Dynamic transcription of ubiquitin genes under basal and stressful conditions and new insights into the multiple *UBC* transcript variants

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Abstract

Ubiquitin (Ub) is a small 76-amino acid protein that is engaged in many different pathways within the cell, including protein turnover. During proteotoxic stress, when the demand of clearing damaged/misfolded proteins strongly increases, cells activate Ub gene transcription to face the need of extra ubiquitin. This paper shows the contribution of the four ubiquitin coding genes (*UBB*, *UBC*, *UBA52*, *RPS27A*) to the ubiquitin RNA pool under basal and stressful conditions. Our results reveal that *UBC* and *RPS27A* represent the major fraction of the Ub transcriptome in different cell lines, but when converted to the coding potential, polyubiquitin genes *UBC* and *UBB* mainly contribute to determine the intracellular ubiquitin content under basal conditions. Both the polyubiquitin genes *UBB* and *UBC* are upregulated upon proteasome inhibition and oxidative stress, with markedly higher responses from the *UBC* promoter. A similar output, with lower fold-inductions, is detected in heat-stressed cells, with *UBC* acting as the main contributor to thermotolerance. By contrast, upon these stressors, the levels of *UBA52* and *RPS27A* mRNAs remain unchanged. Remarkably, UV irradiation fails to induce Ub genes transcription, but rather seems to act at the post-transcriptional level, by stabilizing ubiquitin mRNAs at UV doses which induce rapid degradation of other RNA molecules. Moreover, the evidence that the *UBC* core promoter contains multiple transcription start sites and their responsiveness to stress, are here reported for the first time.

1. Introduction

Ubiquitin (Ub) is a versatile 76 amino acid protein that plays important roles inside cells. First characterized for tagging proteins to degradation by the proteasome [1], it is now known to be also involved in processes as varied as signal transduction, endocytosis, transcription and DNA repair [2]. To perform its functions, Ub is linked to target substrates through an isopeptide linkage formed by a three-step enzymatic reaction: activation-conjugation-ligation, catalyzed by E1, E2 and E3 enzymes, respectively [3]. This post-translational modification can lead to a monoubiquitination or, reiterating the conjugation process with ubiquitin internal lysines, polyubiquitination of the target substrates. Therefore, ubiquitin exists inside cells as free Ub (readily available for ubiquitination), conjugated Ub (covalently attached to substrates) and free unanchored Ub chains. These forms are in dynamic equilibrium, since conjugation and de-conjugation are continuously ongoing processes.

Like other post-translational modifications, ubiquitination is indeed reversible. A class of enzymes is in charge of this process: deubiquitinating enzymes (DUBs) recycle ubiquitin chains in order to maintain free ubiquitin pool [4, 5].

Although ubiquitin is an abundant protein, representing up to 5% of total proteins, the pool of free unconjugated ubiquitin is surprisingly small: this means that, despite its pervasive roles in many cell functions, Ub is not produced in excess. Free ubiquitin levels in a cell are maintained by recycling Ub from its target substrates, when its function is completed, by the rates of Ub degradation and by transcriptional control at four different genetic loci [6, 7]. De novo ubiquitin synthesis is achieved in humans thanks to four genes: monomeric Ub-ribosomal fusion genes, *UBA52* and *RPS27A*, that encode one Ub unit fused to a ribosomal protein, and polyubiquitin genes, *UBB* and *UBC*, which harbor 3-4 and 9-10 tandem repeats of Ub coding units, respectively [8-10]. While the Ub-ribosomal fusion genes (also referred to as *UBA* genes) are constitutively expressed and contribute to fulfill the ubiquitin demand in basal conditions, the polyubiquitin genes have long been known to play a role in stress-response and to be induced when cells are exposed to threats such as heat shock, starvation, DNA damaging agents, proteasome inhibitors, oxidative stress and so on [11-14].

Although *UBB* and *UBC* are transcriptionally upregulated in response to cell stress, both polyubiquitin products also appear to contribute to basal ubiquitin levels, as demonstrated by the phenotypic consequences of targeted homozygous deletion of *UBC* and *UBB* in mice [15-17].

Disruption of polyubiquitin gene *UBC* leads to mid-gestation embryonic lethality, probably due to an impairment in fetal liver development, which can be only partially rescued by ectopic expression of Ub. Moreover, the decreased Ub content upon *UBC* disruption is not compensated by upregulation of the other poly- or mono-ubiquitin genes, suggesting that they are functionally not redundant [15, 18]. On the other hand, homozygous deletion of *UBB* in mice caused infertility and adult-onset hypothalamic neurodegeneration with metabolic and sleep abnormalities [16, 17, 19]. On the whole, these results prove that a proper expression of polyubiquitin genes is required for normal cell survival and development in mammals. This is in contrast with evidence found in yeast, where the deletion of the stress-inducible polyubiquitin gene UBI4 did not affect cell viability under normal growth conditions, but caused an increased sensitivity towards different stressors, like heat shock and starvation [11].

In the present paper, we investigate the contribution of the four Ub genes to the total Ub transcriptome in basal conditions in different cell lines, highlighting the polyubiquitin genes importance for normal cell growth. We also evaluate the responsiveness of Ub genes to different cellular threats (proteasome inhibitors,

oxidative stressors, heat-shock, UV) in HeLa cells. Despite the role of specific Ub genes in facing different cell challenges has been previously investigated, studies addressing the whole Ub genes response to different environmental hostile conditions that provoke cellular stress are lacking. Data presented herein provide evidence of the transcriptional program mounted by HeLa cells to increase the ubiquitin supply, needed to counteract both proteotoxic and genotoxic stress. Our results suggest that the ubiquitin-related transcriptional response to stress is cell-type dependent rather than a general response occurring with standard features, in reaction to all types of stress, in all cellular contexts.
Intriguingly, herein we report for the first time the evidence of ubiquitin C mRNA variants, arising from multiple transcription start sites and we characterize and directly compare their expression under basal and stressful conditions.

2. Materials and Methods

2.1 Cell lines

All the cell lines used in this study were purchased from the American Type Culture Collection (ATCC) and maintained at 37°C in a humidified 5% CO_2 atmosphere. Cervical cancer cell lines HeLa (HPV18), Caski and SiHa (HPV16) and the p53-mutated C33A were cultured in RPMI 1640 medium, while the osteosarcoma cell line U2OS was maintained in McCoy's 5A. Both media were supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin.

2.2 Stress treatments

HeLa cells (~1 x 10^6) were plated in 60 mm dish plates the day before treatment in order to achieve an 80% cell confluency. Cells were challenged with different stressors described underneath. The proteasome inhibitor MG132 (VWR international s.r.l. Italy; stock solution 50 mM in dimethyl-sulfoxide, DMSO) was diluted in complete medium at a final concentration of 20 µM for 4 h. The vehicle DMSO, at the same dilution, was used as control. Exposure to oxidative stress was performed by incubating cells with Sodium Arsenite added to the serum-containing medium at a final concentration of 80 µM for 4 h at 37°C. UV treatment was accomplished removing medium from cells and subjecting them to UV radiation at 254 nm at 10, 50, 100, 400, 800 J/m^2 using a UVC500 (Amersham Pharmacia Biotech, Piscataway, USA). After irradiation, the cells were re-added of their medium and incubated 4 and 24 h at 37°C before the recovery. For mRNA decay studies, actinomycin D (Act D, 5 µg/ml) or the vehicle DMSO were added to cells upon irradiation and maintained 4 h at 37°C, before harvesting. The heat shock treatment was conducted in an incubator at 43°C for 1 h followed by 1 h recovery at 37°C. Untreated cells (NT) were used as control.

2.3 Cell viability assay

Cell viability of HeLa exposed to UV irradiation was evaluated by the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega s.r.l., Milano, Italy) according to the provided protocol. The MTS working solution was incubated for 4 h at 37°C. The amount of soluble formazan produced by cellular reduction of MTS was measured by reading the absorbance at 490 nm, with background subtraction at 630 nm.

2.4 RNA extraction and Reverse Transcription quantitative Real-Time PCR (RT-qPCR)

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA, USA) and 1 µg of total RNA was reverse-transcribed with the SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), as indicated in the standard protocol. cDNAs were used as templates in SYBR green quantitative Real-Time PCR (qPCR) assays, performed with the Hot-Rescue Real-Time PCR kit (Diatheva s.r.l., Fano, Italy). PCR reactions were prepared and run as described earlier [20] using ABI PRISM 7700 Sequence Detection System platform (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express version 2.0 and were purchased from Sigma-Genosys (Ltd, Haverhill, UK). Primer sequences are listed in Table 1. For all primer pairs, the PCR protocol consisted of 95°C for 10 min followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 30 s at 72°C. Relative expression data were calculated with the $2^{-\Delta\Delta C}{}_T$ method [21]. For absolute quantification, plasmid DNA bearing the different targets, amplified and purified by standard procedures, were linearized and quantified at 260 nm using the Nanodrop ND-1000 System (NanoDrop Technologies, Wilmington, DE). From each plasmid, serial dilutions from 10^7 to 10^1 copies were prepared and used as standards in the qPCR assay. After the run, the melting curve of each amplicon was examined to determine the specificity of the product. Amplification plots were analyzed using SDS 1.9.1 software (Applied Biosystems) and expression data were calculated with the standard curve method. The copy number of the different ubiquitin mRNAs (*UBB*, *UBC*, *UBA52*, *RPS27A* and *UBC*^{var}) was normalized to

the copy number of β2-microglobulin (B2M) transcripts. Fold-inductions were calculated versus untreated control cells.

2.5 Rapid amplification of cDNA ends (RACE)

UBC transcription start sites were identified by 5'-RACE following the protocol of the GeneRacer kit (Invitrogen) with slight modifications. One microgram of human total RNA, isolated as above from control and MG132-stressed HeLa cells, was first treated with calf intestinal phosphatase to dephosphorylate non-mRNA and truncated mRNA. Thereafter, the 5'-cap structure from full-length mRNA was removed by incubation with tobacco acid pyrophosphatase (TAP) at 37°C for 1 h. The 5'-phosphate left on decapped mRNA was then ligated to 0.25 μg of GeneRacer RNA Oligo (provided with the kit) at 37°C for 1 h with 5 units of RNA ligase. Next, reverse transcription was carried out at 42°C for 50 min using 200 units of SuperScript II reverse transcriptase to create RACE-ready cDNA. For all subsequent PCR reactions, a *UBC*-specific primer and a GeneRacer primer were combined. Sequence of primers used for RACE are shown in Table 1. 5'-RACE was performed using the Advantage 2 Polymerase Mix (Clontech, Palo Alto, CA) and the following touchdown amplification protocol: 5 cycles of 30 s at 94°C and 1 min at 72°C, 5 cycles of 30 s at 94°C, 30 s at 70°C and 30 s at 72°C, and finally 27 cycles of 30 s at 94°C, 30 s at 69°C and 30 s at 72°C. The amplicons from the first PCR were employed as the template for the nested PCR, performed by using the GeneRacer 5′ Nested Primer and a nested *UBC*-specific reverse primer (see Table 1). The amplification protocol was as above, except for the annealing temperature which was decreased up to 60°C. 3'-RACE was achieved with the GeneRacer 3' primer and a *UBC*-specific forward primer which binds to the 3'-UTR, using the following touchdown protocol: 5 cycles of 30 s at 94°C, 30 s at 70°C and 30 s at 72°C, 5 cycles of 30 s at 94°C, 30 s at 68°C and 30 s at 72°C, and finally 27 cycles of 30 s at 94°C, 30 s at 63°C and 30 s at 72°C. The PCR products were gel-purified and directly sequenced; alternatively they were cloned into the PCR Blunt TOPO Vector (Invitrogen). Thirteen clones of each type were picked for sequence analysis using the dideoxy-termination method and the PE310 capillary sequencer (Perkin Elmer).

2.6 Cell lysate preparation, solid phase immunoassay and Western immunoblotting analysis

Cells (HeLa, SiHa, Caski, C33A and U2OS) were lysed with buffer [50 mM Tris-HCl, pH 7.8; 0.25 M sucrose, 2% (w/v) SDS, 10 mM N-ethylmaleimide (NEM) supplemented with fresh complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and phosphatase (1 mM NaF, 1 mM Na3VO4) inhibitors]. The lysates were boiled for 2 min, then sonicated (50 Watt/15 sec) and centrifuged at 12000 x g for 10 min at room temperature; protein concentration in the soluble fraction was measured using the Lowry method [22]. Lysates were used for quantitation of the ubiquitin content, by solid phase immunoassay, performed as previously described [23]. Blots were immunostained with the rabbit polyclonal anti-ubiquitin antibody (kindly provided by Prof. A. L. Haas (Dept. of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans). Detection and densitometric quantitation were performed in a Chemidoc apparatus (BioRad, Hercules, CA) equipped with the Quantity One software. R^2 was 0.98–0.99 for standard curves with ubiquitin. For Western blot, equal amounts of whole-cell extracts from untreated and stressed HeLa cells, were subjected to SDS-PAGE followed by transfer to nitrocellulose membrane (0.2 mm pore size; BioRad), and analyzed with primary antibodies against ubiquitin (described above), HSF2 (H-300; Santa Cruz Biotechnology Inc.) and β-actin (A 2066; Sigma-Aldrich, Steinheim, Germany). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, CA). Peroxidase activity was revealed with the enhanced chemiluminescence detection method (ECL Fast Pico, Immunlogical Sciences, Rome, Italy).

2.7 Statistics

The statistical analyses were conducted using SPSS Statistic 20 (IBM). One way Anova was used to calculate significance.

3. Results

3.1 Ubiquitin genes contribution to ubiquitin pool in basal conditions

In order to examine the level of ubiquitin in five commonly used cell lines under basal conditions, solid phase immunoassay was employed, using purified bovine ubiquitin as standard. The level of total Ub in unstressed cells (expressed as μg Ub/mg protein) was similar for the osteosarcoma cell line U2OS (4.13±0.005, mean±SEM) and the HPV18 positive cell line HeLa (4.74±0.12), while in the HPV16 positive cells SiHa and Caski and the p53 mutated cell line C33A the ubiquitin content was almost doubled (8.44±0.085, 7.81±0.04 and 8.34±0.08, respectively).

To determine the contribution of Ub genes to cellular Ub transcriptome, a reverse transcription quantitative Real-Time PCR (RT-qPCR) analysis of Ub transcripts was carried out in three out of the five cell lines investigated, namely HeLa, SiHa and U2OS. Primers targeting the various Ub transcripts were designed over the 5'- or 3'-UTR sequences of the four ubiquitin genes. β2-microglobulin (B2M) mRNA was used to normalize expression data.
Results from HeLa cells show a predominance of the Ub-ribosomal fusion gene *RPS27A* (whose mRNA represents 36% of total Ub transcripts), followed by *UBC* (~24%), while *UBB* and *UBA52* equally contribute to the remaining 40% of Ub transcripts (Fig. 1). The pattern is, on the whole, maintained in the other cell lines tested, although a higher percentage of *UBC* was detected both in U2OS (35%) and SiHa (39%): this occurred at the expense of *UBB* and *UBA52* contribution for the U2OS, while in SiHa both Ub fusion ribosomal mRNAs were less represented (Fig. 1). If we multiply these percentages for the number of ubiquitin moieties each gene codes for (3 for *UBB* and 9 for *UBC*), assuming a similar translation efficiency for the various Ub mRNAs, a predominance of *UBC* over the other Ub genes, for the contribution to total ubiquitin protein clearly arises. *UBC* is expected to account for the 64% of total Ub pool in HeLa and its central role as a source of ubiquitin in basal conditions is even more evident in the U2OS and SiHa, where the theoretical *UBC* encoded fraction reaches the 77%. Behind *UBC*, the strongest contribution to Ub production is from the other polyubiquitin gene, *UBB*, in HeLa and SiHa, while in the U2OS *UBB* and *RPS27A* equally contribute to Ub levels.

3.2 Ubiquitin genes contribution to ubiquitin pool under proteotoxic stress conditions

To evaluate the Ub genes responsiveness under proteotoxic stress conditions, HeLa cells were challenged with different stressors, known to induce accumulation of unfolded/damaged proteins and the activation of the UPS system: the proteasome inhibitor MG132, Sodium Arsenite and Heat shock. The transcriptional response of the four Ub genes was evaluated by qPCR: the copy number of each Ub transcript, normalized to the housekeeping B2M mRNA copies, was expressed as fold-change versus the not treated control sample (NT). The bar graphs in Fig. 2 allow a direct comparison of the transcriptional behavior of mono- and polyubiquitin genes upon the different cell insults, known to provoke proteotoxic stress. The two polyubiquitin genes show responsiveness to the proteasome inhibitor MG132, although the fold-induction versus untreated cells was significantly higher for the *UBC* (mean±SEM: 5.83±0.38) than for the *UBB* (2.16±0.09) transcript (Fig. 2A). The expression data refer to HeLa cells incubated with 20 μM MG132 for 4 h: this time point was selected from time-course experiments (where the inhibitor was maintained for 30 min, 2, 4 and 8 h) because the transcriptional induction of Ub responsive genes was marked and no cell toxicity was detectable. As a control, cells were incubated with the vehicle DMSO, which has given results not statistically different from those of untreated sample (not shown). Exposure to MG132 failed to induce transcription of the Ub-ribosomal fusion genes (Fig. 2A).
In this paper we also evaluated how the four Ub genetic loci respond to heat-induced stress, when more Ub is needed for the clearance of damaged/unfolded proteins [24]. In HeLa cells, upon 1 h incubation at 43°C followed by 1 h recovery at 37°C, the mRNA levels of both polyubiquitin genes were upregulated, whereas the levels of *RPS27A* and *UBA52* mRNAs remained unchanged (Fig. 2B). Heat-induced upregulation of *UBC* was slightly more prominent (1.92±0.03-fold change versus unstressed cells) than that of *UBB* (1.35±0.15-fold induction versus control sample). In thermally shocked HeLa cells we confirmed the different transcriptional behavior of Ub genes, although the stress response of *UBC* and *UBB* was very mild, respect to the one found in literature [14], probably because HeLa cells are more heat-resistant. On the other hand, in experiments where the transcriptional response was monitored at different and longer time points after the heat-shock insult or where the heat shock was performed at higher temperatures (44-45°C), we did not observe an increase in the upregulation of *UBB* and *UBC* genes (data not shown).
We next sought to investigate the changes of ubiquitin transcriptome upon oxidative stress conditions. Cycling HeLa cells were treated with 80 μM Sodium Arsenite for different times, up to 12 hours: the peak of Ub transcriptional response was detected at 4 h incubation at 37°C. The concentration of $NaAsO_2$ was chosen in order to maximize the stress response without inducing cell death. At the selected time point, cells were harvested and assayed by RT-qPCR of Ub genes. As for the MG132 treatment, we found upregulation of both polyubiquitin genes with the predominant stress response exhibited by *UBC* than by *UBB* (mean±SEM fold-induction versus untreated cells: 4.87±0.38 and 2.30±0.25, respectively; Fig. 2C). The ubiquitin-ribosomal fusion gene expression did not change in response to oxidative stress (Fig. 2C).
A whole picture of the relative contribution of each Ub gene to the ubiquitin transcriptome in HeLa cells, in basal and stressful conditions, is given in Fig. 2D. The copy number of the mRNAs generated by the four Ub loci was determined by RT-qPCR as in Fig. 1, and expressed as percentage, setting the total Ub transcripts of untreated control cells as 100%. Exposure of cells to the proteasome inhibitor MG132 or the oxidative agent Sodium Arsenite led to a marked, similar increase in total Ub transcripts (235 and 212% versus the control sample). In both conditions, the predominant contribution was exhibited by the polyubiquitin gene

UBC (~137 and 115% upon MG132- and Arsenite-triggered stress), followed by *UBB* (43 and 46%, respectively) (Fig. 2D). The contribution of the hybrid genes *RPS27A* and *UBA52* remained unchanged upon both stress challenges (Fig. 2D). Indeed, as discussed above, *UBC* and *UBB* were the only ubiquitin genes upregulated at the transcriptional level, upon proteasome and oxidative stress (Fig. 2A and 2C). In heat-treated cells, the total Ub mRNA level raised to 135% respect to the basal condition (set at 100%) and this increased transcript level is contributed by the four genes as follows: *UBC* (45%), *UBB* (27%), *RPS27A* (42%) and *UBA52* (21%), with a statistically significant raise (versus NT sample) only for the polyubiquitin genes (Fig. 2D). To better appreciate the effective contribution of each Ub gene to the total Ub content upon proteotoxic stressors, mRNA levels were converted to Ub-coding potential. If we take into account the actual number of Ub moieties encoded by each transcript, the theoretical total Ub content raises ~4.3-, 3.7- and 1.7-fold following proteasome (MG132), oxidative (Sodium Arsenite) and heat stress, respectively. Due to its high-coding potential, *UBC* translation gives the most significant contribution to total Ub levels under proteome stress conditions. The second relevant contribution is from *UBB*, while the *UBA* genes, which code for only one Ub per transcript and are not induced upon these stresses, are expected to contribute only to a minimal fraction of total Ub.
To determine whether and how upregulation of *UBC* and *UBB* in stress-challenged HeLa cells affects Ub pools, we performed immunoblot analysis with an anti-Ub antibody. As shown in Figure 2E, the levels of Ub conjugates significantly increased with all the stressors applied, indicating that the ubiquitin conjugating machinery is functional and not compromised. Accumulation of ubiquitinated proteins may arise not only from a higher Ub conjugation rate, needed to remove damaged/unfolded proteins, but also from a reduced protein degradation: besides proteasome inhibitors, severe or sustained oxidative stress may impair proteasomal activity as well [25]. To ascertain if the Ub-dependent proteolytic machinery indeed functions properly under the stress environments investigated, the cellular content of HSF2 protein, a short-lived transcription factor whose steady-state level depends on the UPS activity, was also determined [26]. As revealed by immunoblotting analysis, HSF2 accumulates only in cells treated with the proteasome inhibitor MG132, while in heat-shock and Arsenite-exposed cells, HSF2 levels are similar to or even lower than those detected in untreated control cells, which is consistent with a fully functional UPS (Fig. 2E).

3.3 Ubiquitin genes response under UV-induced genotoxic stress conditions

In 1992, Nenoi reported, for the first time, the UV-induced accumulation of polyubiquitin C transcript in HeLa cells, by performing a Northern blot analysis of total RNA purified from cells treated with different UV-C doses and harvested at different times after irradiation [27].
Starting from this background information, in the present work, we used qPCR for a more sensitive analysis of ubiquitin genes response upon UV challenge. HeLa cells were treated with 10, 50 and 100 J/m^2 of UV-C light, and recovered at 4 h and 24 h post-irradiation, time points selected from the literature for early and late regulated genes [28], although Nenoi found that *UBC* accumulation after irradiation was rapid and transient, with a peak at 2.5 h and a decrease to a control level by 7 h after UV treatment [27]. No change in Ub genes expression was detected at these different UV doses at the two time points investigated, with respect to mock-treated cells (data not shown). To assess the efficacy of the treatment, we amplified as positive control interleukin 8 (IL-8), which was found to be a UV inducible gene in two whole genome expression studies [28, 29]: IL-8 mRNA markedly accumulated (~7.2-fold) 24 h after HeLa exposure to 100 J/m^2. In the next experiments, HeLa cells were irradiated with higher UV doses (400 and 800 J/m^2), besides 100 J/m^2, as above, and samples collected 4 h after treatment. A statistically significant transcriptional response (~3- and 6.4-fold induction) of *UBC* mRNA was achieved in cells challenged, respectively, with 400 and 800 J/m^2 UV light, respect to sham-irradiated counterparts (Fig. 3A). *UBA52* and *RPS27A* showed a statistically significant induction (5.3- and 5.8-fold, respectively) only upon the highest UV dose (Fig. 3A). However, a cytotoxicity with marked cell detachment was observed at both the highest doses applied, as detected by MTS cell viability assay, where the absorbance of formazan product in irradiated cells, expressed as percentage of the NT value, decreased in a dose-dependent manner (Fig. 3B). Moreover, in the 400 and 800 J/m^2-irradiated cells, where ubiquitin induction was detected, the level of β2-microglobulin mRNA, used to normalize expression data, drastically drop to 29 and 13% of the level measured in the mock-treated cells, respectively (Fig. 3C). Other housekeeping targets were assayed (Glyceraldehyde-3-Phosphate Dehydrogenase [GAPDH], beta glucuronidase [GUSB], Peptidylprolyl Isomerase A [PPIA], Hypoxanthine phosphoribosyltransferase 1 [HPRT1] and 18S rRNA) with similar outputs (not shown). By comparison, upon these UV doses, the level of the ubiquitin transcripts (with the only exception of *UBB*) showed a slower decrease and the most stable was *UBC* mRNA (Fig. 3C). This finding suggests that the apparent upregulation of the Ub genes upon UV stress is more reasonably the consequence of the UV-mediated stabilization of these mRNAs. To directly test if the UV stressor indeed affects mRNA stability, we repeated the UV irradiation at 400 and 800 J/m^2 in the presence of the inhibitor of transcription actinomycin D (Act D) or the vehicle DMSO. As shown in Fig. 3D, in the mock-irradiated cells treated with Act D, the *UBC* mRNA

level drops ~ 9-fold respect to the value detected in the transcriptionally unblocked cells. The UV irradiation significantly raised the residual *UBC* transcript level upon cell challenge with Act D, and this effect seems to be dose-dependent. This means that UV-C light acts at the post-transcriptional level, stabilizing Ub mRNAs.

3.4 Multiple initiation sites for UBC transcription and their responsiveness to stress

By chance, while performing the experiments for this report, we found a recent paper by Oh et al., where a couple of primers annealing to the upstream promoter region were used for the detection of *UBC* mRNA upon knockdown by siRNA technology [30]. In particular, the oligonucleotides amplified a fragment of 259 bp, ranging from nt -257/+2 respect to the "canonical" transcription start site (TSS) first putatively identified and then confirmed by primer extension, by Nenoi et al. [31]. This prompt us to amplify this *UBC* region: qPCR on cDNA from untreated HeLa cells, performed with a primer pair circumscribing the -156/-66 promoter sequence gave a product of the expected length, but with a Ct value 8 units higher than the one detected with the 3'-UTR matching primers. This means that a longer *UBC* transcript variant indeed exists, but it corresponds to ~0.32% of the total *UBC* mRNA level. Similar results were obtained using as a template cDNAs from U2OS and SiHa cells, harvested in basal conditions. To assess if this new *UBC* mRNA isoform (from here on referred to as UBC^{var}, for variant) somehow plays a role in stressful conditions, we extend qPCR analysis to cDNAs prepared from HeLa cells challenged with the different stressors, described above. Fig. 4A shows a statistically significant induction of the UBC^{var} mRNA in all the stressful conditions, with the exception of UV irradiation; the highest upregulation was detected upon Arsenite-triggered oxidative stress (~34-fold induction versus NT). Noteworthy, the fold-induction of the UBC^{var} transcript is markedly higher than the one detected with primers matching to the 3'-UTR sequence with all the stressors tested, and the percentage of this newly identified isoform raised accordingly, achieving the 2.03% of total *UBC* mRNA in cells incubated with Sodium Arsenite (Fig. 4B); however, this fraction seems yet negligible from a biological point of view.

To exactly localize the transcription start site of the UBC^{var} message, we used 5'-RACE, performed on total RNA extracted from both control and MG132-treated HeLa cells, because of the marked stress responsiveness of the new transcript isoform. A strong predominant band of about 100 bp and a faint smear of higher molecular weight bands were obtained after amplification with the GeneRacer 5'-primer provided with the kit and the *UBC* (+53) 3'-primer (Fig. 5A and 5B). The well-defined 5'-RACE product was subsequently cut, purified from agarose gel and either subcloned before sequencing or directly used for sequence analysis (Fig. 5B). From a total of 13 clones and direct sequencing of the PCR product one sequence stop, which corresponds to the transcription initiation site, was detected and located at the +1 position described by Nenoi, which is ~25-nt downstream of the TATA box element [31]. The same output was obtained with the RNA extracted from MG132-stimulated cells. This transcription start site was marked as TSS1 (Fig. 5C). To zoom in on the minor faint bands observed in Fig. 5B, a second 5'-RACE was done using GeneRacer 5' nested and *UBC* (-66) as forward and reverse primer, respectively. The nested PCR produced two discrete bands of very similar molecular weight for the control RNA sample, with the addition of a third lower band in the MG132-treated sample. The bands were purified, cloned and analyzed by sequencing as above. Three further transcription start sites were identified (Fig. 5C) and located 172, 201 and 241 nucleotides upstream from the start site defined by Nenoi [31] and provided in the published cDNA sequence (GenBank accession no. AB009010.1). The nested 5'-RACE was repeated three times and similar results were obtained. Since these end points are separated by more than a few nucleotides, they are very likely multiple initiation sites for *UBC* gene transcription and not a bias of the 5'-RACE technique that was used in this report. These further transcription start sites were named TSS2, TSS3 and TSS4, from the closest to the TSS1 to the 5'-most (Fig. 5C). The commonly found end point for the three TSS identified was an adenine residue, which means that transcription initiation more likely happens specifically at this position. Interestingly, the TSS2 was detected only in the *UBC* transcripts purified from MG132-stressed cells, suggesting that the trans-acting factor(s) involved in this stress response might favor the assembly of the transcriptional machinery over this sequence.

To define the 3'-end of the *UBC* transcript, we performed 3'-RACE with the *UBC*f primer (the one used for expression studies and binding to the 3'-UTR) and the GeneRacer 3' oligo as the reverse primer (Fig. 5A). One prominent marked band, migrating between the 147- and 190-bp bands of the DNA molecular weight marker loaded aside, was detected for both RNA samples (NT and MG132) (Fig. 5B). Sequence analysis confirmed the published data for the 3'-end of the message, with differences among the clones sequenced due only to the length of the poly-A tail, which ranged from ~20 to ~80 nt (Fig. 5C).

4. Discussion

Because of its pervasive role in many aspects of the cell life, ubiquitin is very abundant, accounting for 0.1-5% of total protein concentration in eukaryotic cells [32-34]. In this study, we examined the Ub content of five

different cell lines, three of which are HPV-positive. The Ub pool quantified in HeLa cells is in strict accordance with our previous data [23] and the one determined by others by indirect competitive ELISA [34]. The U2OS osteosarcoma cells have a similar Ub content as HeLa, while the other cell lines present an almost doubled ubiquitin level. On the whole, these data suggest that the positive or negative HPV status does not account for the different Ub content among the cellular models investigated. Since ubiquitin, in mammals, is coded by four genes with different coding potential, we analyzed their relative expression in three out of the five cell lines tested, by the qPCR approach. The pattern of Ub genes expression is largely shared by the different cell samples and shows a predominance of *RPS27A* and *UBC* transcripts, but if we take into account the number of Ub moieties generated by each transcript, a clear supremacy of *UBC* arises. Two key aspects are worth considering: first, the composition of the Ub transcriptome does not completely account for the different Ub content detected by the immunoassay, highlighting that multiple layers of regulation indeed affect the final gene product amount. Second, data obtained confirm that the polyubiquitin C gene contributes to a marked fraction of the Ub supply in the different cell lines investigated, in basal conditions, although it is notably known as a stress-responsive gene [11]. Our results support the conclusions of Ryu et al. that a functional *UBC* gene is needed for normal cell cycle progression, besides stress tolerance [15].

If ubiquitin homeostasis is important for normal cellular functions, maintenance of free ubiquitin above certain threshold levels is strictly required for cellular survival upon different stress conditions [6, 35]. To investigate whether the four Ub genetic loci play specific, non-redundant roles in stress responses in mammals, we systematically evaluated the Ub genes responsiveness in HeLa cells challenged with four different stressors known to induce proteotoxic or genotoxic stress and/or activation of the UPS system: the proteasome inhibitor MG132, Sodium Arsenite, Heat shock and UV irradiation.

Stressing HeLa cells with the proteasome inhibitor MG132 significantly upregulated polyubiquitin gene expression, with the higher induction exhibited by *UBC*. MG132 failed to induce transcription of the Ub-ribosomal fusion genes, which are notably known to contribute in maintaining cellular Ub pools in basal conditions rather than to participate in the stress response [9, 12]. Our data are in accordance with the literature, with the exception of evidences reported by Ryu and coworkers: in mouse embryonic fibroblasts (MEFs) exposed to the proteasome inhibitor ALLN, all four Ub genes were activated, including *UBA* genes [15]. Moreover, among polyubiquitin genes, upregulation was higher for *UBB* than for *UBC* [15]. However, the different outputs may be due to the different cellular models investigated and/or (less probably) to the different stressor applied.

It has been well documented that upon heat-stress, damaged and unfolded/aggregated proteins accumulate and generate an increased demand of Ub for protein clearance [24]. Moreover, the heat-shock-induced accumulation of multi-ubiquitinated proteins has also been observed and ascribed to the activation of the ubiquitinating enzyme system [36]. The higher Ub demand is met by an induced synthesis of Ub, which relies on transcriptional activation of Ub coding genes [12, 37]; however, in the first reports, the analysis of Ub mRNA levels, mainly focused on the poly-Ub coding transcripts [37], was performed by Northern blot, which doesn't allow the accurate and selective quantification obtained by the qPCR assay used herein. In this study, upregulation of polyubiquitin genes (mainly of *UBC*) was found to be primarily responsible for maintaining Ub homeostasis during heat shock in HeLa cells, although the fold-induction was not marked. A similar activation (1.6-fold increase) for the *UBC* gene was detected by Ryu et al. in MEFs exposed to mild and lethal heat shock, with and without preconditioning [15]. The cited evidences and data herein discussed suggest that, although upregulation of polyubiquitin genes represents a general response mounted in reaction to heat stress to face the increased Ub needs, the entity of *UBB* and *UBC* transcriptional induction, as well as the reversible/lethal heat shock boundary, are mainly cell-type specific. To date, the heat-triggered induction of the Ub-ribosomal fusion genes has not been documented. From a molecular point of view, this can be explained by the presence of heat shock elements (HSEs) in the promoters of polyubiquitin genes *UBB* and *UBC*, but not of monoubiquitin genes *RPS27A* and *UBA52* [14, 31]. Heat shock factors (HSFs) binding to HSEs are the master regulators of transcription under protein damaging stress conditions and HSF1/2-driven induction of polyubiquitin genes, which is a rapid means to increase Ub levels, has recently been addressed [14; Crinelli, submitted paper].

An adequate ubiquitin supply is required for cells to cope with oxidative stress as well: misfolded proteins that are generated need to be polyubiquitinated and targeted to the proteasome for degradation. However, the UPS itself is modulated by oxidative conditions, with the proteasome being more susceptible to oxidative damage respect to the Ub conjugating enzymes [25]. Exposure of HeLa cells to 80 μM $NaAsO_2$ leads to the transcriptional activation of both polyubiquitin genes and results in a significant increase of Ub conjugates. Arsenite-induced accumulation of Ub conjugates is an indicator of the upregulated Ub-conjugating activity, because of the increased substrate availability for ubiquitination, and occurs under mild oxidative stress conditions [25]. Oxidative inactivation of the proteasome, prior to inactivation of the ubiquitination system, may also account for the accumulation of Ub conjugates [25]. However, the proteasomal activity was not impaired in our experimental system, since the cellular content of HSF2, which is a known UPS target, did

not raise in Arsenite-treated cells, as instead occurs in cells challenged with the proteasome inhibitor MG132. The same Arsenite concentration was used on CHO and myeloma cells to study the regulation of UPS-mediated degradation upon oxidative cytosolic stress [38]. A marked increase in polyubiquitin conjugates in Arsenite-treated cells was detected, but no gene expression data were provided to explain how the higher Ub demand is met during proteotoxic stress [38]. The question was addressed in the paper of Fernandes et al. where endothelial cells were exposed to glucose oxidase-produced hydrogen peroxide and the mRNA levels of the Ub genes detected by qPCR: only *UBB* was upregulated in response to oxidative stress [39]. This evidence is in disagreement with both our data on HeLa cells and those reported by others on mouse embryonic fibroblasts exposed over a 12 h period to different oxidative stressors, including $NaAsO_2$ [40]. Both polyubiquitin genes were found to respond to all the oxidative stressors tested with a slight prevalence of *UBB* at the last time point [40]. On the other hand, in our cellular model the prominent response at transcriptional level was given by the *UBC* gene, respect to *UBB* and occurred upon 4 h incubation with the oxidant.

UV light is the most important environmental insult with which cells face; ultraviolet-B and C photons can cause substantial cellular damages to biomolecules, as it is well established for DNA. Besides the mutagenic, DNA damaging and cell cycle disturbing actions of UV, the ultraviolet stress response involves the transcriptional regulation of many target genes [28, 29, 41].

In 1992, Nenoi reported that *UBC* clearly increased with UV treatment, in a dose-dependent manner, while *UBA* and *UBB* genes were not changed noticeably [27]. However, the signals generated by *UBA* and *UBB* probes in the autoradiogram overlapped, making any conclusion difficult. In this study we found that both *UBC* and Ub-ribosomal mRNAs were stabilized upon UV irradiation. In the same conditions, several housekeeping transcripts were degraded. From a biological point of view, this means that UV-C light acts at the post-transcriptional level, through the stabilization of Ub mRNAs, as a strategy for cells to face the increased ubiquitin demand. This is not quite surprising since Han et al. reported that various apoptogenic stimuli induce the Ub-ribosomal fusion proteins by stabilizing their mRNAs, rather than by transcriptional induction of their encoding genes [42]. Thus, it can be hypothesized that UV challenge causes genotoxic injuries which in turn trigger different DNA damage response pathways: most of them rely on ubiquitination of specific protein targets, that change depending on genomic location and time within the cell cycle [43]. UV-damaged cells are thus required to exhibit a heightened capacity of the Ub system and might cope with the UV-triggered higher ubiquitination demand through the stabilization of three out of four ubiquitin coding mRNA species.

In summary, HeLa cells deal with the need to maintain the pool of free ubiquitin during and after stress, by the selective transcriptional or post-transcriptional regulation of the four Ub genetic loci, depending on the type of stress (proteotoxic versus genotoxic) to be faced. A deeper knowledge of the pathways triggered by the different stressors, as well as of the downstream effectors modulating the Ub transcriptome is needed to understand the different transcriptional responses of the poly- and mono-Ub genes.

The study of the Ub transcriptome performed lead us to highlight the presence of multiple transcription start sites in the *UBC* gene. The use of multiple TSS is widely spread in the human genome [44] and ubiquitin genes are not an exception: multiple start sites have indeed been described for the *RPS27A* gene [45] and also for the chicken testis polyubiquitin gene UbI [46]. However, as far as we know, afterwards the study of Nenoi in 1996 [31], that sets the hallmarks of the *UBC* gene structure, including the TSS, and our recent molecular studies [20, 47, 48] no other investigations aimed to define the boundary between the true promoter and the transcribed region of *UBC* gene were performed. Yet, the gene records for *UBC* available in the GenBank database, provide different information as to the primary transcript length, with the untranslated exon 1 ranging from 64 bp (GenBank: D63791.1; AB009010.1) to 455 bp (GenBank: NM_021009.6). Results obtained by RACE demonstrate that the human polyubiquitin C gene harbors three further transcription initiation sites, that were mapped, respectively, 172, 201 and 241 nucleotides upstream of the main transcription start site preliminarily described, characterized by a canonical TATA box at -25 position [31]. Functional characterization of these multiple TSS revealed the large predominant activity exhibited by the TATA-dependent initiation site, mainly in basal conditions. Interestingly, the strong upregulation of the UBC^{var} mRNA driven by the upstream TSS, in stressed HeLa cells, induces to speculate that a convenient assembly of the transcription apparatus more closer to the HSEs identified in this promoter (Crinelli, submitted paper) is favored.

5. Conclusions

Maintenance of ubiquitin above certain threshold levels is a priority for cells in order to perform the multiple Ub-dependent functions under normal or stress conditions. However, the understanding of the mechanism(s) involved in the control of Ub homeostasis and the contribution of the four Ub genes to Ub pools are still open questions.

Data herein reported highlight the importance of polyubiquitin genes, besides Ub-ribosomal fusion genes, in maintaining Ub pool in basal conditions in different cell lines, with a predominant role for *UBC*, because of the highest Ub coding potential.
We also demonstrate that polyubiquitin genes are mainly charged of providing cells with the extra-source of ubiquitin needed during proteotoxic stress. The extent of upregulation was shown to vary depending on the type of stress induced. Further experiments are warranting to meet the many unsolved questions as how Ub demanding processes communicate to the chromatin the need to upregulate gene transcription and, what's more, which are the molecular buttons that link the various stress challenges to the selective Ub gene transcriptional response.
Moreover, the ubiquitin transcriptome does not change upon UV-triggered genome stress. Nevertheless, cells deal with the need to maintain their own ubiquitin, required to counteract the biological consequences of genotoxic insults, by the stabilization of Ub mRNAs.
Finally, our work reports, to our knowledge for the first time, the identification of multiple transcription start sites in the *UBC* promoter, that account for the *UBC* transcript variants that we analyzed for both their basal expression and responsiveness to different stressors. Further investigation is warranted to understand the biological significance of these additional TSS and characterize them in an unbiased manner. For now, the provided evidence adds a little piece of knowledge to the *UBC* gene card and highlights that designing primers in the 5'-upstream region respect to the TATA-box linked TSS, is not a proper way of analyzing *UBC* gene activity.

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Table 1. Oligonucleotides used for quantitative Real-Time PCR and RACE

qPCR			
Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')
UBC-f	GTGTCTAAGTTTCCCCTTTTAAGG	*UBC*-r	TTGGGAATGCAACAACTTTATTG
UBB-f	CTTTGTTGGGTGAGCTTGTTTGT	*UBB*-r	GACCTGTTAGCGGATACCAGGAT
UBA52-f	CTGCGAGGTGGCATTATTGAG	UBA52-r	GTTGACAGCACGAGGGTGAAG
RPS27A-f	TCGTGGTGGTGCTAAGAAAAGG	RPS27A-r	TTCAGGACAGCCAGCTTAACCT
UBC(-156)-f	AGGACGGGACTTGGGTGACT	*UBC*(-66)-r	CGCAGAATCGCCGAGAAG
B2M-f	GCCTGCCGTGTGAACCAT	B2M-r	CATCTTCAAACCTCCATGATGCT

RACE			
Forward Primer	Sequence (5’ to 3’)	Reverse Primer	Sequence (5’ to 3’)
GeneRacer 5′	CGACTGGAGCACGAGGACACTGA	*UBC*(+53)-r	CACAGCGATCCACAAACAAGAACC
GeneRacer 5′ Nested	GGACACTGACATGGACTGAAGGAGTA	*UBC*(-66)-r	CGCAGAATCGCCGAGAAG
UBC-f	GTGTCTAAGTTTCCCCTTTTAAGG	GeneRacer 3′	GCTGTCAACGATACGCTACGTAACG

f, forward; r, reverse.
Numbers in parenthesis refer to the main transcription start site of *UBC* gene identified as +1.

Figure legends

Fig. 1. Contribution of Ub genes to the total Ub transcriptome in different cell lines in basal conditions.
UBB, *UBC*, *UBA52* and *RPS27A* mRNA levels were determined by RT-qPCR, normalized to B2M mRNA levels and expressed as percentage of total Ub mRNA level, set as 100. Data are expressed as the mean±SEM of five independent determinations.

Fig. 2. Transcriptional response of Ub genes upon different stress treatments and Western Immunoblotting.
HeLa cells were challenged with MG132 (A), Heat shock (B) and Sodium Arsenite (C) as detailed under Materials an Methods. *UBB*, *UBC*, *UBA52*, and *RPS27A* mRNA levels were measured by RT-qPCR, normalized to B2M mRNA and expressed as fold-induction versus the mock-stressed cells (NT), set equal to 1. In (D) *UBB*, *UBC*, *UBA52* and *RPS27A* mRNA levels were expressed, as in Fig. 1, as percentage versus the total Ub mRNA level of untreated control cells, set as 100. The values are mean±SEM of a minimum of four biological replicates. Asterisks denote statistical significance versus the experimental control (NT): *$P<$ 0.05; **$P<$ 0.01; ***$P<$ 0.001. (E) Whole protein extracts were obtained from HeLa cells submitted to Heat shock (HS), treated with the proteasome inhibitor (MG132), or incubated with Arsenite (As) as detailed in the text. Untreated cells were used as control (NT). Protein extracts (3 μg) were separated by SDS-PAGE onto 14% gels for ubiquitin detection; 10 μg of total proteins were run in parallel onto 8% gels for HSF2 and actin staining. Molecular weight markers are indicated on the left.

Fig. 3. Analysis of the Ub transcriptome upon UV-induced cellular stress.
(A) HeLa cells were challenged with the indicated UV doses or left untreated. *UBB*, *UBC*, *UBA52* and *RPS27A* mRNA levels were measured by RT-qPCR, normalized to B2M mRNA and expressed as fold-induction versus the mock-stressed cells (NT), set equal to 1. (B) MTS cell viability assay was performed on HeLa challenged with UV, as indicated. All the values are expressed versus the NT sample, set as 100. (C) Decay of B2M and Ub mRNAs upon UV irradiation: mRNA levels, analyzed as above, are referred to the level of mock-irradiated sample, set equal to 1. (D) Stability of *UBC* mRNA in control cells (NT) and in cells exposed to UV radiation (400 and 800 J/m^2), with addition of actinomycin D (Act D) or vehicle DMSO. Data are expressed as residual fraction versus the corresponding transcriptionally unblocked sample, set equal to 1. For A-B, the values are mean±SEM of a minimum of four biological replicates. Asterisks denote statistical significance versus the experimental control (NT): *$P<$ 0.05; **$P<$ 0.01; ***$P<$ 0.001. For C-D, the histograms are the mean of two separate experiments, with similar outputs.

Fig. 4. Basal expression and stress responsiveness of the *UBC* transcript variant (*UBC*^{var}).
(A) RNA from HeLa cells challenged with the indicated stressors or left untreated (NT) was used in the RT-qPCR assay with primers matching to the 5'-upstream *UBC* promoter region. Data, analyzed as in Fig. 2, are shown as fold-induction versus unstressed cells, set as 1, and represent the mean±SEM of four independent experiments. Asterisks indicate statistical significance between each stress condition and the control sample: *$P<$ 0.05; **$P<$ 0.01; ***$P<$ 0.001. (B) *UBC*^{var} mRNA is shown as percentage (mean±SEM) of total *UBC* mRNA, set as 100 and detected with primers annealing to the 3'-UTR sequence. Similar results were obtained in three separate experiments.

Fig. 5. Multiple transcription start sites are present in the *UBC* gene.
5'-RACE and 3'-RACE were used to identify the 5'- and 3'-end of *UBC* transcript, respectively. (A) The diagram at the top provides a schematic representation of the *UBC* gene, showing the promoter region and the downstream exon structure. Black arrows stand for primers used for 5'-RACE; gray arrows stand for primers of 5'-nested RACE; empty arrows indicate the positioning of primers used for 3'-RACE. (B) The left and middle agarose gels show, respectively, the PCR products from 5'- and 5'-nested RACE. The gel on the right shows the results of 3'-RACE. The pUC19 DNA/MspI (HpaII) (Fermentas) was used as a molecular weight marker. (C) Partial sequences of the core promoter and 3'-region of *UBC* gene. Rightward arrows indicate the transcription start sites obtained by 5'-RACE and named from TSS1 to TSS4. Position of the major transcription start site (TSS1) is denoted +1. The reverse arrow marks the 3'-end of the *UBC* transcript, before the poly(A) tail. The TATA box is highlighted; the start and stop codons that set the boundaries of the Ub coding sequence (only partially shown) are in bold and underlined. Boxes define the sequence of primers used to amplify the *UBC*^{var} mRNA.

FIGURE 1

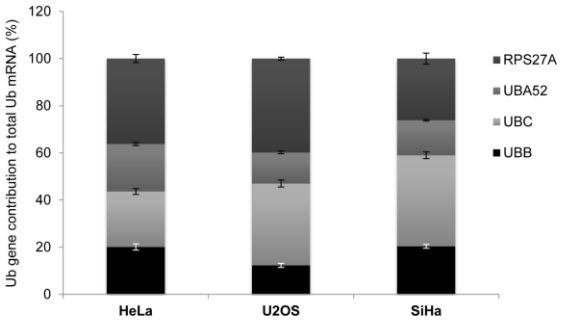

Ub gene contribution to total Ub mRNA (%)
120
100
80
60
40
20
0
HeLa
U2OS
SiHa
RPS27A
UBA52
UBC
UBB

FIGURE 2

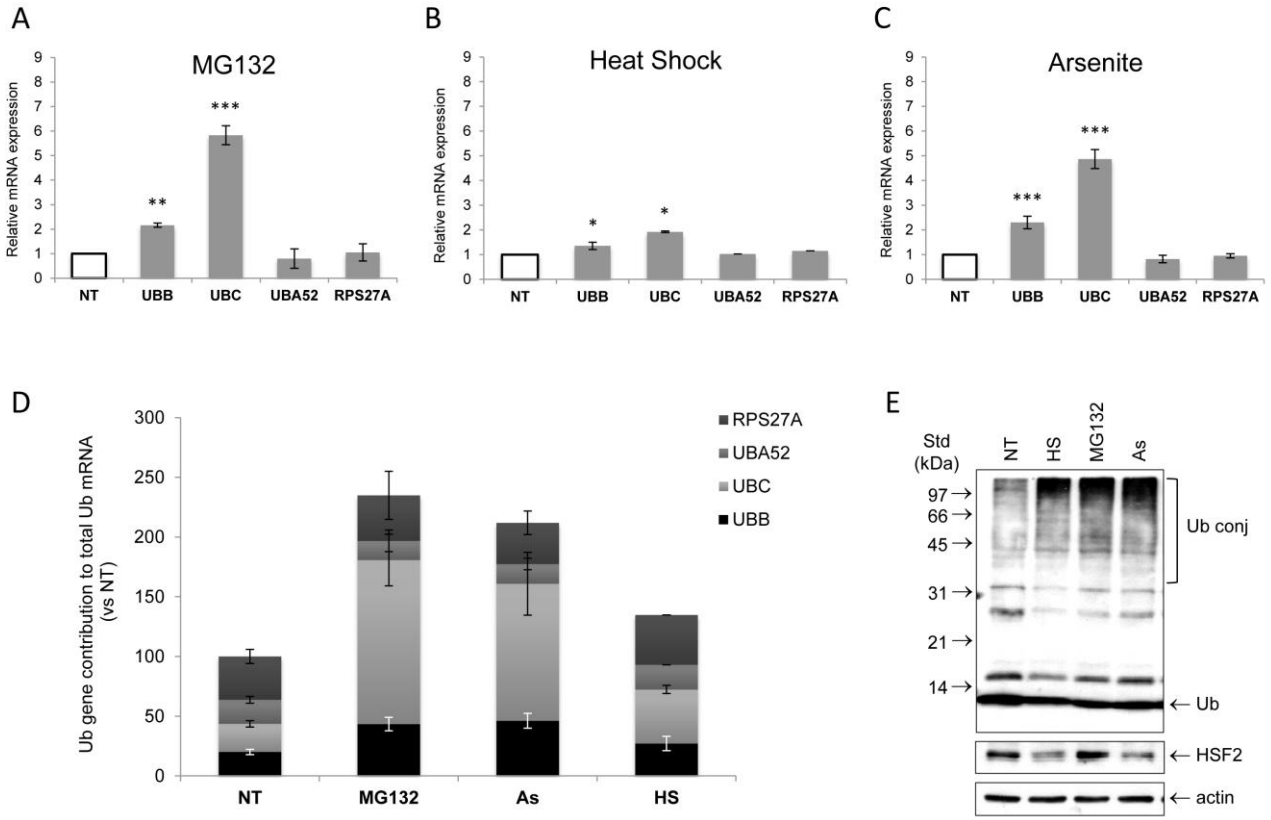

A
MG132
Relative mRNA expression
NT
UBB
UBC
UBA52
RPS27A
B
Heat Shock
C
Arsenite
D
Ub gene contribution to total Ub mRNA (vs NT)
RPS27A
UBA52
UBC
UBB
NT
MG132
As
HS
E
Std (kDa)
NT
HS
MG132
As
97
66
45
31
21
14
Ub conj
Ub
HSF2
actin

FIGURE 3

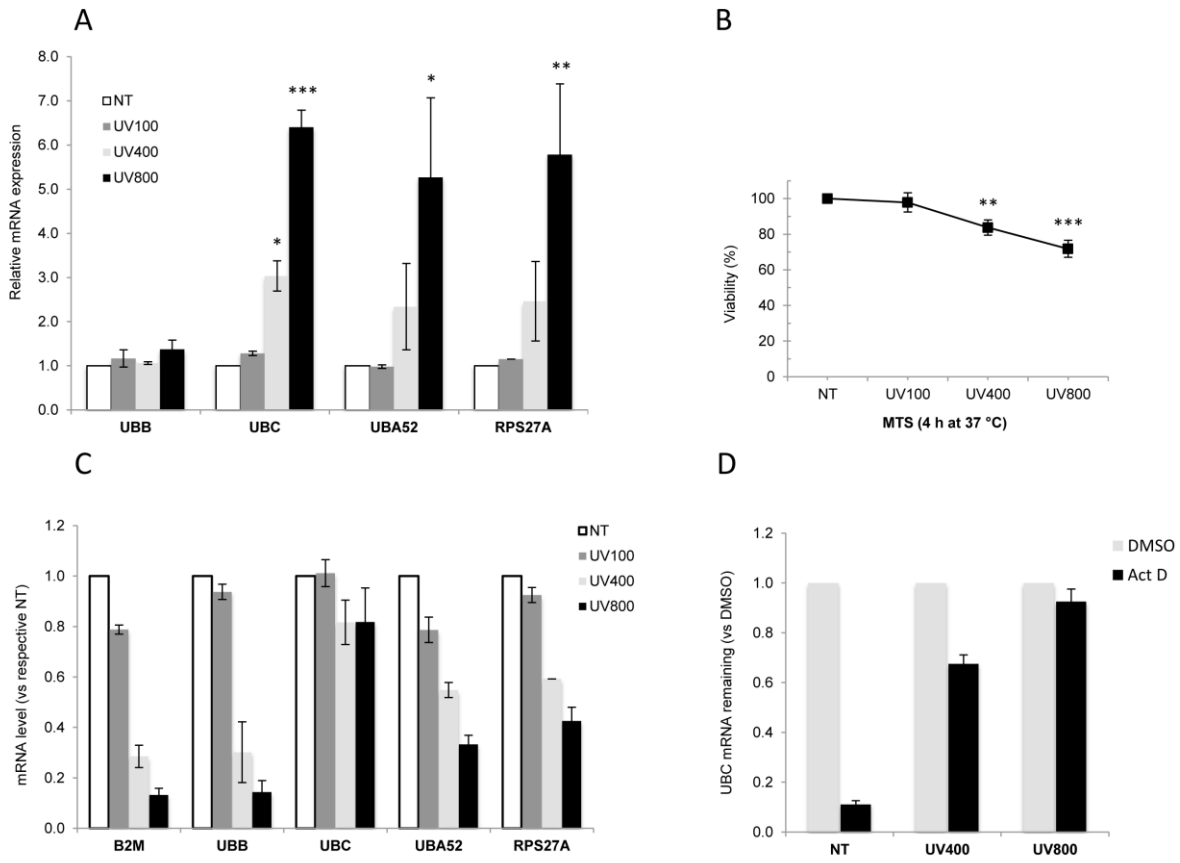
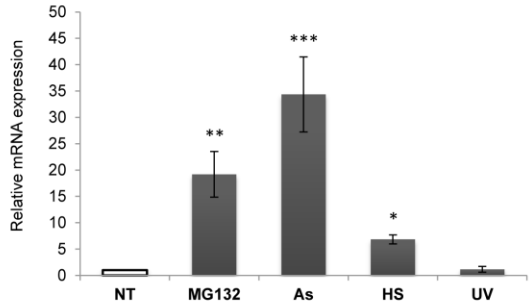
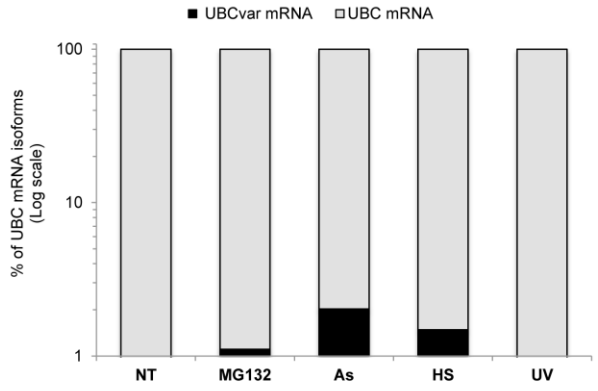

A
Relative mRNA expression
NT
UV100
UV400
UV800
UBB
UBC
UBA52
RPS27A
B
Viability (%)
MTS (4 h at 37 °C)
C
mRNA level (vs respective NT)
B2M
D
UBC mRNA remaining (vs DMSO)
DMSO
Act D

FIGURE 4

A

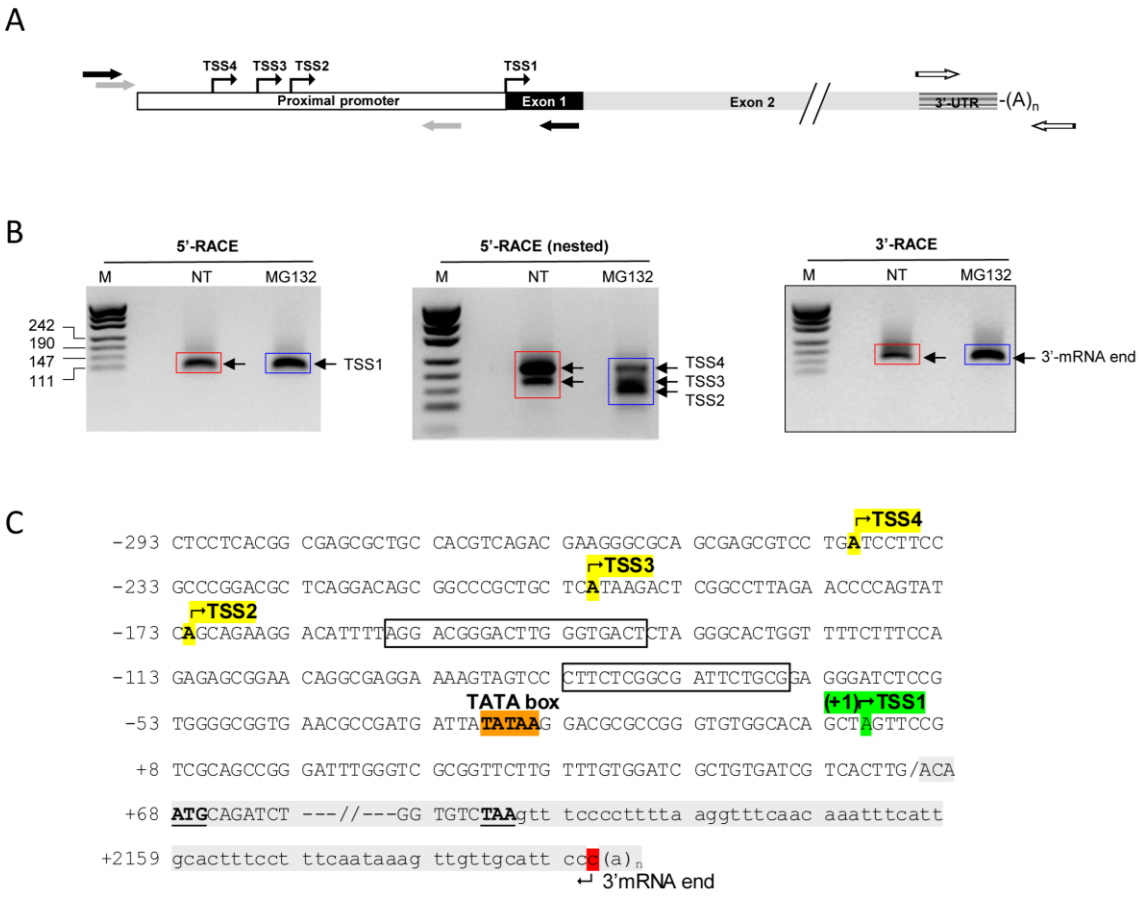


B



UBC^{var} [% of total UBC mRNA (mean ± SEM)]				
0.32 ± 0.02	1.11 ± 0.34	2.03 ± 0.36	1.48 ± 0.18	0.06 ± 0.03
NT	**MG132**	**As**	**HS**	**UV**

FIGURE 5


A
TSS4
TSS3
TSS2
TSS1
Proximal promoter
Exon 1
Exon 2
3'-UTR
-(A)n
B
5'-RACE
M
NT
MG132
242
190
147
111
TSS1
5'-RACE (nested)
M
NT
MG132
TSS4
TSS3
TSS2
3'-RACE
M
NT
MG132
3'-mRNA end
C
-293 CTCCTCACGG CGAGCGCTGC CACGTCAGAC GAAGGGCGCA GCGAGCGTCC TGATCCTTCC
TSS4
-233 GCCCGGACGC TCAGGACAGC GGCCCGCTGC TCATAAGACT CGGCCTTAGA ACCCCAGTAT
TSS3
-173 GAGCAGAAGG ACATTTTAGG ACGGGACTTG GGTGACTCTA GGGCACTGGT TTTCTTTCCA
TSS2
-113 GAGAGCGGAA CAGGCGAGGA AAAGTAGTCC CTTCTCGGCG ATTCTGCGGA GGGATCTCCG
TATA box
-53 TGGGGCGGTG AACGCCGATG ATTATATAAG GACGCGCCGG GTGTGGCACA GCTAGTTCCG
(+1)
TSS1
+8 TCGCAGCCGG GATTTGGGTC GCGGTTCTTG TTTGTGGATC GCTGTGATCG TCACTTG/ACA
+68 ATGCAGATCT ---//---GG TGTCTAAgtt tccccttta aggtttcaac aaatttcatt
+2159 gcactttcct ttcaataaag ttgttgcatt cc(a)n
3'mRNA end