MOLECULAR AND CELLULAR BIOLOGY, May 2005, p. 3535–3542 Vol. 25, No. 9
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.9.3535–3542.2005

Inactivation of CtIP Leads to Early Embryonic Lethality Mediated by G_1 Restraint and to Tumorigenesis by Haploid Insufficiency

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Received 16 November 2004/Returned for modification 25 December 2004/Accepted 3 February 2005

CtIP interacts with a group of tumor suppressor proteins including RB (retinoblastoma protein), BRCA1, Ikaros, and CtBP, which regulate cell cycle progression through transcriptional repression as well as chromatin remodeling. However, how CtIP exerts its biological function in cell cycle progression remains elusive. To address this issue, we generated an inactivated *Ctip* allele in mice by inserting a *neo* gene into exon 5. The corresponding $Ctip^{-/-}$ embryos died at embryonic day 4.0 (E4.0), and the blastocysts failed to enter S phase but accumulated in G_1, leading to a slightly elevated cell death. Mouse NIH 3T3 cells depleted of Ctip were arrested at G_1 with the concomitant increase in hypophosphorylated Rb and Cdk inhibitors, p21. However, depletion of Ctip failed to arrest $Rb^{-/-}$ mouse embryonic fibroblasts (MEF) or human osteosarcoma Saos-2 cells at G_1, suggesting that this arrest is RB dependent. Importantly, the life span of $Ctip^{+/-}$ heterozygotes was shortened by the development of multiple types of tumors, predominantly, large lymphomas. The wild-type *Ctip* allele and protein remained detectable in these tumors, suggesting that haploid insufficiency of *Ctip* leads to tumorigenesis. Taken together, this finding uncovers a novel G_1/S regulation in that CtIP counteracts Rb-mediated G_1 restraint. Deregulation of this function leads to a defect in early embryogenesis and contributes, in part, to tumor formation.

CtIP is a yet poorly understood protein that was initially discovered as a cofactor of transcriptional corepressor CtBP by yeast two-hybrid screening (34). Subsequent efforts have demonstrated that CtIP serves as an interacting partner for two retinoblastoma tumor suppressor family proteins, Rb and p130 (8, 27), and breast tumor suppressor BRCA1 (22, 38, 42), as well as members of the Ikaros family encoding Kruppel-like zinc finger proteins that regulate early hemolymphopoiesis (19).

CtIP is an 897-amino-acid protein that is ubiquitously expressed in different types of cells (8, 42). Apart from specific motifs used to bind Rb (LECEE) and CtBP (PLDLS), two coiled-coil domains of CtIP are also recognized. The one at the N terminus is used for homodimerization of CtIP (7). Interestingly, judging by sequence similarity, the *CtIP* gene so far has only been found in higher eukaryotic organisms, including *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*, with significant sequence divergence, implying a late participation during evolution.

The interaction between CtIP and CtBP is believed to play an important role in the CtIP-mediated and CtBP-dependent transcriptional repression. CtBP, identified as a C-terminal binding protein of human adenovirus E1A protein (2, 33), could function as a transcriptional corepressor in a histone deacetylase (HDAC)-dependent or -independent manner and may play critical roles during the early development and oncogenesis (3). Like CtBP, CtIP also possesses transcriptional repression activity when artificially fused to GAL4 DNA binding domain and coexpressed with reporter gene driven by a simian virus 40 promoter with upstream GAL4 binding sites (27). On the other hand, Rb family proteins bind CtIP at the LECEE motif, and mutation of this site disrupts their interaction. Using the same transcription reporter assay, mutations of the binding domain of Rb prevent the association of CtIP and fail to repress transcription (27). However, it remains unclear whether binding of CtIP to Rb has a role other than transcriptional repression.

In addition to Rb family proteins, BRCA1 can also physically and functionally interact with CtIP/CtBP complex. The N-terminal 133 to 369 amino acids of CtIP mediate its interaction with the C-terminal repeats (BRCT) of BRCA1 (42, 45). Significantly, tumor-associated mutations in BRCT domains abolish this specific interaction (22, 42, 46). DNA damage agents such as ionizing irradiation induce the hyperphosphorylation of CtIP at serine 664 and 745 by ATM kinase (23). And this event is suggested to modulate BRCA1 for the transcriptional activation of p21 and GADD45 upon DNA damage (22, 23), although the detailed mechanism remains to be clarified.

Ikaros family proteins are transcriptional repressors, which can interact with the HDAC-recruiting factors, Sin3 and Mi-2b, and exhibit HDAC-dependent repression activity (20). In addition, Ikaros can also directly bind to CtBP, Rb, and CtIP, which can interact with the basal transcription factor TFIIB (19). That may explain how Ikaros repression works through an HDAC-independent pathway.

Those observations deduced from studies of other proteins

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suggest that CtIP serves as a corepressor for transcriptional repression. However, the actual biological function of CtIP remains unclear. Especially, systematic study of CtIP loss of function has not been reported yet. In this communication, we show that the *Ctip*$^{-/-}$ embryos die at embryonic day 4.0 (E4.0) as blastocysts fail to enter S phase and accumulate in G_1, leading to an elevated cell death. *Rb*$^{+/+}$ cells, but not *Rb*$^{-/-}$, depleted of Ctip are arrested at G_1, suggesting that this arrest is RB dependent. Importantly, the life spans of *Ctip*$^{+/-}$ heterozygotes are shortened by the development of multiple types of tumors, predominantly, large lymphomas. This finding uncovers that CtIP is a key protein in early embryogenesis, and the loss of one allele contributes, in part, to tumor formation.

MATERIALS AND METHODS

Abbreviations. Histone deacetylase; FACS, fluorescence-activated cell sorting; MEF, mouse embryonic fibroblast; E, embryonic day; BudR, bromodeoxyuridine; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling; GFP, green fluorescent protein; MOI, multiplicity of infection; DAPI, 4′,6-diamidino-2-phenylindole.

Cell lines and tissue culture. NIH 3T3, *Rb*$^{+/+}$, and *Rb*$^{-/-}$ mouse embryonic fibroblasts (MEF), and Saos-2 and SR5 human osteosarcoma cells were cultured in high-glucose Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS) at 37°C in an incubator with 10% CO_2.

Construction of targeting vectors and establishing the knockout mouse. The *Ctip* gene was isolated by screening a λDASH mouse genomic library derived from the 129/Sv mouse strain (kindly provided by Tom Doetschman, University of Cincinnati), using a 2.7-kb cDNA fragment of human *CtIP* as probe. A 9.8-kb BamHI fragment of the mouse *Ctip* gene containing exon 5 was subcloned into the pBluescript SK vector. A SalI site was generated in the middle of exon 5 by site-directed mutagenesis for inserting 1.6 kb of *pgkneopA* cassette flanked by XhoI sites in antisense orientation. This construct was used to generate ES clones for producing chimeric mice according to procedures described previously (21).

Embryo collection and PCR genotyping. *Ctip* heterozygotes were used for timed pregnancy experiments. For embryos older than E8.5, the visceral yolk sac was collected and subjected to PCR genotyping. For embryos between E4.5 and E8.5, embryos inside the uterus were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Embryonic tissues stained with Mayer's hematoxylin and eosin (H&E) were microdissected from slides for genotyping. The E3.5 blastocysts were flushed from maternal uterus for analysis. PCR genotyping was performed using the primer A (5′-ACA GGT TAA GAG CAG GAT TGT GTG A-3′) and B (5′-ATA TGA AAG GGA ACT CAC TCA GCT C-3′), which flank the exon 5 and generate a 144-bp fragment from the wild-type *Ctip*, while primer C (5′-TGC ACG AGA CTA GTG AGA CGT GCT A-3′), which resides at PGK promoter, and primer A together generate a 388-bp fragment from the targeted *Ctip* allele.

In vitro blastocyst culture, BrdU incorporation, immunostaining, and TUNEL assay. Blastocysts were isolated from pregnant females at E3.5, and an outgrowth assay was performed as previously described (24).

For BrdU incorporation, blastocysts were cultured for 1 day and then labeled with BrdU in medium for 6 h. Blastocysts were then fixed and incubated with anti-BrdU antibody (Amersham Biosciences) or rabbit polyclonal anti-CtIP antibody (C21) at 4°C overnight. After three washes in PBS, blastocysts were incubated with fluorescence-conjugated secondary antibody at room temperature for 30 min, followed by DAPI staining. Blastocysts were mounted on slides and examined under fluorescence microscopy (Zeiss Axioplan 2 imaging). Slides were demounted later, and blastocysts were collected and lysed for PCR genotyping.

For phospho-histone H3 immunostaining, blastocysts were collected, fixed, and then incubated with anti-phospho-histone H3 (ser10) antibody (6G3; Cell Signaling Technology). A TUNEL assay (24) was performed on freshly collected and fixed blastocysts, using an in situ cell death detection kit (Roche Applied Science) according to the manufacturer's instructions.

***Ctip* siRNA construction.** A *Ctip* siRNA expression cassette was constructed by inserting a DNA fragment derived from two annealed synthetic oligonucleotides (5′-GGC GTC ATC TCC GGT ATT TGC TCA AGA GAC AAA TAC CGG AGA TGA CGC CCT TTT TA-3′ and 5′-AGC TTA AAA AGG GCG TCA TCT CCG GTA TTT GTC TCT TGA GCA AAT ACC GGA GAT GAC GCC-3′) into pBS-U6 vector (37). The BamHI fragment containing the U6-siRNA construct was released and subcloned into pAdTrack vector, which carries a GFP-expressing cassette, resulting in pAd-Ctip-siRNA. The adenovirus was then produced following the protocol as described previously (13). Human *CtIP* siRNA construct was generated under the regulation of U6 promoter by inserting an oligonucleotide sequence from 2275 to 2295 (GGG AGC AGA CCT TTC TCA GTA) of human *CtIP* into U6 RNAi vector to form pBS/U6-CtIPi. The RNAi expression cassette was then inserted into a pATG vector (28), which directs *GFP* expression, and that was named pCtIP-RNAi.

Immunoblotting and FACS analysis. Antibodies against p21 (F5 from Santa Cruz Biotechnology), Rb (Rb245), and CtIP (19E8) were used for Western blotting. Fluorescence-activated cell sorting (FACS) analysis for NIH 3T3 cells infected with *CtIP*-siRNA or *GFP* adenovirus was carried out using FACScalibur as instructed by the manufacturer (BD Biosciences).

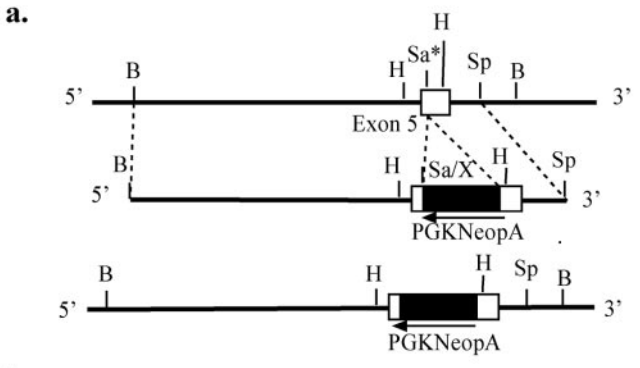
Histology and immunohistochemistry. Collected tissues were fixed in 4% paraformaldehyde and processed through paraffin embedding following standard procedures. Sections were stained with H&E for histopathological evaluation. Immunostaining was performed following the protocol described in a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). For antigen retrieval, slides were heated for 20 min in 10 mM citrate buffer (pH 6.0) in a microwave oven. The antibodies used were CD45R/B220 (BD Biosciences, San Diego, CA), Mac2 (Cadarlane, Ontario, Canada), and CD3 (DAKO, Carpinteria, CA).

RESULTS

Absence of wild-type *Ctip* alleles leads to early embryonic lethality. The biological function of CtIP is yet poorly understood except that it can interact with several tumor suppressor proteins. To address its roles in mouse development, we generated an inactivated *Ctip* allele in embryonic stem (ES) cells by inserting a *pgkneopA* cassette at the position equivalent to amino acid residue 109 in the human CtIP protein (Fig. 1a). Recombinant ES cell clones were identified by Southern blotting (Fig. 1b), and the correct clone was used to generate the chimeric mice and, subsequently, heterozygous mice.

While examining the in vivo effect of homozygous mutations, we found no *Ctip*$^{-/-}$ mice in 94 offspring produced by the intercross of *Ctip*$^{+/-}$ heterozygotes, indicating that they died in uterus. To define the time of death, *Ctip*$^{+/-}$ females conceived by intercrosses were sacrificed, and the fetuses at different gestation times from E4.5 to E12.5 were examined. At all the time points, there were no normal conceptuses with the *Ctip*$^{-/-}$ genotype, but, instead, many resorbed embryos were observed (Fig. 1c). To precisely pinpoint the differences between the wild-type and *Ctip*-null embryos, intact decidual swellings from *Ctip*$^{+/-}$ intercrosses obtained between E4.5 and E5.5 were examined. Both the wild-type and heterozygous embryos showed normal growth and elongation of the egg cylinder, while *Ctip*$^{-/-}$ embryos failed to form egg cylinders and degenerated in the uterus (Fig. 1d), suggesting that Ctip is essential for very early embryonic development.

Inner stem cells of *Ctip* null blastocysts arrest at G_1. To explore the reason why *Ctip*$^{-/-}$ embryos die so early, we employed blastocyst outgrowth in culture as an alternative method to study early developmental event. While 41 cultured *Ctip*$^{+/+}$ and *Ctip*$^{+/-}$ blastocysts gave rise to adherent sheets of trophoblastic giant cells and outgrowth of the inner cell mass, 14 *Ctip*$^{-/-}$ blastocysts showed no outgrowth but retained intact zona pellucida (an example is shown in Fig. 2a), suggesting that *Ctip*$^{-/-}$ blastocysts failed to hatch from the eggshell. We then tested whether the inner cell mass underwent apoptosis by a TUNEL assay (24). On average, the total number of cells per *Ctip*$^{-/-}$ blastocyst was slightly lower than that of the wild type



c.

Age	Litters	Total Number	Genotype +/+	Genotype +/-	Genotype -/-	Resorbed
4-week	16	94	35	59	0	NA
E9.5-12.5	7	54	12	25	0	17
E8.5	4	32	8	14	0	10
E7.5	4	33	8	17	0	8
E6.5	5	40	9	21	0	10
E5.5	5	38	10	19	0	9
E4.5	1	8	2	5	0	1
E3.5	6	43	9	25	9	0

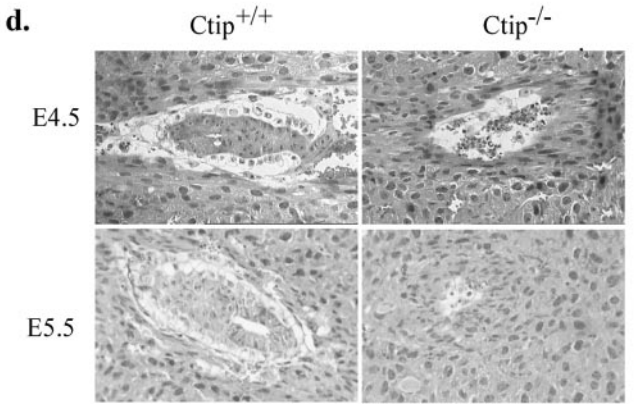


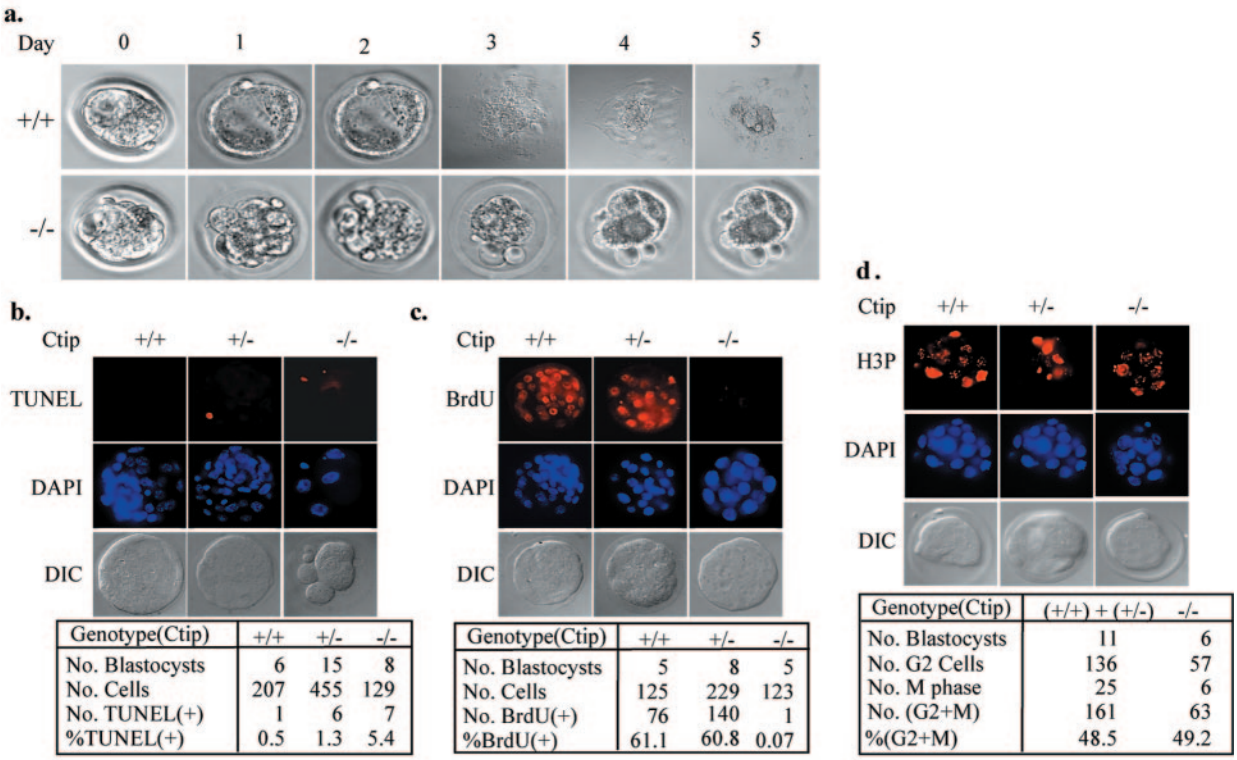
FIG. 1. Targeted disruption of Ctip mouse locus leads to embryonic lethality. a, Knockout strategy. A SalI site in exon 5 was introduced by site-directed mutagenesis, and a *pgkneopA* cassette was inserted into this site. Only partial genomic DNA is shown. b, Identification of the recombinant ES clones by digesting the genomic DNA with BamHI and probing with the 0.5-kb SpeI-BamHI fragment. Lane 2 indicated a correct recombinant ES cell clone. c, Genotype analysis of the progenies from *Ctip*$^{+/-}$ heterozygous intercrosses. d, Histological sections of wild-type and *Ctip*$^{-/-}$ mutant embryos grown in utero. The embryos inside the uterus were fixed, sectioned, and stained with H&E. Left panels, wild-type mouse embryos at E4.5 (top) and E5.5 (bottom) stage; right panels, *Ctip*$^{-/-}$ embryos at E4.5 (top) and E5.5 (bottom) stage. At E4.5, the *Ctip*$^{-/-}$ embryo is mostly resorbed, while at E5.5 day, the *Ctip*$^{-/-}$ embryo is nearly completely resorbed.

or heterozygotes, whereas the number of dead cells was slightly higher in *Ctip*$^{-/-}$ blastocysts than that of the wild type or heterozygotes. These differences were subtle and may not entirely account for the dramatic phenotype seen in the in vitro blastocyst culture (Fig. 2b). To seek for an additional explanation for the phenotype, we analyzed cell cycle profiles for inner stem cells in blastocysts by BrdU uptake at S phase and by immunostaining with anti-phospho-histone H3 (H3P) antibody at G_2 or M phase. Essentially, all the *Ctip*$^{-/-}$ blastocysts failed to incorporate BrdU, while 61% of inner stem cells of the wild type or heterozygotes were labeled with BrdU, suggesting that *Ctip*$^{-/-}$ blastocysts failed to enter S phase (Fig. 2c). However, *Ctip*$^{-/-}$ blastocysts contained numbers of cells at G_2 and M phases similar to those of the wild-type or heterozygotes (Fig. 2d). Taken together, these results suggest that Ctip is essential for the S-phase entry in inner stem cells. Without Ctip, these cells are arrested at G_1, which may eventually lead to cell death. This phenotype is much severer than those observed in mice with null genotype of *Ctbp* (14), *Brca1* (12, 24), *Ikaros* (9), or *Rb* (21), all of which interact with Ctip.

***Ctip* siRNA knockdown leads to G_1 arrest in mouse fibroblasts.** To explore the mechanism of how CtIP functions in S-phase entry, we attempted to establish *Ctip*$^{-/-}$ cells in culture for this pursuit. However, the early lethality of *Ctip*$^{-/-}$ embryo disables the establishment of mouse embryonic fibroblasts (MEF) for further study. To circumvent this difficulty, we constructed two adenoviruses carrying either *GFP* expression cassette only or the additional hairpin oligonucleotide for *Ctip* siRNA expression to deplete Ctip. Infection of NIH 3T3 cells with *Ctip*-siRNA adenovirus, but not control virus, at 10 multiplicities of infection (MOI) efficiently abrogated Ctip expression within 24 to 48 h (Fig. 3a). To determine whether the consequence of Ctip depletion by siRNA would be similar to that observed in vivo, we performed BrdU uptake assay in the time course after infection. Cells infected with the control adenovirus incorporated BrdU vigorously, while those infected with Ctip-siRNA virus failed to do so. The differences in BrdU incorporation between these two were obvious at 48 to 72 h postinfection (Fig. 3b and c). Similarly, by FACS analysis, we found that the population at G_1, but not S or G_2/M, increased significantly in *Ctip*-siRNA virus-infected cells compared to those infected with the control virus (Fig. 3d). Consistently, protein markers for G_1 phase, namely, the cdk inhibitor p21, and hypo-phosphorylated form of Rb increased following *Ctip*-siRNA virus infection compared with the control (Fig. 3e). Cells depleted of Ctip eventually led to cell death after 6 days (Fig. 3f and g), further supporting the observation described for the early *Ctip*$^{-/-}$ embryos.

To test whether this effect was solely caused by Ctip inactivation, we ectopically expressed human CtIP, which was resistant to the mouse Ctip-siRNA due to the variation in primary sequences, in NIH 3T3 cells prior to *Ctip*-siRNA virus infection (Fig. 4a). As shown in Fig. 4b, expression of human CtIP rescued these cells from G_1 arrest mediated by *Ctip*-siRNA virus infection. These results demonstrate unambiguously that Ctip-depleted cells fail to enter S phase and are arrested in G_1, a phenotype similar to that observed in vivo.

CtIP facilitates S entry by releasing Rb-mediated G_1 restraint. It is known that CtIP interacts with RB (8, 27), an essential regulator for G_1/S progression (4, 11). Therefore,



Genotype(Ctip)	+/+	+/-	-/-
No. Blastocysts	6	15	8
No. Cells	207	455	129
No. TUNEL(+)	1	6	7
%TUNEL(+)	0.5	1.3	5.4

Genotype(Ctip)	+/+	+/-	-/-
No. Blastocysts	5	8	5
No. Cells	125	229	123
No. BrdU(+)	76	140	1
%BrdU(+)	61.1	60.8	0.07

Genotype(Ctip)	(+/+) + (+/-)	-/-
No. Blastocysts	11	6
No. G2 Cells	136	57
No. M phase	25	6
No. (G2+M)	161	63
%(G2+M)	48.5	49.2

FIG. 2. *Ctip*$^{-/-}$ blastocysts failed to hatch in vitro and arrested in G_1. a, *Ctip*$^{+/+}$ blastocysts showed normal outgrowth and hatched during in vitro culture (top panels), while *Ctip*$^{-/-}$ blastocysts showed no outgrowth but retained the intact outer membranes (bottom panels). b, Blastocysts cultured for 1 day were subjected to a TUNEL assay by labeling with BrdU. Inner embryonic stem cells were stained with anti-BrdU antibodies and DAPI for nuclei DNA. The results of the TUNEL assay were summarized in the table. The same blastocysts as described for panel b were subjected to the BrdU uptake assay (c) or immunostained with anti-phospho-histone H3 (H3P) to determine the status of G_2/M phases (d). The results of these two assays were summarized in each table following the photos.

Ctip may be required for removing G_1 restraint mediated by Rb. To test this possibility, we performed *Ctip* siRNA knockdown using a pair of isogenic *Rb*$^{-/-}$ and *Rb*$^{+/+}$ MEFs. Infection of *Rb*$^{-/-}$, but not *Rb*$^{+/+}$, MEF with *Ctip*-siRNA adenoviruses had little influence on S-phase entry, suggesting that depletion of *Ctip* expression in *Rb*$^{-/-}$ cells failed to arrest cells at G_1 (Fig. 5a and b). Instead, infection of both MEFs with GFP adenoviruses did not show any difference in S-phase entry (data not shown). Consistently, G_1 protein markers, p21, and hypophosphorylated form of Rb were accumulated following *Ctip*-siRNA virus infection in *Rb*$^{+/+}$ MEFs, while no Rb or p21 expression was detected in *Rb*$^{-/-}$ MEFs (Fig. 5c). Similar phenomena were observed in a pair of human osteosarcoma cells, RB-deficient Saos-2 and RB-reintroduced SR5 (11), transfected with plasmids expressing human *CtIP* siRNA (Fig. 5a and d). These results, taken together, suggest that CtIP is required to remove RB-mediated G_1 restraint for the progression from G_1 to S phase.

The life span of *Ctip*$^{+/-}$ heterozygotes is shortened by multiple tumor formation. The essential role of Ctip in regulating S-phase entry allows us to speculate that deregulation of Ctip will have a significant impact on mouse development. To investigate this possibility, a cohort of more than 30 *Ctip*$^{+/-}$ mice was studied longitudinally. *Ctip*$^{+/-}$ mice died around day 625, while the life span of homozygous *Ctip*$^{+/+}$ mice from similar genetic crosses was about 780 days (Fig. 6a). These mice, once in the moribund state, had succumbed to multiple tumors, predominantly, large lymphomas of both B and T cells (Fig. 6b and c), suggesting that *Ctip* is a bona fide tumor susceptibility gene.

To test whether CtIP contributes to tumorigenesis following the established recessive mechanism (18, 31), we then performed microdissection to isolate those tumor cells and analyzed the *Ctip* genotype by PCR. In seven out of seven tumor samples, they retained the wild-type allele of *Ctip* (Fig. 7a). Similarly, 10 out of 10 tumors, in addition to 10 normal tissue specimens, were stained positively with anti-Ctip antibodies. A pair of normal lymph node and lymphoma cells are shown in Fig. 7b, indicating that the remaining wild-type allele is expressed. Thus, the tumor formation in these mice may be attributed to haploid insufficiency of *Ctip*.

DISCUSSION

In this communication, we generated an inactivated *Ctip* allele in mice to address how CtIP exerts its biological function during mouse development. We showed that the *Ctip*$^{-/-}$ embryos died very early, as the blastocysts failed to enter S phase, leading to a slightly elevated cell death. Consistently, depletion of Ctip arrested *Rb*$^{+/+}$, but not *Rb*$^{-/-}$, MEF or human osteosarcoma Saos-2 cells at G_1, suggesting that this arrest is RB dependent. Importantly, *Ctip*$^{+/-}$ heterozygotes had their life

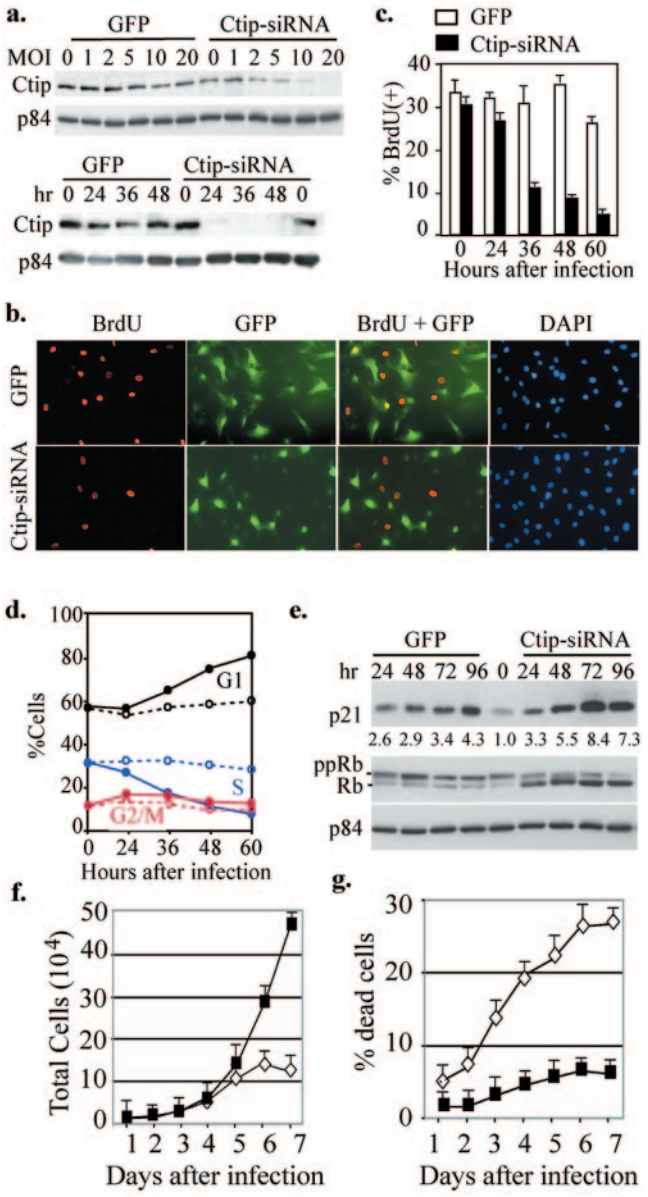


FIG. 3. Depletion of Ctip by infection with adenovirus carrying Ctip siRNA lead to inhibition of S-phase entry. a, Infection with Ctip-siRNA adenovirus abrogated Ctip expression in a dose- and time-dependent manner. NIH 3T3 cells were infected with different MOI of GFP or Ctip-siRNA adenovirus for 48 h, and the corresponding cell lysates were prepared for Western analysis and probed with anti-Ctip or anti-p84, serving as internal loading control (upper panel). Similarly, NIH 3T3 cells were infected with 10 MOI and harvested at different time points for Western blotting analysis as described above (lower panel). b, Cells were labeled with BrdU for 4 h after infection with 10 MOI of GFP or Ctip-siRNA adenovirus for 48 h and stained with anti-BrdU antibody. c, Graphic quantitative summary of cells labeled with BrdU at different time points after infection with adenoviruses as described in panel b. d, Graphic summary of data from FACS analysis of cells collected at various time points after infection with GFP (dashed lines) or Ctip-siRNA viruses (solid lines). e, NIH 3T3 cells were infected with 10 MOI of GFP or Ctip-siRNA and harvested at different time points for Western blotting probed with anti-p21, Rb, or p84 antibodies. In cells depleted of Ctip, Rb became hypophosphorylated and p21 accumulated. f and g, NIH 3T3 cells were infected as indicated for panel e and cells were collected and stained with trypan blue. The numbers of both total (f) and trypan blue-positive cells were counted. The percentages of cell death are plotted in panel g.

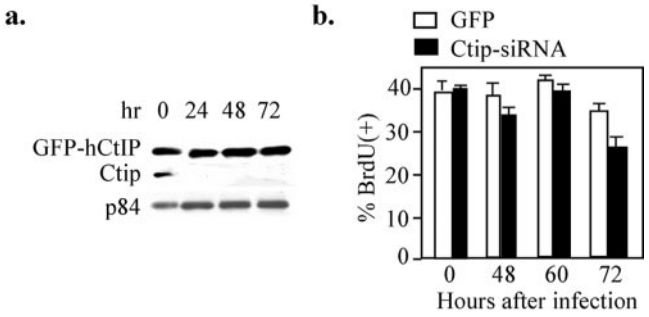


FIG. 4. Expression of the human CtIP restored proliferation of NIH 3T3 cells with mCtip knocked down. a, Human GFP-CtIP fusion protein stably expressed in NIH 3T3 cells is resistant to Ctip-siRNA depletion. A stable clone of NIH 3T3 cells expressing human GFP-CtIP fusion protein was established and infected with Ctip-siRNA adenoviruses. Cell lysates prepared from the infected cells were analyzed by Western blotting probed with anti-CtIP and anti-p84 antibodies. b. The stable clone of NIH 3T3 cells was labeled with BrdU after infection with 10 MOI of GFP or Ctip-siRNA for different time intervals, and the results of BrdU incorporation are summarized.

spans shortened by the generation of tumors, predominantly large lymphomas. The wild-type *Ctip* allele and protein remained detectable in these tumors, implicating that haploid insufficiency of *Ctip* leads to tumorigenesis. These findings suggest that deregulation of CtIP mediated S-phase entry leads to a defect in early embryogenesis and contributes, in part, to tumor formation.

Ctip interacts with Ctbp (34), Brca1 (42, 46), Ikaros (19), and Rb (8, 27). The *Ctip* null phenotype is much severer than those observed in mice with null genotype of those interacting partners. *Ctip*$^{-/-}$ embryos die at E4 because the blastocysts fail to enter S phase, leading to an elevated cell death. On the other hand, the *Ctbp1* null mice survive 20 days postnatally, while the *Ctbp2*-null are embryonically lethal at E10.5 (14). *Brca1*$^{-/-}$ or *Rb*$^{-/-}$ mouse embryos die at E6.5 or E14.5 (12, 21, 24), respectively, while *Ikaros*$^{-/-}$ mice are viable (9). Thus, CtIP plays a critical factor in very early embryogenesis.

The role of Ctip in G_2/M checkpoint control has been reported previously (23). It was postulated that the interaction between Ctip and BRCA1 is essential for this checkpoint activity. However, whether Ctip plays roles in different cell cycle stage remains unclear. We observed that the depletion of Ctip in MEFs leads to G_1 arrest, suggesting an important role of Ctip in G_1 regulation. Intriguingly, our recent preliminary results revealed that depletion of Ctip in different cell types generated defects at distinct cell cycle stages, suggesting that the role of Ctip in cell cycle progression may manifest differentially depending on cell status.

On the basis of the result that depletion of Ctip arrested *Rb*$^{+/+}$, but not *Rb*$^{-/-}$, MEFs at G_1 (Fig. 5), the Rb deficiency, at least in part, mitigates the effect of Ctip deficiency in this cell type. It was previously proposed that CtIP has an adaptor role in connecting Rb family proteins for recruiting corepressor CtBP (27). If this is the case, how this Ctip adaptor counteracts Rb in G_1 restraint is intriguing. Since RB plays diverse, but concerted, roles in G_1/S transition, CtIP may serve as an adaptor to recruit different factors, which counter Rb action in this context. For example, RB is mainly inactivated through hyper-

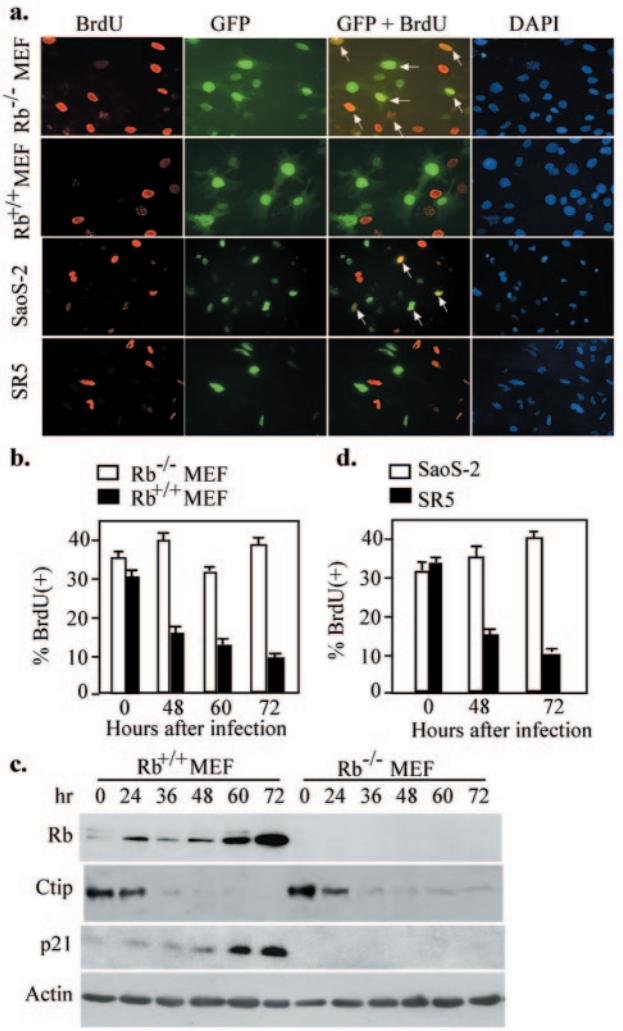
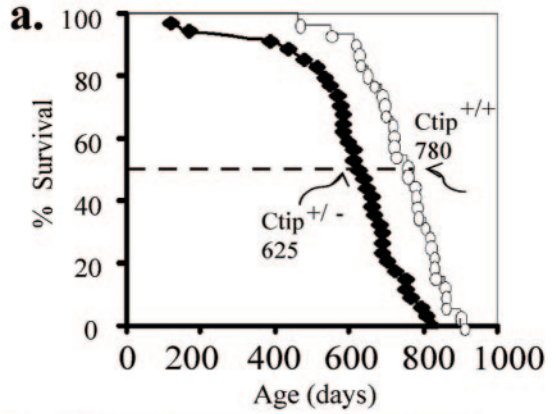


FIG. 5. Ctip-mediated G_1 control is Rb dependent. a, Immunofluorescence staining of BrdU uptake in the pair of $Rb^{+/+}$ and $Rb^{-/-}$ MEFs (×400) infected with Ctip-siRNA adenovirus or in the other pair of human osteosarcoma cells (×200), Saos-2, and SR5, transiently transfected with pCtIP-RNAi plasmids. Cells were labeled with BrdU 48 h after infection or transfection. b, Summary of BrdU incorporation of $Rb^{+/+}$ and $Rb^{-/-}$ MEFs at 48, 60, and 72 h after infection. c, The infected $Rb^{+/+}$ and $Rb^{-/-}$ MEFs were collected for Western blotting probed with antibodies against CtIP, Rb, p21, and actin. d, Summary of the BrdU-positive cells of Saos-2 and SR5, after transfection with either pCtIP-RNAi plasmid for 48 or 72 h.



b.

Tumor type	Percentage
B cell lymphoma	45 (9/20)*
T cell lymphoma	10 (2/20)
Polymorphic lymphoma	5 (1/20)
Histiocytic sarcoma	10 (2/20)
Soft tissue sarcoma	5 (1/20)
Angiosarcoma	5 (1/20)
Spleen hyperplasia	10 (2/20)
Leiomyoma	5 (1/20)
Endometrial hyperplasia	10 (2/20)
Gastric squamous papilloma	5 (1/20)
Kidney cyst	5 (1/20)
Total	90 (18/20)

* number in parentheses indicates the number of mice with neoplasm out of total number of mice

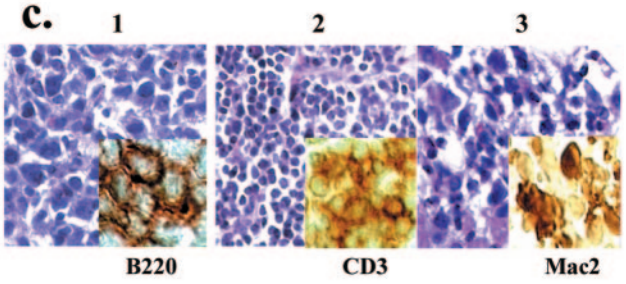


FIG. 6. Life span and tumor spectra of *Ctip* heterozygous mice. a. Loss of one Ctip allele shortened the life span of $Ctip^{+/-}$ compared with $Ctip^{+/+}$ mice. b. Tumor incidence and spectra of $Ctip^{+/-}$ mice. c. Lymphatic malignancies developed in $Ctip^{+/-}$ mice. H&E staining was used to visualize tumor histology. In addition, antibodies specific for the distinctive tumor antigens were used (see inset). For example, B-cell lymphoma (1), T-cell lymphoma (2), and histiocytic sarcoma (3) were detected with anti-B220, anti-CD3, and anti-Mac2, respectively.

phosphorylation by cyclin D/CDK4/6 and cyclin E/CDK2 complexes at G_1/S transition (4); however, why the presence of those kinases capable of hyperphosphorylation of RB is not sufficient for cells to enter S phase when CtIP is depleted is not known. One possible explanation is that CtIP represses the transcription of Cdk inhibitor p21 (22). In the absence of CtIP, p21 becomes derepressed and inhibits Cdk activity, leading to Rb hypophosphorylation as shown in Fig. 3 and 5. However, increased expression of p21 in $Rb^{-/-}$ MEF was not observed even when Ctip was depleted, suggesting that activation of p21 expression by Ctip requires Rb. Consistently, Rb has been reported to activate p21 transcription through Sp1 and Sp3 (6). When Rb is not available for transactivation, p21 is not expressed and the repression function of Ctip becomes insignificant. In this regard, Ctip may recruit a repressor to counteract the Rb-mediated coactivation of p21. However, not all the Rb-null cells fail to express p21 (35), suggesting that a much more complicated mechanism for regulating p21 exists.

In addition, active Rb has been shown to repress E2F-regulated S-phase genes, such as MCMs, DNA polymerase subunits, and deoxynucleoside triphosphate synthetic enzymes (25). If Ctip is critical for S-phase entry, such repression by Rb/E2F has to be released by the presence of Ctip. In this

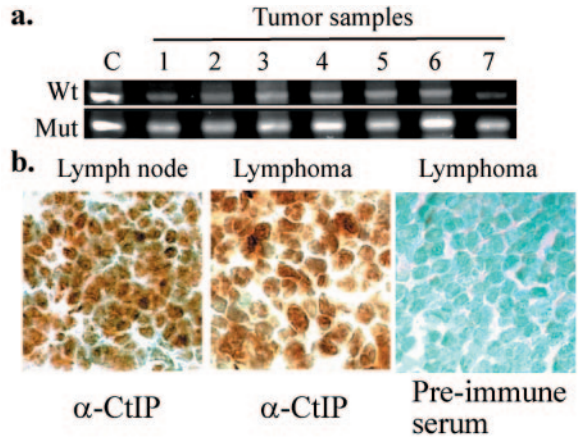


FIG. 7. Tumors derived from the *Ctip* heterozygotes contain an active wild-type allele of Ctip. a. PCR analysis of microdissected tumor samples showed the presence of the wild-type *Ctip* allele. b. Positive immunostaining with anti-Ctip antibodies, but not with preimmune antibodies, of normal lymph node and lymphoma from $Ctip^{+/-}$ mice. Specimens were counterstained with methylene green (×630).

situation, Ctip has to recruit activators to remove the repression of Rb. However, there are very few transcriptional processes involved in early blastocyst division, albeit depletion of Ctip renders blastocysts defective in entering S phase. Thus, additional mechanism for CtIP in negatively regulating RB at the G_1/S transient may exist.

In addition to the transcriptional repression function, RB also has several functions involving DNA replication and chromatin remodeling (47). DNA replication is initiated at specific chromatin elements referred to as replication origins or replicons, on which origin recognition complex could load and in turn sequentially recruit other replication components, including Cdc6, Cdt1, and Mcm2-7, to assemble the prereplication complex. Initiation of replication is then triggered by kinase activities of cyclin-dependent kinase and Cdc7/Dbf4 and by association of other replication factors at G_1/S transition (1). Since RB is able to directly inhibit DNA replication by binding to DNA replication factors such as RFC, MCM7, and DNA polymerase α (29, 36, 39), CtIP may be essential for removing RB from replication origin to release RB restraint for DNA replication and S-phase entry. This possibility is under vigorous investigation.

Besides the synthesis of DNA, it has become clear that the chromatin structure maintaining the epigenetic information is critical for cell cycle progression (26). Immediately before or after replication, a subset of proteins that could modify and remodel the DNA and nucleosomes, such as DNA methyltransferase DNMT1 and chromatin assembly factor CAF1, are recruited in the vicinity of replication fork to alter chromatin domains for a variety of DNA processing, including replication, repair, and recombination (5, 15, 44). RB binds to DNMT1, which may contribute to the repression activity (32). Thus, it is likely that binding of CtIP to RB may be essential for restraining RB from the repression status, although the precise mechanism remains to be elucidated.

Interestingly, the *Ctip* heterozygous mice succumbed to multiple tumors, predominantly, large lymphomas of both B and T cells (Fig. 6b and c), suggesting that Ctip is a bona fide tumor susceptibility gene. In those tumor cells, they retained the wild-type allele of *Ctip* (Fig. 7a) and were stained positively with anti-Ctip antibodies (Fig. 7b), indicating that the remaining wild-type allele is active. Thus, the tumor formation in these mice may be attributed to haploid insufficiency of *Ctip*. This phenomenon has been seen in several cases, including TGFβ1 and Dmp1 (16, 40).

It is intriguing why reduction in Ctip expression predisposes mice to tumor formation. Since the interacting partners of Ctip including Rb and BRCA1 as well as Ikaros are tumor susceptibility proteins (21, 41, 43), disturbance of the delicate regulation by reducing the availability of the other significant partner may have a similar consequence in tumorigenesis. For example, reducing Ctip, which counteracts Rb in mediating G_1/S progression, will increase active form of Rb and disturb Rb-mediated G_1 to S progression. It was reported that expression of a hypophosphorylated form of Rb in mice leads to breast carcinoma, perhaps through reducing apoptosis, with low incidence (17). The G_2/M checkpoint defect and the genomic instability resulting from the limited BRCA1/CtIP interaction could also trigger tumorigenesis in principle (23), although *Brca1* heterozygous mice rarely have an increased tumor incidence (12, 24; W. H. Lee et al., unpublished). Thus, tumor predisposition in *Ctip* heterozygotes is not unexpected. However, it is surprising to learn that most $Ctip^{+/-}$ mice developed multiple tumors, predominantly lymphoma, at an age as early as 6 months. Ikaros may mediate the tissue specificity of developing large lymphoma in Ctip heterozygotes, because this transcriptional repressor is a key regulator for lymphocyte development and malignancy (9, 30, 41). Interestingly, the transcriptional repression activity of Ikaros, similar to Rb, is modulated by phosphorylation in G_1 (10). CtIP may counteract RB as well as Ikaros to release the restraint at G_1. Although the detailed mechanism is waiting for further elucidation, the finding described above uncovers, for the first time, a novel G_1/S regulation in that CtIP directly counteracts RB restraint. Disturbing this regulation will lead to severe consequences involving embryonic lethality and tumorigenesis.

ACKNOWLEDGMENTS

We thank Houyi Wang and Andrea Nikitin for their technical contribution at the early stage of the project and Saori Furuta for her critical reading.

This work was supported by grants from the National Institutes of Health (CA 94170 to W.-H.L. and CA 85605 to P.-L.C.) and a postdoctoral fellowship from Susan G. Komen Foundation to F.L.

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