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Short communication

CHIP E3 ligase regulates mammalian senescence by modulating the levels of oxidized proteins

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ABSTRACT

Senescence can be induced by various stressors including oxidative stress. It has been reported that CHIP (C-terminus of Hsp70-interacting protein) ligase is induced during senescence while CHIP$^{-/-}$ mice exhibit accelerated aging. Here, we explore the effects of modulating CHIP expression on mammalian senescence. We demonstrate that CHIP silencing induces premature senescence that is accompanied by elevated levels of oxidized proteins. On the contrary, ectopic expression of CHIP leads to oxidized proteins levels reduction. Moreover, we reveal that CHIP$^{-/-}$ mouse fibroblasts have an impaired ubiquitin proteasome system. Taken together, we propose that CHIP influences cellular senescence by modulating the oxidative load.

Normal mammalian fibroblasts undergo a limited number of divisions in culture and progressively reach a state of irreversible growth arrest, a process termed replicative senescence. Cellular senescence can also result from exposure to different stressors including oxidative stress (Chen et al., 1998) and inhibition of critical pathways such as the proteasome-mediated degradation (Chondrogianni et al., 2008). The C-terminus of Hsp70-interacting protein (CHIP) is a "quality control protein" being implicated in both these processes (Murata et al., 2003). CHIP is a chaperone-associated E3 ligase, which possesses U-box-dependent ubiquitin ligase activity as well as co-chaperone/chaperone activity (Connell et al., 2001). Various studies have shown that CHIP facilitates the switch from chaperone-mediated folding/maturation to proteasome-mediated degradation via ubiquitination (McClellan et al., 2005; Marques et al., 2006). CHIP is also induced by various stresses (Al-Ramahi et al., 2006; Dikshit and Jana, 2007; Jana et al., 2005; Meacham et al., 2001) and, interestingly, CHIP knock out mice exhibit reduced longevity associated with an accelerated aging phenotype (Min et al., 2008). Following these studies, we have recently reported that CHIP ligase is gradually activated during cellular senescence and this facilitates targeting of CHIP substrates to the proteasome. Moreover, we have shown that the reduced levels of molecular chaperones in combination with the elevated levels of CHIP during replicative senescence lead to the chaperone-dependent degradation of p53, a key modulator of senescence (Sisoula et al., 2011).

Given the above, in this study we have explored the effects of CHIP expression modulation on mammalian fibroblasts senescence. In specific, we have silenced CHIP using siRNA technology and we have assessed the effects on the proliferation of human embryonic lung fibroblasts HFL-1. As shown in Fig. 1, CHIP silencing results in premature senescence as shown by the irreversible cellular growth arrest (Fig. 1A), the induction of p16 (Fig. 1B) and the dramatic accumulation of senescent β-gal positive cells (Fig. 1C). In order to evaluate the dependence of CHIP-mediated premature senescence on the oxidative load, HFL-1 cells were co-treated with siCHIP and the antioxidant N-acetyl-cysteine (NAC) (Fig. 1A). We have found that NAC can cause partial/minimal rescue effect on the proliferation of siCHIP-transfected cells, by delaying premature senescence approximately 10 days in siCHIP/NAC-treated cells. Next, we have determined the levels of intracellular oxidized proteins following manipulation of CHIP expression. As shown in Fig. 2A, CHIP silencing results in accumulation of oxidized proteins *per se* in HFL-1 fibroblasts. On the contrary, ectopic expression of CHIP led to reduction of oxidized proteins in HFL-1 cells treated with H_2O_2 (Fig. 2B, lane 5 versus lane 4). Taking into account the co-chaperone role of CHIP ligase (Connell et al., 2001), we analyzed the possibility the

Abbreviations: CHIP, C-terminus of Hsp70-interacting protein; cpds, cumulative population doublings; E3, ubiquitin ligase enzyme; GFP, green fluorescent protein; MEFs, mouse embryonic fibroblasts; NAC, N-acetyl-cysteine; siRNA, small interfering RNA; UFD, ubiquitin fusion degradation; UPS, ubiquitin-proteasome system; 17AAG, 17-allylaminogeldanamycin.

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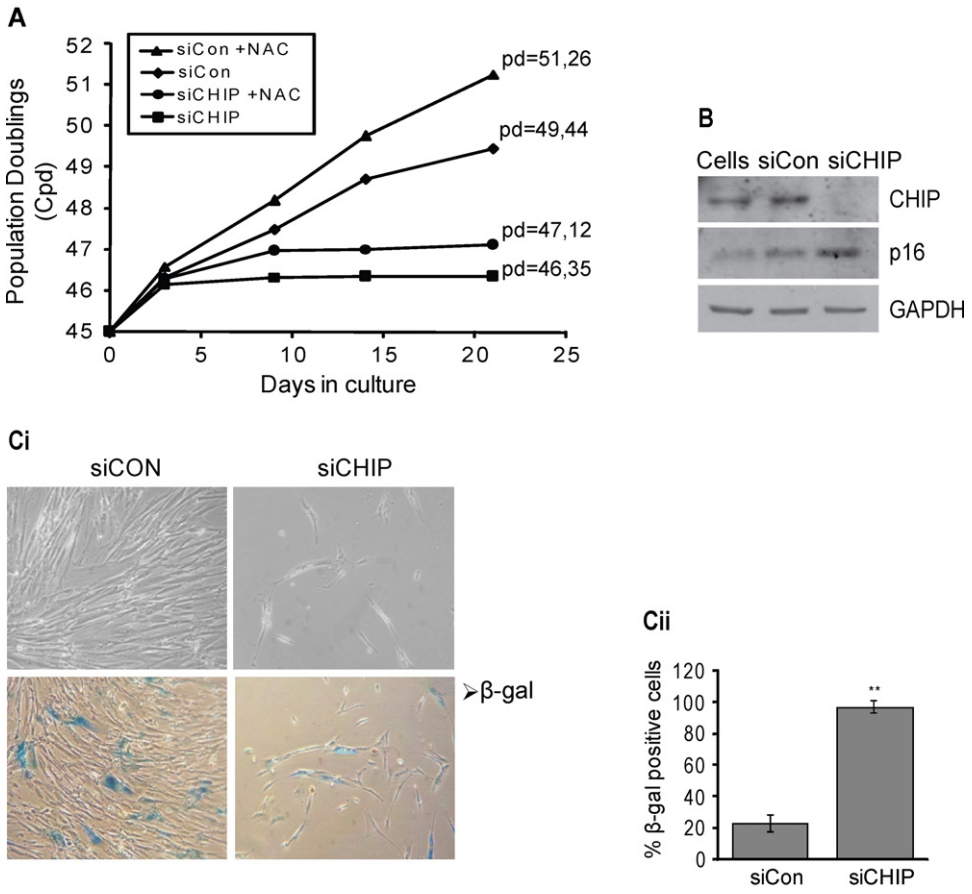

Fig. 1. Knockdown of CHIP expression results in induction of premature senescence in HFL-1 fibroblasts. (A) Number of cumulative population doublings (cpds) of HFL-1 cells transfected with 75 nM siCHIP or scrambled siRNA (siCon), using Lipofectamine 2000 (Invitrogen Ltd., Carlsbad, CA, USA), constantly every 3 days for up to 21 days. For co-incubation with the antioxidant NAC, 2 mM NAC were added in the cultures for the appropriate time periods. Numbers of the graph show the pd measured on the 21st day of the treatment. Data presented are representative of at least three independent trials by using two different sets of CHIP-specific siRNA oligonuceotides (SMARTpool; Dharmacon, Lafayette, CO, USA). (B) Immunoblot analysis of CHIP ligase (anti-CHIP antibody was purchased from Abcam Plc, Cambridge, UK) in non-, siCon- and siCHIP-transfected cells at the end of the treatment. GAPDH levels were used as loading control, whereas p16 confirmed the senescent status (anti-GAPDH and anti-p16 antibodies were purchased from Santa Cruz Biotechnology, CA, USA). (Ci) Representative photographs of cells (upper panel) and cells following β-galactosidase activity staining (lower panel), as also (Cii) percentage of β-galactosidase positive cells. Values represent the average of three independent experiments $\pm$ SD and the asterisks report statistical significance at $p < 0.01$ (**).

observed differences in the levels of oxidized proteins to be due to CHIP-chaperones interaction. It is well established that CHIP is a bona fide interaction partner of the major cytoplasmic chaperones, namely the heat shock proteins Hsp90/Hsp70 (Ballinger et al., 1999). When the cellular proteins adopt unfolded conformations, CHIP is the responsible ligase for inducing their ubiquitination and subsequent proteasomal degradation. In particular, Hsp90 is responsible for the proper folding of these proteins and when its activity is low, the unfolded proteins will be escorted to the proteasome for degradation (Neckers and Neckers, 2005). Thus, the geldanamycin analogue 17AAG, an Hsp90 inactivator, which blocks Hsp90 activity (Stebbins et al., 1997) and therefore, the proper folding of its substrates, was used. As shown in Fig. 2C, inactivation of Hsp90 did not affect the oxidative load of the cells (Fig. 2C, lane 2 versus lane 1 and lane 6 versus lane 4) in comparison with the elevated levels of oxidized proteins because of the CHIP knockdown (Fig. 2C, lane 5 versus lane 3).

To further confirm these findings, similar experiments were also performed in wild type mouse embryonic fibroblasts (MEFs) as well as in MEFs derived from knock out ($CHIP^{-/-}$) mice (Min et al., 2008). These cell lines were transfected with two different fluorescent reporters, namely UFD-GFP and GFP-CL1. UFD-GFP and GFP-CL1 reporters are green fluorescent protein (GFP)-based proteasome substrates consisting of specific degradation signals, which behave as "modulator domains" that can target proteins for ubiquitination and proteasomal degradation, fused to the GFP. Specifically, in the case of the ubiquitin fusion degradation (UFD)-GFP reporter, an N-terminal ubiquitin moiety fused to the GFP open reading frame functions as an acceptor for poly-ubiquitin chains, which results in ubiquitination and proteasomal degradation (Dantuma et al., 2000). The second reporter has been generated using the CL-1 degradation signal, a small 16-amino-acid motif, the introduction of which converts GFP into a small-lived proteasome substrate (Bence et al., 2001). Accumulation of these GFP-substrates indicates impaired ubiquitin-proteasome system (UPS) in the transfected cells (Neefjes and Dantuma, 2004). As shown in Fig. 3A, $CHIP^{-/-}$ MEFs exhibit a defective UPS in comparison to wild type MEFs as assayed by both reporters. Usage of the proteasome inhibitor, MG132, further enhances this defect in $CHIP^{-/-}$ MEFs. Moreover, and in agreement with the data reported here in human HFL-1 cells, $CHIP^{-/-}$ MEFs show elevated levels of oxidized proteins as compared to wild-type cells (Fig. 3B). Finally, overexpression of CHIP in wild-type MEFs results in decreased levels of oxidized proteins *per se*, as well as following treatment with H_2O_2 or metal catalyzed oxidation (Fig. 3Ci). Furthermore, cells overexpressing CHIP can cope better with oxidative stress as judged by their morphology after $FeCl_3$ treatment (Fig. 3Cii).