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p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes

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Cellular senescence irreversibly arrests proliferation in response to potentially oncogenic stress. Senescent cells also secrete inflammatory cytokines such as IL-6, which promote age-associated inflammation and pathology. HMGB1 (high mobility group box 1) modulates gene expression in the nucleus, but certain immune cells secrete HMGB1 as an extracellular Alarmin to signal tissue damage. We show that nuclear HMGB1 relocalized to the extracellular milieu in senescent human and mouse cells in culture and in vivo. In contrast to cytokine secretion, HMGB1 redistribution required the p53 tumor suppressor, but not its activator ATM. Moreover, altered HMGB1 expression induced a p53-dependent senescent growth arrest. Senescent fibroblasts secreted oxidized HMGB1, which stimulated cytokine secretion through TLR-4 signaling. HMGB1 depletion, HMGB1 blocking antibody, or TLR-4 inhibition attenuated senescence-associated IL-6 secretion, and exogenous HMGB1 stimulated NF-κB activity and restored IL-6 secretion to HMGB1-depleted cells. Our findings identify senescence as a novel biological setting in which HMGB1 functions and link HMGB1 redistribution to p53 activity and senescence-associated inflammation.

Introduction

Cellular senescence suppresses cancer by arresting the proliferation of cells at risk for malignant transformation (Campisi, 2001; Prieur and Peeper, 2008). Many potential oncogenic events initiate senescence, including telomere dysfunction, severe DNA damage, oncogenes, and disrupted chromatin. These events activate tumor suppressor pathways governed by p53 and pRB, transcriptional regulators that establish and maintain the senescence

arrest (Herbig et al., 2006; Campisi and d'Adda di Fagagna, 2007). p53 arrests the cells primarily by inducing the p21 gene, which inhibits cyclin-dependent kinases and cell cycle progression; pRB imposes the arrest primarily by assembling repressive chromatin at pro-proliferative genes.

The senescence arrest was long considered a cell-intrinsic process (Hayflick, 1965). However, recent findings identified secreted proteins that partly reinforce the arrest in an autocrine manner (Kortlever et al., 2006; Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008). These proteins comprise a larger senescence-associated secretory phenotype (SASP), featuring growth factors, proteases, and inflammatory cytokines that affect neighboring cells in a paracrine manner (Parrinello et al., 2005; Bavik et al., 2006; Coppé et al., 2008, 2010b). The senescence response might be antagonistically pleiotropic (Campisi, 2003). That is, it protects organisms from cancer early in life, but the accumulation of senescent cells in late life can drive age-related disease, including, ironically, cancer. Because both cancer and aging are fueled by inflammation (Coussens and Werb,

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Figure 1. **HMGB1 relocalizes in SEN human cells.** (A) Whole-cell lysates (WC) of 10^5 WI-38 human fibroblasts, PRE or made SEN by X-irradiation (XRA), replicative exhaustion (REP), p16^{INK4a} overexpression (p16) or oncogenic RAS, were analyzed for HMGB1 and actin (control) by Western blotting. (B) IMR-90 fibroblasts described in A were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Parallel cells were labeled with BrdU

2002; Franceschi, 2007), the SASP can, in principle, account for the antagonistic pleiotropy.

It is not clear how the nuclear events that regulate the senescence arrest relate to the SASP. Neither pRB nor p53 are required for the SASP, and p53 deficiency amplifies the SASP (Coppé et al., 2008, 2011). However, DNA damage response (DDR) proteins that act upstream of p53 (ATM, NBS1, CHK2, H2AX) are required for the SASP (Rodier et al., 2009, 2011). Still, it is not known how senescence-inducing stimuli cause the widespread changes in gene expression required for the SASP.

HMGB1 is an abundant, evolutionarily conserved non-histone protein (Goodwin et al., 1973) that bends DNA to provide transcription factor access to promoter regions (Grosschedl et al., 1994). One such factor is p53 for which HMGB1 increases DNA binding and transactivation activity (Jayaraman et al., 1998; Banerjee and Kundu, 2003). HMGBI has a surprising additional function—as a secreted protein (Wang et al., 1999a) with a central role in inflammation caused by cell or tissue damage (Bianchi, 2007; Yamada and Maruyama, 2007). HMGB1 passively leaks from necrotic, but not apoptotic, cells, is actively secreted by pathogen-stimulated macrophages, and mediates the potentially lethal inflammation caused by endotoxins (Gardella et al., 2002). Extracellular HMGB1 binds cell surface RAGE (receptor for advanced glycation end-products) and TLRs (toll-like receptors) to initiate signaling that culminates in the expression of inflammatory cytokines such as interleukin (IL)-6 (Park et al., 2003, 2006; Lotze and Tracey, 2005; Schiraldi et al., 2012). Recent findings suggest HMGB1 complexes with extracellular molecules such as single-stranded DNA or IL-1β to promote inflammation (Ivanov et al., 2007; Tian et al., 2007; Sha et al., 2008).

HMGB1 is an Alarmin family member. Alarmins function intracellularly, but upon cellular stress or damage are actively secreted, whereupon they provoke an innate immune response. HMGB1 is also a component of damage-associated molecular patterns (DAMPs), which act and reside intracellularly but are secreted upon cellular damage. Extracellular DAMPs induce a sterile inflammatory response (Bianchi, 2007, 2009; Andersson and Tracey, 2011).

We show that HMGB1 is secreted by senescent cells very early after a senescence-inducing stimulus, before development of the SASP. In contrast to the SASP, HMGB1 secretion depended on p53, but not ATM, and disrupted HMGB1 levels induced a p53-dependent arrest. Importantly, secreted HMGB1 was essential for optimal secretion of IL-6 and MMP-3, prominent SASP components.

Results

Senescent human cells secrete HMGB1

We examined normal human fibroblasts (WI-38) before (PRE) and after we induced them to senesce (SEN) by X-irradiation

(10 Gy; XRA), replicative exhaustion (REP), overexpression of the p16^{INK4a} tumor suppressor (p16; which activates pRB), and oncogenic RAS^{V12} (RAS). Each stimulus caused a striking loss of intracellular HMGB1, determined by Western blotting (Fig. 1 A). As expected (Dimri et al., 1995; Coppé et al., 2008), each stimulus induced senescence, determined by enlarged morphology (not depicted), low BrdU incorporation (growth arrest), and senescence-associated β-galactosidase (SA-β-Gal) activity (Fig. 1 B).

By immunostaining, HMGB1 was abundant in PRE nuclei (Fig. 1 B), as reported for most cells (Falciola et al., 1997). However, SEN cells had barely detectable nuclear HMGB1 (some had faint cytoplasmic staining; Fig. 1 B). Reduced nuclear HMGB1 occurred in several SEN human fibroblast strains (fetal lung, neonatal foreskin, placenta), as well as mammary and prostate epithelial cells (Fig. S1, A–D), indicating it is not strain- or cell type–specific. Further, it was not due to necrosis. SEN cells are viable and metabolically active (Campisi and d'Adda di Fagagna, 2007), and no senescence inducer caused cell loss or deterioration, as determined by visual inspection and release of lactate dehydrogenase into the culture media (Fig. 1 C). Thus, loss of nuclear HMBG1 is a hallmark of SEN cells, regardless of inducer or tissue of origin. In this regard, HMGB1 differs from high mobility group A (HMGA) proteins, which accumulate on chromatin in SEN cells (Narita et al., 2006).

To determine whether SEN cells secrete HMGB1, we used ELISAs and Western blotting to analyze whole-cell (WC) lysates and conditioned media (CM) from cells induced to senesce by XRA or REP (Fig. 1, D and E). In the case of XRA, which induces senescence synchronously, we detected high levels of HMGB1 in CM 24 h after irradiation (Fig. 1 D). In contrast to certain other cells in which secreted HMGB1 is in exosomes or insoluble fractions (Liu et al., 2006), HMGB1 secreted by SEN cells was predominantly soluble (Fig. S1 E). Pellet fractions of CM from SEN and PRE cells contained similar HMGB1 levels, even after treatment with detergent to disrupt possible exosomes (Fig. S1 E). Similar results were obtained using CM from REP SEN cells (Fig. 1 E). Thus, nuclear HMGB1 loss and its secretion occurred 1 d after XRA, preceding the SASP and SA-β-Gal expression, which initiate after ∼3–5 d and peak after 7–10 d after XRA (Coppé et al., 2008). A lower XRA dose (0.5 Gy), which causes modest DNA damage but not senescence (Rodier et al., 2009), did not cause HMGB1 relocalization (not depicted).

We conclude that HMGB1 redistribution from the nucleus to the extracellular space is a common, early response to senescence-causing stimuli.

Senescent mouse cells lose nuclear HMGB1 in culture and in vivo

HMGB1 activities are conserved throughout mammalian evolution (Dumitriu et al., 2005), so we asked whether SEN mouse cells also secrete HMGB1. Immunostaining showed abundant

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(48 h) and stained for SA-β-Gal activity; values shown are percentage of cells. Bar, 10 µM. (C) Cytotoxicity was determined by lactate dehydrogenase in CM from IMR90 cells. Error bars are the standard deviation of triplicate samples. (D) At the indicated time after XRA, we analyzed CM from IMR90 cells for HMGB1 by ELISA. Shown is the fold increase over cells made quiescent by culture in 0.2% serum for 3 d. Error bars are the SEM of triplicate samples. (E) CM and whole-cell lysates (WC) were collected from PRE and duplicate REP IMR-90 cells and analyzed by Western blotting for HMGB1 and actin. Expression relative to actin is shown below each lane.

nuclear HMGB1 in PRE mouse embryo fibroblasts (MEFs); upon senescence induced by XRA, nuclear HMGB1 was barely detectable (Fig. 2 A). Further, Western blotting showed WC lysates from XRA SEN MEFs contained little HMGB1 compared with PRE MEFs (Fig. 2 B), whereas CM from SEN MEFs contained abundant HMGB1 (Fig. 2 C).

To determine whether senescence depleted nuclear HMGB1 in vivo, we immunostained tissues from unirradiated mice and mice given whole-body XRA (9 Gy), which causes persistent hallmarks of senescence (DNA damage foci; p16[INK4a] expression) in several mouse tissues (Rodier et al., 2009; Le et al., 2010). HMGB1 was largely nuclear in unirradiated kidney cells, with <10% showing no or only faint nuclear staining (Fig. 2 D). 1 wk after XRA, ∼80% of kidney cells had faint nuclear and/or detectable cytoplasmic HMGB1 staining (Fig. 2, D and E). We also examined the brain after XRA. As reported (Müller et al., 2004), unirradiated brain stained weakly for HMGB1, but the staining was mostly nuclear; after XRA, nuclear staining diminished (Fig. S1 F).

Because SEN cells increase with age in mammalian tissues (Dimri et al., 1995; Herbig et al., 2006; Wang et al., 2009), we examined kidneys from young (4 mo) and old (32 mo) mice for nuclear HMGB1 (Fig. 2 F). Qualitatively, kidneys from old mice appeared to contain more cells that lacked nuclear HMGB1. Quantitatively, sera from old mice contained on average significantly more HMGB1 than sera from younger animals (Fig. 2 G; 4 and 12 mo; P < 0.001).

Our data support the idea that SEN cells increase with age, and validate loss of nuclear HMGB1 as a conserved feature of SEN mouse cells in culture and in vivo.

Senescent cells secrete acetylated HMGB1

HMGB1 secretion by macrophages depends on acetylation, which prevents nuclear reentry and allows packaging into secretory lysosomes (Gardella et al., 2002; Raucci et al., 2007). To determine whether acetylation regulates HMGB1 localization in SEN cells, we incubated PRE human fibroblasts with trichostatin A (TSA), a protein deacetylase inhibitor that promotes rapid loss of nuclear HMGB1 in mouse macrophages (Bonaldi et al., 2003), and immunostained with pan-acetyl-lysine (Ac-Lys) and HMGB1 antibodies 4 h later. Control cells showed prominent nuclear HMGB1 and moderate Ac-Lys staining, as expected; TSA increased Ac-Lys staining and markedly reduced nuclear HMGB1 staining (Fig. 3 A). To determine whether HMGB1 secreted by SEN cells was acetylated, we immunoprecipitated CM from PRE or SEN cells with the Ac-Lys antibody, and analyzed precipitates for HMGB1 by Western blotting. HMGB1 in CM was acetylated, whether secreted at low levels by PRE cells or high levels by SEN cells (Fig. 3 B).

Senescent cells actively export nuclear HMGB1

Acetylated HMGB1 is actively exported from the nucleus in monocytes and macrophages (Gardella et al., 2002; Raucci et al., 2007). To determine whether this was true in SEN cells, we used leptomycin B (LMB) to inhibit CRM1-dependent nuclear export. LMB prevented HMGB1 nuclear export in monocytes (Bonaldi

et al., 2003). Untreated SEN cells immunostained weakly for nuclear HMGB1, but strongly for persistent DNA damage (53BP1) foci (Rodier et al., 2009) (Fig. 4, A and B). After a 4-h exposure to low-dose LMB (20 nM), the nuclei retained 53BP1 foci and gained robust nuclear HMGB1 staining (Fig. 4, A and B). These data indicate that SEN cells continually synthesize HMGB1, which transiently occupies the nucleus, but then is actively exported.

High (2 µM) LMB doses and long exposures increase nuclear p53 and induce senescence (Smart et al., 1999). The low (20 nM) dose used here eventually increased nuclear p53 (Fig. S2 A); after 6 d, cells senesced, as assessed by arrested growth and SA-β-Gal activity (Fig. S2 B). Growth arrest and SA-β-Gal occurred despite strongly retained nuclear HMGB1 (Fig. S2 A), indicating these phenotypes do not depend on nuclear HMGB1 loss.

To rule out the possibility that LMB induced apoptosis, which could account for HMGB1 nuclear retention, we immunostained cells treated with LMB or staurosporine (positive control) for active caspase-3, a hallmark of apoptosis (Fig. 4 C). Greater than 80% of staurosporine-treated cells stained positive (left panel), in contrast to <2% of LMB-treated cells (right panel). Thus, LMB-treated cells retained nuclear HMGB1 owing to inhibited nuclear export, not apoptosis. Finally, western analysis and ELISAs showed that LMB-treated SEN cells failed to secrete HMGB1 (Fig. 4, D and E). In contrast, H_2O_2 at a dose (0.5 mM) that caused necrosis also caused HMGB1 release, as expected (Raucci et al., 2007), which was unaffected by LMB (Fig. 4 F).

HMGB1 depletion or overexpression induces senescence

To determine the significance of HMGB1 relocalization, we depleted HMGB1 in human fibroblasts (IMR-90, HCA2) by RNAi using lentiviruses carrying no insert or either of two short hairpin RNAs (shRNAs) that reduced intracellular HMGB1 protein levels by >90% (Fig. 5, A and B; Fig. S3, A and B). HMGB1-depleted cells underwent senescence, as judged by arrested growth (Fig. 5 C), senescence-associated heterochromatin foci (SAHF) formation (Narita et al., 2003), and SA-β-Gal activity (Fig. 5, B and C; Fig. S3 B). HMGB1 depletion also caused senescence in human mammary epithelial cells (HMECs) and MEFs (Fig. S4, A–D), indicating the response was not restricted to human fibroblasts.

These findings are significant for two reasons. First, although IMR-90 cells senesce with SAHF (Narita et al., 2003), these structures are not a universal feature of SEN cells (Kosar et al., 2011). Indeed, HCA2 cells senesce without SAHF (Fig. S3 B; REP). Thus, HMGB1 depletion induced SAHF in cells that do not form these structures. Second, the consequence of HMGB1 depletion differed markedly from that of HMGA depletion, which reduced the senescence response to oncogenic RAS or p16[INK4a] overexpression (Narita et al., 2006).

Many tumor cell lines and tumors express high levels of HMGB1 (Brezniceanu et al., 2003; Ellerman et al., 2007), suggesting HMGB1 might be an oncogene that allows bypass of senescence signals. To test this idea, we used lentiviruses to overexpress HMGB1 in IMR-90 (Fig. 5, D and E) and HCA2 (Fig. S3, C, D, and F) cells. Surprisingly, overexpression also induced
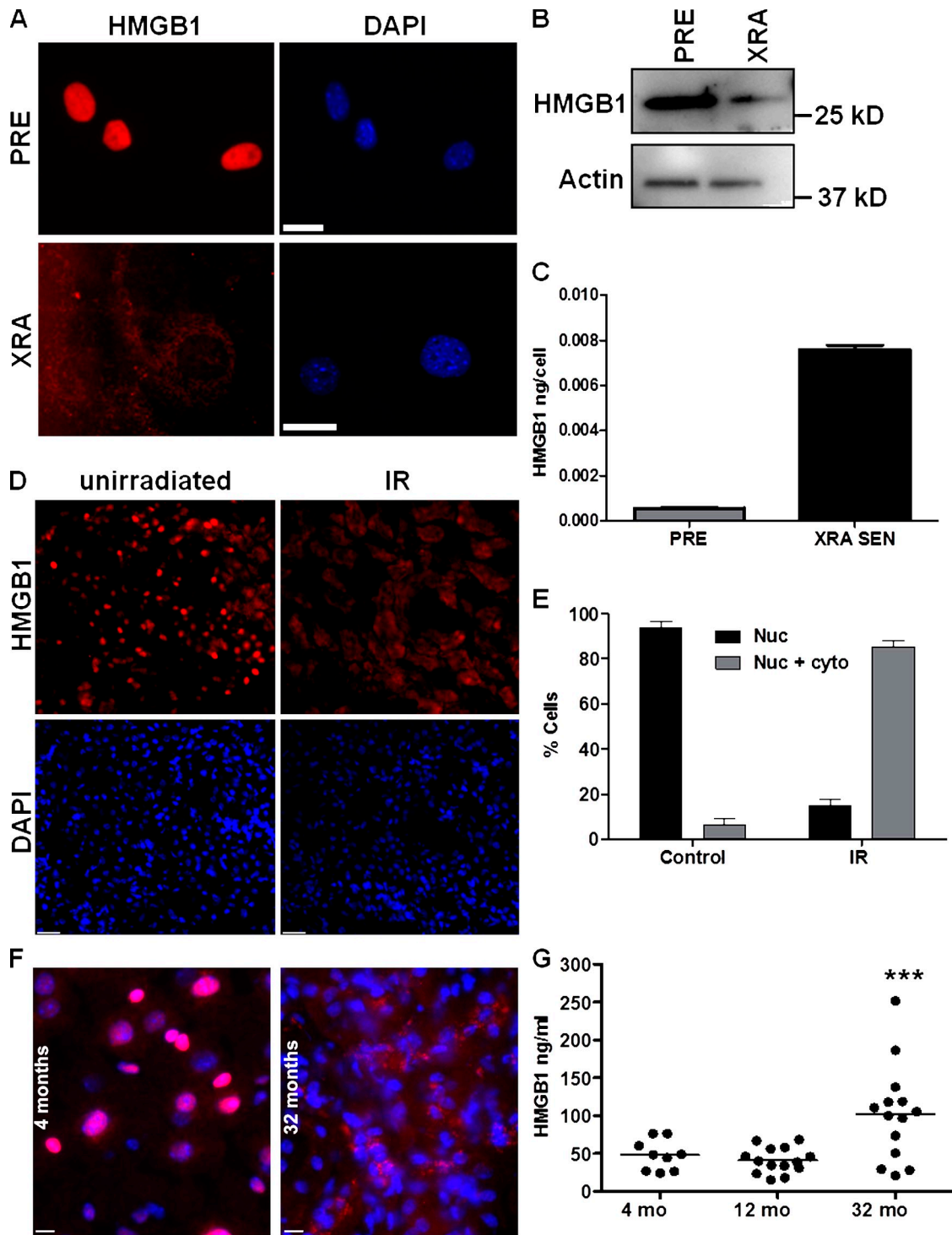
Figure 2. **HMGB1 relocalizes in SEN mouse cells.** (A) PRE or SEN (XRA) MEFs were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bar, 10 μM. (B) Lysates from PRE or SEN (XRA) MEFs were analyzed by Western blotting for HMGB1 and actin (control). (C) CM from PRE or XRA cells were analyzed for HMGB1 by ELISA. Error bars are the average of duplicate samples. (D) 6–8-wk-old C57BL/6 mice were unirradiated (control) or sublethally irradiated (IR). Kidneys collected 1 wk later were immunostained for HMGB1 (red); nuclei were stained with DAPI (blue). Bar, 25 μM. (E) Kidneys from mice in B were immunostained for nuclear (Nuc), or nuclear + cytoplasmic (Nuc + Cyto) HMGB1. More than 200 nuclei were scored blinded from 2–3 control or irradiated (IR) mice; error bars are SEM. (F) Kidneys from 4- and 32-mo-old C57BL/6 mice were immunostained for HMGB1 (red); nuclei were counterstained with DAPI (blue). Bars, 10 μM. (G) Sera from mice at the indicated ages were assayed for HMGB1 by ELISA. Kruskal-Wallis test was used to determine significance (***, $P < 0.001$).

senescence, as determined by arrested growth, SAHF, and SA-β-Gal (Fig. 5, E and F; Fig. S3 E). Overexpression similarly induced senescence in HMECs and MEFs (Fig. S4, B and D).

HMGB1 depletion or overexpression stimulated p53 expression to levels found in cells induced to senesce by XRA or REP (Fig. 5 G; Fig. S3 G).

Figure 3. **Senescent cells secrete acetylated HMGB1.** (A) WI-38 cells were given PBS (−, control) or 200 ng/ml trichostatin A (TSA) (+) for 4 h, and immunostained with pan-acetyl-lysine (green)– and HMGB1 (red)–specific antibodies. Nuclei were stained with DAPI (blue). Bar, 20 µM. (B) CM from PRE and SEN (XRA) IMR-90 cells were immunoprecipitated by mouse IgG (control) or acetyl-lysine (Ac-L) antibody. Precipitates and 5% of unprecipitated (Input) CM were analyzed by Western blotting for HMGB1. % total = amount of acetylated HMGB1 immunoprecipitated compared with total HMGB1 in CM.
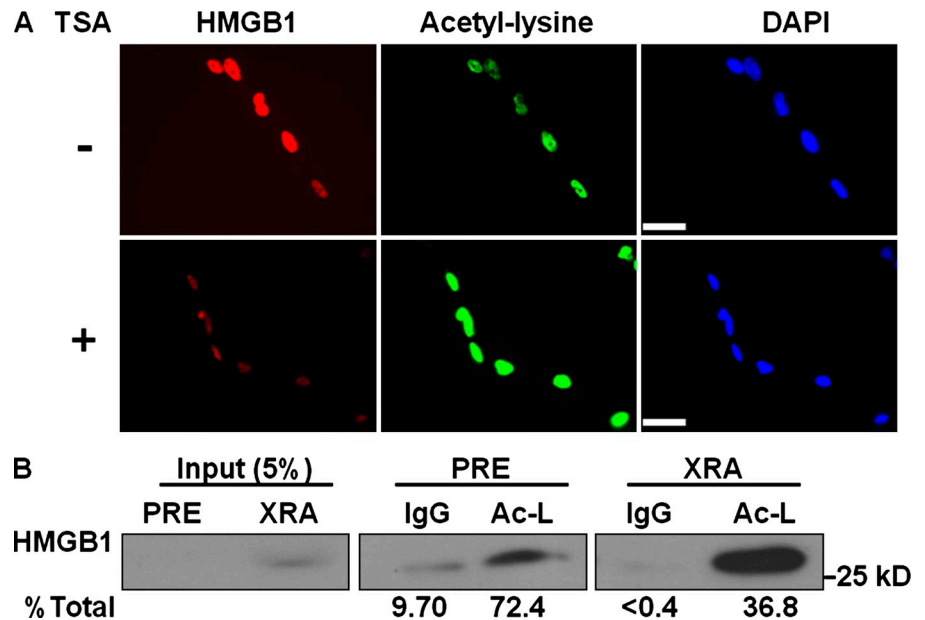
Senescence is a stress response, although not all stressors induce senescence (Suzuki and Boothman, 2008). To examine whether disrupted HMGB1 levels caused a general stress response, we monitored heat shock protein 70 (HSP70). Although heat shock (42°C) robustly increased HSP70 expression, disrupted HMGB1 stoichiometry failed to do so (Fig. S3 H). Further, heat shock did not stimulate HMGB1 secretion (Fig. S3 I). Thus, altered HMGB1 expression, whether reduced or elevated, caused senescence.

IL-6 secretion by SEN cells requires HMGB1 expression

Because extracellular HMGB1 stimulates inflammatory cytokine secretion by immune cells (Andersson et al., 2002; Dumitriu et al., 2005; Yamada and Maruyama, 2007), and such cytokines are SASP components (Coppé et al., 2008, 2010b), we asked whether senescence caused by altered HMGB1 expression increased cytokine secretion. Despite inducing growth arrest, SAHF, SA-β-Gal (Fig. 5 C), and p53 expression (Fig. 5 G), HMGB1-depleted SEN cells secreted little IL-6 (Fig. 5 H), a key SASP component. By contrast, HMGB1-overexpressing SEN cells secreted both HMGB1 (Fig. 5 K; Fig. S4 E) and IL-6 (Fig. 5 H).

Because SEN cells express IL-6 owing primarily to NF-κB transcriptional activity (Freund et al., 2011), we measured NF-κB transcriptional activity in HMGB1-overexpressing or -depleted HCA2 cells, with or without shRNA against RelA, an essential NF-κB subunit. As a positive control, TNF strongly induced RelA-dependent NF-κB activity (Fig. 5 I). Consistent with the IL-6 ELISA results (Fig. 5 H), NF-κB activity was robust in HMGB1-overexpressing cells, but not HMGB1-depleted cells (Fig. 5 I). HMGB1 overexpression increased cytoplasmic HMGB1 immunostaining and secretion (Fig. 5, J and K). Secreted HMGB1 had no effect on the growth arrest because cells remained arrested in the presence of an HMGB1-blocking antibody (Fig. 5 L). Thus, altered HMGB1 expression induced senescence, but only depletion, not overexpression, suppressed NF-κB activity and IL-6 secretion.

HMGB1 relocalization does not depend on p16^{INK4a}

Both HMGB1 overexpression and depletion increased p53 to SEN levels (Fig. 5 G; Fig. S3 G). However, only HMGB1 depletion induced p16^{INK4a}, and only in high p16-expressing IMR-90 (Fig. 5 G), not low p16-expressing HCA2 cells (Fig. S3 G). Further, altered HMGB1 expression did not induce SAHF in p16^{INK4a}-deficient HMECs, despite altering heterochromatin in MEFs (Fig. S4 C). Thus, p16^{INK4a} may have little or no role in the senescence response to perturbed HMGB1 expression.

To test this possibility, we depleted IMR-90 cells of p16^{INK4a} by RNAi (Fig. 6 A). When induced to senesce by XRA, the cells lost nuclear/intracellular HMGB1 (Fig. 6, B and C), expressed SA-β-Gal, and arrested growth in response to HMGB1 overexpression or depletion (Fig. 6 D; and unpublished data). As observed for RAS-induced senescence (Narita et al., 2003), efficient SAHF formation required p16^{INK4a}. Altered HMGB1 expression induced SAHF in 50–70% fewer p16^{INK4a}-depleted cells compared with control cells (Fig. 6 E). Further, loss of nuclear HMGB1 and HMGB1 secretion occurred when senescence was induced by p16^{INK4a} overexpression (Fig. 6 F; Fig. S4 E), which does not cause a SASP (Coppé et al., 2011), suggesting that HMGB1 relocalization and the SASP are regulated differently.

HMGB1 relocalization requires p53 activity

To determine the role of p53, we expressed a dominant-negative p53 peptide (GSE22; Ossovskaya et al., 1996) or depleted p53 by RNAi. HMGB1 was abundant in CM from HCA2 fibroblasts expressing p53, but low upon GSE22 expression, mirrored by a commensurate increase in HMGB1 in WC lysates (Fig. 7 A). HMGB1 ELISA confirmed that GSE attenuated HMGB1 secretion in XRA SEN cells (Fig. 7, B and E). Low p16^{INK4a}-expressing cells such as HCA2 resume growth (revert) upon p53 inactivation (Beauséjour et al., 2003). To test whether HMGB1 retention upon p53 loss was due simply to resumed proliferation, we depleted p53 in cells expressing high levels of p16. Depletion of p53 produced retention of HMGB1 after senescence induced by
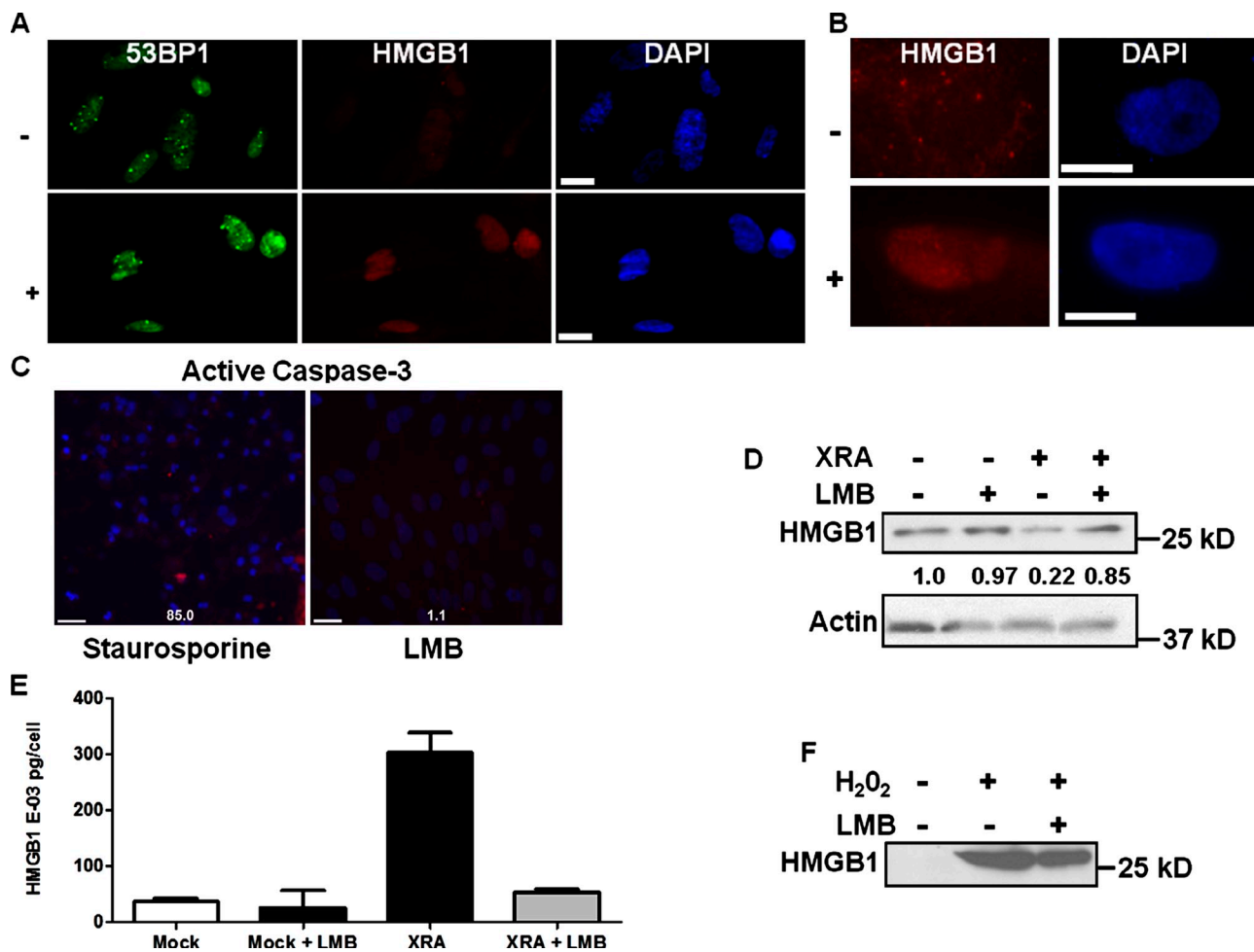
Figure 4. HMGB1 is actively exported from senescent cell nuclei. (A) SEN (XRA) HCA2 cells were cultured for 3 h with DMSO (−, control) or 20 nM leptomycin B (LMB) (+) and stained for HMGB1 (red) and 53BP1 (green). Nuclei were stained with DAPI (blue). Bar, 10 μM. (B) REP SEN HCA2 cells were cultured as in A and stained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bar, 10 μM. (C) HCA2 cells were given 5 nM staurosporine or 20 nM LBM for 6 h and stained for active caspase-3 (red; % positive cells shown). Nuclei were stained with DAPI (blue). Bar, 10 μM. (D) PRE (XRA−) or SEN (XRA+) IMR-90 cells were given DMSO (−) or 20 nM LMB (+) for 3 h. Lysates were analyzed for HMGB1 and actin (control) by Western blotting. Expression normalized to actin and relative to PRE cells (1.0) is indicated. (E) CM from D were analyzed for HMGB1 by ELISA. Error bars = SEM of triplicate samples. (F) HCA2 cells were cultured without or with 0.5 mM H_2O_2 for 2 h, washed, and incubated for 16 h with DMSO or LMB. CM were analyzed by Western blotting for HMGB1. Data are representative of at least two independent experiments.

X-irradiation, which was confirmed using an HMGB1 ELISA of CM (Fig. 7 D). Thus, senescence-associated nuclear HMGB1 loss and secretion required p53 function, in contrast to the SASP.

Senescence caused by altered HMGB1 expression is p53-dependent

We confirmed that some cancer cells overexpress HMGB1 (Brezniceanu et al., 2003) using a panel of normal and tumor-derived human breast cells (unpublished data), which appears at odds with HMGB1 overexpression inducing senescence. However, the cancer lines we tested were all p53 defective. To determine whether senescence caused by altered HMGB1 expression was p53 dependent, we overexpressed or depleted HMGB1 in cells in which p53 was unaltered (+) or depleted by RNAi (−) (Fig. 7 F). As reported (Narita et al., 2003), RAS-induced senescence was p53 independent (Fig. 7 F). However, senescence caused by HMGB1 overexpression or depletion was p53 dependent, as determined by SA-β-Gal activity (Fig. 7 F), growth arrest (not depicted), and SAHF formation (Fig. 7 G).

Further, in the absence of p53 function, cells that overexpress HMGB1 secreted very little HMGB1 (Fig. 7 H), in contrast to the SASP, which is more robust in p53-deficient cells (Coppé et al., 2008).

ATM is dispensable for HMGB1 secretion

Although the SASP does not require p53 activity, it requires DDR signaling (Coppé et al., 2008; Bhaumik et al., 2009; Rodier et al., 2009, 2011; Le et al., 2010), and cells lacking ATM, a key DDR component, senesce with much reduced cytokine secretion (Rodier et al., 2009). We therefore examined HMGB1 localization in HCA2 and ATM-deficient fibroblasts (AT2SF, AT5823). PRE AT cells had less nuclear HMGB1 immunostaining than HCA2 cells (Fig. 8 A), which LMB prevented (not depicted). After XRA-induced senescence, HCA2 cells lost nuclear staining as expected, but AT cells stained similarly to unirradiated cells (Fig. 8 A). Unirradiated AT cells incorporated less BrdU and expressed more SA-β-Gal than HCA2 cells, yet both cell types responded to XRA by reduced BrdU incorporation and
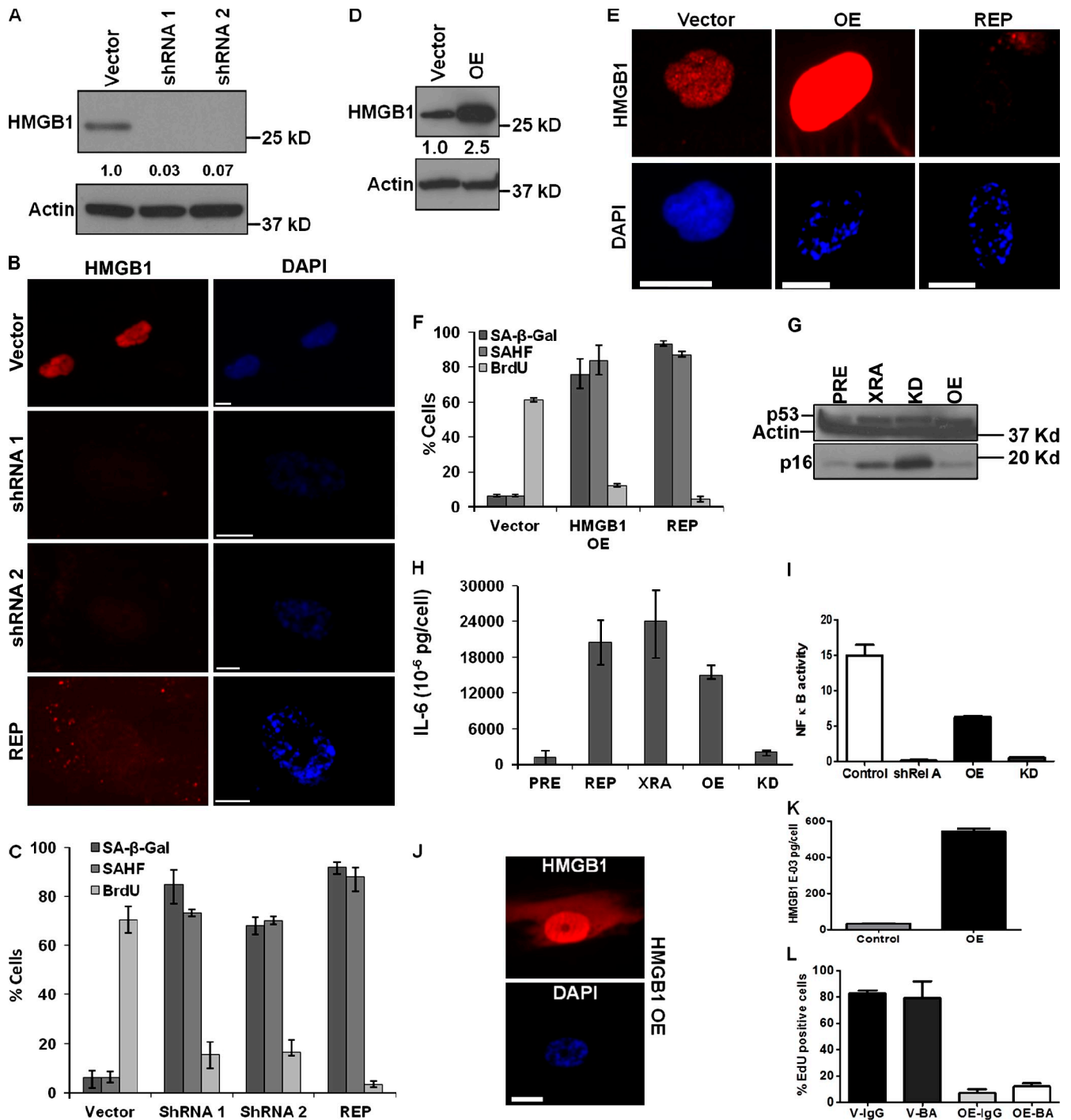
Figure 5. **HMGB1 depletion or overexpression induces senescence.** (A) Lysates from IMR-90 cells infected with lentiviruses carrying no insert (Vector) or HMGB1 shRNAs (shRNA1, 2) were analyzed for HMGB1 and actin (control) by Western blotting. Shown is expression level relative to Vector. (B) Cells in A were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). REP SEN cells are shown for comparison. Bar, 10 µM. (C) Cells in B were scored for SA-β-Gal, BrdU (24 h pulse), and SAHF. Error bars = SEM of two independent experiments. (D) Lysates from IMR-90 cells infected with lentivirus expressing no insert (Vector) or HMGB1 (OE) were analyzed for HMGB1 and actin (control) by Western blotting. Shown is expression level relative to Vector. (E) Cells in D were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). REP SEN cells are shown for comparison. Bar, 10 µM. (F) Cells in E were scored for SA-β-Gal, BrdU (24 h pulse), and SAHFs. REP SEN cells are shown for comparison. Error bars = SEM of two independent experiments. (G) Lysates from PRE IMR-90 cells or cells induced to senesce by XRA (XRA), HMGB1-overexpression (OE), or HMGB1-depletion (KD) were analyzed for p53, p16^{INK4a}, and actin (control) by Western blotting. (H) CM from PRE or SEN (REP, XRA, and HMGB1 OE or KD) IMR-90 cells were analyzed for IL-6 by ELISA. Shown is a representative of two experiments. Error bars = SEM of duplicate determinations. (I) HCA2 cells expressing an NF-κB luciferase reporter were infected with lentiviruses carrying GFP shRNA (control), RelA shRNA, HMGB1 cDNA (OE), or HMGB1 shRNA (KD). 7 d after selection, shGFP and shRel A cells were given TNF for 24 h in serum-free media. Cells were lysed and luciferase activity measured (fold change over cells given BSA (control) protein). One-way ANOVA was used to analyze groups; $P < 0.001$. (J) Cells infected with HMGB1 OE lentivirus were stained for HMGB1 (red) and nuclei (DAPI; blue). Bar, 10 µM. (K) CM from cells infected with insertless (control) or HMGB1-expressing (OE) lentiviruses were assayed for HMGB1 by ELISA. Error bars = SEM of duplicate determinations. (L) IMR-90 cells infected with lentiviruses carrying no insert (V) or overexpressing HMGB1 (OE) were cultured for 5 d with mouse IgG or HMGB1 blocking antibody (BA) and assessed for EdU incorporation. Error bars = SEM of duplicate determinations.
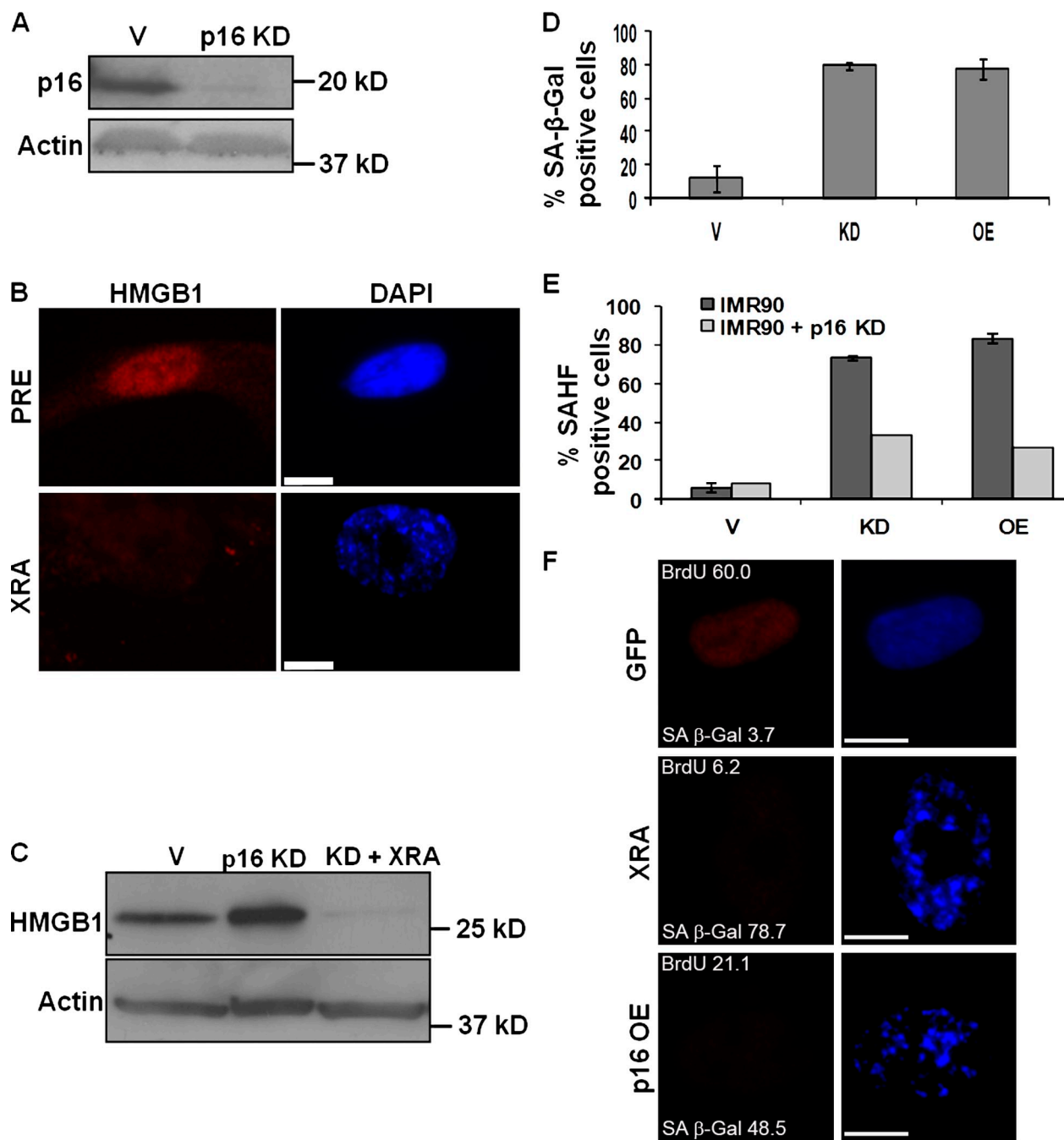
Figure 6. **p16^INK4a does not regulate HMGB1 in SEN cells.** (A) Lysates from IMR-90 cells infected with lentiviruses carrying no insert (V) or p16^INK4a shRNA (p16 KD) were analyzed for p16^INK4a and actin (control) by Western blotting. (B) PRE and SEN (XRA) IMR-90 cells depleted of p16^INK4a were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bars, 10 µM. (C) Lysates from PRE IMR-90 cells or cells infected with lentiviruses carrying no insert (V) or p16^INK4a shRNA (p16 KD). p16 KD cells were made senescent by X-irradiation (XRA) and analyzed 10 d later. Cells were analyzed for HMGB1 and actin (control) by Western blotting. (D) IMR90 cells depleted of p16^INK4a were infected with lentiviruses carrying no insert (V), HMGB1 cDNA (OE), or HMGB1 shRNA (KD) and stained for SA-β-Gal. Error bars = SEM of two independent experiments. (E) IMR-90 cells expressing or depleted of p16^INK4a were infected with lentiviruses carrying no insert (V), HMGB1 shRNA (KD), or HMGB1 cDNA (OE). After 10 d, cells were scored for SAHFs. Error bars = SEM of two independent experiments. (F) IMR-90 cells infected with lentiviruses carrying GFP (control) or p16 (OE) were scored for SA-β-Gal and BrdU incorporation (24 h pulse) and immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). XRA-induced SEN cells are shown for comparison. Bars, 10 µM.

increased SA-β-Gal expression (Fig. 8 B). Consistent with the immunostaining, PRE AT cells secreted more HMGB1 than PRE HCA2 cells. Further, SEN AT cells secreted more HMGB1 than SEN HCA2 cells, which was unaltered by senescence (Fig. 8 C). We also expressed shRNAs against GFP (control) or ATM in

HCA2 cells. ATM depletion reduced intracellular HMGB1 to levels present in AT cells (Fig. 8 D). Although reasons for the high HMGB1 secretion and low intracellular HMGB1 in AT cells are unknown, ATM deficiency clearly did not prevent loss of nuclear HMGB1 or its secretion.
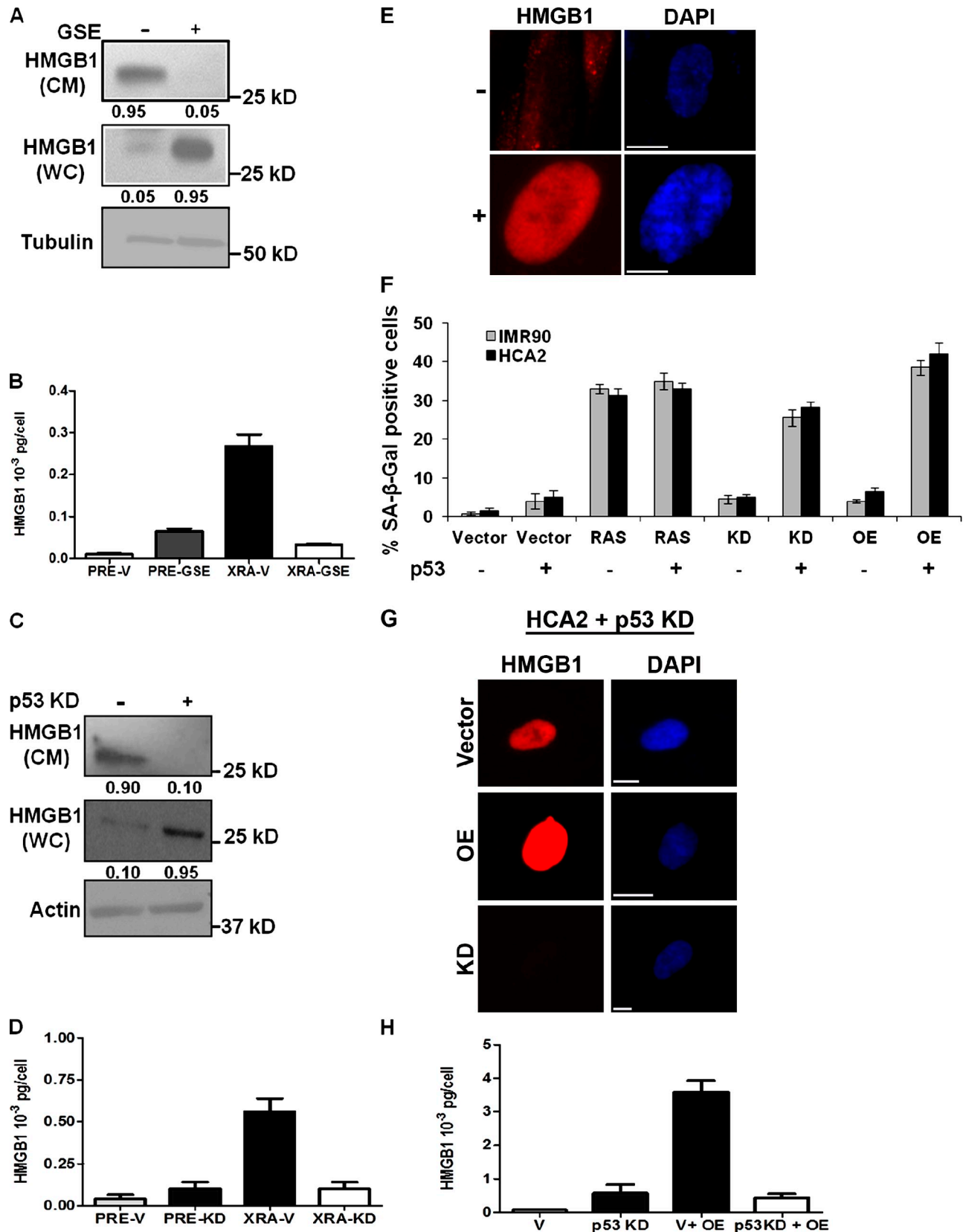
Figure 7. **HMGB1 relocalization and senescence caused by altered HMGB1 expression requires p53.** (A) Lysates (WC) and CM from XRA SEN HCA2 cells infected with control (−) or GSE (+) expressing lentiviruses were analyzed for HMGB1 and tubulin (control) by Western blotting. The fraction of detectable protein is given below each lane. (B) CM from PRE or SEN (XRA) HCA2 cells expressing control (V) or GSE-expressing lentiviruses were analyzed for HMGB1 by ELISA. (C) Lysates (WC) or CM from XRA SEN IMR90 cells infected with control (−) or shp53 (+) expressing lentiviruses were analyzed for HMGB1 and actin (control) by Western blotting. The fraction of detectable protein is given below each lane. (D) CM from PRE or SEN (XRA) IMR90

Intracellular and extracellular HMGB1 regulate the SASP

Stressed cells secrete DAMPs, including HMGB1 (Bianchi, 2007; Lotze et al., 2007), which also function intracellularly. HMGB1 preferentially binds euchromatin (Falciola et al., 1997). Although SEN cells secreted HMGB1, we detected low levels in the nucleus (Fig. 9 A). In all SEN cells examined, residual nuclear HGMB1 localized to areas of light DAPI staining, indicative of euchromatin, and was excluded from SAHF (Fig. 9 A). This result, and our finding that >95% HMGB1 depletion (Fig. 5 A) caused senescence without a SASP, suggested that low levels of nuclear HMGB1 were necessary for the SASP. To test this idea, we expressed shRNAs against GFP or HMGB1 in SEN cells, and measured secretion of IL-6 and MMP3, two SASP factors. HMGB1 depletion reduced IL-6 and MMP3 secretion (>90%; Fig. 9 B). Thus, the SASP requires HMGB1 expression, but not high levels in the nucleus.

To test whether the SASP requires extracellular HMGB1, we cultured PRE or SEN cells with a control or HMGB1 blocking antibody. At a low antibody concentration (2 µg/ml), IL-6 secretion was unaffected (Fig. 9 C). However, at higher concentrations (4 and 8 µg/ml), the blocking, but not control, antibody reduced IL-6 secretion two- to threefold, indicating that optimal IL-6 secretion by SEN cells also requires extracellular HMGB1.

The redox status of reactive HMGB1 cysteines distinguishes its cytokine-inducing and chemokine activity (Venereau et al., 2012; Yang et al., 2012). To determine the redox status of HMGB1 secreted by SEN cells, we collected CM from PRE and SEN cells 24 h and 7 d after XRA, and analyzed CM before (−) or after (+) addition of dithiothreitol (DTT). As expected, PRE CM contained very little HMGB1. In the absence of DTT, we detected a rapidly migrating (oxidized) form, which migrated more slowly after DTT reduction (Fig. 9 D). Thus, SEN cells secreted oxidized HMGB1, the form that stimulates cytokine secretion (Venereau et al., 2012), consistent with our finding that an HMGB1-blocking antibody attenuated IL-6 secretion by SEN cells.

Extracellular HMGB1 signals via TLR4 and NF-κB, and is inhibited by intracellular HMGB1

HMGB1 released by immune cells signals primarily through the RAGE and TLR4 receptors (Klune et al., 2008; Tang et al., 2010). RAGE-blocking antibodies did not alter IL-6 secretion by SEN cells (unpublished data). However, lentiviral-delivered shRNA against TLR-4, which reduced TLR4 mRNA ∼50%, as well as CLI-95, a small molecule inhibitor of TLR-4 signaling (Kawamoto et al., 2008), both significantly reduced IL-6 secretion by SEN cells (Fig. 9 E). Thus, in SEN cells, extracellular HMGB1 signals IL-6 secretion primarily through TLR4.

We also cultured cells depleted of HMGB1 with recombinant HMGB1 (rHMGB1). The rHMGB1 was active because immunostaining showed it promoted NF-κB nuclear localization to the same extent as TNF (Fig. 9 F), as demonstrated for mouse cells (Palumbo et al., 2007). We incubated HMGB1-depleted cells with BSA (negative control), TNF or IL-1α (positive controls), or HMGB1. After 24 h, TNF and IL-1α stimulated IL-6 secretion, regardless of HMGB1 status, as expected (Ammit et al., 2000; Orjalo et al., 2009). rHMGB1 failed to stimulate IL-6 secretion in PRE cells, despite inducing nuclear localization of NF-κB (Fig. S5). As expected, TNF and IL-1α induced both NF-κB translocation and IL-6 secretion by PRE cells. In contrast, rHMGB1 stimulated both NF-κB translocation and IL-6 secretion in HMGB1-depleted cells, as did TNF and IL-1α (Fig. 9 G). Further, using a luciferase reporter to measure NF-κB transcriptional activity (Freund et al., 2011), TNF and IL-1α stimulated transcription (Fig. 9 H) regardless of the presence of endogenous HMGB1, but rHMGB1 stimulated NF-κB transcriptional activity only in HMGB1-depleted cells (Fig. 9 H).

The data suggest that IL-6 secretion by SEN cells is stimulated by extracellular HMGB1, but inhibited by high HMGB1 levels in the nucleus. Thus, development of the SASP likely requires both loss of nuclear HMGB1 and gain of extracellular HMGB1.

Discussion

Cellular senescence prevents the proliferation of potential cancer cells (Campisi, 2001). Several markers identify senescent cells, although none are completely unique to the senescent state. These markers include an enlarged morphology, SA-β-Gal, SAHF (in some cells; Campisi and d'Adda di Fagagna, 2007), persistent DNA damage foci (Rodier et al., 2009, 2011) and the SASP (Coppé et al., 2008), all of which require 3–7 d to develop. We show that HMGB1 relocalization is an early response to senescence cues, occurring within 24–48 h of XRA, which delivers a synchronous senescence signal. Several human and mouse cells lost nuclear HMGB1 and secreted it upon entering senescence, suggesting that loss of nuclear HMGB1 can be used as another (imperfect) marker of senescent cells.

HMGB1 is a founding member of the Alarmin family. Alarmins function both inside and outside the cell, the latter in response to damage or stress (Bianchi, 2007). Necrotic cells passively leak HMBG1, but immune cells actively secrete this protein to amplify inflammatory stimuli such as those initiated by lipopolysaccharide, interferons, or nitric oxide (Wang et al., 1999a,b; Semino et al., 2005). Our finding that SEN cells also actively secrete HMGB1 before other well characterized senescence-associated changes suggests loss of nuclear HMGB1 and

cells expressing control (V) or shp53 (KD) lentiviruses were analyzed for HMGB1 by ELISA. (E) REP SEN HCA2 cells infected with control (GFP; −) or GSE (+) expressing lentivirus were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bars, 10 µM. (F) IMR-90 and HCA2 cells expressing (+) or depleted of p53 (−) were infected with a lentivirus carrying no insert (Vector), oncogenic RAS, HMGB1 shRNA (KD), or HMGB1 (OE), and scored for SA-β-Gal 3 d later. Error bars = SEM of two independent experiments. (G) Cells in B were infected with lentiviruses carrying no insert (Vector), HMGB1 (OE), or HMGB1 shRNA (KD). 6 d after infection, cells were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bars, 10 µM. (H) CM from cells infected with lentiviruses carrying no insert (V), shp53 (p53 KD), and/or HMGB1 (OE) were analyzed for HMGB1 by ELISA. Error bars = SEM of triplicate samples.
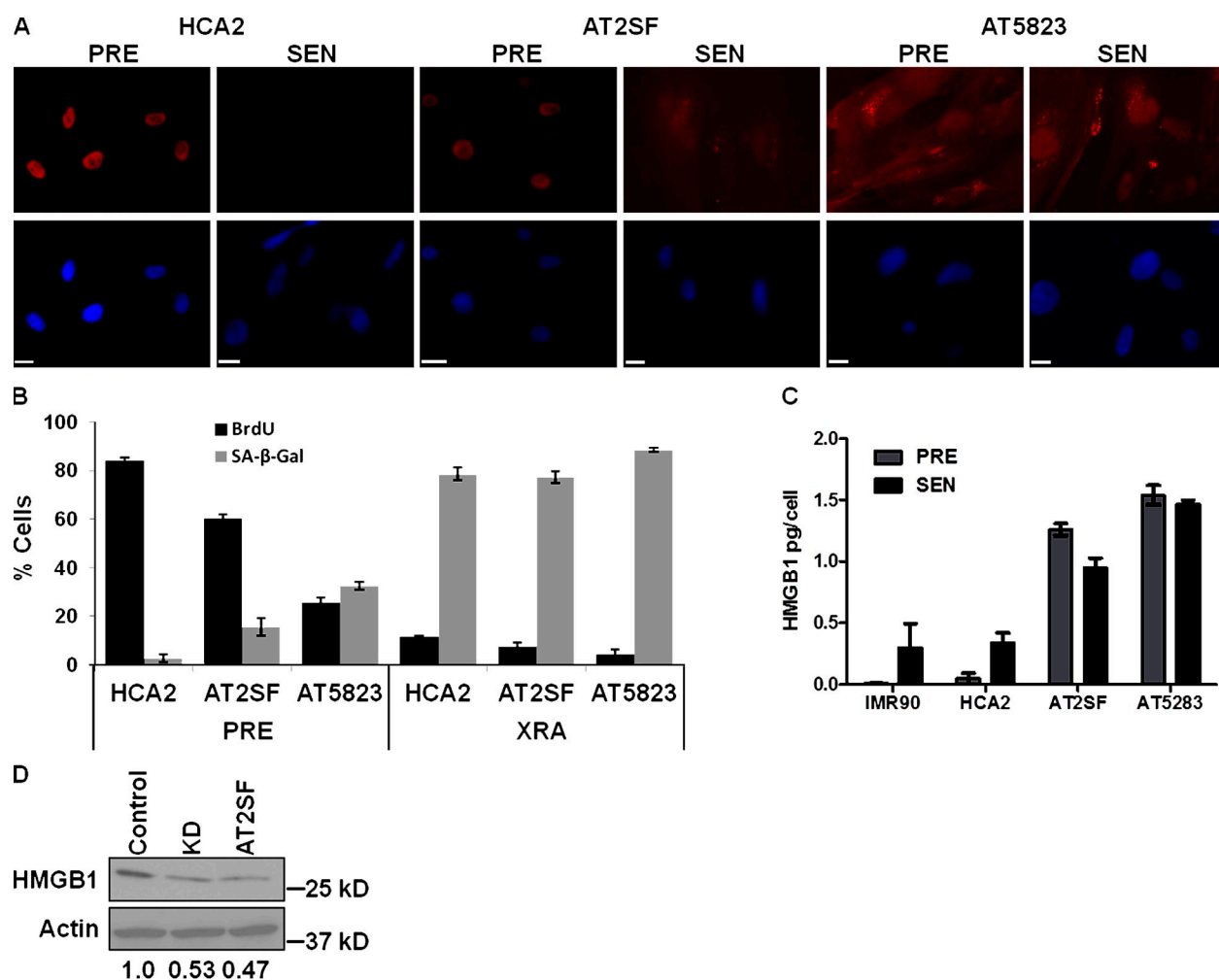
Figure 8. **ATM is dispensable for HMGB1 secretion.** (A) PRE or SEN (XRA) HCA2 cells and two A-T derived fibroblast strains (AT2SF, AT5283) were immunostained for HMGB1 (red). Nuclei were stained with DAPI (blue). Bars,10 µM. (B) Cells in A were scored for SA-β-Gal and BrdU incorporation (24-h pulse). Error bars = SEM of two experiments. (C) CM from cells in A were assessed for HMGB1 secretion by ELISA. Error bars = SEM of two experiments. (D) Lysates from HCA2 cells infected with lentiviruses carrying GFP (Control) or ATM (KD) shRNA and lysates from A-T fibroblasts (AT2SF) were analyzed for HMGB1 and actin by Western blotting. HMGB1 signals were normalized to actin, and are shown relative to shGFP.

its secretion initiates the SASP and, hence, the inflammatory activity of senescent cells (Coppé et al., 2010a; Freund et al., 2010). HMGB1 secretion may also initiate the removal of senescent cells by innate immune cells, which has been described in mouse models of tumorigenesis and liver fibrosis (Xue et al., 2007; Krizhanovsky et al., 2008).

The redox state of HMGB1 determines whether it stimulates inflammatory cytokine secretion or acts as a chemoattractant to recruit immune cells (Venereau et al., 2012). In culture, senescent cells secreted oxidized HMGB1, the form that stimulates cytokine secretion. In vivo, senescent cells may initially secrete oxidized HMGB1, which then becomes reduced to promote immune cell recruitment. Although HMGB1 secretion and other mechanisms may stimulate removal of senescent cells by the immune system, senescent cells nonetheless accumulate with age and at sites of age-related pathology. Consistent with this finding, we detected elevated levels of circulating HMGB1 in old mice.

The SASP, a hallmark of many senescent cells (Coppé et al., 2008), is restrained by p53 (Coppé et al., 2008) and stimulated by ATM (Rodier et al., 2009). HMGB1 secretion differed

from the SASP in several ways. First, HMGB1 secretion preceded the secretion of SASP components. This finding reinforces the idea that senescence entails a series of defined events (Rodier and Campisi, 2011), similar to apoptosis (Green, 2005). Second, unlike the SASP, loss of nuclear HMGB1 and HMGB1 secretion required p53 activity. p53-deficient cells did not secrete or lose nuclear HMGB1 in response to senescence-inducing stimuli, nor did they cease growth upon altered HMGB1 expression. Third, unlike the SASP, HMGB1 secretion did not require ATM activity, suggesting HMGB1 release is not a DNA damage response. Consistent with this idea, HMGB1 was secreted by cells induced to senescence by p16[INK4a] overexpression, which do not experience DNA damage or express a SASP (Rodier et al., 2009; Coppé et al., 2011). Thus, regulation of HMGB1 secretion is distinct from regulation of the SASP.

Early loss of an abundant chromatin architectural protein like HMGB1 might drive changes that lead to the senescence growth arrest and SASP. But it is also possible that earlier senescence-associated chromatin changes drive loss of nuclear HMGB1. For example, after 6 d in TSA, which alters chromatin,
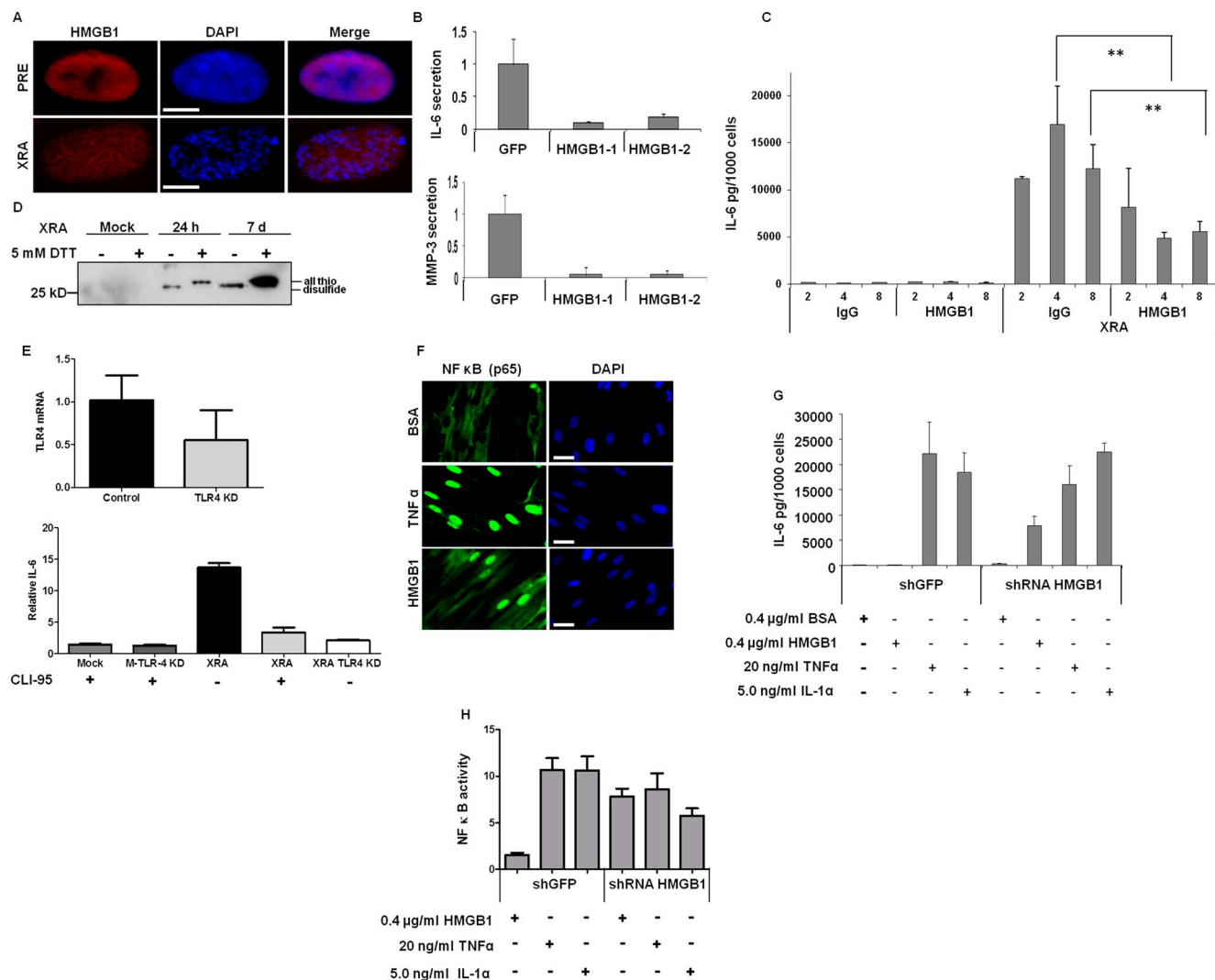
Figure 9. **HMGB1 regulates SASP factors.** (A) PRE and SEN (XRA) WI-38 cells were immunostained for HMGB1 (red). DAPI stained the nuclei (blue). Images were uniformly enhanced to detect HMGB1 in SEN cells. Bar, 10 µM. (B) XRA SEN WI-38 cells were infected with lentiviruses carrying GFP or either of two HMGB1 (HMGB1-1, HMGB1-2) shRNAs. 4 d later, CM was collected and analyzed by ELISA for IL-6 and MMP-3. Shown are fold changes relative to shGFP. Error bars = SEM of two experiments. (C) PRE or SEN (XRA) HCA2 cells were incubated with 2, 4, and 8 µg/ml mouse IgG or HMGB1 blocking antibody. CM were analyzed by ELISA for IL-6. Error bars = SEM of triplicate samples. **, P < 0.02. (D) CM was collected from IMR90 cells that were mock irradiated, or 24 h or 7 d after irradiation. CM were heated in the presence (+) or absence (−) of 5 mM DTT, then analyzed by Western blotting. Positions of reduced (all thiol) and oxidized (disulfide) HMGB1 are indicated. (E) IMR90 cells were infected with control or shTLR-4 expressing lentiviruses and assessed for relative TLR-4 mRNA and IL-6 secretion. IL-6 secretion was assessed in mock (M) or X-irradiated (XRA) SEN cells expressing no insert or TLR-4 shRNA (TLR4 KD), or cultured in the presence (+) or absence (−) of CLI-95, a TLR-4 inhibitor. Shown is the fold expression relative to mock-irradiated cells without CLI-95. Error bars = SEM of triplicate samples. (F) IMR90 cells were cultured with 400 ng/ml BSA, 20 ng/ml TNF, or 400 ng/ml recombinant HMGB1 for 20 min and immunostained for NF-κB (p65 subunit; green). DAPI stained the nuclei (blue). Bar, 10 µM. (G) HCA2 cells infected with shGFP or shHMGB1-expressing lentiviruses were selected for 7 d and cultured for 24 h with indicated concentrations of BSA, recombinant HMGB1, TNF, or IL-1α. CM were analyzed by ELISA for IL-6. Error bars = SEM of triplicate samples. (H) HCA2 cells expressing an NF-κB luciferase reporter were infected with lentiviruses expressing shGFP or shHMGB1. 7 d after selection, cells were cultured for 24 h with the indicated proteins in serum free media at the concentrations in G. Cells were lysed and luciferase was measured. Luciferase levels are fold changes over cells incubated with BSA. One-Way ANOVA was used to analyze the groups; P < 0.001.

cells lost nuclear HMGB1, as reported for monocytes (Bonaldi et al., 2003), and ceased growth, but <15% of cells expressed SA-β-Gal and adopted a senescent morphology. Because HMGB1 binds DNA loosely and transiently (Scaffidi et al., 2002), early chromatin remodeling during senescence may release HMGB1. ATM-deficient cells also show chromatin alterations, as well as levels of reactive oxygen species (ROS; Kim and Wong, 2009) that can provoke loss of nuclear HMGB1 (Tang et al., 2010), which may explain why A-T fibroblasts secreted HMGB1 independent of senescence-inducing stimuli.

Our findings are consistent with a model in which HMGB1 regulates the SASP at two levels: secreted HMGB1 stimulates the SASP through TLR4 signaling and NF-κB transcriptional activity, whereas high levels of nuclear HMGB1 suppress the SASP and NF-κB transcriptional activity. Thus, HMGB1 depletion reduced both nuclear and secreted HMGB1 in senescent cells, and reduced the secretion of SASP components, similar to HMGB1 blocking antibodies. Further, although HMGB1 depletion and overexpression both induced growth arrest, SA-β-Gal, and SAHF, only overexpression caused HMGB1 secretion, NF-κB

transcriptional activity, and IL-6 secretion. Further, exogenous HMGB1 stimulated NF-κB translocation but not transactivation activity or IL-6 secretion in PRE or HMGB1-depleted cells. Notably, PRE and HMGB1-depleted cells retained the ability to secrete IL-6 in response to TNF and IL-1α, indicating that HMGB1 is not required for cells to respond to other inducers of cytokine secretion.

Extracellular HMGB1 is thought to contribute to age-related pathologies such as cardiovascular disease and cancer by stimulating chronic inflammation, a characteristic of aging tissues (Kohno et al., 2009; Tang et al., 2010; Andersson and Tracey, 2011). Likewise, the SASP is thought to contribute to a variety of age-related pathologies, also by driving the sterile inflammation that is hallmark of aging tissues (Campisi and d'Adda di Fagagna, 2007; Coppé et al., 2010a; Freund et al., 2010). At present, the precise relationships between secreted HMGB1, the SASP, and age-related pathology remain to be determined.

Materials and methods

Cells and cell culture
HCA2 (O. Pereira-Smith, University of Texas Health Science Center, San Antonio, TX), BJ (American Tissue Type Collection), IMR-90, WI-38, IMR91(L) and IMR91(S) (Coriell Repository, Camden, NJ), and placental fibroblasts (A. Krtolica, StemLifeLine, Inc.) were cultured in DMEM plus 10% FBS and penicillin/streptomycin at 3% oxygen and 10% CO_2. AT2SF and AT5823 were cultured in MEM plus 15% FBS and penicillin/streptomycin at ambient oxygen and 5% CO_2. Human mammary epithelial cells (M. Stampfer, Lawrence Berkeley National Laboratory, Berkeley, CA) were cultured in complete MEGM (Lonza) at ambient oxygen and 10% CO_2. Prostate epithelial (Coriell Repository) cells were cultured in PrEC complete media (Lonza) at ambient oxygen and 5% CO_2. MEFs were isolated from C57BL/6 13.5-d embryos, and cultured in 3% O_2 (Parrinello et al., 2003).

Senescence induction, SA-β-Gal, and BrdU labeling
We induced senescence by exposing subconfluent cultures to 10 Gy x-rays; control cultures were mock irradiated. Alternatively, we used lentiviruses to express no insert (control), p16INK4a, Ha-RASV12, or HMGB1 cDNAs, or shRNAs against GFP (control) or HMGB1, or allowed cells to undergo replicative exhaustion. PRE cells were used 1–2 d after mock irradiation or infection; SEN cells were used 7–10 d after irradiation or infection. Cells were considered PRE if >70% incorporated BrdU over a 2-d interval and <10% expressed SA-β-Gal. Cells were considered SEN after 7–10 d; <10% incorporated BrdU and >80% expressed SA-β-Gal (Dimri et al., 1995). We detected SA-β-Gal as described previously (Dimri et al., 1995) or using a commercial kit (BioVision). In brief, cells were fixed in 4% paraformaldehyde, washed, and incubated with detection reagent (provided by manufacturer) at 37°C overnight. We measured DNA synthesis using a modified BrdU labeling kit (Roche) or the EdU proliferation assay (Life Technologies).

Immunofluorescence
Cells in 4- or 8-well chamber slides were immunostained as described previously (Davalos and Campisi, 2003). In brief, cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton, and blocked with 10% goat or donkey serum before incubating overnight at 4°C with primary antibody in 5 or 10% serum. After washes with PBS, we incubated samples with Alexa Fluor 488– or 594–conjugated secondary antibodies for 30 min at room temperature, followed by three washes with PBS. The final wash contained 0.1 mg/ml DAPI. We mounted slides in Vectashield and viewed by epifluorescence. Mouse tissue was processed in coplin jars using the same protocol. We viewed samples using a microscope (model BX60; Olympus) and either a 100× UPlanFl 1.3 NA lens with oil or a 40× UPlanFl 0.5 NA lens without oil. Images were acquired with a charge-coupled device camera (Spot Flex color camera; Diagnostic Instruments, Inc.) and captured using SPOT imaging software (Diagnostic Instruments, Inc.). Any image modifications were applied to the entire image and all relevant fields and samples processed with Photoshop CS2 (Adobe).

Antibodies and chemicals
Rabbit (BD or Proteintech) or mouse (R&D Systems) anti-HMGB1 antibodies were used for Western blots, and rabbit (BD or Abcam) antibodies or DPH1.1 (a kind gift from K.J. Tracey, Feinstein Institute for Medical Research, Manhasset, NY) for immunofluorescence. We also used mouse anti-actin (EMD Millipore), anti-tubulin (Sigma-Aldrich), anti–acetyl-lysine, anti-53BP1 (EMD Millipore), anti-p16 (Neomarkers), anti-p53 (Santa Cruz Biotechnology, Inc.), anti–NF-κB (Santa Cruz Biotechnology, Inc.), and rabbit anti-active caspase-3 (Cell Signaling Technology) primary antibodies. Secondary goat antibodies conjugated to Alexa Fluors (488, 594) were from Molecular Probes. We used DAPI to stain nuclear DNA. H_2O_2, trichostatin A, leptomycin B, staurosporine, recombinant HMGB1, BSA, and TNF were from Sigma-Aldrich. IL-1α was from R&D Systems.

ELISA and cytotoxicity
CM were collected from cells cultured in 0.2% serum for 24 h, filtered, and stored at −80°C. Cell number was determined and each sample was normalized for cell number. We used ELISA kits to measure IL-6 (R&D Systems) and HMGB1 (IBL International, Chondrex), and a lactate dehydrogenase kit (Takara Bio Inc.) to measure cytotoxicity, in each case following the manufacturer's instructions.

Mouse sera
Young and old mouse (C57BL/6) tissue and sera were obtained from the National Institute on Aging rodent tissue bank.

Western analysis
Whole-cell lysates were prepared in 5% SDS or RIPA buffer (EMD Millipore; Davalos and Campisi, 2003). Cells were cultured in 0.2% serum before collecting CM. The medium was filtered and either precipitated with trichloroacetic acid or concentrated with Centricon centrifugal filter units (EMD Millipore) to 100×. Western analyses were performed as described previously, loading either 30 µg protein or concentrated medium equivalent to $3–6 \times 10^5$ cells per lane (Davalos and Campisi, 2003).

Irradiation
Cells were irradiated with a total dose of 10 Gy at 0.8 Gy/min using a Pantak x-ray generator (320 kV/10 mA with 0.5 mm copper filtration). 6–8-wk-old C57BL/6 mice (Charles River) were total body irradiated at the sublethal dose of 9 Gy (30 Gy/min) using a Gammacell 220 and ^{60}Co as a source. Brain and kidney were collected 1 wk after irradiation.

NF-κB assay
Cells expressing the Cignal NF-κB Lentiviral Reporter (CLS-013L; SA Biosciences) were infected with insertless, HMGB1-overexpressing or HMGB1 shRNA-expressing lentiviruses, and incubated with recombinant BSA, TNF, IL-1α, or HMGB1 for 24 h in serum-free media. NF-κB activity was measured as described previously (Freund et al., 2011). In brief, cells were lysed and assayed for luciferase using the Luciferase Assay System (E1500; Promega). All values were normalized to cell number.

Expression vectors and siRNA
The following sequences were used to target the indicated proteins by RNAi: HMGB1-#1, 5′-GAGGCCTCCTTCGGCCTTC-3′; HMGB1-#2, 5′-GTTGGTTCTAGCGCAGTTT-3′; p53, 5′-GACUCCATRGGUAAUCUAC-3′ (Nair et al., 2005); p16INK4a, 5′-CGCTGTGGCCCTCGTGCTGATGCTA3′ (Beauséjour et al., 2003; Narita et al., 2003); TLR-4, 5′-ATATTAAGG-TAGAGAGGTGGC-3′ (OpenBiosystems, TRCN0000056894); RELA, 5′-AACTCATCATAGTTGATGGTG-3′ (OpenBiosystems, TRCN0000014686; Freund et al., 2011); and ATM, 5′-TGAAGATGGTGCTCATAAA-3′ (Open Biosystems, TRCN0000039948; Rodier et al., 2009). We also used a sequence verified HMGB1 cDNA (TC118802; Origene) cloned into a modified lentiviral vector (670–1) that conferred puromycin resistance (Campeau et al., 2009). To inactivate p53, we used the genetic suppressor element (GSE) 22 (Gudkov et al., 1993; Beauséjour et al., 2003).

Online supplemental material
Fig. S1 shows that HMGB1 relocalization occurred in senescent cells from multiple tissue types. Fig. S2 demonstrates that extended incubation of fibroblasts with leptomycin B induced senescence without HMGB1 secretion. Fig. S3 shows overexpression or depletion of HMGB1 induced senescence in low expressing p16 HCA2 fibroblasts. In addition to growth arrest and senescence-associated β-galactosidase activity, HMGB1-mediated senescence induced associated heterochromatin foci in HCA2 and senescence did not occur in response to stress. Fig. S4 shows that HMGB1 overexpression and deletion induced senescence in MEF and HMEC. p16-mediated senescence

does not exhibit a SASP, but promoted HMGB1 secretion. Fig. S5 shows rHMGB1 induced NF-κB nuclear relocalization in the presence or absence of endogenous nuclear HMGB1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201206006/DC1.

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