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Mice Heterozygous for Germline Mutations in Methylthioadenosine Phosphorylase (MTAP) Die Prematurely of T-cell Lymphoma

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Abstract

Large homozygous deletions of 9p21 that inactivate *CDKN2A*, *ARF*, and *MTAP* are common in a wide variety of human cancers. The role for *CDKN2A* and *ARF* in tumorigenesis is well established, but whether *MTAP* loss directly affects tumorigenesis is unclear. *MTAP* encodes the enzyme methylthioadenosine phosphorylase, a key enzyme in the methionine salvage pathway. To determine if loss of *MTAP* plays a functional role in tumorigenesis, we have created an *MTAP*-knockout mouse. Mice homozygous for a *MTAP* null allele (*Mtap^{lacZ}*) have an embryonic lethal phenotype dying around day 8 post-conception. *Mtap/Mtap^{lacZ}* heterozygotes are born at Mendelian frequencies and appear indistinguishable from wild-type mice during the first year of life, but they tend to die prematurely with a median survival of 585 days. Autopsies on these animals reveal that they have greatly enlarged spleens, altered thymic histology, and lymphocytic infiltration of their livers, consistent with lymphoma. Immunohistochemical staining and FACS analysis indicate that these lymphomas are primarily T-cell in origin. Lymphoma infiltrated tissues tend to have reduced levels of *Mtap* mRNA and MTAP protein, and unaltered levels of methyldeoxycytidine. These studies show that *Mtap* is a tumor suppressor gene independent of *CDKN2A* and *ARF*.

Keywords

Cancer; Tumor Suppressor Gene; Methionine; Embryonic Lethal

Introduction

A quarter century ago, Toohey first recognized that certain murine malignant hematopoietic cell lines lacked methylthioadenosine phosphorylase (MTAP) activity (1). MTAP is a

metabolic enzyme in the methionine salvage pathway that converts the polyamine byproduct 5'-dideoxy-5'-methylthioadenosine (MTA) into adenine and methylthioribose-1-phosphate and is expressed in all tissues throughout the body (2,3). Loss of MTAP has been shown to result in increased accumulation of MTA, a known inhibitor of S-adenosylmethionine-dependent methyltransferases (2,4). In addition, loss of MTAP is known to affect the production of polyamines due to upregulation of ornithine decarboxylase (5).

Today we know that loss of MTAP is frequent in a large number of different human tumors including leukemias, lymphomas, mesothelioma, lung carcinoma, pancreatic carcinoma, squamous cell carcinoma, biliary tract cancer, glioblastoma, osteosarcoma, and neuroendocrine tumors (6–18). Loss rates range from 14% to 100% depending on the tumor type and the method used to assess MTAP loss.

MTAP is frequently inactivated in human tumors by large homozygous deletion of the 9p21 region where both *MTAP* and the *CDKN2A/ARF* tumor suppressor gene complex are located (19). Since these deletions generally inactivate *CDKN2A/ARF* as well as *MTAP*, it has been hypothesized that loss of *MTAP* in tumors was simply due to it being a co-incident bystander. However, there is a growing body of data that suggests this may not be the case. Re-expression of *MTAP* in *MTAP*-deleted MCF-7 breast cells results in loss of anchorage independent growth *in vitro*, and loss of tumor formation *in vivo* when these cells were injected subcutaneously in SCID mice (20). In addition, expression of *MTAP* in a *MTAP*-deleted melanoma cell line resulted in reduced invasion as measured in a Boyden Chamber assay (21). Finally, in non-small cell lung cancer and in astrocytomas, loss of *MTAP* has been observed in cells that retain *CDKN2A/ARF* (8,9). Taken together, these observations suggest that *MTAP* may function as a tumor suppressor gene in its own right.

To test the idea that *MTAP* is a tumor suppressor gene, we have engineered a mouse with a germline mutation in the mouse *MTAP* gene (*Mtap*). We found that homozygosity for *MTAP* results in an early embryonic lethal phenotype, while heterozygosity often results in premature death by T-cell lymphoma. Our findings support the view that *MTAP* is a bona fide tumor suppressor gene.

Materials and Methods

Creation of *MTAP*-knockout mouse

RRK081 cells (SV129/Ola background) heterozygous for the *Mtap^{lacZ}* allele were obtained from Bay Genomics (<http://baygenomics.ucsf.edu>) and injected into C57BL6 blastocysts by microinjection. Eighty 3.5-day-old blastocyte embryos were then implanted into pseudo-pregnant C57BL6 mice. Four chimeric offspring were obtained and two exhibited germline transmission. Heterozygous offspring from these mice were then used for intercrosses to generate homozygous offspring, and were repeatedly backcrossed to C57BL6. Mice were fed Harlan Tekland 2018SX diet *ad libitum*. For survival analysis, mice were monitored daily and Kaplan-Meier analysis was performed using GraphPad Prism 4.0. All studies were approved by the Fox Chase Cancer Center animal use committee.

Genotyping

Mtap^{lacZ} was genotyped using two different methods. Initially genotyping was done at the RNA level. RNA was extracted from tail snips using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription-PCR was done using the SuperScript One-step Reverse Transcription-PCR kit (Invitrogen) and oligos 5' CGGTGAAGATTGGAATAATTGGTG-3' (sense), 5'-GACAGTATCGGCCTCAGGAAGATCG (antisense) and 5'-

CTGTCAATGAATTGGTCAATGACCATG-3' (antisense) for the amplification of *Mtap*⁺ and *MTAP*^{lacZ} bands of 322 bp and 421 bp, respectively. After an initial reverse transcription phase at 52°C for 30 min, PCR amplification of cDNA was performed with an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 5 min.

Later, a DNA based assay was developed using primers 5'CAGGACAAATTGTGGGTC-TAAG-3' (forward), 5'-GCAACAAGGACAGCCCAAGGAGATC-3' (reverse 1) and 5'-ACTTGAGACCTTCTTTCTGGTCTTTC-3' (reverse 2) to obtain either a *Mtap* band of 381 bp (with reverse 2) or a *Mtap*^{lacZ} band of 289 bp (with reverse 1). PCR reactions were carried out in a total volume of 25 µl reaction mixture containing 100 ng genomic DNA as a template, 1× PCR buffer (10 mM Tris-HCl, PH 8.0/50 mM KCl/1.5 mM MgCl₂), 250 µM of each of four deoxynucleoside triphosphates, 10 ng each of sense and antisense primers, and 2.5 units of Taq DNA polymerase (Invitrogen). The thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s.

Timed Matings

Female and male animals were paired in the evening and checked early in the morning for the presence of a vaginal plug. If a plug was observed, animals would be sacrificed after 7.5, 8.5, 9.5, and 12.5 days.

Immunohistochemistry

Autopsied materials were fixed in buffered formalin and embedded in paraffin. The paraffin blocks were cut into 5-µm-thick sections that were placed on positively charged slides. The sections were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then done in 10 mmol/L sodium citrate (pH 6.0) in a microwave for 10 min. The endogenous peroxidase activity was blocked by immersing the slides in 3% H₂O₂ in PBS for 30 min. After 30 min of incubation with goat blocking serum, slides were incubated with a 1:250 dilution of MTAP rabbit polyclonal antibodies (kindly provided by Dr. Schramm, (22)) or a 1:150 dilution of CD3 rabbit polyclonal antibodies (Dako, Carpinteria, CA) at 4°C overnight followed by incubation with goat anti-rabbit biotinylated secondary antibody for 30 min at room temperature followed by streptavidin peroxidase for 30 min at room temperature. 3,3'-Diaminobenzidine (Sigma-Aldrich, St. Louis, MO) substrate chromogen was applied for 4 min until the brown color developed. In the case of CD45, we used 1:200 dilution of mouse monoclonal biotinylated antibodies (BD Biosciences, San Jose, CA) and did not use secondary antibody. The slides were counterstained with hematoxylin and mounted with Permount (Fischer Scientific, Pittsburgh, PA).

FACS analysis

FACS analysis on spleen from euthanized animals was performed as previously described (23). Analysis was performed using a BD Biosciences LSR II/DiVa flow cytometer, equipped with three laser excitation (405, 488, 630 nm). Staining combinations were performed on single cell suspensions of spleen and included FI anti-CD8 (53-6), PE anti CD3 (500A-A2), APC anti-CD4 (GK1.5), Cy5PE TCR beta (H57-597). All reagents were made in the laboratory of Richard R. Hardy, except for DX5 and TCR-beta, which were purchased from eBioscience.

Southern Analysis

Genomic DNA was extracted using DNA isolation kit (Puregene) from spleens of diseased and normal animals. Approximately 20 µg of genomic DNA was digested using restriction

enzyme *HindIII* (for T cell lineage clonality assays of the TCR β locus), and probed according to standard Southern blot protocol with TCR β probe (3'J β 2 probe for the J β 2 cluster, (24)). Genomic DNA from tissue or cell lines with unrearranged germ line configuration of each antigen receptor locus was used as negative controls.

Real-time PCR

MTAP, *p16* and *β -actin* were measured using Taqman technology on an ABI Prism 7900 Sequence Detection System. Total RNA was extracted using the PicoPure RNA kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. First-strand cDNA was generated from total RNA from each sample using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were prepared in triplicate for each gene using Taqman Gene Expression Master Mix and the following Taqman Gene Expression Assays (Applied Biosystems): *Mtap* (Mm01257901_m1), *Cdkn2a/Arf* (Mm00494449_m1) and *β -actin* (Mm00607939_s1). Plates were loaded and reactions were cycled using Taqman universal cycling conditions according to the manufacturer's instructions. During thermal cycling, the threshold cycle (C_t) was determined for each sample by taking the average of the three replicates. The average C_t value for the control gene *β -actin* was subtracted from the average C_t value for each target gene (*Mtap* and *cdkn2a/Arf*) to normalize the amount of sample RNA added to the reaction. The comparative C_t (C_t) method was used to determine the level of the target gene mRNA in heterozygous animals samples relative to the level found in the normal sample (Applied Biosystems User Bulletin #2, October 2001): relative quantification = 2^{-C_t} , where C_t = average C_t (diseased) – average C_t (normal).

MTAP enzyme assay

MTAP enzyme activity in the liver extract was measured as described previously (20). A unit of MTA phosphorylase activity is defined as the enzyme amount that catalyzes the formation of 1 μ mol of adenine/min under the conditions of the assay described.

CGH analysis

According to the manufacturer's protocol for Agilent Oligonucleotide Array-based CGH for Genomic DNA Analysis Version 4.0 (Agilent Technologies, Santa Clara CA), three micrograms of high quality genomic DNA was digested with restriction endonucleases, *AluI* and *RsaI* and digested genomic DNA was labeled using Agilent Genomic DNA Labeling Kit PLUS. Test and reference DNA samples were labeled with either cyanine 5- or cyanine 3-dUTP, according to the manufacturer's protocol. Cyanine 5- and cyanine 3-labeled DNA products were then purified using Microcon YM-30 filtration devices. The DNA yield and level of dye incorporation were measured using the ND-1000 Spectrophotometer. Appropriate cyanine 5- and cyanine 3-labeled DNA sample pairs were combined and then mixed with mouse Cot-1 DNA, Agilent 10X Blocking Agent, and Agilent 2X Hybridization Buffer. The labeled target solution was hybridized to Agilent's 244K Mouse Genome CGH microarray (G4415A) using SureHyb chambers. After hybridization the microarrays were washed and dried according to the procedures described in Agilent's protocol. Microarray slides were scanned immediately using an Agilent microarray scanner. Data for individual features on the microarray were extracted from the scan image using Agilent's Feature Extraction (FE) Software. Output files from FE were subsequently imported into Agilent's CGH data analysis software, CGH Analytics for DNA copy number analysis.

Methyldeoxycytidine Quantitation

Percent of methyldeoxycytidine present in genomic DNA was determined by LC-MS/MS as described (25).

Results

Homozygosity for *MTAP^{lacZ}* causes early embryonic lethality

RRK081 embryonic stem cells, containing a gene-trap vector inserted between exon 3 and exon 4 of the mouse *Mtap* locus (*Mtap^{lacZ}*; Supplemental Figure 1), were injected into mouse blastocysts derived from C57BL/6 animals and the embryos were implanted in pseudopregnant females. Four chimeric offspring were obtained and these animals were then bred to C57BL/6 animals to establish germline transmission. Two of the chimeras exhibited germline transmission.

Heterozygous *Mtap^{lacZ/+}* offspring were then intercrossed to determine the phenotype of a homozygous animal. We failed to obtain a single homozygous offspring out of 165 that were genotyped. Analysis of the heterozygous animals for MTAP enzyme activity in the liver showed that these animals had on average 31% activity compared to the wild-type animals (n=6 per group, p<0.0003), indicating that the insertion allele is a complete loss of function mutation. Taken together, these findings indicate that lack of MTAP activity results in an embryonic lethal phenotype.

We performed timed mating experiments to determine exactly when during development *Mtap^{lacZ}* homozygotes were being lost (Supplemental Table 1). We failed to observe any homozygous embryos present in the uteri at post-conception day 12.5 and day 9.5 mothers and also found a large number of empty uterine sacs. However, we were able to identify homozygous embryos present in the uteri of day 8.5 and day 7.5 animals. Homozygous embryos present at day 8.5, were much smaller and misformed compared to control heterozygous embryos (Fig. 1a). Both in size and appearance they appeared to resemble day 8 embryos (Theiler stage 12 as opposed to Theiler stage 13 (26)) and were probably in the process of reabsorption. Examination of day 7.5 embryos for MTAP expression by immunohistochemistry reveals that MTAP is expressed ubiquitously throughout the embryo at this time (Figure 1b). These results indicate that *Mtap^{lacZ/Mtap^{lacZ}}* homozygotes are lost early in embryonic development, around post-conception day 8.

Mtap^{lacZ} heterozygotes die prematurely of severe lymphoproliferative disease

Mtap^{lacZ/+} heterozygotes are born at Mendelian frequencies and have no obvious abnormalities. We observed no difference in weight gain between heterozygotes and control animals during the first six months of life (Supplemental Fig. 2a). Because MTAP is involved in methionine and polyamine metabolism, we examined the serum amino acid profiles of overnight fasted *Mtap^{lacZ/+}* and *+/+* animals. We observed no significant differences in the 27 compounds that were measured (Supplemental Fig. 2b).

To determine if there might be any long term detrimental effects of heterozygosity for *Mtap^{lacZ}*, we monitored a cohort of *Mtap^{lacZ/+}* heterozygotes and sibling *+/+* controls for up to 20 months and found that there was a significant difference in the survival of heterozygous animals compared to their wild-type siblings (Fig. 2a). Heterozygotes start dying as early as 200 days and have a median survival of 585 days. We performed necropsies on 21 of these animals and found that at least 60% of the animals showed marked splenomegaly, and often enlargement of the liver and thymus as well (Fig. 2b). Examination of spleen, liver, and thymus sections by H and E staining indicated that the animals suffered from severe lymphoproliferative disease resembling lymphoma (Fig. 2c). In addition, in one animal we also observed hepatocellular carcinoma in the liver.

Characterization of lymphoma cells present in *Mtap^{lacZ}* heterozygotes

To more fully characterize the lymphoma present in *Mtap^{lacZ/+}* animals, we performed immunohistochemical analysis of tissues from heterozygous and sibling wild-type animals. Spleen, thymus and liver were stained with the T-cell marker CD3 or the B-cell marker CD45. In the spleens of all of the 14 heterozygous animals diagnosed with lymphoma at autopsy, we observed increased CD3+ cells compared to control spleen and reduced numbers of CD45+ cells (Fig. 3a). Infiltration of CD3+ cells was also observed in the livers of some of the heterozygous animals (Supplemental Fig. 3). In animals with enlarged thymuses, we observed an expansion of the CD3+ population and a large decrease in the number of CD45+ staining cells (Figure 3b). The increase in CD3+ cells and the decrease in CD45+ cells indicates that *Mtap^{lacZ/+}* animals are succumbing to T-cell lymphoma.

To further characterize the T-cell expansion, we examined the T-cell population in the spleen of aged heterozygous and control animals for CD4 and CD8 content using FACS analysis. For these experiments we sacrificed 11 *Mtap^{lacZ/+}* heterozygotes (average age 713 days) and six +/+ controls (average age 684 days). In these sacrificed animals, we did not observe gross enlargement of the spleen as observed in the naturally deceased animals, and histopathological examination of the spleen showed that most of these animals had much milder lymphoid hyperplasia. However, despite this fact, we found that 4 of the 11 heterozygous animals had CD4+ levels above the 95% confidence range determined by the mean of the control animals (Fig. 4a). We did not observe any animals with CD8 levels outside the control range. These results indicate that heterozygous animals are predisposed to develop expansions of a CD4+ T-cell population.

To determine if the T-cell expansions were monoclonal, we performed southern blot analysis on spleen or thymus DNA isolated from 17 aged heterozygous and 4 sibling wild-type animals using a probe specific for the Db2-Jb2 cluster at the TCR- β locus (Fig. 4b). Five of the 17 heterozygous samples came from autopsy material derived from animals that had exhibited marked splenomegaly and had died of natural causes, while the remaining samples came from the healthy euthanized animals that had mild lymphoid hyperplasia used for the FACS analysis described above. In three of the five autopsy animals (60%) we found evidence of monoclonality, but we did not observe any evidence of monoclonality in the sacrificed animals. These findings show that the majority of heterozygous animals died of monoclonal T-cell lymphoma, but that the appearance of monoclonality occurs late in the disease process.

Loss of MTAP expression in lymphoma cells

We next examined if expression of the wild-type *Mtap* allele in *Mtap^{lacZ/+}* mice was reduced in tissues showing lymphoproliferative disease. First, we performed immunohistochemistry using an anti-MTAP polyclonal antibody on thymus, spleen, and liver from an animal with severe lymphoma and compared staining to a wild-type healthy control animal (Fig. 5a). We observed significantly reduced MTAP staining in all three tissues compared to the control animal, showing that MTAP expression was reduced in all the lymphoid infiltrated tissue. In addition, we examined the spleens of nine other animals that died of lymphoma, and in all cases observed reduced MTAP staining compared to control animals (data not shown).

To determine if down-regulation of MTAP was caused by reduced *Mtap* mRNA, we quantitated *Mtap* RNA by qRT-PCR. Initially, we examined MTAP expression in 5 three-month old *Mtap^{lacZ/+}* and five +/+ control animals that showed no evidence of lymphoid hyperplasia in the spleen. As expected, we found that the heterozygous animals had significantly lower *Mtap* mRNA than the +/+ controls (Fig. 6a). We then examined *Mtap* mRNA levels in the spleens of 13 older (sacrificed) *Mtap^{lacZ/+}* animals with lymphoid hyperplasia. In 12 of the 13 cases we observed *Mtap* mRNA levels below the 95% confidence interval defined by the

younger *Mtap^{lacZ/+}* animals. To eliminate the possibility that *Mtap* mRNA levels decreased with age in healthy animals, we also measured *Mtap* mRNA in five older *+/+* animals without evidence of lymphoid hyperplasia. We did not observe a difference in MTAP mRNA compared to the younger *+/+* animals, indicating that MTAP expression is not lost simply due to aging.

Since chromosomal deletion is a major mechanism for inactivation of *Mtap* in human tumors, we performed comparative genome hybridization analysis (CGH) on DNA isolated from the spleen of three *Mtap^{lacZ/+}* animals exhibiting severe lymphoid hyperplasia. In only one of the three samples (A7) did we see evidence for loss of the *Mtap* region of mouse chromosome 4 (Fig. 5b). This finding suggests that the reduced expression of *Mtap* mRNA observed above may be occurring mostly through epigenetic mechanisms.

Loss of MTAP expression is independent from loss of CDKN2A expression

Since *MTAP* and *CDKN2A/ARF* are often inactivated together in human tumors by large deletions, we also examined *Cdkn2a/Arf* mRNA in the spleens of animals showing lymphoid hyperplasia by qRT-PCR. We found no difference in *Cdkn2a/Arf* mRNA levels in young sibling *+/+* and *Mtap^{lacZ/+}* animals, indicating that the *Mtap^{lacZ/+}* insertion did not effect basal *Cdkn2a/Arf* expression. In older *+/+* animals lacking hyperplasia, we observed a 20% decrease in *Cdkn2a/Arf* expression compared to the younger controls, suggesting some loss of *Cdkn2a/Arf* expression occurs as a result of aging. However, in the older *Mtap^{lacZ/+}* animals we found large variability in *Cdkn2a/Arf* expression. In seven animals we observed significant elevations in *Cdkn2a/Arf* mRNA, while in seven others we observed reduced expression. The lack of correlation between *Cdkn2a/Arf* and *Mtap* mRNA levels in these samples suggests that loss of *Mtap* expression is driving lymphoma formation independent of *Cdkn2a/Arf* in these mice.

Loss of MTAP does not affect global deoxycytidine methylation

One possible mechanism by which loss of MTAP might promote tumorigenesis is by affecting cellular methylation reactions. To determine if loss of MTAP affected cellular methylation, we measured the amount of methyldeoxycytidine present in the DNA isolated from the enlarged spleens of *Mtap* heterozygotes and compared it to spleen DNA isolated from control animals. We did observe a slight decrease in the percentage of DNA incorporated methyldeoxycytidine in control vs. *Mtap* heterozygous animals, but the difference was not statistically significant ($n=5$, 4.3 ± 0.08 vs. 4.04 ± 0.45 , $P=0.33$).

Discussion

In the experiments described above, we have characterized the phenotype of mice homozygous and heterozygous for a null allele of the *Mtap* locus. Aged mice heterozygous for *Mtap^{lacZ}* die prematurely with enlarged spleens, thymuses, and livers that have large numbers of CD3+ T-cells invading the affected organs. These findings show that germline heterozygosity for *Mtap^{lacZ}* predisposes animals to develop T-cell lymphoma, and suggests *Mtap* may be acting as a tumor suppressor gene. If *Mtap* were acting as a tumor suppressor gene, then according to Knudson's two-hit hypothesis (27) tumor cells should inactivate the remaining wild-type gene. Supporting this idea, we observed reduced *Mtap* mRNA in 12 of 13 heterozygous animals examined with splenic lymphoid hyperplasia. Interestingly, in the one animal that had high levels of *Mtap* mRNA, immunohistochemistry indicated a significant loss of *Mtap* protein in affected tissue (see Fig. 5a). This finding suggests that *Mtap* expression can be lost due to transcriptional as well as post-transcriptional mechanisms.

In human tumor derived cell lines the major mechanism of *MTAP* inactivation involves large homozygous deletions of the 9p21 region. These deletions generally affect both *MTAP* and the *CDKN2A/ARF* locus, located approximately 80 Kb away. Examination of *Cdkn2a/Arf* mRNA

by qRT-PCR in the spleens of animals with lymphoid hyperplasia indicate that seven out of 12 animals that had low *Mtap* mRNA had *elevated* levels of *Cdkn2a/Arf*, strongly indicating that in *Mtap^{lacZ}* heterozygous mice, most *Mtap* inactivation is not occurring by large deletions that co-delete *CDKN2A/ARF*. Consistent with this idea, we only observed evidence for homozygous deletion in one of the three mice examined by CGH. These findings support the view that *Mtap* has tumor suppressor qualities independent of *CDKN2A/ARF* and that inactivation of the wild-type *Mtap* allele is occurring primarily by epigenetic mechanisms in these animals. Epigenetic silencing of *MTAP* via promoter hypermethylation has been previously demonstrated to occur in human gastric and lung cancers (28,29).

In humans, loss of *MTAP* has been observed in a wide variety of tumors, including both solid and hematological malignancies. With the exception of a hepatocellular carcinoma observed in one animal, *Mtap/Mtap^{lacZ}* animals only developed T-cell lymphomas. In older heterozygous animals that have not yet died, we observed mild T-cell lymphoproliferative disease that was not clonal, suggesting that loss of *Mtap* might stimulate T-cell proliferation. The increased number of T-cells might increase the chances that a malignant clone may appear that would subsequently then kill the animals. The observation that germline mutations in tumor suppressor genes cause different phenotypes in mice and humans have been observed previously. For example, heterozygosity for *Nf2* tumor suppressor gene results in increased risk of neurologic tumors in humans, but predispose mice to a wide variety of tumors including lymphoma, lung adenocarcinoma, hepatocellular carcinoma and sarcomas (30).

Despite the fact that human and mouse cells lacking *MTAP* appear to grow well in culture, mice homozygous for *Mtap^{lacZ}* are inviable and die early in embryogenesis, around post-conception day 8. Just before this time (post-conception day 7.5) *MTAP* appears to be expressed in all cells throughout the embryo. The day 8 homozygous embryos are severely deformed and much smaller than wild-type embryos suggesting that loss of *MTAP* may be affecting processes involved in mouse organogenesis. Early embryonic lethality in mice homozygous for null mutations has been observed for several mouse tumor suppressor genes including *Rb*, *Wt1*, *Apc*, *Nf2* and *Brca1* (30). Our findings are also consistent with those published by (31), which also found that *Mtap* was an essential gene in mice.

A possible mechanism by which loss of *MTAP* promotes tumorigenesis may be related to its effect on altered methionine metabolism and methylation. Tumor cells appear to have disrupted methionine methylation as manifested by their inability to grow in media in which methionine is replaced by its immediate precursor homocysteine (32). It has been hypothesized that the increased methionine requirements of tumor cells may be due to the high levels of transmethylation observed in tumor cells (33). Interestingly, drugs that inhibit DNA methylation reactions, such as ethionine or 5-Azacytidine, are also pro-tumorigenic. In theory, loss of *MTAP* could effect DNA methylation by two different mechanisms; either intracellular accumulation of the methyltransferase inhibitor MTA, or by reduction in the levels of S-adenosylmethionine due to the lack of a functional methionine salvage pathway. In the experiments reported here, we did not find a significant difference in the methylation status of enlarged spleen DNA isolated from *Mtap^{+/-}* heterozygotes and *Mtap^{+/+}* control animals. However, these studies are limited by the fact that DNA samples were isolated from total tissue and presumably contain a mixture of *Mtap^{-/+}* stromal cells and *Mtap^{-/-}* tumor cells. Although not statistically significant, it is interesting to note that on average the DNA from *Mtap^{+/-}* spleens had slightly reduced methylation and more variability than DNA from the control animals.

In summary, our studies support the idea that *Mtap*, which encodes a basic metabolic enzyme, is a tumor suppressor gene that is essential for normal mouse development. The mice developed

should be useful tools in elucidating the mechanism by which loss of *Mtap* contributes to tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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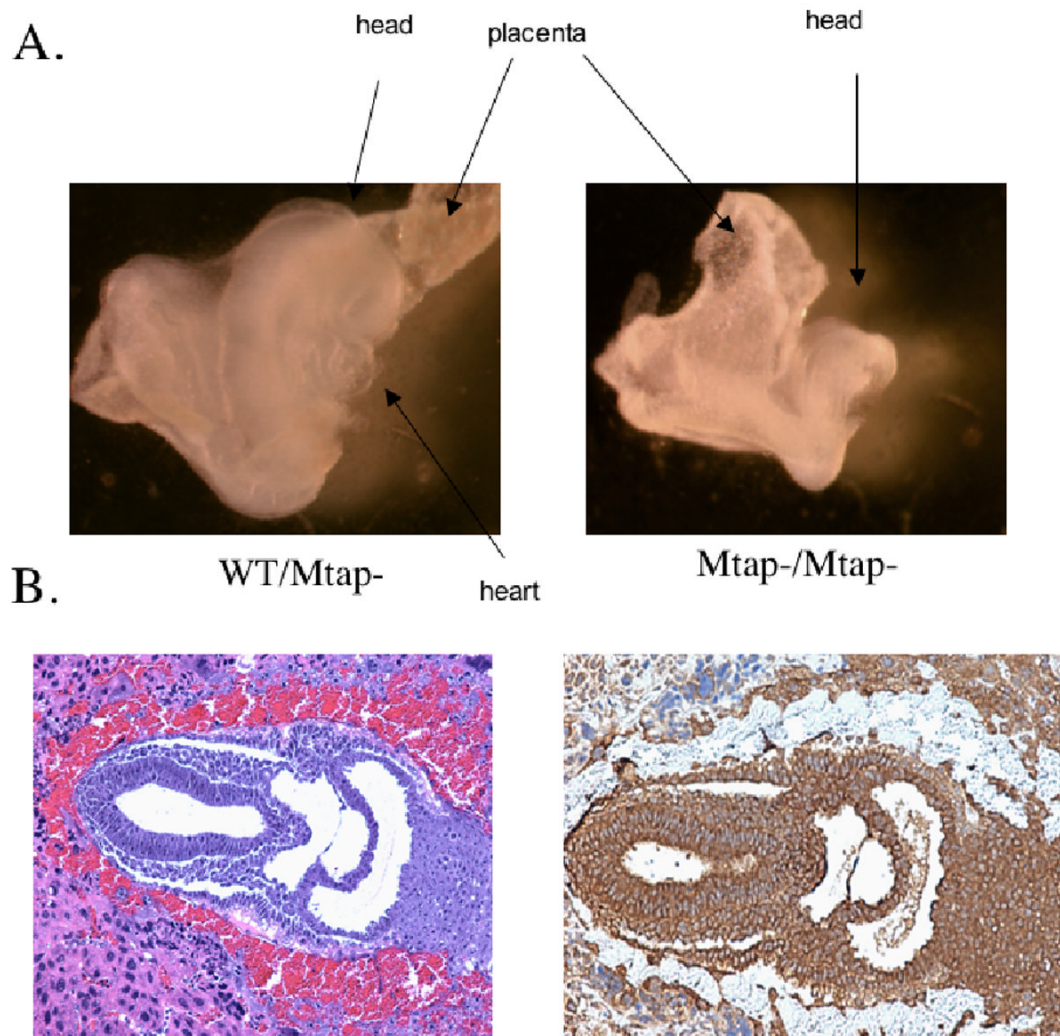


Figure 1.

Mtap is essential during embryogenesis. *A*, Photograph of sibling day 8.5 *Mtap^{lacZ/+}* and *Mtap^{lacZ/Mtap^{lacZ}}* embryos. Photographs taken with 40× magnification. *B*, Wild-type day 7.5 embryos sectioned and stained with H and E (left) or with an anti-MTAP antibody (right). Photographs taken at 400× magnification.

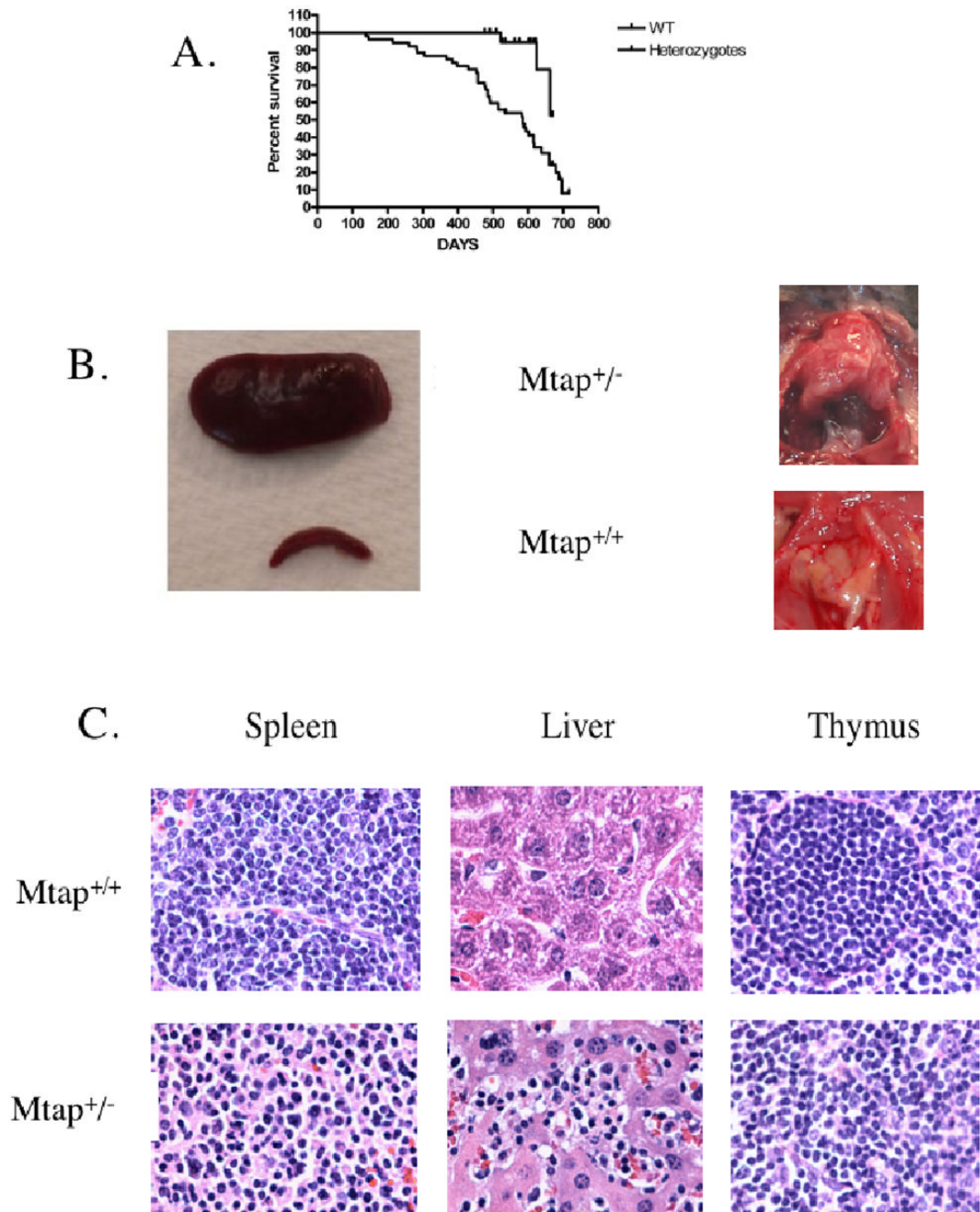


Figure 2. *Mtap^{lacZ/+}* animals die prematurely of lymphoproliferative disease. **A**, Sibling wild-type ($n=23$) and heterozygous animals ($n=57$) were monitored daily and followed for up to 20 months. Tick marks indicate censored observations. Comparison of survival curves using a Logrank test gives a $P<0.0005$. **B**, Spleens and thymus at necropsy of an *Mtap* heterozygote and wild-type control. **C**, Pathology from a control and a heterozygous diseased animal. Sections of spleen, liver, and thymus were stained with Hematoxylin and Eosin and were viewed under $400\times$ magnification. Note the lymphocyte infiltration in the liver and the loss of the follicular architecture in the thymus of heterozygous animals.

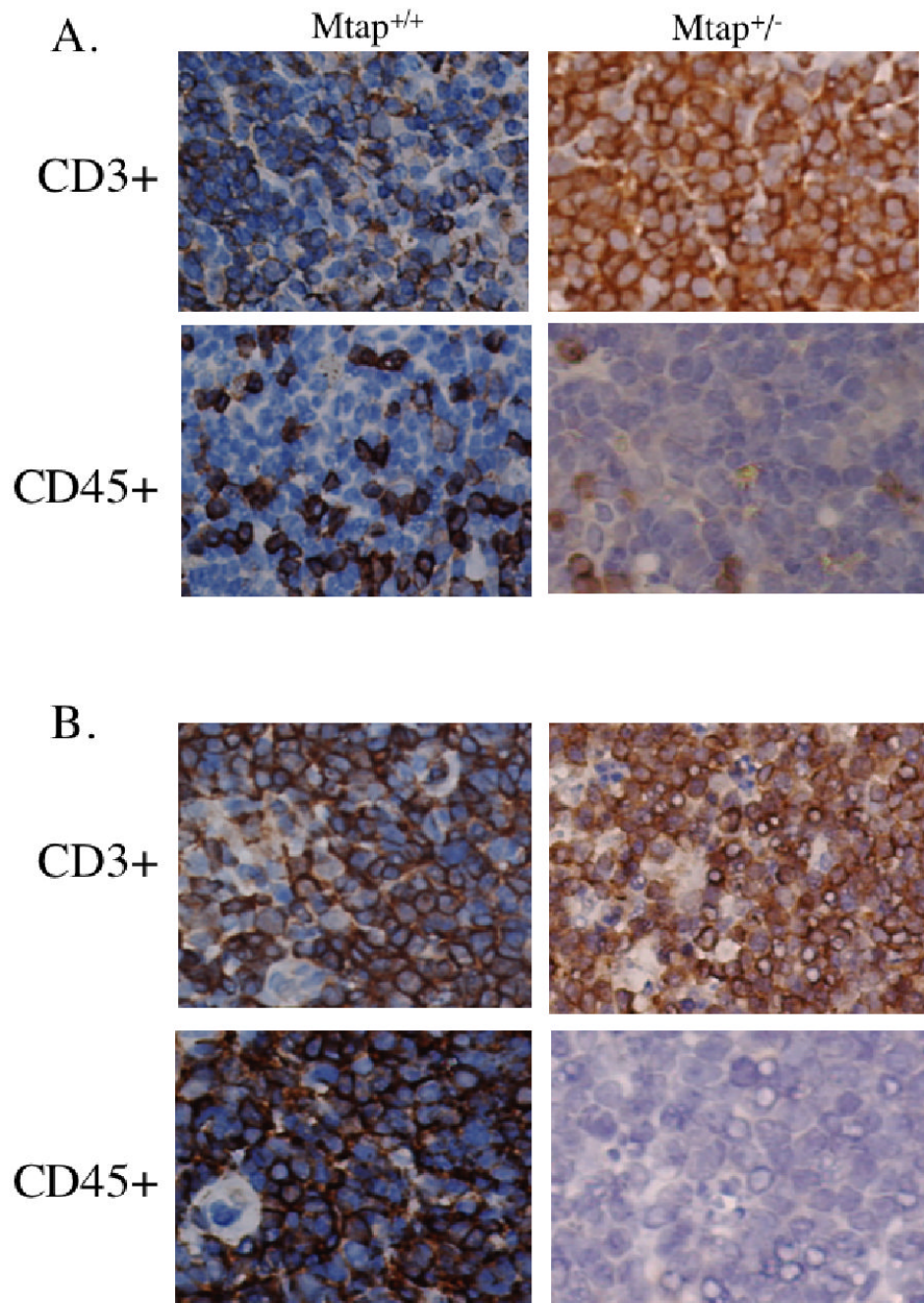


Figure 3. Proliferation of CD3+ T-cells in spleen and thymus. *A*, Spleen of an aged control (sacrificed at 525 days) and *Mtap*^{lacZ/+} animal (deceased at 260 days) stained with either antibody to CD3 + or CD45+ viewed at 400× magnification. *B*, The thymus of the same two animals as above.

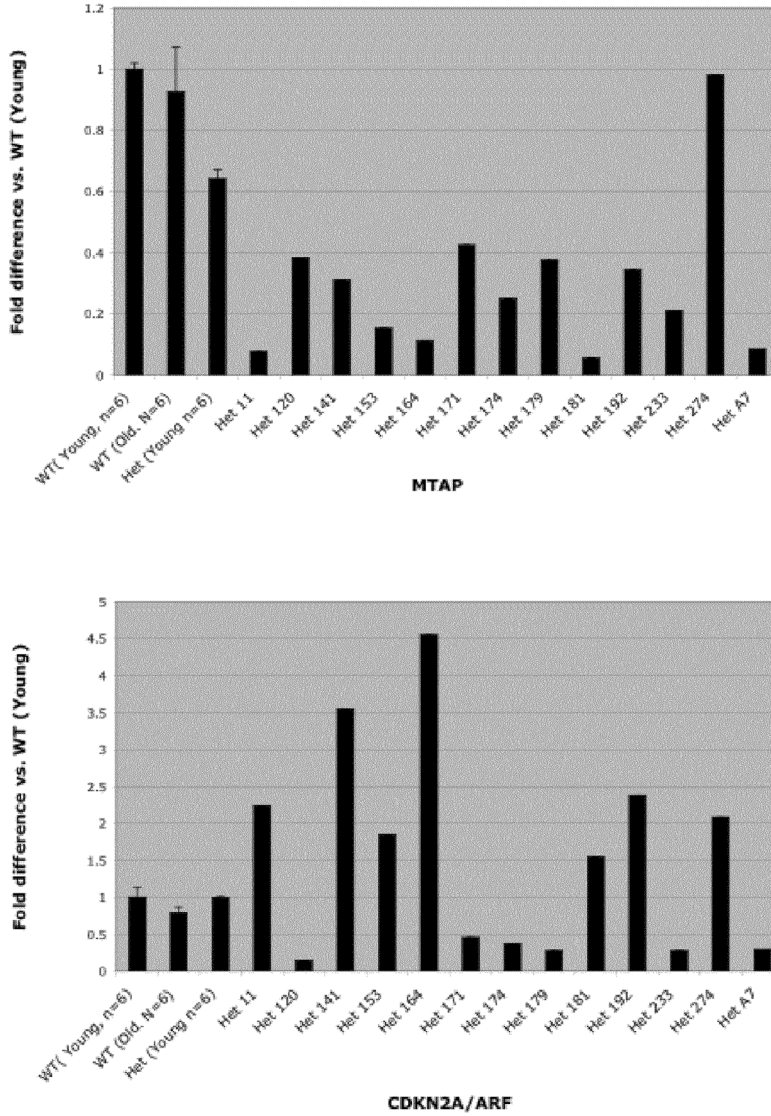


Figure 6. qRT-PCR of *Mtap* and *Cdkn2a/Arf* in aged *Mtap^{lacZ/+}* animals. *A*, MTAP mRNA was quantitated by qRT-PCR (see Methods). Reference ranges were established using young wild-type animals, young heterozygous animals and old wild type animals and the 95% confidence intervals are shown (leftmost three columns). Other columns show relative *Mtap* mRNA levels in each of 13 aged heterozygotes with evidence of lymphoproliferative disease. *B*, Identical to *A*. except that *Cdkn2a/Arf* message was measured.