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Control of the reversibility of cellular quiescence by the transcriptional repressor HES1

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Abstract

The mechanisms by which quiescent cells, including adult stems cells, preserve their ability to resume proliferation after weeks or even years of cell cycle arrest are not known. We report that reversibility is not a passive property of non-dividing cells, because enforced cell cycle arrest for a period as brief as four days initiates spontaneous, premature and irreversible senescence. Increased expression of the gene encoding the basic helix-loop-helix protein HES1 was required for quiescence to be reversible, because HES1 prevented both premature senescence and inappropriate differentiation in quiescent fibroblasts. In some human tumors the HES1 pathway was activated, which allowed these cells to evade differentiation and irreversible cell cycle arrest. We conclude that HES1 safeguards against irreversible cell cycle exit both during normal cellular quiescence, and pathologically in the setting of tumorigenesis.

Reversibility is a defining characteristic of cellular quiescence: in contrast to cells in other non-proliferating states, including terminal differentiation and senescence, only quiescent cells normally retain the ability to resume proliferation. Cells entering each of these arrested states stop the cell division cycle by increasing the abundance of cell-cycle inhibitory proteins, such as cyclin-dependent kinase (CDK) inhibitors (1–5), yet it is only in quiescent cells that this block to proliferation can be reversed. Expression of CDK inhibitors is sufficient to enforce a non-dividing state (1), and depletion of these proteins can disrupt quiescence in many cells, including hematopoietic stem cells (6,7). However, ectopic expression of CDK inhibitors does not recapitulate the transcriptional signature of quiescent cells (8), which suggested that cell cycle arrest and cellular quiescence are not functionally equivalent.

The amount of the CDK inhibitor $p21^{Cip1}$ (p21) is increased in fibroblasts that become quiescent in response to serum starvation or cell-cell contact (Fig. S1A). To determine if regulated expression of p21 would induce a reversible, quiescent-like cell cycle arrest, we used retroviral-mediated gene transduction to introduce into proliferating early passage human lung fibroblasts a p21 expression cassette flanked by loxP sites (loxp-p21) (Fig. S2A). Expression of p21 from this cassette efficiently blocked S phase entry (Fig. 1A). Four days later we reversed the increase in p21 abundance by infecting the cells with a vector expressing a cre recombinase-green fluorescent fusion protein (cre-GFP). Six days later more than 95% of the cells showed fluorescence from GFP, and the expression of p21 had returned to the baseline level found in proliferating cells (Fig. 1B). Nevertheless, these cells failed to reenter the cell cycle (Fig. 1A),

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expressed increased amounts of the senescence-associated enzyme β-galactosidase (Fig. 1C), and formed senescence-associated heterochromatin foci (SAHF) (Fig. 1J). As a control, we also transduced cells with an empty loxp vector and arrested them by contact inhibition for four days. After infection with cre-GFP more than 95% of the cells showed fluorescence from GFP. These cells resumed proliferation efficiently after release from contact inhibition (Fig. 1D), and did not display a senescent-like morphology. These experiments showed that sustained (four days or longer) expression of p21 induced an irreversible senescent-like state. We thus explored the mechanism by which quiescent cells avoid this fate despite their constitutive expression of p21.

We previously used gene expression profiling to observe that the transcriptional repressor Hairy and Enhancer of Split1 (HES1) is transcriptionally regulated in quiescent fibroblasts, but not in fibroblasts that have undergone cell cycle arrest in response to ectopic expression of CDK inhibitory proteins (8). We confirmed the increased abundance of HES1 mRNA in quiescent human fibroblasts by quantitative real-time polymerase chain reaction (PCR). Compared to proliferating fibroblasts, serum-deprived or contact-inhibited cells expressed 12.2 fold and 8.6 fold higher amounts of HES1 mRNA, respectively, whereas cells that were arrested by p21 did not increase transcription of the HES1 gene (Fig. S1B). We thus tested whether HES1, which is part of a large chromatin modification complex (9–11), might influence the reversibility of cellular quiescence.

To test whether HES1 is sufficient to prevent senescence associated with prolonged cell cycle arrest, we first transduced early passage human lung fibroblasts with the following HES1 constructs: wtHes1, wtHes1-estrogen receptor fusion protein (wtHes1-ER), ΔbHes1 (a mutant defective in DNA binding), and dnHes1 (a dominant negative HES1) (Fig. S2B, (12)). dnHES1 retains the DNA binding and dimerization domains of HES1, but lacks a C-terminal WRPW domain, which mediates the interaction between HES1 and its co-repressors (10,11). dnHes1 competes with the endogenous HES1 for binding to DNA and therefore prevents the chromatin modifications caused by endogenous HES1 and its associated proteins. We then stably transduced these cells with the loxp-p21 vector, and induced p21 expression and cell cycle arrest for four days. Cells were then transduced with cre-GFP to restore p21 expression to baseline, or maintained with p21 expression at high levels (Fig. 1E). Six days after downregulation of p21, cells expressing ΔbHes1, dnHes1 or wtHes1-ER (− 4-hydroxytamoxifen), failed to reenter the cell cycle and displayed a senescent phenotype. In contrast, cells expressing wtHes1 or wtHes1-ER (+4-hydroxytamoxifen) resumed proliferation after the removal of p21, and few cells showed β-galactosidase expression (Fig. 1F and G). Wild-type HES1 also suppressed the appearance of a senescent morphology in cells maintained with high amounts of p21 (Fig. 1H, I and J). However activation of the conditional Hes1-ER construct with 4-hydroxytamoxifen did not reverse senescence in cells that had already undergone this phenotypic transition (Fig. 1G and I).

These experiments showed that HES1 was sufficient to prevent senescence associated with ectopic expression of p21, but is it necessary to prevent senescence in naturally quiescent cells? After ten days of quiescence induced by serum-deprivation, control fibroblasts resumed proliferation after stimulation with serum (Fig. 1K). In contrast, fibroblasts that expressed dnHES1 during the ten days of culture without serum became morphologically senescent, expressed senescence associated β-galactosidase, and failed to resume proliferation after addition of serum (Fig.1K and L). Thus, HES1 controls the reversibility of cellular quiescence by blocking an alternative fate characterized by irreversible cell cycle arrest.

Premature senescence in normal human fibroblasts also occurs in response to oncogene activation, and is thought to represent a physiological pathway for tumor suppression (2). To test whether HES1 influences oncogene-induced senescence, we transduced human fibroblasts

(CCL153 and WI-38) with an empty vector, or a construct expressing wtHes1. An activated oncogenic Ras gene (H-RasV12) was then introduced into these cells (13). Within ten days of H-RasV12 expression, vector cells expressed increased amounts of the senescence-associated enzyme β-galactosidase, and formed senescence-associated heterochromatin foci (SAHF). HES1 suppressed these senescent phenotypes (Fig. 2A, B and C), without affecting the abundance of Ras or p16 (Fig. 2D).

Quiescent fibroblasts are resistant to terminal differentiation and permanent cell cycle arrest induced by ectopic expression of the myogenic transcription factor MyoD, whereas fibroblasts arrested after ectopic expression of a CDK inhibitor p21 are fully MyoD responsive (8). This is another example of how quiescent cells actively maintain the ability to reversibly enter and exit the cell cycle. We now find that expression of HES1 in primary human fibroblasts is both necessary and sufficient to inhibit MyoD-induced differentiation. We sequentially transduced cells with a vector expressing a MyoD-estrogen receptor fusion protein (MyoD-ER) and a vector expressing wild-type HES1 (wtHes1), or dnHes1. Cells were then induced to differentiate by exposure to β-estradiol. Expression of myosin heavy chain (MHC) RNA and protein, molecular markers for muscle differentiation, were detected in control proliferating fibroblasts but not in proliferating fibroblasts expressing wtHes1 (Fig. 3A and B; (14)). Quiescent fibroblasts did not express MHC in response to MyoD (Fig. 3A–C; (8)). However, expression of dnHes1 allowed quiescent fibroblasts to undergo myogenic differentiation in response to MyoD. Thus, MHC RNA and protein accumulated in dnHes1 cells but not in control cells (Fig. 3A and B), and production of MHC was also detected by immunostaining with an antibody to MHC (Fig. 3C). Furthermore, dnHes1 cells fused to form multinucleated myotubes within 72 hours of MyoD-ER activation (Fig. 3C).

The WRPW domain of HES1 binds to Transducin-Like Enhancer of split 1 (TLE1), which functions as a co-repressor by recruiting histone deacteylases (9–11). Compared to proliferating fibroblasts, cells that were made quiescent after deprivation of serum or by contact inhibition expressed 6.2 fold and 3.1 fold higher amounts of TLE1, respectively, whereas cells that were arrested by p21 did not up-regulate TLE1 (Fig. S1C). We tested whether TLE1, like HES1, was necessary to suppress differentiation in quiescent fibroblasts. We sequentially transduced primary human fibroblasts with a vector expressing MyoD-ER and a vector expressing either shRNA-TLE1 or a control shRNA. Expression of shRNA-TLE1 specifically reduced TLE1 expression by 64%, but did not decrease the abundance of closely related TLE2 (Fig. S3A and B). Cells were deprived of serum for 4 days and then transferred to differentiation medium containing β-estradiol. Control fibroblasts did not undergo myogenic differentiation, whereas quiescent shRNA-TLE1 cells expressed MHC mRNA and protein, and fused to form multinucleated myotubes within 72 hours (Fig. S3C and D).

Expression of HES1 can be increased by Notch-dependent and Notch–independent pathways (15–18). Exposure of quiescent fibroblasts to a pharmacological inhibitor of Notch signaling, the γ-secretase inhibitor DAPT (19), decreased the mRNA abundance of HES1 (Fig. S4A). Furthermore, DAPT-treated quiescent fibroblasts accumulated MHC within 72 hours of activation of MyoD (Fig. S4B). Therefore, activation of the Notch pathway appears to at least partially contribute to accumulation of HES1 and maintenance of a non-differentiated state in quiescent fibroblasts.

Tumor cells might adopt pathways normally used by quiescent cells to suppress entry into irreversibly arrested states (8). Indeed, our results showed that HES1 may contribute to tumorigenesis by suppressing oncogene-induced senescence. Rhabdomyosarma (RMA) is an aggressive skeletal muscle tumor of childhood, which originates from myogenic progenitor cells (20). RMA's constitutively express MyoD, but myogenesis is blocked by unknown mechanisms (21). HES1 mRNA amounts were increased 4.6 fold to 68.9 fold in 21 out of 21

primary RMA's of varying histology, location and stage. The abundances of HES1 mRNA and protein were increased in 3 out of 3 RMA cell lines (Fig. 4 A and B). We transduced a human RMA cell line, RHJT, with a vector expressing dnHes1 or an empty vector control. After transfer to differentiation medium containing a low amount of serum, we observed a large time-dependent accumulation of MHC in RHJT cells expressing dnHes1 compared to that in control RHJT cells (Fig. 4C). Expression of dnHes1 also gradually reduced proliferation and promoted differentiation of RHJT cells in growth medium containing 10% serum. Both dnHes1 cells and control cells were maintained in growth medium and passaged every 3 days. After two weeks in culture, 55% of control cells became labeled with a brief pulse of 5-bromo-2-deoxyuridine (BrdU) whereas only 15% of dnHes1 cells showed incorporation of BrdU (Fig. 4D). In cells expressing dnHes1, exit from the cell cycle was accompanied by cell fusion to form multinucleated myotubes that expressed large amounts of myosin heavy chain (MHC) (Fig. 4E and F). Exposure of RHJT cells to 5 uM DAPT to inhibit Notch signaling reduced the expression of HES1 by 70%, and after transfer of cells to differentiation medium, expression of MHC was increased in DAPT-treated cells (Fig. S4C and D). These results suggest that Notch signaling may contribute to tumor initiation and/or progression in rhabdomyosarcomas through increased expression of HES1 and suppression of MyoD-dependent differentiation.

In conclusion, we showed that HES1 maintained the reversibility of quiescence by controlling the choice between different out-of-cycle states. Inactivation of HES1 caused non-dividing fibroblasts to spontaneously enter into an irreversible senescent state, or allowed them to terminally differentiate in response to specific signals. Terminal differentiation and cellular senescence are both associated with the repackaging of nuclear DNA into stable heterochromatic structures that repress the expression of proliferation-related genes (22,23). These heterochromatic foci do not assemble in reversibly arrested, quiescent fibroblasts (22). We propose that HES1, which is part of a large chromatin-modifying complex (9–11), may counteract the heterochromatin assembly pathways present in irreversibly arrested cells. This hypothesis is supported by our demonstration that the HES1 DNA binding and co-repressor recruiting activities were required to prevent senescence and suppress differentiation in quiescent fibroblasts. Furthermore, we showed that expression of HES1 suppressed the formation of senescence-associated heterochromatin foci. Quiescent hematopoeitic progenitor cells also upregulate HES1 (12), suggesting that this function might be general.

Increased amounts of HES1 have been observed in ovarian carcinomas, breast cancers, non-small cell lung cancers, meningiomas and medulloblastomas (24–29). HES1 expression is also increased in the stem-like cells in breast ductal carcinoma in situ (DCIS), and is associated with recurrence at 5 years after surgery (26). We showed in human rhabdomyosarcomas that the effect of pathological over-production of HES1 was to cause these tumors cells to become refractory to cell cycle arrest and terminal differentiation, and that inactivation of HES1 led to spontaneous tumor cell differentiation. The ability of HES1 to determine the fate of quiescent cells may underlie, at least in part, the widespread role of the Notch and HES1 pathways in maintaining stem cell lineages, regulating tissue regeneration, and promoting tumorigenesis.

Supplementary Material

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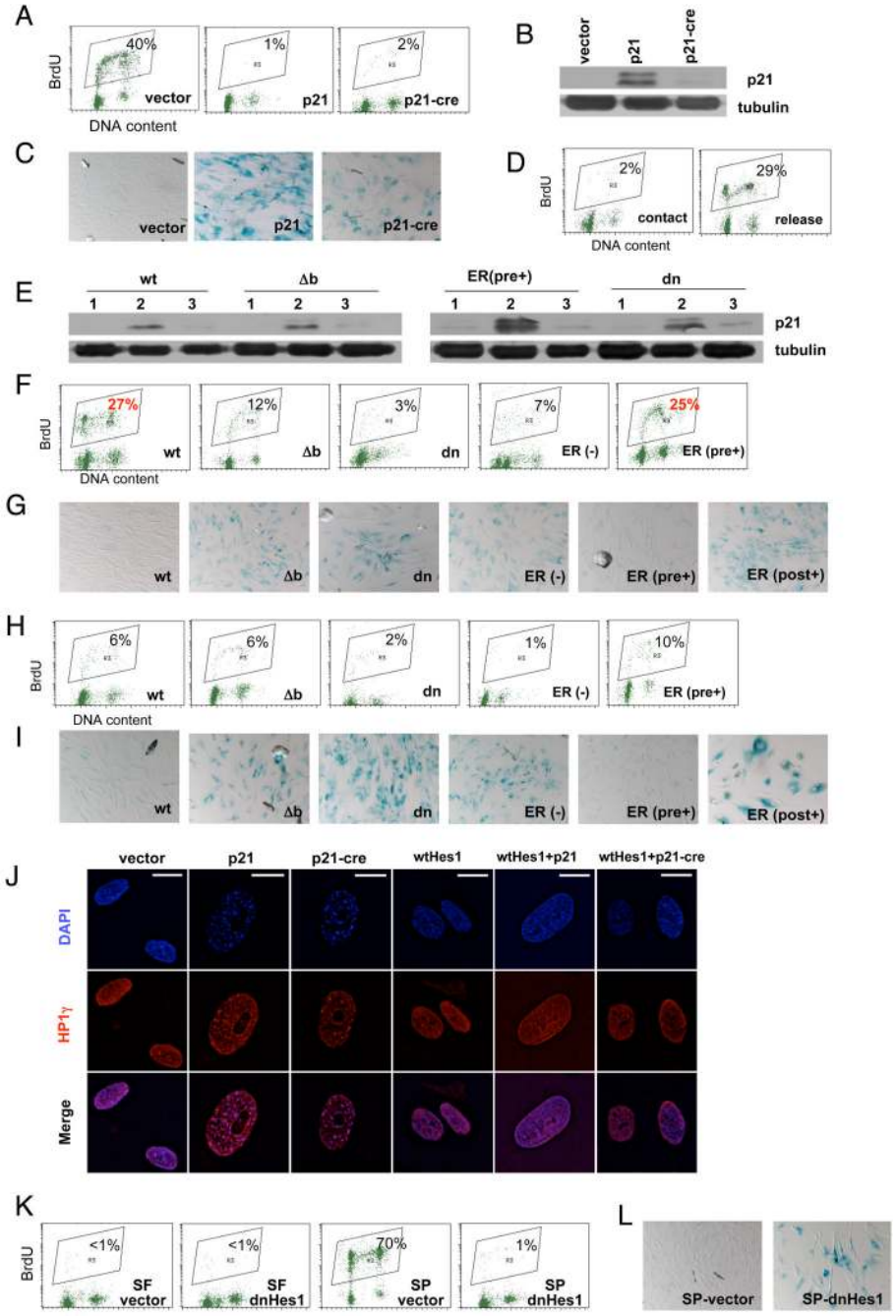

Fig. 1. Suppression of p21-initiated senescence by HES1

(A–C) Human fibroblasts (HFs) were transduced (day 0) with a $p21^{Cip1}$ expression cassette flanked by loxP sites (p21) or a loxp vector (vector). On day 4, p21 expressing cells were transduced with a vector expressing cre recombinase and green fluorescent fusion protein (p21-cre). On day 10, bromodeoxyuridine (BrdU) was added for 6 hours. **(A)** The percentage of BrdU positive cells is indicated. **(B)** Abundance of p21 protein and **(C)** expression of senescence-associated β-galactosidase (SA-β-gal). **(D)** HFs were transduced with an empty vector, contact inhibited for four days (contact), then infected with cre-GFP and re-plated at lower density (release). The percentage of BrdU positive cells is shown. **(E–I)** HFs were transduced with wild-type HES1 (wt), wild-type HES1-estrogen receptor fusion protein (ER),

DNA-binding mutant HES1 (Δb), or dominant-negative HES1 (dn), and then transduced with p21-loxP. 4-hydroxytamoxifen was added to wtHes1-ER cells prior to p21 transduction (pre +), nine days after p21 transduction (post+), or not applied (−). Four days after p21 transduction, cells were either transduced with cre-GFP **(F,G)** or maintained with high p21 levels **(H,I)**. **(E)** Expression levels of p21; lanes 1: control; 2: p21; 3: p21 + cre. **(F,H)** Cells were incubated with BrdU for 6 hours. **(G,I)** expression of SA-β-gal. **(J)** Cells were stained with DAPI and an antibody to HP1γ. **(K)** HFs were transduced with dnHes1 or an empty vector, serum deprived for ten days (SF) and then re-stimulated with full-serum medium (SP). BrdU was addded for 24 hours. The percentage of BrdU positive cells is indicated. **(L)** Expression of SA-β-gal.

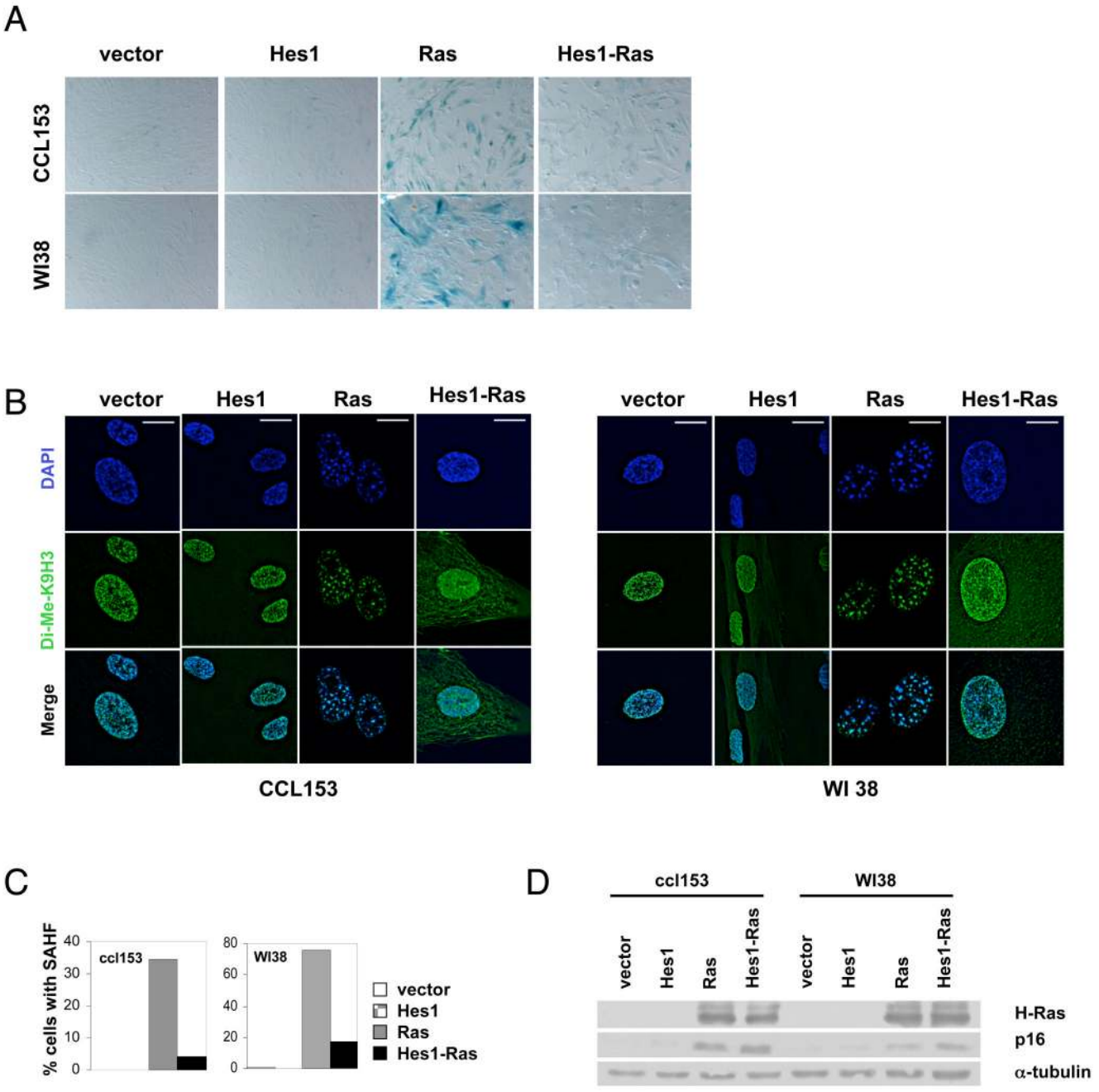

Fig. 2. Suppression of oncogene Ras-induced senescence by HES1

Primary human fibroblasts (CCL153 and WI-38) were transduced with an empty vector or wild-type Hes1. Cells were then transduced with an activated form of H-Ras. Ten days after H-Ras expression, **(A)** cells were stained for senescence associated β-galactosidase; **(B)** cells were stained with DAPI and an antibody to di-Me-K9H3. Scale bars represent 20 μm. **(C)** Percentage of cells with senescence-associated heterochromatin foci (SAHF). Results were quantitated by counting 100 cells on each slide, from two independent experiments. **(D)** Abundance of H-Ras and p16 protein.

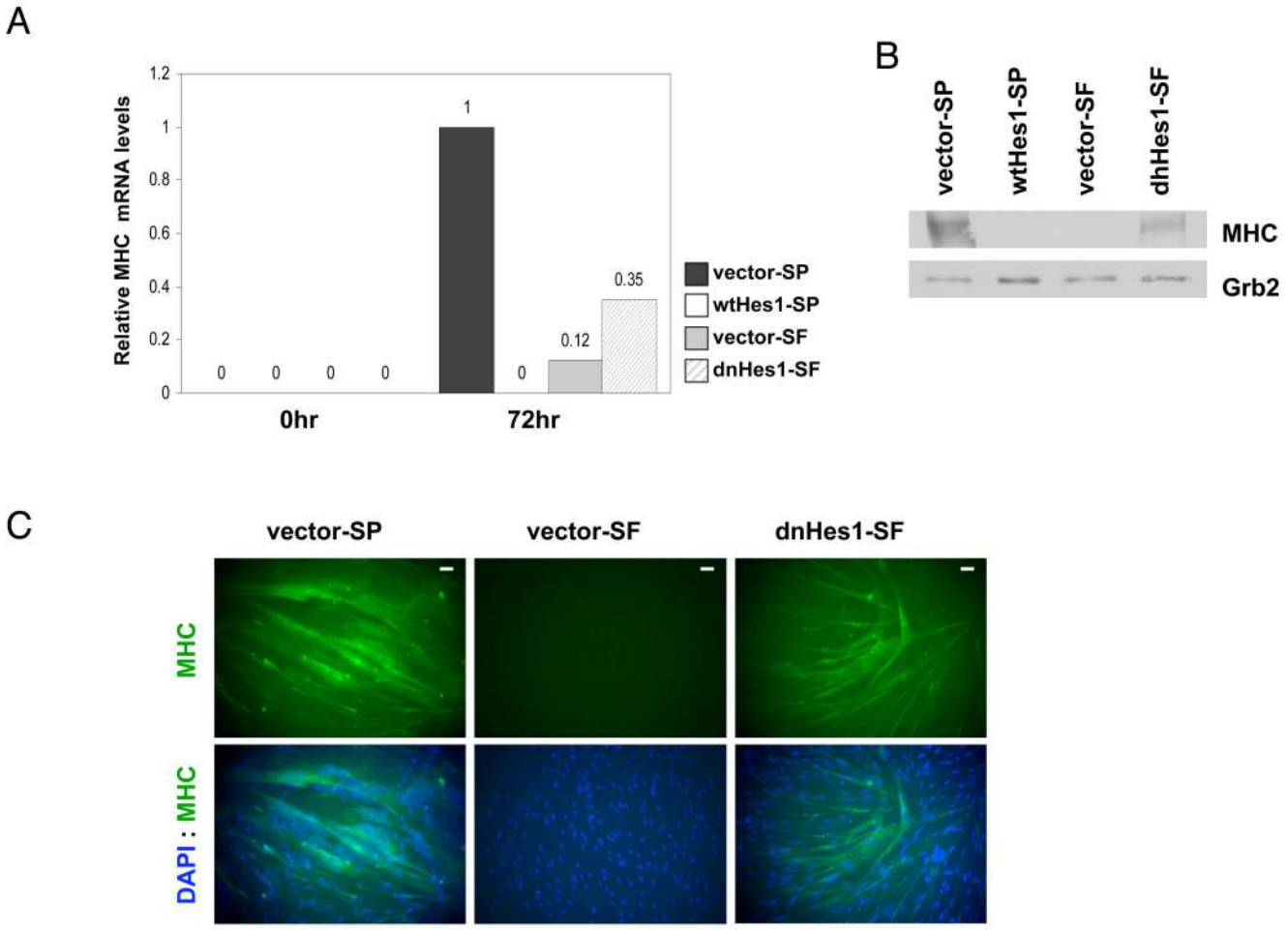

Fig. 3. Suppression of MyoD-induced muscle differentiation by HES1

Primary human fibroblasts (CCL153) were sequentially transduced with a MyoD-estrogen receptor fusion protein (MyoD-ER) and then with an empty vector (vector), wtHes1, or dnHes1. Proliferating cells were induced to differentiate by β-estradiol (SP), or were made quiescent for 4 days by serum starvation (SF) before induction of differentiation. **(A)** At 0 and 72hr after addition of β-estradiol, myosin heavy chain (MHC) RNA was measured by real-time PCR normalized to GAPDH levels. The fold change relative to differentiated vector cells is plotted. Data are the average of duplicates. RNA amounts below the detection limit are indicated as "0". **(B)** The induction of MHC at 72 hrs post-differentiation is shown by immunoblot; and **(C)** by immunostaining using an anti-MHC antibody. MHC is green; nuclei are blue (DAPI). Scale bars represent 20 μm.

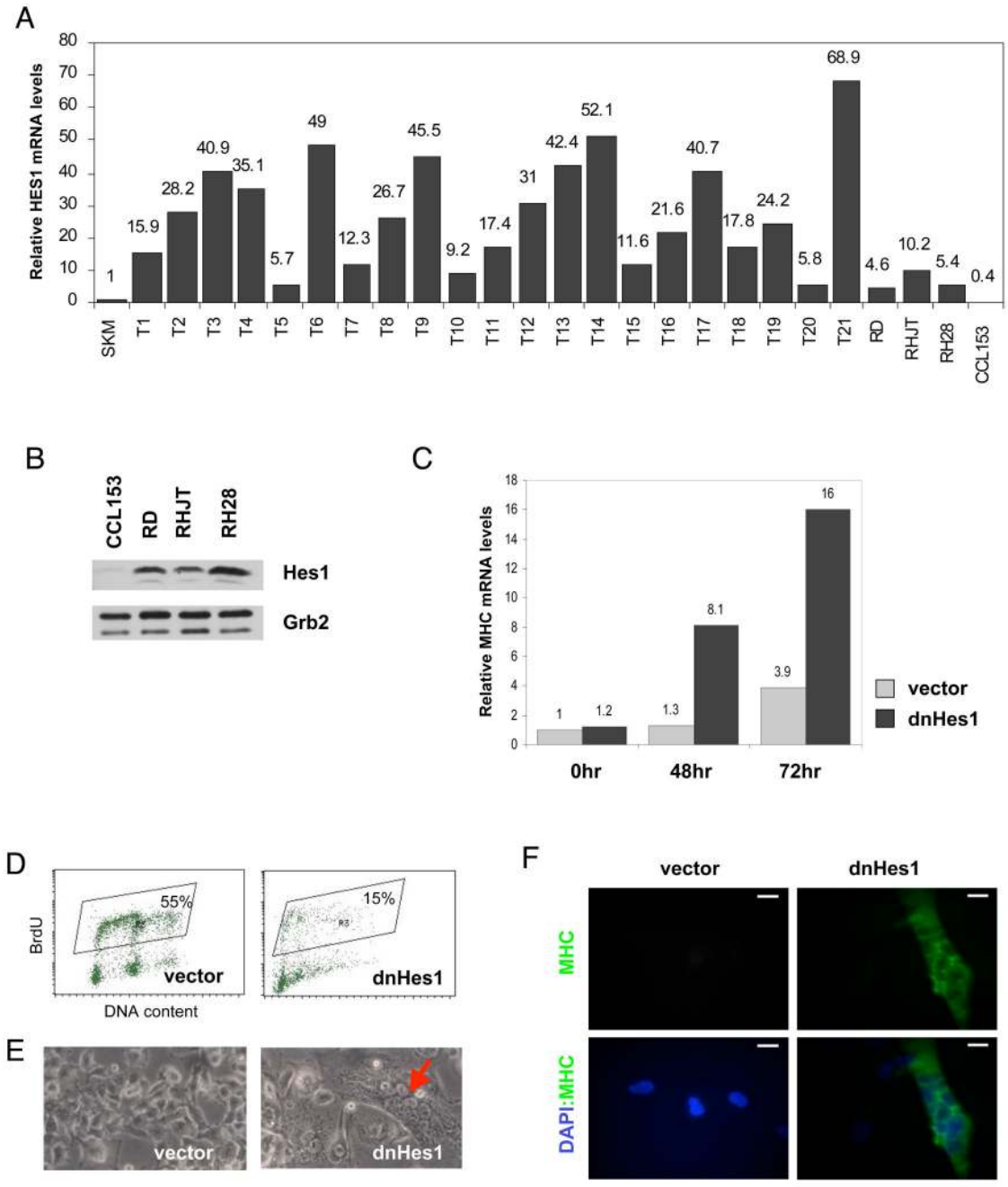

Fig. 4. Expression and function of HES1 in rhabdomyosarcomas

(A) The expression of HES1 was measured by real-time PCR (as above) in human rhabdomyosarcoma primary tumors (T1–T21) and cell lines (RD, RHJT and RH28), in control skeletal muscle (SKM), and in primary human fibroblasts (CCL153). Fold-change compared with control SKM is plotted. Data are the average of duplicates. **(B)** Abundance of HES1 protein in rhabdomyosarcoma cells. **(C)** RHJT cells were transduced with dnHes1 or vector control, and then transferred to low-serum media to induce differentiation. At the indicated time points MHC expression was measured as above. Fold-change compared with control cells is plotted. Data are the average of duplicates. **(D)** RHJT cells were transduced with dnHes1 or vector control, and were maintained in growth medium for two weeks. Cells were then

incubated with BrdU for 6 hours and analyzed by flow cytometry as above. The percentage of BrdU positive cells is indicated. **(E)** RHJT cells expressing dnHes1 formed multi-nucleated myotubes (red arrow); and **(F)** expressed MHC (green). Nuclei are blue (DAPI).Scale bars represent 20 μm.