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Quantification of oxidative stress in live mouse embryonic fibroblasts by monitoring the responses of polyubiquitin genes

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ABSTRACT

Stress-regulated polyubiquitin genes in mammals are expected to be upregulated under oxidative stress conditions. In order to assess gene regulation via the conventional method, the isolation of RNA molecules or the transfection of reporter constructs into cells is frequently required. If the stress response within cells can be monitored in a reversible manner with minimal manipulation, the study of the stress response pathways will become much easier. Herein, we have developed a simple fluorescence plate reader-based assay to monitor the stress responses of polyubiquitin genes in mouse embryonic fibroblasts, in which one allele of the ubiquitin-coding region of the polyubiquitin gene *Ubb* or *Ubc* was replaced by the *eGFP-puro* cassette, thereby placing GFP expression under the control of the endogenous polyubiquitin gene promoter. Using this simple assay, we established that both mammalian polyubiquitin genes are upregulated upon oxidative stress with slightly higher responses from the *Ubb* promoter. The principal advantage of this assay is that it allows for the monitoring of stress responses of polyubiquitin genes without disrupting cellular growth; this assay can therefore be applied repeatedly to the same cells. Furthermore, by calculating the increase in fluorescence deriving from newly synthesized GFP upon stress, which can be regarded as a *bona fide* polyubiquitin gene stress response, we were able to determine and directly compare the concentrations of various oxidative stressors that induce the similar cellular stress levels. Therefore, this simple assay may also be employed in the screening of potentially toxic reagents that induce the stress response pathways.

1. Introduction

The exposure of cells to environmental insults such as oxidative stress results in the activation of adaptive stress response pathways, increasing cellular stress resistance and the probability of survival [1]. Upon exposure to environmental toxicants or cellular stressors that generate reactive oxygen species (ROS), transducers such as mitogen-activated protein kinases (MAPKs) are activated by detecting signals from stress, and modify downstream sensors such as Kelch-like ECH-associated protein 1 (Keap1) and/or transcription factors such as NF-E2-related factor 2 (Nrf2) [2,3]. The activated transcription factors induce the upregulation of stress-responsive target genes to protect cells against toxicity. Among these potential target genes are the stress-regulated polyubiquitin genes, which, in addition to the constitutive monomeric ubiquitin (Ub) ribosomal fusion genes, contribute significantly to the maintenance of cellular steady-state Ub levels [4–9]. Under stress conditions, the cellular demand for Ub is increased to correct protein misfolding or to degrade damaged proteins [10,11]; otherwise, accumulations of these nonnative protein conformers prove toxic to the cells. In budding yeast, the stress-inducible polyubiquitin gene, *UBI4*, has been shown to be upregulated in response to oxidative stress [12]. Although *UBI4* is dispensable under normal growth conditions, the loss of *UBI4* has been demonstrated to induce a dramatic increase in oxidative stress sensitivity, thus suggesting that the polyubiquitin gene plays an important role in increasing cellular Ub levels to allow them to survive under toxic stress conditions. Interestingly, there are two different polyubiquitin genes in mammals, *Ubb* and *Ubc*, which are composed of 3–4 and 9–10 tandem repeats of Ub-coding units, and they are both required for metazoan development as well as survival under stress conditions [13–15], although *UBI4* in yeast is required only under stress conditions [10]. *Ubb* is highly expressed in the gonads and brain, and the loss of *Ubb* results in infertility in both male and female mice [15], as well as hypothalamic neurodegeneration [13]. *Ubc* is

Abbreviations: MEFs, mouse embryonic fibroblasts; Ub, ubiquitin; GFP, green fluorescent protein; eGFP, enhanced GFP.

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expressed at relatively high levels in the liver, and the loss of *Ubc* leads to embryonic lethality presumably due to defective proliferation of fetal liver cells [14].

It is generally believed that the levels of cellular Ub are increased profoundly by the upregulation of polyubiquitin genes [16,17], which generate an Ub adduct that is rapidly hydrolyzed to monomeric Ub, although it has also been suggested to be increased by the activity of an enzyme that converts existing Ub chains to monomeric Ub [18]. Therefore, it is anticipated that the upregulation of polyubiquitin genes may function as a favorable indicator of adaptive stress responses, and the level of upregulation may be correlated with stress severity, although cell death may occur under severe stress conditions. However, it has yet to be determined whether or not the upregulation of polyubiquitin genes represents a general cellular response occurring in reaction to all types of stress, and also whether or not *Ubb* and *Ubc* are regulated differently under different stress conditions, or even after exposure to different toxic reagents that induce the same stress response pathway. Additionally, a precise determination of the concentrations of toxic reagents that can induce the anticipated stress response levels may profoundly facilitate the screening of potentially toxic reagents. Therefore, it is clearly necessary to develop a simple and reliable assay method to monitor the upregulation of polyubiquitin genes under stress conditions.

Herein, we report the development of a simple fluorescence plate reader-based assay designed to determine cellular stress levels by monitoring the response of the polyubiquitin genes *Ubb* and *Ubc*, using $Ubb^{+/-,eGFP\text{-}puro}$ and $Ubc^{+/-,eGFP\text{-}puro}$ mouse embryonic fibroblasts (MEFs), in which one allele of the polyubiquitin gene is knocked-out and the *eGFP-puro* cassette is knocked in to the same locus; thus, GFP expression is under the control of the endogenous *Ubb* or *Ubc* promoter [14,15]. The principal advantage of this assay involves its ability to monitor the stress response of polyubiquitin genes in "live" MEFs, which can be repeatedly employed without necessitating the harvesting of cells for the isolation of RNA for RT-PCR or Northern blot analysis, or the transfection of cells with reporter constructs. Via this simple, rapid, and reliable method, we demonstrated that both *Ubb* and *Ubc* are upregulated upon oxidative stress, and that the upregulation of *Ubb* is slightly more prominent than that of *Ubc*. Intriguingly, the upregulation of polyubiquitin genes was shown to vary depending on the types of stress induced. Finally, we determined and directly compared the concentrations of various oxidative stressors that induce similar polyubiquitin gene responses.

2. Materials and methods

2.1. Preparation of MEFs

$Ubc^{+/-,eGFP\text{-}puro}$ (or simply $Ubc^{+/-}$) and $Ubb^{+/-,eGFP\text{-}puro}$ (or simply $Ubb^{+/-}$) mice were generated as previously described [14,15], and maintained in plastic cages with *ad libitum* access to food and water. All animal procedures were approved by the University of Seoul Institutional Animal Care and Use Committee (UOS IACUC). Mouse embryonic fibroblasts (MEFs) were prepared from 13.5-day post-coitum mouse embryos, as described previously [14].

2.2. Fluorescence plate reader assay

Sixteen hours after plating out 2×10^4 MEFs (passage numbers between 3 and 6) into each well of a 96-well plate, the MEFs were treated with chemicals as indicated in the text, in the presence or absence of 50 μg/ml of emetine. Every 3 h and for up to 12 h, the medium (DMEM supplemented with 10% fetal bovine serum, 20 mM glutamine, and penicillin/streptomycin) was temporarily removed and washed twice in PBS, after which the fluorescence was measured using a fluorescence plate reader (Molecular Devices SpectraMax M2^e) prior to the addition of medium back into each well. Fluorescence (RFU; relative fluorescence unit) was read from the bottoms of the plates with excitation and emission wavelengths of 485 nm and 525 nm, respectively. To reduce the background fluorescence, an emission cutoff filter of 515 nm was employed. The entire measurement procedure in each case took only a few minutes, thus minimizing the disruption of cellular growth.

3. Results and discussion

3.1. Development and optimization of the fluorescence plate reader-based assay to monitor the stress response of polyubiquitin genes in MEFs

We first attempted to develop a simple assay for measuring the stress responses of polyubiquitin genes in MEFs over the course of the culture period. To this end, we initially optimized and streamlined the procedure by which the levels of GFP fluorescence in MEFs could be measured without stress induction (Fig. 1A top, solid lines). We observed higher background GFP fluorescence levels from the $Ubc^{+/-,eGFP\text{-}puro}$ MEFs relative to the $Ubb^{+/-,eGFP\text{-}puro}$ MEFs; this is consistent with the observation that *Ubc* contributes much more profoundly than that of *Ubb* to the total levels of Ub in MEFs, as demonstrated by the results of quantitative real-time RT-PCR (data not shown). For an easy comparison, we set the initial GFP fluorescence levels in both MEFs as 100%, and the relative GFP fluorescence levels in each well were monitored over a 12-h period (Fig. 1A). In both MEFs, the levels of GFP fluorescence increased slightly over time (approximately 20% increase over 12 h) (Fig. 1A top, solid lines). This slight increase in GFP fluorescence may be attributable to the observed increase in cell numbers over time and/or to the increase in GFP synthesis over degradation. To distinguish between these two possibilities, we initially normalized GFP fluorescence levels to the number of cells per well; we then treated cells with emetine in order to block the synthesis of new proteins. When GFP fluorescence levels were normalized to the cell number, we found that GFP fluorescence levels per cell remained almost constant over the 12-h incubation period (Fig. 1A bottom, solid lines). Therefore, the slight increase in GFP fluorescence observed over time (per well) is principally attributable to cell proliferation during the culture period. In the presence of emetine, however, the levels of GFP fluorescence were reduced over time (Fig. 1A top and bottom, dotted lines). In fact, with emetine, the numbers of cells in each well remained almost constant over the 12-h culture period (data not shown); this indicates that the previously made GFP, which has a half-life of over 10 h [19], undergoes a gradual degradation (Fig. 1A top and bottom, dotted lines). Therefore, the observed differences in GFP fluorescence in the absence and presence of emetine (per well) are largely the result of the newly synthesized GFP, in addition to the contribution of the increase in cell numbers (ΔGFP fluorescence per well in Fig. 1C), although the differences in GFP fluorescence in the absence and presence of emetine (per cell) appear to be exclusively attributable to the newly synthesized GFP (ΔGFP fluorescence per cell in Fig. 1C).

We subsequently treated MEFs with 10 μM MG-132 for up to 12 h to induce stress responses by inhibiting proteasomal degradation. The stress responses of polyubiquitin genes under proteasomal inhibition conditions may function as a positive control for the assay, as we have already demonstrated via quantitative real-time RT-PCR that the transcript levels of *Ubb* and *Ubc* in MEFs are increased significantly after exposure to 10 μg/ml of ALLN for 24 h [14]. As anticipated, under proteasomal inhibition conditions, GFP fluorescence levels from both MEFs were increased signifi-

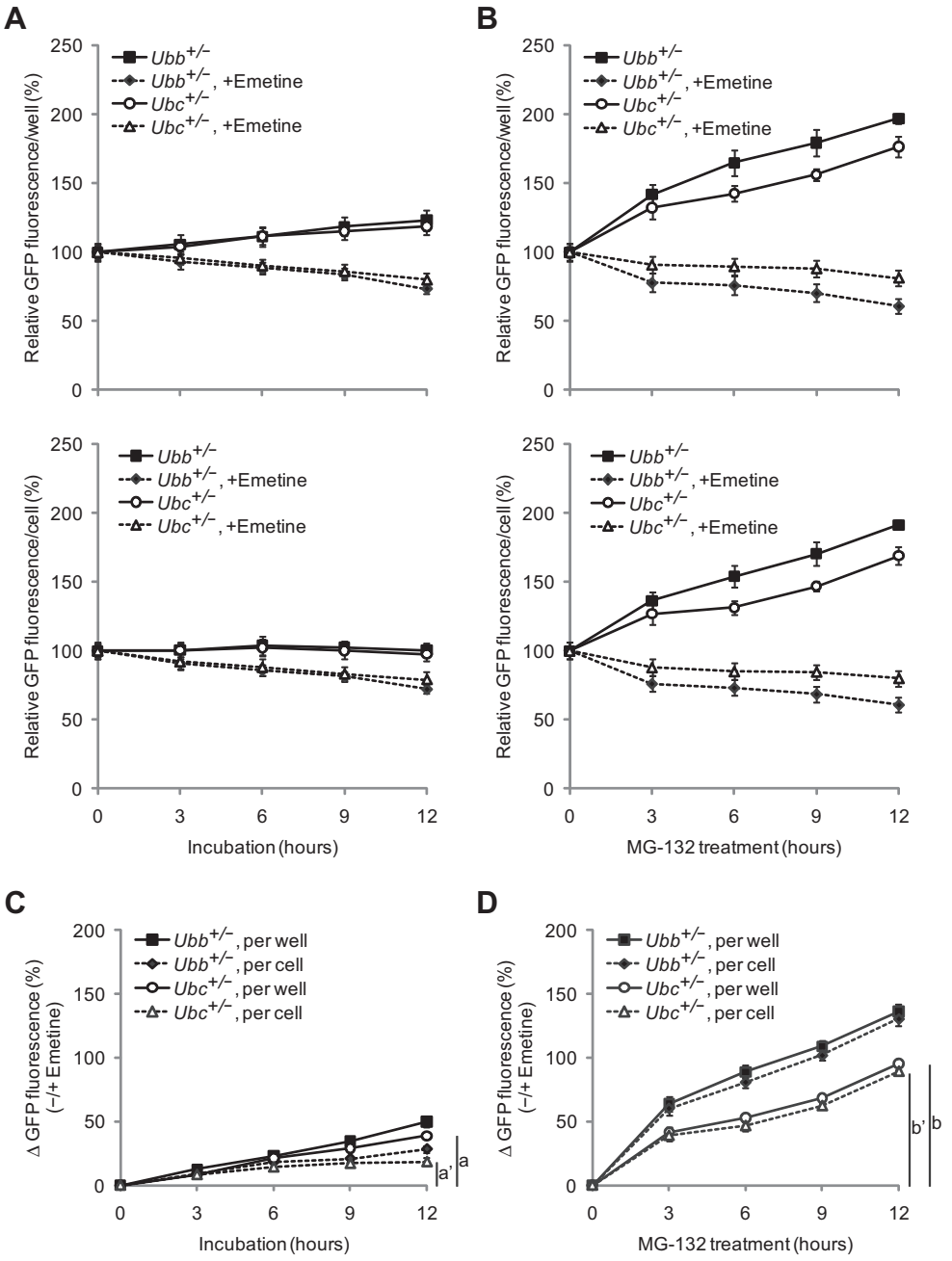

Fig. 1. The development of a fluorescence plate reader-based assay to monitor the stress responses of polyubiquitin genes in MEFs. (A–B) Sixteen hours prior to treatment with 10 μM MG-132 (or vehicle), 2×10^4 MEFs were plated out into each well of a 96-well plate. Upon and following MG-132 or vehicle treatment in the presence or absence of 50 μg/ml of emetine, at 3-h intervals, the medium was temporarily removed and the fluorescence was assessed using a fluorescence plate reader (top). To determine the fluorescence levels per cell, the fluorescence levels were normalized to the cell number at each time point (bottom). The initial fluorescence levels in both $Ubb^{+/-,eGFP\text{-}puro}$ and $Ubc^{+/-,eGFP\text{-}puro}$ MEFs were set to 100%. (C–D) ΔGFP fluorescence in the absence and presence of emetine is a simple representation of newly synthesized GFP, which is used to directly measure the transcriptional upregulation of *Ubb* or *Ubc*. The differences in ΔGFP fluorescence (−/+emetine) value b–a (per well) or b′–a′ (per cell) at 12 h in $Ubc^{+/-,eGFP\text{-}puro}$ MEFs, which roughly (per well) or exactly (per cell) correspond to the increase in fluorescence attributable to newly synthesized GFP under stress conditions, is reflective of the *bona fide* stress responses of *Ubc* by transcriptional upregulation under proteasomal stress. The stress responses of *Ubb* can also be calculated similarly. All data are expressed as the means ± SEM from four different $Ubb^{+/-,eGFP\text{-}puro}$ or $Ubc^{+/-,eGFP\text{-}puro}$ MEFs with passage numbers between 3 and 6.

cantly over time (Fig. 1B top, solid lines), and was completely abolished in the presence of emetine (Fig. 1B top, dotted lines). Similar results were observed when GFP fluorescence levels were normalized to the cell number (Fig. 1B bottom), and thus the differences in GFP fluorescence observed in the absence and presence of emetine with MG-132 treatment can be regarded as solely attributable to the contribution of the newly synthesized GFP (ΔGFP fluorescence per well or per cell in Fig. 1D). Therefore, the increase in ΔGFP fluorescence (−/+emetine) with MG-132 treatment over the non-treated controls (e.g., for *Ubc*, b–a (per well) or b′–a′ (per cell)) is reflective of the *bona fide* stress response of polyubiquitin genes by transcriptional upregulation under stress conditions.

3.2. Monitoring the oxidative stress response of polyubiquitin genes in "live" MEFs

In an effort to make the assay both simple and rapid, we decided to monitor GFP fluorescence levels per well, not on a per-cell basis,

which otherwise would have made the assay quite cumbersome, necessitating the counting of cells in each well of the 96-well plates. As the ΔGFP fluorescence (−/+emetine) levels of the non-treated controls at 12 h of incubation were slightly higher on a per-cell basis (see Fig. 1C), it is possible that we may have slightly underestimated the increase in fluorescence under stress without normalization to the cell number; however, we deemed that this extra step could be omitted to keep the assay extremely simple, without compromising the results. To validate our assay, we first treated MEFs with 10 μM MG-132 for 12 h, with monitoring of GFP fluorescence levels every 3 h, and then calculated the increase in fluorescence due to newly synthesized GFP, which is a direct indicator of the stress responses of polyubiquitin genes (Fig. 2A). Both polyubiquitin genes continued to be upregulated throughout the 12-h incubation period, with higher stress responses exhibited by *Ubb* than by *Ubc*, which is consistent with the previous findings of quantitative real-time RT-PCR analysis [14].

We next monitored the responses of polyubiquitin genes in MEFs in the presence of oxidative stressors over a 12-h period. We treated MEFs with 500 μM paraquat, 10 μM $NaAsO_2$, or 1 mM H_2O_2 for 12 h and monitored GFP fluorescence levels every 3 h (Fig. 2B–D). The concentrations of oxidative stressors were chosen in order to maximize the stress responses without inducing cell death. All of the oxidative stressors that we tested herein showed a gradual upregulation of polyubiquitin genes over the 12-h incubation period (Fig. 2B–D). However, we were unable to determine which polyubiquitin gene preferentially responds to oxidative stress, although it was very clear that *Ubb* preferentially responded to proteasomal stress in MEFs (see Fig. 2A). Intriguingly, the response of polyubiquitin genes upon stress depends on the type of stressors employed. While the upregulation of polyubiquitin genes was observed under proteasome inhibition, autophagy inhibition (data not shown), and oxidative stress conditions, when MEFs were treated with 5 μM thapsigargin, which induces ER stress, the upregulation of polyubiquitin genes was very minimal (Fig. 2E). This result was confirmed by measuring the levels of GFP fluorescence in MEFs treated with up to 10 μM thapsigargin via flow cytometry (data not shown). When MEFs were treated for 12 h with higher concentrations of thapsigargin, many of the cells underwent morphological changes and began to float on the plates, which presumably implies that the cells made the decision to undergo apoptosis rather than resisting the applied stress. It remains to be determined why polyubiquitin genes respond well to specific types of stress, but not to others; further investigations will be

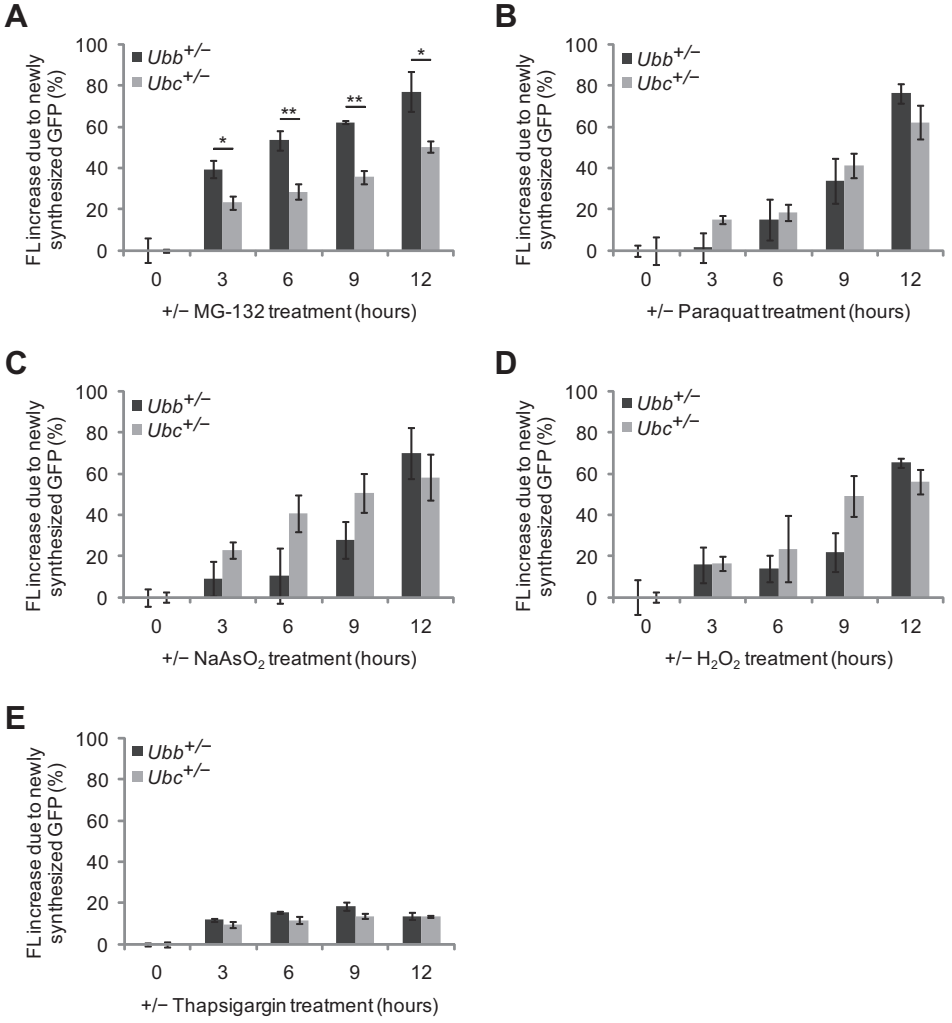

Fig. 2. Monitoring the oxidative stress responses of polyubiquitin genes in "live" MEFs. (A–E) Sixteen hours prior to 10 μM MG-132, 500 μM paraquat, 10 μM $NaAsO_2$, 1 mM H_2O_2, 5 μM thapsigargin, or vehicle treatment, MEFs were plated out into each well of a 96-well plate. Upon and following stressor or vehicle treatment in the presence or absence of 50 μg/ml of emetine, at 3-h intervals, the medium was temporarily removed and the fluorescence was measured on a per-well basis. Increases in FL (fluorescence) due to newly synthesized GFP, a *bona fide* stress response of polyubiquitin genes under stress conditions, was calculated as shown in Fig. 1. All data are expressed as the means ± SEM from four different $Ubb^{+/-,eGFP\text{-}puro}$ or $Ubc^{+/-,eGFP\text{-}puro}$ MEFs with passage numbers between 3 and 6. $^{*}P < 0.05$; $^{**}P < 0.01$ (two-tailed unpaired Student's *t*-test).

required to elucidate the mechanisms underlying the activation of stress response pathways resulting in the upregulation of the polyubiquitin genes.

3.3. Determination of the concentrations of oxidative stressors for the anticipated stress response of polyubiquitin genes

We employed this assay to assess the responses of MEFs against a variety of oxidative stressors with a concentration range of 10-fold difference for 12 h, and subsequently determined the increase in ΔGFP fluorescence (−/+emetine) over non-treated controls (Fig. 3). The increase in ΔGFP fluorescence (−/+emetine) against various concentrations of stressors was plotted, and the curve was fitted to the exponential equation $y = y_0 + a\,(1 - e^{-bx})$. Using these dose-dependence curves, it proved possible to calculate the concentration of stressors with the anticipated stress responses of polyubiquitin genes. For example, the treatment of MEFs with 2.57 μM MG-132, 106 μM paraquat, 4.70 μM $NaAsO_2$, or 235 μM H_2O_2 for 12 h induced the upregulation of *Ubb* by 50% as a stress response (Fig. 3A–D, black dotted arrows). On the other hand, the treatment of MEFs with 8.81 μM MG-132, 230 μM paraquat, 7.20 μM $NaAsO_2$, or 617 μM H_2O_2 for 12 h was required to induce the upregulation of *Ubc* by 50% (Fig. 3A–D, grey dotted arrows). This suggests that *Ubb* may respond preferentially to oxidative stress in MEFs, although in most cases this response did not reach a statistically significant level. Thus, lower concentrations of stressors are required to upregulate *Ubb* than *Ubc* to achieve similar levels of polyubiquitin gene upregulation. This result is consistent with our previous observation, via quantitative real-time RT-PCR, that the fold increase in the level of *Ubb* transcripts was substantially higher than that of the *Ubc* transcript level under proteasome inhibition [14] or heat shock conditions (Ryu et al., unpublished data). Although proteasomal and oxidative stressors markedly upregulated both the *Ubb* and *Ubc* polyubiquitin genes upon the application of stress conditions, thapsigargin at dosages up to 5 μM exerted no major effects on the upregulation of polyubiquitin genes under stress (Fig. 3E).

Although we anticipated originally that *Ubb* and *Ubc* might respond differently to different types of stress, we found that these polyubiquitin genes generally do not distinguish cellular stress in MEFs, and also that no distinct preferred upregulation of one polyubiquitin gene to another occurred in response to a specific stress,

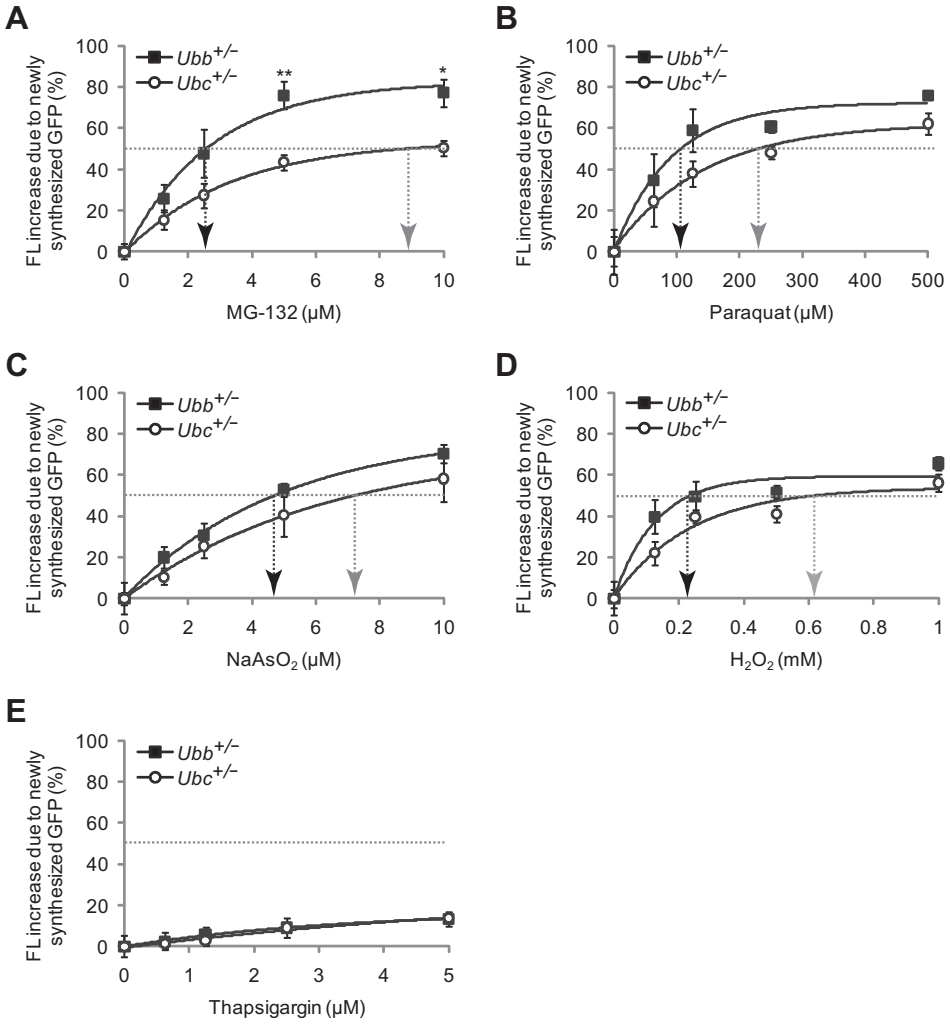

Fig. 3. Determination of the concentrations of oxidative stressors for the anticipated stress responses of polyubiquitin genes. (A–E) Sixteen hours prior to MG-132, paraquat, $NaAsO_2$, H_2O_2, thapsigargin, or vehicle treatment at the indicated concentrations, MEFs were plated out into each well of a 96-well plate. After 12 h of treatment with stressor or vehicle in the presence or absence of 50 μg/ml of emetine, the medium was removed and the fluorescence was measured on a per-well basis. The increase in FL (fluorescence) attributable to newly synthesized GFP was calculated as described in Fig. 1. Black and grey dotted arrows indicate the concentrations of stressors that induce the upregulation of *Ubb* and *Ubc* by 50% under stress, respectively. All data are expressed as the means ± SEM from four different $Ubb^{+/-,eGFP\text{-}puro}$ or $Ubc^{+/-,eGFP\text{-}puro}$ MEFs with passage numbers between 3 and 6. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. $Ubc^{+/-,eGFP\text{-}puro}$ MEFs (two-tailed unpaired Student's *t*-test).

although *Ubb* appears to be preferred to a slight degree. However, this slight preference toward *Ubb* does not necessarily demonstrate that *Ubb* contributes more than *Ubc* to the increase in cellular Ub levels under stress conditions, simply because the basal expression level of *Ubb* is lower than that of *Ubc* (about 2/3 based on background GFP fluorescence) and the number of tandem Ub-coding units is higher in *Ubc* than in *Ubb* (9 vs. 4 in mice); thus, the contribution of *Ubc* toward total Ub levels is more than twice that of *Ubb*, even with similar transcriptional activation levels. In fact, MTT cell viability assay demonstrated that, although both $Ubb^{-/-}$ and $Ubc^{-/-}$ MEFs exhibited the enhanced sensitivity to oxidative stress relative to wild-type MEFs, $Ubc^{-/-}$ MEFs were more sensitive than $Ubb^{-/-}$ MEFs, thus suggesting that *Ubc* contributes more than *Ubb* to the increase in cellular Ub levels under oxidative stress conditions, and confirming that Ub homeostasis is important to exert stress resistance (Ryu et al., unpublished data).

In sum, although the upregulation of polyubiquitin genes may not be a completely universal feature of stress responses in MEFs, oxidative stressors can induce the upregulation of both polyubiquitin genes, thus suggesting that the upregulation of the polyubiquitin gene is a useful oxidative stress indicator. Additionally, this simple assay not only enables us to monitor the stress responses of polyubiquitin genes in live MEFs, but also to determine the concentrations of oxidative stressors that can induce the anticipated levels of cellular stress.

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