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Molecular dissection of the interaction between HIC1 and SIRT1

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ABSTRACT

HIC1 (Hypermethylated in Cancer 1) is a tumor suppressor gene frequently epigenetically silenced in human cancers. *HIC1* encodes a transcriptional repressor involved in the regulation of growth control, cell survival and DNA damage response. The deacetylase SIRT1 regulates the repressive capacity of HIC1 in several fashions. First SIRT1 interacts with the BTB/POZ domain of HIC1 to form a transcriptional repression complex that prevents the transcription of *SIRT1* itself. SIRT1 is also responsible of the deacetylation of the lysine 314 of HIC1 that allows its subsequent SUMOylation which in turn favors its interaction with the NuRD complex. To better understand the interplay between HIC1 and SIRT1, we performed co-immunoprecipitation experiments to define the domains essential for the HIC1/SIRT1 interaction. We demonstrated that the isolated four last zinc fingers of HIC1 were capable to interact with SIRT1 and that the amino-acids 610–677 of SIRT1 encompassing the ESA region of the deacetylase were crucial for the HIC1/SIRT1 interaction and HIC1 deacetylation. Finally we demonstrated that this interaction mainly depends on CKII-mediated phosphorylation of SIRT1 serine 659/661 which occurs upon DNA damage. Therefore, our results demonstrate that the activating acetylation to SUMOylation switch of HIC1 is favored by genotoxic stresses to regulate the DNA damage response.

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1. Introduction

HIC1 (Hypermethylated in Cancer 1) is a tumor suppressor gene located in 17p13.3, a chromosomal region frequently hypermethylated or deleted in cancerous tissues [1]. HIC1 is widely expressed in healthy tissues whereas *HIC1* is epigenetically silenced in many human cancers [1,2]. This tumor suppressor gene encodes a transcriptional repressor that is composed of three main functional domains: a N-terminal BTB/POZ protein–protein interaction domain (Broad complex, Tramtrack and Bric à brac/POx viruses and Zinc finger), a central region which is not phylogenetically well conserved except for several conserved polypeptides and a C-terminal domain containing five *Krüppel*-like C_2H_2 zinc fingers that allow the specific binding of the protein onto HIC1 responsive elements (HiRE, GGCA consensus) in the promoter of its target genes [3]. To date 11 HIC1 target genes have been identified of which *SIRT1* [4–6]. SIRT1 is the main member of the sirtuin family of NAD^+-dependent protein deacetylases. Seven human sirtuins (SIRT1–SIRT7) have been identified and are all characterized by a highly conserved deacetylase domain in the central part of the protein [7]. SIRT1 is a major regulator of transcription on the one hand through deacetylation of histones (H3K9, H3K14, H4K16, H1K26), on the other hand by deacetylating several transcription factors and coregulators like p53, FOXO family members or the DNA repair and anti-apoptotic factor Ku70 [8]. We previously demonstrated that SIRT1 can deacetylate the lysine 314 located in the central region of HIC1 which is embedded in a SUMOylation consensus site ($MK^{314}HEP$) [9]. This SUMOylation of HIC1 is essential for its activity since its abolition (K314R or E316A mutants) diminishes the transcriptional repression potential of HIC1 [9] and the recruitment of the NuRD complex on HIC1 target genes [6]. Moreover, Chen et al. also demonstrated that SIRT1 can interact with the BTB/POZ domain of HIC1 [4]. This HIC1/SIRT1 complex binds to the *SIRT1* promoter to repress its transcription and to regulate p53-dependent apoptotic DNA damage response. Consistent with the fact that *HIC1* [1,10,11] and *SIRT1* [12] are both p53 target-genes these authors proposed the existence of a complex regulatory loop between HIC1, SIRT1 and p53 whose perturbation results in tumor progression as shown in Knock-out animal models [13] and in human cancers [14].

Herein we demonstrated that the four last zinc fingers of HIC1 were also capable to interact with SIRT1 and that the amino-acids 610–677 of SIRT1 including the ESA (Essential for SIRT1 deacetylase Activity) region were required for its interaction with HIC1 and for HIC1 lysine 314 deacetylation. Finally, our results proved that the HIC1/SIRT1 interaction is strongly favored by CKII-mediated SIRT1 phosphorylation that occurs upon DNA damages suggesting that HIC1 could be activate by SIRT1 to regulate the DNA damage response.

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2. Materials and methods

2.1. DNA constructs

The full length FLAG-HIC1 and the different HIC1 deletants (BTB-CR, BTB, 5ZF-Ct, 5ZF, 4ZF) containing an N-terminal FLAG epitope cloned in the pcDNA3 expression vector have been described previously [3,15,16]. The 5ZF C493S, C521S, C549S and C577S point mutants were generated by a two-round PCR strategy and verified by sequencing as previously described [3]. The pcDNA3-SIRT1 expression vector was a kind gift from Tony Kouzarides (University of Cambridge, United Kingdom). The FLAG tagged full length and SIRT1 deletion mutants (Δ1 to Δ10) and the HA tagged DBC1 were respectively kindly provided by Frank Dequiedt (Faculty of Agronomy, Gembloux, Belgium) and Zhenkun Lou (Mayo Clinic College of Medicine, Rochester, USA).

2.2. Cell culture and transfection

HEK 293T cells were maintained in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal calf serum and non essential amino acids. Cells were transfected in OptiMEM (Gibco) by the PEI (Euromedex) method as previously described [12] in 100-mm dishes with 2.5 μg of DNA. Cells were transfected for 6 h and then incubated in fresh complete medium.

2.3. Co-immunoprecipitation assays

Co-immunoprecipitation experiments were performed as previously described [6]. Briefly, 48 h after transfection, cells were rinsed in cold phosphate-buffered saline (PBS) and lysed in cold IPH buffer (50 mM Tris [pH 8], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, protease inhibitor cocktail [Roche]). Cell lysates were cleared by centrifugation (14,000 rpm, 4 °C, 15 min). The supernatants were pre-cleared by incubation with 20 μl of protein A/G sepharose beads (Amersham Biosciences) (1 h, 4 °C) and then incubated overnight with 2 μg of antibody. Then, 20 μl of protein A/G beads were added for 30 min. The beads were washed four times with IPH buffer. Bound proteins were eluted by boiling in Laemmli sample buffer.

2.4. Western blotting and antibodies

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE healthcare). After 1 h of blocking in PBSM (PBS with 5% milk), the membranes were incubating overnight at 4 °C with specific primary antibodies in PBSTM (PBSM with 0.1% Tween) and washed three times with PBSN (PBS with 0.1% NP-40). The membranes were next incubated for 1 h at room temperature with secondary antibodies coupled to peroxydase (Amersham) in PBSM, washed three times in PBSN and revealed by chemiluminescence.

The anti-HIC1 2563 and anti-K314 acetyl-HIC1 antibodies have been previously described [6]. Anti-FLAG antibody (M2) was purchased from Sigma–Aldrich, anti-Gal4 and anti-SIRT1 (H300) from Santa-cruz biotechnology and anti-HA from Babco.

3. Results and discussion

When they deciphered the regulatory feedback loop between p53, SIRT1 and HIC1, Chen et al. demonstrated that the HIC1 BTB/POZ domain co-immunoprecipitated with SIRT1 [4]. However, these authors did not investigate the putative interaction between SIRT1 and others regions of HIC1, in particular the ZFs region. To test this, we transfected HEK 293T cells with full length SIRT1 together with FLAG-tagged HIC1 proteins corresponding to the N-terminal and C-terminal moieties, respectively. Therefore, expression vectors for the BTB/POZ domain and the central region of HIC1 (BTB-CR) or the ZFs region plus the C-terminal part of the protein (5ZF-Cterm) were co-transfected with SIRT1 (Fig 1A) and we performed co-immunoprecipitation experiments. As expected, we could detect the previously published interaction between the BTB-POZ domain of HIC1 and SIRT1 [4], as shown with the BTB-CR (Fig 2A) construct. In addition, our results also clearly demonstrated that SIRT1 co-precipitated with the 5ZF-Cterm region and that this interaction was stronger than with the full-length HIC1 or the BTB-CR region (Fig 2A).

We then co-immunoprecipitated SIRT1 and FLAG-tagged fusion proteins corresponding to the 5ZF region or the last four ZFs (4ZF) of HIC1. These 4 ZFs are separated by a typical H/C link and are involved in the sequence specific DNA binding of HIC1 whereas the first ZF is not necessary [3] (Supplementary Fig. S1). We found that the four last zinc fingers of HIC1 are sufficient to mediate the HIC1/SIRT1 interaction (Fig 2B). Like HIC1, BCL6 (B-cell lymphoma 6) belongs to the BTB/POZ family of transcriptional repressors. BCL6 can recruit HDAC5, another histone deacetylase through its last 4 ZFs in a fashion that does not rely on the integrity of a single ZF [17]. Therefore, we wanted to know if this was also the case for HIC1. To test this, we generated point mutants of ZF2, ZF3, ZF4 and ZF5 in which a cysteine residue has been replaced by a serine so that the ZF architecture is disrupted (Fig 1A right). These mutants were all affected in sequence-specific DNA-binding as expected from the Zinc Finger recognition code proposed by Choo and Klug [3,18] (Supplementary Fig. S1). However, when the same mutants were tested for their ability to co-precipitate SIRT1 the results showed that SIRT1 interacts with the 5ZF wild type region and with the different mutants with the same affinity demonstrating that the SIRT1/HIC1 interaction does not rely on the integrity of a single ZF (Fig. 2C). So, our results reveal that the HIC1/SIRT1 interaction requires the BTB/POZ domain but also the four last ZFs of HIC1. This complex interaction could suggest that HIC1 could recruit the SIRT1 deacetylase as a two-edged sword to ensure transcriptional repression. Firstly, in the vicinity of chromatin SIRT1 could act through epigenetic mechanisms and deacetylate histones, notably H3K9 and H4K16 to "lock" chromatin [19], thus contributing to HIC1 target-gene promoter repression, including *SIRT1* itself [4,6]. Secondly, the deacetylation/SUMOylation switch induced by the SIRT1/HDAC4 complex activates HIC1 repression potential by favoring the recruitment of the NuRD complex on a subset of its target genes [6,9].

Reciprocally, to define the SIRT1 domains required for the interaction with HIC1, we next performed co-immunoprecipitation experiments between full length HIC1 and a set of SIRT1 deletants (Fig 1B). We demonstrated that the Δ4 (amino-acids 214–295), Δ5 (amino-acids 296–377) and Δ8 (amino-acids 542–609) deletants were more potent HIC1 interactors than the full length SIRT1 (Fig 3A). The region encompassing the amino-acids 214–377 of SIRT1 correspond to the domain required for the interaction of SIRT1 with DBC1 (Deleted in Breast Cancer 1), a negative regulator of the deacetylase [20,21]. We hypothesized that DBC1 could interfere with HIC1/SIRT1 interaction. To answer this question, we performed co-immunoprecipitation between HIC1 and SIRT1 in presence of increasing amount of DBC1 and showed that DBC1 does not interfere with the HIC1/SIRT1 interaction (Fig 3B). Secondly, we found that the Δ9 deletant lacking the amino-acids 610–677 does not interact with HIC1 (Fig 3A) and is not able to deacetylate HIC1 on lysine 314, as the catalytic domain deletant Δ5 (Fig 3C). This last result is in close agreement with the previously identified ESA (Essential for SIRT1 deacetylase Activity) region encompassing the amino acids 631–655 of murine SIRT1 [22]. Indeed, in this study the authors demonstrated that a mutant form of murine SIRT1 lacking the ESA region was devoid of deace-

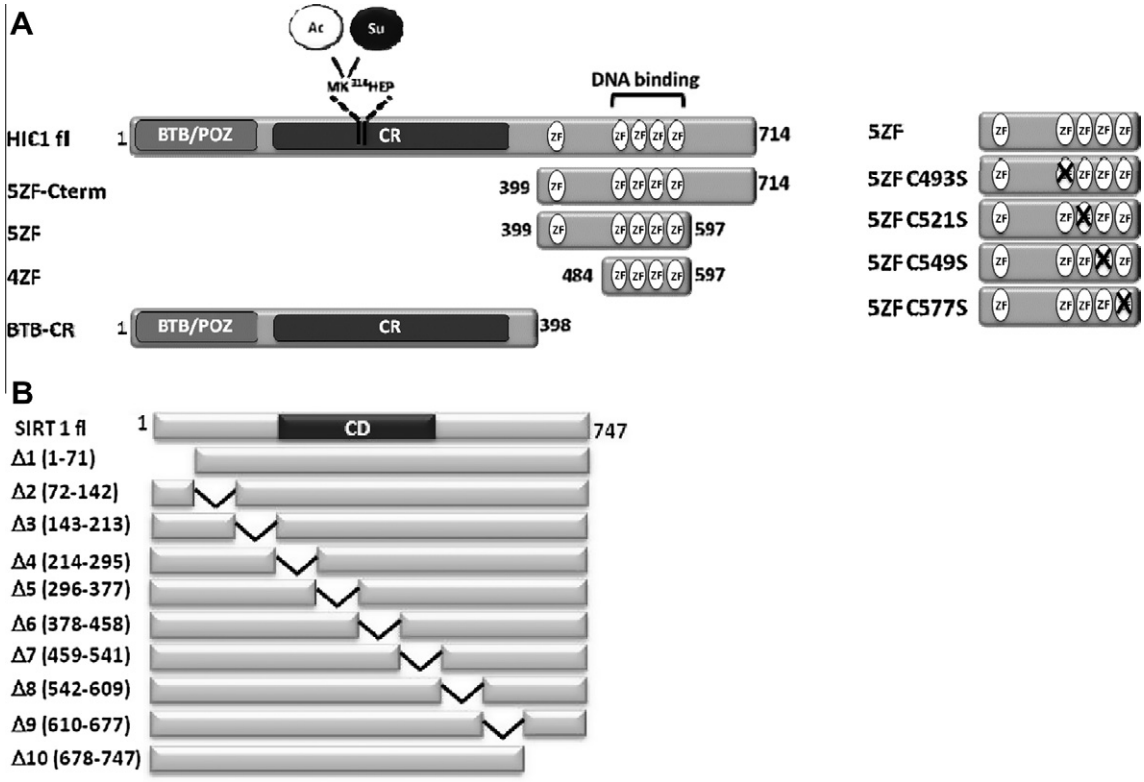

Fig. 1. Schematic representation of the different constructs used in this study. (A) *Left*: Schematic representation of the full length HIC1 (HIC fl) and its deletion mutants. BTB/POZ: Broad complex, Tramtrack and Bric à brac/POx viruses and Zinc finger; CR: Central region; ZF: Zinc finger. Ac: Acetyl; Su: SUMO *Right*: Schematic representation of the HIC1 Zinc fingers point mutants. (B) Schematic representation of the full length SIRT1 (SIRT1 fl) and its deletion mutants. CD: catalytic domain.

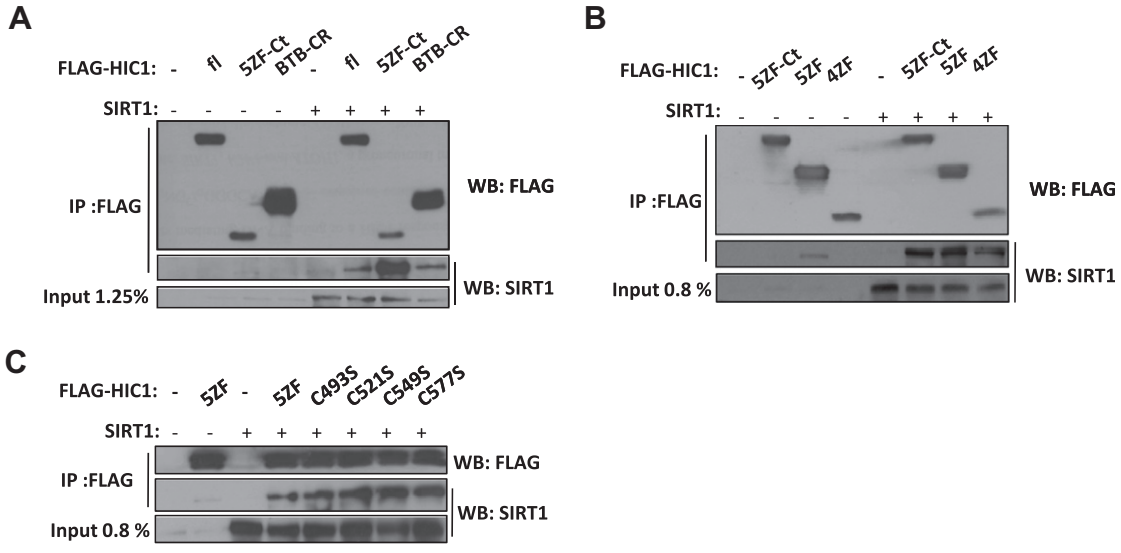

Fig. 2. The BTB/POZ domain and the four last zinc fingers of HIC1 are required for the HIC1/SIRT1 interaction. (A–C) HEK 293T cells were transfected with the indicated expression vectors. Forty eight hours after transfection, cells were lysed and extracts were submitted to co-immunoprecipitation with the anti-FLAG antibody. The immunoprecipitates as well as 1.25% or 0.8% of the whole cell lysate (input) were then analyzed by Western blotting (WB) with the indicated antibodies.

tylase activity against either Ac-p53 or native Ac-histone H3 and that this region increases the interaction between the deacetylase core of SIRT1 and the target substrate. So our results demonstrate that the amino-acids 610–677 are crucial for the HIC1/SIRT1 interaction and subsequent deacetylation of HIC1. Therefore, these results suggest that the ESA region is conserved in human SIRT1 and could be involved in the interaction with a wide range of SIRT1 substrates.

Mouse SIRT1 has been found to be activated upon DNA damage through its CKII-mediated phosphorylation of 4 Serine residues (S154, S649, S651 and S683) which are included in an evolutionarily-conserved CKII consensus [23]. This CKII mediated phosphorylation increases SIRT1 substrate binding activity and consequently the deacetylation of several targets including p53. Notably S649 and S651 in murine SIRT1 correspond to the serine 659 and 661 of human SIRT1 and are absent in the SIRT1 Δ9 mu-

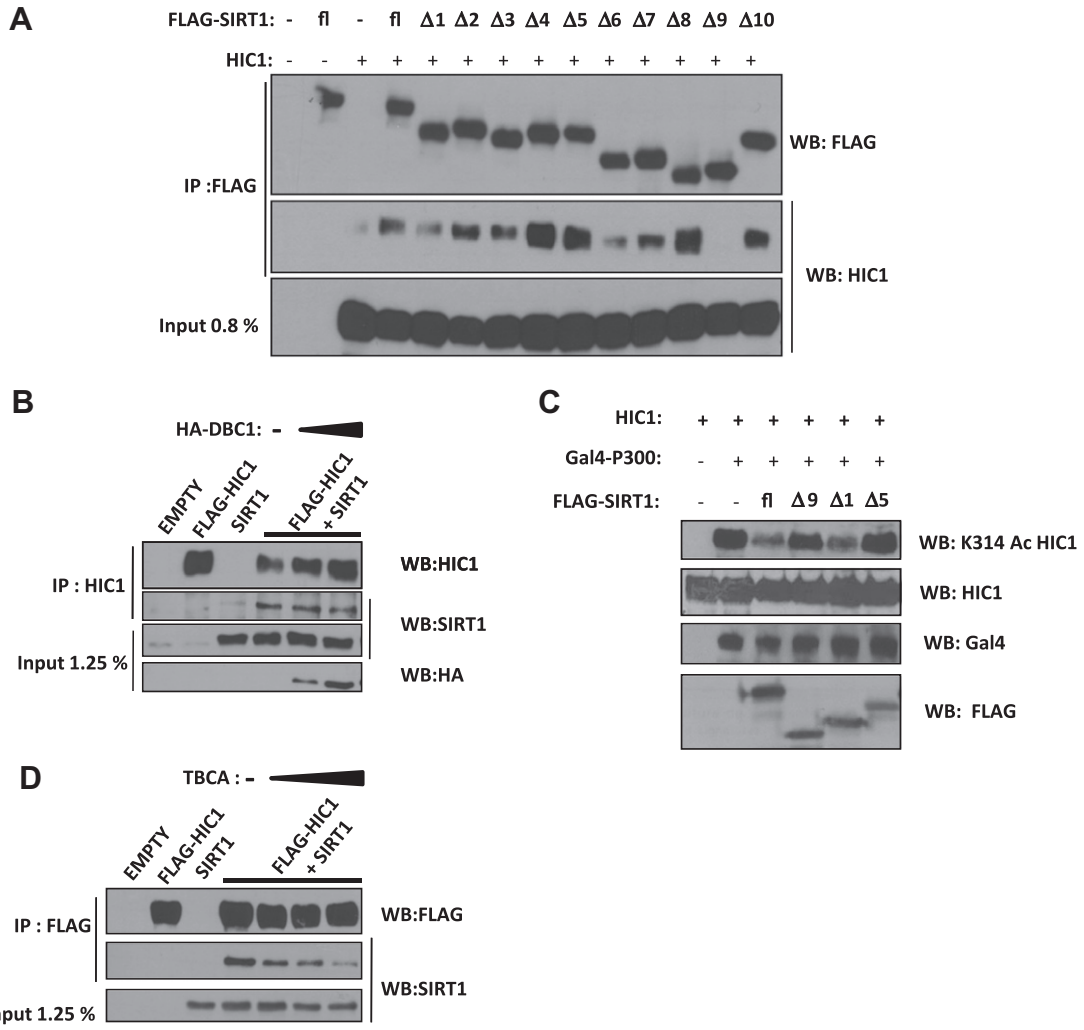

Fig. 3. The SIRT1/HIC1 interaction and the subsequent deacetylation of HIC1 require the amino-acids 610–677 of SIRT1 and depend on the CKII-mediated phosphorylation of SIRT1 serine 659 and/or 661. (A) HEK 293T cells were transfected with the indicated expression vectors. Forty eight hours after transfection, cells were lysed and extracts were submitted to co-immunoprecipitation with the anti-FLAG antibody. The immunoprecipitates as well as 0.8% of the whole cell lysate (input) were then analyzed by Western blotting (WB) with the indicated antibodies. (B) HEK 293T cells were co-transfected with FLAG-HIC1 and SIRT1 in the presence of increasing amount of HA-DBC1. Forty eight hours after transfection, co-immunoprecipitation was performed as in A. (C) HEK 293T were co-transfected with HIC1, the acetylase p300 together with the full length SIRT1 or different deletants of the deacetylase. Forty eight hours after transfection, cells were lysed and the acetylation of HIC1 on lysine 314 was checked by Western blot using the anti K314Ac-HIC1 specific antibody. (D) HEK 293T were co-transfected with FLAG-HIC1 and SIRT1. Thirty six hours after transfection, cells were treated with increasing amount of CKII inhibitor overnight (10, 20 or 30 μM TBCA, Sigma–Aldrich). Forty eight hours after transfection, co-immunoprecipitation was performed as in A.

tant which did not interact with HIC1 (Fig. 3A). For these reasons, we decided to test the influence of human SIRT1 phosphorylation on the HIC1/SIRT1 interaction. We thus investigate the interaction between HIC1 and SIRT1 in the presence of increasing concentrations of TBCA, a CKII inhibitor (Fig 3D). Results showed a dose-dependent decrease in HIC1/SIRT1 interaction upon TBCA treatment (Fig 3D). This result together with the lack of interaction with SIRT1 Δ9 strongly suggests that HIC1/SIRT1 interaction depends on the prior phosphorylation of SIRT1 S659 and/or S661 by CKII.

In conclusion, several pieces of evidence suggest that HIC1 is a central actor of the DNA damage response [24]. First, a functional p53 responsive element (PRE) has been found in the promoter region of *HIC1* demonstrating that HIC1 is a direct p53 target gene [1,10,11]. Chen et al. demonstrated that HIC1 collaborated with SIRT1 to regulate the p53-dependent apoptotic DNA damage response in part through the direct repression of the *SIRT1* promoter itself [4] but also, our results suggest that HIC1 could be activated upon DNA damages by its SIRT1-mediated deacetylation followed by its subsequent SUMOylation.

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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.2012.04.026.

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