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Mouse embryonic fibroblasts null for the Krüppel-like factor 4 gene are genetically unstable

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Abstract

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor with tumor suppressive activity in colorectal cancer. Here, we investigated whether KLF4 is involved in maintaining genetic stability in mouse embryonic fibroblasts (MEFs) isolated from mice wild type (+/+), heterozygous (+/−), or homozygous (−/−) for the *Klf4* alleles. Compared to $Klf4^{+/+}$ and $Klf4^{+/-}$ MEFs, $Klf4^{-/-}$ MEFs had both a higher level of apoptosis and rate of proliferation. Quantification of chromosome numbers showed that $Klf4^{-/-}$ MEFs were aneuploid. A higher number of $Klf4^{-/-}$ MEFs exhibited γ-H2AX foci and had higher amounts of γ-H2AX compared to controls. Cytogenetic analysis demonstrated the presence of numerous chromosome aberrations including dicentric chromosomes, chromatid breaks, and double minute chromosomes in $Klf4^{-/-}$ cells but in few, if any, $Klf4^{+/+}$ or $Klf4^{+/-}$ MEFs. Approximately 25% of $Klf4^{-/-}$ MEFs exhibited centrosome amplification in contrast to the less than 5% of $Klf4^{+/+}$ or $Klf4^{+/-}$ MEFs. Finally, only $Klf4^{-/-}$ MEFs were capable of anchorage-independent growth. Taken together, these findings demonstrate that MEFs null for the *Klf4* alleles are genetically unstable, as evidenced by the presence of aneuploidy, chromosome aberration and centrosome amplification. The results support a crucial role for KLF4 in maintaining genetic stability and as a tumor suppressor.

Keywords

aneuploidy; centrosome amplification; cell cycle; chromosome aberrations; γ-H2AX; KLF4

Introduction

Krüppel-like factor 4 (KLF4) belongs to the Krüppellike factor family of zinc-finger-containing transcription factors that are involved in diverse biological and pathobiological conditions (Dang *et al*., 2000b; Bieker, 2001; Black *et al*., 2001; Kaczynski *et al*., 2003). Expression of KLF4 is enriched in epithelial tissues including the intestine and epidermis (Garrett-Sinha *et al*., 1996; Shields *et al*., 1996). In the intestinal epithelium, KLF4 is highly expressed in the postmitotic, differentiated epithelial cells (Shields *et al*., 1996; McConnell *et al*., 2007). *In vitro*, overexpression of KLF4 leads to growth arrest by activating key checkpoints in the cell cycle (Shields *et al*., 1996; Chen *et al*., 2001). Similarly, KLF4 has been shown to exert a checkpoint function following DNA damage (Zhang *et al*., 2000; Yoon *et*

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al., 2003; Yoon and Yang, 2004). Consistent with its role as a checkpoint protein, expression of KLF4 is often reduced in tumors such as colorectal cancer and gastric cancer (Dang *et al*., 2000a; Zhao *et al*., 2004; Wei *et al*., 2005; Kanai *et al*., 2006; Ghaleb and Yang, 2008). The reason for such reduction has been shown to be due to loss of heterozygosity of the *KLF4* locus or hypermethylation of the *KLF4* promoter in a subset of colorectal cancer, which leads to the conclusion that KLF4 is a tumor suppressor in colorectal cancer (Zhao *et al*., 2004).

The *in vivo* functions of KLF4 have been demonstrated by studies of mice with targeted deletion of the *Klf4* gene, which showed that KLF4 is important for the barrier function of the skin (Segre *et al*., 1999) and terminal differentiation of goblet cells in the colon of newborn mice (Katz *et al*., 2002). However, the effect of KLF4 on epithelial cell tumorigenesis could not be assessed in these models as mice homozygous for *Klf4* deletion die within 1 day after birth (Segre *et al*., 1999; Katz *et al*., 2002). Experiments involving conditional *Klf4* mutant mice did show that loss of *Klf4* from the gastric mucosa resulted in epithelial hyperplasia, confirming the antiproliferative activity of KLF4 *in vivo* (Katz *et al*., 2005). Recently, it was shown that the intestinal tumor burden was increased when mice heterozygous for the *Klf4* alleles were crossed with the $Apc^{Min/+}$ mice, indicating for the first time that KLF4 has a tumor suppressive effect in the intestine *in vivo* (Ghaleb *et al*., 2007b).

A hallmark of cancer is the presence of genetic instability, frequently manifested as aneuploidy (Rajagopalan and Lengauer, 2004; Ganem *et al*., 2007; Weaver and Cleveland, 2007). One of the contributing factors of genetic instability is abnormal amplification of centrosomes, which increases the frequency of mitotic defects (D'Assoro *et al*., 2002; Fukasawa, 2005, 2007). Centrosome amplification has been demonstrated in numerous human cancers (Lingle and Salisbury, 1999; Ghadimi *et al*., 2000; Lingle *et al*., 2002; Mayer *et al*., 2003; Salisbury *et al*., 2004; Chng *et al*., 2008). A key mechanism responsible for centrosome amplification is the loss of the tumor suppressor p53. Thus, mouse embryonic fibroblasts (MEFs) null for the *p53* alleles contain abnormal centrosome number and genetic instability as demonstrated by the presence of aneuploidy (Fukasawa *et al*., 1996, 1997; Tarapore *et al*., 2001; Tarapore and Fukasawa, 2002). As such, *p53*-null status confers to MEFs a growth advantage and a capacity for transformation (Harvey *et al*., 1993). These studies therefore underscore the critical role of p53 in the maintenance of centrosome duplication and genetic stability.

Because KLF4 has been shown to be a crucial mediator of p53 in the DNA damage response (Zhang *et al*., 2000; Yoon *et al*., 2003; Yoon and Yang, 2004; Ghaleb *et al*., 2005) and is both necessary and sufficient in preventing centrosome amplification following γ radiation-induced DNA damage (Yoon *et al*., 2005), we sought to determine the role of KLF4 in maintaining genetic stability using MEFs isolated from *Klf4*-null embryos. We show that $Klf4^{-/-}$ MEFs exhibit evidence of increased DNA damage, chromosome aberrations, centrosome amplification, aneuploidy, and capacity for transformation. These results implicate a critical role for KLF4 in the maintenance of genetic stability.

Results

Klf4-null MEFs are aneuploid

We first examined the growth characteristics in culture of MEFs isolated from days 13.5 $Klf4^{+/+}$, $Klf4^{+/-}$ and $Klf4^{-/-}$ mouse embryos. As seen in Figure 1a, the growth rates of MEFs of all three genotypes were nearly identical. However, $Klf4^{-/-}$ MEFs had a higher level of apoptosis than $Klf4^{+/+}$ and $Klf4^{+/-}$ cells as measured by the proportion of cells in the sub-G_1 population upon cell-cycle analysis (Figure 1b). Consistent with this finding, $Klf4^{-/-}$ MEFs had a higher amount of cleaved caspase-3 compared to $Klf4^{+/+}$ MEFs (Supplementary Figure S1), following treatment with tumor necrosis factor-α (TNF-α) at a concentration that induces apoptosis in MEFs (Takada *et al*., 2007). This result is also similar to the previous finding that

Klf4$^{-/-}$ MEFs are more prone to γ-irradiation-induced apoptosis than *Klf4*$^{+/+}$ MEFs (Ghaleb *et al*., 2007a).

In addition to having a higher level of apoptosis than *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ cells, *Klf4*$^{-/-}$ MEFs had a higher rate of DNA synthesis as measured by the level of incorporation of bromodeoxyuridine (BrdU; Figure 1c and Supplementary Figure S2). This would explain the similar growth rates of MEFs of the three genotypes despite the increased apoptosis in *Klf4*$^{-/-}$ MEFs. Western blot analysis of *Klf4*$^{-/-}$ MEFs showed an absence of Klf4 and p21, and a strong induction of p53 and cyclin E when compared to *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ cells (Figure 1d). These results are consistent with previous reports that KLF4 activates expression of p21 (Zhang *et al*., 2000; Chen *et al*., 2001) and represses that of p53 (Rowland *et al*., 2005) and cyclin E (Yoon *et al*., 2005).

Upon flow cytometric analysis of the cell-cycle profiles of the MEFs, we noticed that *Klf4*$^{-/-}$ MEFs had a slight shift to a higher DNA content than *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ MEFs (Supplementary Figure S3). This trend was more apparent in late-passage cells (P50) compared to early-passage cells (P20) (Supplementary Figure S3). We therefore measured the number of chromosomes in cells derived from the three genotypes. As seen in Figure 2, although *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ MEFs contained a similar distribution of chromosome numbers between the 35–44 and 75–84 ranges at both early and late passages, *Klf4*$^{-/-}$ MEFs consistently had higher numbers of chromosomes with many cells displaying greater than 85 chromosomes per cell regardless of passage numbers. Even in very early passage cells (P2), a greater proportion of *Klf4*$^{-/-}$ MEFs had more than 85 chromosomes per cell when compared to *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ MEFs (Supplementary Figure S4). These results demonstrate that *Klf4*$^{-/-}$ MEFs are aneuploid.

Klf4-null MEFs exhibit evidence of DNA damage and chromosome aberrations

Previous reports indicate that KLF4 is crucial for the cell-cycle checkpoint functions in response to DNA damage (Zhang *et al*., 2000; Yoon *et al*., 2003; Yoon and Yang, 2004; Ghaleb *et al*., 2005). To determine whether cells null for *Klf4* exhibit evidence of increasing DNA damage, we performed immunostaining ofMEFs for the presence of γ-H2AX foci, a marker for the DNA damage response (Rogakou *et al*., 1998). As can be seen in Figure 3a and Table 1, while 16±0.7and 21±1.4% of the *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ MEFs, respectively, were positive for the presence of γ-H2AX foci, 81±1.4% of the *Klf4*$^{-/-}$ cells were positive. The increase in γ-H2AX foci formation in *Klf4*$^{-/-}$ MEFs was confirmed by western blot analysis of γ-H2AX in the three different cell types (Figure 3b).

We then performed cytogenetic analysis of the MEFs. As seen in Figure 4, many of the *Klf4*$^{-/-}$ MEFs exhibited a myriad of chromosome aberrations including dicentric chromosomes, chromatid breaks and double minute chromosomes. In contrast, such aberrations were rare in either *Klf4*$^{+/+}$ or *Klf4*$^{+/-}$ cells (Table 2). These results indicate that deletion of *Klf4* in MEFs leads to genetic instability.

Loss of *Klf4* in MEFs results in centrosome amplification

A previous report showed that KLF4 is both necessary and sufficient in preventing centrosome amplification following γ-irradiation-induced DNA damage in the human colon cancer cell line HCT116 (Yoon *et al*., 2005). To determine whether *Klf4* plays a role in regulating centrosome duplication inMEFs, we performed immunostaining for γ-tubulin. As seen from the results in Figure 5, approximately 25% of the *Klf4*$^{-/-}$ MEFs contained three or more centrosomes per cell, indicating centrosome amplification. In contrast, relatively few *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ cells exhibited centrosome amplification. A similar centrosome amplification was

noted in very early passage (P2) *Klf4*$^{-/-}$ MEFs (Supplementary Figure S5). These results indicate that Klf4 is involved in the maintenance of centrosome stability in MEFs.

Klf4-null cells are capable of anchorage-independent growth

To address whether *Klf4*$^{-/-}$ cells acquired a transformed property, we examined anchorage-independent growth of MEFs in soft agar. As seen in Figure 6, *Klf4*$^{-/-}$ but not *Klf4*$^{+/+}$ or *Klf4*$^{+/-}$ MEFs were capable of forming colonies in soft agar. This result suggests that loss of *Klf4* renders a transformed phenotype to the MEFs.

Discussion

The control of normal growth process and maintenance of genetic stability requires a complex interacting network of regulatory factors. Genetic instability is commonly present in cancer because of mutation in the genes encoding these regulatory factors (Lengauer *et al*., 1998). Genetic instability can occur at the level of chromosomes (often manifested as chromosomal instability or CIN) or at the level of nucleotide (often manifested as microsatellite instability or MIN). Aneuploidy, defined as aberrant chromosome numbers, is thought to develop as a result of CIN. The observation that cancer cells harbor aneuploidy was made almost a century ago by Theodor Boveri. Although the exact cause of CIN has not been clearly established, many pathways and processes have been implicated such as chromosomal segregation, checkpoint control and centrosome duplication. Recent studies suggest that aneuploidy acts both to promote tumorigenesis and as a tumor suppressor (Weaver and Cleveland, 2006, 2007).

KLF4 is a member of the Krüppel-like factor family that exhibit important regulatory functions in diverse physiologic processes (Dang *et al*., 2000b; Bieker, 2001; Black *et al*., 2001; Kaczynski *et al*., 2003). Expression of KLF4 is often enriched in tissues that undergo rapid turnover such as the intestine and the epidermis (Garrett-Sinha *et al*., 1996; Shields *et al*., 1996). Studies suggest that one of the functions of KLF4 in the intestine is to maintain cells in a quiescent state (Shields *et al*., 1996; Ghaleb *et al*., 2005). This is supported by the observation that KLF4 exerts a cell-cycle checkpoint effect in part by acting as a transcriptional activator of the cyclin-dependent kinase inhibitor, p21 (Chen *et al*., 2001). As such, KLF4 safeguards the G_1/S and G_2/M checkpoints and mediates the checkpoint functions of p53 following DNA damage (Zhang *et al*., 2000; Yoon *et al*., 2003; Yoon and Yang, 2004).

The current study demonstrates that MEFs null for the *Klf4* gene exhibit genetic instability as evidenced by the presence of aneuploidy, increasing DNA damage, chromosomal aberrations, centrosome amplification and anchorage-independent growth (Figures 2–6). This does not appear to be a consequence of prolonged propagation in culture as *Klf4*$^{-/-}$ MEFs at a stage as early as passage 2 exhibit evidence of genetic instability manifested by a trend toward aneuploidy and centrosome amplification (Supplementary Figures S4 and S5). Many of these properties such as aneuploidy and centrosome amplification are similar to those observed in MEFs null for the *p53* alleles (Harvey *et al*., 1993; Fukasawa *et al*., 1996, 1997). Similarly, *p53*-null mice are susceptible to radiation-induced carcinogenesis and accumulate chromosome breakage (Lee *et al*., 1994). The findings of our study are therefore consistent with the fact that KLF4 is a downstream mediator of p53 function (Zhang *et al*., 2000). Moreover, unlike *p53*$^{-/-}$ MEFs, which exhibit an increased rate of proliferation (Harvey *et al*., 1993) when compared to control cells, *Klf4*$^{-/-}$ MEFs proliferate at a similar rate as *Klf4*$^{+/+}$ and *Klf4*$^{+/-}$ MEFs (Figure 1a). This is because of the combined effect of both an increased rate of apoptosis and proliferation in *Klf4*$^{-/-}$ MEFs when compared to controls (Figures 1b and c). The susceptibility to apoptosis of *Klf4*$^{-/-}$ cells is likely because of the absence of p21 (Figure 1d), which has been shown to be an inhibitor of both p53-dependent and -independent apoptosis (Gartel and Tyner, 2002). Consistent with these findings, *Klf4*$^{-/-}$

MEFs are more susceptible to apoptosis following treatment with TNF-α (Supplementary Figure S1) or γ-irradiation (Ghaleb *et al*., 2007a) than $Klf4^{+/+}$ MEFs. These results are also consistent with previous studies that KLF4 exhibit antiapoptotic activity in a context-dependent manner (Rowland *et al*., 2005; Rowland and Peeper, 2006; Ghaleb *et al*., 2007a).

It is of interest to note that the level of p53 is elevated in $Klf4^{-/-}$ MEFs in comparison to $Klf4^{+/+}$ and $Klf4^{+/-}$ cells (Figure 1d). This result is consistent with the previous report that KLF4 acts as a transcriptional repressor of p53 (Rowland *et al*., 2005). However, despite the relatively high level of p53, $Klf4^{-/-}$ MEFs exhibit genetic instability in a manner similar to $p53^{-/-}$ MEFs. These results are suggestive that KLF4 is downstream from p53 in the ability of p53 to maintain genetic stability.

The centrosome is the major microtubule-organizing center of animal cells and plays a fundamental role in cell division and cell polarity (Kirschner and Mitchison, 1986a, b). Centrosome amplification is often observed in cancers and is thought to contribute to cancer development (Fukasawa, 2005, 2007). This is illustrated by the finding that *p53*-null cells exhibit centrosome amplification (Fukasawa *et al*., 1996). We previously showed that KLF4 is necessary and sufficient in preventing centrosome amplification following γ-irradiation-induced DNA damage (Yoon *et al*., 2005). Here, we show that a significant fraction of $Klf4^{-/-}$ MEFs exhibit evidence of spontaneous centrosome amplification, at both passage 20 (P20) (Figure 5) and passage 2 (Supplementary Figure S5). We attribute this observation to the elevated level of cyclin E in $Klf4^{-/-}$ MEFs (Figure 1d). Cyclin E is a critical factor that controls the duplication of centrosome and its overexpression has been shown to result in centrosome amplification (Tokuyama *et al*., 2001; Hinchcliffe and Sluder, 2002; Tarapore *et al*., 2002; Kawamura *et al*., 2004; Hanashiro *et al*., 2008). The increased cyclin E level in $Klf4^{-/-}$ MEFs is consistent with our previous report that KLF4 suppresses cyclin E (Yoon *et al*., 2005). We presume that the resultant centrosome amplification in $Klf4^{-/-}$ MEFs is a contributing factor to the genetic instability in cells lacking *Klf4*. However, the presence of increased DNA damage and chromosomal aberration in $Klf4^{-/-}$ MEFs would suggest that KLF4 may be involved in the regulation of DNA repair. Alternatively, overexpression of cyclin E has been shown to lead to the formation of double-stranded DNA breaks because of replication fork collapse (Bartkova *et al*., 2006). Lastly, the mechanism by which *Klf4* deletion results in aneuploidy is an open question although, again, deregulated cyclin E has been shown to induce chromosome instability (Spruck *et al*., 1999). It is also of interest to note that among the target genes suppressed by KLF4, some function to control the spindle assembly checkpoints (Chen *et al*., 2003). This coupled with the recent finding that overexpression of certain spindle assembly checkpoint genes promotes aneuploidy and tumorigenesis (Sotillo *et al*., 2007) would suggest that KLF4 may be involved in controlling genetic stability by regulating the spindle assembly checkpoint.

In summary, we provide direct evidence that the absence of KLF4 results in genetic instability and subsequent transformation. This supports a tumor suppressive role for KLF4 in certain tumors as previously observed. Further investigation of the mechanism by which KLF4 controls genetic stability will provide new information on how KLF4 functions as a tumor suppressor.

Materials and methods

Isolation of MEFs and cell culture

Mice heterozygous for the *Klf4* alleles ($Klf4^{+/-}$) on a C57BL/6 background (Katz *et al*., 2002) were crossbred. MEFs that are wild type ($Klf4^{+/+}$), heterozygous ($Klf4^{+/-}$), or null ($Klf4^{-/-}$) for *Klf4* were derived from day 13.5 embryos using the 3T3 protocol as previously described (Todaro and Green, 1963). Briefly, 10^6 MEFs were plated on 10-cm dishes and

maintained in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin at 37 °C in atmosphere containing 5% CO_2. Cells were passed every 3 days at a density of 10^6 cells per 10-cm dish. Unless otherwise specified, experiments were performed on cells at P20.

Cell proliferation and soft-agar assays

For cell proliferation assay, cells were seeded onto six-well plates at a density of 10^5 cells per well. On a daily basis, cells were trypsinized and counted using a Bright-Line Hemacytometer (Sigma, St Louis, MO, USA). For anchorage-independence assay, MEFs were seeded at a density of 5×10^4 cells per plate in triplicate in 5-cm soft-agar dishes (0.5 and 0.3% bottom and top agar, respectively). The cells were fed fresh media (DMEM with 10% FBS) every 3 days until foci were counted 21 days later.

Bromodoxyuridine uptake studies

Cells were seeded onto coverslips overnight until 70–80% confluence. Cells were pulsed with BrdU for 30 min at a final concentration of 100 μM. Following incubation with BrdU, cells were fixed in cold methanol for 20 min at −20 °C and then rehydrated in phosphate-buffered saline (PBS). Briefly, 800 μl of 2M HCl was added to each wall and incubated at room temperature for 30 min. Cells were washed twice for 5 min in 1ml of 0.1M sodium borate (pH 8.5) and washed for 5 min in PBS before blocking in 2% bovine serum albumin (BSA)/PBS for 1.5 h at room temperature. Anti-BrdU was diluted at 1:50 in 2% BSA/PBS and added to each coverslip and incubated at 4 °C overnight. Coverslips were washed three times in PBS for 5 min and antibody–antigen complexes were detected with Alexa Fluor 488-conjugated goat antimouse antibody diluted 1:500 in 2% BSA/PBS and incubated at room temperature for 1 h. Cells were then washed four times with PBS and counterstained with 4′,6-diamidino-2-phenylindol (DAPI) for 5 min at room temperature in the dark. Finally, cells were washed five times with PBS and mounted in Prolong Antifade kit (Invitrogen), and visualized with a Zeiss 510 confocal microscope. Each experiment was performed in triplicate, and 200 cells were counted per replicate.

TNF-*α* treatment

MEFs (1×10^5) were plated onto six-well plates 1 day before addition of TNF-α. Cells were then treated or not with 40 ng/ml TNF-α (Sigma) for 18 h. TNF-α-induced cell death was measured by western blot using cleaved caspase-3.

Flow cytometry

Cell-cycle analysis was performed as previously described (Yoon *et al*., 2003). Cells were rinsed in PBS, trypsinized and resuspended in DMEM containing 10% FBS. Pelleted cells were fixed in 70% with ethanol in PBS and incubated at −20 °C overnight. The fixed cells were pelleted and resuspended in PBS that contain 50 μg/ml propidium iodide, 50 μg/ml RNase A, 0.1% Triton X-100 and 0.1mM ethylene diaminetetraacetic acid at room temperature for 30 min before analysis. Cell-cycle profile analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cytogenetic analysis

Cytogenetic analysis by metaphase spreading of MEFs was performed following standard protocols (Lee *et al*., 1990) with slight modifications. In brief, cells were initially plated in DMEM containing 10% FBS until they reached 60–70% confluency. Cells then were incubated in the presence of 0.1 μg/ml colcemid (Invitrogen, Carlsbad, CA, USA) for 4 h to induce metaphase arrest, centrifuged and resuspended in 75mM potassium chloride for 10 min. Cells were then fixed with freshly prepared methanol:acetic acid (3:1, v/v) solution drop wise

whereas the tubes were vortexed at low speed. The cells were collected by low-speed centrifugation (800 r.p.m.) for 5 min. The cell suspension was then spread onto glass slides then air-dried. Slides were aged at 60 °C overnight before the addition of DAPI. Metaphase spreads images were acquired using an Axioskop 2 plus microscope (Zeiss, Thornwood, NY, USA) equipped with an AxioCam MRc5 CCD camera (Zeiss). The numbers of chromosomes in metaphase (n = 100 cells) from each genotype were counted and analysed.

Centrosome and γ-H2AX immunostaining

Mouse embryonic fibroblasts grown on coverslips were washed with PBS. They were then fixed with cold 100% methanol at −20 °C for 20 min. Cells were then washed three times in PBS before blocking in PBS/0.3% BSA for 1 h at room temperature. FITC-conjugated γ-tubulin antibody was added to final concentration of 10 μg/ml in blocking solution and incubated for 1 h. Cells were then washed three times with PBS and counterstained with DAPI for 5 min at room temperature in the dark. Finally, cells were washed five times with PBS and mounted in Prolong Antifade kit (Molecular probe), and visualized with a Zeiss 510 confocal microscope. Immunostaining for γ-H2AX was carried out as previously described (Dalton *et al*., 2007).

Western blot analysis

Protein extraction and western blot analysis were as previously described (Yoon *et al*., 2003). The membranes were immunoblotted with primary antibodies against KLF4, p53, p21, cyclin E and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), γ-H2AX (Upstate Biotechnology, Billerica, MA, USA) and cleaved caspase-3 (Cell Signaling, Danvers, MA, USA). The blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The antibody–antigen complex was visualized by ECL chemiluminescence (Amersham, Pittsburgh, PA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BrdU
: bromodeoxyuridine

BSA
: bovine serum albumin

CIN
: chromosomal instability

DMEM
: Dulbecco’s modified Eagle’s medium

FBS
: fetal bovine serum

KLF4

Krüppel-like factor 4

MEFs

mouse embryo fibroblasts

PBS

phosphatebuffered saline

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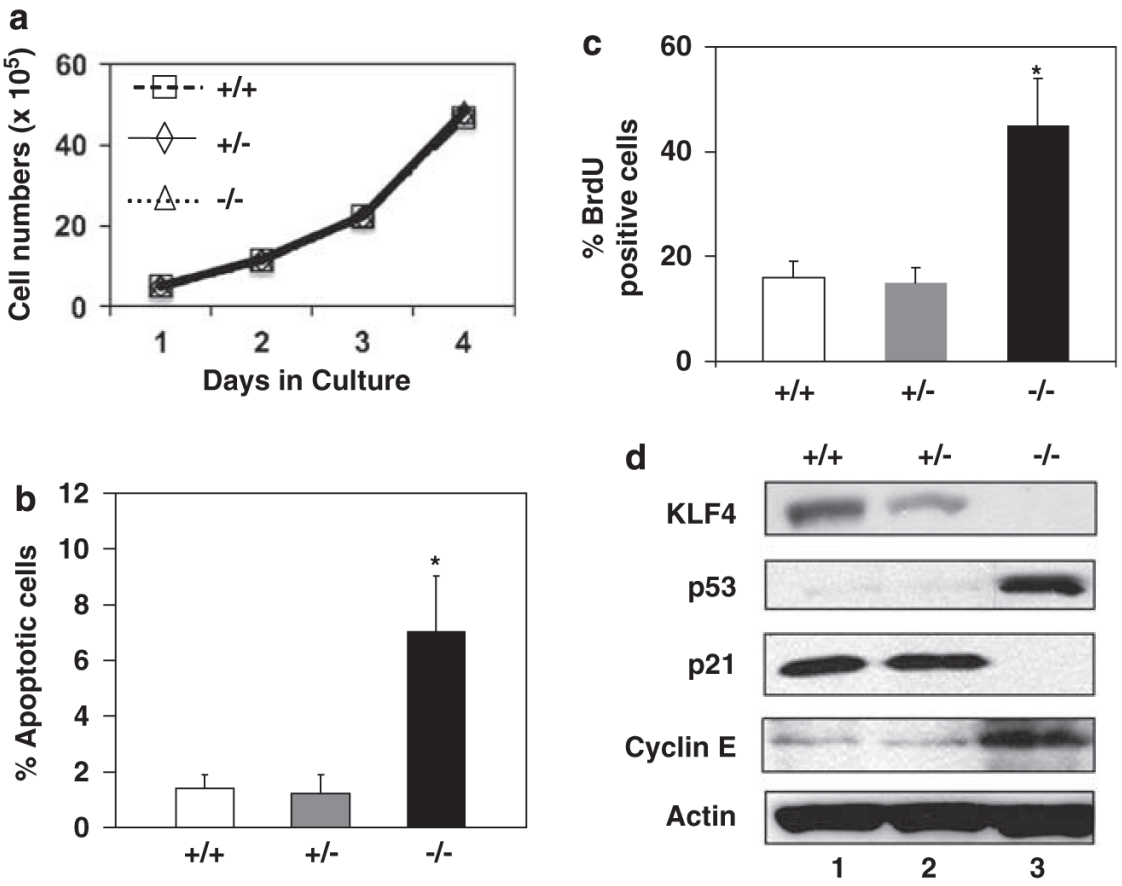

Figure 1.

Growth characteristics of *Klf4*$^{+/+}$, *Klf4*$^{+/-}$ and *Klf4*$^{-/-}$ mouse embryonic fibroblasts (MEFs) in culture. (**a**) Cells were plated at 10^5 cells per 60-mm plate. Three plates were counted at each time point and the values represent the mean number of cells per dish. $N = 3$. (**b**) The percentages of apoptotic cells 1 day after seeding were measured from the sub-G_1 population of cells during flow cytometry. $N = 3$; *$P<0.05$ compared to *Klf4*$^{+/+}$ MEFs. (**c**) DNA synthesis was measured by the incorporation of bromodeoxyuridine (BrdU) into replicating cells. Shown are the percentages of cells that stained positive for BrdU. $N = 3$; *$P<0.05$ compared to *Klf4*$^{+/+}$ MEFs. (**d**) Western blot analysis of KLF4, p53, p21, cyclin E and β-actin of proteins isolated from cells at 1 day after seeding. Shown are the representative results of four separate experiments.

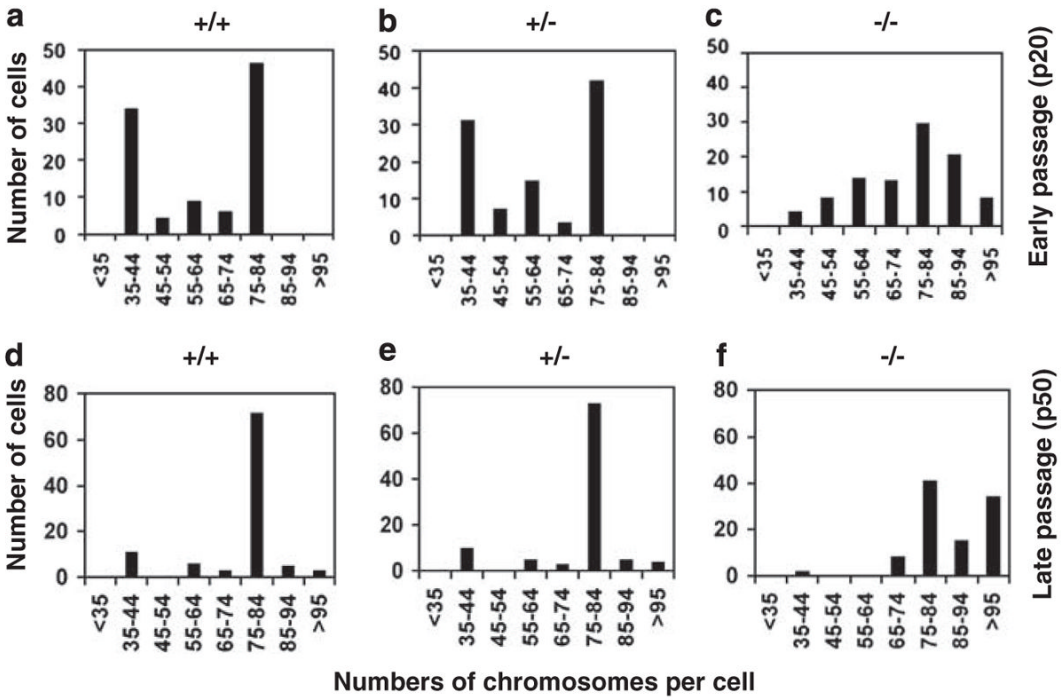

Figure 2.

Determination of chromosome numbers in mouse embryonic fibroblasts (MEFs). Karyotyping was performed 1 day after seeding in early passages (P20; **a**–**c**) and late passages (P50; **d**–**f**) MEFs. Spreads from 100 cells were examined per genotype.

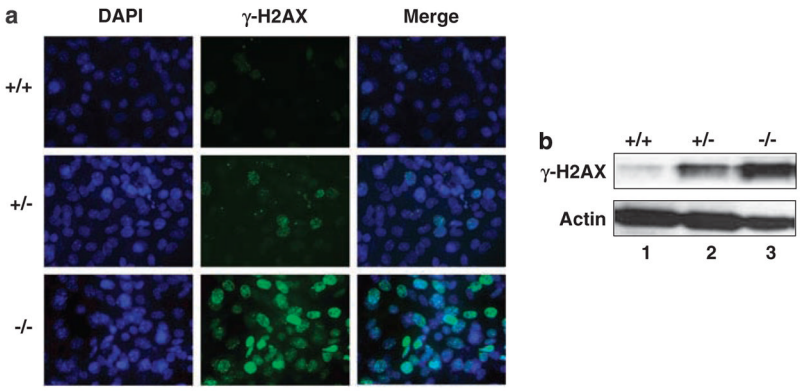

Figure 3.

Immunostaining and western blotting for γ-H2AX in mouse embryonic fibroblasts (MEFs). (**a**) Immunostaining was carried out on γ-H2AX in MEFs of the three different genotypes. 4′, 6-Diamidino-2-phenylindol (DAPI) stain (blue) was used to visualize nuclei. Shown is a representative result of three independent experiments. (**b**) Western blot analysis was carried out using an antibody against γ-H2AX or β-actin. Shown is a representative result of three experiments.

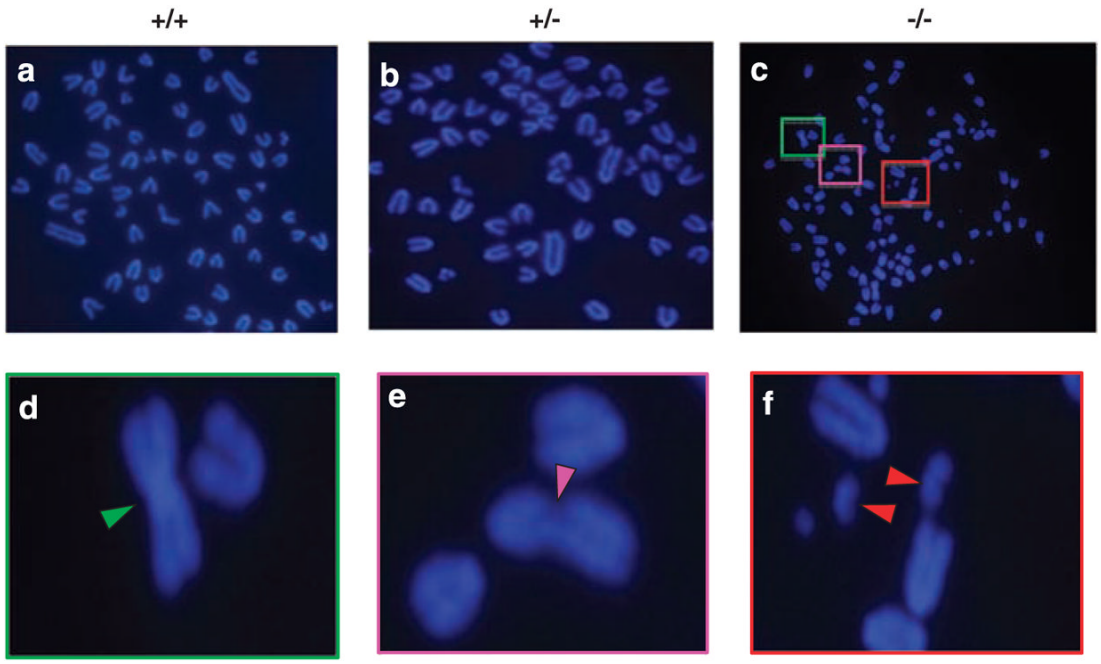

Figure 4.

Cytogenetic analysis of mouse embryonic fibroblasts (MEFs). Cytogenetic analysis was carried out in metaphase chromosome spreads prepared from MEFs of the three genotypes. Shown is a typical result of three independent experiments for *Klf4*$^{+/+}$ (**a**), Klf4$^{+/-}$ (**b**) and Klf4$^{-/-}$ (**c**) MEFs. The colored boxes illustrate a magnified view of three different chromosome aberrations: (**d**) dicentric chromosome, (**e**) chromatid breaks and (**f**) double minute chromosomes. Arrowheads point to the aberrant chromosomes.

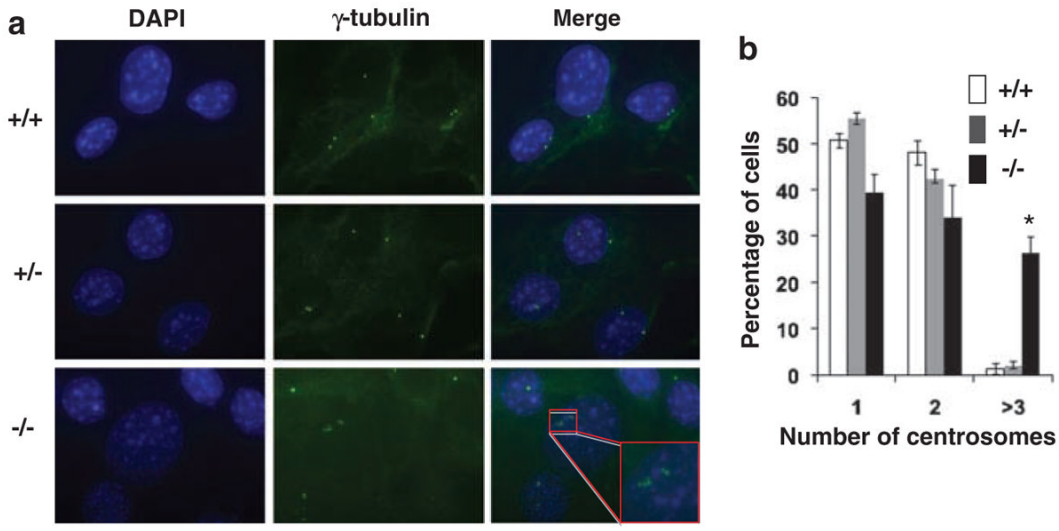

Figure 5.

Centrosome staining of mouse embryonic fibroblasts (MEFs). (**a**) Centrosome staining was carried out with an antibody against γ-tubulin (Yoon *et al*., 2005). 4′,6-Diamidino-2-phenylindol (DAPI) stain (blue) was used to visualize the nuclei. Shown is a typical result of four independent experiments. The insert shows a cell with abnormal number (≥3). (**b**) Histogram showing quantification of centrosome numbers in MEFs with the three genotypes. In total, 100 cells were counted per cell type per experiments. $N = 5$; $^{*}P<0.005$ compared to $Klf4^{+/+}$ cells.

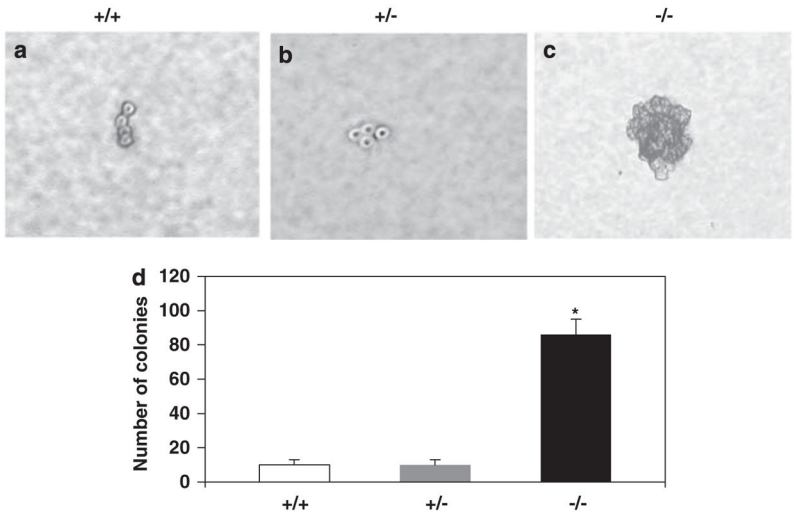

Figure 6.

Anchorage-independent growth assays of mouse embryonic fibroblasts (MEFs). MEFs with the three genotypes were seeded in soft agar and formation of colonies determined 3 weeks later. A typical photomicrograph is shown for $Klf4^{+/+}$ (**a**), $Klf4^{+/-}$ (**b**) and $Klf4^{-/-}$ (**c**) MEFs. Note that $Klf4^{+/+}$ and $Klf4^{+/-}$ cells typically grew to a four-cell stage as shown in the figure but failed to form any colonies. (**d**) Quantification of the number of colonies formed in soft agar. Shown are the average numbers of colonies per 10-cm plate. $N = 3$; $*P<0.01$ compared to $Klf4^{+/+}$ MEFs.

Table 1

Quantification of γ-H2AX stain in MEFs[a]

MEF genotype	Total no. of cells counted	No. of cells positive for γ-H2AX	% of cells positive for γ-H2AX
Kl4$^{+/+}$	210	34	16±0.7
Klf4$^{+/-}$	275	58	21±1.4
Klf4$^{-/-}$	264	214	81±1.4*

Abbreviation: MEF, mouse embryonic fibroblast.

[a] $N = 3$ for each genotype.

* $P<0.05$ compared to *Klf4*$^{+/+}$ or *Klf4*$^{+/-}$ MEFs.

Table 2

Quantification of chromosome aberrations in MEFs[a]

MEF genotype	% of cells with dicentric chromosomes	% of cells with chromatid breaks	% of cells with double minute chromosomes
Kl4$^{+/+}$	2±0.7	1±0.7	3±0.7
Klf4$^{+/-}$	4±0.7	2±0.7	3±1.4
Klf4$^{-/-}$	34±1.4*	10±0.7*	40±1.4*

Abbreviation: MEF, mouse embryonic fibroblast.

[a] One-hundred cells of each cell type were examined for the presence of chromosome aberrations. N=3 for each genotype.

* p<0.05 compared to *Klf4*$^{+/+}$ or *Klf4*$^{+/-}$ MEFs.