1-1-2017

Regulator Of Cell Cycle (Rgcc) Expression During The Progression Of Alzheimer's Disease

Scott E. Counts

Elliott J. Mufson
Barrow Neurological Institute, elliott.mufson@dignityhealth.org

Cell Transplantation, Vol. 26, pp. 693–702, 2017
Printed in the USA.

0963-6897/17 $90.00 + .00
DOI: https://doi.org/10.3727/096368916X694184
E-ISSN 1555-3892
www.cognizantcommunication.com

Regulator of Cell Cycle (RGCC) Expression During the Progression of Alzheimer's Disease

Scott E. Counts*†‡ and Elliott J. Mufson§¶

*Department of Translational Science and Molecular Medicine, Michigan State University, Grand Rapids, MI, USA
†Department of Family Medicine, Michigan State University, Grand Rapids, MI, USA
‡Hauenstein Neurosciences Center, Mercy Health Saint Mary's Hospital, Grand Rapids, MI, USA
§Department of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, USA
¶Department of Neurology, Barrow Neurological Institute, Phoenix, AZ, USA

Unscheduled cell cycle reentry of postmitotic neurons has been described in cases of mild cognitive impairment (MCI) and Alzheimer's disease (AD) and may form a basis for selective neuronal vulnerability during disease progression. In this regard, the multifunctional protein regulator of cell cycle (RGCC) has been implicated in driving G_1/S and G_2/M phase transitions through its interactions with cdc/cyclin-dependent kinase 1 (cdk1) and is induced by p53, which mediates apoptosis in neurons. We tested whether RGCC levels were dysregulated in frontal cortex samples obtained postmortem from subjects who died with a clinical diagnosis of no cognitive impairment (NCI), MCI, or AD. RGCC mRNA and protein levels were upregulated by ~50%–60% in MCI and AD compared to NCI, and RGCC protein levels were associated with poorer antemortem global cognitive performance in the subjects examined. To test whether RGCC might regulate neuronal cell cycle reentry and apoptosis, we differentiated neuronotypic PC12 cultures with nerve growth factor (NGF) followed by NGF withdrawal to induce abortive cell cycle activation and cell death. Experimental reduction of RGCC levels increased cell survival and reduced levels of the cdk1 target cyclin B1. RGCC may be a candidate cell cycle target for neuroprotection during the onset of AD.

Key words: Mild cognitive impairment (MCI); Alzheimer's disease (AD); Cell cycle; Regulator of cell cycle (RGCC); Nerve growth factor (NGF); Apoptosis

INTRODUCTION

Several lines of evidence suggest that cell cycle reactivation occurs in postmitotic neurons in Alzheimer's disease (AD) and its putative prodromal stage, mild cognitive impairment (MCI). The AD brain is characterized by neuronal expression of cell cycle regulatory proteins[1–3] and DNA replication[4–6], whereas we have demonstrated the presence of the cell cycle proteins proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin B1 in neurons in vulnerable brain regions in subjects with MCI[7]. Mechanistically, a link has been established between unscheduled cell cycle reentry and neuronal apoptosis, suggesting a pathogenic mechanism for neuronal selective vulnerability[8–13]. Moreover, the activation of several cell cycle-related kinases, including cdc2/cyclin-dependent kinase 1 (cdk1), cdc2-like kinase, cdk2, and cdk5, has been shown to phosphorylate tau and promote tau aggregation[14–17].

The mechanisms underlying aberrant neuronal cell cycle reentry during the onset of AD have not been firmly established, but various stressors such as DNA damage and neurotrophin dysregulation have been proposed[18–22]. Notably, the tumor suppressor protein p53, which induces cell cycle arrest and DNA repair in damaged proliferating cells, facilitates apoptosis when the neuronal milieu is presented with toxic insults[23–25]. Although the link between p53, cell cycle dysregulation, and apoptosis is unclear, p53 induces the expression of the multifunctional protein regulator of cell cycle (RGCC)[26,27], which is highly expressed in many cancerous tissues[28]. RGCC has been shown to either induce S phase entry and mitosis or promote differentiation in nonneuronal cells, which appears to be context dependent[27–32]. Whether RGCC dysfunction might represent a novel pathway linking aberrant cell cycle activation and apoptosis in neurons during the progression of AD remains undetermined. In the

Received September 29, 2016; final acceptance January 9, 2017. Online prepub date: November 30, 2016.
Address correspondence to Scott E. Counts, Ph.D., College of Human Medicine, Michigan State University, 333 Bostwick Avenue NE, Grand Rapids, MI 49503, USA. Tel: 616-234-0997; Fax: 616-234-0990: E-mail: scott.counts@hc.msu.edu

present study, we measured RGCC mRNA and protein levels in frontal cortex samples obtained postmortem from individuals who died with an antemortem diagnosis of no cognitive impairment (NCI), MCI, or AD. We also tested whether RGCC expression impacted cell survival in an in vitro experimental paradigm for neuronal cell cycle-induced apoptosis.

MATERIALS AND METHODS

Subjects

Brain tissues from NCI ($n=14$), MCI ($n=11$), and mild/moderate AD ($n=11$) cases from both genders were obtained from participants in the Rush Religious Orders Study, a longitudinal clinical pathologic study of aging and AD in elderly Catholic clergy[33]. Demographic, clinical, and neuropathological characteristics of the subjects are summarized in Table 1. Details of cognitive evaluations and diagnostic criteria have been extensively published[33–36]. Briefly, a team of investigators performed annual neuropsychological performance testing including the Mini Mental State Exam (MMSE) and 17 additional neuropsychological tests referable to five cognitive domains: orientation, attention, memory, language, and perception. A Global Cognitive Score (GCS), consisting of a composite *z*-score calculated from this test battery, was determined for each participant. A board-certified neurologist with expertise in the evaluation of the elderly made the clinical diagnosis based on impairments in each of the five cognitive domains and a clinical examination. The diagnosis of dementia or AD met recommendations by the joint working group of the National Institute of

Table 1. Clinical, Demographic, and Neuropathological Characteristics by Diagnosis Category

	Clinical Diagnosis				
Characteristics	NCI ($n=14$)	MCI ($n=11$)	AD ($n=11$)	*p* Value	Pairwise
Comparison					
Age (years) at death				0.1*	–
Mean ± SD	83.9 ± 4.5	84.4 ± 5.2	86.2 ± 5.1		
Range	76–92	72–91	78–95		
Number (%) of males	6 (43%)	6 (54%)	6 (54%)	0.5†	–
Years of education				0.1*	–
Mean ± SD	19.1 ± 2.9	18.9 ± 4.3			
Range	15–22	8–24	14–21		
Number (%) with ApoE ε4 allele	2 (14%)	2 (18%)	5 (45%)	0.01†	AD > NCI, MCI
MMSE				<0.0001*	NCI, MCI > AD
Mean ± SD	28.1 ± 0.9	26.8 ± 2.6	15.1 ± 7.7		
Range	26–29	22–30	0–27		
Global cognitive score				<0.0001*	NCI, MCI > AD
Mean ± SD	0.0 ± 0.3	−0.37 ± 0.4	−1.8 ± 0.6		
Range	−0.5 to 0.4	−1.2 to 0.3	−2.8 to −0.7		
Postmortem interval (h)				0.3*	–
Mean ± SD	4.7 ± 2.9	6.0 ± 3.3	5.4 ± 3.4		
Range	2.2–12.0	2.7–13.0	2.7–12.0		
Distribution of Braak scores				0.1*	
0	0	0	0		
I/II	5	4	2		
III/IV	8	6	6		
V/VI	1	1	3		
NIA-Reagan diagnosis (likelihood of AD)				0.2*	
No AD	0	0	0		
Low	6	6	5		
Intermediate	6	5	5		
High	2	0	1		
CERAD diagnosis				0.2*	
No AD	3	4	3		
Possible	3	2	2		
Probable	6	3	5		
Definite	2	2	1		

*Kruskal–Wallis test, with Bonferroni correction for multiple comparisons.
†Fisher's exact test, with Bonferroni correction for multiple comparisons.

Neurologic and Communicative Disorders and Stroke/AD and Related Disorders Association (NINCDS/ADRDA)[37]. The MCI population was defined as subjects who exhibited impairment on neuropsychological testing but did not meet the criteria for AD or dementia. These criteria for MCI are consistent with those used by others in the field[38].

Tissue samples were accrued as previously reported[34,39,40]. At autopsy, tissue from one hemisphere was immersion fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer (pH 7.2) for 24–72 h at 4°C. Tissue slabs from the opposite hemisphere were frozen at −80°C prior to collection of frontal cortex samples for quantitative polymerase chain reaction (qPCR) and biochemical analysis. A series of fixed tissue sections were prepared for neuropathological evaluation including visualization and quantitation of neocortical and hippocampal amyloid plaques and neurofibrillary tangles (NFTs) using antibodies directed against the Aβ peptide (Aβ; 4G8; Covance, Princeton, NJ, USA), tau (PHF1; a gift from Dr. Peter Davies)[33,40], as well as thioflavine-S (Sigma-Aldrich) histochemistry and a modified Bielschowsky silver stain (components from Fisher Scientific, Pittsburgh, PA, USA). Additional sections were stained for Lewy bodies using antibodies directed against ubiquitin and α-synuclein. Exclusion criteria included argyrophilic grain disease, frontotemporal dementia, Lewy body disease, mixed dementias, Parkinson's disease, stroke, and hippocampal sclerosis. A board-certified neuropathologist blinded to the clinical diagnosis performed the neuropathological evaluation. Neuropathological criteria were based on National Institute on Aging (NIA)-Reagan, Consortium to Establish a Registry for Alzheimer's Disease (CERAD), and Braak staging[41–43]. Amyloid burden and apolipoprotein E (ApoE) genotype were determined for each case as described previously[33,40].

qPCR

Total RNA from frozen frontal cortex (Brodmann area 10) samples was extracted using guanidine-isothiocyanate lysis (PureLink; Ambion, Waltham, MA, USA), and RNA integrity and concentration were verified using Bioanalysis (Agilent Technologies, Santa Clara, CA, USA). Samples were assayed on a real-time (RT)-PCR cycler (ABI 7500; Applied Biosystems, Foster City, CA, USA) in 96-well optical plates as described previously[44–47]. qPCR was performed using TaqMan hydrolysis probe primer sets (Applied Biosystems) specific for amplification of the following human transcripts: *rgcc* (probe set Hs00204129_m1), tumor protein p53 (*tp53*; Hs01034249_m1), and *cdk1* (Hs00938777_m1). A primer set specific for human glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) (Hs02758991_g1) was used as a control housekeeping transcript. For PC12 cell culture experiments (see below), total RNA was extracted and assayed as described above using primers specific for rat cyclin b1 (ccnb1; probe set Rn01494180_g1; Applied Biosystems) and rat *gapdh* (Rn01775763_g1). The Δ–Δ Ct (ddCT) method was employed to determine relative levels of each amplicon[44–46,48]. Variance component analyses revealed relatively low levels of within-case variability, and the average value of the triplicate qPCR products from each case was used in subsequent analyses. Alterations in PCR product synthesis were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction for post hoc comparison. The level of statistical significance was set at $\alpha = 0.05$ (two sided).

Western Blotting

Frozen frontal cortex tissue samples from the same cases used for qPCR were sonicated in ice-cold homogenization buffer [20 mM Tris, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 10% sucrose, pH 7.4] containing protease inhibitors (2 mg/ml leupeptin, 0.01 U/ml aprotinin, 1 mg/ml pepstatin A, 1 mg/ml antipain, 2.5 mg/ml chymostatin, 10 mM benzamidine, 0.1 mM PMSF, 0.4 mg/ml TPCK, 0.4 mg/ml TLCK, 0.4 mg/ml soybean trypsin inhibitor, 0.1 mM sodium fluoride, and 0.1 mM sodium orthovanadate). All chemicals were purchased from Sigma-Aldrich. Samples were prepared by centrifugation at $100 \times g$ for 10 min at 4°C. The protein concentration of the resulting S1 supernatant was determined by the Bradford method (Bio-Rad, Hercules, CA, USA), which uses bovine serum albumin (BSA) as the protein standard. Sample proteins from the S1 fraction were denatured in sodium dodecyl sulfate (SDS; Fisher Scientific) loading buffer to a final concentration of 5 mg/ml. Proteins (25 μg/sample) were separated by SDS polyacrylamide gel electrophoresis (10%; Lonza, Basel, Switzerland), transferred to Immobilon-FL membranes (Millipore, Billerica, MA, USA), blocked in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 (Fisher Scientific) and 2% nonfat milk, and then incubated overnight at 4°C with rabbit polyclonal antiserum to RGCC (1:500; Novus Biologicals, Littleton, CO, USA). Blots were then incubated for 1 h with near-infrared-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antiserum (IRDye 680LT; 1:10,000; Licor, Lincoln, NE, USA) and analyzed on an Odyssey imaging system (Licor). Following imaging, the membranes were stripped and reprobed with a mouse monoclonal β-actin antibody (1:20,000; Millipore) overnight followed by a 1-h incubation with near-infrared-labeled goat anti-mouse IgG secondary antiserum (IRDye 680LT; 1:10,000; Licor) and Odyssey imaging. Signals for RCGG were normalized to β-actin for quantitative analysis[34,47,49].

PC12 Cell Culture

PC12 cultures (a gift of Dr. Richard Burry, Ohio State University, Columbus, OH, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (Gibco, Grand Island, NY, USA), 5% FetalClone I bovine serum (Hyclone, Logan, UT, USA), and 1% penicillin/streptomycin (Gibco). Cultures were plated at 10 K/cm^2 onto Matrigel-coated dishes (1%; Collaborative Biomedical; Becton Dickinson, Frankin Lakes, NJ, USA) in DMEM with 1.5% serum. PC12 cultures were grown for 1 week in the presence of 400 pm (~50 ng/ml) mouse 7S nerve growth factor (NGF; Alomone Labs, Jerusalem, Israel). Media were replaced on days 3 and 5 in vitro. On day 7, PC12 cells were rinsed and incubated with 50 nM RGCC or scrambled siRNA (Origene, Rockville, MD, USA)/1% Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) in OptiMEM (Gibco)/400 pm NGF for 18 h[48], then rinsed and exchanged into DMEM/1.5% serum without NGF for 48 h. Cultures were assayed for cell viability using the LIVE/DEAD assay (Thermo Fisher Scientific, Waltham, MA, USA). Sister cultures were analyzed for cyclin B1 (*ccnb1*) expression, as described above.

Statistical Analysis

Demographic variables (Table 1) were compared among clinical diagnostic groups by Kruskal–Wallis or Fisher's exact tests with Bonferroni correction for pairwise comparisons. Transcript levels (qPCR), protein levels (Western blotting), and cell viability measures were compared among groups by one-way ANOVA with Bonferroni post hoc testing. The level of statistical significance was set at $p<0.05$. RGCC protein levels across diagnostic groups were tested for associations with clinical and pathological variables using Spearman rank correlations. The level of statistical significance was set at $p<0.01$.

RESULTS

Subject Demographics

The clinical diagnostic groups did not differ by age, gender, years of education, or postmortem interval (Table 1). There were significantly more subjects with an ApoE 4 allele in the AD (45%) group than in the NCI (14%) or MCI (18%) group. AD cases had significantly lower MMSE scores compared to both NCI and MCI ($p<0.001$), whereas the latter two groups did not differ statistically (Table 1). GCS *z*-scores were significantly lower in the AD compared to the NCI and MCI cases ($p<0.0001$). Subjects in the different clinical diagnostic groups displayed considerable overlap with respect to pathological diagnostic criteria. Pathological examination revealed that 64% of NCI, 64% of MCI, and 82% of AD cases were classified as Braak stages III–VI. Using

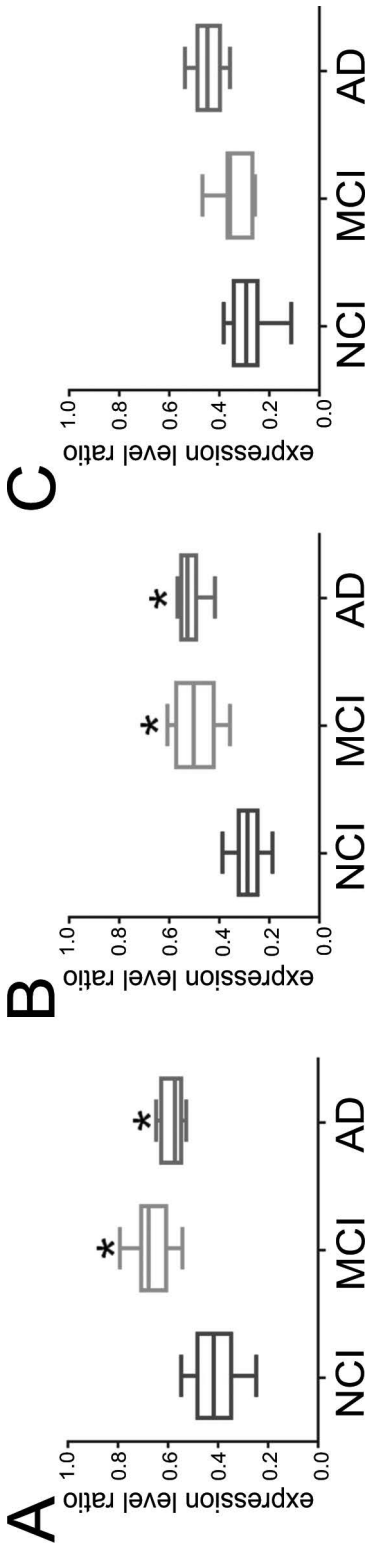

Figure 1. p53 (*tp53*) and protein regulator of cell cycle (*rgcc*) gene expression levels are increased in mild cognitive impairment (MCI) and Alzheimer's disease (AD). Box plots show relative expression levels of (A) *tp53*, (B) *rgcc*, and (C) cdc/cyclin-dependent kinase 1 (*cdk1*) normalized to glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) levels (mean ± max/min; arbitrary units) in total RNA derived from no cognitive impairment (NCI), MCI, and AD cases. $^*p<0.05$ versus NCI, via one-way analysis of variance (ANOVA) with Bonferroni post hoc comparisons.

the NIA-Reagan criteria, 57% of NCI, 45% of MCI, and 55% of AD cases were classified as intermediate to high likelihood of AD (Table 1). For CERAD diagnosis, 57% of NCI, 45% of MCI, and 55% of AD cases received a diagnosis of probable/definite AD. Statistical analysis did not reveal any differences in pathology among the NCI, MCI, and AD groups.

RCGG Expression Levels in MCI and AD

qPCR analysis was performed to quantify RGCC (*rgcc*), p53 (*tp53*), and CDK1 (*cdk1*) gene expression levels in frozen frontal cortex tissue samples accrued from NCI, MCI, and AD subjects (Fig. 1). A significant ~55%–60% increase in *rgcc* transcript levels was measured in MCI compared to NCI cases ($p<0.05$), whereas *rgcc* levels were upregulated by ~50% in AD compared to NCI ($p<0.05$) (Fig. 1A). By contrast, *tp53* expression levels were significantly increased by ~55%–60% in MCI and AD compared to NCI ($p<0.05$) (Fig. 1B), whereas there were no differences in *cdk1* expression across the diagnostic groups (Fig. 1C).

To test whether RCGG protein levels were also upregulated in the MCI and AD cases, quantitative Western blotting was performed on tissue extracts from the same cases (Fig. 2). RGCC immunoreactivity (~15-kDa band) was higher in the MCI and AD frontal cortex compared to NCI (Fig. 2A). Quantitative analysis of the Western blots showed that normalized RGCC protein levels were significantly increased by ~50%–55% in MCI and AD ($p<0.05$). Spearman rank correlations showed no association between RGCC protein levels and age, gender, PMI, or ApoE status (data not shown). By contrast, increased RGCC protein levels were associated with poorer cognitive performance as measured by the MMSE ($r=0.39$, $p=0.002$) and GCS ($r=0.43$, $p=0.005$), but not with Braak, NIA-Reagan, or CERAD neuropathological criteria.

Inhibition of RGCC Protects PC12 Cells From Nerve Growth Factor Withdrawal

Neuronotypic differentiation of rat PC12 cells with NGF, followed by NGF deprivation in low/no serum, results in aberrant cell cycle entry and apoptosis[8,50–53]. In order to assess whether RGCC might play a role in neuronal apoptosis related to cell cycle reentry, we differentiated PC12 cells and then treated the cultures with *rgcc*-specific siRNA or scrambled control siRNA prior to NGF withdrawal (Fig. 3). There was an overall ~75% decrease in the cell survival of PC12 cultures subjected to NGF withdrawal compared to cultures maintained on NGF ($p<0.01$). By contrast, *rgcc* downregulation rescued the PC12 cultures from NGF deprivation, resulting in an ~25% decrease in cell survival compared to NGF-maintained cultures (Fig. 3A). To assess the extent of cell cycle activation in the cultures, we used qPCR to measure expression levels of cyclin b1 (*ccnb1*), a CDK1 binding partner that is downregulated during NGF-induced differentiation and upregulated during NGF withdrawal and apoptosis of PC12 cells[54,55]. There was an overall ~80% increase in cyclin B1 levels in PC12 cultures subjected to NGF withdrawal compared to cultures maintained on NGF ($p<0.05$). By contrast, *rgcc* downregulation prevented cyclin B1 upregulation during NGF deprivation (Fig. 3B).

DISCUSSION

For over two decades, the concept of "abortive mitosis" has been noted as a cellular mechanism of apoptosis during development and neuronal cell death in neurodegenerative disease[3,56,57]. With respect to AD, it has been proposed that deleterious events such as the loss of neurotrophic support needed to maintain terminal differentiation, or neuronal DNA damage from oxidative stress, result in the transition from a quiescent G_0 cell cycle

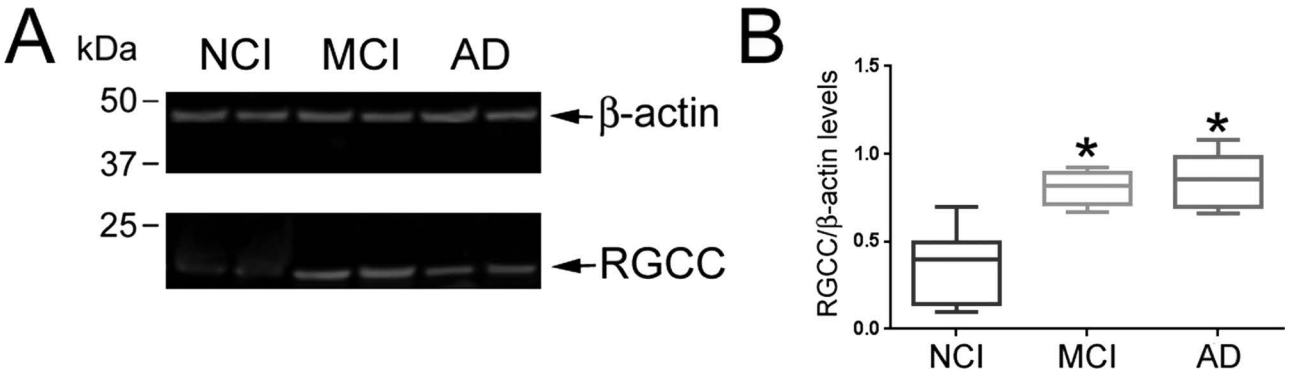

Figure 2. Regulator of cell cycle (*rgcc*) protein levels are increased in mild cognitive impairment (MCI) and Alzheimer's disease (AD). (A) Representative Western blot shows greater RCGG immunoreactivity (~15 kDa) in tissue extracts derived from MCI and AD cases compared to no cognitive impairment (NCI) cases; levels of β-actin were equivalent across samples. (B) Box plots show relative quantitative measurements of RGCC immunoreactivity normalized to β-actin signals (mean ± max/min; arbitrary units) in the three diagnostic groups. *$p<0.05$ versus NCI, via one-way analysis of variance (ANOVA) with Bonferroni post hoc comparisons.

stage into an unscheduled attempt at DNA replication and mitosis[4,7,18–20]. The consequent loss of genomic and cellular homeostasis ultimately triggers programmed cell death[3,58,59]. Moreover, the activation of several cell cycle kinases, normally under tight regulatory control in postmitotic neurons, can lead to tau hyperphosphorylation and aggregation into NFTs[14–17]. Hence, the cell cycle continues to represent a viable target for disease-modifying therapies for AD[21,58,59]. Here we provide evidence that the cell cycle regulatory protein RGCC is upregulated in MCI and AD, correlates with global cognitive decline, and may be involved in facilitating aberrant cell cycle reentry induced by neurotrophin loss in differentiated neurons, suggesting that RGCC may be a candidate cell cycle target for neuroprotection during the onset of AD. This report may also add another provocative link to the potential mechanistic interrelationship between cell transformation in cancer and selective vulnerability in neurodegenerative disease. These diseases share many molecular pathogenic processes, including oxidative and inflammatory stress, proteostatic stress, and metabolic dysregulation[21,60–64], and it has been postulated that these pathways lead to either clonal expansion in proliferating cells or clonal elimination in terminally differentiated cells such as neurons[65].

The functional and mechanistic repertoire of RGCC activity has not been fully elucidated. It was originally discovered as the RGC-32 response gene during complement activation of rat oligodendrocytes[26]. RGCC physically associates with and activates CDK1, a key kinase involved in the G_1/S and G_2/M phase transitions[26,29,30]. However, RGCC has also been implicated in diverse functions such as cellular differentiation, inflammation, vascular remodeling, and insulin resistance[32,66–70]. Interestingly, RGCC was identified as a transcriptional target and mediator of p53 tumor suppression in glioma cells[27]. In neurons, the p53 protein possesses multifactorial properties regulating DNA damage, cell cycle control, and apoptosis[71,72]. Given the evidence that p53 protein is upregulated and possibly dysregulated due to structural modifications in MCI and AD[73–75], we investigated whether RCGG was also upregulated in these disease stages and whether it could potentially play a role in neuronal cell cycle dysfunction and/or apoptosis. In this regard, we validated p53 upregulation[74] but also found that RGCC was upregulated in the frontal cortex in MCI and AD. By contrast, transcripts encoding the RGCC-regulated cell cycle protein CDK1 were stable during disease progression despite a trend ($p=0.07$) for upregulation, consistent with the notion that RGCC regulates CDK1 activity rather than expression[30].

The functional consequences of RGCC upregulation in MCI and AD subjects are unclear, but its role in cell cycle activation led us to test whether this upregulation

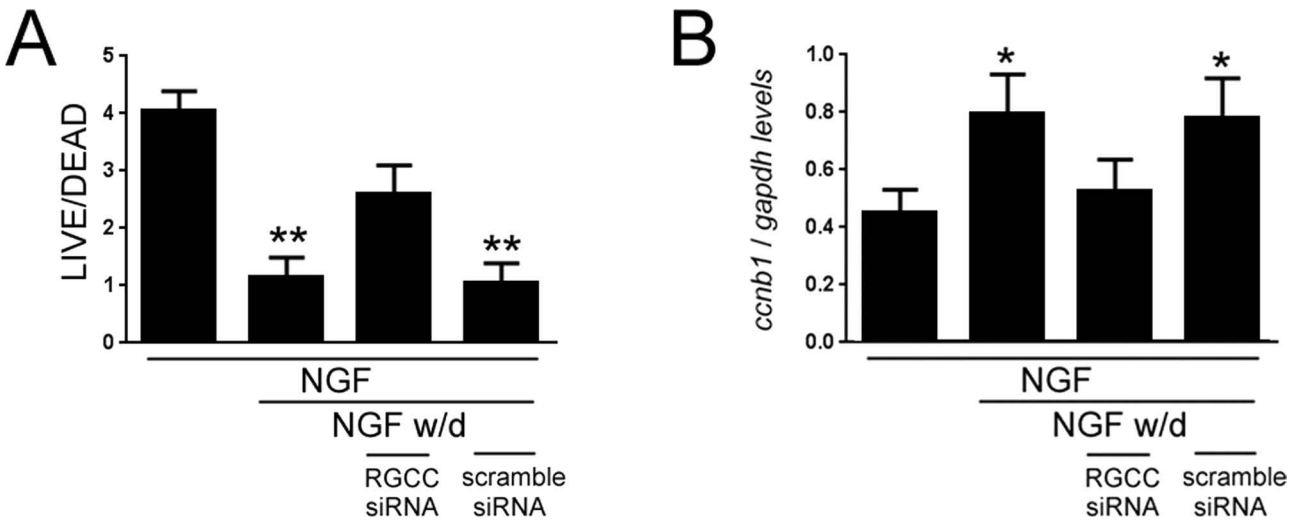

Figure 3. Regulator of cell cycle (*rgcc*) inhibition rescues PC12 cells from cell death induced by nerve growth factor (NGF) withdrawal. (A) Bar graph shows relative levels of cell survival as measured by the LIVE/DEAD assay [mean±standard deviation (SD); arbitrary units] for PC12 cultures maintained on NGF, deprived of NGF for 48 h, deprived of NGF for 48 h in the presence of *rgcc* siRNA, or deprived of NGF for 48 h in the presence of scrambled control siRNA. (B) Bar graph shows relative levels of cyclin b1 (*ccnb1*) transcript levels as measured by quantitative polymerase chain reaction (qPCR) (mean±SD; arbitrary units) for PC12 cultures maintained on NGF, deprived of NGF for 48 h, deprived of NGF for 48 h in the presence of *rgcc* siRNA, or deprived of NGF for 48 h in the presence of scrambled control siRNA. *$p<0.05$; **$p<0.01$ versus NGF, via one-way analysis of variance (ANOVA) with Bonferroni post hoc comparisons.

could reflect a deleterious event promoting "abortive mitosis" and neuronal vulnerability. To this end, we used the PC12 cell culture model as a well-established system for NGF-mediated neuronotypic differentiation and NGF withdrawal-mediated cell cycle reactivation and apoptosis[8,50–53]. Using *rgcc* and scrambled sequence control siRNA, we found that *rgcc* knockdown protected PC12 cells from NGF withdrawal and prevented the upregulation of the CDK1 binding partner cyclin B1[54,55], suggesting that RCGG participates in cell cycle reactivation and cell death within the context of deficient neurotrophin signaling.

A central concept underlying the selective vulnerability of neurons in AD is that they are dependent on neurotrophins such as NGF and brain-derived neurotrophic factor (BDNF) for survival[76–78]. NGF and BDNF are derived from proNGF and proBDNF precursor proteins, and these mature peptides interact with their cognate high-affinity receptors TrkA and TrkB, respectively, for prosurvival functions[77,79,80]. By contrast, proNGF and proBDNF have higher affinity for the pan neurotrophin receptor $p75^{NTR}$ and elicit prodeath signals[81]. Notably, we found that cortical TrkA protein levels were selectively reduced in mild AD compared to $p75^{NTR}$ [49], whereas cortical proNGF levels were elevated in MCI and AD compared to NCI[82]. Hence, increased cortical proNGF in combination with reduced cortical TrkA expression may result in enhanced binding between proNGF and $p75^{NTR}$, potentially shifting away from prosurvival NGF signaling to apoptotic signaling. Likewise, levels of BDNF and TrkB are decreased in vulnerable brain regions in MCI and AD[39,83]. This observation, combined with the presence of cell cycle proteins within vulnerable brain regions in MCI and mild AD[4,7], suggests that neurotrophin receptor imbalance promotes a loss of neurotrophic support and unscheduled cell cycle reentry and apoptosis during the prodromal stages of AD. In this regard, whereas cell cycle abnormalities have been linked to in vitro and in vivo amyloid and tau pathology[84–86], we did not find a significant association between RGCC levels and neuropathological diagnostic criteria. This may be due to the lack of significant differences in Braak, NIA-Reagan, or CERAD scores among the diagnostic groups (Table 1). On the other hand, they may suggest that neurotrophic imbalances affect RGCC and other cell cycle events independent of plaque or tangle burden. The extent to which increased RGCC levels denote its involvement in neurotrophin-mediated mitotic cell death cascades in the MCI and AD brain is a question for future study. Furthermore, given the involvement of p53 in neuronal apoptosis following NGF withdrawal[87], it will be interesting to explore whether a p53-RGCC-CDK1/cyclin B cascade mediates aberrant cell cycle activation in postmitotic neurons. If so, this pathway may present a novel therapeutic target for disease modification during the progression of AD.

ACKNOWLEDGMENTS: We are grateful for the altruism of the Religious Orders Study participants. This study was supported by the National Institutes of Health (NIH) grants PO1AG14449, RO1AG043375, R21AG026032, and R21AG042146; the Saint Mary's Foundation; Miles for Memories of Battle Creek, MI; and Barrow Neurological Institute Barrow and Beyond. The authors declare no conflicts of interest.

REFERENCES

1. Busser J, Geldmacher DS, Herrup K. Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. J Neurosci. 1998;18(8):2801–7.
2. Vincent I, Jicha G, Rosado M, Dickson DW. Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. J Neurosci. 1997; 17(10):3588–98.
3. Vincent I, Rosado M, Davies P. Mitotic mechanisms in Alzheimer's disease? J Cell Biol. 1996;132(3):413–25.
4. Yang Y, Geldmacher DS, Herrup K. DNA replication precedes neuronal cell death in Alzheimer's disease. J Neurosci. 2001;21(8):2661–8.
5. Bonda DJ, Evans TA, Santocanale C, Llosa JC, Vina J, Bajic V, Castellani RJ, Siedlak SL, Perry G, Smith MA, Lee HG. Evidence for the progression through S-phase in the ectopic cell cycle re-entry of neurons in Alzheimer disease. Aging (Albany NY) 2009;1(4):382–8.
6. Mosch B, Morawski M, Mittag A, Lenz D, Tarnok A, Arendt T. Aneuploidy and DNA replication in the normal human brain and Alzheimer's disease. J Neurosci. 2007;27(26):6859–67.
7. Yang Y, Mufson EJ, Herrup K. Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease. J Neurosci. 2003;23(7):2557–63.
8. Farinelli SE, Greene LA. Cell cycle blockers mimosine, ciclopirox, and deferoxamine prevent the death of PC12 cells and postmitotic sympathetic neurons after removal of trophic support. J Neurosci. 1996;16(3):1150–62.
9. Freeman RS, Estus S, Johnson EM Jr. Analysis of cell cycle-related gene expression in postmitotic neurons: Selective induction of cyclin D1 during programmed cell death. Neuron 1994;12(2):343–55.
10. Herrup K, Busser JC. The induction of multiple cell cycle events precedes target-related neuronal death. Development 1995;121(8):2385–95.
11. Park DS, Levine B, Ferrari G, Greene LA. Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J Neurosci. 1997;17(23):8975–83.
12. Frade JM. Unscheduled re-entry into the cell cycle induced by NGF precedes cell death in nascent retinal neurones. J Cell Sci. 2000;113(Pt 7):1139–48.
13. Malik B, Currais A, Soriano S. Cell cycle-driven neuronal apoptosis specifically linked to amyloid peptide Abeta1-42 exposure is not exacerbated in a mouse model of presenilin-1 familial Alzheimer's disease. J Neurochem. 2008;106(2):912–6.
14. Noble W, Olm V, Takata K, Casey E, Mary O, Meyerson J, Gaynor K, LaFrancois J, Wang L, Kondo T, Davies P, Burns M, Veeranna, Nixon R, Dickson D, Matsuoka Y,

Ahlijanian M, Lau LF, Duff K. Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron 2003; 38(4):555–65.
15. Baumann K, Mandelkow EM, Biernat J, Piwnica-Worms H, Mandelkow E. Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. FEBS Lett. 1993;336(3):417–24.
16. Bennecib M, Gong CX, Grundke-Iqbal I, Iqbal K. Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain. FEBS Lett. 2000;485(1):87–93.
17. Paudel HK. Phosphorylation by neuronal cdc2-like protein kinase promotes dimerization of Tau protein in vitro. J Biol Chem. 1997;272(45):28328–34.
18. Arendt T, Bruckner MK, Mosch B, Losche A. Selective cell death of hyperploid neurons in Alzheimer's disease. Am J Pathol. 2010;177(1):15–20.
19. Silva AR, Santos AC, Farfel JM, Grinberg LT, Ferretti RE, Campos AH, Cunha IW, Begnami MD, Rocha RM, Carraro DM, de Braganca Pereira CA, Jacob-Filho W, Brentani H. Repair of oxidative DNA damage, cell-cycle regulation and neuronal death may influence the clinical manifestation of Alzheimer's disease. PLoS One 2014;9(6):e99897.
20. Swerdlow RH, Burns JM, Khan SM. The Alzheimer's disease mitochondrial cascade hypothesis. J Alzheimers Dis. 2010;20(Suppl 2):S265–79.
21. Webber KM, Raina AK, Marlatt MW, Zhu X, Prat MI, Morelli L, Casadesus G, Perry G, Smith MA. The cell cycle in Alzheimer disease: A unique target for neuropharmacology. Mech Ageing Dev. 2005;126(10):1019–25.
22. Zivkovic L, Spremo-Potparevic B, Siedlak SL, Perry G, Plecas-Solarovic B, Milicevic Z, Bajic VP. DNA damage in Alzheimer disease lymphocytes and its relation to premature centromere division. Neurodegener Dis. 2013;12(3): 156–63.
23. Behrens MI, Lendon C, Roe CM. A common biological mechanism in cancer and Alzheimer's disease? Curr Alzheimer Res. 2009;6(3):196–204.
24. Nakanishi A, Minami A, Kitagishi Y, Ogura Y, Matsuda S. BRCA1 and p53 tumor suppressor molecules in Alzheimer's disease. Int J Mol Sci. 2015;16(2):2879–92.
25. Vaghefi H, Neet KE. Deacetylation of p53 after nerve growth factor treatment in PC12 cells as a post-translational modification mechanism of neurotrophin-induced tumor suppressor activation. Oncogene 2004;23(49):8078–87.
26. Badea TC, Niculescu FI, Soane L, Shin ML, Rus H. Molecular cloning and characterization of RGC-32, a novel gene induced by complement activation in oligodendrocytes. J Biol Chem. 1998;273(41):26977–81.
27. Saigusa K, Imoto I, Tanikawa C, Aoyagi M, Ohno K, Nakamura Y, Inazawa J. RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest. Oncogene 2007;26(8):1110–21.
28. Vlaicu SI, Cudrici C, Ito T, Fosbrink M, Tegla CA, Rus V, Mircea PA, Rus H. Role of response gene to complement 32 in diseases. Arch Immunol Ther Exp. (Warsz) 2008;56(2):115–22.
29. Badea T, Niculescu F, Soane L, Fosbrink M, Sorana H, Rus V, Shin ML, Rus H. RGC-32 increases p34CDC2 kinase activity and entry of aortic smooth muscle cells into S-phase. J Biol Chem. 2002;277(1):502–8.
30. Fosbrink M, Cudrici C, Tegla CA, Soloviova K, Ito T, Vlaicu S, Rus V, Niculescu F, Rus H. Response gene to complement 32 is required for C5b-9 induced cell cycle activation in endothelial cells. Exp Mol Pathol. 2009;86(2): 87–94.
31. Oram SW, Liu XX, Lee TL, Chan WY, Lau YF. TSPY potentiates cell proliferation and tumorigenesis by promoting cell cycle progression in HeLa and NIH3T3 cells. BMC Cancer 2006;6:154.
32. Tegla CA, Cudrici CD, Nguyen V, Danoff J, Kruszewski AM, Boodhoo D, Mekala AP, Vlaicu SI, Chen C, Rus V, Badea TC, Rus H. RGC-32 is a novel regulator of the T-lymphocyte cell cycle. Exp Mol Pathol. 2015;98(3):328–37.
33. Bennett DA, Wilson RS, Schneider JA, Evans DA, Beckett LA, Aggarwal NT, Barnes LL, Fox JH, Bach J. Natural history of mild cognitive impairment in older persons. Neurology 2002;59(2):198–205.
34. Counts SE, Nadeem M, Lad SP, Wuu J, Mufson EJ. Differential expression of synaptic proteins in the frontal and temporal cortex of elderly subjects with mild cognitive impairment. J Neuropathol Exp Neurol. 2006;65(6): 592–601.
35. Mufson EJ, Chen EY, Cochran EJ, Beckett LA, Bennett DA, Kordower JH. Entorhinal cortex beta-amyloid load in individuals with mild cognitive impairment. Exp Neurol. 1999;158(2):469–90.
36. Perez SE, He B, Nadeem M, Wuu J, Scheff SW, Abrahamson EE, Ikonomovic MD, Mufson EJ. Resilience of precuneus neurotrophic signaling pathways despite amyloid pathology in prodromal Alzheimer's disease. Biol Psychiatry 2015;77(8):693–703.
37. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984;34(7):939–44.
38. Petersen RC, Doody R, Kurz A, Mohs RC, Morris JC, Rabins PV, Ritchie K, Rossor M, Thal L, Winblad B. Current concepts in mild cognitive impairment. Arch Neurol. 2001;58(12):1985–92.
39. Ginsberg SD, Alldred MJ, Counts SE, Cataldo AM, Neve RL, Jiang Y, Wuu J, Chao MV, Mufson EJ, Nixon RA, Che S. Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer's disease progression. Biol Psychiatry 2010;68(10):885–93.
40. Mufson EJ, Chen EY, Cochran EJ, Beckett LA, Bennett DA, Kordower JH. Entorhinal cortex beta-amyloid load in individuals with mild cognitive impairment. Exp Neurol. 1999;158(2):469–90.
41. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 1991;82(4):239–59.
42. Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Thies B, Trojanowski JQ, Vinters HV, Montine TJ. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement. 2012;8(1):1–13.
43. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991;41(4):479–86.

44. Alldred MJ, Che S, Ginsberg SD. Terminal continuation (TC) RNA amplification without second strand synthesis. J Neurosci Methods 2009;177(2):381–5.
45. Counts SE, He B, Che S, Ikonomovic MD, DeKosky ST, Ginsberg SD, Mufson EJ. Alpha7 nicotinic receptor up-regulation in cholinergic basal forebrain neurons in Alzheimer disease. Arch Neurol. 2007;64(12):1771–6.
46. Ginsberg SD. Transcriptional profiling of small samples in the central nervous system. Methods Mol Biol. 2008; 439:147–58.
47. Beck JS, Mufson EJ, Counts SE. Evidence for mitochondrial UPR gene activation in familial and sporadic Alzheimer's disease. Curr Alzheimer Res. 2016;13(6):610–4.
48. Weinberg RB, Mufson EJ, Counts SE. Evidence for a neuroprotective microRNA pathway in amnestic mild cognitive impairment. Front Neurosci. 2015;9:430.
49. Counts SE, Nadeem M, Wuu J, Ginsberg SD, Saragovi HU, Mufson EJ. Reduction of cortical TrkA but not p75(NTR) protein in early-stage Alzheimer's disease. Ann Neurol. 2004;56(4):520–31.
50. Counts SE, Lah JJ, Levey AI. The regulation of presenilin-1 by nerve growth factor. J Neurochem. 2001;76(3):679–89.
51. Greene LA. Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. J Cell Biol. 1978;78(3):747–55.
52. Mesner PW, Epting CL, Hegarty JL, Green SH. A timetable of events during programmed cell death induced by trophic factor withdrawal from neuronal PC12 cells. J Neurosci. 1995;15(11):7357–66.
53. Bianco MR, Berbenni M, Amara F, Viggiani S, Fragni M, Galimberti V, Colombo D, Cirillo G, Papa M, Alberghina L, Colangelo AM. Cross-talk between cell cycle induction and mitochondrial dysfunction during oxidative stress and nerve growth factor withdrawal in differentiated PC12 cells. J Neurosci Res. 2011;89(8):1302–15.
54. Gao CY, Zelenka PS. Induction of cyclin B and H1 kinase activity in apoptotic PC12 cells. Exp Cell Res. 1995;219(2):612–8.
55. Yan GZ, Ziff EB. NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. J Neurosci. 1995;15(9): 6200–12.
56. Lee S, Christakos S, Small MB. Apoptosis and signal transduction: Clues to a molecular mechanism. Curr Opin Cell Biol. 1993;5(2):286–91.
57. Ucker DS. Death by suicide: One way to go in mammalian cellular development? New Biol. 1991;3(2):103–9.
58. Smith MA, Nunomura A, Zhu X, Takeda A, Perry G. Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease. Antioxid Redox Signal. 2000;2(3): 413–20.
59. Katsel P, Tan W, Fam P, Purohit DP, Haroutunian V. Cell cycle checkpoint abnormalities during dementia: A plausible association with the loss of protection against oxidative stress in Alzheimer's disease [corrected]. PLoS One 2013;8(7):e68361.
60. Audas TE, Audas DE, Jacob MD, Ho JJ, Khacho M, Wang M, Perera JK, Gardiner C, Bennett CA, Head T, Kryvenko ON, Jorda M, Daunert S, Malhorta A, Trinkle-Mulcahy L, Gonzalgo ML, Lee S. Adaptation to stressors by systemic protein amyloidogenesis. Dev Cell 2016; 39(2):155–168.
61. Bennett DA, Leurgans S. Is there a link between cancer and Alzheimer disease? Neurology 2010;74(2):100–1.
62. Driver JA. Inverse association between cancer and neurodegenerative disease: Review of the epidemiologic and biological evidence. Biogerontology 2014;15(6):547–57.
63. Harris RA, Tindale L, Cumming RC. Age-dependent metabolic dysregulation in cancer and Alzheimer's disease. Biogerontology 2014;15(6):559–77.
64. Vilchez D, Saez I, Dillin A. The role of protein clearance mechanisms in organismal ageing and age-related diseases. Nat Commun. 2014;5:5659.
65. Heintz N. Cell death and the cell cycle: A relationship between transformation and neurodegeneration? Trends Biochem Sci. 1993;18(5):157–9.
66. An X, Jin Y, Guo H, Foo SY, Cully BL, Wu J, Zeng H, Rosenzweig A, Li J. Response gene to complement 32, a novel hypoxia-regulated angiogenic inhibitor. Circulation 2009;120(7):617–27.
67. Cui XB, Luan JN, Ye J, Chen SY. RGC32 deficiency protects against high-fat diet-induced obesity and insulin resistance in mice. J Endocrinol. 2015;224(2):127–37.
68. Tang R, Zhang G, Chen SY. Response gene to complement 32 protein promotes macrophage phagocytosis via activation of protein kinase C pathway. J Biol Chem. 2014;289(33):22715–22.
69. Xu R, Shang C, Zhao J, Han Y, Liu J, Chen K, Shi W. Knockdown of response gene to complement 32 (RGC32) induces apoptosis and inhibits cell growth, migration, and invasion in human lung cancer cells. Mol Cell Biochem. 2014;394(1–2):109–18.
70. Zhao P, Gao D, Wang Q, Song B, Shao Q, Sun J, Ji C, Li X, Li P, Qu X. Response gene to complement 32 (RGC-32) expression on M2-polarized and tumor-associated macrophages is M-CSF-dependent and enhanced by tumor-derived IL-4. Cell Mol Immunol. 2015;12(6):692–9.
71. Culmsee C, Mattson MP. p53 in neuronal apoptosis. Biochem Biophys Res Commun. 2005;331(3):761–77.
72. Lanni C, Racchi M, Memo M, Govoni S, Uberti D. p53 at the crossroads between cancer and neurodegeneration. Free Radic Biol Med. 2012;52(9):1727–33.
73. Buizza L, Cenini G, Lanni C, Ferrari-Toninelli G, Prandelli C, Govoni S, Buoso E, Racchi M, Barcikowska M, Styczynska M, Szybinska A, Butterfield DA, Memo M, Uberti D. Conformational altered p53 as an early marker of oxidative stress in Alzheimer's disease. PLoS One 2012;7(1):e29789.
74. Cenini G, Sultana R, Memo M, Butterfield DA. Elevated levels of pro-apoptotic p53 and its oxidative modification by the lipid peroxidation product, HNE, in brain from subjects with amnestic mild cognitive impairment and Alzheimer's disease. J Cell Mol Med. 2008;12(3):987–94.
75. Perluigi M, Barone E, Di Domenico F, Butterfield DA. Aberrant protein phosphorylation in Alzheimer disease brain disturbs pro-survival and cell death pathways. Biochim Biophys Acta 2016;1862(10):1871–82.
76. Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature 1997;389(6653):856–60.
77. Counts SE, Mufson EJ. The role of nerve growth factor receptors in cholinergic basal forebrain degeneration in prodromal Alzheimer disease. J Neuropathol Exp Neurol. 2005;64(4):263–72.

78. Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci. 2001;24:1217–81.
79. Mufson EJ, Counts SE, Perez SE, Ginsberg SD. Cholinergic system during the progression of Alzheimer's disease: Therapeutic implications. Expert Rev Neurother. 2008;8(11):1703–18.
80. Barbacid M. Structural and functional properties of the TRK family of neurotrophin receptors. Ann NY Acad Sci. 1995;766:442–58.
81. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science 2001;294(5548):1945–8.
82. Peng S, Wuu J, Mufson EJ, Fahnestock M. Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer's disease. J Neuropathol Exp Neurol. 2004;63(6):641–9.
83. Peng S, Wuu J, Mufson EJ, Fahnestock M. Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. J Neurochem. 2005;93(6):1412–21.
84. Bloom GS. Amyloid-beta and tau: The trigger and bullet in Alzheimer disease pathogenesis. JAMA Neurol. 2014; 71(4):505–8.
85. Seward ME, Swanson E, Norambuena A, Reimann A, Cochran JN, Li R, Roberson ED, Bloom GS. Amyloid-beta signals through tau to drive ectopic neuronal cell cycle reentry in Alzheimer's disease. J Cell Sci. 2013;126(Pt 5): 1278–86.
86. Varvel NH, Bhaskar K, Patil AR, Pimplikar SW, Herrup K, Lamb BT. Abeta oligomers induce neuronal cell cycle events in Alzheimer's disease. J Neurosci. 2008;28(43): 10786–93.
87. Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD. p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol. 1998;143(6):1691–703.