A role for c-Abl in cell senescence and spontaneous immortalization

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Abstract c-Abl is a proto-oncogene that is essential for mouse development and tissue homeostasis. Misregulation of c-Abl, as seen in the constitutively active BCR-ABL, is the leading cause of human chronic myeloid leukemia. However, how the Abl proteins execute their functions still remains largely unknown. Here, we report an important role for c-Abl in replicative senescence and immortalization by regulating the expression of two tumor suppressors that induce cellular

senescence, p53 and p16^{INK4a}. Using primary mouse embryonic fibroblasts (MEFs), we show that *c-Abl*^{−/−} cells were more resistant to immortalization than wild-type cells using a standard 3T3 or 3T9 protocol. We could only immortalize three out of nine *c-Abl*^{−/−} MEF cultures even when we increased the number of starting cells. This resistance was attributed to premature senescence and reduced survival in senescent *c-Abl*^{−/−} cells due to an increase in p16^{INK4a} and p53 expression. Deleting

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p53 allows *c-Abl*^{−/−} *p53*^{−/−} MEFs to bypass senescence to be spontaneously immortalized. Cell immortalization, but not senescence, was generally accompanied by mutations in p53 in both wildtype and *c-Abl*^{−/−} MEFs, although the spectrum is different from that of human tumors. The role for c-Abl in regulating cell senescence and immortalization might explain some of the developmental defects in *c-Abl*^{−/−} mice and how BCR-ABL transforms cells.

Keywords Senescence · Abl · p16 · p53

Introduction

Replicative senescence is a state of permanent cell cycle arrest after primary cells have exhausted their proliferation potential (10–20 divisions for mouse embryonic fibroblasts (MEFs) and about 60 divisions for human primary cells) (Hayflick 1965). Senescent cells in culture are metabolically active and remain in senescence until some of the cells accumulate mutations that allow escape from senescence and immortalization (Sherr and DePinho 2000; Shay et al. 1991). While senescence of human cells is typically caused by telomere attrition and entry into crisis, it is unclear what triggers murine cell senescence as they have long telomeres (Harley et al. 1990; Sharpless and DePinho 2004; Wu and Zakian 2010). There is increasing evidence that cell senescence acts as a protective cellular mechanism, beyond its traditional role in tissue and organism aging, and serves as a barrier to cell transformation and cancer development (Beausejour and Campisi 2006; Collado et al. 2005; Herbig et al. 2006; Jeyapalan et al. 2007; Shen 2011). Acquisition of the ability to escape from senescence is hence a prerequisite for cancer development. Numerous studies indicate that cell senescence is controlled by the p53–p21^{Cip1/Waf1} pathway and the p16^{INK4a}–Rb pathway, two most dominant tumor suppression pathways (Ben-Porath and Weinberg 2005; Lundberg et al. 2000; Kuilman et al. 2010). p53 deficiency results in an escape from senescence and spontaneous immortalization in mouse cells, while p16^{INK4a} deficiency delays the onset of senescence. In some cell types, both p53 and p16^{INK4a} are required for onset of senescence (Kuilman et al. 2010; Feng et al. 2011). Up-regulation of p53 or p16^{INK4a} results in senescence, and an increase in p16^{INK4a} protein levels has been observed in aging tissues in vivo, indicating that cell senescence

underlies the aging process in vivo (Jeyapalan et al. 2007; Krishnamurthy et al. 2006; Molofsky et al. 2006).

Another important initiator of cancer formation is the activation of oncogenes that results from genetic aberrations, including point mutations, chromosomal translocations. An example of this is a reciprocal *t*(9;22) translocation that results in the expression of fusion protein BCR-ABL. BCR-ABL is a constitutively active tyrosine kinase and is found in 95 % of the chronic myeloid leukemia (CML) patients and in 5–10 % of the acute lymphoblastic leukemia patients (Wong and Witte 2004; Hantschel and Superti-Furga 2004). CML is a clonal myeloproliferative disease that originates from an abnormal pluripotent bone marrow stem cell. Some of the known mechanisms by which BCR-ABL causes CML include promoting cell proliferation, preventing apoptosis, and altering cell adhesion via multiple signaling pathways such as the Ras-MAPK and the PI3K pathways (Wong and Witte 2004). The proto-oncogene of BCR-ABL is c-Abl, which encodes a tyrosine kinase that is tightly regulated. Various studies suggest that c-Abl plays an important role in receptor-mediated signaling pathways including TGFβ, PDGF, and in pathways that are elicited by genotoxic stress and oxidative stress (Van Etten 1999; Wang 2004; Pendergast 2002). Both genotoxic and oxidative stress, as well as some oncogenes and cytokines, are known to induce pre-mature aging in cells and organisms (Serrano and Blasco 2001; Lu and Finkel 2008; Parrinello et al. 2003; Rodier et al. 2009; Serrano et al. 1997; Kuilman et al. 2008). It has been shown that genotoxic stress can activate c-Abl, as well as BCR-ABL, and is involved in cell proliferation and apoptosis, likely through p53 and p73 (Wang 2004; Cao et al. 2003).

While BCR-ABL has been shown to inhibit apoptosis, c-Abl's role in cell death is still unclear. On one hand, several studies indicate that the overexpression of c-Abl causes apoptosis, and that DNA damage-induced apoptosis is compromised in MEFs deficient for c-Abl, suggesting a pro-apoptotic role for c-Abl (Van Etten 1999; Wang 2004; Li 2005; Wang et al. 2011). On the other hand, neuronal cells deficient for c-Abl and Arg (Abl-related-gene), the homolog of c-Abl, show massive apoptosis during early development, although Arg knockout mice are normal (Koleske et al. 1998). MEFs deficient in c-Abl and Arg show increased cell death in response to oxidative stress, and osteoblasts deficient in c-Abl show hypersensitivity to oxidative stress (Cao et al. 2003; Li et al. 2004). Furthermore, pre-B and pro-B cells isolated from c-Abl knockout mice are also hypersensitive to IL-7

deprivation-induced apoptosis (Wang 2004). Thus, discrete results regarding the roles of c-Abl in cell death and survival are obtained from cell-based studies, mouse studies, and CML patient studies. Mouse genetic studies indicate that c-Abl is essential for early development and survival. *c-Abl*^{−/−} mice showed a higher rate of perinatal lethality, decreased fertility, runtedness, immunodeficiency, spleen and thymus atrophy, and osteoporosis (Schwartzberg et al. 1991; Tybulewicz et al. 1991; Li et al. 2000). It appears that c-Abl deficiency leads to some ageing-related phenotypes, which is in accordance with an anti-cell death role for c-Abl. Yet, the molecular mechanisms by which c-Abl deficiency results in these developmental defects are not fully understood.

To further understand the function of c-Abl, we compared the growth potential and immortalization of primary MEFs that are isolated from *c-Abl*^{−/−} embryos and their control littermates. Our results indicate that c-Abl deficiency promotes cell senescence and obstructs immortalization, most likely through a decrease in cell proliferation capability and in cell survival rate. While cell senescence is accompanied with an up-regulation of p16^{INK4a}, immortalization is caused by inactivation of p53 through mutations. This study uncovers a novel function of c-Abl, which potentially explains BCR-ABL's role in transforming hematopoietic stem cells and c-Abl's role in mouse development.

Materials and methods

Primary MEFs isolation and culture

c-Abl^{−/−}, *p53*^{−/−}, and *c-Abl*^{−/−} *p53*^{−/−} mice (The Jackson Laboratory) were crossed to C57BL/6 six times. Primary MEFs were isolated from 13.5-day mouse embryos and were cultured according to the modified 3T3/3T9 protocol (Harvey and Levine 1991; Hayflick and Moorhead 1961). Briefly, the heart, liver, and spleen are removed from the embryos and the remaining tissue is passed through a syringe and trypsinized for one hour. The cells are then plated into a 6-cm plate (passage 0), and transferred into a 10-cm plate (passage 1) when confluent. The cells are then plated into four 10-cm plates (passage 2) and when confluent, seeded 10⁶ (3T3) and 3×10⁶ (3T9) cells/10-cm plates (passage 3) for continuous culture. Wildtype and *c-Abl*^{−/−} MEF are derived from heterozygous crosses of *c-Abl*^{+/-} mice

and genotyping is carried out using PCR. The presence of c-Abl protein is further confirmed by Western blot analysis. Each *c-Abl*^{−/−} and WT MEF pair used for immortalization was littermates and was passaged in parallel. Immortalized *c-Abl*^{−/−} *Arg*^{−/−} and control wild-type MEFs are obtained from Dr. Kolesky at Yale University. Primary MEFs were cultured in Dulbecco's modified Eagle's medium, containing 10 % fetal bovine serum, 1 % glutamine, and 2 % Pen-Strep. Cells were taken at different passages for reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis.

Western blot analysis

Whole cell lysates were prepared using RIPA buffer with 0.1 % SDS, 1 % NP40, 0.25 % NaDOC, 0.4 % NaF, 2 % Na₃VO₄, protease inhibitors, and quantified using Bio-Rad protein quantification assay. The protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane, and detected using enhanced chemoluminescence system (Amersham). The following primary antibodies are used: p53 (2524, Cell Signaling), p-p53 (9284S, Cell Signaling), c-Abl (sc-131, Santa Cruz Biotechnology), p21^{Cip1/Waf1} (sc-471, Santa Cruz Biotechnology), p16^{INK4a} (sc-1207, Santa Cruz Biotechnology), Id1 (sc-27187, Santa Cruz biotechnology), β-actin (sc-81178, Santa Cruz biotechnology), and tubulin (sc-5286, Santa Cruz biotechnology).

RT-PCR and DNA sequencing

Total RNA was extracted from cells of different passages using the Trizol reagent, and was converted to cDNA with proofreading *Taq* polymerase (Roche) using primers designed at the 5'UTR and 3'UTR of the murine p53 mRNA. Primer sequence:

5'UTR: 5'GCTTCAGTTCATTGGGACCATC3';
3'UTR: 5'CAGCAGAGACCTGACAACTATC3'.

p53 PCR product was then subcloned into TA vector, transformed into DH5α, and individual colonies are picked to send for sequencing. Second pair of primer sequence for p53 sequencing:

5'CAGGGCAACTATGGCTTCCA3'
5'CATCACCATCGGAGCAGCGC3'

Genomic DNA sequencing

Genomic DNA is extracted from cells using Trizol reagent and was used as template to amplify the genomic DNA fragments that correspond to the mRNA that have mutations. The PCR product is then subcloned into DH5 α and sent for sequencing.

TUNEL assay

Apoptosis of the cells in senescence and early passages was detected with a TUNEL assay kit (DeadEnd™ Fluorometric TUNEL System, G3250, Promega) following the manufacturer's protocol.

Cell senescence assay

Cellular senescence was determined with a kit from Cell Signaling (#9860) following the manufacturer's protocol. Briefly, MEFs cultured at different passages were fixed and Senescence-associated beta-galactosidase (SA- β -Gal) activity was detected at pH 6.0. Cells with distinctive blue color were counted and the percentage of senescent cells was presented.

Image acquisition, quantitation of Western blot, and statistical analysis

All the presented data have been repeated at least three times, with similar results being obtained. The results from one experiment were presented. For quantitation, Western blot results were scanned with a molecular dynamics scanning densitometer. Statistical analysis was performed using Student's *t* test. Significant association was defined when $P < 0.05$ as compared with control.

Results

c-Abl^{−/−} MEFs were resistant to spontaneous immortalization

To examine c-Abl's role in spontaneous cell immortalization, we adopted the 3T3 and 3T9 protocols to culture primary MEFs that were isolated from *c-Abl*^{−/−} and control littermate embryos from a cross of *c-Abl*^{+/−} mice. Freshly isolated MEFs were plated into a 6-cm plate (passage 0) and transferred into a 10-cm plate (passage 1) when confluent. The cells were further

plated into four 10-cm plates (passage 2) and when confluent, they were used to compare the growth potential. 3T3 cells were seeded at 10⁶ cells/10 cm plate (passage 3) and passaged every 3 days for continuous culture. We started off with the same number of cells for both wildtype and *c-Abl*^{−/−} MEFs, at three plates each. While the wildtype cells readily proliferated to large numbers, smoothly entered senescence at about passage 8, and were easily immortalized after about 17 passages in culture, *c-Abl*^{−/−} MEF cultures showed a significant decrease in proliferation capacity before reaching senescence. Moreover, we failed to immortalize the *c-Abl*^{−/−} MEFs with the 3T3 protocol due to a rapid decrease in cell numbers during senescence and an eventual collapse of the culture (Fig. 1). We reasoned that by starting with more cells, we might be able to immortalize the mutant cells. We repeated the experiment with the 3T9 protocol (seeded at 3 × 10⁶ cells/10 cm plate), starting at three plates each (data not shown). While nine independently derived wildtype cell cultures could all be immortalized, we only managed to immortalize three out of nine *c-Abl*^{−/−} cultures. Taken together, these results indicate that c-Abl plays a critical role in senescence and immortalization of MEFs.

Fig. 1 *c-Abl*^{−/−} MEFs showed premature senescence and a lack of immortalization. *c-Abl*^{−/−} and wildtype MEFs (from the same litter) were cultured with a 3T3 method. The cell division number at each passage was plotted. It was obvious that *c-Abl*^{−/−} MEFs entered senescence earlier than their wildtype counterparts and only WT cells were immortalized

Premature senescence and cell survival defects

What are the possible causes behind the defects in immortalization of *c-Abl*^{-/-} MEFs? Analysis of cell proliferation revealed two defects: a reduction in the initial cell proliferation capacity and a reduction in the cell survival rate during the senescence phase. We found that at the start of p3, *c-Abl*^{-/-} MEFs doubled 5.28 times as compared with 7.09 for wildtype MEFs before they entered senescence in 3T3 assays. In 3T9 assays, *c-Abl*^{-/-} MEFs doubled 4.74 times as compared with 5.72 for control wildtype MEFs in 3T9 assays (Fig. 2a, b). Since *c-Abl*^{-/-} and wildtype MEFs showed little difference in doubling time in p1–p3 cultures (data not shown), these results suggest that *c-Abl*^{-/-} underwent premature senescence. SA-β-Gal assay confirmed that *c-Abl*^{-/-} cultures showed more senescent cells than control wildtype cultures (Fig. 2c). Furthermore, we found that in the senescence phase, *c-Abl*^{-/-} MEFs tended to die out during continuous passaging as compared with wildtype MEFs. We also observed an increase in the percentage of apoptotic cells in *c-Abl*^{-/-} senescent cultures (Fig. 2d). This result is consistent with previous reports showing that *c-Abl*^{-/-} *Arg*^{-/-} MEFs and *c-Abl*^{-/-} osteoblasts are more susceptible to oxidative stress-induced cell death (Cao et al. 2003; Li et al. 2004). Thus, c-Abl seems to play a protective role against apoptosis during senescence.

Morphological changes

During the passaging of the mutant and wildtype MEFs, we observed a difference in cell morphology between the two cultures. This morphological difference between *c-Abl*^{-/-} and wildtype MEFs in either 3T3 or 3T9 cultures is not very obvious in early passages (Fig. 3a, b). However, in later passages, especially when the cells are in senescence (p7–p15, p12 cultures were shown), *c-Abl*^{-/-} MEFs show a phenotype that is distinct from that of wildtype. While the wildtype cells are evenly distributed on the culture plates and exhibit a large and flattened morphology, typical of senescent cells, *c-Abl*^{-/-} cells did not. Instead, they tend to be larger, more elongated with a spindle shape, and to aggregate (Fig. 3a, b). This morphology is maintained throughout the passages of senescence phase in *c-Abl*^{-/-} MEFs, while wildtype cells go through senescence and were eventually immortalized (Fig. 3a). As c-Abl is involved in cytoskeleton remodeling and cell spreading, this might also be one of the reasons why *c-Abl*^{-/-} cells

Fig. 2 *c-Abl*^{-/-} MEFs showed a reduced number of doubling before entering senescence and showed an increase in apoptotic cells. **a** The cell doubling data were derived from Fig. 1a. **b** The cell doubling data were derived from 3T9 procedure. **c** *c-Abl*^{-/-} MEFs showed an increase in the percentage of senescent (SA-β-Gal) cells. *Left*, picture of SA-β-Gal-positive cells in *c-Abl*^{-/-} and wildtype cultures. Percentage of senescent cells was counted in ten views. *Right*, quantitation data (n=3). **d** *c-Abl*^{-/-} MEFs showed an increase in the percentage of apoptotic cells in the senescent cell cultures but not in the pre-senescence cell cultures. *Left*, TUNEL-positive cells in *c-Abl*^{-/-} and wildtype cultures. Percentage of TUNEL-positive (FITC) cells was counted in ten views. *Right*, quantitation data (n=3)

are unable to take on the spread-out morphology during senescence (Hantschel and Superti-Furga 2004). This morphological change is also reminiscent of cell senescence induced by genotoxic stress or BRAF^{E600}, which

typically causes the cells to become spindle-shaped (Kuilman et al. 2010; Michaloglou et al. 2005; Dhomen et al. 2009).

Possible roles for p16^{INK4a} in MEF senescence

Cell senescence is believed to be initiated and maintained by the p16^{INK4a}-Rb pathway and the p53–p21^{Cip1/Waf1} pathway (Shay et al. 1991). We then analyzed the protein levels of these molecules in MEF cultures at different stages, and found that while p16^{INK4a} protein level was low in p1–p4 cultures and it was dramatically elevated at later stages of culture (Fig. 4 and data not shown). Unexpectedly, the protein levels of p53 and p21^{Cip1/Waf1} were slightly decreased at later stages of the cultures in both *c-Abl*^{−/−} and control MEFs (Fig. 4), suggesting that p16^{INK4a}, but not p53 or p21^{Cip1/Waf1}, might play a more active role in the initiation of senescence in MEFs. More importantly, *c-Abl*^{−/−} cells showed elevated levels of p16^{INK4a}, p53, and p21^{Cip1/Waf1} compared with wildtype cells, indicating that up-regulation of these molecules in the absence of c-Abl might contribute to the reduced proliferation capacity and premature senescence of *c-Abl*^{−/−} MEFs. The role for c-Abl in premature senescence and defective immortalization was likely to be partially mediated by the up-regulation of p53 as MEFs deficient for both c-Abl and p53, like *p53*^{−/−} MEFs, bypassed senescence and were directly immortalized (Fig. 4b).

Immortalization was accompanied by loss of function mutations in p53

Earlier studies showed that MEF immortalization is accompanied by mutations in p53, as judged by their migration alterations on SDS-PAGE gels (Serrano et al. 1997; Harvey and Levine 1991; Hainaut and Hollstein 2000). To confirm a link between MEF immortalization and p53 mutations and to determine whether c-Abl has an effect on p53 mutations during immortalization, we sequenced the coding sequence of p53 in early, senescent, and immortalized MEFs in the presence or absence of c-Abl. Total RNA was isolated from *c-Abl*^{−/−} MEFs and wildtype cultures of different passages. p53 cDNA was synthesized using RT-PCR and subcloned into TA vectors. Six to ten colonies were sequenced with two pairs of primers and the results were summarized in Table 1 and in the [Electronic supplementary material](#). We observed that there were few mutations in the early passage and senescent cell cultures regardless of the

status of c-Abl. Alternatively, it is possible that p53 mutations are not easy to identify in dividing cell cultures because cells with mutant p53 do not have a growth advantage over normal cells and are thus inadequately represented in cell cultures. However, p53 mutations are present in almost all immortalized MEFs. Sequencing the corresponding genomic DNA fragments after PCR amplification revealed that point mutations, small fragment deletion, or insertions occurred in the genome DNA but not the big fragment deletions which are likely derived from alternative splicing (data not shown). This warrants further investigation. In all the mutations identified in this study, we found deletions, missense mutations, and insertions, which are predicted to affect the transcription activity of p53 (Fig. 5).

The loss of function mutation of p53 is usually accompanied by down-regulation of p53 target genes such as p21^{Cip1/Waf1}. Comparison of the protein levels of p21^{Cip1/Waf1} in primary, senescent, and immortalized MEFs confirmed severe decrease in p21^{Cip1/Waf1} in immortalized cells (Fig. 6a). However, p16^{INK4a} was markedly up-regulated in immortalized MEFs (Fig. 6A). This is likely due to the loss in p53 function as well, as previous studies have shown that p16^{INK4a} is up-regulated in *p53*^{−/−} MEFs as well as in the tissues of *p53*^{−/−} mice (Leong et al. 2009). Western blot analysis of the re-run samples confirmed that the protein levels of p53 were greatly accumulated in two of immortalized MEFs (Fig. 6b). This biochemical evidence also supports that p53 function is lost in immortalized MEFs.

To further verify the above findings, we analyzed MEFs derived from *c-Abl*^{−/−} *Arg*^{−/−} mice, as Arg is the only homologue of c-Abl in mouse. c-Abl and Arg double knockout mouse is embryonic lethal at 10.5 dip, accompanied by a defect in neural tube closure and massive apoptosis (Koleske et al. 1998). Only one pair of *c-Abl*^{−/−} *Arg*^{−/−} and control MEFs were available due to early lethality of the embryos, which were spontaneously immortalized. Analysis of p53 expression revealed that these two cells expressed p53 of different sizes (Fig. 6c). To determine p53 activation in these cells, we treated them with adriamycin, a genotoxic and chemotherapeutic drug that generates double and single stranded DNA breaks, to activate p53. It was found that the immortalized control MEFs expressed a truncated form of the p53 protein (about 44 kD), which is phosphorylated and stabilized modestly in response to DNA damage (Fig. 6c). However, this p53 is less

Fig. 3 *c-Abl*^{-/-} MEFs showed an altered morphology in senescence. Wildtype and *p53*^{-/-} MEFs were cultured with the 3T3 (a) or 3T9 protocol (b) and the morphology of the cells at different stages was shown. The pictures were taken the 2nd day after seeding

functional as the protein level of p21^{Cip1/Waf1} was markedly reduced in this cell culture. In the immortalized *c-Abl*^{-/-} *Arg*^{-/-} cells, p53 could be phosphorylated in response to DNA damage. However, it could not be further stabilized by DNA damage. This p53 still retains some transcription activity as there was a considerable

level of p21^{Cip1/Waf1} expressed, yet p21^{Cip1/Waf1} induction in response to DNA damage was minimal, suggesting that this mutant p53 might have reduced activity. The p53 cDNA from the mutant and control cells was subcloned and sequenced. The p53 in the immortalized control cells carried a single nucleotide deletion that led to a C-

Fig. 4 *c-Abl*^{-/-} showed elevated expression of p16^{INK4a} and p53. **a** Wildtype and *c-Abl*^{-/-} MEFs were cultured with the 3T3 protocol. At different stages, cell cultures were harvested and the expression of p53, p21^{Cip1/Waf1}, and p16^{INK4a} was analyzed by Western blot. **b** Deficiency of p53 could rescue the premature phenotype of *c-Abl*^{-/-} MEFs and immortalize the cells

terminal truncated protein (aa 246). For *c-Abl*^{-/-} *Arg*^{-/-} cells, a 405C to G mutation was found, which led to a C132W mutation. Sequencing of the genomic DNA of WT and *c-Abl*^{-/-} *Arg*^{-/-} confirmed the deletion (data not shown). This result, although limited due to the unavailability of double knockout mice, suggest that immortalization of *c-Abl*^{-/-} *Arg*^{-/-} and normal MEFs are also accompanied by p53 mutations.

Discussion

This study uncovers an important role for c-Abl in senescence and immortalization of primary MEFs. First, c-Abl appears to be critical for MEF immortalization. While all wildtype MEFs can be immortalized by either 3T3 or 3T9 culture, none of the five *c-Abl*^{-/-} cultures was immortalized with 3T3 protocol and only three of the nine *c-Abl*^{-/-} MEF cultures were immortalized with the 3T9 protocol. Secondly, c-Abl deficiency results in a modest but significant reduction in cell proliferation capacity before they reach senescence. A similar phenomenon has been observed in *c-Abl*^{-/-} osteoblasts (Kua et al., unpublished results). Thirdly, c-Abl deficiency results in a change in cell morphology, which is more evident in senescent cells. *c-Abl*^{-/-} MEFs look different from the wildtype cells and they tend to aggregate in late passages. This may be related to c-Abl's role in regulating cytoskeleton remodeling or due to a different kind of cellular senescence

Table 1 Summary of p53 mutations in immortalized normal and *c-Abl*^{-/-} MEFs

genotypes	Mutations in p53 gene	cDNA mutation	Genomic DNA mutation	Supplementary figures
<i>c-Abl</i> ^{+/+}	C932 deletion. Frame shift after aa 310	+	+	S1
	A386G mutation. Missense K129R mutation	+	+	S2
	15 bp insertion after nt 765. Truncation after aa 257	+	+	S3
	17 bp deletion from nt 549. Frame shift after aa 183	+	+	S4
	110 bp deletion from nt 664. Frame shift after aa 211	+	—	S7
	G151T mutation. Truncation at aa 50	+	+	S5
	322 bp deletion after nt 660. Frame shift after aa 320	+	—	S10
	79 bp insertion after nt 910. Frame shift after aa 303	+	—	S8
<i>c-Abl</i> ^{-/-}	283 bp deletion after nt 93. Frame shift after aa 31	+	—	S9
	C415T mutation. P139S missense mutation	+	+	S6
<i>c-Abl</i> ^{+/+} <i>Arg</i> ^{+/+}	C739 deletion. Truncated by 50 aa after aa 246	+	+	S11
<i>c-Abl</i> ^{-/-} <i>Arg</i> ^{-/-}	C405G mutation. C132W mutation	+	+	S12

“+” means that the mutation was detected in cDNA or Genomic DNA, “—” means that the mutation was not detected in genomic DNA

Fig. 5 A diagram showing the domain structure of mouse p53 and the mutations identified in this study. TAD transactivation domain, PRR proline-rich region, OD tetramerization domains, CTD the carboxyl terminus

(Kuilman et al. 2010; Pendergast 2002). Fourthly, we found that senescent *c-Abl*^{-/-} MEFs showed reduced survival rates. While wildtype senescent cells could be passaged many times to be immortalized, *c-Abl*^{-/-} MEFs diminish rapidly in numbers. The above defects are expected to contribute to the differences in cell immortalization. Taken together, our results suggest that c-Abl plays a positive role in promoting cell growth and immortalization, similar to BCR-ABL (Wong and Witte 2004).

Cell senescence occurs to most types of primary cells and spans a broad range of research from tissue degeneration/aging to tumorigenesis (Beausejour and Campisi 2006; Jeyapalan et al. 2007). While both of the two pathways—p53–p21^{Cip1/Waf1} pathway and p16^{INK4a}–Rb pathway—control cell senescence, we found here that in MEFs, the levels of p53 and p21^{Cip1/Waf1} did not increase with continuous passaging of the cells. Instead, they are down-regulated modestly in senescent MEFs. On the contrary, p16^{INK4a} seems to show an up-regulation in senescent cells, suggesting that p16^{INK4a} might play a more active role than p21^{Cip1/Waf1} in initiating senescence in normal MEFs (Kuilman et al. 2010). More importantly, *c-Abl*^{-/-} senescent MEFs is associated with a greater increase in p16^{INK4a} protein levels as compared with wildtype senescent cells. These results suggest that c-Abl might regulate p16^{INK4a} expression to modulate MEF senescence, potentially explaining the premature senescent phenotype in the *c-Abl*^{-/-} cells.

In addition, *c-Abl*^{-/-} MEFs also showed an increase in p53 and p21^{Cip1/Waf1} protein levels as compared with wildtype cells. This is different from the positive role that c-Abl plays in DNA damage-induced p53 stabilization and activation (Wang 2004; Shaul 2000; Meltser et al. 2011), and suggests that c-Abl might regulate p53 expression in a context dependent manner. An example of a context dependent gene regulation in DNA damage is the control of p21^{Cip1/Waf1} expression by Atm. While *Atm*^{-/-} cells

show diminished p21^{Cip1/Waf1} induction upon genotoxic stress, these cells also show marked elevation of p21^{Cip1/Waf1} protein levels at the basal level (Barlow et al. 1997; Xu and Baltimore 1996). This complex regulation under different cellular contexts could be a reason behind the seemingly inconsistent results that were obtained with regards to c-Abl's function in cell cycle control and apoptosis. Given that p53 also plays a critical role in initiating/maintaining senescence and apoptosis, up-regulation of p53 might contribute to the reduced proliferation potential and the reduced survival rate in *c-Abl*^{-/-} MEFs, which together might constitute the main reason why *c-Abl*^{-/-} MEFs are resistant to immortalization. This is also supported by our observation that *c-Abl*^{-/-} p53^{-/-} MEFs do not undergo senescence and are spontaneously immortalized.

Our studies further support previous findings that p53 plays a critical role in MEF immortalization. By sequencing p53 cDNA clones from *c-Abl*^{-/-} and wildtype cells at early passage, senescent and immortalized stages, we found that p53 mutations are always detected in immortalized cells, but rarely in early passage or senescent cells. More interestingly, we identified several p53 insertions and deletions in the DNA binding domain, the C terminus, and the N terminus of the protein. Some of the big fragment deletions are likely derived from splicing errors. These mutations appear to render p53 to be less active, as judged by the change in p21^{Cip1/Waf1} expression and the elevation of the p53 protein levels. This observation, together with the fact that p53 deficiency automatically leads to cell immortalization in MEFs, reinforce the concept that p53 inactivation is the main underlying mechanism for cell immortalization (Serrano et al. 1997; Harvey and Levine 1991). How these p53 mutations are generated in the senescent cells needs further investigation.

It is worth noting that the p53 mutations we identified during MEF spontaneous immortalization, together with the mutations identified in immortalization of Hupki

Fig. 6 Comparison of the protein levels of senescence controlling genes in pre-senescence, senescence, and immortalized MEFs of *c-Abl^{+/+}*, *c-Abl^{-/-} Arg^{-/-}*, and wildtype MEFs. **a** Wildtype and *c-Abl^{-/-}* MEFs were cultured with the 3T3 protocol. At stages of pre-senescence, senescence, and immortalized, cell cultures were harvested and the expression of p53, p21^{Cip1/Wafl}, and

p16^{INK4a} was analyzed by Western blot. **b** Re-run of the samples described in 6A with an adjustment of the loading orders. **c** Altered expression of p53 and in *c-Abl^{-/-} Arg^{-/-}* MEFs. Wildtype and *c-Abl^{-/-} Arg^{-/-}* MEFs were stressed with adriamycin for different periods of time and the protein levels of p53 and p21^{Cip1/Wafl} were determined by Western blot

(human p53 knock-in) MEFs (Whibley et al. 2010), are not characteristic of mutations identified in human

tumors (transformed cells). The mutations present in human cancers are predominantly transition point

mutations (73.6 %, IARC p53 database) distributed along the DNA binding domain of p53, leading to the loss or decrease of p53 transcription activity (Petitjean et al. 2007). In most of the immortalized MEFs of our study, we recovered more deletions or insertions of p53, some of which may be due to aberrant splicing. In spontaneously immortalized Hupki cells, about half do not display disrupted p53, and in the MEFs that do have mutations, G to C transversions predominate (Whibley et al. 2010). These results suggest that the DNA sequence of p53, especially the none-coding sequences, might have an influence on p53 mutations.

In summary, this study provides evidence that c-Abl plays an important role in cell senescence and immortalization, and that c-Abl might execute this function by regulating the expression of the two predominant tumor suppressors p16^{INK4a} and p53. The decrease in cell proliferative capacity and in survival caused by c-Abl deficiency might underlie some of the phenotypes observed in c-Abl deficient mice, including runtedness, lymphopenia, osteoporosis, and reduced post-natal survival, all of which are features of aging. The role for c-Abl in cell immortalization may be one of the mechanisms by which BCR-ABL promotes the development of CML.

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