Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase

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Background: Telomeres consist of repetitive (TTAGGG) DNA sequences that are maintained by the multisubunit telomerase ribonucleoprotein. Telomerase consists of an RNA, which serves as template for the sequence tracts, and a catalytic subunit that functions in reverse transcription of the RNA template. Cloning and characterization of the human catalytic subunit of telomerase (hTERT) has supported a role in cell transformation. How telomerase activity is regulated, however, is largely unknown.

Results: We show here that hTERT associates directly with the c-Abl protein tyrosine kinase. We also found that c-Abl phosphorylates hTERT and inhibits hTERT activity. Moreover, our findings demonstrate that exposure of cells to ionizing radiation induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism. The functional significance of the c-Abl–hTERT interaction is supported by the demonstration that cells deficient in c-Abl show telomere lengthening.

Conclusions: The ubiquitously expressed c-Abl tyrosine kinase is activated by DNA double-strand breaks. Our finding of telomere lengthening in c-Abl-deficient cells and the functional interactions between c-Abl and hTERT support a role for c-Abl in the regulation of telomerase function.

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Telomerase is a ribonucleoprotein complex that elongates telomeres [1,2]. Telomeres contain distinctive repeats of guanine-rich sequences that are replicated by DNA-dependent DNA polymerases and by telomerase-dependent synthesis of telomeric DNA from an RNA template [3]. Cells deficient in telomerase have short telomeres as a consequence of failure to synthesize telomeric DNA ends [4,5]. The demonstration that expression of the telomere catalytic subunit hTERT and activation of telomerase activity can extend the life span of normal human cells has suggested that decreases in telomere length contribute to senescence [6,7]. Studies in mice lacking telomerase RNA have also shown that telomeres function in maintaining genomic stability [8]. Because telomerase activity is low in most somatic cells, telomeres shorten as cells progress through replicative cycles [9–13]. Telomere shortening in cells continues until crisis and the escape of immortal cells that have reactivated telomerase [14–19]. Other studies have shown that telomerase activity is detectable in human tumors [10,20,21]. Moreover, recent work has shown that ectopic expression of hTERT in combination with two oncogenes results in tumorigenesis of normal human epithelial and fibroblast cells [22].

is tightly regulated in cells [23,24]. c-Abl associates with the DNA-dependent protein kinase (DNA-PK) complex [25] and with the product of the gene mutated in ataxia telangiectasia (ATM) [26,27]. The catalytic subunit of DNA-PK (DNA-PKcs) and ATM are members of a family of phosphatidylinositol (PI) 3-kinase-like enzymes involved in regulation of the cell cycle, recombination, control of telomere length and the DNA damage response [28–30]. c-Abl is activated by DNA-PK and ATM in cells exposed to ionizing radiation and other DNA-damaging agents [25–27,31]. Cells deficient in DNA-PK or ATM are hypersensitive to ionizing radiation [32–34], and c-Abl-deficient cells are resistant to DNA-damage-induced apoptosis [35,36]. The available evidence indicates that c-Abl confers growth arrest and proapoptotic responses to DNA damage by mechanisms that depend partly on p53 and its homolog p73 [35,37–41]. It also functions as an upstream effector of the Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase pathways [26,42–44]. Other studies have implicated c-Abl-dependent inhibition of PI 3-kinase in the apoptotic response to DNA damage [45]. These findings have supported a role for c-Abl in converting DNA damage into signals that control cell behavior.

Because c-Abl is activated by DNA double-strand breaks [31] and proteins involved in the repair of these lesions function in telomere control [46–48], we investigated whether c-Abl interacts with telomerase. We found that c-Abl phosphorylates hTERT and that c-Abl functions as a negative regulator of hTERT activity.

Results and discussion

The c-Abl protein tyrosine kinase is tightly regulated and expressed in 293T and MCF-7 cells [39]. To investigate whether c-Abl associates with the hTERT catalytic subunit, lysates from 293T cells cotransfected with c-Abl and a haemagglutinin-epitope-tagged version of hTERT (HA–hTERT) were subjected to immunoprecipitation with anti-c-Abl antibody. Analysis of the immunoprecipitates with anti-HA antibody showed binding of c-Abl and hTERT (Figure 1a). The reciprocal experiment, in which anti-HA immunoprecipitates were analyzed by immunoblotting with anti-c-Abl antibody confirmed coimmunoprecipitation of c-Abl and hTERT (Figure 1b). Similar findings were obtained in HeLa cells cotransfected with c-Abl and hTERT (data not shown). To assess whether endogenous c-Abl associates with endogenous hTERT, lysates from hTERT-expressing MCF-7 cells [7] were subjected to immunoprecipitation with anti-c-Abl antibody. Analysis of the resulting precipitates by immunoblotting with anti-hTERT showed constitutive binding of endogenous c-Abl and hTERT (Figure 1c). The finding that endogenous hTERT is detectable in anti-c-Abl immunoprecipitates from MCF-7 cells stably overexpressing a kinase-inactive form of c-Abl, c-Abl(K-R), also indicated that the association was independent of the c-Abl kinase function (Figure 1c). To extend these findings to other cell types, lysates from hTERT-expressing 293T cells [7] were subjected to immunoprecipitation with anti-hTERT and the precipitates were analyzed by immunoblotting with anti-c-Abl antibody. As in MCF-7 cells, c-Abl and hTERT also associated constitutively in 293T cells (Figure 1d). Taken together, these findings indicate that c-Abl associates with hTERT and that this interaction is independent of the kinase function of c-Abl.

To confirm the association of c-Abl and hTERT, lysates from 293T cells transfected with HA–hTERT were incubated with glutathione-S-transferase (GST) fusion proteins containing c-Abl (GST–c-Abl) or the Src homology 3 domain of c-Abl (GST–Abl SH3). Analysis of the adsorbates with anti-HA antibody showed binding of hTERT to GST–c-Abl and GST–Abl SH3 (Figure 2a, left panel and data not shown). By contrast, there was no detectable binding of hTERT to a GST–Grb2 fusion protein that contains the amino-terminal SH3 domain (Figure 2a, right panel). The c-Abl SH3 domain binds to proline-rich sequences with the consensus PXXXXPXXP (in single-letter amino acid code where X is any amino acid) [49,50]. The identification of one potential sequence for c-Abl SH3

Figure 1
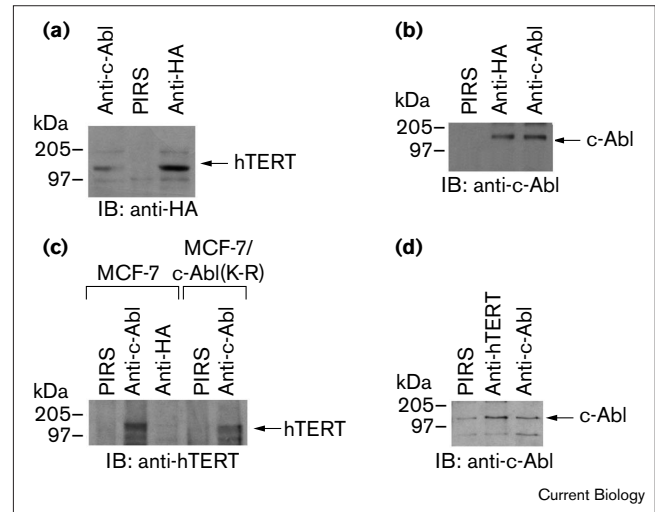
Interaction of c-Abl with hTERT. **(a)** 293T cells were transiently transfected with wild-type c-Abl and HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-c-Abl antibody, anti-HA antibody or preimmune rabbit serum (PIRS). The precipitates were analyzed by immunoblotting (IB) with anti-HA antibody. **(b)** 293T cells were transiently transfected with HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-c-Abl, anti-HA, or PIRS. The precipitates were analyzed by immunoblotting with anti-c-Abl antibody. **(c)** Total cell lysates from MCF-7 and MCF-7/c-Abl(K-R) cells were subjected to immunoprecipitation with PIRS or anti-c-Abl antibody or anti-HA antibody and analyzed by immunoblotting with anti-hTERT antibody. **(d)** Lysates from 293T cells were subjected to immunoprecipitation with PIRS, anti-hTERT, or anti-c-Abl antibody and analyzed by immunoblotting with anti-c-Abl antibody.

binding in hTERT (PSTSRPPPRP; amino acids 308–316) suggested that there is a direct interaction between these two proteins. To define regions involved in the interaction between c-Abl and hTERT, four fragments derived from hTERT (Figure 2b) were synthesized as amino-terminal GST fusion proteins. Lysates from 293T cells were incubated with 5 μg GST–hTERT-1, -2, -3, or -4. Analysis of the adsorbates with anti-c-Abl demonstrated selective binding of c-Abl to the fragment GST–hTERT-2 containing the consensus proline-rich motif (Figure 2c). To confirm that the interaction between c-Abl and hTERT is direct, anti-HA immunoprecipitates from HA–hTERT-transfected cells were subjected to SDS–PAGE and transferred to a nitrocellulose filter. Analysis of the filter by incubation with purified GST–Abl SH3 and immunoblotting with anti-GST antibody showed binding of c-Abl to HA–hTERT (Figure 2d). These findings demonstrate that c-Abl directly associates with hTERT.

To assess whether c-Abl phosphorylates hTERT, we incubated purified kinase-active c-Abl or the kinase-inactive form c-Abl(K-R), with immunopurified hTERT in the presence of $[\gamma\text{-}^{32}P]ATP$. Analysis of the products by

Figure 2
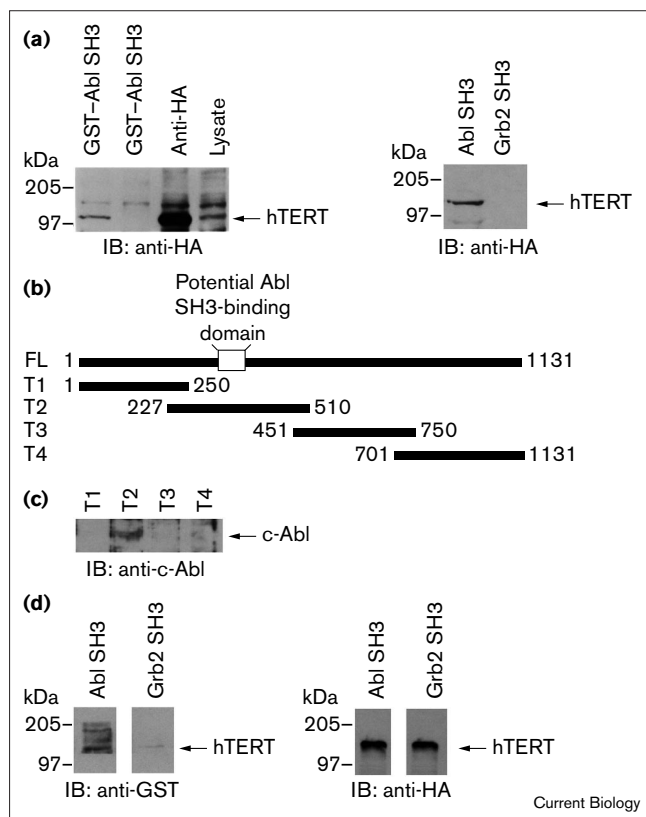
Direct interaction of hTERT with c-Abl. **(a)** The left panel shows results for 293T cells transiently transfected with vector or HA–hTERT. Lysates from HA–hTERT-transfected cells were incubated with GST–c-Abl SH3 (lane 1) or immunoprecipitated with anti-HA antibody (lane 3). Lysates from vector-transfected 293T cells were separately incubated with GST–c-Abl SH3 (lane 2). Lysate (lane 4) and precipitated proteins were analyzed by immunoblotting (IB) with anti-HA antibody. The right panel shows results for 293T cells transiently transfected with HA–hTERT. Total cell lysates were incubated with GST–c-Abl SH3 or GST–Grb2 SH3 fusion proteins bound to glutathione–sepharose. The precipitates were analyzed by immunoblotting with anti-HA antibody. **(b)** Schematic representation of full-length hTERT (FL) and hTERT deletion mutants (T1–T4). **(c)** Lysates from 293T cells were incubated with GST–hTERT-1 (T1), GST–hTERT-2 (T2), GST–hTERT-3 (T3) and GST–hTERT-4 (T4). Precipitated proteins were analyzed by immunoblotting with anti-c-Abl antibody. **(d)** 293T cells were transiently transfected with HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-HA antibody. The precipitated proteins were resolved by SDS–PAGE and transferred to nitrocellulose filters. The filters were incubated with purified eluted GST–c-Abl SH3 or GST–Grb2 SH3 fusion proteins. After incubation, the filters were analyzed by immunoblotting with anti-GST antibody (left panels) and anti-HA antibody (right panels).

autoradiography showed that hTERT was a substrate for c-Abl *in vitro* (Figure 3a). To find out whether c-Abl phosphorylates hTERT *in vivo*, 293T cells were transiently cotransfected with HA–hTERT and vector, c-Abl, or c-Abl(K-R). As a control, 293T cells were transfected with c-Abl in the absence of HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-HA and

Figure 3
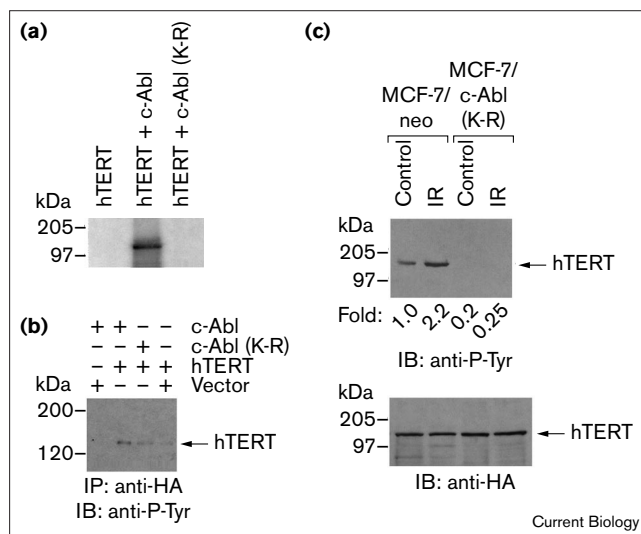
Phosphorylation of hTERT by c-Abl. **(a)** 293T cells were transiently transfected with HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-HA antibody and the protein precipitates were incubated with buffer (lane 1), purified kinase-active c-Abl (lane 2), or purified kinase-inactive c-Abl(K-R) (lane 3) in the presence of [γ^{32}-P]ATP for 15 min at 30°C. The phosphorylated proteins were analyzed by SDS–PAGE and autoradiography. **(b)** 293T cells were transiently cotransfected with HA–hTERT and vector, c-Abl, or c-Abl(K-R). As a control, 293T cells were transfected with c-Abl in the absence of HA–hTERT. Anti-HA antibody immunoprecipitates (IP) were analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody (anti-P-Tyr). **(c)** MCF-7/neo and MCF-7/c-Abl(K-R) cells were transiently transfected with HA–hTERT. After transfection, cells were exposed to 20 Gy ionizing radiation (IR) or left untreated (control) and harvested after 2 h. Nuclear lysates were isolated and subjected to immunoprecipitation with anti-HA antibody and analyzed by immunoblotting with anti-P-Tyr antibody (upper panel) and anti-HA antibody (lower panel). The results are also expressed as fold induction (mean ± SD of three independent experiments).

analyzed by immunoblotting with anti-P-Tyr antibody. In contrast to vector or c-Abl(K-R), overexpression of wild-type c-Abl correlated with tyrosine phosphorylation of hTERT (Figure 3b).

Because c-Abl is activated by DNA damage [31], we investigated whether genotoxic stress affects the interaction between c-Abl and hTERT. Parental MCF-7 cells and MCF-7 expressing c-Abl(K-R) (MCF-7/c-Abl(K-R)) cells were transiently transfected with HA–hTERT. After transfection, cells were exposed to ionizing radiation and nuclear lysates were subjected to immunoprecipitation with anti-HA antibody. The protein precipitates were analyzed by immunoblotting with anti-P-Tyr antibody. Exposure of MCF-7 cells to ionizing radiation was associated with increases (approximately 2.5-fold) in tyrosine phosphorylation of hTERT (Figure 3c). Moreover, ionizing radiation had no detectable effect on tyrosine phosphorylation of hTERT in MCF-7/c-Abl(K-R) cells (Figure 3c).

These findings demonstrate that ionizing radiation induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism.

The functional significance of the interaction between c-Abl and hTERT was investigated by assay of telomerase activity in 293T cells that had been transiently cotransfected with HA–hTERT and vector, c-Abl, or c-Abl(K-R). Anti-HA immunoprecipitates were assayed for telomerase activity (Figure 4). The telomerase function of HA–hTERT was inhibited in cells cotransfected to express c-Abl compared with that in cells expressing c-Abl(K-R). To define further the role of c-Abl in the regulation of telomerase activity, HA–hTERT was transfected into mouse embryo fibroblasts (MEFs) from wild-type (c-Abl$^{+/+}$) and c-Abl$^{-/-}$ mice. Analysis of anti-HA immunoprecipitates showed that, compared with c-Abl$^{+/+}$ cells, there was a significant increase in telomerase activity in c-Abl$^{-/-}$ cells (Figure 4). These findings indicate that phosphorylation of hTERT by c-Abl is associated with inhibition of telomerase activity.

To investigate further the involvement of c-Abl in the regulation of telomerase activity, early-passage MEFs deficient in c-Abl (derived from mice with targeted *c-abl* disruption) [51] were assayed for telomere length. Telomere repeat length was assessed by hybridization of two distinct fluorescently labeled peptide nucleic acid (PNA) oligomer probes, one for telomeres and the other for the centromere, to metaphase chromosomes (Figure 5). Compared with c-Abl$^{+/+}$ MEFs, the c-Abl$^{-/-}$ cells showed increases in telomere length. Quantification of telomere length by assessment of telomere/centromere ratios in ten independent metaphase spreads show significantly higher ratios for c-Abl$^{-/-}$ cells, than for c-Abl$^{+/+}$ cells (Table 1). These findings provide further support for regulation of telomerase activity by the c-Abl kinase.

In *Saccharomyces cerevisiae*, the Rap1p protein binds to telomeric DNA and negatively regulates telomere length [52–54]. The function of Rap1p in telomere regulation is mediated by Rap1-interacting factors, Rif1 and Rif2 [55,56]. Telomeric repeat-binding proteins implicated in regulation of telomere length have been identified in *Schizosaccharomyces pombe* (Taz1p) [57], in human cells (hTRF1) [58] and in Chinese hamster cells (chTRF1) [59]. Our studies demonstrate a distinct mechanism of telomere regulation through the interaction of c-Abl with the hTERT catalytic subunit of telomerase. The finding that c-Abl-mediated phosphorylation of hTERT inhibits telomerase activity supports a function in negatively regulating telomere length. The results also show that DNA damage induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism. These findings are in agreement with the activation of c-Abl in the response to genotoxic stress [25–27] and support a link between

Figure 4
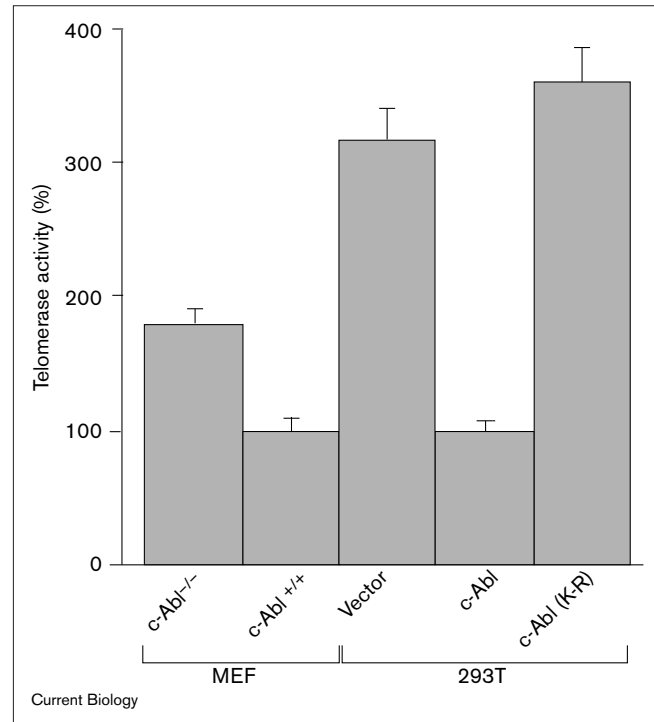
Inactivation of hTERT by c-Abl. MEFs from wild-type (c-Abl$^{+/+}$) and c-Abl$^{-/-}$ mice were transfected with HA–hTERT. 293T cells were transiently cotransfected with HA–hTERT and vector, c-Abl or c-Abl (K-R). Anti-HA antibody immunoprecipitates were assayed for telomerase activity. Telomerase activity is expressed as mean of three independent experiments; error bars indicate SD.

DNA-damage-induced signals and the regulation of telomerase activity. Reports that Ku and the Mre11–Rad50–Xrs2 complex function in regulating telomere length [46–48] have also supported the interaction of DNA-damage-induced pathways and telomere control.

Severe combined immunodeficiency (*scid*) mice that are deficient in DNA-PK have longer telomeres than those of corresponding wild-type mice [60]. By contrast, recent studies have shown that mice lacking poly(ADP-ribose) polymerase (PARP) show telomere shortening [61]. The findings in c-Abl$^{-/-}$ cells support a function for c-Abl in regulating telomere length. Consistent with a role for c-Abl in the negative regulation of hTERT, the c-Abl$^{-/-}$ cells, but not their c-Abl$^{+/+}$ counterparts, show telomere lengthening. The c-Abl$^{-/-}$ cells used in our studies were derived from mice with targeted disruption of *c-abl* at a region corresponding to the tyrosine kinase domain and have no detectable c-Abl expression [51]. By contrast, homozygous *abl^{m1}/abl^{m1}* mutant cells that express the c-Abl–neo fusion protein with loss of the c-Abl carboxyl terminus, but retention of c-Abl kinase activity [62], do not show telomere lengthening (data not shown). The

Figure 5
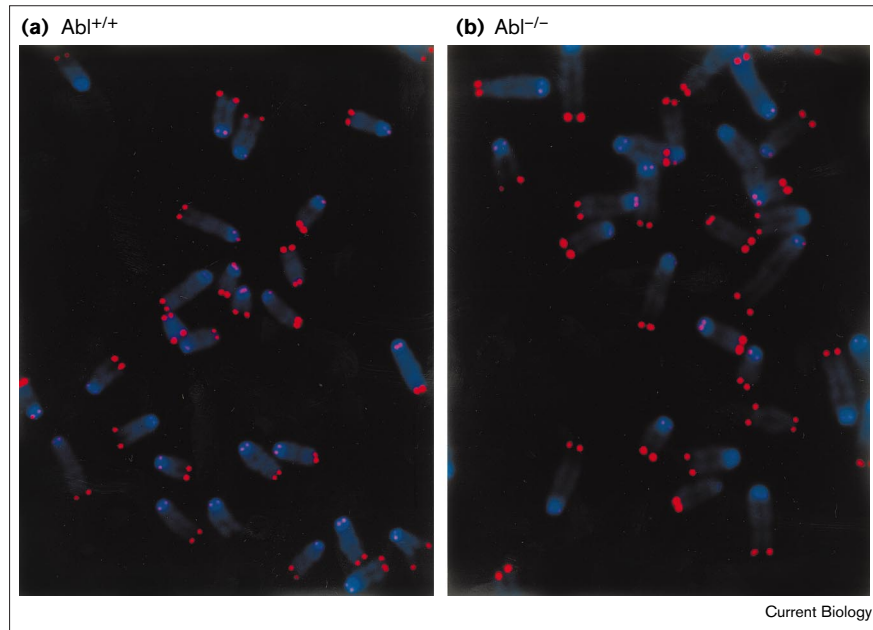
PNA fluorescence *in situ* hybridization to metaphase chromosomal spreads of **(a)** wild-type (Abl+/+) and **(b)** Abl−/− fibroblasts. Two hybridization probes were used, one specific for telomere and one for the centromeric DNA, with standard PNA hybridization conditions. Signal intensities for the red channel (telomere), green channel (centromere) and blue channel (4′,6-diamidino-2-phenylindole (DAPI) stain) were measured by custom software. Values were averaged to compute a ratio of telomere/centromere intensity per metaphase (Table 1). Ratios were used to compute a statistically significant value for each cell type.

c-Abl−/− mice have pronounced defects in spermatogenesis at the pachytene stage [63]. The c-Abl protein is localized at the ends of pachytene chromosomes and therefore may also interact with telomerase in meiotic cells [63]. The finding of telomere lengthening in c-Abl−/− cells and the functional interactions found between c-Abl and

Table 1

Comparisons of telomere length between Abl+/+ and Abl−/− cells.

	Abl+/+				Abl−/−			
Cell number	2n	T	C	T/C	2n	T	C	T/C
1	38	4,858	4,038	1.20	38	5,432	1,061	5.12
2	40	4,621	3,652	1.30	36	7,056	2,365	2.98
3	38	6,922	6,079	1.14	41	5,569	2,256	2.47
4	39	6,608	5,062	1.31	40	7,294	4,638	1.57
5	30	6,631	4,647	1.43	80	5,677	3,429	1.66
6	40	6,329	4,612	1.37	39	7,171	3,382	2.12
7	35	5,829	4,688	1.24	40	6,033	3,824	1.58
8	80	3,553	3,325	1.07	38	7,049	1,891	3.73
9	40	7,574	5,406	1.40	40	8,175	3,562	2.30
10	41	4,825	3,400	1.42	41	7,504	1,844	4.07
Mean		5775	4491	1.29		6696	2825	2.76
SD		1261	896	0.12		944	1105	1.20
CV (%)		21.8	19.9	9.6		14.1	39.1	43.5

T, telomere; C, centromere; 2n, diploid chromosome number; CV, coefficient of variation.

hTERT support a role for c-Abl in the regulation of telomerase function.

Materials and methods

Cell culture

MCF-7, MCF-7/c-Abl(K-R), 293T, c-Abl$^{-/-}$ and c-Abl$^{+/+}$ cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. U-937 cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cultures were irradiated at room temperature by a Gammacell 1000 (Atomic Energy of Canada) with ^{137}Cs source emitting at a fixed dose rate of 0.76 Gy/min as determined by dosimetry.

Transient transfections

293T cells (1×10^6/100 mm culture dish) were plated 24 h before transient transfection with HA–hTERT, wild-type c-Abl, and/or the dominant negative c-Abl(K-R) mutant [64] by calcium phosphate precipitation as described [65]. After incubation for 12 h at 37°C, the medium was replaced and the cells were incubated for another 24–36 h.

Immunoprecipitation and immunoblot analysis

Immunoprecipitations were performed as described [66]. In brief, cells were washed with PBS and lysed in 1 ml lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 1 mM DTT and 10 µg/ml leupeptin and aprotinin). Total cell lysates were subjected to immunoprecipitation with anti-c-Abl antibody (K-12, Santa Cruz Biotechnology), preimmune rabbit serum, anti-HA antibody (Boehringer-Mannheim), or anti-hTERT antibody [67]. The resultant protein precipitates were analyzed by immunoblotting with anti-hTERT, anti-c-Abl, anti-HA or anti-P-Tyr antibody (UG10, UBI).

Generation of hTERT deletion mutants

GST fusions of hTERT fragments 1 (amino acids 1–250), 2 (227–510), 3 (451–750) and 4 (701–1130) were generated by PCR and subcloning into pGEX-4T-1.

Fusion protein binding assays

GST, GST–c-Abl SH3 [49] and GST–Grb2 SH3 (Santa Cruz) fusion proteins were purified by affinity chromatography with glutathione–sepharose beads. Cell lysates were incubated with immobilized GST or GST-fusion proteins for 2 h at 4°C. The resulting protein complexes were separated by SDS–PAGE and subjected to immunoblot analysis with anti-HA antibody. Cell lysates were incubated with GST or GST–hTERT fragments 1–4. The resulting protein complexes were analyzed by immunoblotting with anti-c-Abl antibody.

Far-western analysis

293T cells were transiently transfected with HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-HA. The precipitated proteins were resolved by SDS–PAGE and transferred to nitrocellulose filters. The filters were then incubated with purified eluted GST–c-Abl SH3 or GST–Grb2 SH3 fusion proteins for 1 h at 30°C. The filters were analyzed by immunoblotting with anti-GST or anti-HA antibody.

Phosphorylation of hTERT by c-Abl

Recombinant c-Abl and c-Abl(K-R) were prepared from baculovirus-infected insect cells [68]. 293T cells were transiently transfected with HA–hTERT. Total cell lysates were subjected to immunoprecipitation with anti-HA antibody and the precipitates were incubated with buffer, kinase-active or kinase-inactive c-Abl in the presence of [γ^{32}-P]ATP. The reaction products were analyzed by SDS–PAGE and autoradiography.

Telomerase activity assay

Telomerase activity was measured with the Telomerase PCR ELISA kit (Boehringer-Mannheim) as described [69]. Cell lysates, prepared as described, were subjected to immunoprecipitation with anti-HA antibody. Samples were microfuged and protein concentrations of the supernatant measured (BioRad Protein Assay kit). Cell extracts were incubated at 25°C with biotin-labeled primers. The telomeric repeats added onto the ends of the synthetic primers were amplified by PCR. The denatured products were bound to a streptavidin-coated plate and then hybridized to a digoxygenin-labeled, telomeric repeat-specific probe. The biotin-labeled PCR product was detected with peroxidase-conjugated antibody to metabolize TMB (3,3,5,5-tetramethylbenzidine) and generate a colored reaction product. Sample absorbance at 450 nm was measured by an ELISA reader. Telomerase activity was determined in triplicate with negative and positive controls. A negative control was provided for each lysate by heat inactivation at 95°C for 10 min.

Microscopic evaluation of telomere length

Cells in logarithmic growth phase were cultured in standard media. Metaphase spreads were formed by standard conditions [70]. Hybridizations and analysis were performed as described [71,72]. Because mouse chromosomes are acrocentric, the p-arm associated telomeres colocalize with the centromere and, as such, are often associated with decreases in telomere fluorescence [72].

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