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Research paper

Loss of the ubiquitin conjugating enzyme UBE2E3 induces cellular senescence

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ABSTRACT

Cellular senescence plays essential roles in tissue homeostasis as well as a host of diseases ranging from cancers to age-related neurodegeneration. Various molecular pathways can induce senescence and these different pathways dictate the phenotypic and metabolic changes that accompany the transition to, and maintenance of, the senescence state. Here, we describe a novel senescence phenotype induced by depletion of UBE2E3, a highly-conserved, metazoan ubiquitin conjugating enzyme. Cells depleted of UBE2E3 become senescent in the absence of overt DNA damage and have a distinct senescence-associated secretory phenotype, increased mitochondrial and lysosomal mass, an increased sensitivity to mitochondrial and lysosomal poisons, and an increased basal autophagic flux. This senescence phenotype can be partially suppressed by co-depletion of either p53 or its cognate target gene, $p21^{CIP1/WAF1}$, or by co-depleting the tumor suppressor $p16^{INK4a}$. Together, these data describe a direct link of a ubiquitin conjugating enzyme to cellular senescence and further underscore the consequences of disrupting the integration between the ubiquitin proteolysis system and the autophagy machinery.

1. Introduction

Cellular senescence has emerged as a critical mechanism by which organisms suppress tumor proliferation and maintain tissue homeostasis and optimal wound healing. Paradoxically, the transition of cells to a state of senescence in particular anatomical niches has been implicated in the development of age-related neurodegenerative diseases [4]. The recognition of these dual roles for senescence has led to in-depth investigations of the pathways that drive proliferating cells, or those that are differentiated but have retained a proliferative potential, and even post-mitotic neurons [18] into an irreversible cell cycle arrest and senescence. These studies have uncovered numerous mechanisms by which senescence can be induced and maintained. The pathways range from genomic damage and the accompanying DNA damage response (DDR) to mitochondrial dysfunction associated senescence (MiDAS) to oncogene-induced senescence (OIS) [47]. Perhaps one of the most intriguing facets of how cells become senescent is that the metabolic and phenotypic changes that ensue differ depending on the initiating event. For example, a distinguishing hallmark of DDR-mediated senescence is a senescence-associated secretory phenotype (SASP) characterized by activation of IL-1β, NF-κB, IL-6 and their respective downstream signaling pathways. Yet, this inflammatory arm is not a prominent component of cells that become senescent through MiDAS, which is distinguished by a blunted inflammatory arm of SASP and expression of IL-10, CCL27, and AREG [48].

The ubiquitin (Ub) proteasome system (UPS) is comprised of a network of enzymes and activities that collectively regulate the stability, localization, and function of many intracellular proteins. The central protein of the UPS is Ub, a highly conserved, 76 amino acid polypeptide that is post-translationally conjugated onto cognate substrates. The attachment of Ub to target proteins is mediated by a hierarchal cascade of enzymes consisting minimally of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub protein ligase (E3). The human genome encodes 2 E1s, 40–60 E2s, and 600–1000 E3s and substrate selection is conferred through the pairing of particular E2-E3 combinations (reviewed in [50]). *In vitro* and in vivo evidence established that a single E2 can partner with multiple E3s and vice versa. E3s can be single proteins or multi-subunit complexes.

Over the past decade, additional factors have been identified that facilitate the specificity of Ub conjugation to substrates but the E1-E2-

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E3 axis constitutes the core machinery. Akin to kinases and phosphatases, the ubiquitylation of substrates is countered by the trimming action of de-ubiquitylating enyzmes (DUBs). These enzymes, which are either thiol proteases or metalloenzymes, deconstruct Ub chains and thereby counter the synthetic activity of the E1-E2-E3 conjugation machinery. Substrates can be modified with monoUb or with polyUb chains or with both, and the consequences of ubiquitylation are in turn governed by factors including the number of Ub molecules attached, their configuration and topology, and the binding proteins that recognize monoUb and different forms of polyUb [21,43,49]. The best-studied consequence of polyUb synthesis on target substrates is to deliver the marked protein to the 26 S proteasome for degradation. The 26 S proteasome is a macromolecular assembly of proteases that cleaves substrates to peptides. The resulting peptide fragments are cleaved by cytoplasmic peptidases into amino acids or consumed *en bulk* for hydrolysis by the lysosome.

Over the past decade, studies have converged to reveal that ubiquitylation and the autophagy system cooperate to target damaged and dysfunctional organelles as well as invading bacteria for degradation via the autophagy-lysosomal system (reviewed in [12]). For example, the UPS E3 ligase parkin and its activating partner kinase, PINK1, have been shown to decorate damaged mitochondria with polyUb chains that serve as an initiating signal for elimination of these organelles by a specialized type of autophagy termed mitophagy (reviewed in [16,27]). This and similar discoveries highlight the extent to which Ub integrates the UPS and autophagy systems, and it is within this context that we have been investigating the metazoan enzyme, UBE2E3.

UBE2E3 is an E2 that partners with multiple E3 ligases to conjugate monoUb onto substrates [28]. The enzyme is highly conserved; the mouse and human protein sequences are identical. We reported an essential role for UBE2E3 in cell proliferation as knockdown of the enzyme causes a robust increase in $p27^{Kip1}$ and an accompanying cell cycle exit [32]. More recently, we demonstrated that depletion of the enzyme causes a dramatic redistribution of the normally reticular mitochondrial network [34]. This collapse of the mitochondrial network into a perinuclear tangle is accompanied by a re-localization of the anti-stress transcription factor Nrf2 from the nucleus to the mitochondrial tangle and a concomitant decrease in Nrf2 transcriptional activity [34]. Because cell cycle exit, disruption of mitochondrial homeostasis [48], and mis-localization of Nrf2 [22] have all been independently associated with cellular senescence and premature aging, and are all induced by UBE2E3 knockdown [32–34], we investigated whether the loss of UBE2E3 can drive proliferating cells into senescence.

Here we report that cellular senescence resulting from depletion of UBE2E3 is independent of DNA damage and is characterized by a distinct SASP profile, an increase in mitochondrial and lysosomal mass, a dependence on the expression of the tumor suppressor $p16^{INK4a}$ and on the nuclear expression of p53 and $p21^{CIP1/WAF1}$, and an increased basal autophagic flux. This senescence signature is distinguished from the previously defined DDR, OIR, and MIDAS senescence pathways. Moreover, this work provides the first direct evidence that suppressing the expression of a specific metazoan ubiquitin conjugating enzyme causes cellular senescence.

2. Materials and methods

2.1. Cell culture, siRNA transfections, stable cell lines, starvation

RPE-1 cells were cultured and transfected as described [30] and stable cell lines were constructed as described [30]. RPE-1 cells stably expressing GFP-LC3 were starved in Krebs-Ringer Solution containing Sodium Bicarbonate (Alfa Aesar cat# J67591) and 1 × Pen/Strep for 2 h.

Cell Line	Plasmid	Selection
Mito-Tomato		Puromycin
Mt-mKeima	pCHAC-mt-mKeima was a gift from Richard Youle (Addgene plasmid # 72342)	
GFP-LC3	pBABEpuro GFP-LC3 was a gift from Jayanta Debnath (Addgene plasmid # 22405)	Puromycin
roGFP	Gift from S. James Remington (Univ of Oregon)	Puromycin
Mito-roGFP	Gift from S. James Remington (Univ of Oregon)	Puromycin

2.2. Reagents, chemicals, siRNA, and antibodies

Antibody	Catalog#	Company	IF dilution	Western dilution
LaminB1	Ab16048	ABCAM	1:1000	
pH2A.X S139	05–636	Millipore	1:5000	
p53	SC-126	Santa Cruz	1:1000	1:1000
p-p53 Ser15	9284	Cell Signaling		1:1000
$p21^{CIP1/WAF1}$	2947	Cell Signaling	1:1000	
Actin-HRP	HRP-60008	Proteintech		1:20,000
HMBG1	Ab18256	ABCAM		1:1000

Chemical	Catalog#	Company	Concentration
Etoposide	E1383	Sigma	25 μM
Hydrogen Peroxide	95321	Sigma	1 mM
FCCP	BML-CM120	Enzo Life Sciences	5 μM
TMRE	70016	Biotium	5 nM
MitoTracker Green	M7514	ThermoFisher	200 nM
AndyFluor488 Annexin V	A034	Genecopoeia	5 μg/ml
Lysotracker Red	L7528	ThermoFisher	50 nM
Chloroquine	sc − 205629	Santa Cruz	50 μM

siRNA	Catalog #	Company
siControl	D-001220-01-20	Dharmacon
siUBE2E3	D-008845-02/D-008845-03	Dharmacon
sip53	Sc-29435	Santa Cruz
$sip21^{CIP/WAF1}$	6456	Cell Signaling
$sip16^{INK4a}$	DRHNO-000001	Dharmacon

2.3. Measurement of secreted IL-6

Human IL-6 Quantikine ELISA Kit (Cat# D-6050 R&D Systems) was used as per the manufacturer's recommendation to measure the secreted IL-6 in the media from 400,000 cells over 24 h, 10 days post treatment.