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Arf and p53 act as guardians of a quiescent cellular state by protecting against immortalization of cells with stable genomes

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ABSTRACT

Normal cells undergo a growth-arrested status that is produced by p53-dependent down-regulation of histone H2AX. Immortality is developed after abrogation of the H2AX-diminished state, which is associated with genomic instability (often with tetraploidy) and the induction of mutations in either the *Arf* or *p53* gene. However, the role of Arf in control of H2AX expression and genome stability is still unclear. Here, we show that both Arf and p53 are required for the down-regulation of H2AX and formation of the growth-arrested state. Wild-type (WT) mouse embryonic fibroblasts (MEFs) subjected to tetraploidization with DNA lesions did not undergo mitotic catastrophe-associated cell death and stayed in a growth-arrested state, until immortality was attained with mutations in the *Arf*/*p53* module and recovery of H2AX expression. Whereas tetraploidization was essential for immortalization of WT MEFs, this event was not required for immortalization of MEFs containing mutations in *Arf*/*p53* and these cells still underwent mitotic catastrophe-associated cell death. Thus, WT MEFs are protected from immortalization with genome stability, which is abrogated with tetraploidization and mutation of either *Arf* or *p53*.

1. Introduction

Most cancers that develop in old age are characterized by chromosomal or microsatellite instabilities, as well as mutations in genes, such as those involved in the Arf-MDM2-p53 axis [1–3]. Similar to cancer cells, mouse embryonic fibroblasts (MEFs) acquire immortality associated with genomic instability [4] and mutations in the *Arf*/*p53* module [5]. Although *Arf* and *p53* are part of the same regulatory module, these genes are mutated in a mutually exclusive manner in immortalized MEFs, suggesting that both Arf and p53 are required for protection against cellular immortalization [6,7]. By contrast, the p53-dependent acute response to damage still occurs in p53-proficient cancer cells that contain mutations in *Arf* [8,9]. These findings suggest that normal cells are protected from immortalization by regulation of both Arf and p53, and that this protection mechanism is distinct from the role of p53 in the acute damage response [6].

Because Arf and p53 are critical tumor suppressors, *Arf*- and *p53*-knockout (KO) mice are predisposed to cancer development [10,11]. In addition, transgenic mice with an extra copy of the *Arf* and *p53* genes (super-*Arf*/*p53* mice) show signs of cancer suppression and have extended life spans [5]. Intriguingly, like wild-type MEFs with stable genomes, MEFs from super-*Arf*/*p53* mice are strongly protected against immortalization [5]. These findings imply that the primary function of the Arf/p53 module is control of cellular homeostasis, which contributes to lifespan extension and cancer suppression. By contrast, cells with hyperactive p53 induced by overexpression or acute damage undergo senescence or apoptosis *in vitro* [12–14], and transgenic mice with hyperactive p53 undergo premature aging [15–17]. Furthermore, mutant mice that are unable to induce many of the canonical p53 target genes in response to acute DNA damage retain tumor suppression activity under normal conditions [8,9]. Taken together, these findings suggest that p53 has distinct functions under normal and hyperactivated conditions; the Arf-dependent function of p53 is to control cellular homeostasis under normal conditions, leading to lifespan extension and cancer prevention, and is likely to be distinct from the function of hyperactivated p53 [6].

After serial proliferation, normal cells generally undergo a growth-arrested state associated with diminished levels of H2AX

Abbreviations: CTU, camptothecin; HU, hydroxyurea; KO, knockout; MEFs, mouse embryonic fibroblasts; WT, wild-type.

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[6,7]. These growth-arrested cells are defective in DNA damage repair and are therefore susceptible to the accumulation of unrepaired DNA lesions [18]. In response to aberrantly accelerated growth stimuli, these growth-arrested cells develop DNA replication stress-associated lesions and subsequent genomic instability [4]. Cellular growth retardation and DNA damage repair deficiency are both likely caused by a reduction in histone H2AX levels because cells lacking H2AX also display these characteristics [19–22]. By contrast, transformed or immortalized cells are formed following abrogation of the H2AX-diminished state [6,7].

Down-regulation of H2AX is dependent on p53; the mechanism of regulation presumably involves the Arf/p53 module because the H2AX-diminished and growth-arrested state is not induced in *p53*-KO MEFs or in immortalized MEFs that contain mutations in either *Arf* or *p53* [6,7]. Although the role of p53 in establishment of a quiescent state has been described previously [6,7], the mechanism by which Arf contributes to the down-regulation of H2AX, protection against immortalization, and genomic instability (ploidy) is still unclear.

In this study, we demonstrate that Arf is required for growth arrest associated with reduced levels of H2AX in MEFs. The quiescent state of normal MEFs was abrogated by mutations in either *Arf* or *p53*. Although tetraploidization was not essential for immortalization of *p53*-KO and *Arf*-KO MEFs, tetraploidization of wild-type (WT) MEFs was required to induce mutations in the Arf/p53 module.

2. Materials and methods

2.1. Cell culture

WT, *Arf*-KO, and *p53*-KO MEFs were prepared from Day 13.5 mouse embryos, as previously described [7]. MEFs were cultured as described previously [23] and were passaged using the standard 3T3 protocol [24], unless otherwise indicated. DNA replication stress-associated damage was induced by the treatment of cells with camptothecin (CPT) (Sigma) or hydroxyurea (HU) (Sigma) as indicated in each figure.

2.2. Antibodies and immunoblotting

Antibodies against H2AX (Bethyl Laboratories), γH2AX (Millipore-Upstate), β-actin (AC-74, Sigma), Parp1 (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), and histone H3 (MABI0301, Monoclonal Antibody Institute) were used in this study. Immunoblotting was performed as described previously [23].

2.3. Analyses of the chromosomal status

For analyses of mitotic phase chromosomes, cells were treated with 200 ng/ml nocodazole for 5 h and then mitotic cells were collected. The cells were hypotonically swollen by treatment with 75 mM KCl for 30 min, and then fixed with Carnoy's solution (60% methanol, 30% acetic acid, and 10% chloroform) for 20 min. After changing the fixative once, cells were dropped onto glass slides and air-dried [4]. The slides were stained with 4% Giemsa stain (Merck) for 10 min, washed briefly in tap water, and then air-dried. For FACS analyses of the cellular ploidy status, harvested cells were incubated in PBS containing RNase A (200 μg/ml, Sigma) for 30 min on ice and then stained with propidium iodide (20 μg/ml, Sigma) for an additional 30 min on ice in the dark. The stained cells were analyzed by flow cytometry (Beckman Coulter).

3. Results

3.1. Arf-KO MEFs do not undergo H2AX-diminished growth arrest

To determine the role of Arf in the establishment of a H2AX-diminished and growth-arrested state, experiments were performed using primary WT and *Arf*-KO MEFs. Unlike WT MEFs, the *Arf*-KO MEFs did not undergo growth arrest and continued to

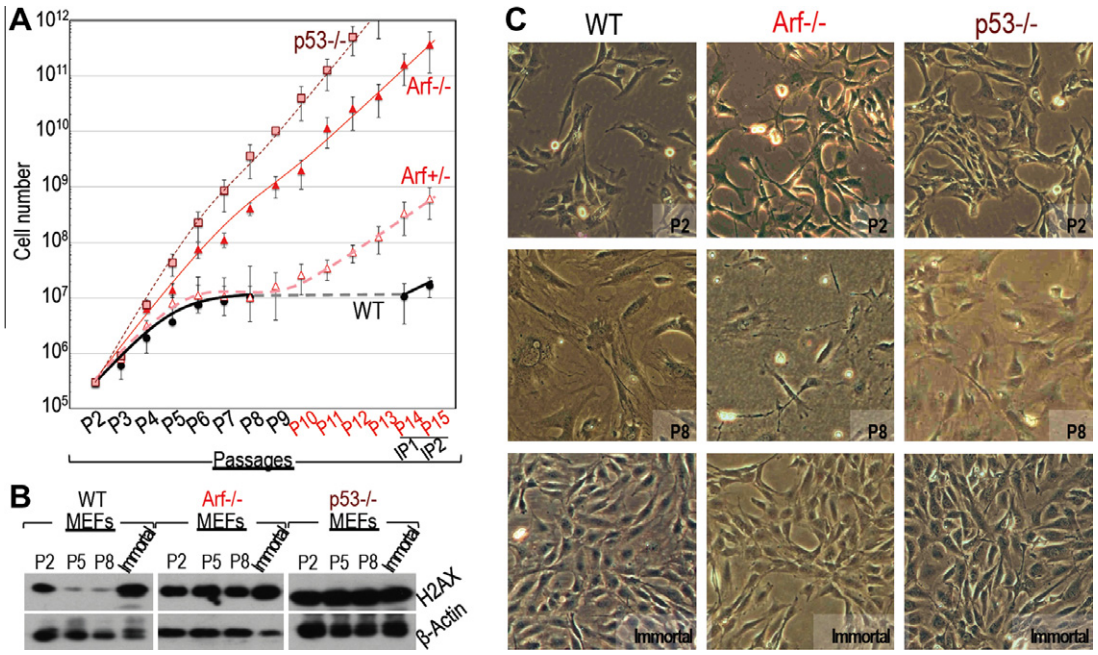

Fig. 1. Arf-KO MEFs do not undergo H2AX-diminished growth arrest. (A) Growth curves of MEFs (WT, $Arf^{+/-}$, $Arf^{-/-}$, and $p53^{-/-}$) cultured under a standard 3T3 passage protocol. Unlike WT and $Arf^{+/-}$ MEFs, $Arf^{-/-}$ MEFs continuously grew and developed immortality. Data show the mean ± SD of $n = 3$ independent experiments. IP1 and IP2 indicate Immortal Passage 1 and 2 for WT MEFs. (B) Immunoblot analysis of histone H2AX expression in passage 2 (P2), P5, P8, and immortalized MEFs (WT, $Arf^{-/-}$, and $p53^{-/-}$). Expression levels of β-actin were used as a loading control. Unlike WT MEFs, $Arf^{-/-}$ and $p53^{-/-}$ MEFs failed to form the H2AX-diminished state. (C) Morphologies of P2, P8, and immortalized WT, $Arf^{-/-}$, and $p53^{-/-}$ MEFs. Similar to WT MEFs, $Arf^{-/-}$ and $p53^{-/-}$ MEFs displayed a senescent morphology before acquiring the immortalized morphology.