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Synthetic embryonic lethality upon deletion of the ER cochaperone p58^{IPK} and the ER stress sensor ATF6α

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ABSTRACT

The unfolded protein response (UPR) is activated as a consequence of alterations to ER homeostasis. It upregulates a group of ER chaperones and cochaperones, as well as other genes that improve protein processing within the secretory pathway. The UPR effector ATF6α augments—but is not essential for—maximal induction of ER chaperones during stress, yet its role, if any, in protecting cellular function during normal development and physiology is unknown. A systematic analysis of multiple tissues from *Atf6α−/−* mice revealed that all tissues examined were grossly insensitive to loss of ATF6α. However, combined deletion of ATF6α and the ER cochaperone p58^{IPK} resulted in synthetic embryonic lethality. These findings reveal for the first time that an intact UPR can compensate for the genetic impairment of protein folding in the ER *in vivo*. The also expose a role for p58^{IPK} in normal embryonic development.
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1. Introduction

Disruption of protein folding in the endoplasmic reticulum (ER) is sensed by ER-resident transmembrane proteins which initiate signaling cascades collectively known as the unfolded protein response (UPR) [1]. Three pathways, initiated by ATF6α, IRE1α, and PERK, characterize the canonical UPR, and each culminates in the production of a transcriptional activator [2]. IRE1α is conserved amongst eukaryotes and catalyzes splicing of an mRNA encoding the transcription factor XBP1 in vertebrates [3–5]. PERK is metazoan-specific, and phosphorylates the translation factor eIF2α to transiently depress general protein synthesis but also to stimulate translation of a subset of mRNAs, including that of the transcription factor ATF4 [6–8]. ATF6α is likewise metazoan-specific, and is cleaved by regulated intramembrane proteolysis to liberate an active transcription factor [9,10].

The IRE1α and PERK pathways have roles in embryonic or postnatal development. Deficiency of IRE1α or XBP1 compromises fetal liver development [11,12], and liver-specific rescue of XBP1 results in postnatal exocrine pancreas insufficiency [13]. PERK ablation leads to postnatal loss of insulin-producing pancreatic β cells [14]. While the IRE1α and PERK pathways converge upon transcriptional regulation of genes that improve ER protein folding and processing [3,5,6], both can also regulate genes involved in process unrelated to ER folding such as membrane biosynthesis and lipid metabolism [15–18].

In contrast to ablation of the IRE1α or PERK pathways, deletion of ATF6α does not result in prenatal or neonatal lethality and *Atf6α−/−* mice are fertile and viable [19,20]. Microarray experiments have suggested that the direct targets of ATF6α are largely restricted to genes encoding ER chaperones and cochaperones, ER-associated degradation factors, and other genes with direct roles in protein folding and processing [19,21]. Its function is partially compensated by its paralog ATF6β, and chaperone insufficiency appears to underlie the embryonic lethality of *Atf6α−/−; Atf6β−/−* animals [20,22]. Perhaps because of this redundancy, to date *Atf6α−/−* mice have only yielded phenotypes in the presence of exogenous superphysiological challenges such as inhibition of N-linked glycosylation, exposure to neurotoxin, exercise, experimental colitis, or maintenance on a high fat diet [23–27]. Thus, the role of ATF6α, if any, in maintaining homeostasis during normal development and physiology is not clear.

The sensitivity of mice lacking ATF6α to inhibition of N-linked glycosylation or experimental colitis is phenocopied in animals lacking the ER-resident DnaJ protein p58^{IPK}/ERDJ6 [23,24]. While this protein was originally identified as a negative regulator of the cytosolic eIF2α kinase PKR [28], and thereafter as a negative regulator of PERK [29,30], it has since been found to contain an N-terminal ER targeting sequence, and p58^{IPK} is localized

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essentially quantitatively in the ER lumen; no detectable amounts of the protein are found in the cytosol under either basal or ER stress conditions [31]. In the ER lumen, p58^{IPK} interacts with the HSP70 chaperone BiP/GRP78 [31–33], as would be expected for a DnaJ-family protein [34]. Mice lacking p58^{IPK} are smaller than wild-type littermates, and develop diabetes attributed to compromised pancreatic β cell function [35]. The similar sensitivity to ER stress-inducing agents in animals lacking p58^{IPK} or ATF6α suggests that a major function of p58^{IPK} is to protect the protein folding capacity of the ER, and raises the possibility that p58^{IPK} might serve in this capacity in tissues in addition to the endocrine pancreas, but that the effects of loss of p58^{IPK} function in these tissues are masked by compensatory activation of the UPR.

2. Materials and methods

All protocols for animal use were reviewed and approved by the University Committee on Use and Care of Animals at the University of Iowa. Animals were fed standard rodent chow and housed in a controlled environment with 12 h light and dark cycles. *Atf6α−/−* [19] and *p58^{IPK}−/−* [35] animals have been backcrossed into the C*57BL/6J* strain for >10 generations. For histological analysis of wild-type and *Atf6α−/−* mice, tissues were fixed in formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined in groups matched by age and gender for histological signs of pathology by a veterinary pathologist (A.K.O.) who was blinded to genotype. Multiple regions of each organ were examined. Numbers of mice examined were as follows: female wild-type, aged 7–17 months: 6; female *Atf6α−/−*, aged 7–17 months: 8; male wild-type, aged 17 months: 3; and male *Atf6α−/−*, aged 17 months: 5. Timed intercrosses were dated by detection of a mucus plug, which was considered as E0.5; staging was confirmed by embryo size and developmental features. ADRP immunostaining, injections with tunicamycin, and RNA analysis from liver were performed as described [36].

3. Results

3.1. Atf6α−/− mice are histologically normal

To date, the phenotypes reported for *Atf6α−/−* mice or fish have been associated with exogenous challenges that are likely to compromise ER function and elicit *bona fide* ER stress [23–27]. While *Atf6α−/−* mice are viable, fertile, and grossly normal, it had not previously been reported whether deletion of ATF6α compromises the integrity of any tissue. Thus, we collected multiple tissues from wild-type and age-matched *Atf6α−/−* mice of both genders and of varying ages with an emphasis on older (>1 year) animals, in which

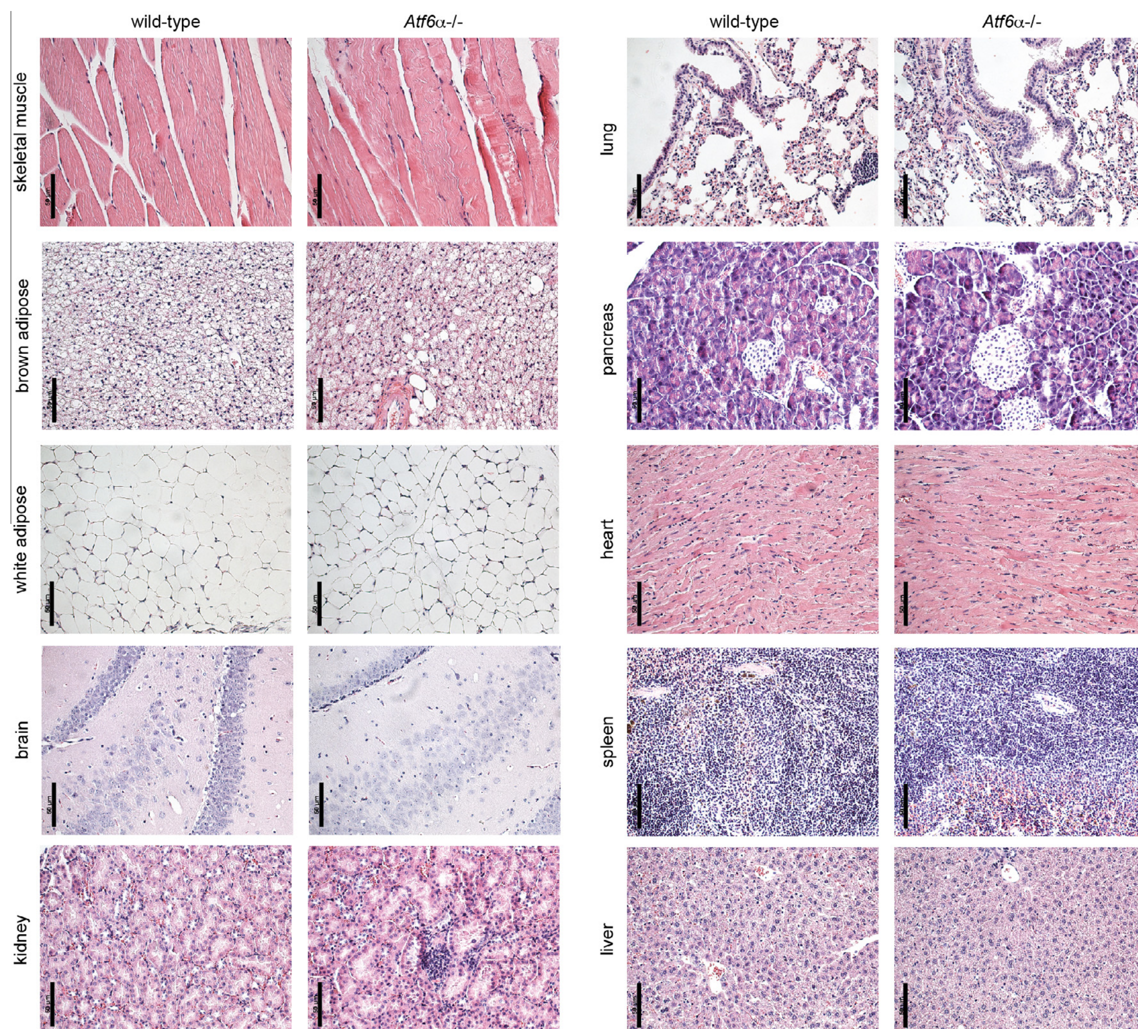

Fig. 1. No histological abnormalities in *Atf6α−/−* mice. The indicated tissues were stained with hematoxylin and eosin and examined for histological signs of pathology by a veterinary pathologist who was blinded to genotype. Multiple regions of each organ were examined. Representative images of the indicated tissues are shown, all from 17 month-old female mice. Results from male mice and from younger female mice were similar. Scale bar = 50 μm.

a modest phenotype might be most likely to be manifested. These tissues—skeletal muscle, brown and white adipose, brain, kidney, lung, pancreas, heart, spleen, and liver—were fixed, paraffin-embedded, stained, and analyzed by a veterinary pathologist (A.K.O.) who was blinded to genotype. In this analysis, no systematic genotype-dependent abnormalities of any sort were observed in any tissue examined. Although multiple regions of each tissue were analyzed, here we show representative images demonstrating that the structural organization of each of these tissues is intact in *Atf6α−/−* mice (Fig. 1). While these findings do not exclude the possibility of phenotypes for *Atf6α−/−* mice in tissues not examined, or by non-histological criteria, they are in clear contrast to the effects of manipulation of other UPR pathways on secretory tissues such as pancreas and liver [11,13,37]. Taken together with the susceptibility of *Atf6α−/−* animals to various exogenous challenges, this result suggests that ATF6α is necessary to protect against superphysiological stressors but is dispensable for normal physiological maintenance. Therefore, to whatever extent normal physiological processes elicit ER stress, the remaining limbs of the UPR are sufficient to protect tissue structure and function.

*3.2. Embryonic lethality upon deletion of ATF6α and p58^IPK^*

Given that the most dramatic metabolic phenotypes in otherwise viable UPR-compromised animals are observed only upon challenge, we reasoned that a phenotype might be elicited in *Atf6α−/−* mice by intercrossing them with mice lacking the ER cochaperone p58^IPK^/ERDJ6. We predicted that deletion of p58^IPK^ would be a sufficient stimulus for eliciting ER stress and hence a phenotype in *Atf6α−/−* animals in whatever tissues p58^IPK^ is important during normal physiology.

To our surprise, homozygous deletion of both genes resulted in synthetic embryonic lethality prior to embryonic day 14.5. Intercrosses among animals heterozygous for both genes yielded no *Atf6α−/−; p58−/−* animals among 255 weaned progeny (Fig. 2A). Intercrosses among animals homozygous for deletion of one gene and heterozygous for the other similarly produced no *Atf6α−/−; p58−/−* animals at weaning (Fig. 2B). To exclude the possibility that double knockout animals were born but then died prior to weaning, timed intercrosses were established using various combinations of heterozygous and homozygous animals (Table S1), with pups analyzed at embryonic day 14.5. We obtained a sufficient number of embryos to conclude that embryonic lethality of *Atf6α−/−; p58−/−* animals occurs prior to E14.5 (Fig. 2C). We did recover one double-nullizygous pup from among two litters of E9.5 embryos (Table S1), which was in the process of being reabsorbed and which provided only enough embryonic material for genotyping.

We next tested whether heterozygosity for *p58^IPK^* would be sufficient to elicit a phenotype in *Atf6α−/−* animals. We found that *Atf6α−/−; p58+/−* animals were grossly normal, were comparably fertile to wild-type animals, and displayed no overt tissue pathology. Histological examination of the pancreas, kidney, and liver—tissues in which a basal or stress-induced phenotype in *p58−/−* animals has been reported [24,35,38]—of these animals was also normal (data not shown).

We then examined the consequence of *p58^IPK^* heterozygosity in an *Atf6α−/−* background in the liver. We chose the liver because *p58^IPK^* is expressed at the highest levels in that tissue [31,39] and because of our interest in the relationship between ER stress and fatty liver—also known as hepatic steatosis. As exogenous ER stress in the liver elicits dysregulation in hepatic lipid metabolism and consequent fatty liver that is dramatically exacerbated in *Atf6α−/−* mice [24,40], we hypothesized that loss of one allele of *p58^IPK^* might be a sufficient ER stress—in the sensitizing context of loss of ATF6α—to lead to hepatic lipid accumulation. However, in contrast to the stark

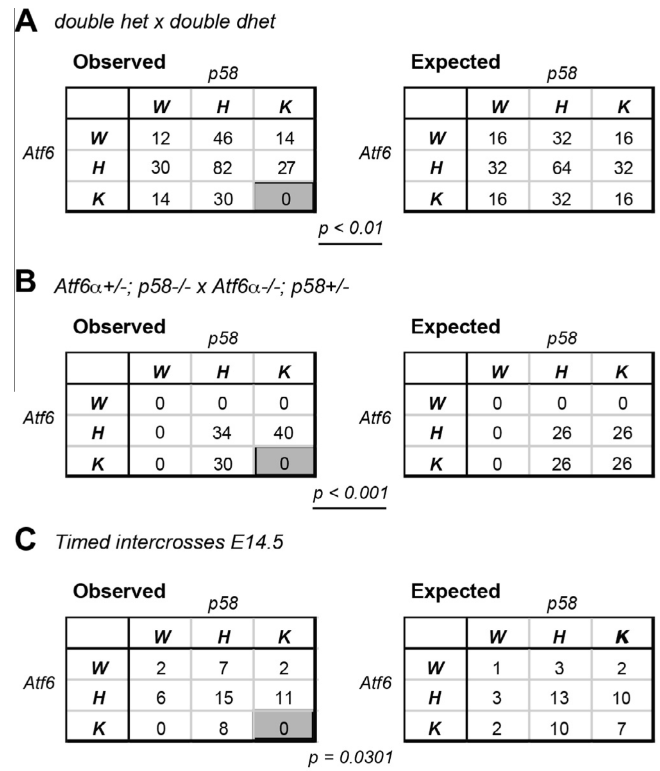

Fig. 2. Combined deletion of ATF6α and p58^IPK^ results in embryonic lethality. (A) *Atf6α+/−; p58^IPK^+/−* animals were intercrossed, and the genotypes of the progeny from multiple litters were recorded. W = wild-type; H = heterozygous; K = knockout. The observed genotype frequencies are given in the table on the left, while the expected frequencies are in the table on the right. *P*-value was determined by Chi-square analysis. Total animal numbers do not correspond because of rounding for the expected group. (B) Same as (A), except *Atf6α+/−; p58−/−* males were crossed to *Atf6α−/−; p58+/−* females and vice versa. (C) Animals of various combinations of *Atf6α* and *p58* genotypes were subjected to timed intercross, and embryos were harvested and genotyped at embryonic day 14.5. The observed and expected preponderances of each genotype from all crosses are shown. The outcome of each cross is given in Table S1.

steatosis seen upon challenge of even wild-type animals with the ER stress-inducing agent tunicamycin (TM), animals lacking both alleles of *Atf6α* and one allele of *p58^IPK^* did not show evidence for accumulated hepatic lipid droplets, as evidenced by immunostaining for the droplet marker protein ADRP (Fig. 3A). Similarly, while exogenous ER stress causes a pronounced suppression of lipid metabolic genes in *Atf6α−/−* animals [24,40,41], *Atf6α−/−; p58+/−* animals showed no significant changes in the basal expression of such genes compared to *Atf6α−/−; p58+/+* animals, nor in expression of the UPR target genes *Bip* and *Chop* (Fig. 3B). Loss of one allele of p58^IPK^ also did not further sensitize *Atf6α−/−* animals to ER stress in the liver; these animals did not evince altered *Xbp1* mRNA splicing nor enhanced upregulation of *Bip* and *Chop* upon challenge with TM (Fig. 3C and D).

4. Discussion

The embryonic lethality of *Atf6α−/−; p58−/−* animals suggests that p58^IPK^ plays a role in embryonic development, the loss of which can be compensated by ATF6α. Several possibilities could account for this lethality: p58^IPK^ might facilitate the folding of one or a small number of developmentally important proteins, and if its deletion induces ER stress, then ATF6α might compensate by upregulating other chaperoning pathways, including those mediated by other ER DnaJ proteins such as ERDJ3 and ERDJ4 [42,43]. Alternatively, p58^IPK^ might contribute in a less specific

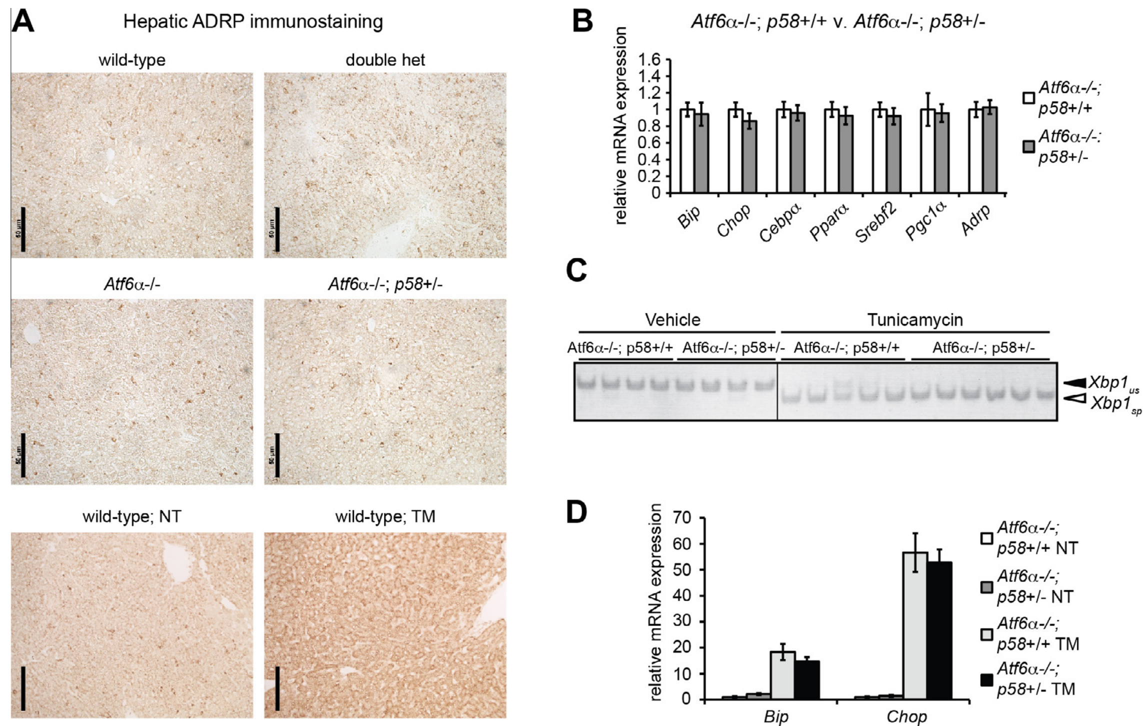

Fig. 3. Heterozygosity for p58[IPK] does not sensitize *Atf6α−/−* animals to ER stress in the liver. (A) Liver sections from animals of the indicated genotypes were examined for lipid droplet accumulation by immunostaining for ADRP. For comparative purposes, the increase in ADRP immunostaining seen upon challenge of wild-type animals with 1 mg/kg TM for 18 h from a separate experiment is shown. (B) Littermate *Atf6α−/−; p58+/+* and *Atf6α−/−; p58+/−* animals of both genders were sacrificed and the expression of UPR target genes and metabolic genes in the liver was determined by qRT-PCR. None of the genes showed statistically significant differences in expression between genotypes. (C) *Atf6α−/−; p58+/+* and *Atf6α−/−; p58+/−* animals were challenged with either vehicle or 250 μg/kg TM. 16 h after treatment RNA was prepared from liver samples and *Xbp1* mRNA splicing was assessed by conventional RT-PCR. Unspliced (us) and spliced (spl) forms are indicated. Image is shown in black-to-white inverted form for greater visual clarity. (D) Same as (C), except the expression of *Bip* and *Chop* mRNA was determined by qRT-PCR from liver samples. Differences in expression between genotypes upon TM treatment were not statistically significant.

way to the overall efficiency of protein folding in one or more tissues; and in the absence of ATF6α, loss of this contribution might result in unameliorated ER stress and either tissue malfunction (e.g., failure to secrete important developmental morphogens) or insurmountable levels of ER stress-induced apoptosis, which would be enhanced when the UPR is compromised. It is also possible that loss of ATF6α produces ER dysfunction and increases the load on the ER, and that an ER lacking p58[IPK] is ill-equipped to handle such increased load—i.e., that ATF6α acts upstream of p58[IPK] in inducing ER dysfunction rather than the other way around. However, given that there is as yet no evidence that loss of ATF6α alone induces ER dysfunction in any tissue or cell type yet studied, here or elsewhere, we think this possibility is unlikely. We also cannot formally exclude that the developmental role of p58[IPK] is exerted through putative regulation of PERK or PKR, but the absence of evidence for detectable cytosolic p58[IPK] militates against this possibility.

The site of p58[IPK] embryonic function might be in the nascent pancreas, the development of which takes place from E8.5-E14.5 [44]. However, the pattern of expression of p58[IPK] in the embryo is unknown, and in the adult it is ubiquitously expressed, suggesting that it might also function broadly in the embryo. Ultimately, a systematic series of timed intercrosses will be necessary to better understand the timing of the lethality, the tissue or tissues affected, and the molecular mechanisms likely at work.

This work is the first to our knowledge to combine deletion of an ER chaperone or cochaperone with that of a UPR signaling zmolecule in a vertebrate. A key conclusion from the synthetic embryonic lethality of *Atf6α−/−; p58−/−* animals is that the UPR in general, and the ATF6α pathway in particular, can compensate for ER chaperone insufficiency and reverse a protein folding defect. Although it is possible that ATF6α accomplishes this by regulating other processes that indirectly improve ER function and so reduce the requirement for p58-dependent folding, the fact that p58[IPK] functions as a cochaperone and that ATF6α is known to regulate chaperone and cochaperone expression makes ATF6α-dependent upregulation of compensatory folding pathways the most likely explanation for survival of *Atf6α+/+; p58−/−* animals. While this conclusion at first glance seems self-evident, both pharmacological (e.g., tunicamycin, thapsigargin, etc.) and physiological (obesity, exercise, etc.) inducers of ER stress perturb cellular function in many ways, and it is conceivable that the UPR could protect cellular function by other means—for example through regulation of protein synthesis rates, calcium homeostasis, glycosylation efficiency, etc. rather than by a UPR pathway that predominantly regulates the expression of chaperones. Thus, while *Atf6α−/−* animals have no apparent basal phenotype, compounding their deletion with that of p58[IPK] elucidates the physiological capacity of UPR-regulated protein folding pathways.

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submit. J.A.G. and D.T.R. conceived and designed the experiments. J.A.G., H.M.T., and D.D.M. performed the experiments. A.K.O. analyzed tissue specimens for pathology. J.A.G. and D.T.R. analyzed data. D.T.R. wrote the paper.

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.2013.11.060.

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