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SUMO modification regulates the protein stability of NDRG1

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ABSTRACT

N-myc Downstream Regulated Gene 1 (NDRG1) is a metastasis suppressor protein which suppresses metastasis without affecting primary tumorigenesis. There have been many reports about the anti-metastatic function of NDRG1 in various cancers. However, the regulatory mechanism of NDRG1 at the protein level has not been studied widely. Here, we found that NDRG1 is posttranslationally modified by Small Ubiquitin-like Modifier (SUMO), preferentially by SUMO-2, and the major SUMO acceptor site of NDRG1 is Lys 14. Using various SUMO-2 modification status mimicking NDRG1 mutants, we characterized the role of SUMO-2 modification on NDRG1. SUMO-2 modification does not affect the subcellular distribution of NDRG1. However, the protein stability of NDRG1 is influenced by SUMO-2 modification. We found that both the wildtype and the SUMO modification site mutant form of the NDRG1 protein were very stable but the protein stability of SUMO-2 fused NDRG1 K14R had dramatically decreased. In addition, the expression of p21 is downregulated by overexpression of SUMO-2 fused NDRG1 K14R mutants. These results indicate that SUMO-2 modification is implicated in the modulation of NDRG1 protein level and function. This novel link between SUMO modification and regulation of NDRG1 could be a therapeutic target for treatment of various metastatic cancers.

1. Introduction

N-myc downstream regulated gene-1 (NDRG1) is a member of the NDRG family which belongs to the α/β hydrolase superfamily, although NDRG1 does not possess hydrolytic catalytic function. NDRG1 is a 43 kDa protein which shows predominant cytoplasmic expression and is highly conserved across a wide range of organisms [1]. The biological function of NDRG1 in cell growth, differentiation, development, lipid biosynthesis and myelination, stress response and immunity has been reported. The implication of NDRG1 in cancer progression and metastasis has been extensively studied. Also, the anti-metastatic function of NDRG1 as a metastasis suppressor protein has been identified in multiple cancers including breast, colon, prostate, and gastric cancers [2–5]. The expression of NDRG1 is regulated by diverse agents such as metal ions, androgens, forskolin, okadaic acid, vitamin A, DNA damage, PTEN, p53, and hypoxia [1]. The modification of NDRG1 by phosphate group has been reported in several reports. Phosphorylation of NDRG1 is concentrated at the C-terminal three decapeptide tandem repeats, each of which contains the residues GTRSRSHTSE [1]. Further, phosphorylation of NDRG1 is accomplished by serum- and glucocorticoid-induced kinase (SGK)-1 and glycogen synthase kinase (GSK)-3β [6]. NDRG1 phosphorylation by SGK-1 was temporally and spatially controlled during the cell cycle [7]. Phosphorylation of NDRG1 at Ser330 and Thr346 by SGK-1 is required for its suppressive action on the NF-κB signaling pathway and CXC chemokine expression and hence suppresses angiogenesis and tumor growth in pancreatic cancer cells [8].

Posttranslational modification by small proteins such as ubiquitin or small ubiquitin-like modifier (SUMO) to the ε-amino group of a lysine residue plays an important role in the functional regulation of target proteins. SUMO is an approximately 11 kDa polypeptide that is structurally, but not functionally, highly similar to ubiquitin [9]. SUMOylation to target proteins is a multistep process involving the E1-activating enzyme complex SAE1/SAE2, the E2-conjugating enzyme Ubc9, and E3 ligases [10]. Attachment of SUMO to the vast majority of known SUMO substrates occurs in the SUMOylation consensus sequence ψKxE (where ψ is a bulky hydrophobic amine acid and x is any reside). However, not all consensus sites are SUMOylated. Notably, SUMOylation has also been reported to occur on non-consensus sites indicating that the consensus tetrapeptide is not an absolute requirement for SUMO attachment [11,12]. In mammals, three SUMO isoforms, designated SUMO-1, SUMO-2, and SUMO-3 are expressed [13]. The mature

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forms of SUMO-2 and SUMO-3 only differ from one another by three N-terminal residues but have only a 50% sequence identity with SUMO-1 [14]. SUMOylation affects the activity, interaction properties, subcellular localization and the stability of target proteins. SUMOylation interacts with another well-characterized posttranslational modification by ubiquitin in many ways [15].

NDRG1 is negatively correlated with tumor progression and survival in multiple cancers and therefore is a promising new target for cancer treatment. To get a better understanding about NDRG1 regulation and function we tried to find a new post-translational modification of NDRG1. In this report, we found that NDRG1 is a new SUMO conjugation target and is preferentially modified by the SUMO-2 isoform. The major SUMO acceptor site of NDRG1 is lysine 14 residue which is not a classical consensus SUMO modification site. To assess the functional consequences of SUMO-2 conjugation on NDRG1 we used several NDRG1 mutants with differential SUMOylation statuses. SUMO-2 conjugation does not affect the subcellular distribution of NDRG1. However, SUMO-2 attachment destabilizes the NDRG1 protein and promotes the ubiquitination of NDRG1. Finally, we found that SUMO-2 modification inhibits the induction of p21 by NDRG1. Taken together, we suggest that novel regulatory mechanism of NDRG1 by SUMO-2 conjugation is important for the regulation of NDRG1.

2. Materials and methods

2.1. Antibodies

Antibodies were purchased from the manufacturers as follows: anti-NDRG1 antibody (ab124689, Abcam), anti-p21 (sc-394, Santa Cruz), anti-Xpress (46-0528, Invitrogen), anti-β-actin (A1978, Sigma–Aldrich).

2.2. Generation of plasmid constructs

A NDRG1 K14R mutant plasmid was generated by site-directed mutagenesis using the KOD-Plus- (TOYOBO). To generate the SUMO-NDRG1 K14R fusion constructs, SUMO (amino acids 1–96) lacking the C-terminal Gly–Gly was amplified by PCR and subcloned into pCMV10. K14R-mutated NDRG1 was cloned downstream of SUMO, and the resulting SUMO fusion constructs were sequenced to confirm the reading frame.

2.3. In vivo ubiquitination assay

HEK293T cells were transfected with His-tagged Ub and the expression plasmids as indicated. Twenty micromolar MG132 was treated for 4 h before harvesting the cells. Forty-eight hours after transfection, cells were lysed in lysis buffer (150 mM NaCl, 25 mM Tris–HCl [pH 7.8], 0.1% NP-40, and 1 mM EDTA) supplemented with protease inhibitor complex mixture (Roche Molecular Biochemicals) and 0.25% SDS. After 5 min boiling, the clarified extracts were incubated with Talon resins for 4 h. The slurry was washed 4 times with wash buffer supplemented with imidazole. After denaturation, the bound proteins were separated by SDS/PAGE and immunoblotted.

2.4. In vivo SUMOylation assay

HEK293T cells were transfected with His-tagged SUMO and the expression plasmids as indicated. Forty-eight hours after transfection, the cells were lysed in lysis buffer (150 mM NaCl, 25 mM Tris–HCl [pH 7.8], 0.1% NP-40, and 1 mM EDTA) supplemented with protease inhibitor complex mixture (Roche Molecular Biochemicals) and 0.25% SDS. After 5 min boiling, the clarified extracts were incubated with Talon resins for 4 h. The slurry was washed 4 times with wash buffer supplemented with imidazole. After denaturation, the bound proteins were separated by SDS/PAGE and immunoblotted.

3. Results

3.1. NDRG1 is a SUMO modification target

To investigate the novel regulatory mechanism of NDRG1 at the protein level, we tested whether NDRG1 is a posttranslational modification target of SUMO. Using His-tagged SUMO constructs, we did a SUMOylation assay of NDRG1. As shown in Fig. 1A, NDRG1 is modified by all three SUMO isoforms. The conjugation level is preferentially high in SUMO-2, thus we used SUMO-2 for NDRG1 modification thereafter. To find the functional lysine residue(s) of NDRG1 that serve as the SUMO acceptor sites, we searched through the putative consensus SUMOylation sequence ψKxE (Fig. S1A) [14]. Each of three lysines of NDRG1 was changed independently to arginine, and the resultant mutants were tested for the ability of *in vivo* SUMO modification (Fig. S1B). All of the three mutants revealed no alteration in SUMO modification of NDRG1. In addition, three lysines to the alanine mutant of NDRG1 (K19, 198, 306R) still

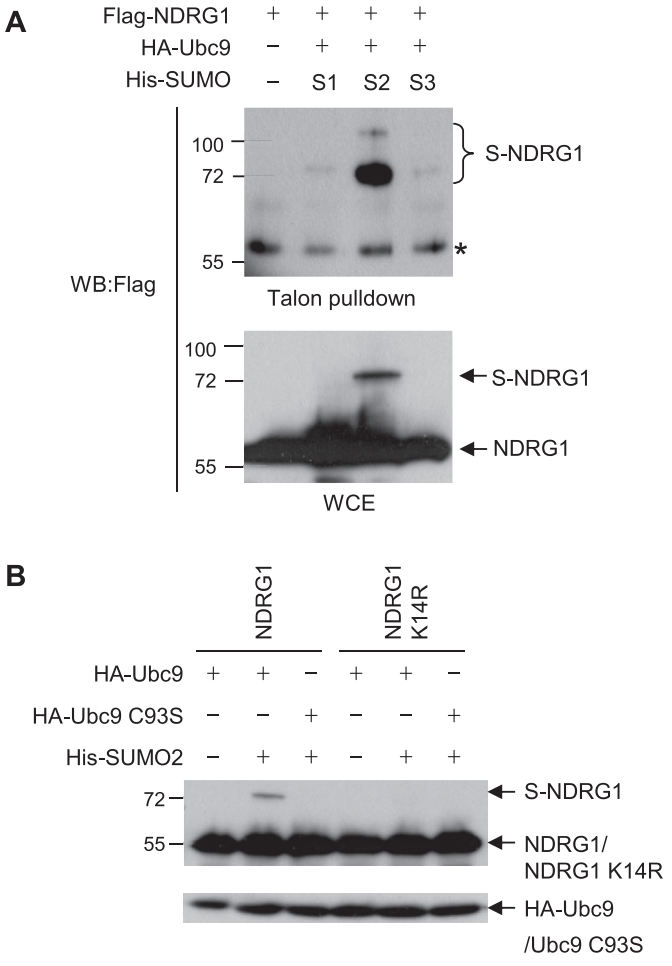

Fig. 1. NDRG1 is a SUMO modification target. (A) HEK293T cells were transfected as indicated. After 48 h, cell lysates were subjected to Talon pulldown followed by an immunoblot with anti-Flag antibody. (B) Wildtype or NDRG1 K14R was transfected with SUMO-2 and Ubc9 or Ubc9 C93S as indicated for *in vivo* SUMOylation. After 48 h, cell lysates were subjected to western blotting with anti-Flag antibody for NDRG1 detection or anti-HA antibody for Ubc9.

retained the ability of SUMO conjugation. To find out the SUMOylation site of NDRG1, we used several fragments of NDRG1 with lysine to alanine mutation for a SUMO conjugation assay (data not shown). We found that the SUMO acceptor site of NDRG1 is Lys 14 which is not the consensus SUMOylation sequence [11]. K14R mutation abrogated NDRG1 from SUMO-2 modification (Fig. 1B). To confirm the high molecular weight band of NDRG1 is the SUMO-2 conjugated NDRG1, we used an Ubc9 active site mutant (Ubc9 C93S) and a C-terminal di-glycine mutant of SUMO-2 which is defective for conjugation (SUMO-2 AA) for the SUMOylation assay (Figs. 1B and S2). There was no SUMO conjugation band of NDRG1 with these two mutants which were used for the SUMOylation assay. Taken together, we concluded that NDRG1 is a new target of SUMO modification and NDRG1 is preferentially modified by SUMO-2 at lysine 14.

3.2. SUMOylation does not affect the subcellular localization of NDRG1

SUMOylation regulates the diverse cellular processes including transcriptional regulation, nuclear transport, DNA repair, and signal transduction [14]. The functional consequences of SUMO modification are diverse between target proteins. To elucidate the effect of SUMO-2 modification on NDRG1, we made several NDRG1 mutants with differential SUMOylation statuses (Fig. 2A). We covalently attached SUMO-2 to the N-terminus of NDRG1 K14R to generate a constitutively SUMOylated form of NDRG1 [16,17]. The subcellular distribution of some target proteins is controlled by SUMO attachment [18]. We examined whether SUMO-2 conjugation on NDRG1 regulates the subcellular localization of NDRG1. Wildtype NDRG1 is predominantly localized in the cytoplasm and the SUMOylation defective NDRG1 mutant (NDRG1 K14R) also retains its location to the cytoplasm. Further, the SUMO-2 fused NDRG1 K14R mutant is still localized in the cytoplasm (Fig. 2B). These results indicate that SUMO-2 modification on NDRG1 does not affect the subcellular distribution of NDRG1.

3.3. NDRG1 is destabilized by SUMO-2 conjugation

It has been reported that SUMO conjugation regulates the protein stability of some target proteins [18]. To investigate the possibility of the regulation of NDRG1 protein stability by SUMO-2 conjugation, we tested the protein stability of NDRG1 mutants after inhibition of protein synthesis by cycloheximide (CHX). The protein level of wildtype and NDRG1 K14R remained unchanged after CHX treatment at the indicated time points. However, the protein stability of SUMO-2 fused NDRG1 K14R was dramatically decreased after CHX treatment (Fig. 3A). In some targets, SUMOylation functions as a signal for polyubiquitination for proteasomal degradation [19]. Thus we tested whether the destabilization of metastasis suppressor protein NDRG1 by SUMO-2 attachment is linked to the ubiquitin conjugation. We found that the degree of ubiquitination decreases at the SUMOylation defective NDRG1 mutant compared to the wildtype. Conversely, SUMO-2 fused NDRG1 K14R shows a

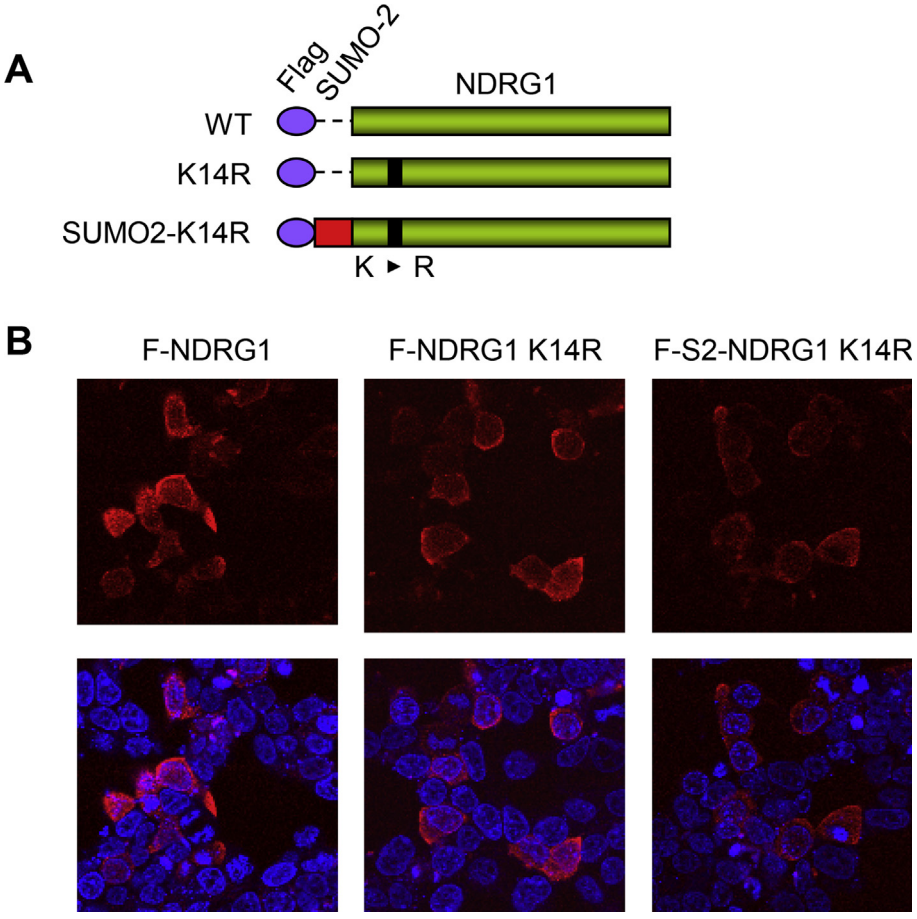

Fig. 2. SUMO-2 conjugation does not affect the subcellular localization of NDRG1. (A) Schematic representations of Flag-tagged NDRG1, NDRG1 K14R, or SUMO-2-fused NDRG1 K14R. (B) Subcellular localization of Flag-tagged NDRG1, NDRG1 K14R, or SUMO-2-fused NDRG1 14R (green). Nuclei were visualized by DAPI staining (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

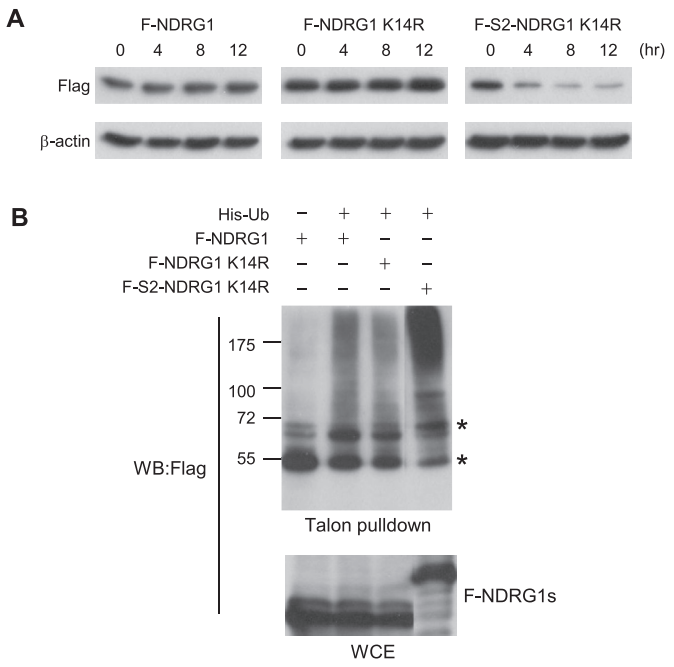

Fig. 3. SUMO-2 conjugation of NDRG1 promotes proteasomal degradation of NDRG1. (A) Flag-NDRG1, Flag-NDRG1 K14R, or Flag-S2-NDRG1 K14R expressed HEK293T cells were treated with cycloheximide (CHX) for indicated times. The same amount of protein from each cell lysate was immunoblotted with anti-Flag antibody. (B) HEK293T cells were transfected as indicated. After 44 h, cells were treated with 20 μM MG132 for the next 4 h. Cell lysates were then subjected to a Talon pulldown followed by an immunoblot with anti-Flag antibody.

dramatic increase of ubiquitination (Fig. 3B). Taken together, we suggest that SUMO-2 conjugation of NDRG1 increases the ubiquitination of NDRG1 and promotes its degradation.

3.4. SUMOylation of NDRG1 suppresses the induction of p21 by NDRG1

There are some reports about the implication of the key cell cycle inhibitor, p21 in the anti-tumoral effect of NDRG1. It has been reported that upregulation of p21 by NDRG1 was suggested as a molecular player in the antimetastatic activity of NDRG1 [20]. NDRG1 overexpression induced cell cycle arrest at the G0/G1 phase accomplished by increased p21 in HCC cells [21]. We characterized the regulation of p21 by SUMO modification of NDRG1 in U2OS cells. p21 is upregulated by wildtype NDRG1 and NDRG1 K14R shows a little more increased p21 protein than wildtype even with the lower expression of NDRG1 K14R than wildtype NDRG1. However, SUMO-2 fused NDRG1 K14R did not upregulate p21 (Fig. 4). This result indicates that SUMO-2 modification of NDRG1 could regulate the anti-tumoral activity of NDRG1 by inhibiting p21 induction.

4. Discussion

In the present study, we explored the posttranslational modification of the metastasis suppressor NDRG1 by SUMO. We found that NDRG1 is preferentially modified by SUMO-2 and the major SUMO-2 acceptor site of NDRG1 was lysine 14 residue which is an atypical site with little sequence similarity to the classical SUMO consensus motif. We created several NDRG1 mutants bearing differential SUMOylation statuses to characterize the effects of SUMO attachment to NDRG1. Firstly, we verified that SUMOylation does not affect the cytoplasmic distribution of NDRG1. However, SUMO-

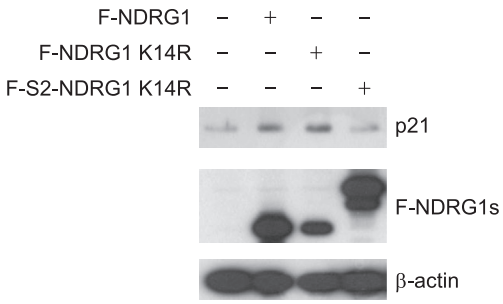

Fig. 4. SUMO-2 modification of NDRG1 inhibits the induction of p21 by NDRG1. Expression plasmids were transfects as indicated in U2OS cells. 48 h later, the protein level of p21 was assessed by western blotting.

2 conjugation destabilizes the NDRG1 protein and promotes the ubiquitination of NDRG1. This means that SUMOylation of NDRG1 functions as a signal for polyubiquitination and degradation. Finally, we found that SUMO-2 conjugation of NDRG1 inhibits the p21 inducing activity of NDRG1. In conclusion, we suggest that SUMO-2 modification of NDRG1 regulates the protein stability of NDRG1 and the p21 inducing activity of NDRG1. Thus, SUMO-2 modification could be implicated in the regulation of the anti-tumoral and growth inhibitory activity of NDRG1.

Anti-tumoral and anti-metastatic function of NDRG1 has been widely investigated [1], yet the posttranslational regulation of NDRG1 has not been studied widely. Important implications of phosphorylation on the functions of NDRG1 including centromere function, cell cycle, angiogenesis, and tumor growth have been revealed [6–8]. In this report, we characterized the novel regulatory mechanism of NDRG1 by SUMO. SUMOylation interacts with another well-characterized posttranslational modification by ubiquitin in many ways [15]. One such interaction is that SUMOylation functions as a signal for polyubiquitination for proteasomal degradation [19,22]. The novel class of ubiquitin ligases termed ubiquitin ligases for SUMOylated proteins (ULS) or SUMO-targeted ubiquitin ligases (STUBL), bearing SUMO interaction motifs, is implicated in the ubiquitination of SUMOylated proteins to target them for degradation by the proteasome [19,23]. PML was shown to be degraded in a SUMO-dependent manner by the proteasome after treatment with arsenic trioxide (ATO) [24]. RNF4, one of the most extensively studied STUBL, was shown to lead to the ubiquitination of SUMOylated PML in response to ATO [25]. In this report, SUMOylation of NDRG1 functions as a signal for polyubiquitination. Thus, we assessed the possibility of RNF4 as an ubiquitin ligase toward SUMOylated NDRG1. However, there was no interaction between NDRG1 and RNF4 (data not shown). SUMO modification is a dynamic process and the conjugation of SUMO can be reversed by the action of SUMO specific proteases (SENPs) [26]. We found that SUMO-2 conjugation of NDRG1 can be removed by SENP2 (Fig. S3). Further study about the implication of SENP2 on the regulation of NDRG1 function remains to be elucidated.

Induction of the cyclin-dependent kinase inhibitor, p21, by NDRG1 is implicated in the anti-metastatic function of NDRG1 [20,21]. In this report, we showed that SUMO-2 conjugation inhibits the induction activity of p21 by NDRG1. At this point, we do not know the underlying mechanism of SUMO-2 modification of NDRG1 on the inhibition of p21 induction. However, destabilization of NDRG1 by SUMO-2 modification could be one possible explanation for the decrease of p21 induction by NDRG1. Thus, we assume that SUMO-2 modification of NDRG1 suppresses the function of NDRG1 as a metastasis suppressor. Taken together, we suggest that novel regulatory mechanism of NDRG1 by SUMO-2 conjugation inhibits the anti-metastatic function of NDRG1 and could be a novel therapeutic target for metastatic cancer treatment.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

J.E.L. and J.H.K. designed the research; J.E.L. performed the research; J.E.L. and J.H.K. analyzed the data; and J.H.K. wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.090.

Transparency document

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