The mouse polyubiquitin gene *UbC* is essential for fetal liver development, cell-cycle progression and stress tolerance

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UbC is one of two stress-inducible polyubiquitin genes in mammals and is thought to supplement the constitutive *UbA* genes in maintaining cellular ubiquitin (Ub) levels during episodes of cellular stress. We have generated mice harboring a targeted disruption of the *UbC* gene. *UbC*^{−/−} embryos die between embryonic days 12.5 and 14.5 *in utero*, most likely owing to a severe defect in liver cell proliferation. Mouse embryonic fibroblasts from *UbC*^{−/−} embryos exhibit reduced growth rates, premature senescence, increased apoptosis and delayed cell-cycle progression, with slightly, but significantly, decreased steady-state Ub levels. *UbC*^{−/−} fibroblasts are hypersensitive to proteasome inhibitors and heat shock, and unable to adequately increase Ub levels in response to these cellular stresses. Most, but not all of the *UbC*^{−/−} phenotypes can be rescued by providing additional Ub from a poly hemagglutinin-tagged Ub minigene expressed from the *Hprt* locus. We propose that *UbC* is regulated by a process that senses Ub pool dynamics. These data establish that *UbC* constitutes an essential source of Ub during cell proliferation and stress that cannot be compensated by other Ub genes. *The EMBO Journal* (2007) **26**, 2693–2706. doi:10.1038/sj.emboj.7601722; Published online 10 May 2007
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Introduction

Ubiquitin (Ub) is a highly conserved 76 amino-acid protein that plays critical roles in the function of eukaryotic cells (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Ub signaling is a covalent process initiated by formation of an

isopeptide bond between the C-terminal carboxyl group of Ub and a free amino group of a substrate, and is terminated by enzymatic cleavage of this linkage (D'Andrea and Pellman, 1998). Ub can be conjugated to itself and the resulting polyubiquitin chains are likely to have distinct roles in Ub-dependent signaling processes (Pickart, 2000; Pickart and Fushman, 2004). Because of its widespread use in intracellular signaling and the large numbers of Ub moieties that may be conjugated to a single substrate, Ub is very abundant, accounting for 0.1–5% of total cellular protein (Ohtani-Kaneko *et al*, 1996; Takada *et al*, 1996; Osaka *et al*, 2003; Ryu *et al*, 2006). Maintenance of cellular Ub at levels sufficient to sustain its multiple cellular functions under all metabolic conditions is therefore of critical importance to cellular survival.

Cellular Ub is comprised of two distinct pools consisting of free Ub and Ub–substrate conjugates. Ub isopeptidases antagonize Ub conjugation and ensure that these pools are in dynamic equilibrium (Wilkinson, 1997). Although conjugated Ub is largely recycled to the monomer pool by isopeptidases associated with the 26S proteasome, steady-state levels of cellular Ub depend on adequate levels of Ub gene transcription to compensate for basal Ub turnover, which has been estimated to range from $t_{1/2} \sim 2$ h in yeast (Hanna *et al*, 2003) to 31 h in mammalian cells (Haas and Bright, 1987). In mammals, Ub is encoded by four Ub genes, two of which (*UbB* and *UbC*) encode Ub polyproteins and two of which (*UbA52* and *UbA80*) encode fusions between Ub and two small ribosomal proteins (Lund *et al*, 1985; Wiborg *et al*, 1985; Baker and Board, 1987, 1991; Finley *et al*, 1989; Redman and Rechsteiner, 1989). Thus, all Ub is translated from fusion proteins that are processed to generate free Ub monomers by ubiquitously expressed Ub-specific proteases. Because Ub fusions, the primary translation products of Ub genes, are generally undetectable in cells, it is assumed that they are rapidly and efficiently processed into Ub monomers which, owing to the exquisite conservation of the Ub gene, are chemically identical.

The polyubiquitin genes in all eukaryotes that have been studied are stress-regulated genes and contain heat-shock elements in their promoter regions (Bond and Schlesinger, 1986; Fornace *et al*, 1989). In yeast, the single polyubiquitin gene (*UBI4*) is dispensable under vegetative growth conditions, suggesting that the *UbA* genes (*UBI1–3*) provide the bulk of cellular Ub (Finley *et al*, 1987). *ubi4* cells, however, are sensitive to a variety of different types of stress and cannot sporulate (Finley *et al*, 1987). Far less is known about the functional organization, regulation and expression of the mammalian polyubiquitin genes. The two polyubiquitin genes *UbB* and *UbC* differ most notably in the number of Ub coding units they contain, arranged in a spacerless, tandem, head-to-tail fashion (Wiborg *et al*, 1985). The *UbB* genes are most similar to yeast *UBI4* in that they contain 3–5 Ub units

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(*UBI4* has 5), whereas the *UbC* gene contains 9–10 Ub units. Both polyubiquitin genes are upregulated by cellular stresses and contain classical heat-shock elements in their promoters (Fornace *et al*, 1989). Despite the broad importance of Ub in virtually every aspect of eukaryotic cell function, surprisingly little is known about the relative contributions and functions of the individual Ub genes and how they are regulated to maintain an adequate supply of Ub in normal and stressed cells. In this study, we have used conventional gene targeting methodology to ablate the single Ub-coding exon of the murine *UbC* gene. While loss of a single *UbC* allele has no apparent phenotype, we find that homozygous deletion of the mouse *UbC* gene causes mouse embryos to die *in utero* in midgestation, most likely due to arrested fetal liver proliferation. Homozygous loss of *UbC* also leads to severely reduced proliferative capacity of mouse embryonic fibroblasts (MEFs), a delay in cell-cycle progression and increased susceptibility to cellular stress.

Results

UbC is essential for fetal development

The murine *UbC* locus consists of two exons spanning 3.5 kb on mouse chromosome 5. To disrupt the *UbC* gene, we constructed a targeting vector to replace the single exon containing the entire *UbC* coding sequence with sequence encoding a promoterless GFP-puromycin-resistance fusion protein (GFP-puro) flanked by *loxP* sites (Figure 1A). Homologous recombinants were generated in embryonic stem (ES) cells by positive selection with puromycin and negative selection (against non-homologous recombinants) using diphtheria toxin. Two independent ES cell clones were isolated, verified by Southern blotting (Figure 1B, upper panels) and injected into C57BL/6J blastocysts to generate six chimeric lines, all of which had identical phenotypes. Heterozygous *UbC*^{GFP-puro} mice were phenotypically normal and were intercrossed to obtain progeny homozygous for the floxed targeted allele. Genotyping of the progeny failed to detect homozygous *UbC*^{GFP-puro} pups implying that *UbC* null embryos die *in utero* (Table I). To establish the timing of embryonic lethality, embryos were harvested at various stages of development and genotyped. Homozygous *UbC*^{GFP-puro} embryos were recovered at the expected Mendelian frequencies up to E11.5 but the fraction of viable homozygous embryos decreased at E12.5 and later ages (Table I). Correspondingly, the number of dead or partially resorbed embryos found *in utero* increased with embryonic age; the vast majority of these were homozygous for the *UbC*^{GFP-puro} allele.

The gross morphological appearance of viable homozygous *UbC*^{GFP-puro} embryos at E13.5 was generally similar to wild-type and heterozygous littermates (Figure 1C). They were pink in color and well vascularized but distinctly smaller in overall size. The only apparent morphological or histological difference was the drastically reduced size of the fetal liver mass evident at E13.5 (Figure 1C, arrow). Histological analysis (Figure 1D) confirmed that livers from E13.5 homozygous *UbC*^{GFP-puro} embryos were severely reduced in size compared to wild-type or heterozygous littermates. These livers were distinguished by reduced hepatic parenchyma and enlarged venous sinusoids (Figure 1D). Both hepatocytes (white arrow, lower) and hematopoietic

precursors (yellow arrow, upper) are evident in homozygous *UbC*^{GFP-puro} livers, although their numbers are reduced (Figure 1D). The hepatic phenotype will be described in greater detail in a forthcoming manuscript.

In principle, the midgestation lethality and liver development phenotypes observed in embryos homozygous for the disrupted *UbC* could be due to the loss of *UbC* coding sequence or to the presence of the GFP-puro selection cassette. To discriminate between these two possibilities, mice heterozygous for the *UbC*^{GFP-puro} allele were bred with mice expressing Zp3-Cre recombinase in order to excise the 'floxed' GFP-puro sequence and generate a true null allele. Although *UbC*^{+/-} progeny of these crosses were obtained at expected Mendelian frequencies, no viable *UbC*^{-/-} pups were obtained. E13.5 *UbC*^{-/-} embryos exhibited the same gross phenotypic characteristics as did those homozygous for the floxed *UbC*^{GFP-puro} allele; notably the smaller overall size and greatly diminished fetal liver mass (Figure 1E). We conclude that the major phenotypes resulting from homozygous *UbC* disruption are due to the loss of *UbC* coding sequence and not the presence of GFP-puro. Because the floxed GFP-puro selection cassette precisely replaces the only coding exon of the *UbC* gene, the recombinant allele, *UbC*^{GFP-puro} represents a complete null and will be referred to in this work as *UbC*^{-/-}. Moreover, the availability of GFP under the control of the murine *UbC* promoter provides a useful reporter of *UbC* transcriptional activity that will be exploited later.

Partial rescue of the *UbC*^{-/-} embryonic phenotype by ectopic expression of hemagglutinin-tagged Ub

To verify that the embryonic lethal phenotype observed in the *UbC* null mice was due to a deficiency in the cellular availability of Ub, we sought to introduce extra copies of Ub into the mouse genome. To generate mice that express epitope-tagged Ub, we employed a gene-targeting strategy that would allow us to incorporate a known number of hemagglutinin-tagged Ub (HA-Ub) copies transcribed under the control of the human *UbC* promoter to ensure ubiquitous expression (Figure 2A). HM-1 ES cells were targeted to reconstitute their disrupted *Hprt* locus (Bronson *et al*, 1996) and allow expression of the engineered HA-Ub gene. Subsequent to the HAT selection, ES cell colonies were screened by Southern blotting for the appropriate recombination (Figure 2B, left panel) and two clones were used for blastocyst injection. Three resulting chimeric mice were bred to C57BL/6J mice to obtain germline transmission.

Homozygous (+ / +) HA-Ub knock-in female mice were then bred to *UbC*^{+/-} males to generate *UbC*^{+/-}; HA-Ub mice (Figure 2C). Although interbreeding of *UbC*^{+/-}; HA-Ub mice did not result in any viable *UbC*^{-/-}; HA-Ub live pups, *UbC*^{-/-}; HA-Ub embryos were observed at expected Mendelian frequencies up to E15.5, and viable *UbC*^{-/-}; HA-Ub embryos were found even at E17.5 (Table II, Figure 2D). *UbC*^{-/-}; HA-Ub embryos appeared normal upon gross histological examination, although they were slightly smaller in overall size. Importantly, the *UbC*^{-/-}; HA-Ub embryos did not exhibit evidence of liver malformation (Figure 2E). These data indicate that both the midgestation embryonic lethality and the liver development phenotypes are partially rescued by providing extra genomic copies of Ub, suggesting that the *UbC* null phenotype is the direct consequence of a deficiency in the abundance or availability of Ub during fetal development.

Figure 1 Targeted disruption of *UbC* locus results in impaired fetal liver development. **(A)** Schematic representation of targeting strategy. From top to bottom: partial restriction map of *UbC* locus, targeting vector, genomic structure of disrupted allele before and after Cre recombination. The position of 5' probe and DTA probe for Southern blotting and the location of PCR primers used for screening homologous recombinants and genotyping are shown. The map is not drawn to scale. **(B)** Southern blot analysis of *SacI*-digested genomic DNA from ES cell clones. Upper left panel: 5' probe detected 9.7 and 5.9 kb fragments from wild-type (wt) and disrupted alleles, in homologous recombinants (hr), but only 9.7 kb fragment from wt allele in non-recombinant, wt ES cells. It also hybridized to linearized 6.8 kb targeting vector (+ vec). Upper right panel: DTA probe detected linearized 6.8 kb targeting vector (+ vec) and >3.9 kb fragment from non-homologous recombinants (nhr), but not from homologous recombinants (hr) or wt ES cells. Lower panel: PCR results for wt (+/+), heterozygous (+/-) and homozygous (-/-) knockout embryos at E13.5 (upper panel) and histology of sagittal embryonic sections stained with H&E (lower panel). Fetal liver is indicated by white arrow. Scale bar, 1 mm. **(C)** Morphology of wt (+/+), heterozygous (+/-) and homozygous (-/-) knockout embryos at E13.5 (upper panel) and histology of sagittal embryonic sections stained with H&E (lower panel). Fetal liver is indicated by white arrow. Scale bar, 1 mm. **(D)** Histology of sagittal embryonic (upper panel) and liver (lower panel) sections from E13.5 embryos. Note the reduced size of *UbC*^{-/-} embryonic liver (indicated with asterisk). Sections were stained with H&E. Hematopoietic precursors (yellow arrow, upper) are stained darkly. Hepatocytes (white arrow, lower) are stained lightly, whereas hematopoietic precursors (yellow arrow, upper) are stained darkly. Upper panel, scale bar, 1 mm; lower panel, scale bar, 50 μm. **(E)** Morphology of wt (+/+), heterozygous (+/-) and homozygous (-/-) knockout embryos at E13.5 with floxed GFP-puro cassette removed by Zp3-Cre recombinase. Fetal liver is indicated by white arrow. Scale bar, 1 mm.

Reduced proliferation and premature senescence in homozygous *UbC* null MEFs

To assess the cellular consequences of loss of the *UbC* gene, MEFs were isolated from E12.5 to E13.5 embryos. Even at early passages, the growth rates of *UbC*^{-/-} MEFs were distinctly reduced compared to MEFs from wild-type or

heterozygous mice (Figure 3A). Quantification of proliferation marker Ki-67 labeling of MEFs suggests that proliferation of *UbC*^{-/-} MEFs was significantly reduced compared to *UbC*^{+/+} MEFs (Figure 3B and C). In addition, *UbC*^{-/-} MEFs exhibited increased senescence-associated β-galactosidase (SA-β-gal) staining (Figure 3B) and enlarged and flat-

Table 1 Embryonic lethality in *UbC*^{-/-} mice

Age	Litters	<i>UbC</i> ^{+/+}	<i>UbC</i> ^{+/-}	<i>UbC</i> ^{-/-}	Dead	Total
E9.5–11.5	4	10	19	7	0	36
E12.5	17	46	66	20	21	153
E13.5	26	21	51	11	21	104
E14.5–15.5	7	18	29	2	13	62
E16.5–17.5	5	9	21	0	8	38
E18.5	2	3	9	0	1	13
Birth	52	95	196	0	0	291

Embryos were dissected at the indicated time of gestation and examined heartbeat for viability. Genotypes were determined by PCR using genomic DNA isolated from embryos. E: embryonic day.

tened morphology (data not shown) implying that homozygous loss of *UbC* leads to premature senescence (Goldstein, 1990; Dimri *et al*, 1995). Neither the proliferation defect (Figure 3A) nor the premature senescence (data not shown) phenotypes were evident in *UbC*^{-/-}; HA-*Ub* MEFs indicating that these phenotypes are the direct consequence of Ub deficiency.

To assess endogenous *UbC* transcriptional activity, we took advantage of the GFP-puro coding sequence that precisely replaces the polyubiquitin coding exon in the *UbC* locus (Figure 1A). GFP-puro fluorescence was significantly higher in *UbC*^{+/-} MEFs compared to wild-type MEFs but was roughly half that of *UbC*^{-/-} MEFs, indicating that steady-state GFP-puro fluorescence correlates well with *UbC*^{GFP-puro} copy number (Figure 3D). We found that GFP-puro fluorescence was slightly but not significantly decreased in *UbC*^{-/-}; HA-*Ub* compared to *UbC*^{-/-} MEFs, suggesting that the additional HA-*Ub* in these cells is not able to significantly repress basal *UbC* transcription. Strikingly, we found that GFP-puro fluorescence increased about three-fold as MEFs were maintained through multiple passages in culture (Figure 3E). This increase was not mitigated by the presence of HA-*Ub* nor was it exacerbated by the loss of the second *UbC* allele, suggesting that transcription of the endogenous *UbC* promoter increases as cells senesce.

Delayed mitotic entry in *UbC*^{-/-} MEFs

To understand the basis of the reduced proliferative capacity observed in *UbC*^{-/-} MEFs, we used flow cytometry to analyze cell-cycle progression (Figure 4). Unsynchronized early-passage *UbC*^{-/-} MEFs displayed a reduced fraction of cells with 2n and an increased fraction with 4n DNA content when compared to wild-type MEFs (Figure 4A), suggesting that *UbC* deletion reduces the length of the G1 phase of the cell cycle with a corresponding increase in the length of G2/M. Correspondingly, there was no difference in the fraction of *UbC*^{-/-} MEFs labeled with an antibody to phospho-histone H3, a marker for M phase, compared with *UbC*^{+/+} or *UbC*^{+/-} MEFs (data not shown), suggesting that the increased fraction of cells at G2/M is likely due to delayed mitotic entry. The fraction of cells with less than 2n DNA content, presumably apoptotic, was significantly increased in *UbC*^{-/-} MEFs (Figure 4A), although this increase does not fully account for the reduced G1 population in *UbC*^{-/-} MEFs (Figure 4A; 3% increase in apoptotic, but 9% reduction in G1). All of the cell-cycle progression phenotypes observed in *UbC*^{-/-} MEFs were fully rescued by coexpression of HA-*Ub*, suggesting that they are likely the consequence of Ub deficiency.

Analysis of GFP-puro fluorescence in *UbC*^{+/-} and *UbC*^{-/-} MEFs by flow cytometry permitted correlation of *UbC* transcriptional activity with cell-cycle progression (Figure 4B–D). GFP-puro levels in cells with 2n DNA content (G1) were comparable to those of the bulk population, with the fluorescence of *UbC*^{-/-} roughly twice that of *UbC*^{+/-} MEFs (Figure 4C). GFP-puro fluorescence was elevated ~2-fold relative to the G1 level in MEFs of all genotypes with 4n DNA content, suggesting that *UbC* transcription is increased in G2 (Figure 4C). Finally, we observed that GFP-puro fluorescence was elevated in *UbC*^{-/-} but not *UbC*^{+/-} MEFs in S phase more than two-fold above the levels in the same cell population in G1 phase (Figure 4D), suggesting that *UbC*^{-/-} MEFs respond to reduced Ub levels by attempting to increase *UbC* transcription in S phase.

Unequal contribution of Ub genes to cellular Ub pools

To directly determine whether the phenotypes arising from homozygous loss of *UbC* are due to reduced levels of Ub, we measured the levels of cellular Ub and mRNA transcripts of the four Ub genes in MEFs (Figure 5). Cellular Ub is partitioned between pools of free Ub monomer and covalent Ub-protein conjugates. Because there are no reagents to accurately determine the concentrations of either of these two forms of Ub without interference with the other, we used a Ub-specific protease (Usp2-cc) to convert all Ub conjugates to Ub monomer and an indirect competitive ELISA method to measure the concentration of Ub monomer in MEFs of the different genotypes (Figure 5A, left panel). This approach, which provides an accurate measurement of total cellular Ub content (Ryu *et al*, 2006), reveals that steady-state Ub levels were reduced by 40% in *UbC*^{-/-} MEFs compared to wild-type, which was fully restored to wild-type levels with coexpression of HA-*Ub*. This rescue can be accounted for by the contribution of HA-*Ub*, and is thus unlikely to be due to increased transcription of other Ub genes (Figure 5A, right panel). Thus, *UbC* contributes significantly to the maintenance of steady-state total Ub levels in cultured early-passage MEFs.

A central role of *UbC* in maintenance of steady-state Ub levels in MEFs was also supported by real-time quantitative RT-PCR analysis of Ub transcripts (Figure 5B). *UbC* transcript levels were reduced by approximately half in *UbC*^{+/-} MEFs and, as expected, were undetectable in *UbC*^{-/-} cells. Although no significant changes in the levels of *UbA* transcripts were observed in MEFs harboring one or two disrupted *UbC* alleles, we observed a significant increase of transcripts from the *UbB* locus in *UbC*^{-/-} cells. Evidently, this increase was not enough to compensate for the loss of *UbC*

Figure 2 Partial rescue of *UbC*^{-/-} phenotypes by ectopically expressed HA-Ub. **(A)** Targeting strategy. From top to bottom: wild-type mouse *Hprt* locus (*Hprt* wt), disrupted *Hprt* locus from HM-1 cells (HM-1) and targeted *Hprt* locus (HA-Ub). Sites recognized by the *Stul* restriction enzyme are indicated. The map is not drawn to scale. **(B)** Southern blot analysis and genotyping by PCR. Left panel: Southern blot analysis after digestion of genomic ES cell DNA with *Stul* restriction enzyme is shown. DNA was used from wild-type ES cells (*Hprt* wt), disrupted *Hprt* containing HM-1 ES cell (HM-1) and a targeted ES cell clone (HA-Ub) that was used for subsequent injections. Right panel: PCR results for C57BL/6J (B6), hemizygous wild-type (-/+) or HA-Ub knock-in (+/+) male mice, and heterozygous (+/-) or homozygous (+/+) HA-Ub knock-in female mice are displayed. **(C)** Breeding strategy to generate *UbC*^{-/-}; HA-Ub mice. Owing to random inactivation of X chromosome in females, rescued phenotype is expected from *UbC*^{-/-}; HA-Ub^{+/+} male or *UbC*^{-/-}; HA-Ub^{+/+} female mice. Both genotypes will be referred to in this work simply as *UbC*^{-/-}; HA-Ub when the gender does not need to be defined. X^{Ub}X^{Ub} = HA-Ub^{+/+} female; X^{Ub}X = HA-Ub^{+/+} female; X^{Ub}Y = HA-Ub^{+/+} male; XY = HA-Ub^{+/+} male. **(D)** Morphology of *UbC*^{+/+}; HA-Ub (+/+, +Ub) and *UbC*^{-/-}; HA-Ub (-/-, +Ub) embryos at E15.5 and E17.5. Scale bar, 2 mm. **(E)** Histology of sagittal liver sections from E15.5 and E17.5 embryos. Liver sections were stained with H&E. Note that no developmental defects are found in *UbC*^{-/-} livers with ectopic expression of HA-Ub. Upper panel, scale bar, 1 mm; lower panel, scale bar, 200 μm.

Table II Effect of HA-Ub on embryonic lethality in *UbC*^{-/-} mice

Age	Litters	<i>UbC</i> ^{+/+} ; HA-Ub	<i>UbC</i> ^{+/-} ; HA-Ub	<i>UbC</i> ^{-/-} ; HA-Ub	Dead	Total
E12.5–13.5	2	6	10	3	0	19
E14.5–15.5	4	8	21	11	0	40
E16.5–17.5	2	2	8	4	3	17
E18.5	1	2	6	0	3	11
Birth	23	58	96	0	0	154

Figure 3 Reduced proliferation and premature senescence of *UbC*^{-/-} MEFs. (A) Proliferation curve of *UbC*^{+/+} (+/+), *UbC*^{+/-} (+/-) and *UbC*^{-/-} (-/-) MEFs with (+Ub) or without ectopic expression of HA-Ub. A total of 2.5×10^4 cells were plated on 12-well plate and counted over a 6-day period. Data are expressed as mean ± s.e.m. from 4–10 MEFs originated from different embryos per genotype. (B, C) *UbC*^{+/+} (+/+) and *UbC*^{-/-} (-/-) MEFs stained with Ki-67 and SA-β-gal. Representative data from passage=5 are shown. Ki-67 positive cells (%) were counted in three MEFs originated from different embryos per genotype. At least four different fields were counted in each MEFs. Scale bar, 100 μm, **P<0.01. (D) GFP fluorescence as an indicator of transcriptional activity of *UbC* gene. Mean GFP fluorescence from population of *UbC*^{+/+} (+/+), *UbC*^{-/-} (-/-) and *UbC*^{-/-}; HA-Ub (-/-, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. (E) Increase of GFP fluorescence in MEFs with passage number. Mean GFP fluorescence from population of *UbC*^{+/+} (+/+), *UbC*^{-/-} (-/-), *UbC*^{+/-}; HA-Ub (+/-, +Ub) and *UbC*^{-/-}; HA-Ub (-/-, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs at each passage. Data are from two MEFs originated from different embryos per genotype at different passage number.

gene due to the low abundance of *UbB* gene in MEFs (Figure 5B). The central role of *UbC* as a source of Ub in MEFs is even more evident when the fact that the polyubi-

quitin genes *UbB* and *UbC* encode, 4 and 9 Ub moieties per transcript, respectively—as opposed to only one for *UbA*⁻ is considered (Figure 5C).

Figure 4 Cell-cycle defect in *UbC*^{-/-} MEFs and its rescue by ectopically expressed HA-Ub. (A) Increased apoptosis, reduced G1 and increased G2/M boundaries in *UbC*^{-/-} (-/-) MEFs (passage = 3) that can be rescued by ectopic expression of HA-Ub in *UbC*^{-/-}; HA-*Ub* (-/-, +Ub) MEFs. Representative cell-cycle profile analyzed by flow cytometry is shown. [#]*P*<0.1 versus wild-type (+/+) MEFs, ^{**}*P*<0.01 versus wild-type (+/+) MEFs. (B) GFP fluorescence of *UbC*^{+/+} (+/+), *UbC*^{+/-} (+/-) and *UbC*^{-/-} (-/-) MEFs as a function of DNA content. GFP fluorescence in cells with 4n DNA content (G2/M) is higher than with 2n DNA content (G1). (C) GFP fluorescence in each phase of cell cycle in MEFs. Mean GFP fluorescence from population of *UbC*^{+/+} (+/+), *UbC*^{-/-} (-/-) and *UbC*^{-/-}; HA-*Ub* (-/-, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. (D) GFP fluorescence in S and G2/M phase is expressed relative to G1 phase. ^{*}*P*<0.05.

Increased sensitivity of *UbC*^{-/-} MEFs to cellular stress

Our data demonstrate that *UbC* contributes importantly to the maintenance of steady-state Ub pools and that *UbC* transcription, as monitored by GFP-puro fluorescence, strongly correlates with the senescence of MEFs, suggesting that *UbC*

transcription is responsive to cellular stress. To determine the extent to which the *UbC* gene contributes to stress tolerance, we investigated whether *UbC*^{-/-} MEFs show increased sensitivity to heat shock (Figure 6A and B). The promoter region of the mammalian *UbC* gene contains a heat-shock

Figure 5 Disruption of *UbC* gene results in reduced cellular Ub content. (A) Indirect competitive ELISA for total Ub (left panel) or HA-Ub (right panel) levels in MEFs. Total cell lysates from *UbC*^{+/+} (+/+), *UbC*^{+/-} (+/-), *UbC*^{-/-} (-/-) and *UbC*^{-/-}; HA-Ub (-/-, +Ub) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean ± s.e.m. from four MEFs originated from different embryos per genotype with triplicate experiments. **P* < 0.05 versus wild-type (+/+) MEFs. (B) Various Ub transcript levels in MEFs. Total RNA was isolated from *UbC*^{+/+} (+/+), *UbC*^{+/-} (+/-) and *UbC*^{-/-} (-/-) MEFs and UbC, UbB, UbA52 and UbA80 mRNA levels were measured by quantitative real-time RT-PCR and normalized to 18S rRNA levels. Data are expressed as mean ± s.e.m. from five MEFs originated from different embryos per genotype. **P* < 0.05 versus the corresponding transcript levels in wild-type (+/+) MEFs. (C) Contribution of Ub genes to total Ub levels. Ub transcript levels in MEFs shown in (B) are normalized by the number of Ub moieties that each Ub transcript generates.

element, and previous studies have shown that UbC transcript levels are increased in response to thermal stress (Fornace *et al*, 1989; Sherlock *et al*, 2001; Murray *et al*, 2004). Exposure of MEFs to mild heat stress (43°C for 30 min) induced heat-shock protein 70 (Hsp70) in both wild-type and *UbC*^{-/-} MEFs to a similar extent (Figure 6A), indicating that the *UbC* gene is not required for activating the heat-shock response. This mild stress, which preconditions cells to survive a subsequent lethal heat shock (45°C) (McMillan *et al*, 1998), resulted in a negligible reduction of viability in MEFs of both genotypes (Figure 6B). Exposure of MEFs to lethal heat shock for 15 or 45 min, without preconditioning, led to a significant reduction in viability that was more pronounced in cells lacking *UbC*. Viability of MEFs of both genotypes was dramatically improved by preconditioning, consistent with our finding (Figure 6A) that cells of both genotypes were able to induce Hsp70 levels to a similar extent following a mild heat stress. However, the extent to which *UbC*^{-/-} MEFs were rescued by preconditioning was significantly reduced compared to wild-type MEFs (Figure 6B). Thus, loss of *UbC* leads to increased sensitivity to lethal heat shock and impairs the acquisition of thermotolerance.

To impose a stress more directly related to the function of the Ub proteasome system (UPS), we investigated the effects of proteasome inhibitors on *UbC*^{-/-} MEFs. In contrast to thermal stress, which activates only heat-shock transcription factor 1 (HSF1) expression (Pirkkala *et al*, 2000), proteasome

inhibitors activate a more extensive heat-shock response that includes activation of HSF2 (Mathew *et al*, 1998). We found that *UbC*^{-/-} MEFs were killed by significantly lower concentrations of ALLN (Figure 6C) or MG132 (data not shown) than were MEFs heterozygous for the *UbC* knockout. ALLN treatment also decreased the fraction of cycling MEFs and increased the fraction of cells with sub-G1 DNA content to a far greater extent in *UbC*^{-/-} MEFs than in wild-type cells (Figure 6D). This effect was not reversed in the HA-Ub background, suggesting that, although the extra copies of Ub provided by this locus were able to rescue most of the observed *UbC*^{-/-} phenotypes, they were not sufficient to compensate for the stress of proteasome inhibitor treatment. Together, these data establish that the *UbC* gene contributes to the response of cells to cellular stress and to thermotolerance.

Activation of Ub gene expression by cellular stress

To assess the contribution of *UbC* to the maintenance of cellular Ub levels under stress, we exploited GFP-puro fluorescence to monitor the effect of heat shock on *UbC* gene expression (Figure 7A). GFP-puro fluorescence in viable *UbC*^{-/-} MEFs was significantly increased ~1.6-fold by even a mild preconditioning heat shock and was not further activated by a lethal heat shock whether or not it was preceded by preconditioning (Figure 7A). It is currently unclear why GFP-puro fluorescence was significantly reduced in viable *UbC*^{-/-} MEFs, following a 45 min lethal heat shock without preconditioning.

Figure 6 Enhanced sensitivity of *UbC*^{-/-} MEFs to heat stress and proteasome inhibition. **(A)** Immunoblot analysis for inducible Hsp70 expression in MEFs before and after exposure to mild heat shock (HS) at 43°C for 30 min and recovery at 37°C for 6 h. Total cell lysates from *UbC*^{+/+} (+/+) and *UbC*^{-/-} (-/-) MEFs (20 μg) were subjected to SDS-PAGE followed by immunodetection with anti-Hsp70 antibody. α-tubulin was used as a loading control. **(B)** Viability of MEFs upon heat stress. *UbC*^{+/+} (+/+) and *UbC*^{-/-} (-/-) MEFs were exposed to heat stress as described in Materials and methods. Preconditioned (PC+) or non-preconditioned (PC-) MEFs were exposed to lethal HS at 45°C for the time as indicated and recovered at 37°C for 24 h. After recovery, the percent of viable cell populations (both adherent and floating) was determined by propidium iodide (PI) staining. Data are expressed as mean ± s.e.m. from 6–7 MEFs originated from different embryos per genotype. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus wild-type (+/+) MEFs with no treatment (PC-, HS 0 min), **P* < 0.05 versus wild-type (+/+) MEFs with the same treatment. **(C)** MTT cell viability assay for MEFs. *UbC*^{+/+} (+/+) or *UbC*^{-/-} (-/-) MEFs were treated with indicated concentration of ALLN for 24 h. At the end of incubation, viability was assessed by the percentage of MTT conversion. Data are expressed as mean ± s.e.m. from three MEFs originated from different embryos per genotype. **P* < 0.05 versus *UbC*^{+/+} (+/+) MEFs. **(D)** Cell-cycle abnormality in *UbC*^{-/-} (-/-) MEFs (passage = 3) and failure of its rescue by ectopic expression of HA-Ub in *UbC*^{-/-}; HA-Ub (-/-, +Ub) MEFs in the presence of proteasome inhibition. MEFs were exposed to 10 μg/ml ALLN (+ ALLN) for 24 h and subjected to cell-cycle analysis by flow cytometry. Representative cell-cycle profile is shown. **P* < 0.05 versus wild-type (+/+) MEFs/+ ALLN.

Figure 7 Effect of heat shock and proteasome inhibitor on *UbC* gene expression and cellular Ub content. (A) Change of GFP fluorescence in *UbC*^{-/-} (−/−) MEFs upon heat stress. MEFs were exposed to heat stress as indicated and mean GFP fluorescence from population of viable (PI-negative) cells was measured. Change of GFP fluorescence relative to no treatment was calculated in each MEFs and expressed as mean ± s.e.m. from six MEFs originated from different embryos. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus MEFs with no treatment. (B) Indirect competitive ELISA for total Ub levels in MEFs before and after exposure to mild heat shock at 43°C for 30 min and recovery at 37°C for 6 h. Total cell lysates from *UbC*^{+/+} (+/+) and *UbC*^{-/-} (−/−) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean ± s.e.m. from 5–6 MEFs originated from different embryos with triplicate experiments. ***P* < 0.01 versus wild-type (+/+) MEFs/no treatment. (C) Immunoblot analysis of ubiquitinated proteins in MEFs exposed to DMSO (−ALLN) or 10 μg/ml ALLN (+ALLN) for 24 h. Total cell lysates from *UbC*^{+/+} (+/+) , *UbC*^{+/-} (+/-) and *UbC*^{-/-} (−/−) MEFs (50 μg) were subjected to SDS-PAGE followed by immunodetection with anti-Ub antibody. β-actin was used as a loading control. Ub_n, ubiquitin conjugates; uH2A, mono-ubiquitinated histone 2A; Ub₁, ubiquitin monomer. (D) Indirect competitive ELISA for total Ub levels in MEFs exposed to DMSO (−ALLN) or 10 μg/ml ALLN (+ALLN) for 24 h. Total cell lysates from *UbC*^{+/+} (+/+) , *UbC*^{+/-} (+/-) , *UbC*^{-/-} (−/−) and *UbC*^{-/-}; HA-Ub (−/−, +Ub) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean ± s.e.m. from four MEFs originated from different embryos per genotype with triplicate experiments. #*P* < 0.05 versus wild-type (+/+) MEFs/−ALLN, **P* < 0.05 versus −ALLN, ***P* < 0.01 versus −ALLN. (E) Transcriptional activation of *UbC* gene under proteasome inhibition as monitored by the increase of GFP fluorescence. GFP fluorescence of *UbC*^{+/+} (+/+) , *UbC*^{-/-} (−/−) and *UbC*^{-/-}; HA-Ub (−/−, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. **P* < 0.05 versus −ALLN.

tioning. Consistent with this increase in *UbC* transcriptional activity and the dominant contribution of *UbC* to the maintenance of Ub pools in MEFs (see Figure 5C), we observed that total Ub levels in wild-type MEFs were significantly increased following a preconditioning heat shock (Figure 7B). Strikingly, heat shock failed to upregulate the already-diminished basal levels of Ub in *UbC*^{-/-} MEFs. Thus, *UbC* activation and the consequent ~1.4-fold increase in total Ub levels is an important contributor to thermotolerance.

By contrast to heat shock, exposure to proteasome inhibitor led to a far larger (~4-fold) increase in total Ub in wild-type MEFs (Figure 7D), which included a substantial increase

in the level of Ub conjugates, as revealed by immunoblotting (Figure 7C). The response of *UbC*^{-/-} MEFs to proteasome inhibitors was severely blunted compared to wild-type MEFs (Figure 7D) with a drastically diminished abundance of Ub conjugates (Figure 7C). Although coexpression of HA-Ub increased steady-state Ub levels in untreated *UbC*^{-/-} MEFs, total Ub levels in *UbC*^{-/-}; HA-Ub MEFs were not increased significantly in response to ALLN (Figure 7D), indicating that the minimal human *UbC* promoter, which drives HA-Ub expression in this strain, is not properly regulated in this ectopic locus. This result may explain the inability of the additional HA-Ub to rescue the cell-cycle abnormality in

Figure 8 Effect of proteasome inhibitor on Ub gene expression. (A) Various Ub transcript levels in MEFs after exposure to DMSO (–ALLN) or 10 μ g/ml ALLN for 24 h (+ ALLN). Total RNA was isolated from *UbC*^{+/+} (+/+), *UbC*^{+/-} (+/-) and *UbC*^{-/-} (-/-) MEFs with or without proteasome inhibition and UbC, UbB, Uba52 and Uba80 mRNA levels were measured by quantitative real-time RT-PCR and normalized to 18S rRNA levels. mRNA levels of wild-type (+/+) MEFs without proteasome inhibition (–ALLN) were arbitrarily assigned as 1 for each transcript. Data are expressed as mean $\pm$ s.e.m. from five MEFs originated from different embryos per genotype. **P* < 0.05 versus wild-type (+/+) MEFs/–ALLN, **P* < 0.05 versus –ALLN, ****P* < 0.001 versus –ALLN. (B) Contribution of Ub genes to total Ub levels with or without proteasome inhibition. Ub transcript levels in MEFs shown in (B) are normalized by the number of Ub moieties that each Ub transcript generates.

UbC^{-/-}; HA-Ub MEFs exposed to proteasome inhibitor (see Figure 6D). The increase in Ub levels in wild-type and *UbC*^{+/-} MEFs exposed to ALLN correlated well with activation of the *UbC* promoter, assessed by GFP-puro fluorescence (Figure 7E). Interestingly, despite the blunted response to proteasome inhibitors of *UbC*^{-/-} MEFs, the nearly two-fold increase in Ub levels suggests that other Ub genes, in addition to *UbC*, might be activated in response to severe stress. Indeed, the increase in total Ub in response to ALLN in wild-type MEFs can be accounted for by increased transcription of all four Ub genes (Figure 8A), although the absolute contribution of *UbC*, normalized for its 9 Ub coding units, is by far the dominant one (Figure 8B). Even though UbB transcript levels are increased dramatically in response to proteasome inhibition, UbB's contribution to total Ub pools in these cells is evidently insufficient to compensate for the loss of *UbC*.

Discussion

The embryonic lethality observed in *UbC*^{-/-} mice establishes an essential role for polyubiquitin gene expression during embryonic development. As the ultimate product of all four

mammalian Ub genes is chemically identical irrespective of its genetic provenance, the simplest explanation for our findings is that all of the observed phenotypes are the consequence of Ub deficiency. This conclusion is strongly supported by the demonstration of reduced steady-state Ub levels in *UbC*^{-/-} MEFs, and by the extensive, if incomplete, rescue of multiple *UbC*^{-/-} phenotypes by expression of six copies of HA-Ub from the *Hprt* locus.

The dramatic impairment of hepatogenesis is the most likely explanation for the midgestation embryonic lethality observed in *UbC*^{-/-} mice. This hypothesis is supported by the finding that HA-Ub largely, if not completely, rescues the hepatogenesis defect in *UbC*^{-/-} embryos and delays embryonic death. The fetal liver is the primary site of definitive hematopoiesis in midgestation mouse embryos. Not surprisingly, genetic disruption of a large and diverse array of genes that control both hematopoiesis and hepatogenesis lead to fetal liver hypoplasia and embryonic death at around E12.5–E15.5 (Dzierzak and Medvinsky, 1995; Zaret, 1998). Between E12.5 and E15.5, the developing liver undergoes massive expansion, primarily to support the developing fetal blood supply (Zaret, 1998). The well-established essential role for Ub-dependent proteolysis in the eukaryotic cell cycle, and our

observation that decreased Ub levels delay mitotic progression in *UbC*^{-/-} MEFs, lead us to speculate that the midgestation lethality observed in *UbC*^{-/-} embryos may be due to a failure to meet the demand for Ub during this period of high mitotic activity.

There are several possible explanations for why the lethality of *UbC*^{-/-} embryos is only partially rescued by HA-Ub. First, the HA-Ub minigene contains 30% fewer Ub moieties per transcript than endogenous *UbC* (6 in the *Hprt*-linked allele versus 9 in the endogenous allele). Therefore, even if transcriptional activity was the same as that from the wild-type locus, the amount of Ub produced from this locus will be less than from endogenous *UbC*. This decreased Ub potential is further exacerbated by the fact that only a single rescued allele is expressed from the X-linked *Hprt* locus. A second concern is that the presence of the HA-epitope tag in the ectopic allele could partially interfere with Ub function by influencing either the stability of the mRNA or protein products, or the susceptibility of conjugates to Ub isopeptidases or processing enzymes (Ellison and Hochstrasser, 1991). Third, it is possible that the strength of the minimal human *UbC* promoter used to drive this construct may be reduced when expressed ectopically. It is unlikely that this ectopically expressed, minimal promoter is able to fully recapitulate the regulatory features of the endogenous *UbC* allele. This possibility is supported by our observation that, while HA-Ub can fully suppress the growth and mitotic phenotypes of unstressed *UbC*^{-/-} MEFs, it fails to completely rescue the defects in MEFs stressed by exposure to proteasome inhibitor. Although each of these possibilities could contribute to the partial rescue, there is no simple way to discriminate among them.

The genomic architecture of Ub genes is highly conserved throughout the eukaryotic domain; all organisms contain at least two genes encoding Ub fusions to small C-terminal extensions and at least one polyubiquitin gene. The *UbA*-type genes (in yeast, *UBI1–3*) encode fusions to small proteins that are components of the large and small ribosomal subunits, underscoring the profoundly conserved linkage between the protein synthesis and protein degradation sys-

tems (Finley *et al*, 1989). In yeast, these genes provide sufficient Ub for vegetative growth but the single polyubiquitin gene, *UBI4*, is essential for survival of most types of cellular stress (Finley *et al*, 1987). Regulation of the two polyubiquitin genes in mammals is less well understood. Like the single *UBI4* polyubiquitin gene in yeast, both *UbB* and *UbC* promoters contain heat-shock elements and their expression is increased in mammalian cells subjected to various types of stress including proapoptotic stimuli (Kugawa and Aoki, 2004), tumor promoters (Finch *et al*, 1992), oxidative stressors and heat shock (Fornace *et al*, 1989; Murray *et al*, 2004). The minimal *UbC* promoter has been widely used to drive transgene expression in mice because of its robust expression in most mouse tissues (Schorpp *et al*, 1996). Unless this gene is strongly repressed in its normal chromosomal locus, it is probable that *UbC*, unlike the prototypical *UBI4* gene in yeast, contributes to maintenance of Ub pools in unstressed cells as well. Indeed, we find that the *UbC* gene contributes a substantial fraction of the Ub transcriptome in most mouse tissues, with liver being notably the highest (Figure 9A). Interestingly, the tissue expression pattern of *UbC* appears to be roughly complementary to that of *UbB* suggesting, consistent with the conclusion, that these two polyubiquitin genes perform non-redundant functions (Figure 9B). Indeed, our data indicate that the loss of *UbC* cannot be compensated by increased transcription of the *UbA* or the *UbB* genes.

Our data establish that *UbC* contributes an especially large proportion of Ub in MEFs, consistent with the observation of growth defects in *UbC*^{-/-} cells. The slow growth phenotype, owing to diminished levels of Ub in cells lacking *UbC*, appears to be due to impaired cell-cycle progression. The UPS plays a well-documented central role in the cell cycle, ensuring the unidirectionality of progression by degrading key modulators including cyclins and cyclin-dependent kinases (Hershko, 2005). The anomalous cell-cycle progression observed in *UbC*^{-/-} MEFs, and its rescue by HA-Ub suggests that the reduced Ub capacity resulting from loss of *UbC* is insufficient for some critical Ub-dependent processes required for mitotic entry, reminiscent of the effects of

Figure 9 Relative contribution of Ub genes to total Ub levels in various mouse tissues. Total RNA was isolated from various tissues in 5-month-old wild-type mice ($n = 7$ for testis; $n = 3$ for all other tissues). *UbC*, *UbB*, *UbA52* and *UbA80* mRNA levels were measured by quantitative real-time RT-PCR and contribution of *UbC* (A) or four different Ub genes (B) to total Ub levels are shown after normalization by the number of Ub moieties that each Ub transcript generates. Data are expressed as mean $\pm$ s.e.m. from the indicated number of tissues.

proteasome inhibitors. Perhaps the principal antiproliferative effect of these drugs, which are increasingly being used as antitumor agents (Teicher *et al*, 1999; Roccaro *et al*, 2006), is through their profound ability to reduce pools of free Ub. The availability of mice defective in *UbC* gene expression and containing a *UbC*-regulated GFP reporter should be an excellent resource to facilitate our understanding of the mechanism of regulation of the Ub system.

Our data also establish that, Ub contributed by *UbC* is important to the ability of MEFs to survive acute lethal heat shock and that upregulation of this gene by the heat shock response contributes to thermotolerance. *UbC* also contributes to the ability of MEFs to survive a challenge with proteasome inhibitors, although the extent to which cellular Ub levels increase in response to the former insult is far more modest (~1.6-fold) than to the latter (~4-fold). This discrepancy cannot be accounted for simply by differences in the strength of the *UbC* transcriptional response (compare Figure 7A and E), which is activated to similar levels by heat shock and proteasome inhibition. Indeed, our data suggest the surprising conclusion that all four of the Ub genes are activated by exposure of MEFs to proteasome inhibitors.

Ub is required for a remarkably diverse set of fundamental cellular processes. Maintenance of an adequate supply of Ub in response to physiological demand is critical for cellular function and survival. Our data suggest that *UbC* constitutes an important, but not sufficient source of Ub during both stressed and unstressed conditions. Further research is needed to understand how Ub levels are sensed within the cell and how the different Ub genes are regulated in order to be able to survive the stress of protein folding.

Materials and methods

Details of construction and husbandry of mouse lines, as well as routine analytical procedures are provided in online Supplementary Information.

Cell-cycle analysis

Asynchronously growing MEFs were trypsinized and fixed in 70% ethanol for 24 h at -20°C, resuspended in PBS (1 × 10⁶ cells/ml) and

treated with RNase A (100 µg/ml) for 30 min at 37°C. Propidium iodide was added to a final concentration of 50 µg/ml and analyzed by FACSCalibur (BD).

Cell viability assay

MEFs were treated with indicated concentration of ALLN for 24 h. At the end of incubation, medium was removed and replaced with MTT working solution (500 µg/ml) and incubated for 4 h at 37°C. The converted dye was solubilized with acidic isopropanol and absorbance of the converted dye was measured at 570 nm with background subtraction at 650 nm.

Heat shock of MEFs

Heat shock of MEFs was performed essentially as described previously (McMillan *et al*, 1998). MEFs (3 × 10⁵ cells) were plated out to 25-cm² flask 36 h before the heat shock experiment. Heat stress was induced by completely submerging flask in the temperature-controlled water bath. To induce thermotolerance, MEFs were preconditioned with mild heat shock at 43°C for 30 min and recovered at 37°C for 6 h. MEFs were then lethally heat-shocked at 45°C for 45 min, followed by recovery at 37°C for 24 h. Alternatively, MEFs were lethally heat-shocked at 45°C for 15 or 45 min without preconditioning and recovered at 37°C for 24 h. MEFs exposed to heat stress (both adherent and floating cells) were trypsinized and resuspended in PBS containing propidium iodide (1 µg/ml) and analyzed by FACSCalibur (BD) to determine the viability of cells.

For immunoblot analysis, preconditioned MEFs were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, with protease inhibitor cocktail from Roche) and total cell lysates were prepared as described in Supplementary data. Total cell lysates (20 µg) were subjected to SDS-PAGE followed by immunoblot detection with monoclonal anti-Hsp70 antibody (SPA-810; Stressgen). Indirect competitive ELISA was performed as described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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