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Epigallocatechin-3-gallate up-regulates tumor suppressor gene expression via a reactive oxygen species-dependent down-regulation of UHRF1

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ABSTRACT

Ubiquitin-like containing PHD and Ring finger 1 (UHRF1) contributes to silencing of tumor suppressor genes by recruiting DNA methyltransferase 1 (DNMT1) to their hemi-methylated promoters. Conversely, demethylation of these promoters has been ascribed to the natural anti-cancer drug, epigallocatechin-3-gallate (EGCG). The aim of the present study was to investigate whether the UHRF1/DNMT1 pair is an important target of EGCG action. Here, we show that EGCG down-regulates UHRF1 and DNMT1 expression in Jurkat cells, with subsequent up-regulation of *p73* and *p16^{INK4A}* genes. The down-regulation of UHRF1 is dependent upon the generation of reactive oxygen species by EGCG. Up-regulation of *p16^{INK4A}* is strongly correlated with decreased promoter binding by UHRF1. UHRF1 over-expression counteracted EGCG-induced G1-arrested cells, apoptosis, and up-regulation of *p16^{INK4A}* and *p73*. Mutants of the Set and Ring Associated (SRA) domain of UHRF1 were unable to down-regulate *p16^{INK4A}* and *p73*, either in the presence or absence of EGCG. Our results show that down-regulation of UHRF1 is upstream to many cellular events, including G1 cell arrest, up-regulation of tumor suppressor genes and apoptosis.

1. Introduction

Human UHRF1, also called ICBP90 [1–4], is an essential protein required for the maintenance of DNA methylation patterns at CpG dinucleotides and of the histone code [5,6]. UHRF1 via its Set and Ring Associated (SRA) domain, exhibits affinity for hemi-methylated DNA, an intermediate generated at the replication fork [7–10]. Interestingly, the SRA domain of UHRF1 is also involved in the interaction with DNMT1 [11]. The SRA domain looks like a hand grasping the double helix, the palm containing the methylated cytosine, and a finger probing the major groove of the DNA for the presence of a methyl group in the opposing strand [8]. This base flipping mechanism has been suggested to create a high-affinity binding site in order to stably target DNMT1 to hemimethylated DNA sites for faithful maintenance methylation [8–10]. Interaction between the SRA domain and DNMT1 may occur via the latter's SRA binding domain, which overlaps with the RFTS domain of DNMT1 [11,12]. Recently, the SRA domain has gained even more interest by its ability to bind to hydroxymethylcytosine, the 6th DNA base [13].

Green tea's anti-cancer properties have been attributed to the polyphenol epigallocatechin-3-gallate (EGCG) [14]. This compound has been reported to up-regulate tumor suppressor genes such as *p16^{INK4A}*, retinoic acid receptor β (*RAR β*), *O^6*-methylguanine methyltransferase (*MGMT*) and human mutL homologue 1 (*hMLH1*) via reversal of hypermethylation of their promoters [15]. Recently, it has been shown that green tea polyphenols induced re-expression of gluthatione-S-transferase pi (GSTP1) through a promoter demethylating mechanism [16]. Furthermore, Annurca apple polyphenols have potent demethylating activity of tumor suppressor genes such as *hMLH1, p14^{ARF}* and *p16^{INK4A}* [17]. EGCG has also been reported to inhibit the enzymatic activity of DNMT1 [15]. More recently, it was shown that EGCG targets DNMT1 expression, which allows subsequent re-expression of *Cip1/p21* and *p16^{INK4A}* [18] and induces apoptosis in several cell types [19–24].

Because UHRF1 expression exhibits anti-apoptotic properties [25] and negatively regulates tumor suppressor genes such as *RB1, p16^{INK4A}* and *BRCA1* [2,11,26–28], we hypothesized that EGCG

Abbreviations: DNMT1, DNA methyltransferase1; EGCG, epigallocatechin-3-gallate; ROS, reactive oxygen species; UHRF1, Ubiquitin-like PHD, Ring finger 1.

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regulate tumor suppressor gene expression by targeting UHRF1 expression. EGCG might then allow re-expression of tumor suppressor genes with subsequent induction of apoptosis. In the present study, we found that EGCG regulates UHRF1 expression as well as that of DNMT1. Notably, EGCG-induced apoptosis and up-regulation of $p16^{INK4A}$ was counteracted by over-expression of wild-type UHRF1 but not by site-specific SRA mutants of UHRF1.

2. Materials and methods

2.1. Chemicals and antibodies

The mouse monoclonal antibody (clone 1RC1C-10) raised against UHRF1 was engineered as described elsewhere [1]. Horseradish peroxydase-conjugated anti-mouse Ab (for mAbs) was from Jackson immunoresearch (West Grove, PA). Horseradish peroxydase-conjugated anti-rabbit Ab (for polyclonal antibodies) was from Cell Signaling (Danvers, MA). The mouse monoclonal anti-p73 antibody was obtained from BD Biosciences (Le Pont de Claix, France). Polyethylene glycol (PEG)-catalase was obtained from Sigma–Aldrich (St-Louis, MO).

The anti-β-tubulin mAb was from Sigma Chemicals (St. Louis, MO) and the anti-DNMT1 mAb (clone 60B1220.1) was from Stressgen (Victoria, BC, Canada). The rabbit polyclonal anti-$p16^{INK4A}$ Ab was from DeltaBiolabs (Gilroy, CA). The rabbit polyclonal anti-VEGF Ab was from Tebu-Bio (Le Perray en Yvelines, France). The superoxide dismutase (SOD) mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was from Alexis Chemicals (Lufelingen, Switzerland). EGCG was from DSM Nutritional Products (Kaiseraugst, Switzerland). Propidium iodide (PI), Annexin 7AAD, Annexin V and Caspase 3/7 kit for Easycyte plus were from Guava Technologies (Hayward, CA).

2.2. Synthesis and over-expression of wild-type and mutants of UHRF1

Wild-type UHRF1 cloned into the pSG5 vector was described elsewhere [1]. Mutant cDNAs, cloned into the pCR-BluntIITOPO [8], were amplified by PCR using high fidelity Phusion DNA polymerase (Finnzymes, Espoo, Finland) and oligonucleotides flanked with the EcoRI (5') and XhoI (3') sites. PCR products were digested with the corresponding restriction enzymes and further cloned into the pCMV2c vector (Sigma–Aldrich) in order to obtain FLAG-tagged UHRF1 wild-type and mutants. The FLAG tag allowed us to verify that the mutants were adequately localized in the nucleus and to check transfection efficiency which ranged from 35% to 45%. All constructs were verified by sequencing (Proteogenix, Oberhausbergen, France).

Transfections were performed in 24-well plates and 80,000 cells using 20 μg/ml of plasmid and lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Four hours, after transfection, cells were treated with EGCG for 24 h and then cells were harvested for analysis.

2.3. Cell culture and western blot

Human T lymphocyte leukemic Jurkat cells were obtained from the America Type Culture Collection (Mannassa, VA) and were grown in RPMI1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin.

Whole cell extracts and western blotting were performed as described elsewhere [1,11]. Proteins from cell lysates were loaded for one-dimensional electrophoresis on SDS–PAGE; 6% acrylamide for the detection of DNMT1, 10% for UHRF1, p73, VEGF, and β-tubulin and 15% for $p16^{INK4A}$. Blots were probed with anti-UHRF1 mAb (0.2 μg/ml), anti-DNMT1 (2 μg/ml), anti-p73 (2 μg/ml), anti-VEGF (1:5,000), anti-β-tubulin (1:25,000) or anti-$p16^{INK4A}$ (1:250). Secondary peroxidase conjugated Abs were diluted 1:10,000. Signals were detected by chemiluminescence using the ECL detection system (Amersham Biosciences Europe GmbH, Saclay, France).

2.4. Apoptosis and cell cycle analysis

Apoptosis and cell cycle analysis were described elsewhere [29]. Briefly, in 96-well cell culture plates, apoptotic and necrotic cells were discriminated by addition of annexin-V and 7-AAD (GUAVA PCA-96 Nexin Kit) or by addition of FLICA (Fluorescent Labeled Inhibitor of Caspases) a reagent that specifically identifies active caspase 3 and 7 (Caspase 3/7 Reagent Kit) and the nuclear DNA stain 7-AAD was included to simultaneously evaluate membrane integrity and cell viability. Cells were run on the capillary cytometer EasyCyte 96 plus (7 parameters) and data analyzed on the Guava CytoSoft™ Express Pro software (Merck/Millipore/Guava Tech., CA).

Cell cycle analysis was performed after cell fixation, prior to staining (CellFix®, BD Biosciences). After addition of Propidium iodide (2 μl/200 μl of 1 mg/ml stock solution) cells were run on the capillary cytometer EasyCyte 96 plus (7 parameters) and data analyzed on the Guava CytoSoft™ Express Pro software (Merck/Millipore/Guava Tech). CytoSoft Express Pro was used to identify the three cell cycle phases and calculate relevant statistics, including population percentages. Population data was displayed in a single parameter histogram with up to four markers. The first marker was used to demarcate apoptotic cells when present. The next three markers were used to delineate G0/G1, S and G2/M phases. Aggregates and G0/G1 phase cell doublets from within parent G2/M populations were eliminated by gating on cytogram of area versus peak PI fluorescence.

2.5. DNA chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed using ChIP-IT Express Enzymatic Magnetic Chromatin Immunoprecipitation kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). A total of 20 μg of Abs were used. Polymerase chain reaction was performed using the protocol recommended for the iQ SYBR Green Supermix from Bio-Rad in a MyIQ thermocycler (Bio-Rad, Hercules, CA). The sequences of the primers for PCR amplification were: $p16^{INK4A}$ promoter sense: 5′-GGGCTCTCACAAC TAGGAA-3′; $p16^{INK4A}$ promoter antisense: 5′-CCAGCAAAGGCGTGT TTGA-3′. Amplicons on agarose gels were visualized by a GeneGenius Bio Imaging System (Syngene/Ozyme, Saint Quentin-en-Yvelines, France) using the GeneSnap software and quantified using the GeneTools software (Syngene/Ozyme).

2.6. Statistical analysis

Data were presented in a bar graph form, expressed as means ± S.E.M. from at least three independent experiments and statistically subjected to the one-way ANOVA test. Significance levels were defined in accordance with the standard notation.

3. Results

3.1. EGCG regulates UHRF1, DNMT1, VEGF, $p16^{INK4A}$, p73α and p73β expression

The present study was undertaken to further decipher the mechanism of action of EGCG. We examined the effect of increasing doses of EGCG on UHRF1, DNMT1, on two tumor suppressor genes, *i.e., p73 and* $p16^{INK4A}$ as well as on vascular endothelial

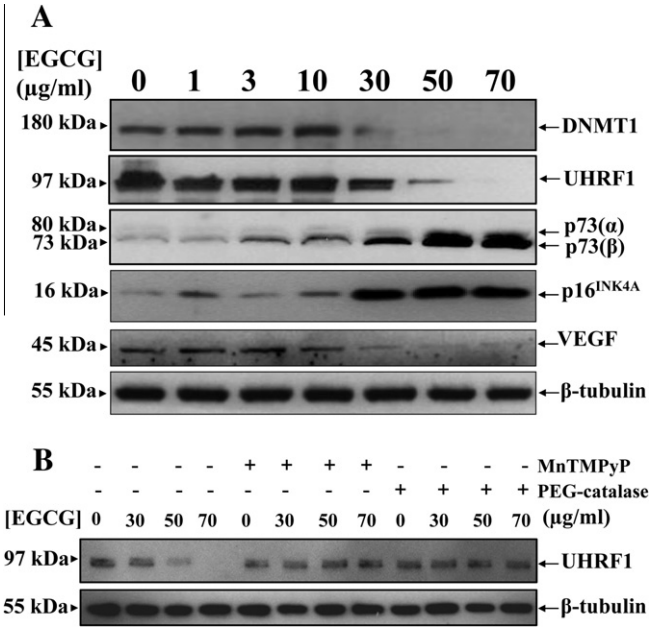

Fig. 1. (A) Effects of EGCG on UHRF1, DNMT1, $p16^{INK4A}$ VEGF and p73 (A). Jurkat cells were treated with different concentrations of EGCG for 24 h. DNMT1, UHRF1, p73, $p16^{INK4A}$, VEGF, and β-tubulin genes expression were analyzed by western blotting. Results were representative of three separated experiments. (B) Effects of MnTMPyP and PEG-catalase on the inhibition of UHRF1 expression induced by EGCG. PEG-catalase (500 U/ml) and MnTMPyP (100 µM) were added to jurkat cells 30 min before the addition of the different concentrations of EGCG. Jurkat cells were collected 24 h later and protein expression was assessed by western-blot. Results were representative of four separate experiments.

growth factor gene (*VEGF*) (Fig. 1). Starting from 30 µg/ml, EGCG induced a fall in the expression of UHRF1, DNMT1 and VEGF (Fig. 1A). The decrease was highly correlated with an enhancement of $p16^{INK4A}$ and p73β expression, and in a lesser extent with p73α.

3.2. EGCG-induced UHRF1 down-regulation is ROS-dependent

Cell permeant MnTMPyP and PEG-catalase, which have ROS scavenger properties, were used to investigate whether ROS are involved in the down-regulation of UHRF1 and thus in re-expression of tumor suppressor genes. The effect of PEG-catalase (500 U/ml) and MnTMPyP (100 µM) in the presence of different concentrations of EGCG was examined (Fig. 1B). In the absence of the compounds, EGCG was efficient in inhibiting UHRF1 expression at concentrations of 50 µg/ml and 70 µg/ml (Fig. 1B). This inhibition was abolished in the presence of either MnTMPyP or PEG-catalase (Fig. 1B), showing that EGCG-mediated UHRF1 down-regulation was ROS-dependent.

3.3. EGCG decreased binding of UHRF1 to the $p16^{INK4A}$ promoter

To check whether UHRF1 is tethered to the $p16^{INK4A}$ promoter in the absence but not in the presence of EGCG, we performed DNA ChIP experiments. PCR amplification of a region of the $p16^{INK4A}$ promoter (191 bp, Fig. 2A) after immunoprecipitation of the chromatin with the anti-UHRF1 mAb was performed. While UHRF1 bound to the $p16^{INK4A}$ promoter in the absence of EGCG, binding was drastically decreased in the presence of 50 µg/ml of EGCG (Fig. 2B). This decrease was found to be significant (Fig. S1). These results showed that by decreasing UHRF1 expression, EGCG directly affected the amount of UHRF1 bound to the $p16^{INK4A}$ promoter, and suggested that this process might be responsible for the up-regulation of the $p16^{INK4A}$ expression.

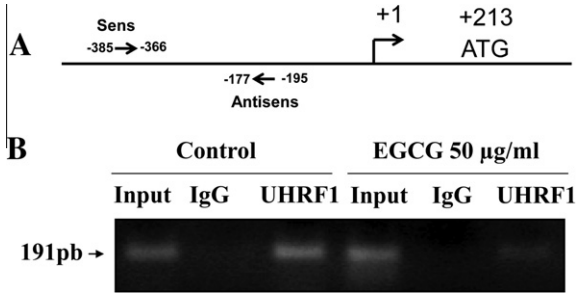

Fig. 2. ChIP assay of the binding of UHRF1 to the $p16^{INK4A}$ promoter in the presence and absence of EGCG. Experiments were carried out on Jurkat cells as described in Materials and methods. Amplified DNA (191 pb), using the primers positioned relative to the transcription start site (+1) and the start codon ATG (A), was analyzed by agarose gel (2%) electrophoresis (B).

3.4. Over-expression of UHRF1 counteracts EGCG-induced cell cycle arrest and apoptosis

Next, we analyzed the effect of EGCG on Jurkat cell proliferation and apoptosis in the absence and presence of over-expressed UHRF1. EGCG enhanced the number of cells in G1 and decreased the number of cells in S-phase and G2/M phase (Fig. S2). Indeed, 74% and 71% of EGCG-treated-Jurkat cells were blocked at G1 phase at 30 µg/ml and 50 µg/ml of EGCG respectively versus 39% without EGCG treatment. In contrast, only 28% and 36% of pSG5/UHRF1-transfected-EGCG-treated-Jurkat cells were blocked in G1 phase at 30 µg/ml and 50 µg/ml of EGCG, respectively. The control plasmid (pSG5), lacking the UHRF1 cDNA, did not modify the effect of EGCG on cell cycle distribution (Fig. S2). These results showed that over-expression of UHRF1 counteracted EGCG-induced G1 arrest.

The effect of UHRF1 over-expression on EGCG-induced apoptosis was checked (Fig. 3). EGCG increased the number of apoptotic cells, an effect that was unchanged in the presence of the control vector (pSG5). In contrast, over-expression of UHRF1 (pSG5/UHRF1) hindered the enhancement of apoptotic cells challenged by EGCG (Fig. 3). This result shows that UHRF1 acts as an anti-apoptotic protein, in the EGCG-induced apoptosis process.

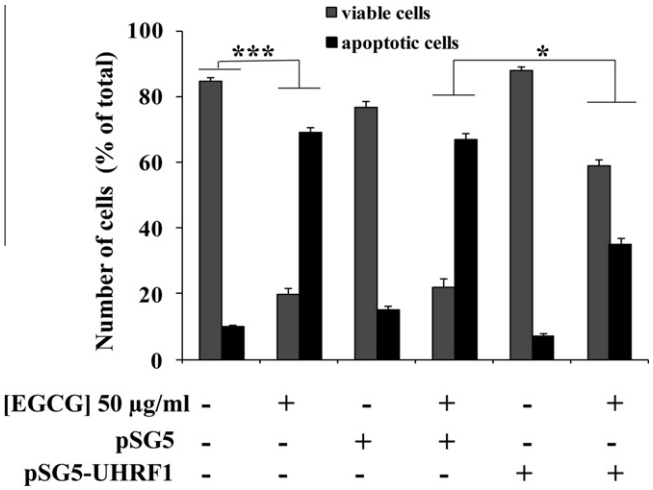

Fig. 3. Apoptosis analysis by FACS for pSGC5 or pSGS5/UHRF1 transfected-EGCG-treated-Jurkat cells. Cell apoptosis rate was assessed by capillary cytometry using the Annexin V-FITC staining assay. The number of apoptotic cells is expressed as percent relative to the total cell number. Values are means ± S.E.M. of three experiments ($n = 3$); statistically significant: $^{*}p < 0.05$, $^{***}p < 0.001$.

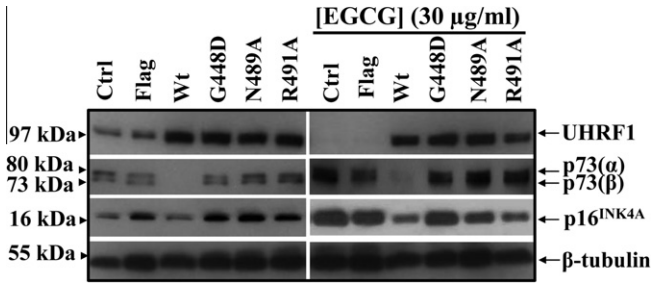

Fig. 4. Effects of SRA mutants on the ability of UHRF1 to silence tumor suppressor genes in the presence or absence of EGCG. Jurkat cells were transfected with 20 µg/ml of wild-type and three mutated forms of UHRF1 at SRA domain (G448D, N489A, and R491A) for 24 h. Tumor suppressor genes ($p16^{INK4A}$ and *p73*) and UHRF1 expressions were analyzed by Western blot.

3.5. The SRA domain of UHRF1 is involved in the silencing of tumor suppressor genes

To determine whether EGCG-induced up-regulation of tumor suppressor genes was dependent on the absence of a functional SRA domain, we constructed three mutants of UHRF1 that targeted the SRA domain, including the "NKR finger" (N489A, and R491A) and the "palm" (G448D). The latter mutation localizes at the entry point of the flipped methylated cytosine binding pocket of the SRA domain. In the absence of EGCG, over-expression of wild-type UHRF1 induced down-regulation of p73α, p73β and $p16^{INK4A}$ (Fig. 4). In contrast, all three UHRF1 mutants were unable to down-regulate p73α, p73β and $p16^{INK4A}$ expression. In the presence of EGCG, over-expression of wild-type UHRF1 decreased $p16^{INK4A}$, p73α and p73β (Fig. 4), whereas UHRF1 SRA mutants were inefficient except for R491A mutant which remained active, as did the wild type, on the $p16^{INK4A}$ expression (Fig. 4).

4. Discussion

The anti-carcinogenic effects of EGCG have been studied extensively *in vitro* and *in vivo* but the precise molecular mechanisms are unclear [30,31]. Recent evidence suggested that epigenetic mechanisms, such as promoter demethylation, could be involved, but actors have yet been identified. DNMT1 was suspected to be a target of EGCG [18,32]. The present study was designed to investigate whether the other main actor in DNA maintenance methylation patterns, UHRF1, when down-regulated, mediates the effects of EGCG. Here, we showed that EGCG down-regulated UHRF1 as well as DNMT1. The down-regulation was absolutely correlated with up-regulation of tumor suppressor genes *p73α, p73β and* $p16^{INK4A}$.

Because UHRF1 controls expression of DNMT1 [11], our results suggest that the mechanism of action of EGCG first involved a down-regulation of UHRF1 with subsequent down-regulation of DNMT1. This may explain why EGCG has DNA demethylation properties [18]. Our study showed that the effect of EGCG was dependent upon the down-regulation of UHRF1 because its over-expression counteracted the effect of EGCG. Moreover, over-expression of UHRF1 mutants was unable to down-regulate $p16^{INK4A}$ and p73α expression. This is in accordance with the fact that UHRF1 requires the SRA domain to bind DNA and that DNMT1 needs UHRF1 to be recruited to chromatin [7,11]. Indeed, when the SRA domain is not functional, UHRF1 cannot localize at methylated CpG, and thus cannot behave as the guide for DNMT1 to show where this latter has to methylate the newly synthesized DNA strand. This is probably a key mechanism in the DNA demethylation process.

EGCG is an anti-oxidant compound *in vitro* but it has pro-oxidant properties in cells [33–35]. Here, we showed that the cellular pro-oxidant properties of EGCG involved down-regulation of UHRF1. This conclusion was supported by the observation that two cell-permeant ROS scavengers were able to inhibit down-regulation of UHRF1 by EGCG. This further supported our UHRF1-dependant model of EGCG action, but the mechanism by which ROS induced down-regulation of UHRF1 was not further investigated. However, recently it was proposed that EGCG could induce p53/p21 expression [36] which are known to down-regulate UHRF1 [27].

The G1/S phase transition requires UHRF1 activity [3] and it can be blocked by EGCG [[37], present study]. In the present study, we showed that UHRF1 over-expression released G1-blocked-EGCG-treated-cells into the S-phase. This raised an important question about the mechanisms by which EGCG induced DNA demethylation and about the kinetics of demethylation in general. Demethylation *per se* is thought to occur via a non-enzymatic process, namely a passive process, where demethylated DNA appears only with newly-synthesized DNA, with cell division. How then can EGCG induce DNA demethylation in spite of cell arrest in G1? Could an active demethylation process be involved? Could an active process involve the recently identified modified cytosines: 5-hmC (5hmC), carboxylcytosine (5caC) and formylcytosine (5fC) [38]? Interestingly, UHRF1 is the first and sole protein, identified so far, capable of sensing or binding 5hmC with an equal preference [13] suggesting that might UHRF1 play a regulatory role in the DNA demethylation process.

In conclusion, our results demonstrate that the mechanism of action of EGCG involves a ROS-dependent down-regulation of UHRF1, decrease of DNMT1 expression, and subsequent up-regulation of tumor suppressor genes. Furthermore, we demonstrate that UHRF1 base flipping is required for the down-regulation of tumor suppressor genes such as $p16^{INK4A}$. EGCG, appears also being a helpful pharmacological tool to down-regulate endogenous UHRF1, allowing thus to investigate, by over-expression studies, the role of the different domain of UHRF1 in the regulation of tumor suppressor gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.087.

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