GFP reporter mouse models of UPS proteolytic function

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Kumarapeli and co-workers presented in the FASEB journal an *in vivo* reporter model for the ubiquitin/proteasome system (UPS) (1). Similarly to the strategy that we used previously for the generation of our transgenic reporter model (2), they generated mice ubiquitously expressing a modified green fluorescent protein (GFP) that is constitutively targeted for proteasomal degradation.

While we welcome new mouse models for the UPS, we regret that the authors, in an attempt to make a direct comparison of the two models based on the literature, misrepresented some data from our earlier study. The authors argue that the proteasome inhibitor provoked in their UPS reporter mice accumulation of the reporter in several tissues that did not respond in earlier experiments with our reporter mice (1). In addition, it is stated that this 'unresponsiveness' of tissues is a serious shortcoming of our reporter system.

The authors correctly state that they used the same proteasome inhibitor (MG262), the same inhibitor concentration (5 μmol/kg) and analyzed the mice at the same time after inhibitor administration (20 hrs), but fail to point out that they injected the inhibitor intravenously while we used intraperitoneal administration (1, 2). We feel it is important to point out to the readers that variations in responsiveness can be alternatively explained as a direct consequence of differences in the bioavailability of the inhibitor due to the route of administration. Moreover, we showed that several primary cultures obtained from our reporter mice (cardiomyocytes, neurons and fibroblasts) properly responded with GFP accumulation to treatment with proteasome inhibitors in vitro (2). Unfortunately, Kumarapeli and co-workers ignored this important observation even though they used the same approach to further validate the responsiveness in their own mouse model (1). We anticipate that, since these mouse models are based on different type of UPS substrates (3), it is indeed likely that there will be variations in the responsiveness of these model substrates (which may reflect how different classes of substrates are handled by the UPS) but would like to emphasize that the data presented by Kumarapeli and co-workers do not allow drawing conclusions on this important issue.

We are pleased to see that the usage of GFP reporter substrates of the UPS is becoming more widespread. *In vivo* UPS models have been instrumental in gaining new insights in pathologic processes as well as actions of proteasome inhibitiors (3–5). We are convinced that, also in the future, the different UPS reporter mice will be important tools to decipher the role of the UPS in various pathologies and are looking forward to detailed studies with UPS reporter mice (6). FJ

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Response from the Authors:

We sincerely welcome the comments by Lindsten et al concerning our recent article (1). As previously indicated, we created our reporter (GFPdgn) mouse model independently in the search for an effective tool to dissect *in vivo* UPS proteolytic function. It was not designed to overcome any pitfalls of Ub^{G76V}-GFP mice. GFPdgn was engineered by the fusion of the degron CL1 to the carboxyl terminus of a GFP. The creation of GFPdgn mice was started immediately after Bence et al

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reported the GFP^{u} reporter for cell culture (2), more than 2 years before the Ub^{G76V}-GFP mice were reported (3). A careful analysis on the testing results from our GFPdgn mice and the data reported for Ub^{G76V}-GFP mice made us feel obligated to discuss the important differences in the suitability of these reporter mice for monitoring in vivo UPS proteolytic function. Indeed, for some of the data reported by Lindsten et al, we provided a likely alternative interpretation that differs from the ones offered by them; but we have never misrepresented any data.

Lindsten et al (3) reported accumulation of Ub^{G76V}-GFP in the liver, small intestine, pancreas, kidney, and a small fraction of the cells in the lung and spleen but no fluorescent cells detected in the brain, heart, and skeletal muscles 20 hours after intraperitoneal (i.p.) injection of MG-262 (5 μmol/kg). It is possible but highly unlikely that the variation in responses is caused by a potential difference in the bioavailability of the inhibitor. This is because MG-262 is cell membrane permeable and easily enters the blood flow when used via i.p. injection. Evidently, Lindsten et al observed a dose-dependent decrease in the chymotrypsin-like activities (CTLA) of the proteasome in the lysates of not only the liver but also the kidney and spleen (3). To produce effects in the kidney, spleen, and lungs, an i.p. injected pharmacological agent needs to get into the systemic circulation first. It is very unlikely that MG-262 absorbed into the systemic circulation was insufficient to inhibit the proteasome in the heart and skeletal muscles while it was evidently effective in the kidney and lungs. Unfortunately, no data were presented by Lindsten et al concerning the CTLA in the heart, skeletal muscles, or the brain in their systemic proteasome inhibition experiments.

To clarify this issue, we have repeated the same proteasomal inhibition experiments with our GFPdgn mice using i.p. injections. As expected, the same degree of CTLA inhibition and GFPdgn accumulation were detected in the heart, brain, and skeletal muscles as we previously observed with intravenous injections. This confirms our proposition that the GFPdgn mice are much better suited to report in vivo UPS proteolytic function in the heart, skeletal muscles, and brain.

Experimental results from *in vitro* cell culture do not necessarily reflect *in vivo* situations and *vice versa*. We did carry out cell culture tests to validate the platform of in vitro use of GFPdgn in adult cardiomyocytes (1); but they were neither intended to nor utilized to validate GFPdgn as a reliable reporter for in vivo UPS proteolytic function.

We agree that substrates carrying different degradation signals are likely handled differentially by the UPS. The objective of a study ultimately dictates its choice of reporter systems to monitor in vivo UPS proteolytic function.

The research into the (patho)physiological significance of the UPS in the heart, skeletal muscles, and brain is inarguably important and the reliable readouts for in vivo UPS proteolytic function in these organs will undoubtedly facilitate the research endeavor. It is gratifying that GFPdgn mice have been successfully used to delineate the deregulation of UPS proteolytic function in the heart of several disease models (1, 4, 5). We anticipate that all sensitive and reliable fluorescence-based UPS reporters will be more extensively employed in further unveiling the regulation of UPS proteolytic function and its roles in physiology and pathology. FJ

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C. elegans as a model for Friedreich Ataxia

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In a recent FASEB paper, Palau and co-workers claimed that lowering frataxin reduces *C. elegans* lifespan (1). Given the importance of frataxin for survival in other organisms, these results may not seem surprising. However, they are clearly in contrast with our previously published findings showing that frataxin RNAi knock down, (both feeding and injection), increases *C. elegans* lifespan (2). Our results reinforce studies showing that inactivation or reduced expression of several different mitochondrial proteins increase lifespan (3, 4). While it is not uncommon for research groups to obtain contrasting results, what captured our attention in the Palau paper was that it misrepresented our experimental procedures and trivially dismissed the significance of our findings.

Both groups utilized RNAi to reduce frataxin expression. Palau and colleagues injected *frh-1* dsRNA directly into the gonads of fourth-larval stage (L4) larvae and followed their offspring's lifespan; we microinjected *frh-1* dsRNA into the gonads of 5-day-old adults and, similarly, examined the lifespan of the offspring of the injected animals. Palau and coworkers incorrectly state (1) that we utilized the injected adult animals for further study and consequently saw "no way to compare both kinds of experiments." Any expert in nematode biology would immediately question such an experimental approach and consequently the validity of any results so obtained.

What then might explain the opposite effects on lifespan observed by the two groups following *frh-1* interference? We can envision at least two explanations for these contrasting results. The most immediate relates to the possibility that Palau's group analyzed their lifespan data differently. Specifically, under some environmental conditions, adult *C. elegans* will often retain their eggs internally resulting in a premature form of death (referred to as "bagging"). This is distinguishable from true age-related death and is generally censored in lifespan analyses. If bagged adults are not ascertained appropriately, mean lifespan will appear to be shortened. Given the egg-laying defective (Egl) phenotype reported by Palau et. al., (and shown in their Figure 2C), internal hatching of progeny was probably a frequent occurrence. It is not clear from their methods section if Palau et. al. censored bagged animals or not. To this end, however, and in agreement with our own results, it is informative that Palau et. al. reported an increase in the lifespan of *frh-1* RNAi-treated animals when lifespan was carried out in the presence of FudR. This chemical sterilizes the animals thus preventing bagging. Given that the authors were aware of our publication, it seems unusual that this data was not shown and not considered further. A second explanation for the divergent results might come from variation in experimental conditions -use of different dsRNA constructs and/or microinjecting worms of different ages. For many genes RNAi efficacy can vary substantially depending on the specific dsRNA construct employed. In the study by Palau et al., dsRNA was generated from a fragment comprised of the entire *frh-1* ORF along with part of the 5'-UTR. In our studies two different dsRNA constructs were used—one based on the *frh-1* cDNA (for feeding experiments) and another based on the full-length genomic fragment (for injection studies). It is also well known that microinjection of dsRNA results in a more drastic reduction of gene expression than simply feeding animals dsRNA via their bacterial diet. In our own published studies on frataxin, we found that microinjection of *frh*-1 dsRNA was more effective than feeding RNAi at causing a reduction in size of offspring (even when compared to animals whose ancestors had been continuously fed RNAi for 3 prior generations). Despite this, and in contrast to the findings of Palau and colleagues, we still observed that the progeny of microinjected animals lived longer, not shorter, than controls. Nevertheless, we suspect that frataxin RNAi knock down is more severe in the progeny of worms injected as L4 than in those injected at 5 days of age, since the latter are approaching the end of their fertile period and the gonad has already aged somewhat by this time. Intriguingly, this would imply that lowering frataxin below a critical threshold is not tolerable by *C. elegans* thus resulting in the shorter lifespans observed by Palau and colleagues.

In a manuscript currently under review, we show that longevity can be modulated by titrating mitochondrial gene expression. In those studies, we unambiguously demonstrate that life extension is observed only under reduced mitochondrial functionality and at greater reductions a shortening of lifespan is observed. Similar threshold effects are apparent in several human mito-

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chondrial-associated diseases. Notably in Friedreich Ataxia, where, the level of expression of frataxin must be below 75% normal in order for patients to present with a pathology; signs and symptoms directly correlate with protein expression level (5). In light of these observations showing mitochondrial threshold effects that dictate lifespan outcome, our experiments and those of Palau and colleagues may not be in conflict and together may in fact provide a better comprehension of both Friedreich Ataxia pathogenesis and mitochondrial control of lifespan. FJ

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