American Journal of Immunology

Original Research Paper

The Arid3a Transcription Factor Rescues Natural and RAS-V12-Induced Senescence Via a Rb-Dependent Pathway

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Article history
Received: 24-09-2017
Revised: 28-09-2017
Accepted: 03-11-2017

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Abstract: Primary cells are protected against oncogenic events by undergoing premature cellular senescence—an irreversible cell cycle arrest activated by mitogenic signaling as well as by overexpression of tumor suppressors, including p16INK4A, p53 and PML. In the human, downregulation of Dril1/E2F-BP1, a transcriptional regulator of E2F, promotes PML-dependent premature senescence and bypass of anti-proliferative signaling by p19Arf/p53/p21Cip1 and p16INK4a to prevent both RasV12-induced and spontaneous senescence. The mouse ortholog, Arid3A/Bright, while highly characterized in B lymphocytes for its function in immunoglobulin transcription and hematopoiesis, had yet to be assessed for a function in growth control. That, along with the considerable sequence/exon structure diversion from its human orthologs, prompted us to evaluate Arid3a in this context. We report that reduction of Arid3a levels in B lymphocytes results in G1/S cell cycle arrest whereas overexpression of Arid3a leads to accumulation of Cyclin E, hyperphosphorylation of pRb, increased transcriptional activity of E2F1 and transformation *in vivo*. Arid3a associates with pRb in chromatin to release HDAC1 from the E2F1 promoter in proliferating cells. Arid3a mutants that fail to associate with pRb neither rescue senescence nor induce proliferation. Our results identify a function for Arid3 in cell cycle progression beyond its previously established role in immunoglobulin gene transcription.

Keywords: Arid3a, Ras, Senescence, Immortalization

Introduction

The idea that somatic mutations contribute to the genesis of malignant lesions led to the cloning and characterization of oncogenes and tumor suppressors (Knudson, 2000; 2001). Activation of oncogenes or inactivation of tumor suppressors leads to a form of cellular senescence that can be distinguished from replicative senescence (or crisis) due to telomere loss (Fridman and Tainsky, 2008; Kuilman and Peeper, 2009). Introduction of ras carrying an oncogenic mutation in its GTPase domain (Ha-rasV12 (Serrano *et al*., 1997a)) into primary fibroblasts results in the induction of various anti-proliferative proteins, including p16INK4a, p21Cip1 and p53 (Kortum *et al*., 2006). The accompanying cell cycle arrest resembles normal senescence, given its irreversible character and the upregulation of senescence-associated markers such as acidic β-galactosidase activity (Dimri *et al*., 1995; Kurz *et al*., 2000). Loss of the alternative INK4a product p19ARF also collaborates with Ha-rasV12 in oncogenic transformation (Kamijo *et al*., 1997) and is required for induction of p53 by Ha-rasV12 (Palmero *et al*., 1998). Members of the retinoblastoma (Rb) tumor suppressor gene family play an important role in protecting cells against the proliferative effects of Ha-rasV12 (Sage *et al*., 2000; Peeper *et al*., 2001).

Not only tumor suppressor gene deficiency, but also co-expression of specific oncogenes can bypass Ha-rasV12-induced senescence and facilitate oncogenic

transformation. For example, c-myc and adenovirus e1a each immortalize primary rodent fibroblasts and collaborate with Ha-rasV12 in oncogenic transformation (Land *et al*., 1983; Ruley, 1983; Weinberg, 1997; 1989). Together, these observations help explain why immortalizing events can disrupt the normal anti-oncogenic response to excessive RasV12 signaling and suggest that premature senescence may act as a tumor-suppressing mechanism (Leal *et al*., 2008; Lleonart *et al*., 2009).

The DRTF1/E2F1 complex functions as a master regulator of G1 to S phase cell-cycle progression (Wong *et al*., 2011; Docquier *et al*., 2012). E2F1 binds preferentially to Retinoblastoma protein (pRb) in a cell-cycle-dependent manner. E2FBP1/DRIL1, the charter member of the 12 membered A/T-rich Interaction Domain (ARID) family (Wilsker *et al*., 2005) was initially characterized (Suzuki *et al*., 1998) as an E2F1 binding factor whose overexpression increases E2F/DP-dependent transcription (Kortschak *et al*., 1998; 2000). E2FBP1 was also shown to repress simplex virus type 1 infection (Fukuyo *et al*., 2011) as well as formation of Promyelocytic Leukemia Nuclear Bodies (PML-NBs) (Fukuyo *et al*., 2004a) which are critical in the control of cellular senescence and stem cell self-renewal (Fukuyo *et al*., 2004; Ferbeyre *et al*., 2000; Pearson *et al*., 2000; Guo *et al*., 2000).

In a parallel set of experiments, Peeper *et al*. (2002) fibroblasts harboring the temperature sensitive mutant of the SV40 large T antigen (tsA58), thereby creating the Btr cell line (Peeper *et al*., 2002; Jat and Sharp, 1989; Tegtmeyer, 1975). Using a retroviral cDNA screen, Peeper *et al*. (2002) isolated human Dril1 (Kortschak *et al*., 1998) as a factor capable of rescuing Btr fibroblasts from RasV12-induced senescence. They further demonstrated that Dril1 over-expression, although independent or downstream of pRb, was capable of both immortalizing and in combination with rasV12, transforming normal mouse fibroblasts in culture and *in vivo*.

For instance, growth of human fibroblasts can be prolonged (up to 30 population doublings) by ectopic expression of the simian virus large T antigen (SV40 large T), which is transcribed early during viral infection by SV40 and associates preferentially with the un- or hypo-phosphorylated form of the retinoblastoma (Rb) tumor suppressor family (Knudson, 2001; Fiers *et al*., 1978; Ludlow *et al*., 1989; Macera-Bloch *et al*., 2002).

The promyelocytic protein PML is involved in the regulation of Ha-rasV12-induced senescence, by influencing the acetylation of p53 (Ferbeyre *et al*., 2000; Pearson *et al*., 2000).

Arid3a-interacting proteins include ubc9 and PIAS1, leading to its paralog, Bdp. Bdp/Arid3b was cloned as a protein that associates directly via its ARID domain with Rb (Numata *et al*., 1999). While E2FBP1/Dril1 is expressed broadly in human tissues, its mouse (m) ortholog, Arid3a/Bright (for B cell regulator of IgH transcription) (Herrscher *et al*., 1995) is expressed exclusively in developing hematopoietic lineages within the embryo and placenta (Rhee *et al*., 2014; 2017), but restricted to B lymphocytes in the adult (Webb *et al*., 1998; Nixon *et al*., 2004). Arid3a, in a complex with Bruton's tyrosine kinase (btk) and TFII-I, binds to specific AT-rich motifs within the nuclear matrix-attachment regions (MARs) of the IgH intronic enhancer (Eμ) resulting in an activated transcription of heavy chain μ chains (Herrscher *et al*., 1995; Kim and Tucker, 2006; Lin *et al*., 2007; Rajaiya *et al*., 2005; 2006; Webb *et al*., 1999; 2000). Arid3a transactivation is dependent on a second, highly conserved, multifunctional domain called REKLES (Kim and Tucker, 2006; Kim *et al*., 2007; Schmidt *et al*., 2009). Motifs within REKLES mediate active cytoplasmic-nuclear shuttling, homo-tetramerization and heterologous protein-protein interactions. REKLES-mediated interactions with Sumo-1 E2 and E3 conjugation enzymes (ubc9 and PIAS-1) led to the finding that a fraction of Arid3a—as with its human homolog (Fukuyo *et al*., 2004) — was Sumo-I modified and partitioned into PML-nuclear bodies (Schmidt *et al*., 2009; Zong *et al*., 2000). Mutation of the single SUMO-I conjugation site in Arid3a had no effect on DNA binding or transcriptional activities, leaving the role of Bright in PML-NB unresolved (Schmidt *et al*., 2009).

While the ARID DNA-binding domains are 98% identical among human and mouse orthologs, the orthologs, in total, are less than 80% conserved (Peeper *et al*., 2001; Herrscher *et al*., 1995). They align particularly poorly from residues 26-240 (just N-terminal to their ARIDs) where 15 gaps are required to achieve an identity/similarity of 75/85% (Wilsker *et al*., 2005). Conserved within this region for mouse, but not for human, is an acidic stretch, which meets consensus requirements for 3 tandem PEST motifs (Wilsker *et al*., 2005). PEST domains have been shown in several cases to target ubiquitin-mediated proteasome degradation (Melchior, 2000). Thus, the human and mouse proteins have considerable opportunity for structure-function based differences.

Here we report that, despite these differences, murine Arid3a immortalizes Mouse Embryonic Fibroblasts (MEFs) and protects them both from spontaneous and Ha-rasV12-induced senescence independent (or upstream) of p19ARF or p53 signaling. This activity of Arid3a is dependent upon cytoplasmic-nuclear shuttling, DNA binding and association with pRb, but not upon Sumo-I modification. Cell cycle progression results from inactivation of the Rb tumor suppressor pathway through accumulation of Cyclin E, hyper-phosphorylation of pRb and activation of E2F1 via loss of promoter-associated HDAC complexes.

Results

Comparisons of Human (h) and Mouse (m) Arid3a to Rescue Senescence in vitro and in vivo

Full-length (1-601) HA-tagged m and hArid3a were engineered, packaged into pBABE-puro retroviruses (Peeper *et al*., 2002) and infected alone or in combination with Ha-tagged RasV12 MEFs. After 1-2 days, cultures were selected with puromycin (2 mg/ml) for an additional 4 days and their ability to proliferate was determined (Fig. 1A). As expected, RASV12 expression led to premature senescence and the cells adopted typical morphology (Fig. 1A), as confirmed by acidic ß-galactasidase staining (Fig. 1B). Focus forming assays showed that, in 2 weeks, hArid3a + Ha-RasV12 rescued cells were morphologically transformed, had lost their ability to stop proliferating upon contact inhibition (Fig. 1C). Although mArid3a + Ha-RasV12 co-expressing MEFs also underwent transformation, there were consistently fewer transformed colonies which required significantly longer time (~3 weeks) to develop. These differences in efficiency were not the result of differences in expression (Fig. 1C), nor in anchorage-independent growth characteristics (data not shown). Both, murine and human Arid3a + RASV12 infected cultures continued to proliferate and bypassed senescence as early as 7 days (passages) post selection. Control cells were senescent whereas mBRIGHT + RASV12 cells were proliferating rapidly (data not shown).

A hallmark of transformed cultured cells is their ability to form tumors in nude mice. Peeper *et al*. (2002) observed tumor formation for DRIL1/hArid3a + Ha-RasV12 transformed MEFs but not for hArid3a alone immortalized MEFs. We employed the identical experiments with mArid3a+Ha-RasV12 transduced lines. Six days after retroviral infection, ~10^6 MEFs were injected subcutaneously into each flank of 12 nude mice. hArid3a+Ha-RasV12 MEFs produced tumors in 10/12 nudes within 10 days (Table 1) hArid3a+Ha-RasV12 MEFs expressing equivalent levels of mArid3a (not shown) formed tumors in nude mice but at a lower frequency (4/13 nudes) and slower rate (12-30 days) than hArid3a+Ha-RasV12 cells.

Therefore, Arid3a from either species can collaborate with Ha-RasV12 to drive cells to transformation in culture and are capable of eliciting tumors in mice, albeit at significantly different penetrance.

Arid3a alone is Sufficient to Immortalize Fibroblasts

As with hArid3a (Peeper *et al*., 2002), mArid3a alone is sufficient to immortalize normal primary MEFs. Upon retroviral transduction with mArid3a, MEFs escaped from natural senescence and these cells could be cultured for at least ~3 months, without loss of proliferation (data not shown). A similar result, also in agreement with (Peeper *et al*., 2002), was observed when mArid3a was ectopically expressed in NIH/3T3 fibroblasts.

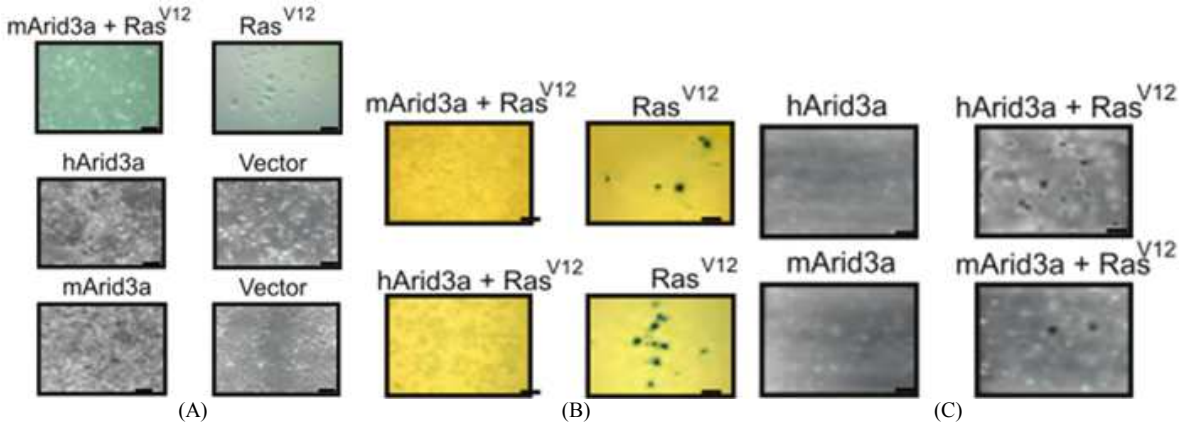


Fig. 1: Mouse (m) and human (h) Arid3a share some but not all features of immortalization and transformation. (A) Expression of either h or mArid3a rescues mouse embryonic fibroblasts (MEF; E15) from Ha-ras^{V12}-dependent morphological senescence. MEFs were infected with retroviruses expressing Ha-Ras^{V12} either with a vector containing no insert (control) or with h or mArid3a. Approximately 2 weeks later, the MEFs were photographed at 40x magnification. (B) Mouse embryonic fibroblasts co-expressing Arid3a and Ha-Ras^{V12} do not stain for senescence-associated b-galactosidase activity. MEFs were infected with retroviruses encoding Ha-ras^{V12} and either murine or human Arid3a (as indicated) and incubated for two weeks in a humidified atmosphere, followed by fixation and overnight staining using a buffer consisting of 1mg/ml X-gal, 120µM $K_3Fe[CN]_6$, 120µM $K_4Fe[CN]_6$, 1mM $MgCl_2$ in PBS at pH 6.0) at 37ºC without CO_2. Senescence-associated β-galactosidase activity is visualized by a blue reaction product. (C) Both m and hArid3a/Ha-Ras^{V12}-expressing mouse embryonic fibroblasts proliferate anchorage-independently but with different efficiencies. MEFs infected and selected as in (a) were analyzed in a focus formation assay. Cells were seeded in duplicate into 6-well plates containing solidified 1% agar and two weeks later, foci were photographed at 40x magnification

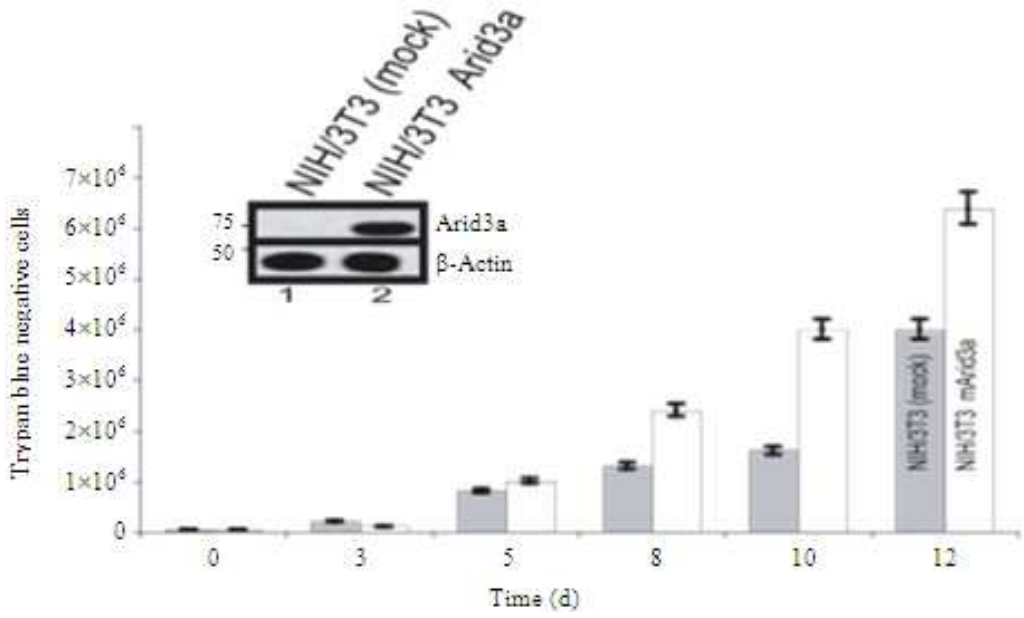


Fig. 2: NIH3T3 fibroblasts stably expressing mArid3a display a growth advantage. NIH/3T3 fibroblasts were infected with a vector containing no insert (control) or with *mArid3a* and subjected to puromycine selection for 5 days. Trypan blue negative cells were counted over the indicated time and Western blotting confirmed presence of mArid3a in infected cells at the end of this experiment. Actin served as loading control

Table 1: Murine and human BRIGHT/RASV12 cells are oncogenic in athymic mice. Nude, athymic mice were injected subcutaneously in both flanks with one million MEFs, expressing cDNAs as indicated. Represented are numbers of tumors per injection, 2 weeks after injection. M or hBRIGHT-only-expressing cells did not form tumors after 5 weeks

Cells	Tumor induction	Latency period
HA-hBRIGHT	0/4	-
HA-hBRIGHT/RASV12	10/10	8 days
HA-mBRIGHT	0/6	-
HA-BRIGHT/RASV12	4/4	12-30 days

Arid3a expression provided a growth advantage (Fig. 2) without altering the cell cycle profile, as assessed by propidium iodide staining (data not shown). It is concluded that Arid3a increases the proliferative rate of cells.

mArid3a Bypasses Ha-RasV12-Induced Senescence Downstream (or Independent) of Traditional Senescence Regulators

Ha-RasV12-induced senescence requires intact p19Arf and the other INK4a locus-encoded regulator, p16INK4a, which accumulate for more than 2 weeks (Palmero *et al*., 1998). We monitored the p19Arf response to RASV12 in the absence or presence of mArid3a retroviruses in whole cell extracts prepared 2 weeks after retroviral transduction (Fig. 3A). No changes were observed, indicating that the cells have become insensitive to elevated levels of both. Because p53 is required for Ha-RasV12-induced senescence (Serrano *et al*., 1997a), we examined whether mArid3a, either in the presence or absence of Ha-RasV12, interfered with basal levels of p53 or one of its transcriptional targets, p21Cip1. Westerns of whole cell lysates showed changes in their accumulation (Fig. 3A) even though mArid3a/Ha-RasV12 cells were rapidly proliferating (Fig. 2A).

Telomere shortening is another factor that contributes to onset of replicative senescence, particularly in human cells (Yang, 2008; Zou *et al*., 2009). Although this effect has not been implicated in oncogenic Ha-RasV12-mediated senescence in human cells (Seger *et al*., 2002), we assayed for its potential contribution to mArid3a rescue in MEFs. Using a TRAP assay, the senescent Ha-RasV12 transduced cells had the same levels of telomerase activity as mArid3a+Ha-RasV12 co-expressing cells (data not shown).

Bypass of RasV12-Induced Senescence by Arid3a Correlates with Induction of Cyclin E1

To identify hArid3a targets, other than the candidates above, we measured expression of cell cycle regulators. Both Cyclins D1 and E were previously shown to cooperate with RasV12 in oncogenic

transformation in vitro and in vivo (Haas *et al*., 1997; Karsunky *et al*., 1999). As shown in Fig. 3B, mArid3a-mediated immortalization of MEFs is accompanied by a gradual upregulation of Cyclin E with no significant effects on Cyclin D1 abundance. The kinetics of Cyclin E induction correlated with the passage, abbreviated post induction (pi) in which mock infected MEFs underwent senescence (Fig. 3B). Both m and hArid3a-expressing fibroblasts continued to proliferate with no apparent lag time (data not shown). Together with (Peeper *et al*., 2002), the results strongly argue that Cyclin E is an essential Arid3a target, which contributes to the immortalizing activity of Arid3a.

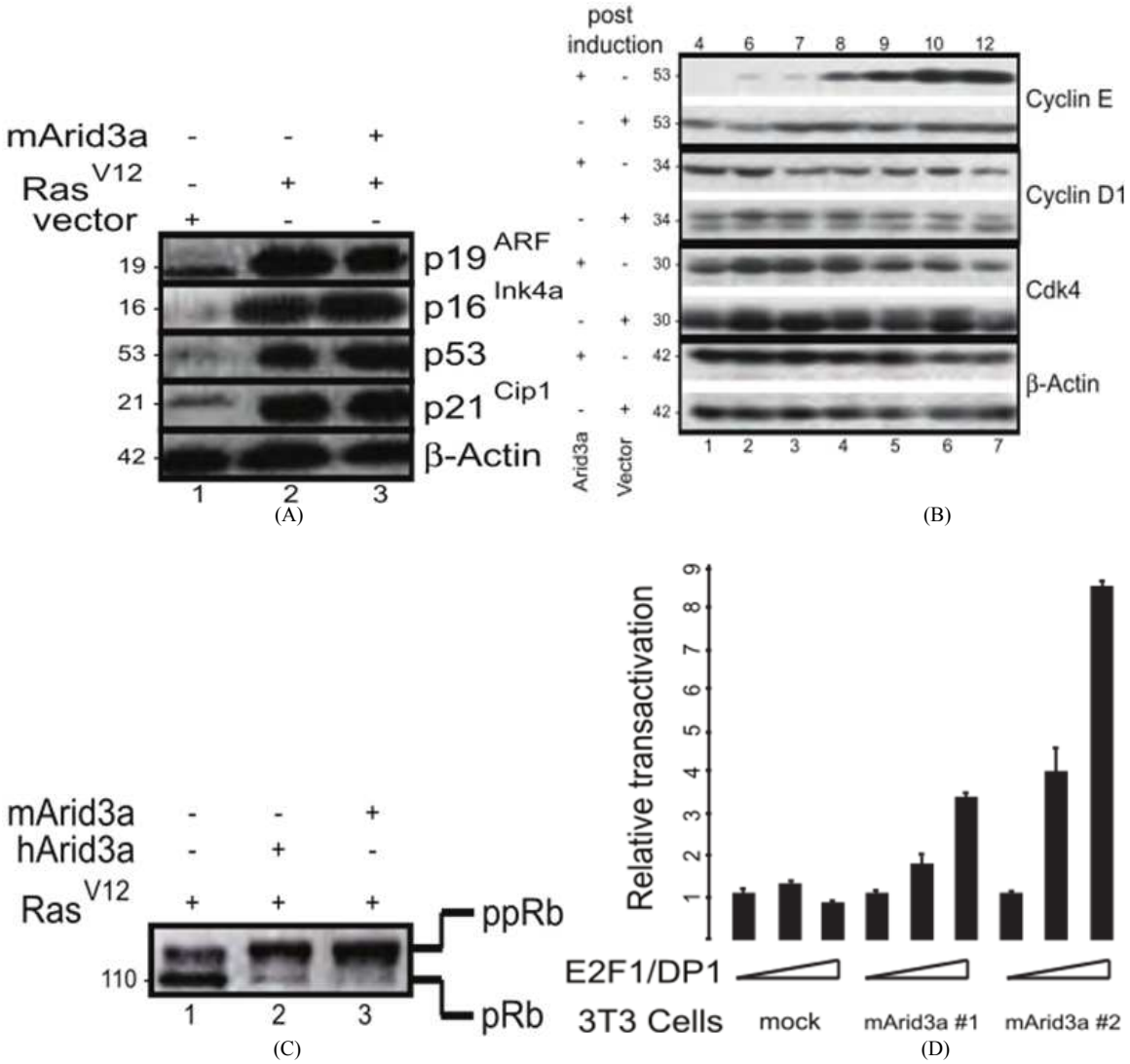


Fig. 3: Ectopic expression of mArid3a correlates with induction of Cyclin E, hyper-phosphorylation of p110Rb and increased activity of the E2F1 transcription factor. (A-C) Ectopic expression of mArid3a correlates with induction of Cyclin E, hyper-phosphorylation of p110Rb and does not prevent *Ha-ras*^{V12}–mediated induction of p19^{Arf}, p16^{INK4a}, p53, or p21^{Cip1} but correlates with induction of Cyclin E and hyper-phosphorylation of p110Rb. Primary MEFs were infected with, and selected for, expression of retroviruses expressing either no insert (vector) or HA-tagged *mArid3a*. MEFs cultures were split twice a week with passages counted post induction, beginning with the first passage after induction. Cell extracts were assayed for protein concentration and 50-80 μg of each were resolved by SDS-PAGE. Western blotting was performed with the indicated antibodies and enhanced chemo-luminescence was used for detection of proteins. (D) mArid3a induces the activity of the E2F1 transcription factor. The independent 3T3 cell pools (established in A-C) were transfected with an E2F-responsive luciferase reporter plasmid, carrying six synthetic E2F1 consensus sites, as well as increasing amounts (indicated by triangles) of expression plasmids encoding E2F1 and its heterodimeric partner DP1. Relative transactivation of the reporter was determined by normalization to a co-transfected Renilla-luciferase internal control and to the reporter activity in the absence of E2F1/DP1. Shown is the mean average of independent triplicates per data point along with standard deviation

Arid3a Induces the Activity of the E2F1 Transcription Factor via pRb Hyperphosphorylation

Cell cycle progression from G1 to S requires sequential hyperphosphorylation of pRb to release its repression of E2F (Harbour *et al*., 1999; Zhang *et al*., 2000; DeCaprio, 2009). As shown in Fig. 3C, the majority of Ha-RasV12 transduced senescent cells contain pRbp mostly in its hypophosphorylated form. Upon co-infection with either h or mArid3a viruses, most of pRb was hyperphosphorylated (ppRb) in agreement with the previous data implying that cells released from senescence had entered S phase.

In addition to loss of p19ARF or p53, Rb-family member deficiency bypasses both normal and Ha-RasV12-induced senescence (Kortum *et al*., 2006; Sage *et al*., 2000; Peeper *et al*., 2002; Dannenberg *et al*., 2000). Several critical G1/S Cyclin promoters, including Cyclin E, are regulated by pRb/E2F1 signaling through hyperphosphorylation of pRb (Ohtani *et al*., 1995; Komori *et al*., 2005; Sebastian *et al*., 2005).

We utilized NIH 3T3 cells to measure the effects of mArid3a on E2F activity to avoid the problem of different rates of cell proliferation seen in senescent vs proliferating MEFs. 3T3 cells are immortal and did not alter their cell cycle kinetics upon mArid3a expression (Fig. 2B). Control or mArid3a transduced 3T3 cells were transiently co-transfected with E2F1 and with an E2F-dependent reporter (Suzuki *et al*., 1998; Ohtani *et al*., 1995). Levels of E2F1, minimally sufficient to stimulate control luciferase activity, produced significant transactivation of the reporter in the two mArid3a-expressing populations (Fig. 3D). These results show that in the presence of mArid3a expression, E2F1 transcriptional activity is enhanced.

Active Nucleo-Cytoplasmic Shuttling, DNA Binding and Lipid Raft Occupancy Positively Correlate with mArid3a's Ability to Rescue Btr Cells from Ha-RasV12-Induced Premature Senescence

To determine the requirements for Arid3a to rescue from natural senescence, we infected Mouse embryonic fibroblasts at passage 3 with wild type and mutant forms of Arid3a (Fig. 4A; see (Kim and Tucker, 2006; Kim *et al*., 2007; Schmidt *et al*., 2009) for details). Two days post-infection, cells were grown under puromycine resistance and Trypan blue negative cells were counted at the time points indicated in Fig. 4B. Whereas wild type Arid3a rescued these cells from natural senescence, the mutant form 401KIKK/AIAA showed similar properties (Fig. 4B and 4C).

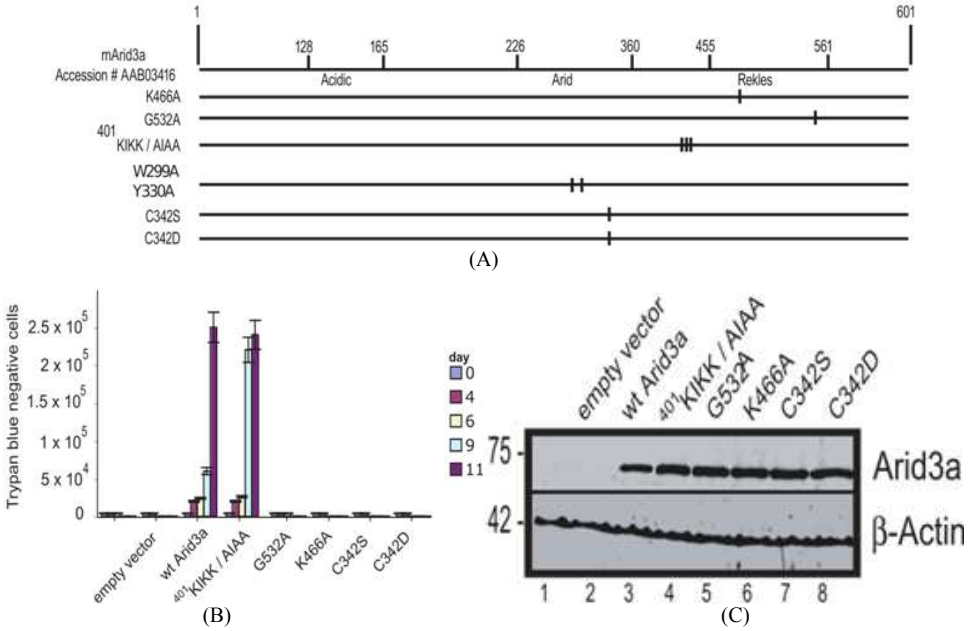


Fig. 4: Cytoplasmic-nuclear shuttling, DNA binding and an intact Arid domain are required for mArid3a to complex with Rb and rescue Btr cells from Ha-ras^{V12}-induced senescence. (A) Schematic of Bright indicating domains and positions of substitution mutations. (B) Mouse embryonic fibroblasts are rescued from senescence by mArid3a. Primary MEF cells were grown near to the end of their natural life span (passage 3), infected with the indicated forms of mArid3a and selected for puromycine resistance for one passage. Trypan blue negative cells were counted. (C) Confirmation of ectopic expression of mArid3a in the transduced MEF cells. Western analysis of whole cell extract at the end of the experiment described in (B) was to confirm expression of Arid3a using Actin as a loading control

To confirm this observation, we employed Btr cells (Peeper *et al*., 2002). Btr cells were grown at the permissive temperature (32°C) and infected with the indicated forms of Arid3a. After two days, the cells were shifted to the non-permissve temperature (39.5°C) and incubated for ten days in a humidified atmosphere. Staining for senescence-associated b-galactosidase activity was performed as described by (Dimri *et al*., 1995), followed by subsequent Eosin counterstaining.

As shown in Fig. 5, all cells are negative for senescence-associated ß-galactosidase activity at the permissive temperature (red color). Only wild type Arid3a and the mutant 401KIKK/AIAA allowed transfectants to grow at the non-permissive temperature; neither the lipid raft localization defective mutant C342S/D (Wilsker *et al*., 2005; Schmidt *et al*., 2009), nor the DNA binding inactive form W299A/Y330A (Nixon *et al*., 2004) allowed transfectants to grow at the non-permissive temperature.

Our co-immunoprecipitation results show that only wild type Arid3a and the mutant 401KIKK/AIAA complex with Rb at 39.5°C (Fig. 6). We conclude that lipid raft occupancy, DNA binding activity and active cytoplasmic-nuclear shuttling are requisite for Arid3a to function as oncogene.

Cytoplasm-Nuclear Shuttling, DNA Binding and Complex Formation with Rb are Features of Oncogenic Arid3a

Full length Arid3a, its characteristic domains (acidic, ARID and REKLES) and the position of substitution point mutations, sumoylation-consensus motif 401KIKK/AIAA, nuclear export deficient G532A, nuclear import deficient K466A, a conservative and con-conservative substitution mutation of the single and conserved cysteine C342S/D along with the dominant negative form W299A/Y330A are summarized in Fig. 4A (Nixon *et al*., 2004; Kim and Tucker, 2006; Kim *et al*., 2007; Schmidt *et al*., 2009).

To determine the requirements for Arid3a to rescue from natural senescence, we used Mouse Embryonic Fibroblast (MEF) cells at the onset of replicative crisis (Kamijo *et al*., 1997) and assayed for the ability of Arid3a and its substitution point mutant forms to rescue from naturally occuring senescence. Two days post-infection, MEF cells were grown under puromycine resistance and Trypan blue negative cells were counted at the time points indicated in Fig. 2B. While wild type Arid3a rescued these cells from natural senescence, only the mutant form 401KIKK/AIAA showed similar effects.

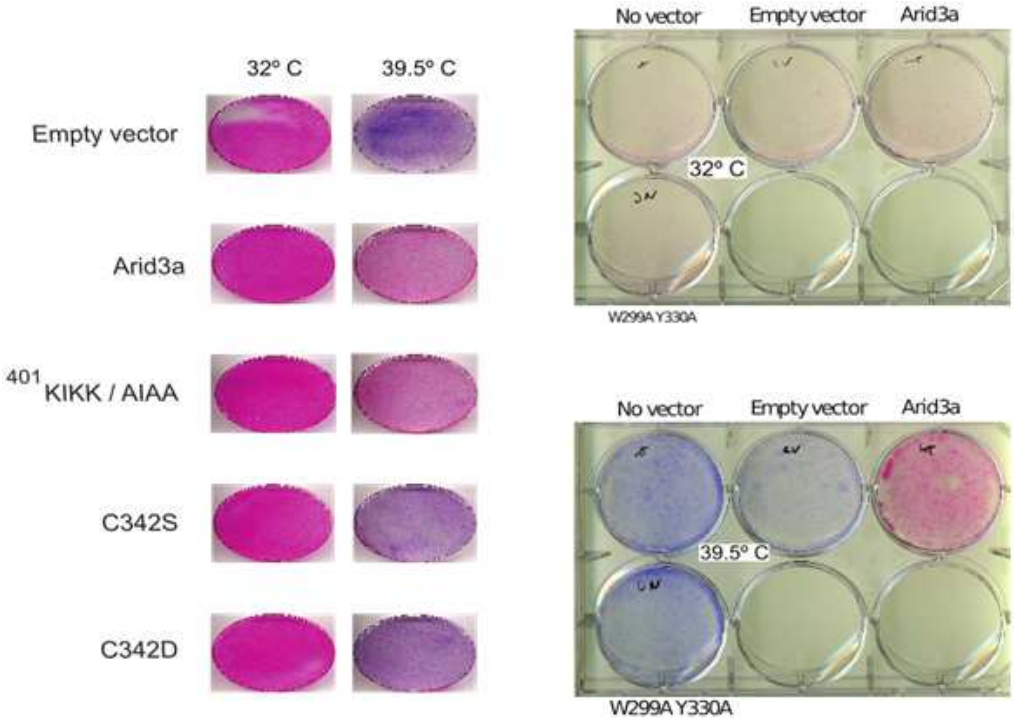


Fig. 5: Requirements of mArid3a to rescue Btr cells from Ha-ras^{V12}-induced senescence. Btr cells were infected with retroviruses encoding the indicated forms of mArid3a and grown at the permissive temperature (32°C) for three days following shift to the non-permissive temperature (39.5°C) for ten days. Cells were fixed and stained for senescence-associated β-galactosidase activity using a buffer consisting of 1mg/ml X-gal, 120μM $K_3Fe[CN]_6$, 120μM $K_4Fe[CN]_6$, 1mM $MgCl_2$ in PBS at pH 6.0) at 37°C without CO_2,as described in Fig. 1, followed by counterstaining with Eosin. Photographs of the individual plates are shown

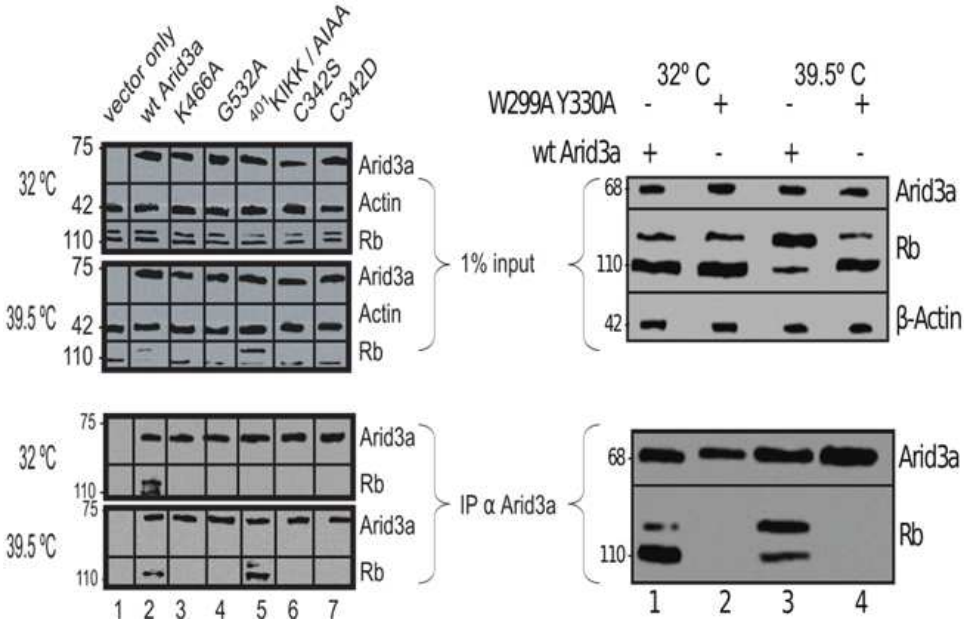


Fig. 6: Cytoplasmic-nuclear shuttling and an intact Arid domain are required for mArid3a to complex with Rb. Btr cells were infected with the indicated retroviruses and treated as described in Fig. 2C. Whole cell lysates were prepared using RIPA buffer (500 mM NaCl; 10mM Tris/Cl pH 8; 0.1% SDS; 5mM EDTA, pH 8; 10x protease inhibitor [Complete tablet, Roche]; 15min on ice) and 0.1% of the input was probed with antibodies against Arid3a, Actin and Rb. Immunoprecipitation of Arid3a was performed using 200µg of pre-cleared extract and 1µg of anti-Arid3a antiserum, followed by precipitation of the complexes with Protein A sepharose and subsequent Western analysis using anti-Arid3a and Rb antisera, respectively

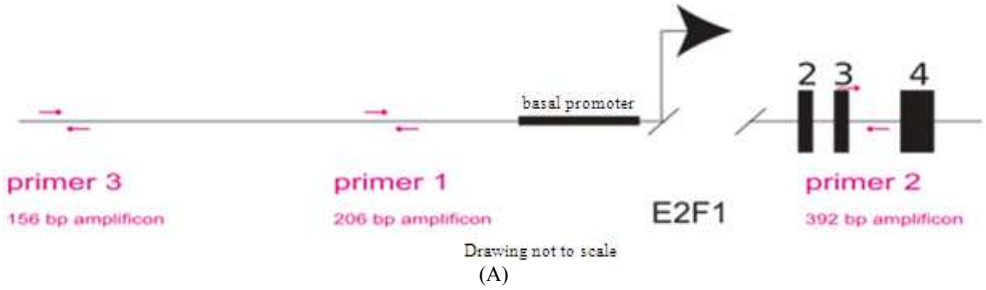
To verify this observation, we utilized Btr cells (Peeper *et al*., 2002) to assay for rescue from Ha-RasV12-induced senescence. Briefly, Btr cells were grown at the permissive temperature (32°C) and infected with the indicated forms of Arid3a. After two days, the cells were shifted to the non-permissve temperature (39.5°C) and incubated for ten days in a humidified atmosphere. Staining for senescence-associated b-galactosidase activity was performed as described by (Dimri *et al*., 1995), followed by subsequent Eosin counterstaining. As shown in Fig. 2C, all cells are negative for senescence-associated b-galactosidase activity at the permissive temperature (red color). Only wild type Arid3a and the mutant 401KIKK/AIAA allowed transfectants to grow at the non-permissive temperature.

Because sequential hyper-phosphoryation of Rb is one hallmark of cell cycle progression, we determined whether hyper-phosphorylation of Rb correlated with Arid3a's ability to bypass Ha-RasV12-induced senescence. In Fig. 2D, we show that only wild type Arid3a and the mutant form 401KIKK/AIAA complex with Rb at 39.5°C and this correlated with presence of Rb species with lower mobility in SDS-PAGE gels, indicative of hyper-phosphorylated Rb forms. We conclude that fully active Arid3a functions as oncogene, e.g., DNA binding activity and cytoplasmic-nuclear shuttling are required for rescue from natural and Ha-RasV12-induced senescence.

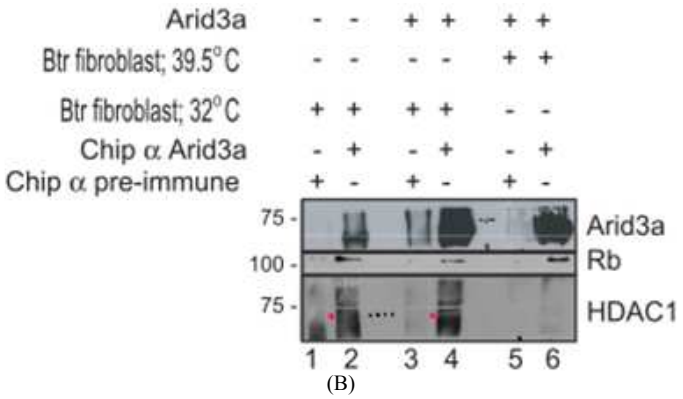
Arid3a Rescues from Senescence via Interaction and Sequestering of Rb-HDAC1 Complexes from the E2F1 Promoter

We found that the transcriptional activity of E2F1 is enhanced in the presence of mArid3a (Fig. 3D). A potential mechanism for this might be pRb sequestration. However, Arid3a does not contain the typical Rb binding pocket. However, its highly similar ARID3A paralogue, BDP, has been shown to interact with pRb through its ARID DNA binding domain, which is 96% identical with that of Arid3a (Wilsker *et al*., 2005; Kortschak *et al*., 1998; 2000; Numata, 1999). Rb binding proteins, Rbp1 and 2, belong to the ARID family, but appear to bind Rb through their LXCXE motifs (Fattaey *et al*., 1993; Binda *et al*., 2006). However, this interpretation was challenged by the fact that 100 mM concentration of Rbp1 peptide containing that motif could not inhibit the Rb-Rbbp1 interaction completely (Numata *et al*., 1999). Based on the Arid3a-BDP sequence identity (Wilsker *et al*., 2005) and our preliminary immunoprecipitation data (Fig. 6), a physical interaction of mArid3a with pRb is probable. Using Chip assays (see Fig. 7A for the landing regions of the primers used and the predicted amplificon size), we found that, in Btr cells at the permissive temperature, Arid3a is found in precipitable complexes on chromatin that contain Rb and Hdac1 (Fig. 7B, arrow pointing to the band in lane 4). Chip PCR

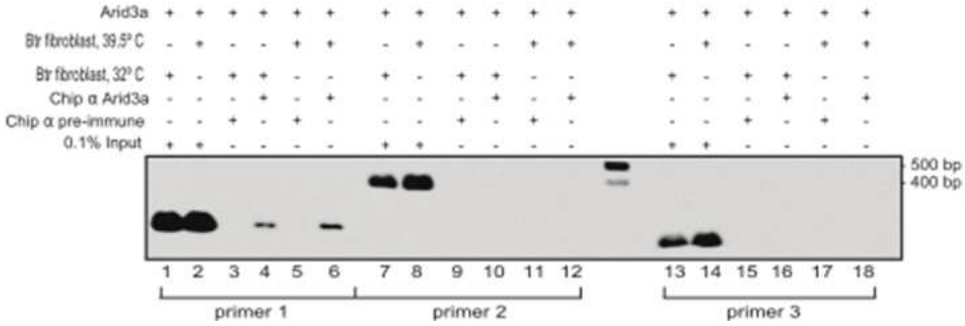
revealed that at Arid3a is detectable at the E2F1 promoter region at the permissive and non-permissive temperature (Fig. 3C, lanes 4 and 6). The inverse experimental approach revealed that HDAC1 is detected in complexes with Arid3a on chromatin only at the permissive temperature (Fig. 7D, lane 4). The corresponding PCR confirmed this finding (Fig. 3E, arrow pointing to the band in lane 4). We conclude that Arid3a can rescue senescence via interaction and sequestering of Rb-HDAC1 from E2F1 promoter.



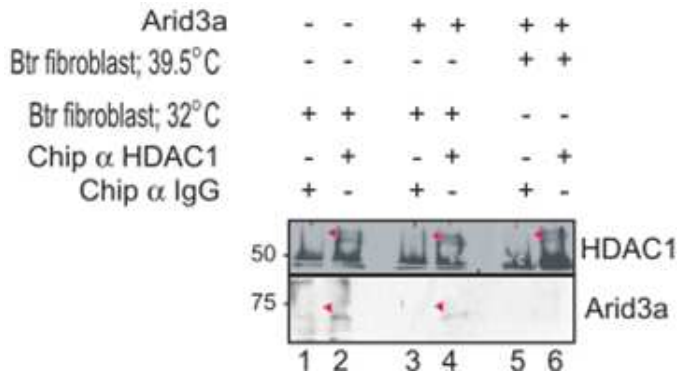
(A)



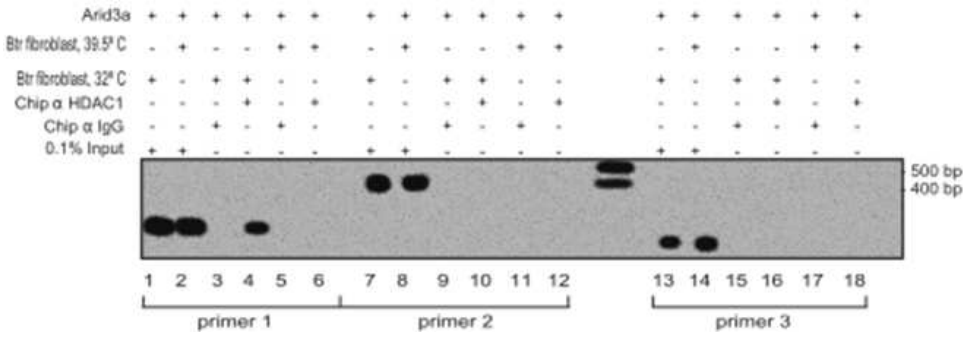
(B)



(C)



(D)



(E)

Fig. 7: Hdac1-associated mArid3a is stripped of the Rb binding region of the E2F1 promoter at the non-permissive temperature in Btr cells. (A) Schematic and not to scale illustration of primer locations and amplificon sizes. (B-D) Hdac1-associated mArid3a is stripped of the Rb binding region of the E2F1 promoter at the non-permissive temperature in Btr cells. Btr cells were infected with empty retroviruses and mArid3a encoding viruses as indicated and grown at the permissive temperature for two days, followed by shift to the non-permissive temperature for ten days. Cells were fixed to cross-link DNA-protein complexes and subjected to DNA shearing, preparation of cross-linked DNA-protein complexes by IP using the antisera indicated. The DNA-protein complexes were then analyzed by Western (B and D) and disrupted by reversion of the cross link and DNA was extracted, followed by PCR and Southern hybridization using radiolabeled primers (C and E)

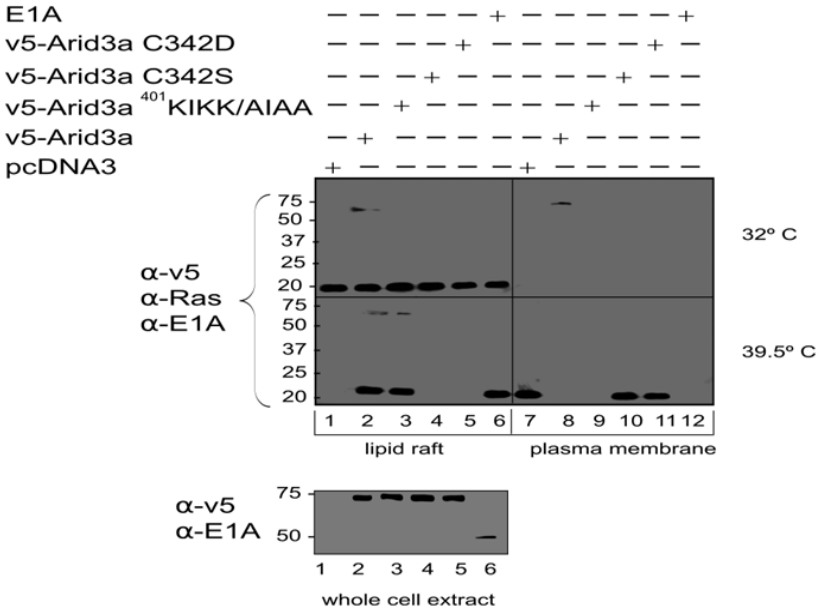


Fig. 8: E1A, Arid3a and the ^{401}KIKK/AIAA substitution mutant retain Ha-ras^{V12} in lipid rafts at the non-permissive temperature 39.5 ºC in Btr cells. Btr cells were infected with retroviruses encoding the indicated products and grown at the permissive temperature for two days following shift to the non-permissive temperature for ten days. Lipid rafts were prepared by separating crude plasma membrane from lipid rafts using a discontinous gradient centrifugation in ice-cold TNE buffer exactly as described in an earlier publication (Schmidt *et al*., 2009). Lipid rafts (buyoant fraction) and plasma membranes (sedimenting fraction) were collected and analyzed by Western blotting using the antibodies indicated. Expression of the targets was confirmed by probing RIPA whole cell extracts with the antibodies indicated

E1A, Arid3a and the Arid3a 401KIKK/AIAA Substitution Mutant Retain Ha-rasV12 in Lipid Rafts at the Non-Permissive Temperature in Btr Cells

Since a considerable fraction of Arid3a resides in the lipid rafts of both B lymphocytes and in Arid3a-transfected non-B cells (Schmidt *et al*., 2009), we sought to determine if localization there was required for senescence rescue. Btr cells were infected with retroviruses encoding E1A, or wild-type Arid3a or Arid3a substitution mutants C342D, C442S and 401KIKK/AIAA. These cells were grown at the permissive temperature for two days following shift to the non-permissive temperature for ten days. Lipid rafts

were prepared by separating crude plasma membrane from lipid rafts via discontinuous gradient centrifugation in ice-cold TNE buffer exactly as previously described (Schmidt *et al*., 2009). Lipid rafts (buoyant fraction) and plasma membranes (sediment fraction) were collected and analyzed by Western blotting with antibodies against E1A, Ras and the V5 tag affixed to the N-termini all Arid3a constructs.

As shown in Fig. 8, only wild type Arid3a and the mutant 401KIKK/AIAA transfectants were capable of growth at the non-permissive temperature; neither the lipid raft localization-defective mutant C342S/D (Wilsker *et al*., 2005; Schmidt *et al*., 2009), nor the DNA binding inactive form W299A/Y330A (Nixon *et al*., 2004) transfectants grow at the non-permissive temperature. We conclude that lipid raft occupancy, DNA binding activity and active cytoplasmic-nuclear shuttling are requisite for Arid3a to function as an oncogene.

Discussion

Players critical to the senescence response (e.g., p19Arf, p53, p16INK4a and the pRb family) are highly controlled and likely represent components of more extensive signaling networks (Fukuyo *et al*., 2004; DeCaprio, 2009). Peeper *et al*. (2002) described the identification of another component, DRIL1/hArid3a, whose orthologue in mice is involved in tissue-specific transcriptional regulation. Here we have compared the properties of the human and mouse genes. In primary murine fibroblasts, both Arid3as bypass not only RASV12-induced senescence, but also spontaneous senescence. Arid3a-dependent immortalization did not require interference with senescence-dependent induction of various key regulators of the senescence program, including p16INK4a, p19Arf, p21Cip1 and p53. This is in contrast to the Bmi1 and Tbx2 proteins, which bypass senescence by down-regulating p19Arf (Jacobs *et al*., 2000). We observed a potentially important species-specific difference in the significantly lower ability for mouse Arid3a + RASV12- MEFs to undergo transformation in culture or to form tumors in mice.

Although RASV12-expressing Rb family-deficient cells show many features of normal cells undergoing RASV12-induced senescence, including up regulation of p16INK4a, p19Arf, p53 and p21Cip1, they continue to proliferate (Peeper *et al*., 2001). Apparently, the anti-proliferative signals elicited by these proteins require pRb family proteins to become effective. It appears that a similar situation exists for Arid3a-dependent bypass of (RASV12-induced) senescence. Arid3a-expressing cells, too, possess intact signaling cascades that are activated upon onset of (RASV12-induced) senescence, including expression of two established, critical regulators of senescence, p19Arf and p16INK4a (Humbey *et al*., 2008). The p53 tumor suppressor remains active and activates its target gene p21Cip1 (Samuelson and Lowe, 1997). Thus, similar to Rb family-deficient cells, Arid3a-expressing cells show a normal biochemical response to RASV12 expression. However, they fail to show the normal biological response; i.e., instead of undergoing replicative arrest, they continue to proliferate.

We found that, mArid3a + RASV12 immortalization of MEFs is correlated with pRb hyper-phosphorylation and accumulation of CYCLIN E. The results of (Peeper *et al*., 2002) showed that hArid3a expression leads to induction of the G1/S CYCLIN E and importantly, that CYCLIN E -/- MEFs are incapable of supporting hArid3a immortalization. CYCLIN E transcription is controlled by pRb family proteins and their effectors, the E2F family of transcription factors (Ohtani *et al*., 1995). CYCLIN E is de-repressed in Rb-deficient fibroblasts (Herrera *et al*., 1996) and collaborates with RASV12 to transform primary cells and to induce tumors (Vidal *et al*., 2002; Pagliarini and Xu, 2003; Deng *et al*., 2005; Labalette *et al*., 2008). Although de-repression of CYCLIN E in Rb-/- MEFs is insufficient for their immortalization, (Peeper *et al*., 2002) further showed that ectopic over-expression of CYCLIN E1 (but not CYCLIN D1) does lead to immortalization of primary fibroblasts. This observation fits well with the reported order of D- and E-type CYCLIN action, where D phosphorylates Rb before E (Leng *et al*., 2002). It also is consistent with the insensitivity of both h and mArid3a cells to increased levels of p16INK4a, which is known to block Rb-dependent proliferation (Lukas *et al*., 1995; Medema *et al*., 1995). Neither our data, nor that of (Peeper *et al*., 2002), eliminate the potential contribution of other CYCLINs. While CYCLINs D and E were the obvious targets, CYCLIN A and even the mitotic CYCLIN B1 have been identified as E2F family member targets (Attwooll *et al*., 2004).

As with mouse Arid3a, simultaneous inactivation of Rb and its family member p107, in the context of the RASV12 oncoprotein, results in loss of proliferative control, but in only modest oncogenicity in mice (Peeper *et al*., 2001). In contrast, hArid3a/DRIL1-RASV12 cells are potently tumorigenic in mice. Perhaps hArid3a deregulates (yet to be identified) factors not affected by mArid3a, which in concert with a deregulated pRb pathway underlies its enhanced transformation and oncogenic activities. Mouse and hArid3a align poorly from residues 26-240 (just N-terminal to their ARIDs) where 15 gaps are required to achieve an identity/similarity of 75/85% (data not shown). Conserved within this region for mouse, but not for human Arid3a, is an acidic stretch, which meets consensus requirements for 3 tandem PEST motifs. PEST domains have been shown in several cases to target both ubiquitin-mediated proteosome degradation (Rogers *et al*., 1986).

Consistent with a role for DRIL1/hArid3a in human oncogenesis is its localization at chromosomal region 19p13.3, a region commonly altered in Non-Hodgkins B cell Lymphoma (NHL) and in breast epithelial malignancies (Mitelman *et al*., 1997). We and others (DP and Rene Benards, personal communication) have observed that hArid3a is highly expressed in malignant colon cell lines (no 19p13 disruptions), as well as in breast cell lines and primary isolates carrying disruptions at 19p13. In both breast and NHL, these 19p13 disruptions associate with aggressive growth and bad prognosis. Over 100 genes have been mapped to this telomeric region (Mitelman *et al*., 1997; Archer *et al*., 2005). While, there are no data in the public sector that link any of these genes to the disruption, it will be informative to determine formally whether the implicated lesion is within hArid3a.

The senescence bypass and E2F activation induced by Arid3a from either species is relatively slow. This is consistent with the view that the two processes are connected, but why so time-consuming? One possibility is that these effects require chromatin remodeling. Consistent with this notion, mArid3a MAR binding within the immunoglobulin heavy chain enhancer leads to bending of DNA up to 90 degrees (Kaplan *et al*., 2001). This was shown to facilitate spatially distant interactions of MARs. In addition, Arid3a has been shown to bind directly to model substrates wrapped into nucleosomal structures. A number of ARID domain-containing proteins have been implicated in chromatin remodeling, including the SWI1 member of the SWI/SNF family (Kortschak *et al*., 1998; Wang *et al*., 2004).

Schmidt *et al*. (2009) correlate presence of a transcription factor (Arid3a) in lipid rafts with modulation events of BCR signaling. Ratliff *et al*. (2014) provide further motivation for mechanistic studies of Arid3a in lieu of its indirect involvement in the pathogenesis of pediatric and adult manifestations of Systemic Lupus Erythematosus (SLE) through regulation of miR125, a member of the down-regulators of Arid3a. This ties in nicely with a report demonstrating that pediatric manifestations of SLE are associated with a higher risk of tumor development as individuals mature (Bernatsky *et al*., 2013). Lipid rafts are recognized as platforms for signaling (Staubach and Hanisch, 2011). Oncogenic RasV12 in lipid rafts strongly enhances phosphorylation of EGF receptors (Casar *et al*., 2009). These observations further correlate with our observation that E1A, as well as Arid3a, localized within lipid rafts at the non-permissive temperature.

Our studies have added yet another Arid3a lipid rafts function-cancer. Targeting lipid rafts for cancer prevention and treatment represent a growing in cancer progression, including cancer cell migration and invasion (Mollinedo and Gajate, 2015) and because cancer-related proteins have been identified in unbiased proteomics analyses of lipid rafts (Lesley *et al*., 1993). The spread of cancer is dependent on cell adhesion as regulation of this metastatic process holds promise for therapeutic intervention. For examine CD44, a principal cell adhesion receptor expressed in numerous malignant cell types (Senbanjo and Chellaiah, 2017), has been recently demonstrated as a lipid rafts occupant (Hryniewicz-Jankowska *et al*., 2014). We showed previously that Arid3a affiliation with lipid rafts requires its palmitoylation at C342 (Schmidt *et al*., 2009). Such a general mechanism has been implicated in breast cancer (Babina *et al*., 2014).

While it may be tempting to speculate about the function of Arid3a in modulation signals in lipid rafts in the presence of Ras in BTR cells at the non-permissive temperature, further experimentation is required to explicate this Bright path cells might follow (Herrscher *et al*., 1995).

Materials and Methods

Expression Vectors

Full-length (1-601) HA-tagged mouse (m) and human (h) Arid3a were cloned as described (Kortschak *et al*., 1998; Herrscher *et al*., 1995) into the eukaryotic CMV-powered expression vector pCR3.1. Inserts were removed and engineered into pBABE-puro retroviruses. Construction of full-length Ha-rasV12 was described (Peeper *et al*., 2002). The sequences of the inserts were confirmed by sequencing of both strands. Both of the original, subcloned Arid3a cDNAs were identical to the published sequence (Kortschak *et al*., 1998; Herrscher *et al*., 1995).

Preparation of MEFs, Cell Culture and Retroviral Infection

Organs and head were removed from a 15 day old mouse embryo and the remaining tissue was washed in Phosphate Buffered Saline (PBS) and minced. After a second PBS wash, the tissue was incubated with 100 µl trypsin/EDTA (Gibco) on ice for 12 hr. The tissue was incubated with 100 µl trypsin/EDTA at 37°C for 30 min, dissociated in complete medium and transferred to a 100-mm dish. MEFs were maintained in DMEM (Gibco) supplemented with 10% FBS (PAA Laboratories) and 0.1 mM β-mercapto ethanol. These MEFs were designated as passage 1 and were maintained in the same media.

Phoenix packaging cells were used to generate ecotropic retroviruses as described (Serrano *et al*., 1997b). MEFs were infected with filtered (0.45 µm) viral supernatant, supplemented with 4-8 µg/ml polybrene. In general, a single infection round of 6 hrs was sufficient to infect at least 90% of the population. Btr cells

(Peeper *et al*., 2002) were maintained at 32°C and infected using viruses as described above.

Proliferation Curves and Cell-Cycle Analysis

To obtain proliferation curves, MEFs were infected with either empty, HA-Arid3a and/or human Ha-ras V12-containing pBABE-puro retroviral vectors (Peeper *et al*., 2002) and after 1–2 days were selected with puromycin (1–3 μg ml^{-1}) for 4 days. After confirming that all mock-infected cells were dead, ~2.5 × 10^4 cells/ well were plated into 12-well plates and cell proliferation was measured calorimetrically as previously described (Mosmann, 1983). For proliferation curves, passage 3 MEFs were infected with retroviral vectors carrying selectable markers. At 1-2 days post infection, they were selected with puromycin (1-3 μg/ml) for 5-7 days. After confirming that all mock-infected cells were dead, the cells were seeded at a density of ~1.5×10^5 cells per 5 cm plate in which they were split twice a week. The proliferation curves were initiated when MEFs neared the end of normal life span (passage 5). Two independent data points were collected in duplicate for each sample. For cell cycle analysis, cells were permeabilized, stained with propidium iodide and the cell cycle distribution was measured by Fluorescence-Activated Cell Sorting (FACS).

Senescence-Associated β-Galactosidase Staining

Senescence-associated β-galactosidase activity was detected as previously described (Dimri *et al*., 1995). Cells were washed once with PBS (pH 7.2), fixed with 1% glutaraldehyde in PBS (pH 7.2) for 30 min at room temperature and washed once in PBS (pH 7.2) supplemented with 1mM $MgCl_2$. Cells were then stained in X-gal solution (1mg/ml X-gal, 120μM $K_3Fe[CN]_6$, 120μM $K_4Fe[CN]_6$, 1mM $MgCl_2$ in PBS at pH 6.0) overnight at 37°C without CO_2. Counterstaining with Eosin (Sigma) was performed according to the manufacturer's instruction.

Western Blotting, IP and Chip

For Western blotting, cell extracts were prepared in NETN lysis buffer (100 mM NaCl, 0.5% NP-40, 50 mM TRIS-HCl pH 8.0, 1 mM EDTA, supplemented with a protease inhibitor cocktail (Boehringer), assayed for protein concentration and 50-80 μg of clarified extract was resolved on SDS-PAGE gels, followed by transfer onto nitrocellulose membranes and probing with antisera, as described (Kim and Tucker, 2006). Primary antibodies used for Western blotting were R562 (Abcam) for p19ARF, ab9113 (Abcam) for the v5 tag, ab28305 (Abcam) for E1A, 3965 (Cell Signaling) for ras, Ab7 (Calbiochem) for p53 and from Santa Cruz, M-156 for p16INK4a, C-19 for p21CIP1, M-20 for Cyclin E, C-22 for Cdk4, H-295 for Cyclin D1 and C-22 for actin. Dr. Julien Sage, Stanford University, kindly provided the anti-Rb antibody (Sage *et al*., 2000). Affinity-purified, rabbit polyclonal anti-mArid3a generated against full-length bacterially synthesized and purified Arid3a has been described (Herrscher *et al*., 1995). Enhanced chemoluminescence (Amersham) was used for detection of proteins. Actin and tubulin served as loading controls for all blots. Chip assays were performed using the Upstate Chip Kit (17-295) according to the manufacturer's instructions (Attema *et al*., 2007). Primer sequences and PCR conditions are available upon request.

Soft Agar Assays and In Vivo Tumorigenesis

Six days post-infection (carried out as described for proliferation curves), cells were mixed with 2 ml 0.4% low-gelling agarose (Sigma type VII) in complete DMEM. Cells (~2.5×104) were seeded into duplicate 6-well plates containing solidified 1% agar (~2 ml) in complete medium. Two weeks later, foci were counted.

The capacity for MEFs to form tumors in athymic nude mice was determined by subcutaneous injection of ~1×106 cells into each flank. Mice were inspected at weekly intervals and when tumors reached 10 mm in diameter.

Dual Luciferase Assays

NIH 3T3 cells in 6 well plates were co-transfected with 100 ng of reporter construct, 100 ng of pRL-TK (Promega Corp., Madison, Wisconsin) as a normalization control and with 800 ng of each eukaryotic expression vector by Fugene 6 reagent (BMB, Germany). Dual luciferase assays were performed 48 hour after transfection using the Dual Luciferase Assay Kit (Promega Corp., Madison, Wisconsin) according to manufacturer's protocols.

Conclusion

Uncontrolled growth is an undisputed hallmark of cancer. With our understanding of the role of oncogenes incomplete, this work used rat fibroblasts that contain a temperature sensitive mutant of SV40 as a study object. With much effort needed to delineate mechanisms involved and applicable tuning loops, our preliminary results nevertheless provide experimental evidence in support of the notion that the transcription factor Arid3a, be it direct or indirect, can be likened as a major player in the exacerbation of cancer risks of adolescents with pediatric manifestations of systemic lupus erythematosus.

Acknowledgements

We thank Drs. Daniel Peeper and Rene Bernards for sharing unpublished data, for helpful discussions and for various reagents, Gary Nolan for retroviral packaging cells, Julien Sage for anti-Rb antiserum and help with the

Chip assays, David Johnson for E2F clones, Ms. Shan Maika, June Harriss and the animal facility for MEF production and help with oncogenicity assays. Chhaya Das and Maya Ghosh provided expert technical assistance and we are grateful for the administrative assistance provided by Paul Das.

Funding Information

Haley O. Tucker, Acknowledges financial support from NIH Grant R01CA31534, Cancer Prevention Research Institute of Texas (CPRIT) Grants RP100612, RP120348; and the Marie Betzner Morrow Centennial Endowment.

Author's Contributions

Christian Schmidt: Designed work, performed experiments, analyzed data, wrote the manuscript and initiated the project.

Dongkyoon Kim: Performed research and analyzed data.

Shawn Mathur, David Covarrubias and Chhaya Das: Performed research.

Mark A. Brown: Analyzed data, wrote the manuscript**.**

Joachim Storsberg: Analyzed data.

Haley O. Tucker: Designed work, analyzed data, wrote the manuscript and initiated the project.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and there are no ethical issues involved.

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