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A heteroallelic *Drosophila* insulin-like receptor mutant and its use in validating physiological activities of food constituents

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ABSTRACT

Here we report an additional *Drosophila* transheterozygote $InR^{GS15311}/InR^{GS50346}$ carrying two different P-element-inducible alleles of insulin-like receptor gene (*InR*). $InR^{GS15311}/InR^{GS50346}$ flies exhibit the following phenotypes previously reported in *InR* and insulin/IGF-1 signaling (IIS) pathway-related gene mutants: small bodies, developmental delay, shortened lifespan, and increased fasting resistance. All of these characteristics are shared among flies carrying mutated genes implicated in the pathway. This heteroallelic combination exhibited fertility but resulted in male semilethality, while females were viable and grew into adults. Furthermore, an experimental model employing the $InR^{GS15311}/InR^{GS50346}$ strain confirmed negligible involvement of royal jelly in IIS. Thus, the heteroallelic *InR* mutant, discovered in this study, will serve as a good model for multiple purposes: investigating the IIS mechanisms; identifying and validating the ingredients that prevent type II diabetes; and screening of food constituents associated with IIS.

1. Introduction

Type II diabetes (T2D), attributed to obesity, is a lifestyle-related disease. The increasing number of patients with type II diabetes, in both developed and developing countries around the world, is a serious burden on today's society [1,2]. T2D is a complex endocrine and metabolic disorder [3]. Clinical and epidemiological studies have indicated that obesity is a major risk factor for T2D, because obesity is associated with an increased risk of developing insulin resistance and impaired glucose tolerance [4,5]. Appropriate dietary habits are important for prevention of diabetes [6], but mechanisms leading to the onset of diabetes are not yet fully understood. Mice are commonly used as experimental models to investigate the mechanisms of diabetes to precisely understand and treat the disease and also to establish appropriate dietary habits [7]. A generally accepted point of view is that insulin-resistant conditions in T2D are caused by defects at one or several levels of the insulin-signaling cascade in skeletal muscle, adipose tissue, and liver, which quantitatively constitute the bulk of the insulin-responsive tissues [8]. Thus, the accumulation of evidence pertaining to mutant animals carrying different mutations in genes associated with insulin/IGF-1 signaling (IIS) is crucial for gaining an insight into the mechanisms of diabetes, and discovery of new mutants will help with advances in this area. Since the IIS pathway is conserved in *Drosophila*, this animal is considered an alternative model for researching the mechanisms of diabetes [9,10]. *Drosophila* has the following structures that are analogous to most of those involved in both human energy metabolism and serve as targets for diabetic complications: heart, brain, kidney (nephrocytes, Malpighian tubules), liver, and adipose tissue, the gastrointestinal tract, and blood (hemolymphs) [10]. A lot of mutant alleles in IIS-related genes have been reported for *Drosophila* [11–14]. In particular, it has been revealed in *Drosophila melanogaster* that insulin-like molecules (known as DILPs in flies) control growth and metabolism, probably via a signaling mechanism operating on a single insulin receptor (*InR*) [15]. Although the insulin receptor gene is not considered responsible for T2D, mutations in both the *InR* gene (*InR*) and T2D are associated with acquisition of insulin resistance. Furthermore, because *InR* plays a role relatively upstream of the IIS pathway, *InR* mutants can serve as useful models of T2D. However, *InR* mutant alleles are often larval lethal, hindering investigation in adult flies [11]. Considering age-related increases in the incidence of T2D, it is desirable to investigate the effects of *InR* mutations in adult *Drosophila*. Therefore, we searched for novel *InR* mutant flies suitable for use in the investigation of T2D. We report here a new *InR* mutant allele, discovered from the existing *Drosophila* stocks, and phenotypes of mutants carrying this allele.

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2. Materials and methods

2.1. Fly stocks and maintenance

Two strains carrying a P-element-inserted allele of *InR* (stock number: 205009; genotype: $y^1\ w^{67c23}$, $P\{w^{+mC} = GSV2\}GS50346/TM3$, $Sb^1\ Ser^1$; and stock number: 206165; genotype: $y^1\ w^{67c23}$, $P\{w^{+mC} = GSV6\}GS15311/TM3$, $Sb^1\ Ser^1$) were obtained from the *Drosophila* Genetic Resource Center (DGRC) and used to construct *y w;* $InR^{GS50346}/TM3\ y^+$ and *y w;* $InR^{GS15311}/TM3\ y^+$ stocks for genetic assessment. $InR^{GS15311}/InR^{GS50346}$ flies were generated by crossing both strains, the population was counted, and adult length, weight, and wing shape were observed under a microscope. A w^{1118} line (stock number: 108479; identical to Iso31, isogenic w^{1118} stock in Bloomington *Drosophila* stock center #5905) was also obtained from DGRC and used as the wild-type experimental control. Flies were raised under a 12 h light-dark cycle at 25 °C and 50% humidity on standard culture medium (standard food; SF) consisting of 10% (w/v) glucose, 7% (w/v) corn meal, 4% (w/v) yeast extract, and 0.55% (w/v) agar medium containing 0.3% (v/v) propionic acid and 0.35% (v/v) butyl *p*-hydroxybenzoate as antifungal agents. Adult flies were fasted by placing them in a single vial containing 1% (w/v) agar medium as described previously [16].

2.2. Western Blotting

Five-day-old adult males were fed or fasted for 12 h, and whole-body extract of each genotype in SDS-buffer was prepared and analyzed using antibodies against dAkt (1:000, #9272, Cell Signaling) and p-dAkt (1:1000, #4054, Cell Signaling). Images were taken with a LAS-3000 mini image analyzer (FUJIFILM, Tokyo, Japan) to analyze the intensity ratio of each band (pAkt/Akt ratio).

2.3. RNA preparation, cDNA synthesis, and quantitative RT-PCR

Five-day-old adult flies were fed or fasted for 24 h, then RNA of each genotype was extracted using RNAzol RT (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) as a second PCR template. Relative quantification of *Thor/4E-BP* gene expression was performed using a set of primers against the *Thor/4E-BP* transcript (5′-GGAGGCACCAAACTTATCTACG-3′, 5′-TTG GACGGCGGAGTTTG-3′) and *β-tublin* [17]. Quantitative RT-PCR was performed on a ROTOR-GENE 6000 (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with SYBR green-based detection of PCR products. Relative quantification of target *Thor/4E-BP* gene expression was done using *β-tublin*.

2.4. Adult longevity assay

A longevity assay was performed as described in our previous study [18]. Briefly, in each lifespan experiment, newly eclosed adult flies (within 8 h) of each genotype were collected, anesthetized with diethyl ether, and separated into virgin males and females. Flies ($n \leqslant 20$) were placed in a single vial containing SF and transferred every 3–4 days to a fresh vial. The number of dead flies was recorded until no living flies remained.

2.5. Fasting resistance

Five-day-old adult flies were transferred to vials with 1% Agar containing no nutrients, and the number of dead flies was counted every hour until no surviving flies remained.

2.6. Freeze-dried fresh royal jelly supplementation

Freeze-dried fresh royal jelly (FDRJ) was provided by Api (Gifu, Japan). Samples were prepared from fresh royal jelly (RJ) from China, processed, and stored at −18 °C. Pooled frozen samples were thawed and filtrated twice for sterilization. After filtration, a vacuum dryer was used to convert liquid RJ into a powder. The dried RJ powder represented FDRJ. The resulting samples were stored at 4 °C until use. FDRJ was added to SF as 2% (w/v) of the final concentration by mixing at 40 °C, dispensing into the glass vials, and allowing to solidify. As an experimental control, whey-supplemented medium (whey; SF plus 2% whey powder, Healthy Best, Osaka, Japan) was prepared in the same manner. $InR^{GS15311}/TM3\ y^+$ males (or virgin females) and $InR^{GS50346}/TM3\ y^+$ virgin females (or males) were kept in vials containing either SF, Whey, or FDRJ for up to 4 h. Parental flies were removed to leave only eggs in the vials. The number of F_1 flies in each vial was counted twice daily from day 9, until all eggs had developed into adult flies.

2.7. Statistical analysis

Statistical analysis was performed using the Student's paired *t*-test for two groups or the Tukey–Kramer post hoc test for three groups. Data are expressed as mean ± SE values. A value of $p < 0.05$ was considered statistically significant.

3. Result and discussion

In our search for *Drosophila* strains carrying an IIS-associated gene mutation (or mutations), we discovered another mutant allele. Briefly, we crossed two strains carrying a P-element-inserted allele of *InR* to create a unique mutant genotype. We found that the resulting transheterozygote progenies were phenotypically similar to known IIS-associated gene mutant strains. Both parent strains carried a homozygous lethal mutation, and transheterozygous F_1 progenies ($InR^{GS15311}/InR^{GS50346}$) displayed a dwarf-adult phenotype (Supplementary Fig. S1). These results are in good agreement with previous findings where mutations of the *InR* or *dilp* genes, which encode the insulin-like peptide ligands, result in reduction of adult body size [13,19,20]. This heteroallelic combination of fertile adult males and fertile adult females differs from that of previous reports on *InR* mutants [11] but resulted in male semilethality in the F_1 generation (Supplementary Fig. S2A). The ratio of the number of $InR^{GS15311}/InR^{GS50346}$ flies to the number of heterozygote $InR^{GS}/TM3\ y^+$ ($InR^{GS15311}/TM3\ y^+$ or $InR^{GS50346}/TM3\ y^+$) flies was 1:4 for F_1 males and 1:2 for F_1 females (Supplementary Fig. S2B), suggesting that the male $InR^{GS15311}/InR^{GS50346}$ transheterozygote died at some point in the developmental process, including embryonic stages, while female transheterozygotes were viable and likely to be free from developmental arrest. We found that the heteroallelic combination $InR^{GS15311}/InR^{GS50346}$ also resulted in a delay in egg-to-adult development time in both males and females (Supplementary Fig. S2C–E). This developmental delay appeared to be a shared characteristic of IIS- and TOR-signaling gene mutants, as it was reported in flies carrying a mutation in the *InR* and *dilp* genes [13,19,20] and also in those carrying another mutant allele (or alleles), such as the $lk6^1/lk6^2$ allele of *LK6*, the functional homolog of mammalian Mnk kinases [21], $chico^1$ allele of *chico*, a *Drosophila* homolog of vertebrate IRS1-4, [12], $Slif^1$ allele of *Slif*, that encodes a putative protein showing strong homology with amino acid permeases of the cationic amino acid transporter (CAT) family [14], and a part of the *Tor* mutant alleles [22].

It was reported that, under fasting conditions, dephosphorylation of Akt is attributed to downregulation of IIS, which is absent in flies with impaired IIS [23,24]. This results in reduced nuclear

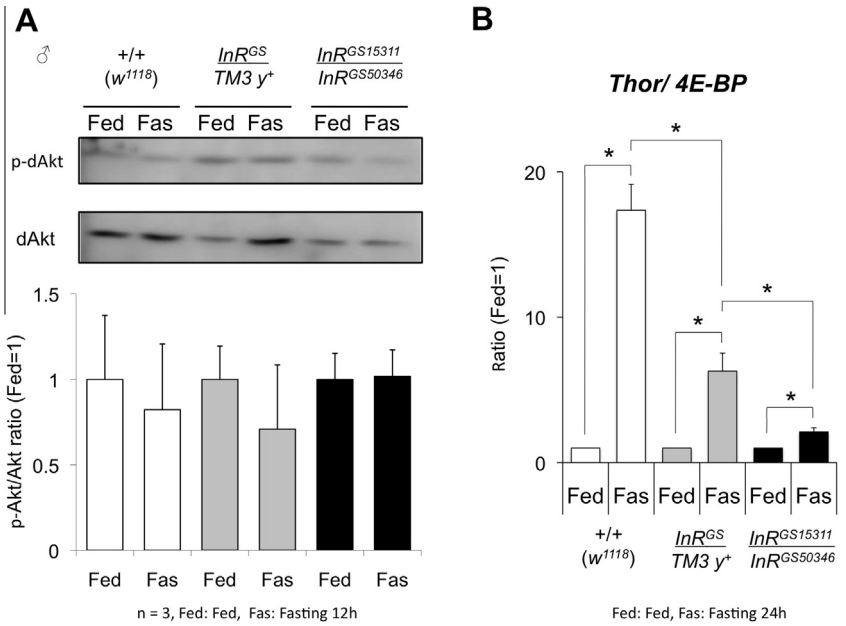

Fig. 1. Impaired IIS in $InR^{GS15311}/InR^{GS50346}$ flies. (A) Western Blotting using antibodies against dAkt and p-dAkt. (B) Quantitative RT-PCR using a set of primers against the *Thor/4E-BP* transcript. Data are expressed as mean ± SE values. *Significant differences are denoted by $p < 0.05$. $n = 3$ groups of 10 animals.

translocation of the forkhead box, sub-group O (FOXO) transcription factor, a substrate-phosphorylated Akt, and consequently, in a lack of upregulation of the FOXO target gene, *Thor/4E-BP* [25]. Indeed, dephosphorylation of Akt and upregulation of the *Thor/4E-BP* gene under fasting conditions, as observed in wild-type and control strains, were absent in $InR^{GS15311}/InR^{GS50346}$ transheterozygotes (Fig. 1), confirming disruption of IIS in these animals carrying two different *InR* mutant alleles. IIS gene mutants are also known to have altered lifespan [26,27]. We found that $InR^{GS15311}/InR^{GS50346}$ flies tended to live shorter lives than the control flies ($InR^{GS}/TM3\ y^+$), and this was more prominent in females than in males (Fig. 2). The lifespan of *InR* mutants can be longer or shorter than that of the control flies depending on the combination of alleles [13], while the lifespan of $InR^{GS15311}/InR^{GS50346}$ appeared to be reduced. On the other hand, fasting resistance was higher in $InR^{GS15311}/InR^{GS50346}$ than in $InR^{GS}/TM3\ y^+$ flies (Fig. 3). High fasting

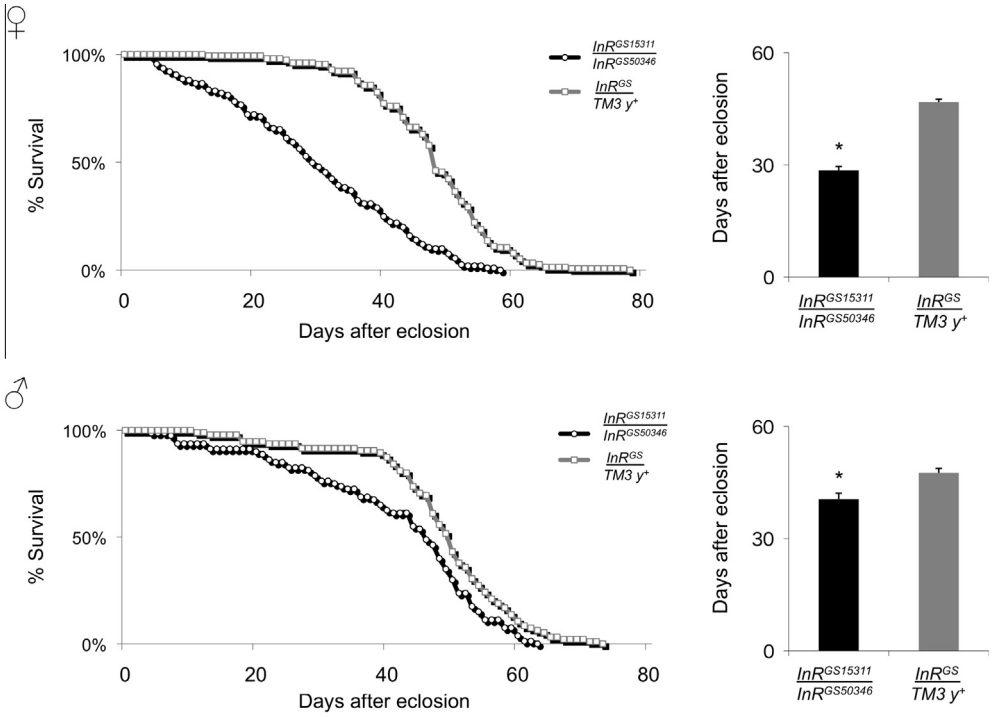

Fig. 2. Reduced lifespan in $InR^{GS15311}/InR^{GS50346}$ flies. Survival curves of the $InR^{GS15311}/InR^{GS50346}$ flies and the control $InR^{GS}/TM3\ y^+$ flies by sex (female, upper left panel; male, lower left panel). The mean lifespan of the female flies (upper right) and the male flies (bottom right). Data are expressed as mean ± SE values. **Significant differences are denoted by $p < 0.001$. Females: $n = 201$ ($InR^{GS15311}/InR^{GS50346}$), $n = 154$ ($InR^{GS}/TM3\ y^+$); males: $n = 80$ ($InR^{GS15311}/InR^{GS50346}$), $n = 95$ ($InR^{GS}/TM3\ y^+$).

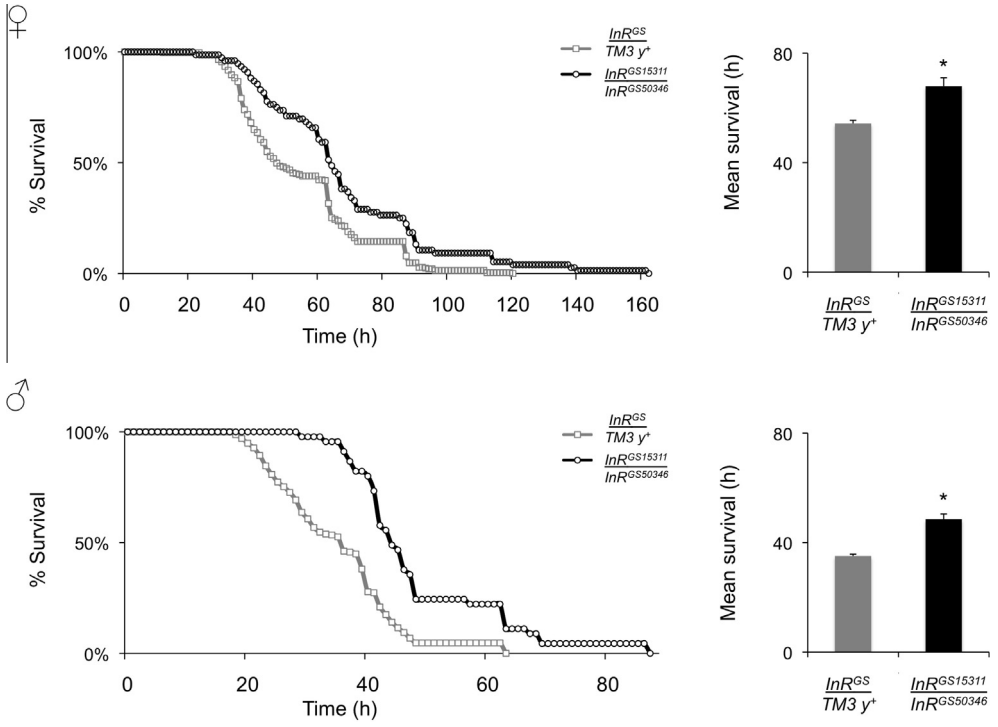

Fig. 3. Enhanced fasting resistance in $InR^{GS15311}/InR^{GS50346}$ flies. Survival curves of female flies with the $InR^{GS15311}/InR^{GS50346}$ genotype or their control $InR^{GS}/TM3\ y^{+}$ genotype (upper left panel) and those of their male counterparts (lower left panel). Mean survival times of females (upper right panel) and males (lower right panel). Data are expressed as mean ± SE values. **Significant differences are denoted as $p < 0.001$. Female: $n = 76$ ($InR^{GS15311}/InR^{GS50346}$), $n = 291$ ($InR^{GS}/TM3\ y^{+}$); males: $n = 45$ ($InR^{GS15311}/InR^{GS50346}$), $n = 234$ ($InR^{GS}/TM3\ y^{+}$).

resistance was attributed to disordered lipid metabolism exhibited by flies carrying the mutated *SH2B* gene, which is an IIS-related gene [24]. Furthermore, the amount of fat cells and lipid storage were shown to correlate with the levels of fasting resistance [28]. Taken together, it is likely that increased fasting resistance was caused by changes in lipid metabolism due to IIS disruption in $InR^{GS15311}/InR^{GS50346}$ flies.

We considered that the $InR^{GS15311}/InR^{GS50346}$ strain might serve as a good experimental model of type II diabetes in addition to being useful for screening food constituents, for the prevention of this disease, and for examining whether these constituents modulate IIS. Royal jelly (RJ), produced by honeybees, contains the bioactive factor royalactin. This substance mediates the developmental rate and number of eggs through an EGFR-mediated mechanism, rather than through IIS, in both fruit flies and honeybees [29]. Meanwhile, *InR* mutants have already been employed to confirm the effect of the dietary ingredients on IIS [30]. Freeze-dried RJ (FDRJ) powder has sufficient bioactivity to progress developmental rate [18]. We thought that if FDRJ contains a biofactor mediated by IIS, a diet supplemented with FDRJ would accelerate the developmental rate in control flies, but not in IIS-interrupted flies. However, developmental rate was increased in not only $InR^{GS}/TM3\ y^{+}$ but also $InR^{GS15311}/InR^{GS50346}$ flies when fed a FDRJ-containing diet (Fig. 4), confirming that the constituents of RJ are unlikely to affect IIS.

In summary, this study found that the transheterozygote $InR^{GS15311}/InR^{GS50346}$ fly is probably an *InR* mutant. $InR^{GS15311}/InR^{GS50346}$ flies shared most phenotypic characteristics, such as small size and slow development, with known *InR* mutants [11]. This heteroallelic combination resulted in male semilethality, but the majority of females were viable and grew into fertile adults. $InR^{GS15311}/InR^{GS50346}$ flies exhibited reduced lifespan and enhanced fasting resistance. Heterozygotes carrying a normal *InR* gene on the balancer chromosome can be produced by crossing, and they

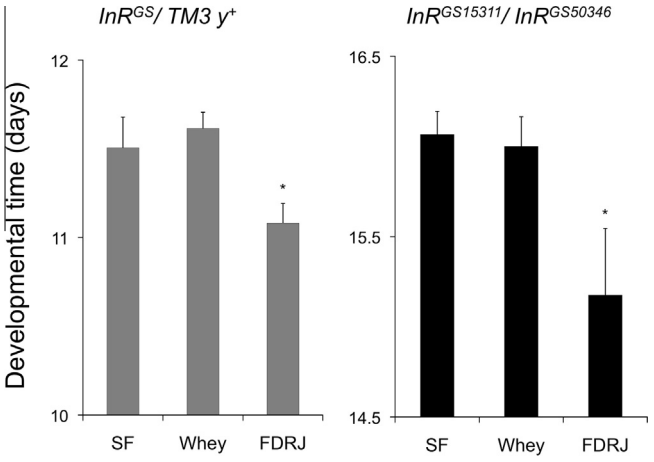

Fig. 4. The $InR^{GS15311}/InR^{GS50346}$ strain as a useful tool for investigating the involvement of food constituents in IIS. SF; standard food, whey; SF supplemented with 2% (w/v) whey, FDRJ; SF supplemented with 2% (w/v) freeze-dried fresh royal jelly. Data are expressed as mean ± SE values. Values significantly different from those of flies reared with control SF are indicated by $^{*}p < 0.05$. $InR^{GS}/TM3\ y^{+}$: $n = 90$ (SF), $n = 152$ (whey), $n = 120$ (RJ); $InR^{GS15311}/InR^{GS50346}$: $n = 15$ (SF), $n = 11$ (whey), $n = 17$ (RJ).

serve as a useful control for the $InR^{GS15311}/InR^{GS50346}$ mutant when examining food constituents, such as RJ (Fig. 4). In other words, the $InR^{GS15311}/InR^{GS50346}$ transheterozygote flies enables simple verification of the involvement of dietary ingredients in IIS as well as their effect on lifespan in adults. Furthermore, the use of parent strains with a substitution of the balancer chromosome with *TM3* y^{+} will provide a useful selection marker y^{+} in the mouth hooks and larval denticles to enable easy separation of $InR^{GS15311}/InR^{GS50346}$ from heterozygous flies, even at the larval stage. We thus

believe that the $InR^{GS15311}/InR^{GS50346}$ will serve as a useful tool for further investigating the mechanisms of T2D and for validating the health claims of functional foods.

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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.2013.02.113.

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