Nucleolar control by a non-apoptotic p53-caspases-deubiquitinylase axis promotes resistance to bacterial infection

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Funding information
Ministry of Science and Technology, Taiwan (MOST), Grant/Award Number: MOST104-2320-B-182-029-MY3; Ministry of Science and Technology, Taiwan (MOST), Grant/Award Number: MOST105-2314-B-182-061-MY4; Chang Gung Memorial Hospital (CGMH), Grant/Award Number: CMRPD1F0442; Chang Gung Memorial Hospital (CGMH), Grant/Award Number: CMRPD1H0021; Chang Gung Memorial Hospital (CGMH), Grant/Award Number: BMRP960; Chang Gung Memorial Hospital (CGMH), Grant/Award Number: CMRPD1H0021.

Abstract
The nucleolus is best known for its cellular role in regulating ribosome production and growth. More recently, an unanticipated role for the nucleolus in innate immunity has recently emerged whereby downregulation of fibrillarin and nucleolar contraction confers pathogen resistance across taxa. The mechanism of this downregulation, however, remains obscure. Here we report that rather than fibrillarin itself being the proximal factor in this pathway, the key player is a fibrillarin-stabilizing deubiquitinylase USP-33. This was discovered by a candidate-gene search of Caenorhabditis elegans in which CED-3 caspase was revealed to execute targeted cleavage of USP-33, thus destabilizing fibrillarin. We also showed that cep-1 and ced-3 mutant worms altered nucleolar size and decreased antimicrobial peptide gene, spp-1, expression rendering susceptibility to bacterial infection. These phenotypes were reversed by usp-33 knockdown, thus linking the CEP-1-CED-3-USP-33 pathway with nucleolar control and resistance to bacterial infection in worms. Parallel experiments with the human analogs of caspases and USP36 revealed similar roles in coordinating these two processes. In summary, our work outlined a conserved cascade that connects cell death signaling to nucleolar control and innate immune response.

Abbreviations: C. elegans: CEP-1, C. Elegans P-53-like protein-1; EGL-1, EGiG Laying defective-1; CED-9, CElI Death abnormality-9; CED-4, CElI Death abnormality-4; CED-3, CElI Death abnormality-3; acCED-3, activated CED-3; Human: USP-33, Ubiquitin Specific Protease-33; FIB-1, FIBrillarin family-1; SPP-1, SaPosin-like Protein family-1; LIN-14 or LIN-28, abnormal cell LINEage-14 or 28; DISL-2, DIS3-Like exonuclease 2 homolog; let-7, LEThal-7; NCL-1, abnormal NuCLeoli-1; GFP, Green Fluorescent Protein; CED-1, CElI Death abnormality-1; ABF-2, AntiBacterial Factor related-2; CNC-2, CaeNorhabditis bacteriaCin-2; LYS-7, LYSozyme-7; ISP-1, Iron-Sulfur Protein-1; NUD-6, NADH Ubiquinone Oxidoreductase-6; USP36, Ubiquitin specific peptidase 36; RNA Pol I, RNA polymerase; CASP3, caspase-3; AOAH, Acyloxyacyl hydrolase; GNLY, granulysin; PSAP, prosaposin; PSAPL1, proactivator polypeptide-like 1; Materials: DIC microscopy, differential interference contrast microscopy; IPTG, Isopropyl β-D-1-thiogalactopyranoside; STA, staurosporine; LDH assay, lactate dehydrogenase assay; CPU, colony-forming unit.

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1 | INTRODUCTION

In addition to arresting cell cycle progression, the bifurcated role of p53 as a tumor suppressor includes activating a programmed cascade of molecules that culminate in cell elimination during development, aging, or catastrophic stresses. The protein network of apoptosis—p53/CEP-1, BH3/EGL-1, Bcl-2/CED-9, Apaf-1/CED-4, and caspases/CED-3—is evolutionarily conserved in humans and Caenorhabditis elegans and tightly regulated during the irreversible induction of cell death. The downstream caspases act proteolytically as the executioner of cell killing and activator of the engulfment process for clearing cell corpse. Notably, the cellular roles of these death-promoting proteases are not exclusive to apoptosis progression, as several recent studies in C. elegans have demonstrated seemingly non-apoptotic roles of caspases/CED-3. CED-4 and CED-3 are known to promote neuronal axon repair and regeneration, and to prolong worm lifespan via lowering ATP expenditure and ROS-induced mitochondrial dysfunction. Additionally, during worm development, CED-3 directly cleaves LIN-14, LIN-28, and DISL-2, which are connected to miRNA processing, stem cell pluripotency, and developmental timing. Intriguingly, CED-4 and CED-3 have recently been shown to protect worms from Salmonella typhimurium infection, thus hinting at a possible connection to pathogen resistance.

The nucleolus is a unique structure in the nucleus that harbors machineries for production of mature ribosomal RNAs and the assembly of ribosomal particles. In line with its role as the factory of protein translation machineries, the nucleolus has direct and profound implications in cellular protein synthesis and molecular homeostasis. Consequently, dysregulated size or abnormal numbers of the nucleolus are frequently observed in many cancer cells, presumably as a response to heightened proliferative and metabolic demands. The tumorigenic link of the nucleolus is further evidenced by the functional interaction with tumor suppressor proteins. For example, in the presence of DNA damage, activated p53 reportedly suppresses RNA Pol I transcription, leading to nucleolar disruption. The notion that the nucleolus acts as intracellular stress sensor is also gaining ground, as its alteration is linked to a multitude of “nucleolar stresses,” including DNA damage, impaired RNA and protein synthesis, nutrient starvation, heat or cold shock, hypoxia, and oxidative stress. Interestingly and perhaps unexpectedly, a role of the nucleolus in microbial resistance has recently emerged, which is characterized by the infection-responsive and pro-survival reduction in expression of a ribosomal RNA-modifying nucleoprotein, fibrillarin. However, how the host cells transduce this nucleolus-associated anti-bacterial signaling remains largely unresolved.

In this study, we identified a regulatory axis of CEP-1-CED-3-USP-33 that is parallel to the previously established let-7-ncl-1-fib-1 cascade in the modulation of nucleolar structure and function in C. elegans. Interestingly, both pathways similarly converge on the nucleolus resident protein fibrillarin. In this capacity, a nucleolar deubiquitinylase (USP36 in human, USP-33 in C. elegans) acts to stabilize fibrillarin, while p53/CEP-1-caspases/CED-3 antagonizes this protein accumulation through caspase cleavage of USP36/USP-33. In addition to nucleolar size enlargement, we found that cep-1 and ced-3 animals exhibit in USP33-dependent manner a greater vulnerability to Staphylococcus aureus and Pseudomonas aeruginosa infection, which was attributable to an altered expression of saposin-like protein 1, spp-1. Together, our findings establish a functional connection of the conserved p53/CEP-1-caspase/CED-3-USP36/USP-33 axis to nucleolar function control and bacterial resistance.

2 | MATERIALS AND METHODS

2.1 | Animal strains

All C. elegans strains were cultured by standard protocols with E. coli (OP50) as food source, as previously described. Strains created in this study and previously established are listed in Table S1.

2.2 | DIC and fluorescence microscopy

Leica DM2500 (Wetzlar, Germany) and Olympus FV10i (Tokyo, Japan) were used to, respectively, acquire DIC and fluorescence images. To precisely discriminate fluorescence intensity between experimental and control worms under the same fluorescence condition, at least five regions of grouped worms were sampled, from which fluorescence intensity was averaged and quantified using Metamorph (Molecular Devices; San Jose, USA) software. Nucleolar size was measured using Photoshop (Adobe; San Jose, USA) software.

2.3 | RNA interference

RNA interference (RNAi) of gene target was done by feeding worms with E. coli expressing specific double-stranded
RNAs, as shown by previous reports.\textsuperscript{25,26} RNAi plasmids were constructed by inserting target gene cDNA into the vector (L4440), followed by transformation into \textit{E. coli} (HT115). Bacteria harboring the RNAi plasmids were grown in LB medium with 100 µg/mL ampicillin and tetracycline at 37°C overnight. After being refreshed in LB medium for 4 hours and further induced by adding 100 µg/mL IPTG for 2 hours, bacteria were allowed to grow on the RNAi plate at 37°C overnight.

2.4 | Immunobloting

All protein samples were boiled in Laemmi sample buffer for 10 minutes and centrifuged at 12,000 \( \times g \), 4°C for 10 minutes. Proteins were separated by 10% or 7% SDS-PAGE and subsequently transferred onto PVDF membranes. Primary antibodies used in this study included GFP (GeneTex; San Antonio, TX, USA), fibrillarin (clone 38F3, Novus; Littleton, CO, USA), Actin (clone C4, Merck Millipore; Temecula, CA, USA), USP36 (polyclone, Thermo Fisher Scientific; Waltham, MA, USA), CASP3 (polyclone, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (monoclone, MyBioSource, San Diego, CA, USA). Detection was done using ECL and UVP system.

2.5 | Real-time quantitative PCR

Total RNAs were isolated from L4 or young adult worms using TRizol. After digestion of genomic DNA by DNase I (Promega; Fitchburg, WI, USA), cDNAs were synthesized from total RNAs by reverse transcription. Each reaction of real-time PCR contained 50 ng cDNA and 1X SsoFast EvaGreen supermix (Bio-Rad Laboratories; Hercules, CA, USA), and was analyzed using the iCycler IQ real-time PCR detection system (Bio-Rad Laboratories). Target gene expression was normalized with the \textit{actin} mRNA level in the respective sample, and presented in fold changes relative to the control group. All primer sequences are listed in Table S2.

2.6 | Caspase-3 activity assay

Worms were lysed by vortex with zirconia beads for 20 5-second pulses on ice. Total protein lysates (100 µg) were then incubated according to the Caspase-Glo 3/7 assay (Promega), followed by activity detection using SpectraMax M2e reader (Molecular Devices; San Jose, CA, USA).

2.7 | Cell-free CED-3 cleavage assay

CED-3 cleavage assay was performed following the standard procedure as described previously.\textsuperscript{9,27} To obtain recombinant proteins as substrates, the \textit{fib-1}, \textit{ced-9}, and \textit{usp-33} cDNAs were first generated by reverse transcription PCR of total RNA collected from N2 worms, and subsequently cloned into pTNT vector (Promega). The \textit{usp-33} point mutant constructs were created using Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). The TNT Quick-Coupled Transcription/Translation Systems (Promega) was used to synthesize with \textsuperscript{35}S-methionine the FIB-1, CED-9, and USP-33 (wild-type, D49A mutant and D285A mutant) proteins. The \textit{ced-3} cDNA was generated also by RT-PCR but cloned into the pET-28a vector. The acCED-3 was expressed from the BL21 (DE3) strain of \textit{E. coli}. All cleavage reactions, with substrates, acCED-3, or inhibitor, were incubated at room temperature for 15-30 minutes, resolved by SDS-PAGE and further analyzed by Typhoon FLA 7000 laser scanner (GE Healthcare; Chicago, IL, USA).

2.8 | \textit{C. elegans} killing assay

The worm-killing assay was performed following standard procedure as described previously.\textsuperscript{23} Young adult worms were transferred to killing plate (tryptic soy agar/TSA medium with \textit{S. aureus} strain HG001, or NGM containing 0.35% peptone with \textit{P. aeruginosa} strain PA14) and scored every 12 hours at 25°C. Animals that died with vulval explosion or crawled off the plate were censored.

2.9 | Cell culture and ectopic gene expression

HeLa cells were cultured in Dulbecco’s modified Eagles’ medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin and streptomycin. Media and reagents were purchased from Thermo Fisher Scientific. Cells were maintained at sub-confluent densities in a 5% CO\(_2\) humidified incubator at 37°C. The USP36 expression plasmid (plasmid ID 22579) was obtained from Addgene. Plasmids were delivered to cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer’s instructions. To induce caspase activity marginally, HeLa cells were treated with low dose (0.5 µM) of staurosporine (Thermo Fisher Scientific) and harvested at the indicated time points. Collected samples were analyzed using western blot and RT-qPCR assays.

2.10 | Apoptosis induction in \textit{C. elegans} by staurosporine treatment

The 1-day adult reporter worms were treated with staurosporine (0.5 µM, 1 µM, and 2 µM) for 2 hours at 20°C in
2.11 Immunostaining

Cells cultured on cover slips were fixed by 4% paraformaldehyde in PBS for 10 minutes on ice and permeabilized using 0.3% Triton-X 100 in blocking buffer (5% normal goat serum in PBS) for 1 hour. The cover slips were sequentially incubated with 1:1000 diluted primary antibodies (anti-USP36 and anti-fibrillarin) and secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and 594 goat anti-rabbit IgG), followed by washes with PBS. Hoechst 33342 (Thermo Fisher Scientific Invitrogen) was used for nuclei counterstaining. All images were captured using Olympus FV10i fluorescence microscopy.

2.12 Bacteria killing and clearance assays

The bacteria killing assay was performed following standard procedure as described previously.23 Human HeLa cells with USP36 overexpression or knockdown were infected by S. aureus (MOI 50) for the indicated time points. Cell viability was measured using LDH assay (Thermo Fisher Scientific) according to manufacturer’s instructions. For monitoring bacteria clearance, THP-1 monocyte cells were transfected with USP36-targeting siRNA (12.5 nM) for 48 hours using Dharmafect-2 (Thermo Fisher Scientific). Differentiation of THP-1 cells into macrophages was then induced by treatment with phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, USA) for 48 hours. To measure pathogen clearance ability of THP-1-derived macrophages, cells were infected S. aureus (MOI 100) for 30 minutes. Extracellular bacteria were subsequently removed from culture by washing with PBS and incubating for 30 minutes in RPMI medium containing 50 µg/mL gentamicin. Infected macrophage cells were then lysed using 0.05% Triton X-100 in PBS, followed by lysates plating on TSA plate for overnight culture. Pathogen clearance ability was measured by counting S. aureus CFU on plates.

2.13 Statistical analyses

All data are presented as mean ± SEM (standard error of mean) or mean ± SD (standard deviation). Statistical analyses were performed with two-tailed Student’s t test for independent samples and with log-rank test for the C. elegans killing assay using GraphPad Prism 6 software (GraphPad, San Diego, USA). Degrees of statistical significance were based on P value, with a value of less than .05 as the cutoff (*P < .05, **P < .01, ***P < .001 and ns, not significant with P > .05).

3 RESULTS

3.1 CEP-1 negatively regulates nucleolar size independent of NCL-1

The dominant role of NCL-1 in nucleolar suppression has been demonstrated through phenotypic characterization of the ncl-1 mutant, which exhibits gain or enlargement of the nucleolar structure.30,31 To screen for an additional suppressor of nucleolar size in C. elegans, we employed a transgenic worm strain, ncl-1(e1942):cguIs1[PFib-1::fib-1::gfp], that expresses ectopic fibrillarin fused with GFP as a reporter in the ncl-1 mutant background.31 In this context, any changes in the nucleolar structure are presumably independent of NCL-1. By virtue of the putative link of cell growth control to the nucleolus, we first focused on p53/CEP-1. Intriguingly, knockdown of cep-1 expression in this transgenic worm via RNA interference (RNAi) feeding resulted in further elevation of FIB-1::GFP reporter protein levels. Quantification of the GFP intensity in fluorescence images (Figure 1A,B) and western blotting (Figure S1A) both revealed greater FIB-1::GFP expression in the cep-1 RNAi knockdown group vs. the mock treatment. To provide further support to phenotypic outcome of cep-1 loss-of-function, we generated a new strain, cep-1(gk138):cguIs1[PFib-1::fib-1::gfp], by crossing the cep-1(gk138) mutant worm with the cguIs1 reporter strain (expressing FIB-1::GFP in the wild-type background) and performed ncl-1 RNAi knockdown. Indeed, loss of ncl-1 in the cep-1(gk138):cguIs1 worms elevated the GFP fluorescence intensity and protein level by about 2.5 folds (Figures 1C,D and S1B). Since the amount of FIB-1 is correlated with the size of the nucleolus,31 this increase in the FIB-1::GFP reporter expression consistently observed in both genetic contexts of cep-1 loss, even in the absence of NCL-1, is highly suggestive of a NCL-1-independent role of p53/CEP-1 in nucleolar size regulation.

To further test this hypothesis, we then measured nucleolar size in germ cells, -2 oocyte and somatic cells, hypodermis 10 cell (hyp10), in worms of various mutant backgrounds. To this end, differential interference contrast (DIC) imaging showed that nucleolar size is enlarged in the oocytes in the cep-1(gk138) and ncl-1(e1942) single mutants as well as in the ncl-1(e1942); cep-1(gk138) double mutant worms (Figure 1E,F). Enlarged nucleoli were also observed in hyp10 cells in the corresponding genetic backgrounds (Figure 1G,H). Conversely, nucleolar
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size in the strain expressing ectopic CEP-1::GFP transgene, cep-1(gk138);cguls1, was smaller in comparison to the wild-type worms (Figure 1E,G, second column; Figure 1F,H). Taken together, these observations indicate that CEP-1 may act as a negative regulator of FIB-1::GFP expression and nucleolar size. Moreover, among the wild-type, single [cep-1(gk138) and ncl-1(e1942)] and double mutant worms tested, the worms with both ncl-1 and cep-1 loss-of-function were found to exhibit the largest nucleoli, reaffirming that CEP-1 acts independently from the NCL-1 pathway.

3.2 | Functional implication of CEP-1-associated caspase cascade in nucleolar size control

After establishing the importance of CEP-1 in nucleolar size regulation, we next sought to examine the connection of downstream apoptosis-related factors—EGL-1, CED-4, and CED-3—to this functional aspect. Depletion of ced-4 by RNAi feeding in the wild-type reporter strain (cguls1) (Figure S2) elevated the GFP fluorescence intensity (Figure 2A,B) and the amount of FIB-1::GFP protein (Figure S2B). However, neither change was detectable when ced-4 RNAi was performed in the cep-1 mutant [cep-1(gk138);cguls1] worms (Figure 2C,D and S2A,C), suggesting that CED-4 function in this capacity is downstream of CEP-1. To further address the functional link of the downstream CED-3, we generated a new strain ced-3(n717);cguls1[Pfib-1::fib-1::gfp] by crossing ced-3 mutant with cguls1 reporter strain, and compared the GFP reporter intensity with that of cguls1. Analogously to the loss of ced-4, ced-3(n717);cguls1 exhibited a higher intensity of GFP fluorescence and FIB-1::GFP expression (Figure S3A-D), thus implicating CED-3 in FIB-1 expression. With respect to nucleolar size, we measured the nucleoli of -2 oocytes and hyp10 cells in

FIGURE 1 CEP-1 acts as a negative regulator of nucleolar size. A, Representative image of the ncl-1(e1942);cguls1 worms with cep-1 (red dotted region) or mock (white dotted region) RNAi shown in the same field. Scale bar: 100 µm. Image shown in inset is magnification of the boxed region. Scale bar: 20 µm. B, Quantitative results of ncl-1(e1942);cguls1 worms, based on the reporter fluorescence intensity in mock vs. cep-1 RNAi (n = 34:33; ***P < .001). C, Representative image of the cep-1(gk138);cguls1 worms with ncl-1 (red dotted region) or mock (white dotted region) RNAi shown in the same field. Scale bar: 100 µm. Image shown in inset is magnification of the boxed region. Scale bar: 20 µm. D, Quantitative results of cep-1(gk138);cguls1 worms, based on the reporter fluorescence intensity in mock vs. ncl-1 RNAi (n = 59:60; ***P < .001). E, Differential interference contrast microscopy analysis of the worms, focusing on -2 oocyte of the gonad E, and the hyp10 cells of the tail regions G. The dotted circles in the images demarcate the nucleoli of cells. Images shown in insets are magnification of the boxed regions. Scale bar: 10 µm. F, H, Quantitative comparison of the overall distributions of nucleolar size in single or double mutants of cep-1(gk138) and ncl-1(e1942), as indicated. Analyses were selectively focused on the -2 oocyte (n = 53:34:46:40:51; ***P < .001) (f) or hyp10 cells (n = 43:27:56:32:47; ***P < .001) (h). The error bars of (b) and (d) are presented as mean ± SEM; the error bars of (f) and (h) are presented as mean ± SD. All data are analyzed using two-tailed Student’s t test.
the egl-1(n1084n3082), ced-4(n1162), and ced-3(ok2734) mutants and compared with those in wild-type and cep-1(gk138) mutants. Figure 2E,F illustrate significant increase in nucleolar size of the four mutants of apoptosis-related genes vs. the wild-type or CEP-1::GFP ectopic-expressed worms. Corresponding to this nucleolar enlargement, the expression levels of FIB-1 protein (Figure 2G,H) and the 26S rRNA (Figure 2I) were markedly higher in mutants of cep-1(gk138), egl-1(n1084n3082), ced-4(n1162), and ced-3(n717) in comparison to the reference strains. Together, these observations provide strong support to the scenario that apoptotic cascade of CEP-1, EGL-1, CED-4, and CED-3 constitutes a functional control checkpoint on FIB-1 expression, rRNA pool, and nucleolar size.

Although CED-3 is directly linked to apoptosis-associated activation of downstream proteases and DNases, we speculated that the role of apoptosis mediators in nucleolar control might be independent of cell death. To resolve this issue, we examined whether CED-3 caspase activity is present in worms in the absence of ongoing apoptosis. In contrast to embryonic, early larval and germline tissues, little or no prominent apoptosis is detectable in the L4 stage worm39,32; worms of this particular developmental stage were then selected for detecting CED-3 caspase activity. In C. elegans, CED-3 is the only caspase that can recognize and target the human caspase-3/7-specific substrate sequence D/EXXD.33 Results from a luminescence assay using specific substrate of caspase-3/7 (Z-DEVD) showed that late L4 worms of wild-type and CEP-1::GFP ectopic-expressed strain exhibited about 200 RLUs (relative luminescence units) of caspase activity (Figure 2J, first column). This activity was further attributed specifically to CED-3 since no discernible caspase activity was evident in all apoptosis-related mutants (Figure 2J). In contrast, CED-3 caspase activities were assayed for whole worms of late L4 (j) or mixed stage (k), with the indicated CED mutant background. Luminescence assay containing specific substrate (Z-DEVD) of the human caspase-3/7 was performed (n, at least three independent experiments). The error bars of (b), (d), and (h-k) are presented as mean ± SEM; the error bars of (e) and (f) are presented as mean ± SD. All data are analyzed using two-tailed Student’s t test.
correlated with apoptosis occurrence, the observation of a basal level of CED-3 caspase activity in non-apoptotic context is indicative of cell death-independent role of caspase cascade in *C. elegans*.

3.3 | CED-3 degrades the FIB-1-stabilizing deubiquitinylase USP-33

To search for a candidate substrate of CED-3, we focused on USP-36, a deubiquitinylase that contains five putative CED-3 cutting sites (17DSAD20, 263DTYD266, 577DSRD576, 1070DDWD1073, and 1074EEFD1077) and reportedly stabilizes fibrillarin in human cells. To then sought to explore the possibility that in *C. elegans* the CEP-1-CED-3 axis mediates FIB-1 protein alteration via targeting USP36 ortholog, USP-33. To provide evidence for the enzyme-substrate relationship between CED-3 and USP-33, cell-free assay was performed and subsequently demonstrated the cleavage of USP-33 (with putative CED-3 cutting sites at 46DKDD49 and 282DILD285) into a smaller fragment (82 to 77 kDa) in the presence of CED-3 (Figure 3A). Two additional observations corroborated the CED-3-specific proteolytic targeting of USP-33: First, the degradation of USP-33 protein was abolished by CED-3-specific inhibitor, zDEVD-fmk. Moreover, when the putative CED-3 cleavage motif in USP-33 was altered by a Asp49Ala (D49A) point mutation, the proteolytic fragmentation of USP-33 was diminished. However, altering another predicted cutting site—by substituting aspartate at the position

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**FIGURE 3** The canonical intrinsic apoptotic pathway mediates nucleolar control through restricting USP-33. A, B, Cell-free CED-3 cleavage assay was performed as described in Materials and Methods. CED-9 (positive control), USP-33 or two CED-3 cleavage site mutant proteins (USP-33 D49A and D285A) were added as substrates in the reaction. zDEVD-fmk is a caspase-specific inhibitor. Black and red asterisks denote full-length and cleaved proteins, respectively. C, Fluorescence microscopy image of *cguls1* worms with usp-33 (red dotted region) or mock (white dotted region) RNAi shown in the same field. Scale bar: 100 µm. Image shown in inset is magnification of the boxed region. Scale bar: 20 µm. D, Fluorescence intensity of the FIB-1 reporter shown in (C) was quantified and comparatively presented for the indicated treatments (n = 30:29; ***P < .001). E, Immunoblotting (top) and quantitative (bottom) analyses of FIB-1::GFP and Actin expression in *cguls1* reporter worms with usp-33 or mock RNAi (n = 3; ***P < .001). F, G, Immunoblotting (f) and quantitative (g) analyses of FIB-1 and Actin expression in wild-type and cep-1::gfp transgenic worms with usp-33 or mock RNAi (n = 3; ns, P > .05, ***P < .001). All error bars in this figure are presented as mean ± SEM. And all data are analyzed using two-tailed Student's *t* test.
285 with alanine—did not influence USP-33 cleavage by CED-3 (Figure 3B). The results showed that USP-33 is targeted by CED-3-mediated proteolysis. To further pinpoint the FIB-1-targeting deubiquitylase in vivo, RNAi experiments were performed on the cguIs1 worms. Results of fluorescence imaging and immunoblotting analysis showed that knockdown of usp-33 decreases the FIB-1::GFP reporter expression (Figure 3C-E). Further knockdown experiment followed by immunoblotting revealed that FIB-1 protein abundance decreased about 30% in usp-33-depleted wild-type worms (Figure 3F,G), confirming that USP-33 positively regulates FIB-1 expression. By contrast, FIB-1 expression remained unchanged when the cep-1::gfp transgenic worms were subjected to usp-33 RNAi knockdown (Figure 3F,G), indicating that this functional aspect of USP-33 may be downstream of CEP-1. Moreover, immunoblotting results showed that altered FIB-1 expression in the cep-1 and ced-3 mutant worms was restored by RNAi depletion of usp-33 (Figure S4A,B). Viewed together, these results are in line with the notion that, rather than acting on FIB-1 per se, the inhibitory effect of CED-3 on FIB-1 expression is exerted via direct turnover of the deubiquitylase USP-33, a FIB-1 stabilizer.

### 3.4 The CEP-1-CED-3-USP-33 axis protects against bacterial infection

Recently, Tiku and colleagues demonstrated that upon bacterial infection worms downregulate fibrillarin as an anti-pathogen protective response; however, the mechanism underlying this fibrillarin reduction remains unresolved. Based on the fibrillarin-targeting regulatory pathway delineated by our study thus far, we thus postulated that CEP-1, CED-3, and FIB-1 would be modulated coordinately when worms are challenged with bacteria. As expected, we found that at 6 and 12 hours post-infection by *Staphylococcus aureus*, the expression of CEP-1 increased by ~2 folds (Figure 4A,B), whereas the activity of CED-3 underwent 1.2-fold and 1.6-fold increases, respectively (Figure 4D). By contrast, the abundance of FIB-1 progressively decreases (by 20% and 50% at 6 hours and 12 hours, respectively; Figure 4A,C). However, this *S. aureus* infection-triggered reduction of FIB-1 expression was not seen in the cep-1(gk138) and ced-3(n717) mutants (Figure S5A,B). We then addressed their roles in microbial immunity by testing whether cep-1(gk138) and ced-3(n717) mutants are more susceptible to *S. aureus* and *Pseudomonas aeruginosa* infection. Killing assay showed that both mutant worms exhibited poorer survival in the presence of these bacteria. Conversely, transgenic worms expressing CEP-1::GFP became more resistant to infection-induced death (Figure 4E,F). Interestingly, in line with the antagonistic relationship to the CEP-1 and CED-3, knockdown of usp-33 gene in the cep-1(gk138) and ced-3(n717) mutants conferred a greater extent of resistance to *S. aureus* and *P. aeruginosa* infection, as illustrated by improved survival rates of the infected worms (Figure 4G,H). We next sought to identify potential effectors that act downstream of FIB-1 reduction during bacterial infection in *C. elegans* by focusing on the antimicrobial peptide genes, which represent the key immune defense effectors in worms. Our quantitative PCR results first showed that *S. aureus* challenge in the N2 worms plenty elevated four antimicrobial peptide genes, *abf-2*, *cnc-2*, *lys-7*, and *spp-1* (Figure 4I). We then found that both of the infection-sensitive mutant worms, cep-1(gk138) and ced-3(n717) expressed significantly reduced level of the saposin-like protein, spp-1 (Figure 4J). Further in line with the role of usp-33 in this pathway, knockdown of usp-33 gene in both of mutant worms restored the spp-1 expression level (Figure 4K,L). Taken together, these results supported the model that the nucleolus-altering CEP-1-CED-3-USP-33 axis is linked to the spp-1-associated restriction of bacterial infection in *C. elegans*, and provided further demonstration that CED-3 is an innate immunity factor.

### 3.5 The nucleolus-linked regulatory axis of p53-caspase-USP36-fibrillarin is conserved in human cells

To substantiate the functional relevance of nucleolar regulatory pathway, we next examined whether the CEP-1-CED-3-USP-33-FIB-1 cascade is also conserved in human cells. Toward this end, we first characterized the effect of human p53 on USP36, which is the worm USP-33 ortholog, and fibrillarin expression. As expected, immunoblotting results showed that ectopic expression of Flag-p53 in HeLa cells resulted in caspase-3 activation and decline in USP36 and fibrillarin expression (Figure S6A). Further consistent with a positive regulation of human USP36 on fibrillarin expression, ectopic-expressed Flag-HA-USP36 in HeLa cells was confirmed to upregulate fibrillarin expression, as shown by western blotting analysis (Figure 5A, lane 4 vs. lane 1). When cells were treated with a low dose of staurosporine, an inducer of p53-caspase-dependent cell death, expression of both USP36 and fibrillarin declined (Figure 5A,C), recapitulating the suppressive role of caspases on these nucleolar proteins. Although our earlier observations in *C. elegans* were based on an apoptosis-free context, we also subjected worms to staurosporine to induce cell death (Figure S7A). Interestingly, we found that the expression of FIB-1 expression was correspondingly decreased in response to staurosporine in wild-type (cguIs1) worms, but not in the ced-3 mutant [ced-3(n717); cguIs1] worms (Figure S7B,C). Finally, we examined the consequences of ectopic USP36 expression and/or staurosporine treatment on three additional
FIGURE 4  The CEP-1-CED-3-USP-33 axis provides bacterial resistance through promoting spp-1 expression in C. elegans. A, N2 worms were infected by S. aureus for the indicated time points. Expression levels of CEP-1 and FIB-1 in were then detected using immunoblotting. B, C, Quantitative analyses of CEP-1 and FIB-1 expression (normalized to Actin) shown in (a) (n = 3, *P < .05, ns, P > .05). D, CED-3 caspase activities were assayed for the worms shown in (a) (n = 3, *P < .05, **P < .01). E, F, Survival analyses were assayed for the indicated worms challenged by S. aureus (e) or P. aeruginosa (f) (n = 72:65:36:93 in (e) and 83:82:78:66 in (f), ***P < .001). G, H, Upon challenge by S. aureus (g) or P. aeruginosa (h), survival analyses were assayed for usp-33 knockdown worms with the indicated mutant backgrounds (n =56:51:70:40 in (g) and 69:78:82:78 in (h), *P < .05, ***P < .001). I, N2 worms were infected by S. aureus for the indicated time points. Infected worms were analyzed by RT-qPCR for antimicrobial peptide mRNA expression. Transcript abundance was normalized to that of actin, and shown relative to the 0 hour group (n = 3; ns, P > .05, *P < .05, ***P < .001). J, Expression levels of endogenous antimicrobial peptide genes in the indicated mutant worms were detected using RT-qPCR analysis. Transcript abundance was normalized to that of actin, and shown relative to control group. Two mutant strains were used: cep-1(gk138) (n = 3; ***P < .001) (k) and ced-3(n717) (n = 6; ***P < .001) (l). All error bars in this figure are presented as mean ± SEM. Data of (b) and (c) were analyzed by using two-tailed Student’s t test, whereas survival assays were analyzed using log-rank test.
FIGURE 5  Expression of USP36 is functionally correlated with p53-dependent caspase-3 activation, nucleolar size, and bacterial resistance in human cells. A, HeLa cells ectopically expressing USP36 (FLAG-HA-tagged) or control (mock) were treated with 0.5 µM staurosporine for the indicated time points. Cells were then harvested for comparative expression profiling. Protein expression of USP36, fibrillarin, CASP3, and GAPDH was analyzed by immunoblotting. B, C, Quantitative results of (a). Immunoreactive signals were quantified and normalized (by Actin) for USP36 (n = 3; ns, P > .05, *P < .05, **P < .001) (b) and fibrillarin (n = 3; ns, P > .05, *P < .05, **P < .001) (c). D, 28S rRNA expression levels of respective samples were determined by RT-qPCR (normalized with Actin) (n = 4; ns, P > .05, *P < .05, **P < .001). E, Immunofluorescence microscopy analysis of fibrillarin and USP36 expression. Images shown in insets are magnification of the boxed regions. Scale bar: 10 µm. F, Quantitative representation of the fluorescence intensity shown in (e), which were normalized by Hoechst intensity of the respective samples (n ≥ 30, **P < .001). G, The nucleolar size in the indicated HeLa cells were quantitatively determined based on images (n ≥ 30, ***P < .001). H, HeLa cells ectopically expressing USP36 (FLAG-HA-tagged) or USP36-targeting siRNA (with the corresponding control) were infected by S. aureus with MOI 50 for 24 hour. LDH assay was performed to monitor extent of cell death (at least three independent experiments, **P < .01). I, Human THP-1 cells treated with USP36-targeting or control siRNA were infected by S. aureus with MOI 100 for the indicated time points (n = 3, *P < .05, ***P < .001). The error bars in this figure are presented as mean ± SEM, except for (g), which is mean ± SD. All data were analyzed using two-tailed Student’s t test.
functional hallmarks of nucleolar activity: expression of large subunit (28S) rRNA (Figure 5D), quantitative distribution of fibrillarin (Figure 5E,F), and nucleolar size (Figure 5G). Our results showed that ectopic USP36 prompted increases in all of these attributes as compared to the control. Conversely, induction of caspases by staurosporine treatment, with or without ectopic USP36 expression, triggered reduction in the levels of 28S rRNA and fibrillarin, as well as smaller nucleolar size. In summary, these results collectively strengthened the p53-caspase-USP36-fibrillarin axis as a pivotal and evolutionarily conserved determinant of nucleolar activity. To address whether this axis also contributes to innate immunity against pathogen infection in human cells, we infected HeLa cells with *S. aureus* and performed cell death analysis by lactate dehydrogenase (LDH) assay. HeLa cells with ectopically expressed USP36 decreased cell survival rate dramatically upon bacterial infection (Figure 5H). In contrast, cells with *USP36* knockdown by siRNA method showed a better cell survival rate to *S. aureus* infection (Figure 5H). Moreover, compared with the control siRNA treatment, *USP36* siRNA knockdown in human THP-1 derived macrophages also enhanced intracellular microbial clearance at 1 and 2 hours post-infection (Figure 5I). Finally, we tested whether the ubiquitinylase-dependent change in saposin expression is also conserved in human. Our results then showed that knockdown of *USP36* in HeLa cells consistently resulted in the elevation of four saposin genes, *AOAH, GNLY, PSAP*, and *PSAPL1* (Figure S8). Taken together, our results indicated that the caspase-USP36-fibrillarin axis constitutes an evolutionary conserved anti-bacterial immune effector function through the nucleolar control.

### 4 | DISCUSSION

As the site of ribosome biogenesis, the nucleolus serves as the starting point for protein and organelle production. Since this functional output is highly correlated with the proliferative and metabolic demand of the cells, dysregulated nucleolar function and structure have frequently been observed in cancers. Functional connection of the nucleolus to cell cycle is further evidenced by a nucleolar surveillance system, in which the cell cycle checkpoint system senses and monitors nucleolar activity. To address whether this axis also contributes to innate immunity against pathogen infection in human cells, we infected HeLa cells with *S. aureus* and performed cell death analysis by lactate dehydrogenase (LDH) assay. HeLa cells with ectopically expressed USP36 decreased cell survival rate dramatically upon bacterial infection (Figure 5H). In contrast, cells with *USP36* knockdown by siRNA method showed a better cell survival rate to *S. aureus* infection (Figure 5H). Moreover, compared with the control siRNA treatment, *USP36* siRNA knockdown in human THP-1 derived macrophages also enhanced intracellular microbial clearance at 1 and 2 hours post-infection (Figure 5I). Finally, we tested whether the ubiquitinylase-dependent change in saposin expression is also conserved in human. Our results then showed that knockdown of *USP36* in HeLa cells consistently resulted in the elevation of four saposin genes, *AOAH, GNLY, PSAP*, and *PSAPL1* (Figure S8). Taken together, our results indicated that the caspase-USP36-fibrillarin axis constitutes an evolutionary conserved anti-bacterial immune effector function through the nucleolar control.
linked to inflammasome formation, embryonic stem cell differentiation, and neuron outgrowth, guidance attraction, and branching. Importantly, as one of the ancient caspases in animals, CED-3 is the sole caspase encoded by *C. elegans* and presumably endowed with the manifold cellular roles not yet diversified by evolution. This notion of “multitasker” is evidently supported by our study, which illustrated CED-3’s roles in both innate immunity and nucleolus control.

In line with p53’s critical role in mediating proliferation cessation in response to genotoxic stresses, loss of p53 function is viewed as a dominant selective determinant underlying malignant transformation. Consequently, agents that elevate p53 tumor-suppressive activity represent potentially viable anti-cancer therapy. This notion is evidenced by multiple small molecule drugs recently developed for activating p53, such as RG7338, which acts to inhibit MDM2-mediated p53 degradation, and PK7088, a small molecule that restores transcriptional function of the p53 Y220C mutant. However, despite their positive effects on p53, the promiscuous mutations that inactivate *TP53* during tumor progression render these drugs ineffective as broad-spectrum therapeutics. Interestingly, our observations of USP36’s pro-nucleolar functions and also its functional antagonism to p53’s growth control suggest that USP36 may be a tumor promoter. Viewed together with the recent findings that USP36 is a stabilizer of c-Myc oncoprotein and its pro-proliferative function, these results hint at the possibility that blocking USP36 activity by drugs or by genetic activation of caspase may be a viable alternative for cancer treatment, even in a *TP53* mutation background.

Furthermore, based on its role in microbial immunity, drugs targeting USP36 could hold additional therapeutic potential as antibiotic substitute for bacterial infection, making this deubiquitinylase an important target in the clinics.
ACKNOWLEDGMENTS
We thank CGC (Caenorhabditis Genetics Center) for providing worm strains, Dr. Mei-Hui Lin and her Ph.D. student Chao-Chin Liu for providing S. aureus strain HG001, equipment and suggestions on the bacterial infection assay and Dr. Cheng-Hsun Chiu for providing P. aeruginosa strain PA14. Special thanks to Hsin-Yu Chen from Dr. Scott Schuyler lab for suggestions on the autoradiograph assay, Dr. Pei-Chien Tsai for statistical analysis consultation, and Drs. Thoru Pederson, Jiahuai Han, Yi-Chun Wu, Bon-Chu Chung, Fu-Tung Liu, Siamon Gordon, Sebastian Fugmann, and Adam Antebi for reading and commenting on this manuscript. This work is supported by grants from the Ministry of Science and Technology of Taiwan (MOST104-2320-B-182-029-MY3 and MOST105-2314-B-182-061-MY4 to BC-MT), Chang Gung Memorial Hospital (CMRPD1F0442, CMRPD1H0021 and BMRP960 to BC-MT, and BMRP742 to SJL) and the Ministry of Education of Taiwan (EMRPD1C0051 to SJL).

AUTHOR CONTRIBUTIONS
Po-Hsiang Chen and Szecheng J. Lo conceived the original idea; Po-Hsiang Chen, Bertradd Chin-Ming Tan, and Szecheng J. Lo designed the experiments; Po-Hsiang Chen, Yi-Tung Chen, Tai-Ying Chu, Tian-Hsiang Ma, and Mei-Hsuan Wu performed the experiments; Po-Hsiang Chen, Hsi-Hsien Lin, Yu-Sun Chang, Bertradd Chin-Ming Tan, and Szecheng J. Lo analyzed the data; Po-Hsiang Chen, Szecheng J. Lo, and Bertrand Chin-Ming Tan wrote the paper.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Chen P-H, Chen Y-T, Chu T-Y, et al. Nucleolar control by a non-apoptotic p53-caspases-deubiquitinylase axis promotes resistance to bacterial infection. The FASEB Journal. 2020;34:1107–1121. https://doi.org/10.1096/fj.201901959R