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ORIGINAL ARTICLE

Low dose IR-induced IGF-1-sCLU expression: a p53-repressed expression cascade that interferes with TGFβ1 signaling to confer a pro-survival bystander effect

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Inadvertent mammalian tissue exposures to low doses of ionizing radiation (IR) after radiation accidents, remediation of radioactive-contaminated areas, space travel or a dirty bomb represent an interesting trauma to an organism. Possible low-dose IR-induced bystander effects could impact our evaluation of human health effects, as cells within tissue are not equally damaged after doses of IR ⩽10 cGy. To understand tissue responses after low IR doses, we generated a reporter system using the human clusterin promoter fused to firefly luciferase (hCLUp-Luc). Secretory clusterin (sCLU), an extracellular molecular chaperone, induced by low doses of cytotoxic agents, clears cell debris. Low-dose IR (⩾2 cGy) exposure induced hCLUp-Luc activity with peak levels at 96 h, consistent with endogenous sCLU levels. As doses increased (⩾1 Gy), sCLU induction amplitudes increased and time-to-peak response decreased. sCLU expression was stimulated by insulin-like growth factor-1, but suppressed by p53. Responses in transgenic hCLUp-Luc reporter mice after low IR doses showed that specific tissues (that is, colon, spleen, mammary, thymus and bone marrow) of female mice induced hCLUp-Luc activity more than male mice after whole body (⩾10 cGy) irradiation. Tissue-specific, non-linear dose- and time-responses of hCLUp-Luc and endogenous sCLU levels were noted. Colon maintained homeostatic balance after 10 cGy. Bone marrow responded with delayed, but prolonged and elevated expression. Intraperitoneal administration of α-transforming growth factor (TGF)β1 (1D11), but not control (13C4) antibodies, immediately following IR exposure abrogated CLU induction responses. Induction *in vivo* also correlated with Smad signaling by activated TGFβ1 after IR. Mechanistically, media with elevated sCLU levels suppressed signaling, blocked apoptosis and increased survival of TGFβ1-exposed tumor or normal cells. Thus, sCLU is a pro-survival bystander factor that abrogates TGFβ1 signaling and most likely promotes wound healing.

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INTRODUCTION

Development of ultrasensitive indicators of biological responses to low doses of ionizing radiation (IR) (for example, <0.1 Gy (10 cGy)) is of paramount importance to eventually understanding how to predict health risks to humans. Low-dose exposures of ⩽10 cGy can occur during space flight, during remediation of radiation-contaminated materials, after radiation accidents or after a dirty bomb. Studies of cellular responses to low doses of IR (⩽10 cGy) are confounded by various factors, such as cell type investigated, radionuclide, the oxygen level in the culture medium, cell cycle stage at the time of exposure, whether primary or immortalized cells are used and whether cells have intact tumor suppressor (for example, p53 or pRb) functions. At 10 cGy, given estimates of DNA lesions created by low linear energy transfer IR exposures, less than four DNA double strand breaks and <100 DNA single strand breaks are expected, suggesting that few DNA damage sensors would be activated. Indeed, most available evidence strongly suggest that only *Ataxia telangiectasia*-mutated kinase (ATM) is activated by DNA damage created by low doses of low linear energy transfer IR doses,[1] probably owing to oxidative stress.[2–4] Indeed, γH2AX foci formation in response to low doses of IR strongly suggest that ATM activation does occur, presumably because of the formation of double strand breaks as a result of replication through unrepaired single strand breaks. To detect these responses and to assess their affects on human health, biodosimeters are needed.

Current biodosimeters under development are mostly based on DNA damage and repair pathways, such as detection of double strand break formation and repair by γ-H2AX foci assessments.[5–7] Unfortunately, the detection and repair of DNA lesions are generally rapidly formed and repaired, leaving such biodosimeters as very limited tools to detect low-dose IR exposures.[5,6] Alternatively, lymphocytes from blood samples or skin/hair samples of affected individuals can be analyzed for micronuclei formation[8,9] or premature chromosome condensation assessments for chromosomal aberrations.[10] Although useful for

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assessing exposure, these assays do not monitor responses of tissues known to be prone to carcinogenesis as a result of low-dose IR exposures. Currently, no developed biodosimeter has been able to assess the human health effects of low doses of IR, but research is ongoing toward that goal, and most likely no one biodosimetry system will be able to solve all needs for assessing human health effects. Nevertheless, development of biodosimeters based on known regulatory functions that can assess exposures in responsive and sensitive tissues in a noninvasive manner, and repeatedly over time, are desperately needed. Biodosimeters that can establish basal responses and monitor damage-induced reporters over time as a function of dose are also desperately needed.

Recently, DNA damage- and senescence-induced secreted proteins (that is, an induced secretome) have been identified as an important response to low doses of IR.[11–13] Identifying low-dose IR-responsive expression pathways that ultimately result in expression of secreted proteins that could have biological impacts on exposed, as well as non-exposed, cells would strongly suggest that not all cells have to be irradiated for entire tissues to respond *in vivo*. To date, few pathways activated *in vivo* in response to low doses of IR have been elucidated. Recently, we delineated a unique pathway of regulation of insulin-like growth factor 1 (IGF-1) expression in response to low doses of IR *in vitro*.[1,14,15] We demonstrated that IGF-1 was repressed by p53/NF-YA complexes in its basal state at one unique Nuclear factor-Y (NF-Y) regulatory site in the IGF-1 promoter, whose binding was lost after low-dose IR exposures in an ATM-dependent manner in cells deficient in p53 function, resulting in secretory clusterin (sCLU) expression. Regulation of IGF-1 promoter activity and ligand expression, and thus sCLU, was rate-limited by p21 and an ill-defined ATM-dependent modification of NF-YA in cells, even though p53 stabilization and protein induction occurred.[1] IGF-1 expression has been linked to cytoprotection of irradiated cells *in vitro* and tissues *in vivo*; however, underlying mechanisms of cytoprotection have not been demonstrated.[16]

sCLU is a known low-dose IR-inducible secreted protein that is regulated by IGF-1 signaling, and can confer significant cytoprotection to cells *in vitro* after exposure to high-dose IR challenges conferring an adaptive response.[14,15] In fact, as numerous cancers constitutively overexpress sCLU as a pro-survival and pro-growth factor, strategies using antisense oligomers[17,18] or small interfering RNA (siRNA) nanoparticles[19] have been developed to enhance chemo- and radiotherapies against human prostate and lung cancers. sCLU can bind and sequester BAX in the endoplasmic reticulum of cells, thereby preventing drug- and radiation-induced apoptosis.[20–22] Thus, low-dose IR-inducible IGF-1-sCLU expression from cells *in vitro* or in tissues *in vivo* could afford significant cytoprotective bystander functions. To date, however, most low-dose IR-induced bystander effects reported support induction of cytotoxic factors that increase damage in non-irradiated cells (reviewed in Snyder[23]). A detailed study of a cytoprotective bystander factor has not been reported.

As we previously linked IGF-1 signaling to expression of pro-survival sCLU levels, we hypothesized that expression of the IGF-1-sCLU axis would: (i) allow development of a potent and ultrasensitive biodosimeter of live cells using the human clusterin (CLU) promoter linked to firefly luciferase (hCLUp-Luc) expression for bioluminescence imaging (BLI); BLI allows for non-invasive temporal quantitative imaging of cells and tissue in real time and greatly increases the sensitivity of detection *in vitro* and *in vivo* and (ii) result in expression of a secreted protein expression axis that would have significant pro-survival bystander effects *in vivo*.

Here, we demonstrate for the first time that cells *in vitro* and transgenic mice *in vivo* containing the hCLUp-Luc reporter can be used as an extremely sensitive and potentially important reporter system to repeatedly image responses of irradiated live cells and tissues. We present evidence that IGF-1-sCLU expression in specific radiation-sensitive tissues *in vivo* is controlled by low-dose IR activation of transforming growth factor (TGF)β1, consistent with prior findings of the activation of this tumor suppressor cytokine protein. The IGF-1-sCLU expression axis is long-lived and extremely responsive to low doses of IR, as well as to other cytotoxic agents,[1] making it ideal for future use as a biomarker for biological responses to low-dose IR exposures. We demonstrate that exogenous reporter responses matched endogenous sCLU protein expression responses in dose-response and temporal kinetics, and that tissues differ in dose- and time-responses to low doses (that is, 2 cGy) versus higher doses of IR. Importantly, induction of sCLU at low doses of IR, from 1 to 100 cGy, were linear for cells *in vitro* and tissue *in vivo*; however, expression of sCLU could not be extrapolated from high doses, and non-linear responses were clearly indicated at doses >1 Gy. Mechanistically, we demonstrate evidence of a negative feedback regulatory bystander loop in which low-dose IR activated TGFβ1-mediated signaling-induced sCLU. However, subsequent elevated levels of sCLU in the media, in turn, suppress TGFβ1 signaling and thereby expression of sCLU protein levels. Homeostatic responses in colon are compared with the apparent non-homeostatic responses in bone marrow in the same animal. Suppression of TGFβ1 signaling by sCLU in the medium ultimately results in protection of TGFβ1-exposed cells from growth suppression, apoptosis and cytotoxicity. The potential importance of this cytoprotective bystander effect in terms of risk to low-dose IR-exposed patients is discussed.

RESULTS

hCLUp-Luc, a low-dose IR-sensitive reporter construct

We previously reported that IGF-1-sCLU expression was induced in human or mouse epithelial cells with long-lived (peaking 24–48 h after exposure) responses to low doses of IR ($\geqslant$2 cGy),[14,15,24,25] but only in cells compromised for p53 function. Importantly, IGF-1R-Src-MAPK-Erk1/2-Egr1 signaling was upstream of CLU promoter transactivation.[14] We hypothesized that the human clusterin promoter linked to a firefly luciferase reporter (hCLUp-Luc) would be a sensitive reporter system capable of responding to very low doses of IR. To generate such a biodosimeter, a 1403-bp hCLU promoter region was cloned upstream from the firefly luciferase reporter, creating hCLUp-Luc. hCLUp-Luc was stably integrated into MCF-7 breast cancer cells, and BLI used to quantify induction. BLI was compared with standard luciferase assays. Responses of cells with integrated hCLUp-Luc directly mimicked endogenous gene expression in dose-response and time-course studies, as reported in MCF-7 cells;[1,14,15] MCF-7 cells have elevated Mdm2 levels that compromise p53 function.[26] Responses of stably integrated hCLUp-Luc MCF-7 cells were identical to various human and mouse cells transiently transfected with hCLUp-Luc to characterize time-course and dose-response expression, using BLI or standard luminometer assessments.

We first optimized BLI of hCLUp-Luc expression from irradiated (0.02–5.0 Gy) stably integrated MCF-7 cells to compensate for cosmic ray background and increase signal/noise ratios in live cells, where repeated measurements in the same cells under optimal cell pellet conditions were made (Supplementary Figures S1A, S1B and S2A–S2D). We then explored expression in dose-response and time-course studies (Figure 1). Log-phase MCF-7 cells were irradiated with various doses of IR (0, 0.2, 0.5, 1.0 and 5.0 Gy), and induction assessed 72 and 96 h (Figures 1a and b at 96 h) later using BLI; sCLU induction involves both ATM-mediated loss of p53/NF-YA binging to the IGF-1 promoter and induction/accumulation of IGF-1 that takes >24 h for the expression cascade to show optimal induction.[1,14,25] Significant hCLUp-Luc promoter activity was noted after 20 cGy, and expression dramatically

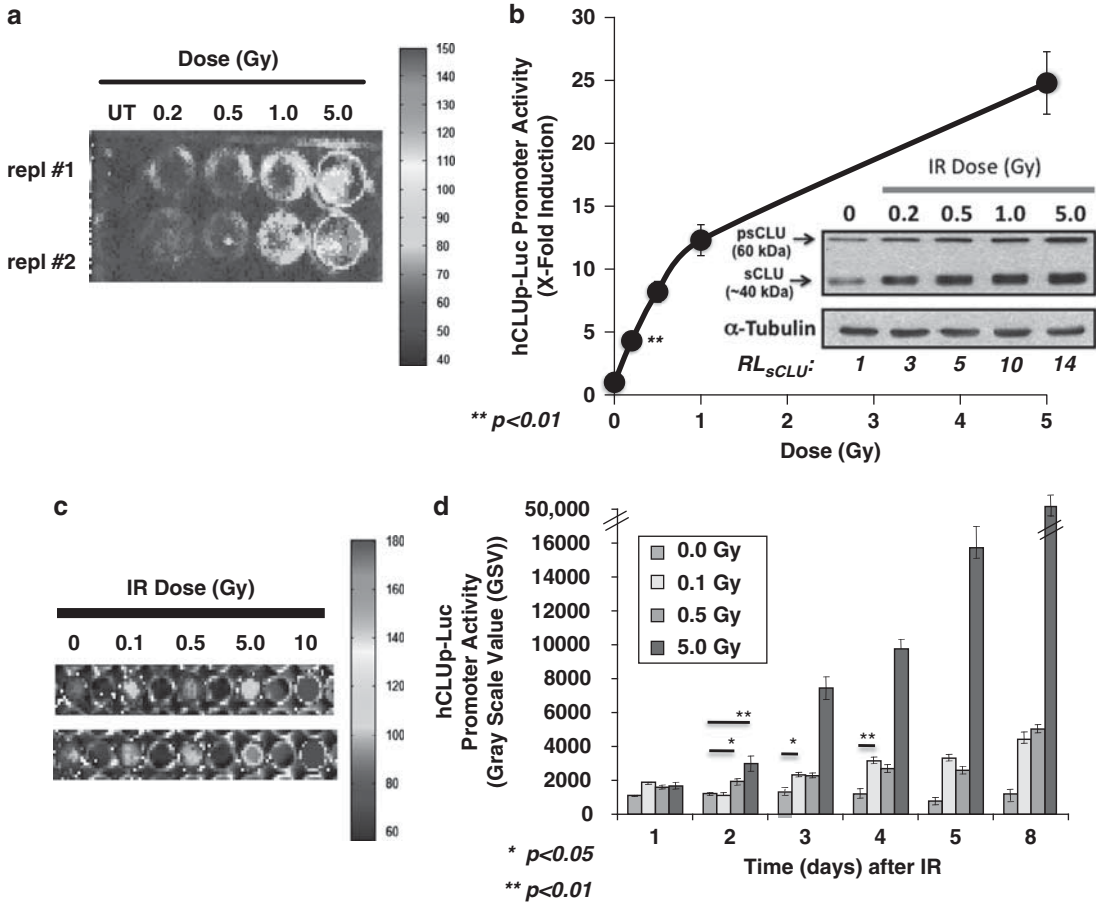

Figure 1. Development of the hCLUp-Luc reporter as a sensitive indicator of low-dose IR exposures. Stable 1403 hCLUp-Luc MCF-7 cells were monitored for time-course and dose-response promoter reporter activities using BLI imaging, where numerous readings were taken from the same living cells. (**a, b**) Dose-response induction of hCLUp-Luc. Significant hCLUp-Luc activity was noted after 0.2 Gy (20 cGy) exposure, with linear *X*-fold increases with IR doses between 0.2 – 1.0 Gy. Inset, endogenous precursor clusterin (psCLU) and sCLU protein expression in MCF-7 hCLp-Luc cells. Relative sCLU levels (RL_{sCLU}) were assessed using NIH Image J assessment of western blots as described.[1] (**c**, **d**) Time-course hCLUp-Luc induction with IR dose. Note that longer times were required for induction as IR doses decrease. Real-time images (**a**, **c**) and corresponding quantitation of three experiments performed in triplicate (**b**, **d**, means ± s.e.) are shown. $*P \leqslant 0.05$; $**P \leqslant 0.01$. Time-course and dose-response increases in hCLUp-Luc activities were identical to endogenous increases in the psCLU, as well as mature sCLU protein expression, in MCF-7 cells.[1,14,24,25]

increased with increasing IR doses (Figures 1a and b). Linear expression with dose ($r = 0.987$) was noted with exposures between 20 and 100 cGy, but hCLUp-Luc reporter activity and sCLU protein expression responses reached plateau levels at doses >1 Gy (Figure 1b, inset); exogenous reporter and endogenous sCLU protein expression were consistently expressed with increasing doses of IR (Figure 1b); in insert, precursor clusterin and sCLU represent related immature and mature sCLU protein forms, respectively, as described.[1] In time-course studies, exposure of MCF-7 cells with 10 cGy caused more than twofold induction of sCLU at 96 h. As cells were exposed to higher doses of IR (up to 5 Gy), hCLUp-Luc promoter induction responses were significantly faster, with approximately threefold induction, 48 h after 5 Gy (Figures 1c and d). Thus, both the magnitude and temporal kinetics of CLU induction were directly proportional to the dose of IR exposure of log-phase MCF-7 cells, consistent with expression of the endogenous CLU gene.[14,15] Similar responses were noted in all mouse and human cells examined, even when transient transfections were used instead of stable clones.[14,15] In general, expression of hCLUp-Luc and sCLU protein expression was linear with dose, up to 1.0 Gy ($r = 0.987$, Figure 1b), but reached plateau levels with higher doses.

p53 represses hCLUp-Luc promoter activity

We previously demonstrated that endogenous sCLU expression was repressed by functional p53.[14,15] Similarly, wild-type p53 HCT116 cells transiently transfected with the hCLUp-Luc promoter reporter and imaged using BLI showed significantly less hCLUp-Luc activity compared with isogenic $p53^{-/-}$ HCT116 cells, where p53 was somatically knocked out (Figure 2a). p53 function can also be affected by alterations in, or over-expression of, PTEN, whose activities enhance or prevent, respectively, the efficacy of Mdm2 to degrade p53.[26] Loss of p53 function would, in turn, allow sCLU expression in stable hCLUp-Luc MCF-7 cells. Indeed, alterations that compromised p53 function (for example, PTENCA or E6 overexpression) led to elevated hCLUp-Luc activities, whereas conditions that enhanced p53 suppressor activity (that is, downregulation of AKT1 with kinase-dead expression or forced overexpression of p53) repressed hCLUp-Luc activities in stable hCLUp-Luc MCF-7 cells, even after exposure to IR using BLI (Figure 2b). Similar responses were noted in HCT116 cells using luminometer assessments (Supplementary Figures S3, S4). Thus, the hCLUp-Luc biodosimeter used in transient or stable transfections emulates endogenous regulation of the CLU gene.

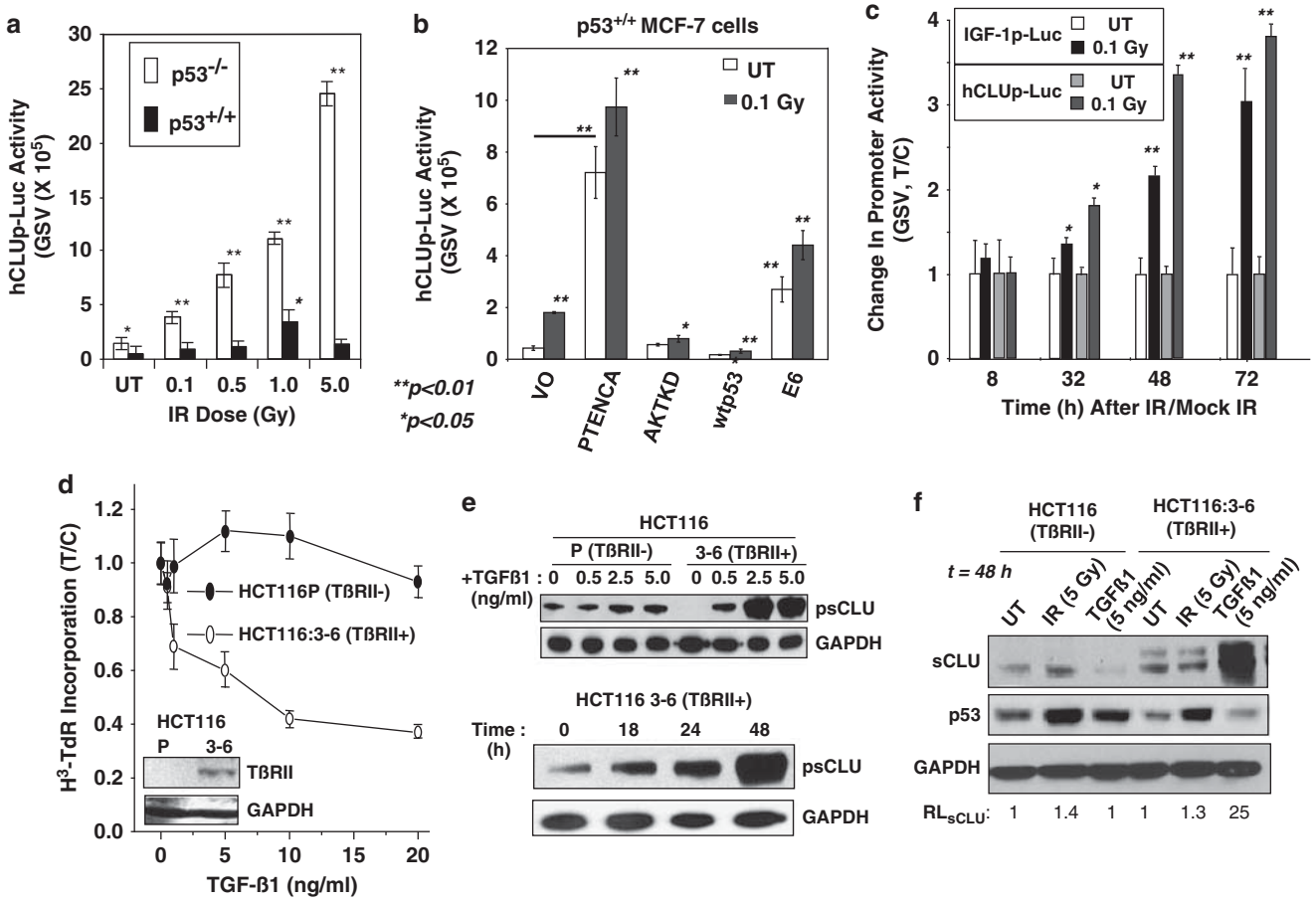

Figure 2. CLU expression is repressed by p53 and upstream factors that alter p53 function. (**a**) Wild-type p53 HCT116 (wtp53) and an isogenic HCT116 cell line in which p53 was somatically knocked out (HCT116 $p53^{-/-}$) were transiently transfected with hCLUp-Luc. Cells were exposed to various IR doses and analyzed for hCLUp-Luc activity using BLI, 48 h after IR. Relative hCLU-Luc expression in Gray Scale Values (GSVs $\times 10^5$) were monitored and results (means $\pm$ s.e.) shown for three experiments repeated in triplicate each. Note slight increases in hCLU-Luc activity in HCT116 $p53^{+/+}$ cells, but significantly greater levels in HCT116 $p53^{-/-}$ cells; there was no difference in overall IR sensitivity in HCT116 $p53^{-/-}$ versus HCT116 $p53^{+/+}$ cells.[27] (**b**) wtp53 MCF-7 cells were co-transfected with hCLUp-Luc and either vector alone, constitutive-active PTEN (PTENCA), dominant-negative AKT1 (AKT kinase-dead, AKTKD), wtp53 or E6 expression vectors. Cells were then irradiated with 0.1 Gy 24 h later and hCLUp-Luc activities monitored 48 h later using BLI. Note that any treatment that functionally inactivated p53 in a direct (that is, E6) or indirect (PTENCA) manner, suppressed hCLUp-Luc activity. In contrast, vectors that express factors that enhance p53 function (that is, wtp53 or AKTKD) suppressed basal and IR-induced hCLUp-Luc activities. Reported are Gray values for treated over control levels, where the level of expression of the mock-irradiated vector only control was set at 1.0. (**c**) MCF-7 cells were transfected with hIGF-1p-Luc or hCLUp-Luc promoter reporters, then mock-treated or exposed to 0.1 Gy 24 h later and analyzed using BLI at various times (8, 32, 48 and 72 h) after IR. Note simultaneous increases in CLU and IGF-1 promoter activities after 0.1 Gy exposure of MCF-7 cells that overexpress endogenous Mdm2. Results (means $\pm$ s.e.) presented are from three separate experiments, each repeated in triplicate. $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$. (**d**) HCT116 (TβRII−) and isogenic HCT116 3-6 (TβRII+) cells were examined for growth arrest responses induced by various TGFβ1 treatments using changes in thymidine (^{3}H-Thyd) incorporation. Note that HCT116 cells were not growth arrested, and maintained an elevated level of ^{3}H-Thyd incorporation in their DNA. In contrast, HCT116 3-6 cells are dramatically growth arrested by TGFβ1, and ^{3}H-Thyd incorporation was significantly suppressed in a 48-h period. Inset shows TβRII expression in HCT116 3-6 cells, but not in HCt116 parental cells. (**e**) Untreated and TGFβ1-exposed cells from (**d**) were examined for induction of sCLU expression with respect to glyceraldehyde 3-phosphate dehydrogenase loading. Note that sCLU induction was noted only in HCT116 3-6 cells (as fast as 18 h after exposure to 5 ng/ml TGFβ1) and that HCT116 cells have a slightly elevated level of sCLU (because of IGF-1 in the serum and constitutive activation of ATM due to its MMR deficiency) compared with HCT116 3-6 cells.[1] (**f**) HCT116 and HCT116 3-6 cells were mock-treated (UN), or exposed to IR (5 Gy) or TGFβ1 (5 ng/ml) and whole cell extracts prepared 48 h later. As both cells are wtp53, neither cell line induce significant levels of sCLU after IR. In contrast, significant sCLU induction, corresponding to loss of p53 steady state levels, was noted in TGFβ1-exposed HCT116 3-6 (TβRII+) cells as previously reported.[26] $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$. Relative levels of sCLU were calculated as described in Figure 1.

Regulation of CLU promoter by upstream IGF-1 signaling

We reported that CLU promoter transactivation and sCLU expression were regulated by upstream IGF-1R signaling.[14] We confirmed that IGF-1 and CLU promoter activities correlated well in temporal expression kinetics after IR (0.1 Gy) using BLI (Figure 2c) in MCF-7 cells. Although the response from the hIGF-1p-Luc reporter was slightly faster than hCLUp-Luc, both promoter reporters were dramatically increased in response to low doses of IR using BLI. Similar data were found by standard luminometer measurements using protein levels or respiratory syncytial virus (RSV)-β-gal (RSV-β-gal) expression standardization.[14] In separate studies, we found that administration of AG1024, an IGF-1R receptor tyrosine kinase inhibitor, dramatically suppressed CLU promoter and sCLU protein expression, whereas IGF-1 promoter and ligand expression were not affected.[1] Thus, hIGF-1p-Luc and hCLUp-Luc reporters were useful indicators of low-dose IR exposures.

sCLU induction by TGFβ1

As p53 suppresses IGF-1-sCLU expression,[1] and TGFβ1 exposure can induce Mdm2 resulting in p53 degradation,[26] we examined sCLU induction after TGFβ1 treatments in HCT116 (TβRII−) versus isogenic HCT116 3-6 (TβRII+) cells. TβRII−deficient HCT116 cells were restored for DNA mismatch repair and TβRII expression, creating HCT116 3-6 cells.[27] In response to various TGFβ1 doses, only HCT116 3-6 cells were dramatically growth arrested compared with HCT116 (TβRII−) cells using changes in ^{3}H-Thymidine incorporation into DNA in a 48-h time period (Figure 2d). Cells in Figure 2d were then examined for changes in expression of sCLU, in which a significant increase (~threefold) in sCLU protein expression (monitored by the ~60 kDa precursor clusterin levels) was found in HCT116 3-6 cells by as little as 0.5 ng/ml TGFβ1 (Figure 2e). In time-course studies, induction of precursor clusterin occurred by 18 h in HCT116 3-6 cells exposed to 5 ng/ml TGFβ1 (Figure 2e, lower panel). Finally, we directly compared mature sCLU induction responses (sCLU) in HCT116 with HCT116 3-6 cells after exposure to mock treatments (UT), IR (5 Gy) or TGFβ1 (5 ng/ml) (Figure 2f). Significant induction of sCLU (>30-fold) was only noted in TβRII+ HCT116 3-6 cells at 48 h, with concomitant and significant loss of steady state p53 levels, consistent with TGFβ1-induced induction of Mdm2 and subsequent p53 degradation.[26] TβRII− HCT116 cells were not responsive to TGFβ1. In contrast, exposure of either cells to IR (5 Gy) failed to induce significant sCLU levels, whereas p53 stabilization was noted in both cells.

A low-dose IR-sensitive transgenic hCLUp-Luc promoter mouse

We then generated a transgenic hCLUp-Luc reporter mouse in an FVB/N genetic background and examined the responses of age (10 week)- and weight (18 g)-matched male and female mice after exposure to whole body low doses of IR (for example, 0.1 Gy). Irradiation of female versus male mice induced BLI expression in specific internal organs of both genders of mice at 24 h. Greater responses were, however, routinely noted in female versus male mice (Figure 3a). Increased expression of hCLUp-Luc activity in female mice may be related to the known testosterone repression of this gene in male mice; the CLU gene is also referred to as testosterone-repressed prostate message-2 (reviewed in Trougakos *et al.* [28]). *Ex vivo* imaging of internal organs of female mice demonstrated dramatic induction of hCLUp-Luc promoter activities in bone marrow, spleen, colon, thymus (Figure 3b) and mammary glands (not shown). In contrast, imaging of muscle, heart, liver or pancreas did not demonstrate significant hCLUp-Luc activities by BLI at any time. BLI or *ex vivo* imaging of control non-irradiated mice or tissue showed little hCLUp-Luc expression.

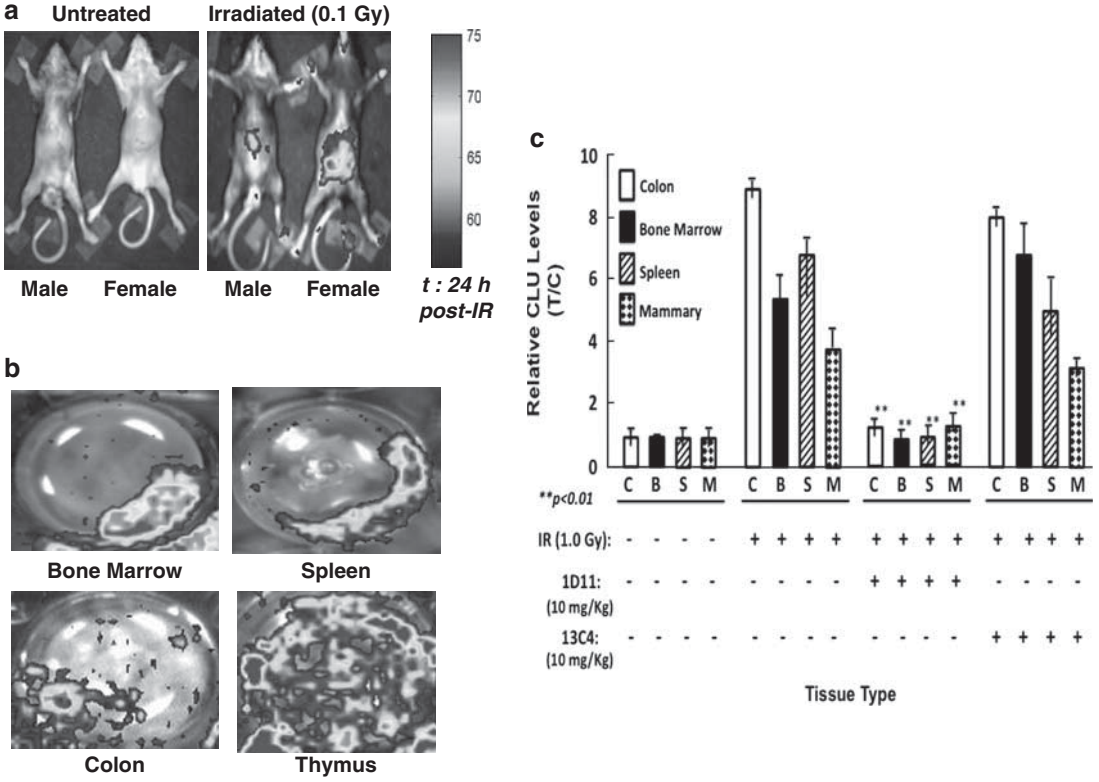

Figure 3. hCLUp-Luc transgenic mice are responsive to low doses of IR in specific hypersensitive tissues. Age (10 weeks)- and weight (18 g)-controlled hCLUp-Luc reporter transgenic FVB/N mice were mock-treated or exposed to 0.1 Gy IR and imaged using BLI 24 h later. (**a**) Images of male and female mice exposed to IR. Mice were exposed to 0.1, 1.0 and 5.0 Gy and at various times intraperitoneally injected with 10 mg/kg luciferin. At 24 h, internal organ hCLUp-Luc responses were noted with all doses used. Images after 0.1 Gy were shown for representation. (**b**) Female mice shown in (**a**) were killed, and internal organs removed and placed in PBS containing 2.5 mg/ml luciferin for BLI *ex vivo*. Note hCLUp-Luc expression was elevated in bone marrow, spleen, thymus and colon at 24 h. Responses from mammary tissue were also noted, but not shown. Responses were not found in muscle, lung, heart or liver. Images are representative of experiments performed at least three times. (**c**) Female mice (three per treatment group) were either mock-irradiated or exposed to whole body IR (10 cGy) as described above, and then injected with or without the α-TGFβ1 (1D11) antibody or a control antibody (13C4), each at 5 mg/kg, and hCLUp-Luc or endogenous sCLU levels monitored by imaging or western analyses 96 h later. sCLU induction at 96 h was blocked by the α-TGFβ1 antibody that blocks all three forms of the TGFβ1 family,[31] whereas the control IgG 13C4 antibody had little effect. Experiments were repeated twice with three[3] animals per treatment group and tissues pooled as indicated for each experiment. Tissues were colon (c), bone marrow (b), spleen (s) and mammary (m).

Induction of sCLU *in vivo* is mediated by TGFβ1

As sCLU can be induced by activated TGFβ1,[29] and low-dose IR exposure is known to activate TGFβ1,[30] we examined whether sCLU responses *in vivo* by hCLUp-Luc and by endogenous sCLU induction by western blotting using excised tissues, as indicated, were prevented by intraperitoneal administration of an α-TGFβ1 (1D11, 5 mg/kg) versus a control IgG antibody (13C4, 5 mg/kg) (Figure 3c). The 1D11 antibody neutralizes all three forms of the TGFβ family.[31] Indeed, both endogenous sCLU, as well as hCLUp-Luc reporter induction, responses were suppressed in colon, bone marrow, spleen and mammary gland tissue by administration of the α-TGFβ1 antibody, whereas the control 13C4 isotype antibody had no effect (Figure 3c).

Induction of IGF-1-sCLU *in vivo* is due to activated TGFβ1 signaling in response to low doses of IR

As p53 can repress IGF-1-sCLU expression after IR, but not in response to TGFβ1 exposure,[32] we suspected that induction of hCLUp-Luc *in vivo* in transgenic mice was due to activation of TGFβ1 signaling after low doses of IR (whole body irradiation (0.1 Gy) of female FVBN mice) (Figures 4a–f). The most responsive tissues within female hCLUp-Luc reporter mice were colon and bone marrow, where temporal induction was monitored after 0.1 versus 1.0 Gy exposures (Figures 4a and b). Interestingly, bone marrow and colon tissue responses differed in their temporal induction and longevity of CLU expression in response to 0.1 Gy treatment, where colon hCLUp-Luc reporter activities and sCLU

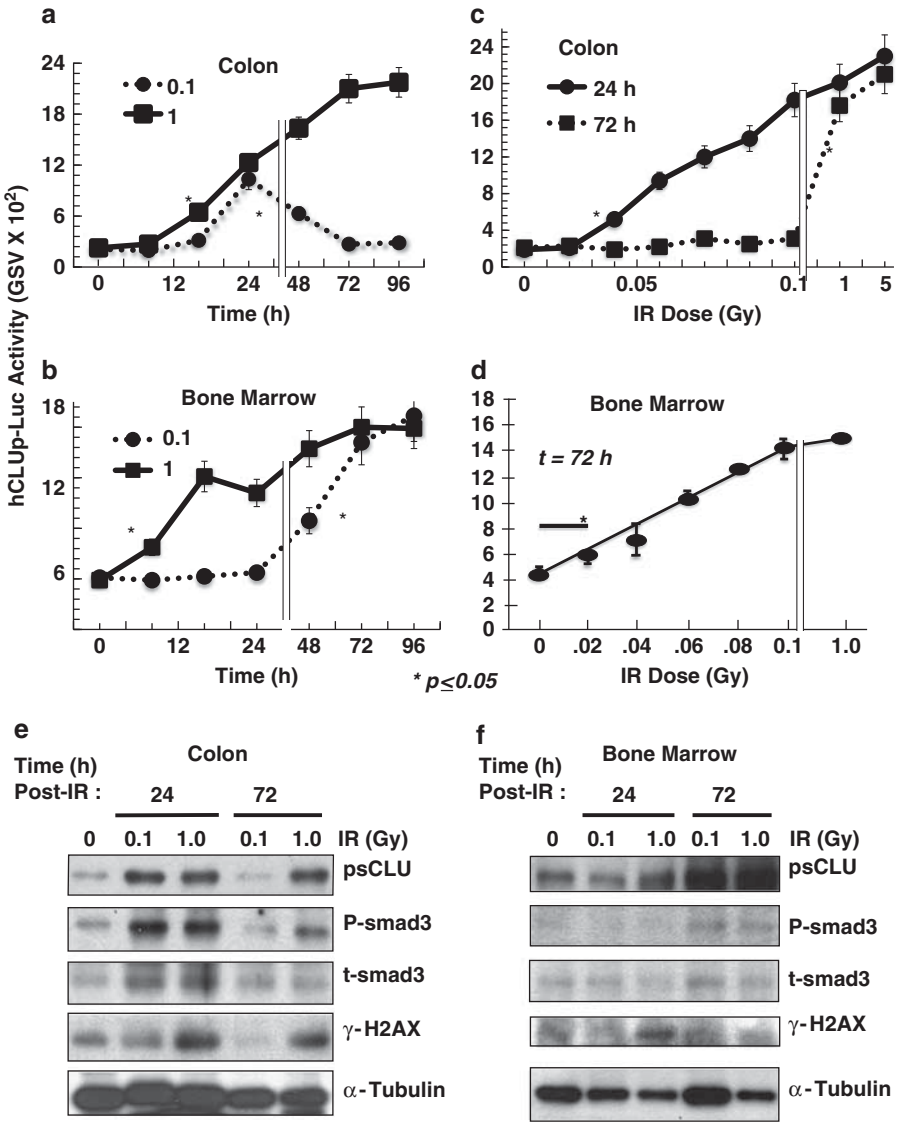

Figure 4. TGFβ1 signaling *in vivo* correlates with sCLU induction after low doses of IR. (**a**, **b**) 10-week-old (18 g) female FVB/N mice were exposed to IR (0.1 or 1.0 Gy) and colon and bone marrow tissues extracted at various times as indicated. (**c**, **d**) Dose-response (0.1–1.0 Gy) studies were also performed in female FVBN hCLUp-Luc transgenic mice and reporter expression monitored at specific times (24 or 72 h) as indicated. (**a**–**d**) Data (means + s.e., $n = 6$) are from experiments performed at least two independent times with three mice per group. Tissues were pooled and then analyzed. (**e, f**) Steady state protein level changes in psCLU and sCLU, phosphorylated or total Smad3 (P-Smad3 or t-Smad3, respectively), γ-H_2AX and α-tubulin were monitored by western analyses. Note increased levels of P-Smad3/t-Smad3, indicative of activated TGFβ1 signaling after 0.1 or 1.0 Gy at 24 or 72 h after IR in both colon and bone marrow tissue samples. Activated Smad signaling (indicated by elevated P-Smad3/t-Smad3 levels) corresponded well with increased sCLU protein levels. Shown are representative western blots for colon and bone marrow from experiments performed three times in triplicate with similar results.

protein expression peaked at 24 h, but waned by 48 h (Figures 4a and e). In contrast, bone marrow sCLU responses were significantly delayed in comparison, showing peak levels 72 h after 0.1 Gy, and remained elevated for at least 96 h after treatment (Figures 4b and f). CLU induction in bone marrow tissue was more rapid after a higher dose (1.0 Gy) of IR (Figures 4b and f), and hCLUp-Luc reporter activities and endogenous sCLU expression levels were linear ($r = 0.965$) with IR doses between 2 and 10 cGy monitored at 72 h (Figures 4d and f). At doses $>0.1-1.0$ Gy, sCLU induction reached plateau levels and were non-linear with IR dose (Figure 4d), as previously reported for endogenous sCLU protein expression.[14] Overall, sCLU induction correlated with the sensitivities of internal organs (bone marrow, spleen, thymus, mammary glands and colon) to whole body IR exposure. TGFβ1 signaling appeared to be involved in CLU gene and sCLU expression responses (Figure 3c). Indeed, western blot analyses of mouse colon (Figure 4e) and bone marrow (Figure 4f) tissue confirmed activation of Smad signaling (elevated phospho-Smad3/total Smad3 levels) with delayed kinetics, consistent with sCLU expression; note that in Figures 4e and f, precursor clusterin is the ~60-kDa precursor form of mature sCLU protein (~40 kDa) that correlated well with CLU promoter activation and messenger RNA expression after exposure to low doses of IR.[14] Thus, IGF-1-sCLU induction *in vivo* appears to be linear with very low doses (10–100 cGy), but non-linear with higher doses of IR. Induction of sCLU levels *in vivo* appears to be the result of activated TGFβ1 signaling in specific organs in response to low doses of IR, responses consistent with prior reports.[33-35] TGFβ1 activation and downstream signaling is likely a mechanism for relieving p53 repression of IGF-1 expression by Smad-activated transcriptional increases in Mdm2.[26]

sCLU suppresses TGFβ1 signaling: a negative feedback repressive effect in tumor as well as normal epithelial cells

We then investigated the physiological role of sCLU in TGFβ1-responsive human colon cancer cells. HCT116 3–6 cells were then knocked down for sCLU expression using siRNA-sCLU or mock-transfected using siRNA-Scr (Scrambled siRNA), and then interrogated for changes in TGFβ1-induced signaling, growth arrest

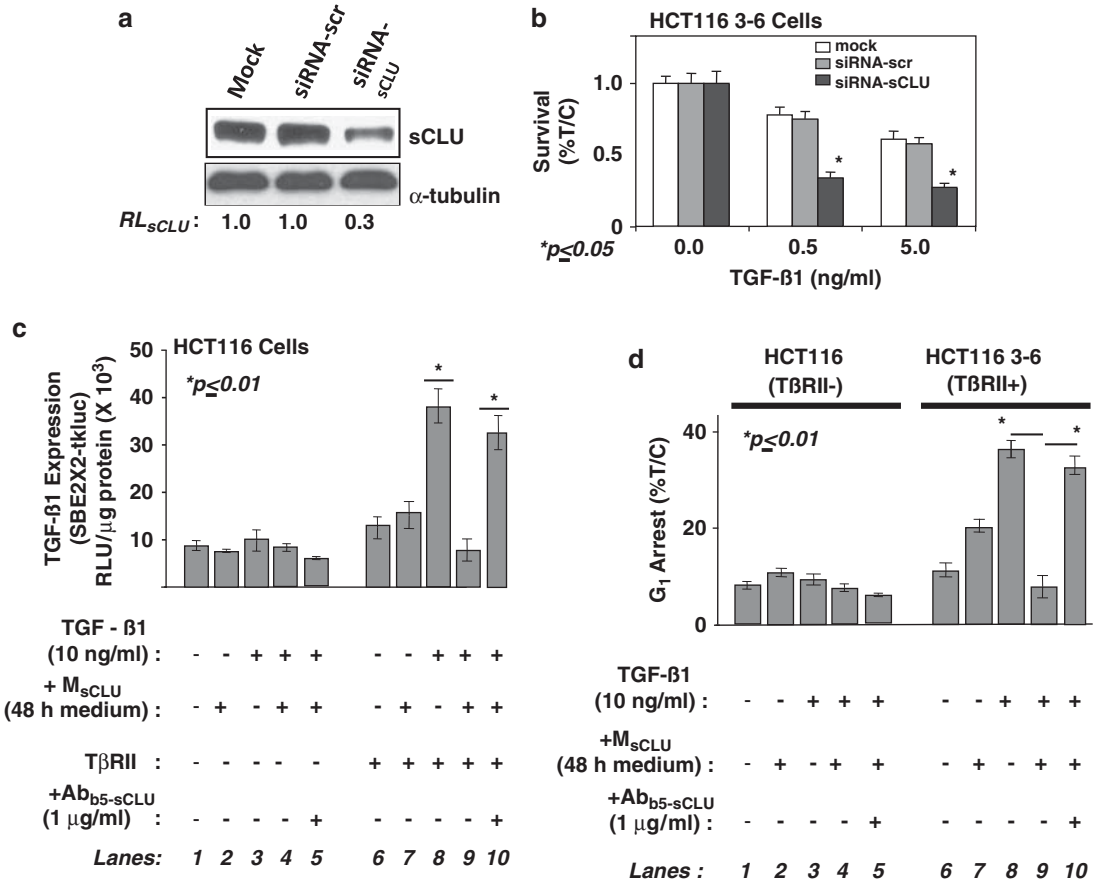

Figure 5. IR-induced sCLU levels confer resistance to TGFβ1 signaling. (**a**) TβRII-responsive HCT116 3–6 cells were transfected with siRNA-Scr or siRNA-sCLU oligomers to mock transfect or knock down sCLU levels, respectively; siRNA-sCLU is specific for the leader peptide in sCLU messenger RNA and did not alter nCLU levels.[14] sCLU levels were knocked down ~70% versus parental or siRNA-Scr transfected HCT116 3–6 cells. (**b**) sCLU knock down cells were significantly more susceptible to TGFβ1-induced lethality by colony forming ability assays. (**c**) Addition of sCLU-containing conditioned medium from IR-treated MCF-7 cells (sCLU in the medium (M_{sCLU})) to TβRII-transfected HCT116 cells repressed TGF-β1 signaling by TGFβ1-responsive SBE2X2-tkLuc reporter activity, which was blocked by b5, a human sCLU-specific antibody ($Ab_{b5-sCLU}$, lanes 8 versus lanes 9 and 10). HCT116 cells were transfected with SBE2X2-tkLuc with or without a TβRII expression vector. Cells were either exposed or not to M_{sCLU} medium from irradiated MCF-7 cells, lacking or containing excess $Ab_{b5-sCLU}$ (1 μg/ml) and treated with TGFβ1 (10 ng/ml). Cells were then monitored 48 h later for TGFβ1 signaling by SBE2X2-tkLuc. (**d**) sCLU suppresses TGFβ1 signaling. Genetically matched TβRII− HCT116 and TβRII + HCT116 3–6 cells were treated with M_{sCLU} medium from irradiated MCF-7 cells and TGFβ1 (10 ng/ml) as in (**c**), but without transfections. TβRII− HCT116 cells were not responsive, and sCLU addition had no affect, whereas HCT116 3–6 cells respond to TGFβ1 by inducing SBE2X2-tkLuc activity that was suppressed by M_{sCLU} (compare lanes 8 with 9). Addition of $Ab_{b5-sCLU}$ (clearing sCLU from the medium) restored TGFβ1 signaling (compare lanes 8–10). Data are representative of three separate experiments repeated in triplicate. *$P < 0.05$.

and lethality (Figure 5). Specific loss (~70% knockdown) of sCLU levels in HCT116 3–6 cells transfected with siRNA-sCLU were noted compared with HCT116 3–6 cells transfected with siRNA-Scr oligomers (Figure 5a). Interestingly, cells knocked down for sCLU were significantly more sensitive to TGFβ1 growth suppression, as well as long-term survival, compared with shRNA-Scr HCT116 3–6 cells (Figure 5b). Conversely, addition of exogenous sCLU in the medium significantly repressed TGFβ1 signaling in TβRII-HCT116 cells transfected with the TβRII receptor (compare lanes 8, 9, Figure 5c). However, TGFβ1 signaling was restored by co-addition of a polyclonal antibody specific to human sCLU ($Ab_{b5-sCLU}$, b5 sCLU antibody, lane 10, Figure 5c). Indeed, sCLU addition prevented TGFβ1-induced G_1 cell cycle checkpoint arrest in TGF-β1-treated TβRII+ HCT116 3–6 cells, which were restored by addition of $Ab_{b5-sCLU}$ (Figure 5d). These data strongly suggested that sCLU suppressed TGFβ1 signaling, representing a negative feedback loop that confers a cytoprotective bystander response.

As the HCT116 and HCT116 3–6 cells that we used for these studies were deficient in Bax expression and lack apoptotic responses after various cytotoxic agents,[36] and sCLU is thought to protect cells from Bax-induced apoptosis,[20,37] we explored responses in life-extended human mammary epithelial cells (HMECs) that were, or were not, knocked down for sCLU expression as in Figure 5 (Figure 6). Indeed, sCLU knockdown (Figure 6a) augmented TGF-β1 lethality (Figure 6b), which correlated with dramatic increases in apoptosis as monitored by terminal deoxynucleotidyl UTP transferase-positive (TUNEL+)-stained cells (Figure 6c). Addition of medium from irradiated MCF-7 cells, which contained elevated levels of sCLU (sCLU in the medium) as in Figure 5, suppressed apoptosis (Figure 6c) and restored the survival of TGFβ1-exposed HMECs (Figure 6b). Addition of excess $Ab_{B5-sCLU}$, which cleared sCLU from medium as monitored by western analyses, prevented sCLU function to block TGFβ1 activity and restored both apoptosis (Figure 6c) and lethality (Figure 6b) caused by TGFβ1 exposure. Thus, as with HCT116 cells (Figure 5), sCLU mediates a pro-survival bystander effect in HMECs by preventing TGFβ1-induced signaling, and therefore, downstream growth arrest, apoptosis and lethality (Figure 6).

DISCUSSION

Although IR exposure is effectively estimated using physical measurements, the actual biomedical implications of IR doses ⩽10 cGy are not known. This is particularly true of patients exposed to low IR doses after radiation accidents, a dirty bomb, remediation of radiation-contaminated areas or exposure to low IR doses during space flight. To better understand the biological effects of low-dose IR exposures, a search is on for specific functional biological response biomarkers. Unfortunately, most DNA damage response biomarkers are short-lived, noted only after high IR doses, and are not amenable to extrapolation to low-dose IR exposures. Among DNA damage sensors (DNA-protein Kinase (DNA-PK), *Ataxia telangiectasia* variant (ATR) and ATM), only ATM activation is reproducibly induced after low doses (⩽10 cGy) of IR (recently reviewed in Criswell *et al.*[38]), and is the basis of most developing γ-H2AX biodosimeters.

We recently linked induction of sCLU after low doses of IR, as well as after low doses of many other DNA-damaging agents in human and rodent cells,[14,15] to the specific induction of IGF-1 by ATM activation[1] or TGFβ1. IGF-1-sCLU expression is hypersensitive to IR, as well as many other cytotoxic stresses, including elevated

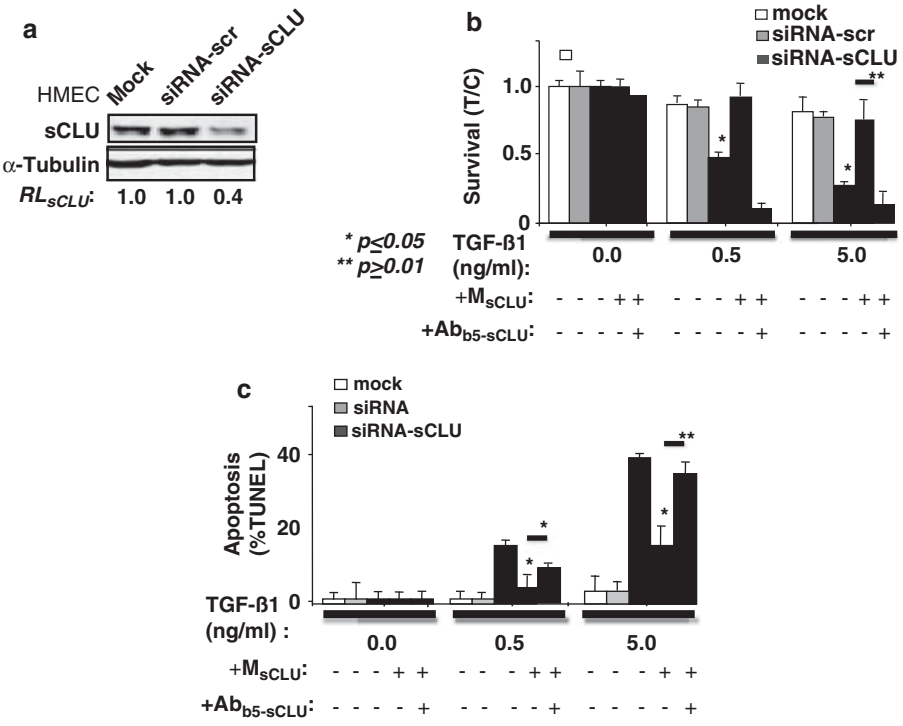

Figure 6. sCLU prevents TGFβ1-induced apoptosis and lethality in HMEC cells. (**a**) Primary HMECs were mock-transfected or transfected with siRNA-Scr or siRNA-sCLU oligomers to knock down sCLU protein expression as described in Figure 5. Specific knockdown of sCLU using this siRNA sequence, with correction using mutant CLU cDNA, was previously determined.[14] sCLU levels were knocked down ~60% in HMECs by siRNA-sCLU, but not significantly altered by siRNA-Scr. Western blots are representative of experiments repeated three times. (**b, c**) Transiently transfected HMECs from (**a**) were analyzed for changes in survival (colony forming ability assays, **b**) and cell death (apoptosis, **c**) before or after various TGFβ1 (ng/ml) exposures, and with or without the presence or absence of M_{sCLU} medium derived from irradiated MCF-7 cells. Treatments with media derived from irradiated MCF-7 cells (M_{sCLU}), cleared or not with $Ab_{b5-sCLU}$ specific for sCLU as described in Figure 5. Data are means ± s.e. from three separate experiments repeated in triplicate.

O_2 exposures and agents that do not damage DNA, but alter osmotic homeostasis.[21] IGF-1-sCLU induction is long-lived and proportional to IR exposures between 10 and 100 cGy *in vitro* (Figure 1) and *in vivo* (Figure 4). Owing to its extreme sensitivity to IR and other cytotoxic agents, we decided to use the hCLUp-Luc reporter to monitor induction kinetics of this low-dose stress-response gene as a first step in the development of a sensitive biodosimeter that may one day be used to assess risk to human health. sCLU expression is complex, with known induction responses after a variety of cell stress events, all culminating in ATM-IGF-1[1] or TGFβ1-Smad-IGF-1 stimulation. Our recent data on endogenous IGF-1-sCLU expression strongly suggested a complex expression axis that is naturally and basally repressed by wild-type p53.[1] Data presented in Figure 2 also indicate that alterations in PI3K and AKT affect sCLU expression, consistent with a role of AKT1 affecting p53 function[39] by altering Mdm2 levels.[26] Thus, this reporter system may be used to tease out regulatory factors that govern its expression, allowing future elucidation of signaling pathways and transcription factors stimulated by low doses of IR or other environmental cytotoxic agents.

As both IGF-1 and sCLU are secreted proteins that have roles in survival (IGF-1 and sCLU) and clearance of cell debris from traumatized tissue (sCLU),[9] we hypothesize that this expression axis has a major role in wound healing and recovery[28,37] *in vivo*. Thus, monitoring IGF-1-sCLU levels may not only allow assessment of the biological effects of low-dose IR exposures, but may one day be used for assessment of normal versus abnormal recovery responses that affect potential health risks to individuals. Our development and initial assessment of this biodosimetry reporter system is but a first step in validating this system. Long-term carcinogenesis experiments, crossing transgenic hCLUp-Luc reporter animals with cancer-susceptible mice (for example, p53-null or mutant K-ras) mice are warranted. Transient IGF-1-sCLU expression may be healthy in normal cells, but constitutive induction *in vivo* may indicate acquired damage-induced or mutation-mediated long-term genetic instability, as recently shown for repair-compromised cells.[1] Responses in colon versus bone marrow after exposure to 10 cGy are intriguing in this context.

As IGF-1-sCLU induction responses of cells and tissues occur on the order of days after exposure, and are directly linked to ATM (γ-H2AX formation downstream) activation, assessing tissue and sera levels immediately after an exposure event, and before induction occurs, would allow establishment of baseline levels. Then, hours and days after presumed low-dose or dose-rate IR exposures, induction of this gene may be used as a valuable assessment of IR doses with biological meaning. Standard curves after various doses of specific agents (for example, IR) can be easily performed (Figure 4). For example, our preliminary studies show that IGF-1-sCLU expression can be induced by low doses of high linear energy transfer IR (for example, iron, Luo *et al*., unpublished data), suggesting that this biodosimetry system can be used to evaluate exposures that organisms might experience in space. As transient transfections of human or mouse cells with the lentiviral-mediated hCLUp-Luc reporter demonstrates the same dose and time dependency of expression as endogenous IGF-1-sCLU, samples from irradiated patients may be directly assessed. The upstream signaling link to ATM or TGFβ1 also means that this system should complement foci formation-based systems that are also under development.

IR induction of IGF-1-sCLU expression by activation of TGFβ1 *in vivo* offers a new wrinkle to an already complex story of IR-ATM-IGF-1-IGF-1R regulation. Previously, exposure of human and rodent cells *in vitro* upregulated IGF-1R levels through ATM activation.[40–42] Furthermore, links between ATM and TGFβ1 exposures were reported, whereby TGFβ1 signaling augmented ATM activation,[33–35] and activation of TGFβ1 by an as yet poorly described reactive oxygen species (ROS) pathway after low doses of IR seem extremely important in carcinogenesis.[30] As p53 repressed IGF-1-sCLU expression[1,14] so efficiently *in vitro*, we were surprised to find the robust upregulation of this expression axis in normal tissue of low-dose IR-exposed mice, particularly in tissues that are typically hypersensitive to IR (Figures 3 and 4). Our data suggest that these induction responses may be mediated by TGFβ1 and its downstream activation of Smad3 signaling. Indeed, evidence for activation of Smad signaling in only tissues expressing hCLUp-Luc reporter activity and sCLU protein expression after low doses of IR were noted (Figures 3 and 4), with tissue induction response kinetics that matched endogenous sCLU protein induction. Our finding of the activation of Smad signaling in tissues of animals exposed to low doses (0.1 Gy) of IR are consistent with prior findings of low-dose IR activation of TGFβ1.[33] By activating TGFβ1 *in vivo*, which most likely further augments ATM activity, p53 function and phosphorylation of NF-YA are promoted through elevated p21 levels that permit IGF-1 promoter induction.[1,32] p21 inhibits cyclin-dependent kinase-induced NF-YA phosphorylation that is required for p53/NF-YA; this has recently been confirmed in lung, colon and breast cancers, and a detailed account of this mechanism by TGFβ1 will soon be reported (Zou and Boothman *et al*., unpublished data). Simultaneously, TGFβ1-activated Smad signaling also results in induction of LEF-1/β-catenin that is required for IGF-1-sCLU induction (Zou and Boothman *et al*., unpublished data). Thus, TGFβ1-activated Smad signaling in tissues hypersensitive to IR induced IGF-1-sCLU expression, presumably required for tissue recovery. Induction in normal tissues is transient and not identical in various exposed tissue (that is, bone marrow versus colon, Figure 4). Thus, different tissues may have different risks after low doses of IR. For example, analyses of mammary and lung tissues after various high linear energy transfer exposures is currently underway in our lab. The danger for cells within specific tissue is that if damage persists, as a result of genetic instability through loss or faulty repair, or permanent mutation, IGF-1-sCLU expression could be permanently turned on. In genetically unstable cells, such as neoplastic-initiated cells, IGF-1-sCLU expression can be dramatically elevated compared with matched stable cells due to loss of p53 function and ATM activation.[1] Permanent IGF-1-sCLU elevation appears to be an early marker of genetic instability in tissues of individuals exposed to low doses of IR or other toxins.

Permanently elevated levels of IGF-1-sCLU in tissues of IR-exposed cells could also indicate an altered microenvironment that may promote tumor growth and progression, including epithelial-to-mesenchymal transition (EMT) activation and metastasis. Irradiated tissue responses may be responding to trauma by inducing IGF-1-sCLU for simultaneous wound healing and cell debris clearance to avoid multiple organ dysfunction.[21] However, formation of genetically unstable cells, or cells with long-lived memory of IR damage would tend to permanently upregulate IGF-1-sCLU expression.[1] Indeed, elevated IGF-1-sCLU expression was noted in cells that have long-lived IR genetic instability, and in cells deficient in double strand break, DNA mismatch, base excision and nucleotide excision repair systems.[1] Such permanent expression of IGF-1-sCLU would enable a changed tissue microenvironment that could enhance the risk of tumor formation and progression. Such responses may be amplified by the downstream ability of sCLU to specifically suppress TGF-β1 signaling (Figures 5 and 6). As TGFβ1 is a natural tumor suppressor, a tissue microenvironment elevated in IGF-1-sCLU may further support tumor growth (after the initial damage-induced TGFβ1-mediated signaling event) through bystander suppression of endogenous Smad signaling, mediated by endogenous TGFβ1 from the stroma. Further research on the pro-survival and pro-metastatic functions of IR-induced IGF-1-sCLU expression, particularly after low-dose IR-exposed genetically altered initiated cells, or from senescent cells, could be a major factor affecting the health risk of specific tissues of individuals

exposed to low doses of IR. This is particularly true in tissues that are relatively hypersensitive to IR, including the colon, thymus, bone marrow, spleen and mammary tissue.

MATERIALS AND METHODS

Chemicals and plasmids

AG1024 (a specific IGF-1 receptor (IGF-1R) tyrosine kinase inhibitor) was obtained from EMD Chemicals (Gibbstown, NJ, USA). IGF-1 was obtained from R&D Systems (Minneapolis, MN, USA). TGFβ1 was obtained from the Sigma/Aldrich Chemical Company (St Louis, MO, USA). Ultrapure luciferin was obtained in bulk from the Case Western or UT Southwestern Cancer Center Imaging Cores and used in luciferase assay reagent assays (Promega, Madison, WI, USA). ^{3}H-Thymidine was obtained from New England Nuclear Co. (Boston, MA, USA). The human 1403 bp CLU promoter fused to luciferase (hCLUp-Luc) was previously described.[14,15,43] The hIGF-1p-luc construct was previously described.[1] Subcloning p53 cDNA into the pcDNA3.1-N-term-Flag construct created flag-tagged CMV-p53 cDNA. Constitutive-active PTEN (PTENCA) and kinase-deficient ATK1 were obtained from Dr Lindsey Mayo (Indiana University, Bloomington, IN, USA).

Antibodies and immunoblotting

Antibodies specific to mouse (M18) and human (B5) sCLU, as well as γ-H2AX, p53 and α-tubulin were purchased from Santa Cruz, as were glyceraldehyde 3-phosphate dehydrogenase and α-tubulin (Senta Cruz, CA, USA) used for loading. Antibodies to phosphorylated Smad3 (p-Smad3, C25A9) or total Smad3 (t-Smad3, C67H9) were obtained from Cell Signaling (Billerica, MA, USA). Immunoblotting was performed as described[14] and relative protein levels quantified from *X* ray films using NIH image J software (NIH, Washington, DC, USA) as described.[14] Anti-TGF-β1 murine monoclonal antibody (1D11) that neutralizes the three TGF-β isoforms,[31] and an isotype-matched control IgG1 monoclonal antibody (13C4, raised against Shigella toxin) were provided by Genzyme Corp. (Framingham, MA, USA).

Cell lines, treatments and survival assays

Human MCF-7 breast cancer cells and a stably transfected 1403 MCF-7 clone (MCF-7 cells containing stably integrated 1403 bp CLU promoter fused to firefly luciferase) were cultured in Dulbecco's modified Eagle medium (BioWhittacker; Walkersville, MA, USA) containing 10% fetal bovine serum (HyClone; Utah, USA). Human HCT116 colorectal carcinoma parental (DNA mismatch repair (MMR)-defective, TβRII−), a corrected clone (HCT116 3–6 cells, MMR+, TβRII+) and p53$^{-/-}$ cell lines were confirmed for p53, MMR and TβRII expression statuses as described,[44,45] and grown in 10% fetal bovine serum-Dulbecco's modified Eagle medium. Growth arrest responses of these cells to TGFβ1 were monitored by changes in ^{3}H-Thymidine incorporation into DNA, using standard protocols. HMECs, life-extended using hTERT and CD4 overexpression, were kindly obtained from Dr David Euhus (UT Southwestern). All cells and their stable derivatives were maintained at 37 °C at 5% CO_2-95% air. For TGFβ1 or IGF-1 treatments, cells were serum-starved (0.5% fetal bovine serum) overnight, and exposed to IGF-1 or TGFβ1 at the indicated doses in normal serum-Dulbecco's modified Eagle medium. Cell irradiations were performed using a JL Shepherd Assoc. (An Fernando, CA, USA) ^{137}Cs Mark I-68 irradiator (3.87 Gy/min), with appropriate shielding to lower dose rate for accurate whole body irradiations at low doses. Mock-irradiated or dimethyl sulfoxide-treated (UT) cells were treated identically to IR- or TGFβ1-exposed cells without cytotoxin treatments. All cells were routinely tested and found free of mycoplasma. Survival was assessed by colony forming ability assays.

Transfections and luciferase assays

Cells were transiently transfected with hIGF-1p-Luc or hCLUp-Luc reporter constructs and RSV-β-gal as a transfection control using Fugene 6 (Roche, Madison, WI, USA) as described.[1,28] Treatments, where indicated, were performed 24 h after transfection. Luciferase activities were analyzed using the Luciferase Assay System (Promega). β-Galactosidase activities were determined using the Galacto-Star reagent (Life Technologies, Carlsbad, CA, USA). All experiments were normalized for protein amounts using Bradford assays (Bio-Rad, Hercules, CA, USA). For generation of CLU promoter-luciferase stable MCF-7 cells, cells were co-transfected with the hCLUp-Luc construct together with twofold excess pcDNA-3 that contained a G418-resistance gene. Cells were then selected with G418 and resistant clones isolated as described.[14] Isolates were then treated with IR, other cytotoxic agents[1,14] or TGFβ1 and analyzed for hCLUp-Luc reporter activities, as well as endogenous sCLU expression. A clone with identical hCLUp-Luc activities monitored by luciferase activities[1,14] and endogenous sCLU protein expression by western blotting (see below) was selected and examined for induction by low doses of IR. HCT116 and HMEC cells were transiently knocked down for sCLU expression using an siRNA-sCLU specifically directed to the coding sequence for the CLU messenger RNA leader peptide sequence as described;[1] specificity of the siRNA using mutated sCLU cDNA was previously demonstrated.[14] Control cells were transfected with siRNA-nonsense/scrambled (shRNA-Scr) sequence. Knock-down of sCLU steady state protein levels were confirmed by western blotting using α-tubulin or glyceraldehyde 3-phosphate dehydrogenase as loading controls.

BLI *in vitro*

BLI imaging was described.[43] Suspensions of log-phase MCF-7 hCLUp-Luc cells were mock- or IR-treated with various doses of IR. Immediately after IR treatment, cells were seeded onto 10 cm or 96-well dishes containing black coatings between wells (Corning Life Sciences, Acton, MA, USA) to eliminate light scatter during imaging, and allowed to grow as needed. Initially, optimization of pelleted cells was performed to obtain the best signal-to-noise ratio for detection of hCLU promoter activity, as high-density suspensions quench luciferase light emissions because of high absorbance rates (Supplementary Figure S1). Cells were harvested by trypsinization 72 h after IR treatment, and suspensions of cells with various concentrations were made in Luciferase Assay Reagent (Promega). Cell suspensions ($\sim 10^7$ cells) were added to wells of a 96-well plate and imaged immediately. However, to maintain the ability to repeatedly image cells on different days, we also optimized imaging of luciferase activity in intact MCF-7 hCLUp-Luc cell monolayers. These experiments were performed at various times after IR[14,43] by adding luciferase assay reagent to cells and mounting plates in a light-tight box using an ultrasensitive CCD camera chilled with liquid nitrogen (Xenogen Vivovision IVIS Lumina Imaging system, San Francisco, CA, USA). As the required integration time for observing CLU promoter activity after very low doses of IR exceeded conventional integration times for CCD camera BLI ($\sim$33 msecs), all images obtained contained random cosmic ray events, resulting in high valued pixel intensities. To eliminate cosmic ray contamination, we developed and implemented a correction algorithm using an adaptive median filtering program for pixel substitution (Supplementary Figure S2). The code was written using MatLab, version 6.5 software (MathWorks, Natick, MA, USA). Both luciferase-induced luminescence and reference plate black and white images were taken. For presentation, images were subsequently processed to generate overlay colored images using MatLab software. To quantify promoter activities, Gray Scale Values were calculated in areas of interest (wells) and background subtracted using NIH Image J software. Experiments were independently repeated three times in triplicate wells. Statistical significance between groups was evaluated using paired Student's *t*-tests.

Human CLU promoter-luciferase (hCLUp-Luc) transgenic mice

A 1403-bp promoter region of the human CLU gene was amplified using PCR and the following primers: 5′-GATCCATTCCCGATTCCTCATCG-3′ and 5′-GCGTTGTGGGCACTGGGAG-3′, located at positions −1403 and −17 of human *clu* promoter, respectively. The high fidelity Elongase enzyme (Life Technologies, Grand Island, NY, USA) was used for the amplifying reaction. After purification by agarose gel electrophoresis, the fragment was phosphorylated using polynucleotide kinase (Fisher Scientific Inc., Hanover Park, IL, USA) and inserted into the SmaI site of the pA3luc

luciferase reporter vector (a generous gift from Dr R Pestell, Thomas Jefferson University, Philadelphia, PA, USA). The resultant hCLUp-Luc DNA construct was sequenced and confirmed accurately by GenBank sequence comparison. hCLUp-Luc was used to generate the stable 1403 MCF-7 cell line.[14] To generate transgenic mice, the hCLUp-Luc fragment ($\sim$3.4 kb) was excised using *Bam*HI, purified by agarose gel electrophoresis and its sequence verified. Purified linear hCLUp-Luc (1 μg) was then submitted to the Case Western Reserve Transgenic Animal Facility, where it was microinjected into fertilized mouse 129/FVBN oocytes and $\sim$300 fertilized oocytes were implanted into FVBN female mice. Tail clippings from offspring $<$3 weeks of age were analyzed using PCR for the presence of 1403 hCLUp-Luc construct, using the following primers: 5′-GCACAGC-TATTCGTGGTGATG -3′, and 5′-GCGTTGTGGGCACTGGGAG-3′ located at positions $-$202 and $-$17 of human *clu* gene, respectively. The resulting 202 bp PCR products were sequenced, identifying human *clu* promoter inserted into the mouse genome. Four pups (three female, one male) positive for 1403 hCLUp-Luc were identified and used as breeding pairs for hCLUp-Luc transgenic FVBN mouse propagation. Southern blot analyses indicated an average of two[2] copies of the 1403 hCLUp-Luc in the genome of each transgenic mouse used for subsequent breeding. Male and female mice were analyzed, but female mice were investigated in more depth because of their higher hCLUp-luc activity induction in specific organs after IR exposure.

BLI *in vivo* and *ex vivo*

Transgenic hCLUp-Luc reporter mice were treated with whole body γ-irradiation (0.02.–5 Gy) using a Shepard Mark Irradiator equipped with a ^{137}Cs source at dose rates: 3.88 Gy/min (for doses $>$0.5 Gy) or 0.42 Gy/min (for doses $<$0.5 Gy). Mice were also mock-irradiated (untreated, UT) under the same conditions. Mice (minimum three per group) were then imaged using BLI.[43] Briefly, male or female FVBN mice were anesthetized using isoflurane, hair was removed by mild shaving and 2.5–3.0 mg D-luciferin was intraperitoneally administered. BLI images of mice were then captured 10–30 mins later using a liquid N_2-cooled Xenogen Vivovision IVIS Lumina Imager for 30 s–2 min. Cosmic radiation interference was eliminated using a correction algorithm developed for these studies as described above. After whole body imaging, mice were killed and tissues extracted, and imaged *ex vivo* using BLI. Photomicrographs shown are representative of experiments performed at least three times with similar results. For time-course and dose-response BLI *ex vivo* imaging studies, transgenic female ($\sim$18 g) hCLUp-Luc reporter FVBN mice (three per group) were used, at least in duplicate, for each datum point. Reported are data from several different experiments performed at different times, and doses and data compiled. Results were highly consistent within and between experiments, with a minimal $n=6$. More importantly, the results were consistent with western blot analyses of specific tissues (see below), indicating that expression signals from the human (hCLUp-Luc) reporter within mouse tissues responded in an identical manner to endogenous sCLU protein expression.

Western blot analyses of irradiated tissues

In separate experiments, mice (three per group) were treated with whole body IR at doses of 0.02 or 1 Gy. Tissues (colon/small intestine, bone marrow, muscle, spleen and lung) were then extracted 24 or 72 h later as indicated and immediately homogenized as described.[43] Tissue homogenates were incubated on ice for 30 min, centrifuged (5000 $\times$ *g*, 15 min, 4 °C) and protein samples diluted to a final concentration of 2 μg/ml. Extracts were combined from each of three mice per group. Proteins were then separated by 8–12% SDS-polyacrylamide gel electrophoresis, and steady state levels analyzed by western blotting. Westerns shown are representative of three experiments with similar results.

Flow cytometry

Cells were fixed with 1% formaldehyde, counterstained with propidium iodide and analyzed for cell cycle changes by fluorescence-activated cell sorting using a Coulter Epics XL flow cytometer (Beckman Coulter Electronics Miami, FL, USA). Data were analyzed using ModFit LT, version 2.0 software (Verify Software House; Topsham, ME, USA).[14,15]

Statistical analyses

All experiments were independently performed at least three times in duplicate, unless otherwise indicated. Student's *t* tests and analysis of variance power analyses were performed with the aid of Dr Jin Xie, Biostatistics Core, Simmons Comprehensive Cancer Center.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)