Proteolysis of MLL family proteins is essential for Taspase1-orchestrated cell cycle progression

Shugaku Takeda,1,7 David Y. Chen,1,7 Todd D. Westergard,1,7 Jill K. Fisher,5,7 Jeffrey A. Rubens,5 Satoru Sasagawa,1 Jason T. Kan,1 Stanley J. Korsmeyer,5,6 Emily H.-Y. Cheng,1,2,3 and James J.-D. Hsieh1,2,4,8

1Molecular Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110, USA; 2Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri 63110, USA; 3Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110, USA; 4Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110, USA; 5Howard Hughes Medical Institute, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA

Taspase1 was identified as the threonine endopeptidase that cleaves mixed-lineage leukemia (MLL) for proper Hox gene expression in vitro. To investigate its functions in vivo, we generated Taspase1−/− mice. Taspase1 deficiency results in noncleavage (nc) of MLL and MLL2 and homeotic transformations. Remarkably, our in vivo studies uncover an unexpected role of Taspase1 in the cell cycle. Taspase1−/− animals are smaller in size. Taspase1−/− mouse embryonic fibroblasts (MEFs) exhibit impaired proliferation, and acute deletion of Taspase1 leads to a marked reduction of thymocytes. Taspase1 deficiency incurs down-regulation of Cyclin Es, As, and Bs and up-regulation of p16ink4a. We show that MLL and MLL2 directly target E2Fs for Cyclin expression. The uncleaved precursor MLL displays a reduced histone H3 methyltransferase activity in vitro. Accordingly, chromatin immunoprecipitation assays demonstrate a markedly decreased histone H3 K4 trimethylation at Cyclin E1 and E2 genes in Taspase1−/− cells. Furthermore, MLLnc/nc;2nc/nc MEFs exhibit impaired proliferation. Our data are consistent with a model in which precursor MLLs, activated by Taspase1, target to Cyclins through E2Fs to methylate histone H3 at K4, leading to activation. Lastly, Taspase1−/− cells are resistant to oncogenic transformation, and Taspase1 is overexpressed in many cancer cell lines. Thus, Taspase1 may serve as a target for cancer therapeutics.

Keywords: Taspase1; MLL; E2F; p16; cell cycle; cancer

Supplemental material is available at http://www.genesdev.org

Received May 15, 2006; revised version accepted July 11, 2006.

Site-specific proteolyses play central roles in many biological pathways exemplified by the sequential activation of blood coagulation factors, the blood pressure-controlled activation of angiotensinogen, the cholesterol-gauged liberation of sterol response element-binding protein [SREBP] from the endoplasmic reticulum [ER] (Brown et al. 2000), the ligand-activated cleavage and subsequent release of the intracellular domain of Notch (Brown et al. 2000), the maturation of the hedgehog signaling molecule (Ye and Fortini 2000), the separation of HCF-1 for proper cell cycle progression (Julien and Herr 2003), and the activation of caspases for cell death execution [Thornberry and Lazebnik 1998]. Identification and characterization of the responsible endopeptidase not only proved critical for a better understanding of such biological processes but also provided targets for intervention of diseases involving specific pathways (Saklatvala et al. 2002).

Taspase1 was originally cloned as the protease that cleaves mixed-lineage leukemia [MLL] at conserved sites [QV(L)D/GXXD] (Hsieh et al. 2003a). Taspase1 encodes a highly conserved 420-amino-acid proenzyme that is intramolecularly processed to generate an active heterodimer (Hsieh et al. 2003a). The Taspase1 heterodimer displays an overall α2/β2 structure and further assembles into an asymmetric α2/β2 heterotrimer (Khan et al. 2005). Taspase1 is an endopeptidase within a family of enzymes possessing an Asparagi-nase_2 homology domain. Other members present in prokaryotes and eukaryotes include the amidohydrolases, L-asparaginase, and glycosylasparaginase. L-aspa-

6Deceased.
7These authors contributed equally to this work.
8Corresponding author.
E-MAIL jhsieh@im.wustl.edu FAX (314) 362-1589.
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1449406.
Taspase1 is a coaptase present in mammals that cleaves MLL family proteins. Taspase1 deficiency leads to homeotic transformations in mice, confirming its participation in Hox gene expression. Remarkably, Taspase1 also regulates the cell cycle, thus controlling organism size. It directly participates in cell cycle gene expression through the MLL–E2F axis. Proteolysis of MLL family proteins by Taspase1 is required to fully activate their histone methyltransferase (HMT) activity. Our study establishes a novel paradigm in which an endopeptidase orchestrates cell cycle progression through site-specific proteolysis.

Results

Generation of Taspase1−/− mice

To assess Taspase1’s functions in a whole organism setting, we generated Taspase1-null mice. A cre-lox gene targeting strategy was employed to delete exon 9 of murine Taspase1, where the active site Thr 234 resides [Fig. 1A]. Successful targeting was verified by Southern blot and PCR analyses [Fig. 1B,C]. Deletion of exon 9 resulted in the disappearance of Taspase1 protein in Taspase1−/− mouse embryonic fibroblasts (MEFs) [Fig. 1D] and whole embryo extracts [data not shown], excluding the possibility of introducing a dominant-negative effect in Taspase1−/− animals. Most importantly, no cleaved MLLc180 was detected in Taspase1−/− cells. In the absence of Taspase1, MLL exists as a full-length precursor protein [MLLcFL] [Fig. 1D]. Although conserved Taspase1 cleavage sequences exist among MLL family proteins, MLL is the only proven substrate. In mammals, there is one additional trithorax homolog, MLL2, which contains all of the architecturally positioned conserved domains as MLL [Fig. 1E; Huntsman et al. 1999]. Although MLL2 has been suggested to undergo proteolysis, it remains undetermined whether it is also processed by Taspase1 [Hughes et al. 2004]. In the absence of Taspase1, no processed MLL2c88 was detected [Fig. 1D]. The site-specific cleavage of MLLcFL and MLL2cFL in Taspase1−/− cellular extracts was successfully restored when recombinant Taspase1 [rTaspase1] was exogenously added [Fig. 1D].
Taken together, Taspase1 is the only protease responsible for the proteolysis of MLL family proteins.

No evident gross abnormalities were observed in mice with haploinsufficiency of Taspase1. Interccrosses between Taspase1−/− mice provided the first assessment of Taspase1’s functions in vivo. A minor embryonic developmental disadvantage was noted among Taspase1−/− newborns [89% of expected Mendelian ratio] (Fig. 1F). Taspase1−/− animals were born with no apparent respiratory distress. However, the majority died at postpartum day 1 or 2 [P1 or P2] with no obvious milk spots, suggesting a feeding defect. This profound early postnatal lethality of Taspase1−/− animals resulted in a 13% survival at 3 wk of age [Fig. 1F].

Taspase1-deficient animals exhibit classic homeotic transformations

Our prior studies indicated a role of Taspase1 in Hox gene expression in vitro. To assess whether Taspase1 deficiency incurs homeotic transformations from altered Hox gene expression, axial skeletons of P1 wild-type, Taspase1+/−, and Taspase1−/− newborns were examined (Fig. 2). Remarkably, Taspase1−/− animals displayed profound skeletal abnormalities with high degrees of penetrance (Fig. 2I). Intermediate phenotypes were also observed among Taspase1+/− animals. Identified abnormalities representing classic homeotic transformations included a broadened and tilted anterior arch of atlas [C1 vertebra] [a.a.a.], a split neural arch of axis [C2 vertebra], a posterior transformation of C7 to T1 with attached outgrowing aberrant ribs, an incomplete segmentation between sternebrae 3 and 4, an anterior transformation of T8 to T7 with resulting additional pairs of sternal ribs, and an anterior transformation of L1 to T13 with resulting 14 pairs of ribs [Fig. 2]. Taken together, Taspase1 confers correct segmental identities.

Taspase1−/− animals and embryos are smaller in size

Remarkably, all Taspase1−/− newborns can be easily identified right after birth because they are markedly smaller in size. Taspase1−/− animals that survived the newborn period were smaller through adulthood, compared with their wild-type and heterozygous littermates [Fig. 3A]. This phenotype commences in uteri. Taspase1−/− embryos are significantly smaller at E18.5 [Fig. 3B]. This abnormality can be detected as early as E14.5 [data not shown]. Such a phenotype is commonly associated with deregulation of signaling pathways controlling cell growth and/or cell proliferation. FACS analyses did not detect any cell size alteration in Taspase1−/− MEFs, compared with wild-type MEFs [Fig. 3C].
Requirement of Taspase1 for the proliferation of MEFs and thymocytes

To further investigate the underlying mechanism by which Taspase1 regulates animal size, we next examined cell proliferation. Compared with wild-type MEFs, the cell number was threefold lower in Taspase1−/− cells after 7 d in culture (Fig. 4A). This difference does not result from increased cell death determined by Annexin V staining (Fig. 4B). Pulse labeling with BrdU demonstrated that Taspase1−/− MEFs have fewer BrdU-positive S-phase cells (18.2 ± 1.5%) than wild-type MEFs (31 ± 2.8%) (Fig. 3C). Taspase1 is abundantly expressed in thymus (Supplementary Fig. S1) where thymocytes undergo maturation and rapid cell division. The profound defects in Taspase1−/− MEFs prompted us to examine its role in thymocyte expansion. We asked whether induced acute deletion of Taspase1 in vivo affects thymocyte proliferation. We employed the MxCre recombinase mouse model in which the expression of Cre recombinase can be induced upon polyinosinic-polycytidylic acid [pI-pC] injection, resulting in a deletion of the floxed [l] conditional allele [Kuhn et al. 1995]. In MxCre+;Taspase1f/− mice, acute deletion of Taspase1 in the thymus (Fig. 4D) led to a 50% reduction of thymus weight (Fig. 4E) and a 54% reduction of total thymocyte number (Fig. 4F), compared with control littermates. The T-cell development in the thymus can be divided into three sequential stages—double-negative (DN), double-positive (DP), and single-positive (SP) stages—based on their cell surface expression of CD4 and CD8. DP cells constitute the majority of thymocytes, resulted from exponential cell division [Paul 2003]. Acute deletion of Taspase1 did not reduce the DN population, but instead, led to a 60% reduction of DP thymocytes (Fig. 4F). Annexin V staining did not detect an increase of cell death in thymocytes upon Taspase1 deletion (Fig. 4G). These data indicate that the removal of Taspase1 in the thymus leads to a profound proliferation block during DP expansion.

Figure 2. Homeotic transformations of axial skeletons in Taspase1−/− mice. Skeletal comparison between wild-type [A–D] and Taspase1−/− [E–H] P1 newborn are illustrated. Bones are stained as red and cartilage as blue with alazarin red and alcian blue, respectively. [A,E] Lateral views of cervical [C] and upper thoracic [T] regions demonstrate deformed a.a.a., split C2 neural arch (arrow), and posterior transformation of C7 with an additional rib [arrowhead] in Taspase1−/− but not wild-type skeletons. [B,F] Magnified frontal views of the a.a.a.. The asterisk denotes deformed and broadened a.a.a. in Taspase1−/− mice. [C,G] Anterior views of the sternum indicate incomplete segmentation between sternebrae 3 and 4 (arrowhead), and an additional pair of sternal ribs fused between sternebra 4 and xiphoid process present in Taspase1−/− but not wild-type newborns. [D,H] Anterior views of lower thoracic and lumbar vertebras demonstrate Taspase1 deficiency resulted in anterior transformation of L1 with one additional pair of ribs (arrow). (I) Summary of skeletal defects observed in Taspase1-deficient newborns.
Deregulated cell cycle gene expression in Taspase1-deficient cells

After establishing an essential role of Taspase1 in the proliferation of both MEFs and thymocytes, we investigated the underlying mechanisms by which Taspase1 regulates cell division. Cell cycle progression is governed by the coordinated assembly of Cyclin/Cdk complexes, through oscillated Cyclin expression, to activate E2Fs for proper expression of essential cell proliferation genes (Dyson 1998; Nevins 2001; Trimarchi and Lees 2002; Blais and Dynlacht 2004; Bracken et al. 2004). Further regulation of the Cyclin/Cdk activities is imposed by Cdk inhibitors (CdkIs) (Sherr and Roberts 1999). Quantitative RT–PCR assays on asynchronous Taspase1−/− MEFs revealed a profound underexpression of E, A, and B, but not D types of Cyclins, a marked overexpression of p16Ink4a, and a moderate elevation of p21, p27, and ARF [Fig. 5A,B]. Similar data were obtained when protein levels were determined [Supplementary Fig. S2]. As p16Ink4a expression is highly elevated, the reduction in expression of Cyclins E, A, and B may simply reflect fewer cycling cells. Alternatively, Taspase1 may directly regulate the Cyclin expression. To discern these possibilities, we cultured MEFs in the presence of BrdU for 24 h to label all of the cycling cells. A 10% increase of quiescent cells was observed in Taspase1−/− MEFs [Supplementary Fig. S3], which cannot be held accountable for the 60% reduction in Cyclin E1 and E2 expression. Accordingly, these data suggest that Taspase1 participates in the regulation of p16Ink4a and Cyclins. To further determine the significance of p16Ink4a overexpression in the observed cell cycle defects of Taspase1−/− cells, we performed RNAi-mediated knockdown of p16Ink4a. Expression of the p16Ink4a short hairpin RNA [shRNA] in Taspase1−/− MEFs reduced the amounts of p16Ink4a to near wild-type levels [Supplementary Fig. S4]. The reduced p16Ink4a expression in Taspase1−/− cells partially rescued the proliferation defects [Fig. 5C,D]. This partial rescue confirms p16Ink4a as an important but not the sole Taspase1 downstream cell cycle regulator. Since p16Ink4a up-regulation is commonly associated with early replicative senescence (Itahana et al. 2001), we performed senescence-associated galactosidase (SA-Gal) assays on passage 2 Taspase1−/− MEFs that were used for all of the assays presented, and did not detect SA-Gal-positive cells [data not shown]. Following a 3T3 protocol, Taspase1−/− MEFs manifested replicative senescence at passage 8, compared with wild-type cells, which senesced at passage 10 [data not shown].

The up-regulated p16Ink4a and down-regulated Cyclin E expression in Taspase1−/− cells predicts a block in G1/S transition, and may pose a higher threshold for G0 to G1 re-entry. To test these hypotheses, primary MEFs were synchronized by serum deprivation, and cell cycle re-entry was determined by Tritium thymidine incorporation. Indeed, upon full serum stimulation, serum-deprived quiescent Taspase1−/− cells displayed a 2-h delay in S-phase entry [Fig. 5E]. As p16Ink4a inhibits D-type Cdk5a, we next determined the activity of D-type Cdk5s in Taspase1−/− cells by examining Rb phosphorylation at Ser 807/811. Taspase1−/− cells displayed a delayed and impaired Rb phosphorylation [Fig. 5F]. This observed delay is similar to that of cells deficient for all three D type Cyclins [D1, D2, and D3] [Kozar et al. 2004]. The marked reduction of total Tritium thymidine incorporation in Taspase1−/− MEFs [Fig. 5E] indicates a significant decrease of cells re-entering the cell cycle, which was further confirmed by long-term BrdU labeling experiments [Fig. 5G]. Sequential induction of Cyclins, namely D, E, and A, followed by B, with consequent activation of respective Cyclin/Cdk complexes, governs the cell cycle progression. We next determined the induction of D, E, and A types of Cyclins after serum restimulation and confirmed the impairment of induction of Cyclins E and A, but not D [Fig. 5H], which is consistent with our prior analyses on asynchronized cells.
**Uncleaved precursor MLL<sup>FL</sup> exhibits impaired histone H3 methyl transferase activity**

MLL is one of the best characterized mammalian methyl transferase that specifically methylates histone H3 at the K4 position that marks active gene expression [Milne et al. 2002, Nakamura et al. 2002, Santos-Rosa et al. 2002]. To investigate how Taspase1 deficiency affects the transcription of cell cycle genes, we focused our attention on the regulation of p16<sup>ink4a</sup>, Cyclin E1, and E2 at the chromatin level to determine whether the known Taspase1 substrate, MLL, directly participates in the cell cycle gene regulation. Indeed, chromatin immunoprecipitation (ChIP) assays demonstrated the occupancy of MLL protein on Cyclin E1 and E2 genes [Fig. 6A]. Consequently, histone H3 K4 trimethylation of target genes was examined to investigate whether noncleavage of MLL altered its methyl transferase activity. A marked reduction of H3 K4 trimethylation at Cyclin E1 and E2 was observed in Taspase1<sup>−/−</sup> cells, which is accountable for their decreased expression (Fig. 6A). As more and more cross-talks between histone modifications are recognized, we tested whether the altered H3 K4 methyl-

ation affects modifications on adjacent K9/14 (Fig. 6A). ChIP assays demonstrated a moderate reduction of histone H3 K9/14 acetylation at Cyclin E1 and E2 genes [Fig. 6A]. The demonstration of a reduction, but not a loss of H3 K4 methylation, suggests that MLL family proteins need to be cleaved by Taspase1 to be fully functional. To examine this possibility, we first immunoprecipitated MLL protein in cellular extracts derived either from wild-type or Taspase1<sup>−/−</sup> MEFs. Bound-end immunoprecipitated MLL was incubated with purified histone H3 and S-Adenosyl-<sup>3H</sup>Methionine before SDS-PAGE analysis. HMT activity was then assessed by autoradiography. A near 50% reduction of HMT activity was observed in Taspase1<sup>−/−</sup> cells, compared with wild-type cells [Fig. 6B]. To ensure that the observed activity reduction is not due to a decreased ability to immunoprecipitate the uncleaved precursor MLL<sup>FL</sup>, we determined the amounts of bead-bound MLL<sup>FL</sup> protein. The poor gel transferring physical property of the 500-kDa MLL<sup>FL</sup> makes it impossible to perform a direct comparison between the immunoprecipitated amounts of MLL<sup>FL</sup> and processed MLL. To overcome this technical difficulty, we incubated bead-bound MLL<sup>FL</sup> with 2 µg of

---

**Figure 4.** Cell cycle defects associated with the loss of Taspase1. (A) Taspase1<sup>−/−</sup> MEFs exhibit impaired proliferation. Replicate cultures of 1 × 10<sup>5</sup> cells per 6-cm dish were plated on day 0, and cells were counted each day for seven consecutive days. Data shown as mean ± SD were obtained from three independent experiments. (B) An- nexin V staining demonstrates similar baseline cell death between wild-type (Wt) and Taspase1<sup>−/−</sup> primary MEFs at passage 2. (C) An- nexin V staining demonstrates similar baseline cell death between wild-type (Wt) and Taspase1<sup>−/−</sup> primary MEFs. Data shown as mean ± SD were obtained from three independent experiments. (C) Taspase1<sup>−/−</sup> MEFs have fewer S-phase cells. BrdU incorporation was determined by FACS analysis, and representave data of three independent experiments are presented. Mean ± SD from three independent experiments are as follows: wild type (Wt) [G1: 48.5 ± 3.2%; S: 31.0 ± 2.8%; G2/M: 22.1 ± 2.9%]; Taspase1<sup>−/−</sup> [G1: 57.9 ± 2.6%; S: 18.2 ± 1.5%; G2/M: 22.1 ± 2.9%]. (D) pl-pC injection induces efficient deletion of Taspase1 in the thymus. The extent of acute deletion of floxed [f] Taspase1 allele was assessed by PCR on genomic DNA obtained from the tail before pl-pC injection and from thymocytes after pl-pC injection [+]. (E) Thymus weight was measured after MxCre induction by pl-pC injections. (F) Numbers of total and subsets of thymocytes after acute deletion of Taspase1 by pl-pC injections are presented. The percentages of DN, DP, and SP thymocytes were obtained by FACS analysis of CD4<sup>+</sup> and CD8<sup>+</sup> expression. Numbers of each subset of thymocytes are obtained by multiplying the total thymocyte number by the respective percentage. (G) Baseline cell death of thymocytes after pl-pC injections was determined by Annexin V staining. Representative data from three independent experiments are presented. Data shown in E and F as mean ± SD were obtained from three mice of each indicated genotype.
rTaspase1 for 6 h to ensure complete processing before SDS-PAGE and anti-MLL Western blot analyses [Fig. 6C]. We confirmed that there were an equal number of moles of MLLFL and processed MLL utilized in our HMT assays [Fig. 6C]. Taken together, the uncleaved precursor MLLFL apparently is hypomorphic. This is in accordance with genetic observations obtained with a Drosophila trithorax hypomorphic E3 (trxE3) allele in which an internal in-frame deletion removes a predicted Taspase1 cleavage site and encodes a noncleavable trithorax (Mazo et al. 1990).

In an attempt to understand how Taspase1 deficiency resulted in the activation of p16Ink4a, ChIP assays on p16Ink4a gene were performed and an increased MLL targeting was observed [Fig. 6A]. However, no significant changes on histone H3 K4 trimethylation and H3 K9/14 acetylation were detected on the p16Ink4a gene [Fig. 6A], supporting the notion that up-regulation of p16Ink4a may not be achieved by MLL-mediated histone modification. MLL has been shown to form complexes with multiple nuclear factors involved in gene regulation [Popovic and Zeleznik-Le 2005]. Increased MLL targeting may facilitate recruiting these factors to p16Ink4a, which leads to its activation. Alternatively, noncleavage of yet identified protein substrate(s) of Taspase1 may contribute to the transcriptional activation of p16Ink4a. It has been shown that PcG protein, Bmi, regulates p16Ink4a expression [Jacobs et al. 1999]. Our findings on MLL targeting to the p16Ink4a gene suggest that both PcG and trxG proteins may coordinately regulate not only Hox genes but also cell cycle genes.

Figure 5. Taspase1 deficiency results in deregulated expression of cell cycle genes. (A,B) Quantitative RT–PCR analyses of Cyclins (A) and CdkIs and ARF (B) on asynchronized primary MEFs are presented. The expression levels in wild-type (Wt) cells are set at 100%. (C,D) Knockdown of p16 in Taspase1−/− MEFs partially rescued the proliferation defect associated with the loss of Taspase1. (C) Triplicate cultures of 1 × 105 cells per 6-cm dish were plated on day 0, and cells were counted each day for seven consecutive days. (D) S-phase cells were determined by pulse labeling with BrdU before being subjected to FACS analysis using FITC-conjugated anti-BrdU antibody. Results are shown as relative to proliferation of wild-type (Wt) cells infected with control shRNA, which was set as 100%. Data shown as mean ± SD were obtained from wild-type and Taspase1−/− primary MEFs infected with lentivirus expressing indicated shRNA. ShRNA against firefly luciferase was used as a negative control. (E) Serum-deprived quiescent primary MEFs were restimulated with medium containing 10% FBS for indicated periods of time. Tritium thymidine uptake was obtained to assess cell cycle re-entry from G0. (F) Taspase1−/− MEFs display delayed and impaired D-type Cdk5-mediated Rb phosphorylation. Primary MEFs at passage 2 were synchronized with serum deprivation before being refed with full serum for the indicated periods of time. Cellular extracts were subjected to Western blot analysis using anti-phosphoRb (Ser807/811) antibody. (G) Taspase1−/− MEFs have profound cell cycle re-entry defects. Primary MEFs at passage 2 were synchronized with serum deprivation before being refed with full serum for 28 h in the presence of BrdU. Cycling cells were assessed with anti-BrdU FITC-conjugated antibody. Representative data from three independent experiments are presented. (H) Taspase1−/− MEFs exhibit delayed and impaired induction of Cyclin E, and A, but not D. Serum-deprived quiescent passage 2 primary MEFs were restimulated with medium containing 10% FBS for indicated periods of time. Expression of cyclins was examined by Western blot analyses.
MLL proteins target different subsets of E2Fs for downstream gene regulation

E2F family proteins are the key molecules in orchestrating the activation of essential cell cycle progression genes including Cyclins E, A, and B (Dyson 1998; Nevins 2001; Trimarchi and Lees 2002; Blais and Dynlacht 2004; Bracken et al. 2004). Since the altered Cyclin gene expression profile in Taspase1-null cells mirrored the defects associated with impaired E2F activities, we envisioned that cleaved MLL might target to these Cyclin genes through E2Fs in that MLL has no sequence-specific DNA-binding ability. Potential interactions between MLL and all six E2F family members were examined by coimmunoprecipitation assays. Remarkably, MLL interacts with E2F2, E2F4, and E2F6 with different affinities [Fig. 7A]. Of note, E2F6 was recently copurified with MLL in the WDR5-associated protein complex (Dou et al. 2005). As MLL2 shares all the conserved domains with MLL, interactions between MLL2 and E2Fs were also tested. Surprisingly, MLL2 binds to a different subset of E2Fs, namely E2F2, E2F3, E2F5, and E2F6 [Fig. 7B]. Distinct interactions between E2Fs and MLLs suggest unique yet redundant roles played by individual MLL proteins in the cell cycle regulation. To further characterize the interaction between MLL and E2F2, deletion mutants of E2F2 were coexpressed with MLL before subjected to coimmunoprecipitation assays. We mapped the marked box and leucine zipper-containing region (amino acids 200–349) of E2F2 that mediates its interaction with MLL [Fig. 7C]. Direct interactions were confirmed by GST pull-down assays. GST-E2F2 [amino acids 210–349] specifically coprecipitated fragments of MLL that encode conserved domains, amino acids 1401–2158 and amino acids 3656–3969 [Fig. 7D]. E2F proteins have been divided into activator E2Fs (E2F1, E2F2, and E2F3a) and repressor E2Fs (E2F3b, E2F4, E2F5, and E2F6). As MLLs interact with both classes of E2Fs, it is conceivable that these interactions may contribute to organized activation of E2F targets in addition to Cyclins. Interestingly, recent studies also revealed the roles of E2F proteins in the expression of Hox genes and E2F6 deficiency leads to homeotic transformations in mice (Storre et al. 2002).

MEFs bearing noncleavable alleles of MLL and MLL2 are impaired in proliferation

The altered histone H3 K4 trimethylation, the direct MLL targeting, and the direct interactions between MLLs and E2Fs support the hypothesis that MLL proteins are crucial substrates for Taspase1 in regulating cell cycle progression. To investigate the biological significance of MLL and MLL2 proteolysis, we generated knock-in mice bearing homozygous non cleavable alleles (nc) of MLL and/or MLL2 in which Taspase1 cleavage sequences are mutated [data not shown]. The absence of individual proteolysis in MEFs derived from MLLnc/nc and/or MLL2nc/nc knock-in mice was confirmed by Western blot analyses [Fig. 8A]. Proliferation curves were obtained with primary MEFs carrying homozygous MLL and/or MLL2 noncleavable alleles [Fig. 8B]. Noncleavage of MLL, but not MLL2 alone, led to a significant proliferation defect. However, the specific role of MLL2 cleavage in cell proliferation was evident in that further impairment in proliferation was observed in cells carrying combined homozygous MLL and MLL2 noncleavable alleles (MLLnc/nc; MLL2nc/nc). However, the proliferation defect appeared more profound in Taspase1−/− than MLLnc/nc; MLL2nc/nc cells. These data raise the possibility that Taspase1 processes additional substrate[s] other than MLL and MLL2 to fully coordinate cell cycle progression. Our data are consistent with a model in which MLLs, following a cleavage-mediated activation by Taspase1, target distinct genetic pathways that control cell proliferation.
Cyclin genes through interactions with E2Fs, resulting in methylation of histone H3 at K4 and activation of these cell cycle progression genes. The up-regulation of p16Ink4a in Taspase1-null cells could be related to non-cleavage of MLLs, but apparently involves a different mechanism.

**Taspase1**<sup>−/−</sup> cells are resistant to oncogenic transformations and Taspase1 is overexpressed in many cancer cell lines

After establishing the importance of Taspase1 in cell proliferation, one critical question is whether Taspase1 plays a role in oncogenesis. To examine this possibility, we tested whether deficiency of Taspase1 confers resistance to oncogenic transformation. **Taspase1**<sup>−/−</sup> cells are significantly more resistant to E1a/Ras, Myc/Ras-, and DNp53/Ras-induced transformation, determined by the colony formation on soft agar [Fig. 8C]. Of note, **Cyclin** E1<sup>−/−</sup>;2<sup>−/−</sup> MEFs also showed resistance to oncogenic transformation [Geng et al. 2003] and mice with increased gene dosage of p16<sup>ink4a</sup>/Arf locus also showed resistance to cancer development [Matheu et al. 2004]. Furthermore, Western blot analyses of the NCI-60 panel of cells demonstrated that Taspase1 is commonly overexpressed in cancer cell lines [Fig. 8D].

**Discussion**

Site-specific proteolysis is involved in multiple biological processes. The identification of the evolutionarily conserved Taspase1 in processing MLL suggests a critical regulation implemented by Taspase1 in the proper execution of affected signaling pathways. Here we report the first loss-of-function genetic analysis of Taspase1 in mice. Taspase1-deficient newborns exhibit classic homeotic transformations, confirming the role of Taspase1 in regulating body patterning. Importantly, our analyses of Taspase1<sup>−/−</sup> mice uncover a surprising connection between Taspase1 and cell proliferation. There are several lines of phenotypic evidence indicating an intrinsic role of Taspase1 in cell cycle control. First, in a whole organism setting, Taspase1-null mice are smaller than their wild-type littermates. Second, MEFs deficient for Taspase1 proliferate poorly. Third, acute deletion of Taspase1 in vivo results in impaired expansion of DP thymocytes, confirming that Taspase1 functions in a cell autonomous fashion. Mechanistic studies revealed a deregulated expression of key cell cycle regulators, including Cyclins E, A, and B, and p16<sup>ink4a</sup>. We show a direct regulation of these genes by Taspase1 through E2Fs for cell cycle control. There are several lines of evidence indicating that Taspase1 plays a role in oncogenesis. To examine this possibility, we tested whether deficiency of Taspase1 confers resistance to oncogenic transformation. **Taspase1**<sup>−/−</sup> cells are significantly more resistant to E1a/Ras, Myc/Ras-, and DNp53/Ras-induced transformation, determined by the colony formation on soft agar [Fig. 8C]. Of note, **Cyclin** E1<sup>−/−</sup>;2<sup>−/−</sup> MEFs also showed resistance to oncogenic transformation [Geng et al. 2003] and mice with increased gene dosage of p16<sup>ink4a</sup>/Arf locus also showed resistance to cancer development [Matheu et al. 2004]. Furthermore, Western blot analyses of the NCI-60 panel of cells demonstrated that Taspase1 is commonly overexpressed in cancer cell lines [Fig. 8D].
Interestingly, recent genetic studies in Drosophila also implicate a positive role played by trithorax, the invertebrate counterpart of vertebrate MLL, in facilitating cell proliferation. Drosophila embryos carrying a null (trxB11) and a hypomorphic allele (trxE3 or trxJY16) of trithorax displayed impaired BrdU incorporation in larval brains (Muyrers-Chen et al. 2004). These hypomorphic alleles encode a noncleavable (trxE3) and a SET domain deleted (trxJY16) trithorax (Breen 1999), indicating the requirement of Taspase1-mediated proteolytic activation and HMT activity for a full trx function. Although MLL has been shown to activate p27Kip1 expression in SV40 trans-
formed MEFs, whether this reflects the physiological function of MLL remains to be determined (Milne et al. 2005; Xia et al. 2005). The early embryonic lethality associated with MLL homozygous disruption precludes detailed studies of its function in the cell cycle (Yu et al. 1995). Our studies on Taspase1−/− and MLLneo/− cells provide clear genetic and biochemical evidence that mammalian MLLs regulate cell cycle progression, which is further modulated by Taspase1.

Layers of modulation between cell proliferation sensors (e.g., receptor tyrosine kinases) and executors (e.g., E2Fs) help integrate signals to organize temporally controlled reciprocal expression of activators (e.g., Cyclins) and inhibitors (e.g., CdkIs), ensuring an accurate cell cycle decision. Taspase1-mediated cell cycle regulation provides a novel paradigm in which a site-specific protease participates in orchestrating these events through cleavage of nuclear factors for gene regulation (Fig. 8E). In the absence of Taspase1, cell cycle progression is deregulated, with decreased Cyclins and increased p16Ink4a. Although Taspase1 deficiency does not affect Cyclin D expression, up-regulated p16Ink4a inhibits D type Cdk complexes. Furthermore, reduced expression of Cyclin E, A, and B affects the activity of respective Cyclin/Cdk complexes. Through panoramic inactivation of different classes of Cyclin/Cdk complexes, Taspase1 inhibition apparently provides a more efficient targeting strategy than those antagonizing individual CdkIs. Importantly, acute deletion of Taspase1 did not lead to lethality in mice, which further validate its potential use as a drug target. Analysis of the requirement for Taspase1 in tumor initiation and/or maintenance using a murine tumor model will further address the feasibility of targeting Taspase1 as anticancer therapeutics.

Taspase1, a member of the Asparaginase_2 family, apparently evolves from enzymes metabolizing asparagine or N-linked glycoproteins to cleave nuclear factors after an aspartate to activate their full transcriptional regulation. Through inactivation of the extent of biological processes regulated by Taspase1, it is tempting to suggest the existence of additional unidentified Taspase1 substrates whose functions are modulated by proteolysis. Systematic identification of such substrates is critical for a full appreciation of the extent of biological processes regulated by Taspase1. Proteases execute their functions through irreversible protein modifications. Thus, their activity tends to be tightly regulated. Although Taspase1 seems to exert its function constitutively, it is foreseeable that such an activity may be regulated under specific circumstances. Future studies on identifying novel Taspase1 substrates, characterizing the biology of proteolysis of each substrate, and investigating the regulation of each proteolytic event will shed light on how Taspase1, an evolutionarily conserved protease, integrates multiple signaling pathways through site-specific proteolysis.

**Materials and methods**

**Targeting the Taspase1 genomic locus**

The murine Taspase1 genomic locus sequences were obtained from the Ensembl database [http://www.ensembl.org]. The targeting construct has a loxP site upstream of exon 9. A loxP-flanked pPGKneo cassette was inserted into the intron between exons 9 and 10. Successfully targeted RW4 embryonic stem (ES) cells [129SvJ] were determined by Southern blot analyses, followed by a transient transfection with CMV-Cre to generate Taspase1−/− ES clones with a deletion of exon 9 and pPGKneo. ES cells with a heterozygous deletion of Taspase1 were injected into C57B/6 blastocysts.

**In vitro cleavage assays and quantitative RT–PCR analyses**

In vitro cleavage assays and quantitative RT–PCR analysis were performed as described with minor modifications (Hsieh et al. 2003a). Fifty micrograms of whole-cell extracts were incubated with 1 μg of purified rTaspase1 before being subjected to Western blot analyses. Oligo sequences are included in the Supplemental Material.

**Skeletal studies**

P1 newborns were sacrificed and stained as described using Alizarin red and Alcian blue for bone and cartilage, respectively (Yu et al. 1995).

**Cell cycle analyses**

Primary MEFs were pulsed with BrdU for 1 h and analyzed by FACSCalibur according to the manufacturer’s manual (BD Pharmingen, FITC BrdU Flow Kit). To assess the quiescent population, asynchronous cells were cultured with BrdU for 24 h before analysis.

**Acute deletion of Taspase1 in vivo**

Mice at 6 wk of age with indicated genotypes were administered with three 400-μg intraperitoneal injections of pl-pC at days 1, 3, and 5. Treated mice were sacrificed and analyzed 7 d after final injection. The acute deletion of Taspase1 in thymus was assessed by PCR analyses.

**Cell cycle re-entry experiments**

Primary MEFs were serum deprived for 72 h in DMEM supplemented with 0.1% fetal bovine serum (FBS) before being refed with full medium supplemented with 10% serum for indicated periods of time and labeled with Tritium thymidine 1 h before harvest. Experiments were performed as described (Kozar et al. 2004). To determine the percentage of cells that actually reentered cell cycle, serum-deprived cells were stimulated with full medium supplemented with 10% serum in the presence of BrdU for 28 h.

**RNAi-mediated knockdown of p16 in MEFs**

ShRNA specifically against mouse p16 [TRCN77816] was purchased from Mission shRNA clones (Sigma) and shRNA against firefly luciferase was used as a control. Wild-type and Taspase1 coordinates cell proliferation
pase1−/− P1 MEFs were infected with lentivirus expressing the indicated shRNA for 2 d before being subjected to an antibiotics selection with puromycin at 1 μg/mL.

**ChIP assays**

ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology). Antibodies utilized include anti-CT MLL, anti-histone H3 [trimethyl K4, abcam 88500], and anti-histone H3 [acetyl K9/K14, Upstate Biotechnology 06-599]. Analyses were performed on ABI 7200 using gene-specific TaqMan probes. A GAPDH probe used as a negative control was previously described (Milne et al. 2005). Probe sequences are included in the Supplemental Material.

**HMT assays and on-bead in vitro proteolysis of MLLFL**

The on-bead HMT assay has been described with minor modifications (Hughes et al. 2004). Three milligrams of cellular extracts from passage 3 asynchronous primary MEFs were incubated with or without 10 μg of anti-CT MLL antibody overnight at 4°C with gentle rocking. Formed immune complexes were precipitated with 60 μL of protein A beads [Amersham Biosciences] for 3 h at 4°C. Bead-bound MLL complexes were split to two tubes: one for HMT assay, and one for on-bead cleavage assay. In brief, HMT assays include bead-bound MLL complexes, 2 μg of histone H3 [Roche], and 2 μL of SAM [Amersham Biosciences]. Bead-bound MLL proteins were washed with cleavage buffer, incubated with 2 μg of rTaspase1 for 6 h to ensure a complete in vitro proteolysis of MLLFL, resolved on SDS-PAGE, and detected by anti-CT MLL antibody.

**Coimmunoprecipitation and GST pull-down assays**

Coimmunoprecipitation assays were performed as described [Hsieh et al. 2003a]. For GST pull-down assays, 5 μg of purified GST or GST-E2F2 [amino acids 201–349] were bound to 30 μL of Glutathione beads [Amersham Biosciences] before incubating with 85S-methionine-labeled MLL fragments [Promega TNT T7 Quick-Coupled Transcription/Translation Kit]. Coprecipitated MLL fragments were subjected to SDS-PAGE and visualized by autoradiography. Pull-down assays were performed in the presence of 0.2% NP40, 140 mM NaCl, 50 mM Tris-HCl [pH 8.0], 100 mM NaF, and 200 μM Sodium Orthovanadate.

**NCI-60 cancer cell lines**

Cell pellets from the NCI human tumor cell line panel were provided by the Developmental Therapeutics Program of the NCI. The Taspase1 expression level was analyzed by ImageGauge [Fujifilm].

**Acknowledgments**

We thank S. Diane Hayward and Lee Ratner for critical comments on this manuscript. We thank Pat Nakatani, Kei-ichiro Ishiguro, Piotr Sicinska, Katarzyna Kozar, Qunyan Yu, and Yang Geng for providing reagents and technical advice. We thank Sheila Stewart for providing the control shRNA plasmid and Susan Holbeck for providing NCI-60 cell pellets. E.H.C. is supported by Searle Scholars Program, Mallinckrodt Jr. Foundation, and NCI Howard Temin Award. This work is supported by Mallinckrodt Jr. Foundation, NCI Howard Temin Award, and CA R01-119008 to J.J.H.
Taspase1 coordinates cell proliferation

and with the hoxc locus. *Mol. Cell* **13**: 587–597.

Huntsman, D.G., Chin, S.F., Muleris, M., Batley, S.J., Collins, V.P., Wiedemann, L.M., Aparicio, S., and Caldas, C. 1999. MLL2, the second human homolog of the *Drosophila* trithorax gene, maps to 1q141.3 and is amplified in solid tumor cell lines. *Oncogene* **18**: 7975–7984.

Itahana, K., Dimri, G., and Campisi, J. 2001. Regulation of cellular senescence by p53. *Eur. J. Biochem.* **268**: 2784–2791.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. 1999. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* **397**: 164–168.

Julien, E. and Herr, W. 2003. Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1. *EMBO J.* **22**: 2360–2369.

Khan, J.A., Dunn, B.M., and Tong, L. 2005. Crystal structure of human Taspase1, a crucial protease regulating the function of MLL. *Structure* **13**: 1443–1452.

Kmita, M. and Duboule, D. 2003. Organizing axes in time and space: 25 years of colinear tinkering. *Science* **301**: 331–333.

Kozar, K., Ciernyehy, M.A., Rebel, V.I., Shigematsu, H., Zagózdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R.T., et al. 2004. Mouse development and cell proliferation in the absence of D-cyclins. *Cell* **118**: 477–491.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. 1995. Inducible gene targeting in mice. *Science* **269**: 1427–1429.

Ma, D., Watanabe, H., Mermelstein, M., Admon, A., Oguri, K., Sun, X., Wada, T., Imai, T., Shiroya, T., Reinberg, D., et al. 1993. Isolation of a CDNA encoding the largest subunit of the Ink4a locus. *Nature* **364**: 419–422.

Mazo, A.M., Huet, D.H., Mozer, B.A., and Dawid, I.B. 1990. The trithorax gene, a *trans*-acting regulator of the bithorax complex in *Drosophila*, encodes a protein with zinc-binding domains. *Proc. Natl. Acad. Sci. USA* **87**: 2112–2116.

McGinnis, W. and Krumlauf, R. 1992. Hox genes and axial patterning. *Science* **256**: 872–930.

McGinnis, W. and Krumlauf, R. 1992. Homeobox genes and developmental processes. *Science* **256**: 872–930.

McNulty, S., Sedkov, Y., Smith, S., Tillib, S., Kraevsky, V., Nakamura, T., Canaani, E., Croce, C.M., and Mazo, A. 2001. Tri-
Proteolysis of MLL family proteins is essential for Taspase1-orchestrated cell cycle progression

Shugaku Takeda, David Y. Chen, Todd D. Westergard, et al.

*Genes Dev.* 2006, 20: Access the most recent version at doi:10.1101/gad.1449406

**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2006/09/05/20.17.2397.DC1

**References**
This article cites 53 articles, 24 of which can be accessed free at:
http://genesdev.cshlp.org/content/20/17/2397.full.html#ref-list-1

**License**

**Email Alerting Service**
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.