protein in myocytes and myotubes by confocal microscopy. Single section through the center of the nucleus showed very weak staining of C3G in myocytes, but prominent staining in the nuclei of differentiated myotubes (Fig. 1f). In addition to an increase in C3G protein level, we also observed an increase in tyrosine phosphorylated C3G (pY514) as cells were induced to differentiate (Supplementary Fig. S2). This was detected under conditions of inhibition of tyrosine phosphatases by pervanadate (PV) treatment, which enables detection of p-tyrosine on proteins. Tyrosine phosphorylation is known to enhance catalytic activity of C3G.

3.2. Overexpression of C3G enhances differentiation of C2C12 cells

The upregulation of C3G in various models of muscle differentiation suggested that it may play an important role in myogenesis. We therefore examined the consequence of C3G overexpression in C2C12 cells grown in GM or DM. Plasmids expressing GFP, or a fusion protein of C3G with GFP were transfected into cells for 96 h and the morphology of GFP positive cells examined. Compared to the fibroblast-like morphology seen in GFP expressing cells, C3G expressing cells were multinucleate, spindle shaped and elongated when cultured in GM. As seen in Fig. 2a, C3G overexpressing cells tended to fuse and form myotubes even in GM. When grown in DM, C3G expression resulted in extensively branched myotubes with multiple nuclei compared to that in GFP expressing myotubes. A significant increase was seen in number of myotubes formed by C3G over-expression (Fig. 2b). C3G induced differentiation was also examined by testing the expression of MHC under conditions of C3G overexpression in C2C12 cells. Western blotting (Fig. 2c) and indirect immunofluorescence (Fig. 2d) showed enhanced MHC levels in C3G expressing cells. The effect of C3G expression on myogenic transcripts, myogenin & MHC in cells cultured in GM was examined by real-time PCR and semi quantitative RT-PCR. At 48 h of C3G infection, levels of both transcripts showed a significant increase (Fig. 2e & f).

We have earlier shown that C3G can initiate signaling independent of its catalytic activity. To examine if the ability of over-expressed C3G to induce differentiation was dependent on its catalytic domain, deletion constructs having only the catalytic domain (ΔN-C3G) or not having the catalytic domain (ΔC-C3G) were examined for their ability to induce myocyte fusion (Fig. 3a). The ΔN-C3G construct is known to show constitutive catalytic activity towards Rap [13] and ΔC-C3G is

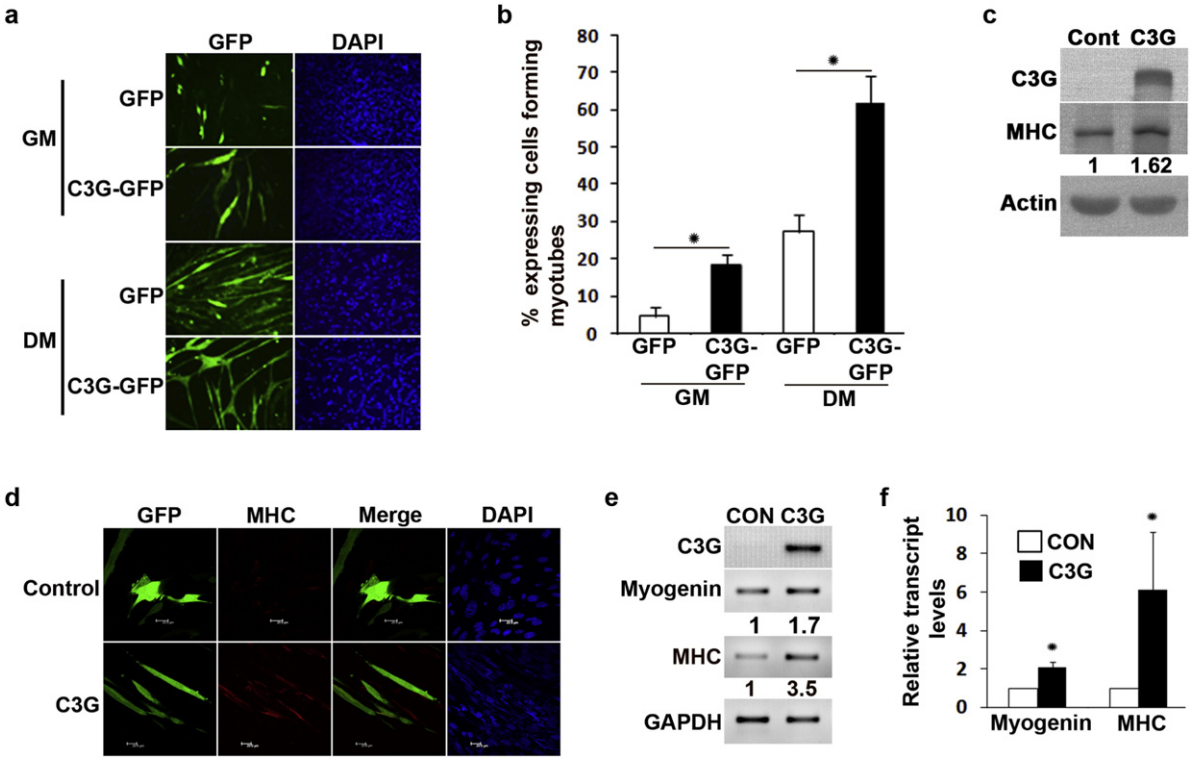

Fig. 2. C3G over-expression promotes myotube formation. (a) C2C12 cells were transiently transfected with either C3G-GFP or GFP, and grown in GM or DM for 96 h, fixed and images captured to show myotube formation. (b) Quantitation of myotube formation (cells with 3 or more nuclei) in GFP and C3G-GFP expressing cells cultured in GM or DM for 72 h.*$p < 0.05$. (c) C3G over-expression increases MHC expression. C3G or pcDNA3.1 plasmids were used to transiently transfect C2C12 cells for 48 h and lysates were subjected to western blotting using C3G, MHC and actin antibodies. Numbers indicate relative change in MHC level upon C3G expression using actin as internal control. (d) Control and C3G adenovirus infected C2C12 cells were grown for 96 h, fixed and stained for MHC. Images were captured using fluorescence microscope. Bar, 20 μm. (e) Myogenin & MHC transcript levels in C2C12 cells expressing control virus or C3G virus for 48 h. Numbers indicate change in transcript level compared to control, normalized with GAPDH. (f) Real time PCR based quantitation of expression of myogenic transcripts in C3G virus expressing cells compared to control. *$p < 0.05$.

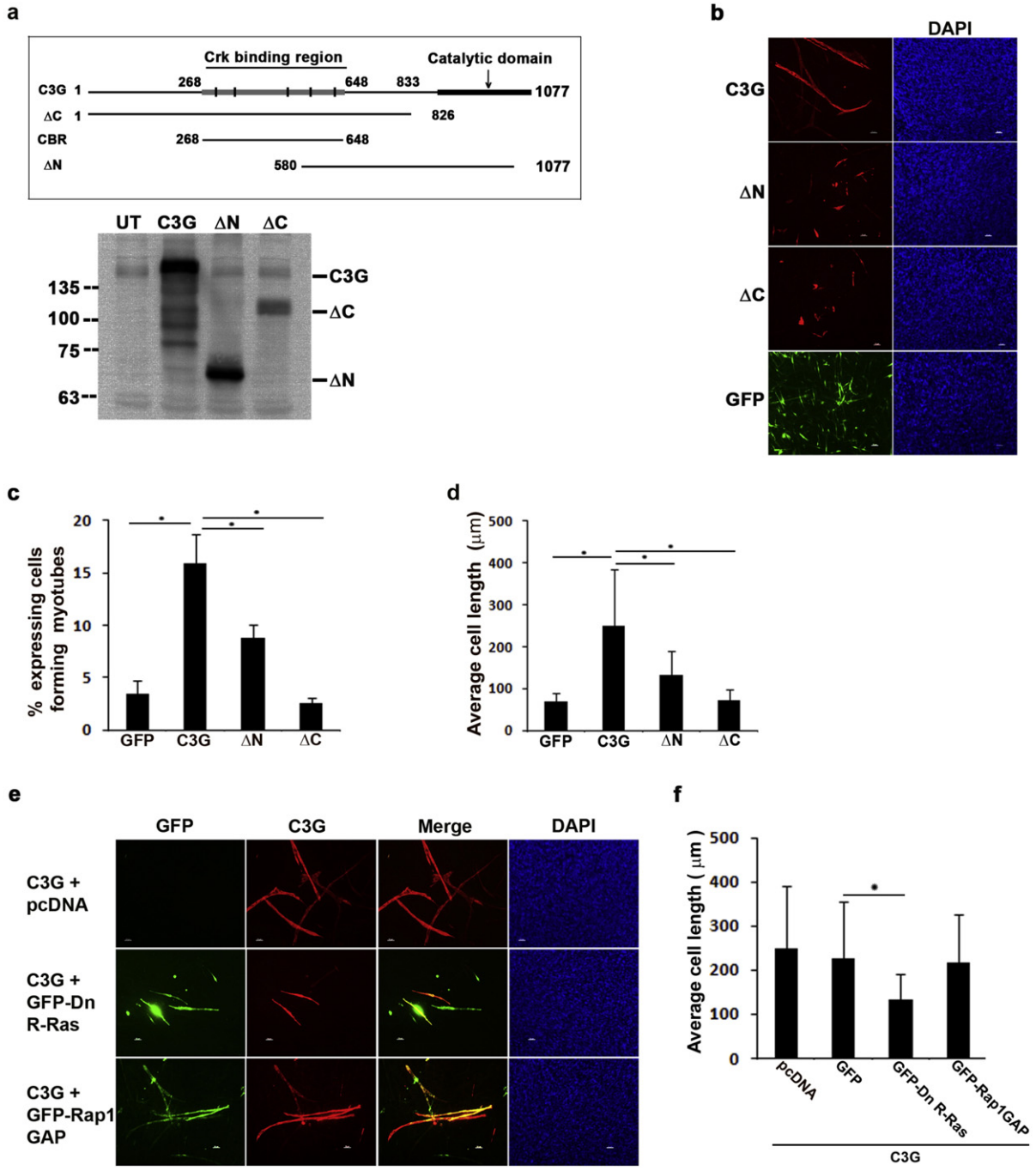

Fig. 3. Catalytic domain of C3G is required for myotube formation. (a) Schematic diagram and expression levels of C3G and its deletion constructs. (b) C2C12 cells transfected with C3G and its deletion constructs, or GFP as control, were cultured for 96 h, fixed and stained for C3G. Images were captured to show morphology of expressing cells. Bar, 50 μm. (c) Quantitation of myotube formation induced by over-expressed C3G and its deletion constructs. Bar diagram shows percentage of expressing C2C12 cells forming myotubes depicted as mean ± s.d. from three independent experiments. *$p < 0.001$. (d) Quantitation of length of cells expressing C3G and its deletion constructs. Bar diagram shows average cell length as mean ± s.d. from three independent experiments. *$p < 0.001$. (e) Involvement of GTPase effectors of C3G, R-Ras & Rap1 in myogenic differentiation. Cells grown in growth medium for 96 h after cotransfection of C3G with either control, GFP-Dn R-Ras (inhibits R-ras signaling), pcDNA, GFP-Rap1GAP (inhibits Rap1 signaling) plasmids were fixed, and stained for C3G. Bar, 50 μm. (f) Length of coexpressing cells in captured images was measured using Carl Zeiss Axiovision Rel 4.7 software. Bar diagram shows average length of expressing cells as mean ± s.d. *$p < 0.0001$.

known to function as a dominant negative (Dn) to inhibit endogenous C3G [27]. It was observed that both the deletion constructs were compromised in their ability to enhance differentiation and cells expressing ΔN and ΔC showed significantly lower myocyte fusion (Fig. 3b, c) as well as reduction in myocyte length (Fig. 3d). In fact, several ΔC expressing cells showed rounded morphology, indicative of apoptosis. These results indicate that both the catalytic domain and protein interaction domain are required for C3G to trigger signaling leading to myocyte fusion. The requirement of catalytic activity of C3G for induction of differentiation supports the observation of enhanced levels of phosphorylated C3G in differentiated cells.

The catalytic activity of C3G appears to be essential, as deletion of the catalytic domain totally blocked its ability to induce differentiation. We therefore examined the involvement of two known GTPase targets of C3G; R-Ras and Rap1 by co-expression of dominant negative constructs (R-Ras43N & Rap1GAP), which function to inhibit signaling mediated by cellular R-Ras & Rap1 respectively. We observed that coexpression of GFP-R-Ras43N with C3G significantly inhibited myotube formation

(Fig. 3e, f). Coexpression of Rap1-GAP, did not affect C3G induced myotube formation.

3.3. C3G is required for myocyte differentiation

Since C3G levels increased during C2C12 differentiation, we directly tested its requirement for myogenic differentiation using small hairpin RNA (ShRNA) mediated knockdown of cellular C3G. C2C12 cells were transfected with control ShRNA (con-Sh) and ShRNA that targets C3G (ShA) and whole cell lysates subjected to western blotting to detect levels of C3G and myogenin. Cells expressing ShA showed more than 50% reduction in C3G levels and also a reduction in myogenin levels compared to control ShRNA expressing cells (Fig. 4a). Similarly, reduction in MHC was observed by using an alternate ShRNA (ShC) (Fig. 5b). Examination of myotube formation under differentiation conditions by staining for MHC showed that ShC expressing cells did not align and fuse to form myotubes efficiently. In fact, detachment from the substratum and extensive cell death was seen in these cells. This was confirmed by examining the actin cytoskeleton and cell death in control and ShC expressing cells, co-transfected with GFP for 48 h. Rhodamine-phalloidin staining showed distinct F-actin filaments and well adhered cells when transfected with control ShRNA, whereas a large number of ShC expressing cells showed loss of actin stress fibers and rounding off (Fig. 4c). Activation of apoptosis in these cells was examined by staining for the presence of cleaved caspase-3 (Cl.-casp3), an indicator of caspase-3 activation and a marker of apoptosis. A majority of ShC expressing cells that showed detachment from the substratum were positive for Cl-casp3 (Fig. 4d). Quantitation of apoptosis seen in cells expressing ShC under conditions of growth in either GM or DM is shown in Fig. 4e. These results indicate that cellular C3G functions to aid myocyte fusion and also enables cell survival.

3.4. C3G regulates Akt activity in C2C12 cells

It is known that apoptosis occurs as myocytes are induced to differentiate to form myotubes under conditions of serum starvation [28].

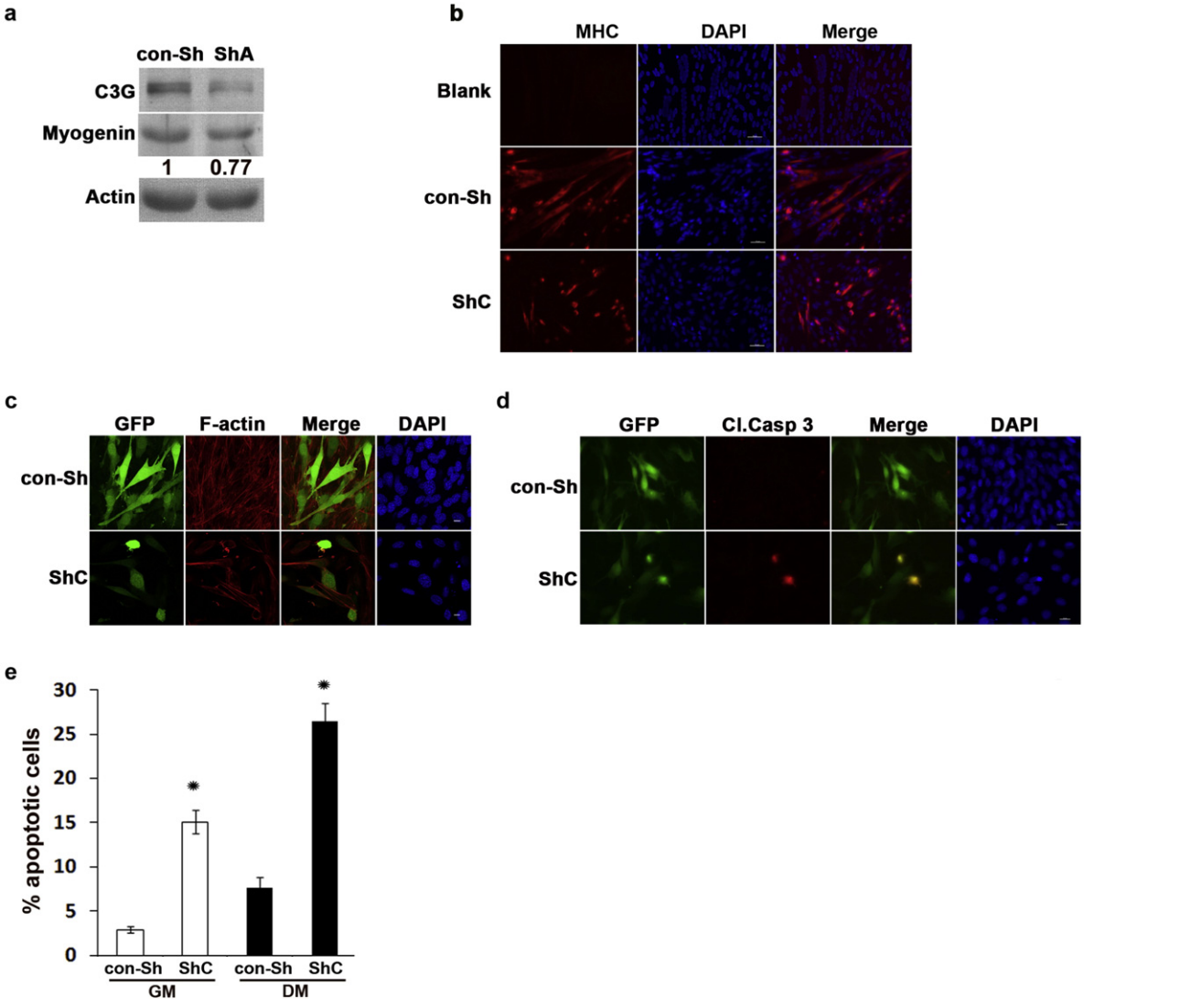

Fig. 4. C3G is required for myotube formation. (a) Knock down of C3G using ShRNA affects myogenin levels. C2C12 cells were transiently transfected with either con-Sh or ShA constructs and lysates subjected to western blotting with indicated antibodies. Numbers indicate relative change in myogenin levels upon knockdown of C3G using actin as internal control. (b) C3G knock down decreases myotube formation. con-Sh or ShC constructs were over-expressed in C2C12 cells for 24 h, followed by differentiation for 72 h, fixed, stained with MHC antibody and images captured. Bar, 50 μm. (c) C3G knock down disrupts actin organization. ShC or con-Sh constructs were cotransfected with GFP in C2C12 cells, grown for 48 h, stained for F-actin with Rhodamine-phalloidin. Images were captured using confocal microscope. Bar, 10 μm. (d) C3G knockdown causes cell death. C2C12 cells were grown for 48 h after cotransfection with either ShC or con-Sh constructs along with GFP, and fixed cells stained for cleaved caspase 3. Images were taken using a fluorescence microscope. Bar, 20 μm. (e) Quantitation of apoptosis in cells expressing con-Sh or ShC along with 20 ng of GFP and grown for 48 h in either GM or DM. Data represents average of % apoptotic cells among GFP positive cells after subtraction of apoptosis in non-expressing cells in the same coverslips. *$p < 0.05$.

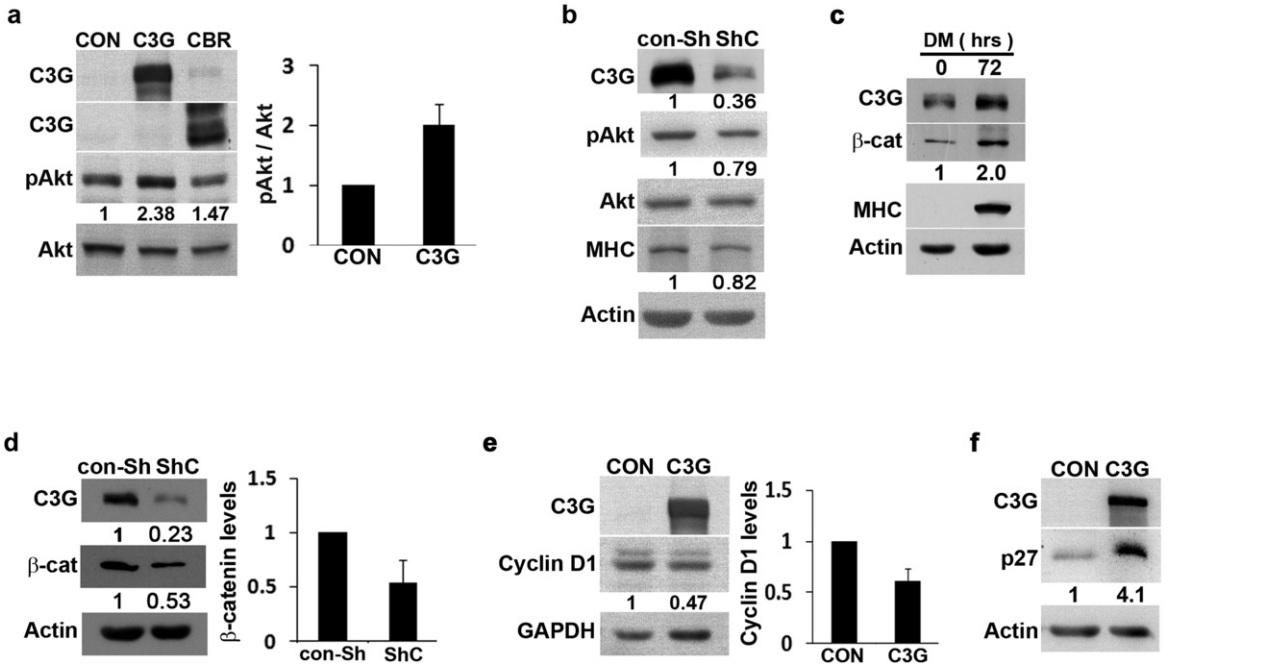

Fig. 5. C3G over-expression activates Akt and decreases cyclin D1 levels. (a) C3G activates Akt. Control, C3G and CBR adenovirus infected C2C12 cells were grown for 72 h. Cell lysates were subjected to western blotting using pAkt, Akt, and C3G antibodies. Numbers indicate relative pAkt levels (adjusted with total Akt). Bar diagram shows relative pAkt levels as mean ± s.d. from three independent experiments. (b) Knock down of C3G decreases pAkt levels. C2C12 cells were transfected with con-Sh and ShC constructs for 48 h and cell lysates subjected to western blotting to detect pAkt, Akt, MHC, C3G and actin using indicated antibodies. Numbers indicate relative change of the respective proteins compared to that seen in controls. (c) β-catenin levels increase during differentiation. Western blot shows β-catenin, MHC and C3G levels in undifferentiated & 72 h differentiated C2C12 cells. Numbers indicate relative β-catenin levels adjusted with actin. (d) Effect of C3G knockdown on cellular β-catenin levels. C2C12 cells were transfected with con-Sh and ShC constructs, for 36 h and cell lysates subjected to western blotting to detect C3G, actin and β-catenin. Relative β-catenin levels from three independent experiments represented as mean ± s.d. are shown in the bar diagram. (e) C3G expression represses cellular cyclin D1 expression. Western blot of lysates from control and C3G adenoviral construct expressing cells (72 h) probed for C3G, cyclin D1 & GAPDH. Bar diagram shows relative cyclin D1 levels averaged from 3 experiments. (f) Induction of p27 in C3G over-expressing cells. Lysates of cells expressing control and C3G adenoviral constructs were subjected to western blotting to detect C3G, p27 and actin.

Therefore, triggers of differentiation also require parallel activation of cell survival signaling. Since Akt activation is a major regulator of cell survival, we examined whether C3G overexpression induces phosphorylation of Akt, an indication of its activation. C2C12 cells were infected with control virus and C3G or CBR overexpressing virus for 72 h and subjected to western blotting to detect p-Akt and Akt. A significant increase in pAkt levels was seen in lysates from cells expressing C3G (Fig. 5a). Cells expressing the deletion construct, CBR showed a small increase in levels of pAkt (compared to C3G). The increase in pAkt levels in response to C3G expression was not particularly dependent on activity of PI3K, as increased pAkt levels were also seen under conditions of wortmannin treatment (Supplementary Fig. S3). pAkt levels increase concomitantly with an increase in C3G levels during C2C12 differentiation (Supplementary Fig. S4a). We therefore examined the consequence of reducing cellular C3G levels on pAkt activation in C2C12 levels. Cells expressing ShRNA targeting C3G show reduced pAkt levels compared to cells expressing control ShRNA (Fig. 5b and Supplementary Fig. S4b). These results suggest that C3G plays a role in myocyte differentiation by regulating activity of Akt.

We have earlier shown that C3G functions as a repressor of β-catenin activity. Since it is known that β-catenin plays an important role in regulating myocyte proliferation vs. differentiation, we examined β-catenin levels in C2C12 cells under conditions of C3G knockdown. Results from others, as well as our own study showed that β-catenin protein levels increase upon myogenic differentiation of C2C12 cells [29] (Fig. 5c & Supplementary Fig. S4a). Reduction of cellular C3G results in significantly lower levels of β-catenin (Fig. 5d) suggesting that regulation of β-catenin function may be one of the mechanisms engaged by C3G to induce differentiation. C3G expressing cells also showed a decrease in cyclin D1 levels (Fig. 5e). These results are in agreement with previous findings which have shown that repression and degradation of cyclin D1 are associated with myogenic differentiation [30,31]. Induction of cell cycle inhibitors, p21 and p27 is a hall mark of cell cycle withdrawal during myocyte differentiation [32]. In addition to repression of cyclin D1, we observed that C3G overexpression results in an increase in p27 levels (Fig. 5f).

3.5. C3G is required for formation of intact focal adhesions

Expression of the central Crk binding region (CBR) of C3G that lacks both N and C-terminal sequences functions as a dominant negative to inhibit endogenous C3G activation [33]. C3G and CBR were expressed in C2C12 cells using adenoviral expression vectors and cultured for 96 h. CBR expressing cells failed to fuse and form myotubes. They showed detachment from the substratum, and change in cell morphology (Fig. 6a) indicating that endogenous C3G function was required for survival and fusion of myocytes. Examination of F-actin in these cells grown for 48 h in growth medium showed loss of F-actin filaments and disruption of intact actin cytoskeleton in CBR expressing cells (Fig. 6b). It was observed that C3G expressing cells show elongation and better alignment with prominent stress fibers compared to control virus expressing cells indicating that C3G expression enables actin cytoskeletal remodeling that favors myoblast elongation and fusion.

Several proteins that play a role in regulating actin remodeling and cell adhesion have been shown to aid myoblast fusion [34]. Earlier studies have implicated a role for C3G in cell adhesion through its localization to focal adhesions (FAs) [35,36]. We therefore examined the presence of C3G at FAs in myocytes and myotubes by examining colocalization with vinculin. It was observed that endogenous C3G was not seen at FA sites in myoblasts but showed partial colocalization with vinculin in myotubes (Fig. 6c). This dynamic localization of C3G suggests its contribution to cell fusion during the formation of myotubes.

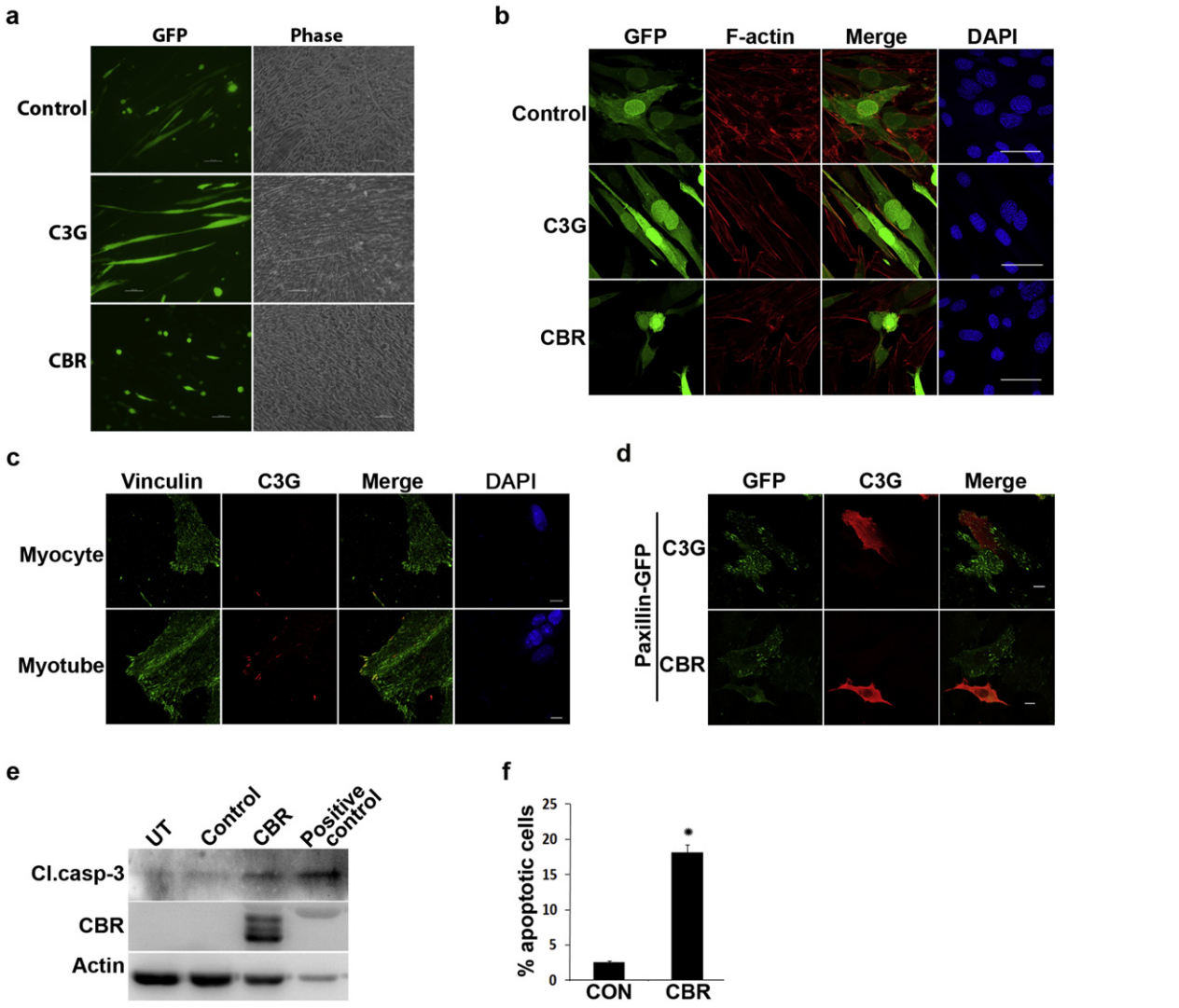

Fig. 6. C3G is required for cell adhesion and survival. (a) CBR over-expression causes cell death and inhibits myotube formation. C2C12 cells infected with control, C3G and CBR adenoviral constructs were grown for 96 h and live cell imaging carried out. Bar, 100 μm. (b) CBR over-expression causes disruption of F-actin filaments. C2C12 cells were infected with control, C3G and CBR adenoviral constructs, grown for 48 h, fixed and stained to detect F-actin with Rhodamine phalloidin. Bar, 50 μm. (c) C3G localizes at focal adhesions in myotubes. Undifferentiated myocytes and 96 h differentiated C2C12 cells (myotubes) were subjected to immunofluorescence with C3G and vinculin antibodies. Images were taken using confocal microscope. Bar, 10 μm. (d) CBR overexpression causes loss of focal adhesions. C2C12 cells cotransfected with paxillin-GFP and C3G or CBR, were grown for 48 h, fixed and followed by staining with C3G antibody. Images were captured using confocal microscope. Bar 10 μm. (e) CBR over-expression causes cell death. C2C12 cells were infected with CBR, or control adenoviral constructs and grown for 48 h before cell lysates were collected to perform western blotting using cleaved caspase 3, C3G and actin antibodies. C2C12 cell lysate collected after 24 h treatment with 20 μg/ml cycloheximide and 30 ng/ml TNFα was used as positive control. (f) Quantitation of apoptosis in cells expressing control and CBR adenovirus for 48 h.

Integrin and FAK dependent signaling is required for myocyte differentiation [37]. We examined if detachment and rounding of CBR expressing cells were due to loss of focal adhesions. C3G and CBR were coexpressed with GFP-paxillin, and FAs in expressing cells examined by GFP signals. FAs are prominently seen in non-expressing cells as well as C3G expressing cells; but CBR expressing cells showed absence of FAs (Fig. 6d), indicating that CBR disrupts formation of FAs which are required for cell adhesion and differentiation. CBR expressing cells also showed condensed chromatin (Supplementary Fig. S5) and higher level of Cl-casp-3 compared to uninfected or control virus infected cells (Fig. 6e) indicating that disabling the function of endogenous C3G results in activation of apoptotic pathways. Quantitation showed a significant increase in cell death in CBR expressing cells compared to control cells (Fig. 6f).

4. Discussion

Our results provide evidence for the role of a GEF, C3G as a determinant of skeletal muscle cell differentiation. The increase in levels of C3G expression seen during differentiation of skeletal muscle cells and our results showing that knockdown of C3G compromises muscle cell differentiation are indicative of the requirement of C3G for muscle development. Additionally, expression of a dominant negative construct that blocks C3G activity, interferes with the differentiation process. The catalytic as well as protein interaction domain of C3G are required, as expression of the independent domains did not promote differentiation. An increase in the catalytically active component of C3G during differentiation and dependence on the activity of R-Ras GTPase, further validate the requirement of catalytic activity for the differentiation process. Our data shows that C3G expression in cells cultured in normal growth medium results in induction of myogenic markers and fusion of cells to form myotubes. C3G is therefore capable of initiating signaling events that result in myogenic gene expression and actin cytoskeletal changes required for cell fusion even in the absence of conditions that favor differentiation to a muscle lineage. Under conditions of C3G expression in cells grown in differentiation medium, we observe increased number and extensive branching of myofibers, indicative of increased

myotube–myotube fusion suggesting that high levels of C3G expression enhance the fusion process.

During differentiation, C3G levels increase up to 48 h after which, levels appear to plateau. Similar kinetics of expression was also observed for myogenin. Increase was also seen at the transcript level indicating that C3G is under transcriptional regulation during induction of differentiation. In silico analysis of upstream sequences of C3G gene, showed the presence of binding sites for myogenic transcription factors, which require experimental validation. C3G expression also results in changes in gene expression of myogenic regulatory factors, suggesting that C3G can signal to enhance levels of these transcripts. This may be one of the mechanisms by which over-expressed C3G can initiate myogenesis even in the absence of differentiation inducing signals.

The high level of C3G protein seen in myotubes was particularly localized to the nuclei suggesting that C3G contributes to the differentiation process by altering specific nuclear functions. Though C3G protein shows the presence of putative nuclear localization signals, this is the first documentation for its presence in the nuclear compartment. Altered chromatin dynamics and changes in gene expression are essential features of myogenesis [38]. Some cytoskeletal regulatory factors like Kindlin2 and smALP undergo nuclear translocation during C2C12 differentiation [39,40]. As a known interacting partner of β-catenin, which has essential nuclear functions in skeletal myogenesis, C3G may also serve a regulatory role by translocating to the nucleus. This remains to be investigated. In addition to an increase in C3G protein levels during differentiation, we also observed an increase in tyrosine phosphorylated C3G which is catalytically active. SFKs and c-Abl interact with and phosphorylate human C3G at Y504 [7,23]. c-Abl and c-Src activity is known to increase upon differentiation of C2C12 cells [41,42] and may be responsible for an increase in catalytically active C3G.

Failure of myoblasts expressing ShRNA targeted to knockdown endogenous C3G to fuse and form multi-nucleated myotubes indicated that optimal level of endogenous C3G is required for myocytes to undergo differentiation. Knockdown of C3G also resulted in lower levels of muscle specific genes suggesting that C3G may be an upstream regulator of myogenic molecules, and therefore activates the differentiation program. Induction of differentiation in myocytes is associated with cell death and activation of cell survival pathways has been a downstream effect of molecules that induce differentiation. Our over-expression and knockdown experiments have shown that C3G signals to activation of Akt. Activation of Akt is required for expression of myogenic factors and cell cycle withdrawal promotes induction of Akt [43]. C2C12 cells do not differentiate when cultured in the presence of Akt inhibitor [44]. Unpublished results from our lab have shown that C3G expression results in Akt activation in human breast cancer cells. Expression of CBRin C2C12 cells also results in a small increase in pAkt levels at 72 h of expression, though these cells do not differentiate. These observations indicate that change in Akt activity is due to expression of C3G, and not a consequence of myogenic differentiation.

Reduction in C3G levels resulted in loss of cells due to cell death, indicated by activation of caspase-3. It therefore appears that C3G activates survival signaling in parallel with activation of the myogenic program. In addition to regulating expression of MHC, C3G was shown to inhibit cyclin D1 and increase levels of the cell cycle inhibitor, p27. Repression of cyclin D1 expression and induction of p27 are associated with cell cycle withdrawal and differentiation [31,45–47]. The effect of C3G on cyclin D1 & p27 levels could be at the transcriptional or post-transcriptional level. This remains to be investigated. The fact that C3G alters Akt activity and expression of myogenic and cell cycle regulators

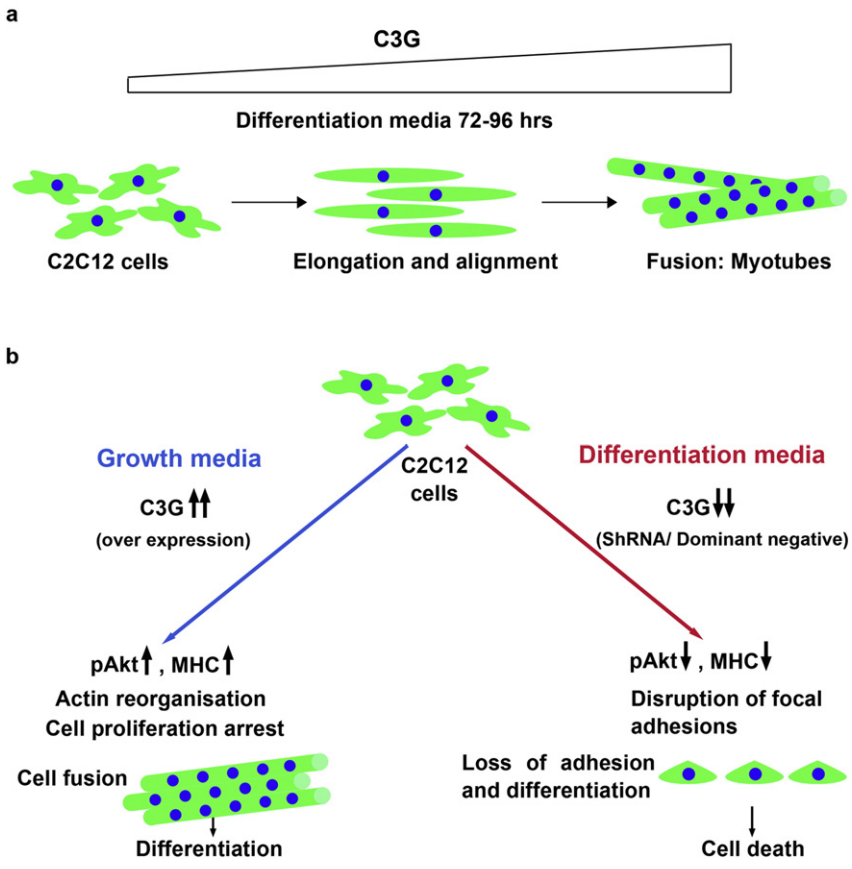

Fig. 7. Schematic figure showing the role of C3G in skeletal muscle differentiation of C2C12 myoblasts. (a) C2C12 cells cultured in differentiation medium for 72–96 h show increase in C3G (transcript and protein) as cells fuse to form myotubes. (b) Overexpression of C3G in C2C12 cells cultured in GM results in an increase in Akt activity and myogenic markers; and altered actin organisation, resulting in cell fusion to form myotubes. On the other hand, reducing C3G function inhibits Akt activity, MHC levels, and myotube formation when cells are cultured in differentiation medium. Compromised C3G function results in disruption of focal adhesions leading to detachment and death of myocytes.

in cells cultured in GM, indicates that changes in these molecules are not due to secondary effects of differentiation.

β-Catenin is required for myogenic differentiation of skeletal muscle as well as C2C12 cells [48,49]. It interacts directly with MyoD to enhance its activity [50]. Our results show that cellular C3G is required for maintaining optimum levels of β-catenin. In epithelial cells C3G represses β-catenin levels by altering its stability and inhibits its activity [20]. Therefore, C3G shows cell type dependent regulation of β-catenin function, and the mechanisms involved need to be investigated. Cell-type dependent regulation of β-catenin is not unusual and has been shown earlier [51].

Of the known targets, C3G engages R-Ras and not Rap GTPase in inducing differentiation. This finding also substantiates an earlier study showing that R-Ras is a positive regulator of skeletal myogenesis [52]. Several small GTPases and their regulatory GEFs and GAPs are involved in myoblast fusion; which is an essential step during muscle development [53]. It is known that cell membrane protrusions propelled by actin, enable cell–cell fusion [54]. The role of C3G in dynamic reorganization of actin to promote filopodia and neurite like extensions has been shown earlier [7,24]. C3G expression resulted in formation of organized F-actin fibers that enables change in shape to spindle-like cells. Since fusion is known to occur only between elongated spindle shaped myocytes, the morphological changes caused by C3G may be contributing to cell fusion. Studies on *Drosophila* wherein the C3G orthologue has been knocked out, show abnormalities in muscle morphology during larval development [18]. The defects are caused by improper attachment of long muscles due to alteration in integrin localization. Enhanced C3G expression was also seen in the rat myocardium in peri-infarct regions and the authors have suggested a role for C3G in post-infarction cardiac muscle remodeling [55].

During myoblast differentiation, vinculin and paxillin are recruited from the cytoplasmic pool into FAs [56]. Our study also shows that C3G localizes to focal adhesions in myotubes and expression of dominant negative C3G results in improper formation of FAs and detachment of cells from the substratum. Similar detachment of cells was also observed upon knockdown of endogenous C3G using ShRNA. C3G interacts with E-cadherin, a molecule that plays an important role in muscle cell membrane fusion [9]. The localization of C3G to FA sites and loss of adhesion seen in cells expressing dominant negative C3G suggests its role in maintaining attachment sites that are required for muscle cell fusion.

We have earlier shown an increase in C3G levels during differentiation of neurons and hematopoietic cells and its requirement for neural differentiation [5,19]. Results of the present study demonstrating requirement of C3G for muscle differentiation raise the possibility of C3G being an important regulator of differentiation of a variety of tissue types during embryonic development. Muscle specific gene transcripts are seen at 8–9 d of development in the mouse embryo [57]. C3G knockout mice die around embryonic d7.5; at which time tissue specific differentiation of various cells is observed. One of the reasons for early embryonic lethality seen in knockout mice could therefore be due to defective differentiation of various tissues. Our results provide insights into the role of C3G in skeletal muscle differentiation which occurs by regulation of molecules involved in myogenic program, cell proliferation arrest, adhesion and survival signaling (Fig. 7).

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

KS is the recipient of a research fellowship from ICMR, India. We thank Dr. Ghanshyam Swarup & Megha Bansal for gift of MEFs and Dr. Bert Vogelstein for gift of adenoviral vectors. This work was supported by grants awarded to VR by Department of Science & Technology, Department of Biotechnology, and Council of Scientific & Industrial Research (BSC0108 and BSC0111), Govt. of India.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.06.015.

References

[1] S.M. Hindi, M.M. Tajrishi, A. Kumar, Sci. Signal. 6 (272) (2013) re2.
[2] M.L. Spletter, F. Schnorrer, Exp. Cell Res. 321 (1) (2014) 90–98.
[3] M. Karalaki, S. Fili, A. Philippou, M. Koutsilieris, In Vivo 23 (5) (2009) 779–796.
[4] M. Peckham, J. Microsc. 231 (3) (2008) 486–493.
[5] V. Radha, A. Mitra, K. Dayma, K. Sasikumar, Biosci. Rep. 31 (4) (2011) 231–244.
[6] B.S. Knudsen, S.M. Feller, H. Hanafusa, J. Biol. Chem. 269 (52) (1994) 32781–32787.
[7] V. Radha, A. Rajanna, A. Mitra, N. Rangaraj, G. Swarup, Exp. Cell Res. 313 (11) (2007) 2476–2492.
[8] R.V. Shivakrupa, C. Sudhakar, G. Swarup, J. Biol. Chem. 278 (52) (2003) 52188–52194.
[9] C. Hogan, N. Serpente, P. Cogram, C.R. Hosking, C.U. Bialucha, S.M. Feller, V.M. Braga, W. Birchmeier, Y. Fujita, Mol. Cell. Biol. 24 (15) (2004) 6690–6700.
[10] T. Ichiba, Y. Kuraishi, O. Sakai, S. Nagata, J. Groffen, T. Kurata, S. Hattori, M. Matsuda, J. Biol. Chem. 272 (35) (1997) 22215–22220.
[11] M. Matsuda, Y. Hashimoto, K. Muroya, H. Hasegawa, T. Kurata, S. Tanaka, S. Nakamura, S. Hattori, Mol. Cell. Biol. 14 (8) (1994) 5495–5500.
[12] R.D. York, H. Yao, T. Dillon, C.L. Ellig, S.P. Eckert, E.W. McCleskey, P.J. Stork, Nature 392 (6676) (1998) 622–626.
[13] T. Ichiba, Y. Hashimoto, M. Nakaya, Y. Kuraishi, S. Tanaka, T. Kurata, N. Mochizuki, M. Matsuda, J. Biol. Chem. 274 (20) (1999) 14376–14381.
[14] C. Guerrero, A. Fernandez-Medarde, J.M. Rojas, J. Font de Mora, L.M. Esteban, E. Santos, Oncogene 16 (5) (1998) 613–624.
[15] S. Tanaka, T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, M. Shibuya, K. Matuoka, T. Takenawa, T. Kurata, K. Nagashima, et al., Proc. Natl. Acad. Sci. U. S. A. 91 (8) (1994) 3443–3447.
[16] Y. Ohba, K. Ikuta, A. Ogura, J. Matsuda, N. Mochizuki, K. Nagashima, K. Kurokawa, B.J. Mayer, K. Maki, J. Miyazaki, M. Matsuda, EMBO J. 20 (13) (2001) 3333–3341.
[17] A.K. Voss, D.L. Krebs, T. Thomas, EMBO J. 25 (15) (2006) 3652–3663.
[18] M. Shirinian, M. Popovic, C. Grabbe, G. Varshney, F. Hugosson, H. Bos, H. Rehmann, R.H. Palmer, PLoS One 5 (3) (2010) e9403.
[19] V. Radha, A. Rajanna, R.K. Gupta, K. Dayma, T. Raman, J. Neurochem. 107 (5) (2008) 1424–1435.
[20] K. Dayma, A. Ramadhas, K.V. Sasikumar, Genes Cancer 3 (9–10) (2012) 564–577.
[21] R. van Amerongen, R. Nusse, Development 136 (19) (2009) 3205–3214.
[22] D. Yaffe, O. Saxel, Nature 270 (5639) (1977) 725–727.
[23] V. Radha, A. Rajanna, G. Swarup, BMC Cell Biol. 5 (2004) 31.
[24] K.V. Dayma, Biochim. Biophys. Acta 1813 (3) (2011) 456–465.
[25] Z. Wu, P.J. Woodring, K.S. Bhakta, K. Tamura, F. Wen, J.R. Feramisco, M. Karin, J.Y. Wang, P.L. Puri, Mol. Cell. Biol. 20 (11) (2000) 3951–3964.
[26] T. Hirata, H. Nagai, K. Koizumi, K. Okino, A. Harada, M. Onda, T. Nagahata, I. Mikami, K. Hirai, S. Haraguchi, E. Jin, O. Kawanami, K. Shimizu, M. Emi, J. Hum. Genet. 49 (6) (2004) 290–295.
[27] A.G. Dupuy, S. L'Hoste, J. Cherfils, J. Camonis, G. Gaudriault, J. de Gunzburg, Oncogene 24 (28) (2005) 4509–4520.
[28] K. Dee, M. Freer, Y. Mei, C.M. Weyman, Cell Death Differ. 9 (2) (2002) 209–218.
[29] S. Tanaka, K. Terada, T. Nohno, J. Mol. Signal. 6 (2011) 12.
[30] A. Alteri, F. De Vito, G. Messina, M. Pompili, A. Calconi, P. Visca, M. Mottolese, C. Presutti, M. Grossi, Cell Cycle 12 (24) (2013) 3781–3790.
[31] W. Yang, Y. Zhang, Y. Li, Z. Wu, D. Zhu, J. Biol. Chem. 282 (6) (2007) 3799–3808.
[32] S.D. Zabludoff, M. Csete, R. Wagner, X. Yu, B.J. Wold, Cell Growth Differ. 9 (1) (1998) 1–11.
[33] A.V. Mitra, Oncogene 29 (32) (2010) 4528–4542.
[34] S. Charrasse, F. Comunale, M. Fortier, E. Portales-Casamar, A. Debant, C. Gauthier-Rouviere, Mol. Biol. Cell 18 (5) (2007) 1734–1743.
[35] A.K. Voss, P. Gruss, T. Thomas, Development 130 (2) (2003) 355–367.
[36] V. Maia, S. Ortiz-Rivero, M. Sanz, J. Gutierrez-Berzal, I. Alvarez-Fernandez, S. Gutierrez-Herrero, J.M. de Pereda, A. Porras, C. Guerrero, Cell Commun. Signal. 11 (1) (2013) 9.
[37] J.W. Han, H.J. Lee, G.U. Bae, J.S. Kang, Cell. Signal. 23 (7) (2011) 1162–1169.
[38] P. Asp, R. Blum, V. Vethantham, F. Parisi, M. Micsinai, J. Cheng, C. Bowman, Y. Kluger, B.D. Dynlacht, Proc. Natl. Acad. Sci. U. S. A. 108 (22) (2011) E149–E158.
[39] L. Cambier, P. Pomies, Biochem. Biophys. Res. Commun. 409 (4) (2011) 628–633.
[40] Y. Yu, L. Qi, J. Wu, Y. Wang, W. Fang, H. Zhang, PLoS One 8 (5) (2013) e63490.
[41] E. Hoerth, R. Kodym, Int. J. Radiat. Biol. 80 (10) (2004) 729–736.
[42] H. Lu, P. Shah, D. Ennis, G. Shinder, J. Sap, H. Le-Tien, I.G. Fantus, J. Biol. Chem. 277 (48) (2002) 46687–46695.
[43] Y. Fujio, K. Guo, T. Mano, Y. Mitsuuchi, J.R. Testa, K. Walsh, Mol. Cell. Biol. 19 (7) (1999) 5073–5082.
[44] L. Shu, P.J. Houghton, Mol. Cell. Biol. 29 (17) (2009) 4691–4700.
[45] D.C. Guttridge, C. Albanese, J.Y. Reuther, R.G. Pestell, A.S. Baldwin Jr., Mol. Cell. Biol. 19 (8) (1999) 5785–5799.
[46] L. Latella, A. Sacco, D. Pajalunga, M. Tiainen, D. Macera, M. D'Angelo, A. Felici, A. Sacchi, M. Crescenzi, Mol. Cell. Biol. 21 (16) (2001) 5631–5643.

[47] A. Di Carlo, R. De Mori, F. Martelli, G. Pompilio, M.C. Capogrossi, A. Germani, J. Biol. Chem. 279 (16) (2004) 16332–16338.
[48] D.D. Armstrong, V.L. Wong, K.A. Esser, Am. J. Physiol. Cell Physiol. 291 (1) (2006) C185–C188.
[49] X.H. Han, Y.R. Jin, M. Seto, J.K. Yoon, J. Biol. Chem. 286 (12) (2011) 10649–10659.
[50] C.H. Kim, H. Neiswender, E.J. Baik, W.C. Xiong, L. Mei, Mol. Cell. Biol. 28 (9) (2008) 2941–2951.
[51] B. Martin, R. Schneider, S. Janetzky, Z. Waibler, P. Pandur, M. Kuhl, J. Behrens, K. von der Mark, A. Starzinski-Powitz, V. Wixler, J. Cell Biol. 159 (1) (2002) 113–122.
[52] J. Suzuki, Y. Kaziro, H. Koide, Oncogene 19 (9) (2000) 1138–1146.
[53] E.H. Chen, B.A. Pryce, J.A. Tzeng, G.A. Gonzalez, E.N. Olson, Cell 114 (6) (2003) 751–762.
[54] K. Shilagardi, S. Li, F. Luo, F. Marikar, R. Duan, P. Jin, J.H. Kim, K. Murnen, E.H. Chen, Science 340 (6130) (2013) 359–363.
[55] L. Wang, G. Li, Z. Wang, X. Liu, W. Zhao, Med. Sci. Monit. Basic Res. 19 (2013) 1–5.
[56] V. Dugina, L. Fontao, C. Chaponnier, J. Vasiliev, G. Gabbiani, J. Cell Sci. 114 (Pt 18) (2001) 3285–3296.
[57] D.G. Edmondson, G.E. Lyons, J.F. Martin, E.N. Olson, Development 120 (5) (1994) 1251–1263.