Germline Mutations in *Mtap* Cooperate with *Myc* to Accelerate Tumorigenesis in Mice

Yuwaraj Kadariya¹, Baiqing Tang¹, Liqun Wang¹, Tahseen Al-Saleem², Kyoko Hayakawa², Michael J. Slifker¹, Warren D. Kruger¹*

¹Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania, Unites States of America; ²Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania, Unites States of America

**Abstract**

**Objective:** The gene encoding the methionine salvage pathway methylthioadenosine phosphorylase (*MTAP*) is a tumor suppressor gene that is frequently inactivated in a wide variety of human cancers. In this study, we have examined if heterozygosity for a null mutation in *Mtap* (*Mtap<sup>lacZ</sup>*) could accelerate tumorigenesis development in two different mouse cancer models, *Eµ*-myc transgenic and *Pten<sup>−/−</sup>*.

**Methods:** *Mtap* *Eµ*-myc and *Mtap* *Pten* mice were generated and tumor-free survival was monitored over time. Tumors were also examined for a variety of histological and protein markers. In addition, microarray analysis was performed on the livers of *Mtap<sup>lacZ/α</sup>* and *Mtap<sup>+/−</sup>* mice.

**Results:** Survival in both models was significantly decreased in *Mtap<sup>lacZ/α</sup>* compared to *Mtap<sup>+/−</sup>* mice. In *Eµ*-myc mice, *Mtap* mutations accelerated the formation of lymphomas from cells in the early pre-B stage, and these tumors tended to be of higher grade and have higher expression levels of ornithine decarboxylase compared to those observed in control *Eµ*-myc *Mtap<sup>+/−</sup>* mice. Surprisingly, examination of *Mtap* status in lymphomas in *Eµ*-myc *Mtap<sup>lacZ/α</sup>* and *Eµ*-myc *Mtap<sup>+/−</sup>* animals did not reveal significant differences in the frequency of loss of *Mtap* protein expression, despite having shorter latency times, suggesting that haploinsufficiency of *Mtap* may be playing a direct role in accelerating tumorigenesis. Consistent with this idea, microarray analysis on liver tissue from age and sex matched *Mtap<sup>+/−</sup>* and *Mtap<sup>lacZ/α</sup>* animals found 363 transcripts whose expression changed at least 1.5-fold (*P*<0.01). Functional categorization of these genes reveals enrichments in several pathways involved in growth control and cancer.

**Conclusion:** Our findings show that germline inactivation of a single *Mtap* allele alters gene expression and enhances lymphomagenesis in *Eµ*-myc mice.

---

**Citation:** Kadariya Y, Tang B, Wang L, Al-Saleem T, Hayakawa K, et al. (2013) Germline Mutations in *Mtap* Cooperate with *Myc* to Accelerate Tumorigenesis in Mice. PLoS ONE 8(6): e67635. doi:10.1371/journal.pone.0067635

**Editor:** Hiromu Suzuki, Sapporo Medical University, Japan

**Received January 7, 2013; Accepted May 20, 2013; Published June 26, 2013**

**Copyright:** © 2013 Kadariya et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by NIH core grant CA06927, NCI grants HL057299, CA131024 (WK), CA129330 (KH), CA145445 (JK) and an appropriation from the Commonwealth of Pennsylvania. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: warren.kruger@fccc.edu.

---

**Introduction**

*MTAP* is a metabolic enzyme in the methionine salvage pathway that converts the polyamine synthesis byproduct 5'-dideoxy-5'-methylthioadenosine (*MTA*) into adenine and methylthioribose-1-phosphate and is expressed in all tissues throughout the body [1,2]. Loss of *MTAP* expression is seen 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 [3–15]. Loss rates range from 14% to 100% depending on the tumor type and the method used to assess *MTAP* loss.

The *MTAP* gene is frequently inactivated in human tumors by large homozygous deletion of the 9p21 region where both the *MTAP* and the *CDKN2A/ARF* tumor suppressor genes are located [16]. In mice, similar deletions occur in the same gene cluster that is located on chromosome 4 [17–19]. Since these deletions generally inactivate *CDKN2A/ARF* as well as *MTAP*, it was initially hypothesized that loss of *MTAP* in tumors was simply due to it being a co-incident bystander. However, a variety of studies now indicate that *MTAP* is a tumor suppressor gene in its own right. Re-expression of *MTAP* protein in *MTAP*-deleted MCF-7 breast adenocarcinoma cells results in loss of anchorage independent growth in vitro and the ability to form tumors when injected into SCID mice [20]. In addition, expression of *MTAP* in an *MTAP*-deleted melanoma cell line or gastric cancer cell line results in reduced invasion and migration in vitro [21,22]. Mice heterozygous for a germline mutation in the mouse *MTAP* gene (*Mtap*) die prematurely of T-cell lymphoma with a mean age of onset of about 18 months [23]. Finally, it was recently reported that humans with germline *MTAP* mutations have Diaphyseal medullary stenosis with malignant fibrous histiocytoma, an autosomal-dominant syndrome characterized by bone dysplasia,
myopathy, and bone cancer [24]. Taken together, these observations suggest that MTAP functions as a tumor suppressor gene independent of CDKN2A/ARF.

A potential mechanism by which loss of MTAP enhances tumor formation involves the link between MTAP and polyamine metabolism. Polyamines are small aliphatic amines essential for cell growth and are elevated in tumors [25]. The rate-limiting enzyme in the production of polyamines is ODC, which catalyzes the conversion of ornithine to putrescine. Deletion of MTAP results in up-regulation of ODC in both yeast and human cells [20,26], and over-expression of ODC is sufficient to transform fibroblasts in vitro and cause increased frequency of skin tumors in a transgenic mouse model [27,28]. More recently, Nilsson et al. demonstrated that ODC was over-expressed in the Eμ-myc transgenic mouse model of lymphoma and that this over-expression was important for lymphomagenesis [29]. Also, it has been shown that the ODC inhibitor DFMO increased tumor-free survival in TH-MYCN mice, which over express MYCN in neural lineages and develop neuroblastomas [30]. Taken together, these studies suggest that over-expression of ODC contributes to transformation by the Myc oncogene.

In the studies described here, we have crossed Mtap<sup>−/−</sup> mice with both Eμ-myc mice and Pten<sup>−/−</sup> mice and have characterized the offspring for tumor formation. There were three distinct goals of these studies: 1) Strengthen the hypothesis that Mtap-loss plays a functional role in tumor formation; 2) Create a mouse model to study the effects of Mtap-loss on tumor formation that had a significantly shorter latency period than the original Mtap<sup>−/−</sup> strain; and 3) Test the hypothesis that loss of Mtap might accelerate tumorogenesis in Eμ-myc mice by causing over expression of ODC.

**Materials and Methods**

**Mouse Breeding and Survival Analysis**

Mtap mice were created and genotyped as previously described [23]. Eμ-myc mice were obtained from the lab of Dr. John Cleveland (Scripps) and genotyped as described in [29]. Pten mice were provided by Dr. Antonio Di Cristofano (Albert Einstein University) and genotyped as described in [31]. All mice were in C57BL6 background.

For Eμ-myc animals, animals were monitored for survival and tumor formation daily by visual inspection and palpation. In these animals, tumor formation was obvious as indicated by swelling and increased body weight. When tumor or distress was detected, the animals were euthanized and necropsied. Pten<sup>−/−</sup> animals were monitored in a similar manner, but sometimes the animals died spontaneously without tumors being detected. In cases where the deceased animal was relatively fresh, necropsies were performed to determine if tumor was present at time of death. In cases where a tumor could not be confirmed at the time of necropsy, animals were censored for the purposes of survival analysis.

**Ethics Statement**

All animal protocols were approved by the Fox Chase Cancer Center IACUC (Protocol #05-06) and done in compliance with NIH guidelines. Animals were monitored daily for signs of distress and suffering. If distress or tumors were detected, animals were euthanized by overdose with isoflurane.

**Immunohistochemistry**

Autopsied specimens were fixed in buffered formalin, embedded in paraffin, and processed as previously described [23]. Rat antibodies directed against mouse CD45R/B220 (BD Biosciences) were used at a 1:200 dilution. Rat antibodies against Ki67 (Dako) were used at a 1:100 dilution. Rabbit ODC polyclonal serum was obtained from Dr. Lisa Chantz (Penn State University) and was used at a concentration of 0.5 ng/μl. For each, visualization was achieved by incubation followed by incubating with either a goat anti-rabbit or anti-rat biotinylated secondary antibody followed by incubation with streptavidin peroxidase and 3,3’-Diaminobenzidine (Sigma-Aldrich) substrate chromogen. Each slide was assessed blindly by a by a trained pathologist specializing in lymphoma (T.A-S) using a 1-6 grading system in which the percentage of cells containing the graded feature (Burkitt’s-like nuclei, Ki67, or ODC) was determined. A grade of 1 equals <10% of cells testing positive, 2 = 10–30%, 3 = 30–50%, 4 = 50–70%, 5 = 70–90%, and 6 <90%.

**FACS Analysis**

FACS analysis on spleen from euthanized animals was performed as previously described [32,33]. Single cell suspensions were made from bone marrow, spleen, lymph node and thymus and stained with fluochrome (FL, PE, APC, Cy7-PE) coupled monoclonal antibodies in various combinations; CD19 (1D3), CD45R/B220 (RA3-6B2), CD95/AA4 (AA4.1), IgM (31.12), IgD (11–26), CD21 (7G6), CD23 (B3B4), CD24 (30F1), CD3 (500A-A2), CD4 (GK1.5), CD8 (53-6), CD5 (53–7.3). Most reagents were made in the laboratory of Richard R. Hardy, except for FL-CD21 from BD Pharmingen, and FL-PNA (peanut agglutinin) from Vector Lab. Analysis was performed using a BD Biosciences LSR II/DeVio flow cytometer, equipped with three-laser excitation (405, 488, 630 nm).

**Quantitative RT-PCR Assay for TdT, Cμ, and Mtap**

For TdT and Cμ analysis, total RNA was prepared by sorting 10⁵ cells into “Solution D,” followed by cDNA preparation as previously described [34]. Gene expression was quantified by real-time PCR, in duplicate, using an ABI7500 thermal cycler, and ABI software was used to determine relative gene expression levels, using β-actin as an internal control.

**Mtap Quantification**

Mtap protein levels were detected by Western blot analysis using a MTAP monoclonal antibody (Santa Cruz Biotechnology) at a 1/1000 dilution. Signal was visualized by SuperSignal West Pico Chemiluminescent kit (Pierce), and signal was quantified using Alpha Innotech image analyzer. All levels were normalized to an alpha-actin internal control. Mtap expression <20% that of control samples was scored as Mtap<sup>−/−</sup>.

**Microarray Experiments**

Livers from 100-day-old male Mtap<sup>−/−</sup> and Mtap<sup>−/−/−</sup> mice were excised and put into RNAlater (Ambion). Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) and further purified by RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. RNA quality was evaluated by electrophoresis on Agilent Bioanalyzer (Agilent). Five μg of total RNA was first transcribed into cDNA using the Invitrogen’s Superscript system with an oligo (dT)24 primer containing a T7 promoter sequence at its 5’ end. After double stranded cDNA synthesis using DNA polymerase I, Biotin-labeled cRNA was generated by in vitro transcription using a GeneChip IVT Labeling Kit (Affymetrix) according to manufacturer’s instructions and then purified using the GeneChip Cleanup Module. Label target was fragmented to a size of 35–200 bases by metal-induced hydrolysis prior to hybridization. Target hybridization was performed on an Affymetrix hybridization oven at
45°C for 16 hours using an Affymetrix GeneChip Mouse Genome 430A 2.0 Array. After hybridization arrays were washed using the Affymetrix fluids station and stained with streptavidin Phycoerythrin according to the Affymetrix protocol. Four bacterial and phage cRNA controls (BioB, Bio C, Bio D and Cre) were included in the hybridization buffer to serve as internal control for hybridization efficiency. Washed arrays were scanned on an Affymetrix GeneChip Scanner 3000. Data was normalized using RMA as previously described [35]. Array data can be accessed in the GEO repository, GSE44539.

Pathway Analysis

For pathway analysis, we selected a set of differentially regulated genes based on the criteria that they exhibited at least a 30% change in mRNA levels and had a p-value < 0.01 (FDR < 0.29). This list, containing 363 probes, was then analyzed using both Web Gestalt Gene Analysis Toolkit V2 [36], and the Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, http://www.ingenuity.com). Both the Web Gestalt and IPA software maps the enriched genes on various canonical pathways and determines if the number of hits in each pathway exceeds those estimated by chance. The Web Gestalt software gives both an unadjusted and an adjusted p-value, where the adjusted p-value represents the false discovery rate. The IPA software ranks each pathway (called networks) by a score that is the negative log of the p-value. The IPA software does not determine a false discovery rate.

Statistics

P-values for contingency tables (2-by-2) were all performed using Fischer’s exact test (2-sided). Survival curve comparisons were assessed using the log-rank test supplied in GraphPad Prism 4.0 (http://www.graphpad.com). For comparison of continuous variables, a Student’s t-test (2-sided) was used.

Results

Mtap^lacZ/+ Decreases Survival in Eμ-myc and Pten^+/− Mice

Homozygous deletion of Mtap leads to early embryonic lethality [20]. However, Mtap^lacZ/+ mice are entirely normal until about 18 months of age when they will start to succumb to T-cell lymphoma. To address whether Mtap could accelerate tumorigenesis in other mouse tumor models, we crossed Mtap^lacZ/+ mice with Eμ-myc transgenic mice. Eμ-myc mice contain a transgene in which the immunoglobulin heavy chain enhancer is driving expression of the Myc oncogene in the B-lineage cells resulting in the development of B-cell lymphomas [37]. We followed a cohort Eμ-myc Mtap^+/+ and Eμ-myc Mtap^lacZ/+ littersmates until they either developed visible lymphoma or died with disease as determined by necropsy. We found that Eμ-myc Mtap^+/+ animals had a median time to tumor formation of 130 days, compared to 87 days for Eμ-myc Mtap^lacZ/+ animals (P<0.001, Fig.1A). These results show that germline heterozygosity for Mtap significantly decreases tumor latency in Eμ-myc mice.

We also examined a second mouse tumor model, Pten^+/−, for interaction with Mtap. Pten (Phosphatase and Tensin Homolog) is a phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate resulting in the formation of phosphatidylinositol (4,5)-biphosphate, which causes inhibition of the AKT signaling pathway. Germline mutation in Pten in humans is associated with Cowden’s disease, characterized by the presence of cutaneous benign hamartomas and high frequency of thyroid and breast cancer [38]. Homozygosity for Pten in mice is embryonic lethal, but heterozygous Pten mice (Pten^+/−) develop a variety of tumors including lymphomas, endometrial tumors, colon cancer, and gonadal tumors that are detectable between six months and one year of age [31,39-40]. We crossed Pten^+/− mice with Mtap^lacZ/+ animals and followed a cohort of Mtap^+/+ Pten^+/− and Mtap^lacZ/+ Pten^+/− for up to 450 days. We observed significantly decreased tumor-free survival in Mtap^lacZ/+ animals compared to Mtap^+/+ (median survival 325 vs. 371 days, P<0.031, Fig. 1B). We found there were significantly more spontaneous deaths in Mtap^lacZ/+ compared to Mtap^+/+ animals (Table 1). The most common type of tumor in both groups was lymphoma (10/32 and 4/32, respectively, Table 2). Other tumors observed included pheochromocytoma, thyroid, breast, and uterine adenocarcinomas. None of the individual tumors showed significant differences in occurrence between Mtap^lacZ/+ and Mtap^+/+ animals, however, there was a significant difference between Mtap^lacZ/+ and Mtap^+/+ in the percentage of necropsied animals in which no lesion was detected (16% vs. 69%, P = 0.0001). These results show that heterozygosity for Mtap decreases survival in Pten^−/− animals.

Mtap^lacZ/+ Increases Grade, Proliferative Capacity, and Odc Expression in Eμ-myc Mice

We next characterized the pathology of the lymphomas in Eμ-myc mice. First, we examined thymus sections from control, Eμ-myc Mtap^+/+, and Eμ-myc Mtap^lacZ/+ animals for a variety of morphological and immunohistochemical features. As expected, staining with anti-bodies to either CD3 (T-cell marker) or CD45R/B220 (B-cell marker) indicated that all the lymphomas in both Eμ-myc Mtap^+/+ and Eμ-myc Mtap^lacZ/+ animals were B-cell neoplasms (not shown). Morphologically, these lymphomas exhibited a spectrum between large cells, with irregular nuclear membranes, vesicular chromatin, and prominent nucleoli (diffuse large B-cell lympho-
ma-like) to others with medium sized cells, relatively fine chromatin and small nucleoli with brisk mitotic activity and apoptosis resembling Burkitt’s lymphoma. Some fell in between resembling “grey zone” lymphoma (Fig. 2A). These features were graded from grade 1 for “no tumor” to grade 6 for the high-grade Burkitt-like lymphoma. Grading of all the samples show that, in general, the tumors observed in Eμ-myc Mtap+/−/+ have a higher grade than those in Eμ-myc Mtap+/−/+ (Fig. 2C). The proliferation marker Ki67 was also examined and scored blindly, and it was found that there were increased numbers of strongly staining cells (up to almost 100%) in Eμ-myc Mtap+/−/+ animals (Fig. 2B–2C). Because loss of MTAP was associated with increased ODC activity in other settings, we stained thymus sections with an antibody to ODC. We observed both a higher percentage of cells expressing ODC and increased intensity of staining in the mouse ODC. We observed that there were increased numbers of strongly staining cells (308 days) (422 days) (325 days) (367 days) (50%) (28.8%) (34.37%) (3.12%) (308 days) (422 days)

Mtap does not Affect the Developmental Stage of the Cell, Giving Rise to the Tumor

Because of both the earlier appearance and the increased grade of the tumor, our next question was whether Mtap+/−/+ altered the transformation stage of the lymphomas in Eμ-myc B cells. To address this question, we performed FACS analysis on tumor-infiltrated tissues including thymus, spleen, lymph node, and bone marrow. As shown in Table 3, we found that, with one exception (mouse 353), all of the lymphoma cells stained positive for CD19, CD45R/B220 and high AA4.1 (CD93) expression and negative for CD5 and CD3, indicating that they are early stage B-cells, either surface IgM− or IgM+, in both Mtap+/+ and Mtap+/−/+ mice. All AA4+ IgM+ cells were IgDlo or IgD−, CD24+−, CD21−, CD23−, in further agreement with their immature B cell stage. All IgM− cells failed to show significant TdT mRNA levels, in contrast to the tight TdT (Terminal deoxynucleotidyl Transferase) expression by the pre B cells [34], and all expressed low levels of cytoplasmic IgM and high surface PNA expression, consistent with pre-B cell stage [41]. Low cytoplasmic IgM level excluded the possibility of IgM− plasmacytoma. Taken together, our data show that the cell of origin of the lymphomas was most likely started from the pre-B stage of development in both Mtap+/+ and Mtap+/−/+ animals.

Table 1. Tumor formation and death in Mtap Pten animals.

| Event                                | Mtap+/−/+ | Pten−/− |
|--------------------------------------|-----------|---------|
| Tumor formation determined by necropsy (median age) | 16/32a    | 9/32b   |
|                                      | (50%)     | (28.8%) |
|                                      | (325 days)| (367 days)|
| Spontaneous death (autolysis)(median age) | 11/32b    | 1/32b   |
|                                      | (34.37%)  | (3.12%) |
|                                      | (308 days)| (422 days)|

Loss of Mtap Protein Expression in Lymphoma Cells

We next examined Mtap expression in lymphoma-infiltrated tissue from 26 Mtap+/−/+ and 17 Mtap+/+ animals by Western blot analysis (Fig. 3A). We found that 13/26 (50%) of the tumors from Mtap+/−/+ mice showed complete loss of MTAP protein compared to 5/17 (29%) of the tumors from Mtap+/+ mice, but this difference was not statistically significant (P = 0.22, Fig. 3b). Given the large difference in tumor latency times between Mtap+/−/+ and Mtap+/+ mice, these findings suggest that a conventional Knudson two-hit tumor suppressor model is not able to fully explain the differences in tumor formation kinetics and tumor severity between Mtap+/−/+ and Mtap+/+ mice.

Comparison of Gene Expression Profiles in Mtap+/+ and Mtap+/−/+ Animals

Given the findings above, we hypothesized that mice heterozygous for Mtap might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtap+/+ animals. Young mice were chosen as we anticipated that there might have phenotypes due to Mtap haploinsufficiency. To test this idea, we performed microarray expression analysis using Affymetrix chips on liver mRNA from a group of young, healthy, age and sex matched Mtap+/−/+ and Mtp...
Figure 2. Pathology of Eμ-myc Mtap+/+ and Eμ-myc MtaplacZ/− mice. A. Representative H and E staining to tumor infiltrated thymus from Eμ-myc Mtap+/+ and Eμ-myc MtaplacZ/− animals viewed under 400X magnification. B. Representative Ki67 and ODC staining from the thymus of control, Eμ-myc Mtap+/+ and Eμ-myc MtpalZ/− mice. C. Histologic grading from H and E, Ki67, and ODC. Grading was performed blinded and evaluated by a board certified clinical histopathologist specializing in hematological tumors (AS). A score of 1 is normal, while a score of 5 was the most severe. Error bars show SD of score for each group.

doi:10.1371/journal.pone.0067635.g002
To explore this further, we selected a group of 363 probes that exhibited at least a 50% change in mRNA levels with \( P_{0.01} \) (FDR, 0.29). Of these, 242 were upregulated and 121 were downregulated in \( \text{Mtap}^{+/+} \) vs. \( \text{Mtap}^{+/+} \). As expected, all four of the probes for \( \text{Mtap} \) were present in the down-regulated group. The remaining 359 probes mapped to 251 unique genes (see Table S1).

| Genotype (all Em-myc) | mouse | CD19 | AA4.1 | PNA | IgM | IgD | CD3 | TdT (qPCR) | C\(_m\) (qPCR) |
|-----------------------|-------|------|-------|-----|-----|-----|-----|-------------|--------------|
| \( \text{Mtap}^{+/+} \) | 370   | +    | +     | ++  | –   | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 322   | +    | +     | ++  | +/– | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 329   | +    | +     | ++  | ++  | +/– | –   | –           | nd nd        |
| \( \text{Mtap}^{+/+} \) | 331   | +    | +     | ++  | ++  | +/– | –   | –           | nd nd        |
| \( \text{Mtap}^{+/+} \) | 336   | +    | +     | ++  | ++  | +/– | –   | –           | nd nd        |
| \( \text{Mtap}^{+/+} \) | 353   | –    | –     | –   | nd  | +   | nd | nd          |
| \( \text{Mtap}^{+/+} \) | 309   | +    | +     | ++  | –   | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 343   | +    | +     | ++  | –   | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 349   | +    | +     | ++  | –   | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 341   | +    | +     | ++  | +/– | –   | –   | –           | +            |
| \( \text{Mtap}^{+/+} \) | 320   | +    | +     | ++  | ++  | +/– | –   | nd nd       |
| \( \text{Mtap}^{+/+} \) | 334   | +    | +     | ++  | ++  | +/– | –   | nd nd       |

To explore this further, we selected a group of 363 probes that exhibited at least a 50% change in mRNA levels with \( P<0.01 \) (FDR <0.29). Of these, 242 were upregulated and 121 were downregulated in \( \text{Mtap}^{+/+} \) vs. \( \text{Mtap}^{+/+} \). As expected, all four of the probes for \( \text{Mtap} \) were present in the down-regulated group. The remaining 359 probes mapped to 251 unique genes (see Table S1).
We searched for functional enrichment of specific pathways of these genes using the Web Gestalt Gene Analysis Toolkit V2 [36]. Mapping our differentially expressed gene set against the biological function annotations in the Gene Ontology database, we found significant enrichment of genes involved rhythmic processes (i.e. circadian rhythm), anti-apoptotic genes, and genes involved in amino acid peptidyl modifications (Table S2). Another interesting group that came up as being enriched were genes involved in immature B-cell differentiation. Using the Kegg database as our functional sorter, we found that several probes mapped to signaling pathways including mTOR signaling, insulin signaling, and adipocytokine signaling, although these enrichments did not achieve statistical significance when correcting for multiple comparisons (Table S3).

We also subjected the same list of to analysis by the IPA software. The top five networks identified were: 1) Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry (score 44); 2) Cancer, Endocrine System Disorders, Hematological Disease (score 31); 3) Cell Morphology, Cancer, Developmental Disorder (score 29); 4) Humoral Immune Response, Protein Synthesis, Hematological System Development and Function (score 25); and 5) Cell-To-Cell Signaling and Interaction, Skeletal and Muscular System Development and Function (score 25). A list of the cancer related genes identified by IPA is shown in Table S4.

The finding of a significant number of cancer related genes in the differentially regulated gene set is consistent with the idea that loss of a single MTAP allele may have protumorigenic affects.

We also examined transcripts of genes known to be involved in polyamine biosynthetic and degradation pathways (Table S5). We found that the transcripts for the polyamine catabolic enzyme spermidine/spermine N1-acetyl transferase 1 (Sat1) were increased in MtaplacZ/+ animals, while the biosynthetic gene spermidine synthase (Srm1) was down regulated.

Discussion
Despite the knowledge that loss of MTAP expression is a relatively frequent event in a variety of different tumor types, the biological importance of MTAP loss in tumorigenesis has only recently started to be addressed. An important motivation for the studies described here was our earlier study in which we showed that mice heterozygous for Mtap died of T-cell lymphoma with a median life expectancy of 18 months [23]. Although this observation supported the idea that Mtap is a bona fide tumor suppressor gene, the long latency of this model makes it impractical for more extensive studies. An additional disadvantage of this model is that often MtaplacZ/+ mice would die of disease without exhibiting obvious external symptoms, making it difficult to get preserved tissue to study. To circumvent these problems, and to further establish that Mtap has tumor suppressor activity, we examined if a germline mutation in Mtap could cooperate and accelerate tumorigenesis in two other mouse tumor models, Em-Myc and Pten<sup>-/-</sup>. These models were chosen both because they have well defined tumor types and because they both have been
used successfully to identify genetic interactions with other tumor suppressor genes such as p53, ARF, and CDN2K [42,43].

Our data clearly show that heterozygosity for Mtap decreases tumor free survival in Eμ-MyC mice, with the median time for detectable tumor formation or death decreasing by 33%. For Pten1/2 mice, we did observe reduced survival, but did not observe a statistically significant increase in tumor formation the necropsied Mtap1/2+ Pten1/2 animals. The reason for this apparent contradiction is that a larger percentage of the Mtap1/2+ animals died spontaneously and the samples were too badly decayed to be necropsied. Whether these animals died of tumors cannot be definitively determined. In retrospect, a proactive necropsy done at a particular time point probably would have been a superior strategy. On the other hand, Eμ-MyC Mtap1/2+ mice developed rapidly forming tumors that were easily detected by observing swollen lymph nodes in the neck of the affected mice. Histopathologic and FACs analysis of the lymphomas indicate that the cells of origin are pre-B and immature B cells, and that this cell type was the same for both Mtap1/2+ and Mtap1/2+ animals. This finding indicates that Mtap1/2+ can cooperate with myc in driving lymphoma formation and that Mtap1/2+ does not alter the developmental stage of the cells giving rise to the lymphoma. However, we found that the tumors from Mtap1/2+ animals were of a higher grade as judged both by cell morphology and staining for the proliferation marker Ki67. This, along with the earlier appearance of the tumors, suggests that loss of Mtap may cause increased tumor aggressiveness.

We also examined the frequency by which Mtap expression was lost in the lymphomas developed in Eμ-MyC Mtap mice. We found that 5/17 tumors (29%) from Mtap+/+ mice had lost Mtap expression compared to 13/26 (50%) from the Mtap+/− animals. Although the frequency of Mtap- tumors appeared to increase in Mtap1/2+ animals, this increase was not statistically significant and is unlikely explain the dramatic decrease in latency time observed in the Mtap1/2+ animals. Rather, this data suggests that Mtap may be acting in a haploinsufficient manner. To develop evidence that germine heterozygosity for Mtap can have phenotypic consequences, we performed microarray experiments examining gene expression profiles in the livers of young age and sex matched Mtap+/+ and Mtap+/− animals. Based on the skewed distribution of P-values of the probes, we estimate that as many as 2048/16716 probes examined (14.4%) may be differentially expressed. Confining ourselves to probes that show at least a 50% difference in expression levels, we identified at least 363 probes representing 251 unique genes. These genes include many genes involved in pathways implicated in cancer development and progression. Because these experiments were done using RNA derived from liver, it is unclear if the genes and pathways identified as being affected by Mtap are directly relevant for the accelerated lymphoma development in these animals. Nonetheless, these experiments clearly show that loss of a single Mtap allele can have significant biological effects.

Previous studies have shown a relationship between loss of Mtap and an up-regulation of ODC, a key enzyme affecting polyamine metabolism [5,20,26]. In the studies described here, we found that the tumors in Eμ-MyC Mtap+/− mice tended to have higher levels of ODC expression than tumors found in Mtap+/− animals. In addition, we found Mtap-dependent differences in the liver mRNA levels of two polyamine metabolic genes (Srt1 and Smt1). Taken together, these observations provide additional support that Mtap-loss affects polyamine metabolism. A possible mechanism by which elevated ODC may contribute to lymphomagenesis may be via its influence on apoptosis. In hematopoietic cell lines, high levels of ODC have been shown to suppress apoptosis by reducing intracellular ROS species [44,45]. However, it should be noted that loss of Mtap might also promote lymphomagenesis by other means as well. In unpublished studies, our lab has found that expression of Mtap in an Mtap deleted osteosarcoma cell line can suppress several tumor related phenotypes without any effect on ODC levels (W.K., unpublished data). Thus, it seems possible that there may be multiple mechanisms by which Mtap-loss promotes tumor formation.

In summary, we have shown here, for the first time, that germline mutations Mtap can cooperate genetically with at least two other cancer causing mutations, Eμ-MyC and Pten+/−, to reduce survival and, in the case of Eμ-MyC, accelerate tumorigenesis. This acceleration does not appear to require the loss of the wild-type Mtap allele, suggesting that loss of a single copy of Mtap may have protumorigenic affects. Consistent with this view is the observation that heterozygosity for Mtap results in large alterations in the liver gene expression profile. Our findings support the view that Mtap-loss is of biological importance in tumorigenesis.

Supporting Information

Table S1 Mtap differentially expressed genes. (XLSX)
Table S2 Gene Ontology Pathways affected by Mtap. (XLSX)
Table S3 Kegg Pathways affected by Mtap. (XLSX)
Table S4 Cancer genes identified by IPA analysis. (XLSX)
Table S5 Analysis of Polyamine Pathway genes. (XLSB)

Acknowledgments

We acknowledge the contribution of the FCCC Genomics, Laboratory Animal, FACS, and Experimental Histopathology Facilities, and A. Kowalczyk, A. Formica, Yue-Sheng Li for technical assistance. We also thank Dr. John Cleveland for providing E-myC mice, Dr. Antonio Di Cristofano for Pten mice, and Dr. Lisa Chantzi for ODC anti-body.

Author Contributions

Conceived and designed the experiments: YK BT KH WDK. Performed the experiments: YK BT MS KH. Analyzed the data: YK BT MS KH. Structural reagents/materials/analysis tools: KH. Wrote the paper: WDK KH.

References

1. Kamatani N, Carson DA (1981) Dependence of adenine production upon polyamine synthesis in cultured human lymphoblasts. Biochim Biophys Acta 675: 344–356.

2. Olopade OI, Pomykala HM, Hages F, Sreen LW, Espinosa R, et al. (1995) Construction of a 2.8-megabase yeast artificial chromosome contig and cloning of the human methylthioadenosine phosphorylase gene from the tumor suppressor region on 9p21. Proc Natl Acad Sci USA 92: 6489–6493.

3. Subhi AT, Tang B, Balsara BR, Altomare DA, Testa JR, et al. (2004) Loss of methylthioadenosine phosphorylase and elevated ornithine decarboxylase is common in pancreatic cancer. Clin Cancer Res 10: 7290–7296.

4. Hustin SR, Hruban RH, Leoni LM, Iacobuzio-Donahue C, Cameron JL, et al. (2005) Homozygous deletion of the MTAP gene in invasive adenocarcinoma of the pancreas and in periampullary cancer: a potential new target for therapy. Cancer Biol Ther 4: 83–86.
5. Schmid M, Malicki D, Nobori T, Rosenbach MD, Campbell K, et al. (1998) Homozygous deletions of methylthioadenosine phosphorylase (MTPA) are more frequent than p16INK4A (CDKN2) homozygous deletions in primary nonsmall cell lung cancers (NSCLC). Oncogene 17: 2699–2703.

6. Brat DJ, James CD, Jedlicka AE, Combsy DC, Chang E, et al. (1999) Molecular genetic alterations in radiation-induced astrocytomas. Am J Pathol 154: 1431–1438.

7. Studler WM, Olopare OI (1996) The 9q21 region in bladder cancer cell lines: large homozygous deletion inactivate the CDKN2A, CDKN2B and MTPA genes. Urol Res 24: 239–244.

8. Garcia-Castelano JM, Villanueva A, Healey JH, Sowers R, Cordon-Cardo C, et al. (2002) Methylthioadenosine phosphorylase gene deletions are common in osteosarcoma. Clin Cancer Res 8: 762–767.

9. Dreyling MH, Roulston D, Bohlender SK, Vardiman J, Olopare OI (1998) Codeletion of CDKN2 and MTPA genes in a subset of non-Hodgkin’s lymphoma may be associated with histologic transformation from low-grade to diffuse large-cell lymphoma. Genes Chromosomes Cancer 22: 72–78.

10. M’Soka T, Nishio K, Taga T, Kato K, Kawasaki H, et al. (2000) Detection of methylthioadenosine phosphorylase (MTPA) and p16 gene deletion in T cell acute lymphoblastic leukemia by real-time quantitative PCR assay. Leukemia 14: 935–940.

11. Hori Y, Hori H, Yamada Y, Carrera CJ, Tomonaga M, et al. (1998) The methylthioadenosine phosphorylase gene is frequently co-deleted with the p16INK4a gene in acute type ashl T-cell leukemia. Int J Cancer 75: 51–56.

12. Karikari CA, Mullendore M, Ezehelen JR, Argani P, Leoni LM, et al. (2005) Homozygous deletions of methylthioadenosine phosphorylase in human biliary tract cancers. Mol Cancer Ther 4: 1869–1879.

13. Chen YJ, Lin SC, Kao T, Chang CS, Hong PS, et al. (2004) Genome-wide profiling of oral squamous carcinoma. J Pathol 204: 326–332.

14. Illei PB, Rusch VW, Zakowski MF, Ladanyi M (2003) Homozygous deletion of CDKN2A and codeletion of the methylthioadenosine phosphorylase gene in the majority of pleural mesotheliomas. Clin Cancer Res 9: 2106–2113.

15. Zeng Y, Cheung TK, Cheung TH, Nobori T, Chang AM (1998) MTPA gene deletion in endometrial cancer. Gynecol Obstet Invest 45: 272–276.

16. Nobori T, Takahayashi K, Tran P, Ovis L, Batova A, et al. (1999) Genomic cloning of methylthioadenosine phosphorylase: a purine metabolic enzyme deficient in multiple different cancers. Proc Natl Acad Sci U S A 95: 6203–6208.

17. Wu CL, Liao YF, Hung YC, Lu KH, Hung HC, et al. (2011) Ornithine decarboxylase prevents dibenzoylmethane-induced apoptosis through repressing reactive oxygen species generation. J of Biochem and Mol Toxic 10.1002/jbt.20391.

18. Kadariya Y, Nishioka J, Nakamura A, Kato-Nakazawa K, Nobori T (2003) Methylthioadenosine phosphorylase gene deletions are common in primary non-small cell lung cancers (NSCLC). Oncogene 17: 5309–5317.

19. Gupta S, Kuhnisch J, Mustafa A, Lhotak S, Schlachterman A, et al. (2009) Mouse models of cystathionine β-synthase deficiency reveal significant threshold effects of hyperhomocysteinemia. FASEB J 23: 883–893.

20. Zhang B, Karios S, Noddy J (2003) WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Res 33: W741–W748.

21. Adams JM, Harris AW, Pinkart CA, Corcoran LM, Alexander WS, et al. (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318: 533–538.

22. Schmitt CA, McCurrach ME, de Stanchina E, Wallace-Brodeur RR, Lowe SW (2000) ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma. Cancer Research 60: 9735–9745.

23. Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, et al. (1999) Mutation of Pten/Mmp1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A 96: 1563–1568.

24. Hori Y, Hori H, Yamada Y, Carrera CJ, Tomonaga M, et al. (1998) The methylthioadenosine phosphorylase gene is frequently co-deleted with the p16INK4a gene in acute type ashl T-cell leukemia. Int J Cancer 75: 51–56.

25. Marton LJ, Pegg AE (1995) Polyamines as targets for therapeutic intervention. Annual Review of Pharmacology and Toxicology 35: 53–91.

26. Babu AL, Diegelman P, Porter CW, Tang B, Lu ZJ, et al. (2003) Methylthioadenosine phosphorylase regulates ornithine decarboxylase by production of downstream metabolites. J Biol Chem 278: 49068–49073.

27. Mosher JA, Dessen H, Skanica M, Luk GD (1993) Transcription of NIH/ST3 cells by ornithine decarboxylase overexpression. Cancer Res 53: 2618–2622.

28. O’Brien TG, Megess L, Gilliard G, Sober AP (1997) Ornithine decarboxylase overexpression is a sufficient condition for tumor promotion in mouse skin. Cancer Res 57: 2630–2637.

29. Nilsson JA, Keller UR, Baulino TA, Yang C, Norton S, et al. (2005) Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. Cancer Cell 7: 433–444.

30. Wu CL, Liao YF, Hung YC, Lu KH, Hung HC, et al. (2011) Ornithine decarboxylase prevents dibenzoylmethane-induced apoptosis through repressing reactive oxygen species generation. J of Biochem and Mol Toxic 10.1002/jbt.20391.