

ORIGINAL ARTICLE

Ectopic expression of methionine aminopeptidase-2 causes cell transformation and stimulates proliferation

LA Tucker, Q Zhang, GS Sheppard, P Lou, F Jiang, E McKeegan, R Lesniewski, SK Davidsen, RL Bell and J Wang

Cancer Research, GPRD, Abbott Laboratories, Abbott Park, IL, USA

Methionine aminopeptidase-2 (MetAP2) processes N-terminal methionine from nascent cellular proteins. Inhibition of MetAP2 has been shown to block angiogenesis and suppress tumor growth in preclinical tumor models. However, the biological role of MetAP2 in cancer is not well understood. We examined the effect of three distinct chemical classes of MetAP2 inhibitors on the growth of a panel of human cancer cells *in vitro*. All MetAP2 inhibitors caused inhibition of tumor cell growth in both anchorage-dependent and, particularly, in anchorage-independent manner. These data prompted us to examine the possible roles of MetAP2 in cancers. Ectopic expression of MetAP2 in NIH-3T3 cells caused transformation, evidenced by the formation of foci in monolayer culture and growth of large colonies in soft agar. Overexpression of MetAP2 in an immortalized bronchial epithelial cell line NL20 accelerated growth. These phenotypes induced by the overexpression of MetAP2 were reversed by the treatment with MetAP2 inhibitors, indicating that the catalytic function of MetAP2 was essential. Accordingly, overexpression of a catalytically inactive MetAP2 resulted in growth retardation of HT1080 tumor cells, suggesting a dominant-negative role of the inactive MetAP2 mutant. Finally, we analysed the expression of MetAP2 in patient cancer samples by immunohistochemistry. Moderate-to-high staining was identified in the majority of breast, colon, lung, ovarian and prostate carcinomas examined. These data suggest that MetAP2 plays an important role in tumor cell growth and may contribute to tumorigenesis.

Oncogene advance online publication, 11 February 2008; doi:10.1038/onc.2008.14

Keywords: MetAP2; transformation; proliferation; IHC

Introduction

Methionine aminopeptidases (MetAPs) play a role in protein co- and/or post-translational modifications by

removing the N-terminal initiator methionine from nascent polypeptides. Inhibition of the MetAP activity could therefore affect protein biological activity, proper cellular localization and turnover, and result in interference with cell signal transduction and cell-cycle progression (Bradshaw *et al.*, 1998; Lowther and Matthews, 2000). For example, covalent attachment of myristate, a 14-carbon saturated fatty acid, to the newly generated N-terminal Gly after the removal of initiator methionine in proteins is an important form of protein modification (Resh, 2004). Protein N-myristoylation is required for membrane binding of many important signal-transduction proteins, including Src family tyrosine kinases, Abl tyrosine kinases, Ser/Thr kinases such as cAMP-dependent protein kinase, phosphatases such as calcineurin B, guanine nucleotide-binding proteins and MARCKS proteins (Resh, 2004). Protein turnover in a given cell follows the N-end rule, which relates the half-life of a protein to the identity of its N-terminal residue (Varshavsky, 2005). Inhibition of MetAP activity could affect protein turnover due to the uncleaved N-terminal methionine. It has been speculated that the antiangiogenesis effect of MetAP2 inhibitors may stem from inhibition of the N-terminal Met–Cys cleavage in a normally short-lived regulator of angiogenesis that is targeted by the N-end rule pathway through its N-terminal Cys residue (Kwon *et al.*, 2002).

MetAP2 is one of the three known MetAPs responsible for the processing of the N-terminal initiator methionine from nascent proteins in cells (Datta, 2000; Lowther and Matthews, 2000; Bradshaw and Yi, 2002; Leszczyniecka *et al.*, 2006). Both MetAP1 and MetAP2 are expressed in many mammalian tissues and cell lines, but only MetAP2 is upregulated during cell proliferation (Wang *et al.*, 2000). MetAP2 is also found in higher concentrations in tumor cells compared with normal cells (Wu *et al.*, 1993; Kanno *et al.*, 2002). Furthermore, MetAP2 has been found to be overexpressed in colon cancers and is associated with metastatic disease progression (Selvakumar *et al.*, 2004). When cultured mammalian cells are starved, the protein level of MetAP2 is reduced significantly; during mitogenic stimulation, cellular MetAP2 protein was rapidly induced (Wu *et al.*, 1993; Wang *et al.*, 2000; Kanno *et al.*, 2002). Reduction of total MetAP2 protein concentrations with antisense treatment in rat hepatoma cells results in suppression of protein synthesis, increased

Correspondence: Dr J Wang, Cancer Research, GPRD, Abbott Laboratories, R47A, AP9-2124, 100 Abbott Park Road, Abbott Park, IL 60064, USA.

E-mail: jieyi.wang@abbott.com

Received 17 September 2007; revised 3 December 2007; accepted 1 January 2008

phosphorylation of eIF-2 α and induction of apoptosis (Datta and Datta, 1999). MetAP2 antisense also induces apoptosis associated with a reduction in telomerase activity and downregulation of Bcl-2 in human mesothelioma cells (Catalano *et al.*, 2001). Knockdown of MetAP2 expression by small siRNA induced significant inhibition of the proliferation of human umbilical vein endothelial cells (Bernier *et al.*, 2005; Yeh *et al.*, 2006). Ubiquitous deletion of the MetAP2 gene resulted in an early gastrulation defect and targeted deletion of MetAP2 specifically in the hemangioblast lineage resulted in abnormal vascular development (Yeh *et al.*, 2006).

The role of MetAP2 in cancer has not been well studied, although this enzyme was identified as the molecular target for the antiangiogenic activity of TNP-470 a decade ago (Griffith *et al.*, 1997; Sin *et al.*, 1997). Fumagillin and TNP-470 are widely known for their antiangiogenic activity by inducing growth arrest of endothelial cells (Ingber *et al.*, 1990; Kusaka *et al.*, 1994), but they also exhibit antiproliferative effects directly on tumor cells (Wang *et al.*, 2003a). In this study, we sought to examine the effect of overexpression of MetAP2 and a catalytically inactive form of MetAP2 in cell culture. Here, we show evidence for the cell-transforming activity of MetAP2 as well as for a dominant-negative function of a MetAP2 mutant. Together with the direct antiproliferative activity of MetAP2 inhibitors in cancer cells and the observation of elevated level of MetAP2 in a variety of primary cancer samples, these data suggest that MetAP2 may play an important role in cancer development.

Results

MetAP2 inhibitors directly inhibit the growth of cancer cells

The prototype MetAP2 inhibitors fumagillin and TNP-470 are known as angiogenesis inhibitors in the

literature (Ingber *et al.*, 1990; Kusaka *et al.*, 1994; Kruger and Figg, 2000), but they also possess antiproliferative activity in certain tumor lines (Wang *et al.*, 2003a). To characterize the sensitivity of tumor cells to MetAP2 inhibition, we tested a panel of tumor lines with three distinct chemical classes of MetAP2 inhibitors: an irreversible inhibitor TNP-470 (Ingber *et al.*, 1990; Griffith *et al.*, 1997; Sin *et al.*, 1997), a bestatin inhibitor A-357300 (Wang *et al.*, 2003a) and a sulfonamide inhibitor A-800141 (Sheppard *et al.*, 2006). In monolayer culture, the growth of these tumor cells was partially inhibited by these MetAP2 inhibitors, with maximal inhibition in the range of 33–80% for all agents (Table 1; Figure 1a). The highest compound concentration tested was 10 μ M, which was at least 100-fold greater than the cellular IC₅₀ values of these inhibitors against MetAP2 activity in cells (Table 1). A-357300 consistently produced the highest maximal inhibition, which may be a result of the lesser selectivity of A-357300 compared with the other MetAP2 inhibitors. HT1080 fibrosarcoma and NCI-H460 lung carcinoma cells were the tumor lines most sensitive to MetAP2 inhibitors. The EC₅₀ (concentration for half-maximal effect for each treatment, not the absolute 50% inhibition) for each inhibitor across these tumor lines was similar (Table 1). For example, the sulfonamide inhibitor A-800141 showed a range of EC₅₀ of 7 to 39 nM in these tumor cells. We also examined the protein level of MetAP2 and p53 in these tumor lines to investigate whether there is a correlation between the sensitivity to MetAP2 inhibitors and expression of these proteins. All tumor lines examined expressed detectable MetAP2 while having various p53 status and growth rates (Table 1; Figure 1b). There was no apparent correlation between response to MetAP2 inhibitors and cellular MetAP2 or p53 expression level/status. These data were not surprising as MetAP2 is expressed in all cells examined and required for proliferation as it was investigated as p67 (Datta, 2000) and in our previous study (Wang *et al.*, 2000). p53 has been reported to

Table 1 Inhibition of the growth of tumor lines by MetAP2 inhibitors *in vitro*

Cell lines	Tissue origin	p53 status	Doubling time (h)	Proliferation on plastic			Colonies in soft agar		
				EC ₅₀ (nM); max inhibition (%)			EC ₅₀ (nM); Max inhibition (%)		
				TNP-470 ^a	A-800141	A-357300	TNP-470	A-800141	A-357300
HT1080	Fibrosarcoma	WT	18	3; 62	26; 62	193; 73	ND	ND	ND
PC3	Prostate	DL	31	39; 37	30; 34	530; 58	1; 90	1; 92	23; 99
NCI-H460	Lung	WT	19	3; 63	11; 61	91; 80	40; 92	20; 85	179; 100
H1299	Lung	DL	23	1; 47	8; 51	28; 63	3; 96	17; 97	136; 100
A549	Lung	WT	25	4; 39	25; 41	160; 54	12; 100	44; 100	278; 96
Calu6	Lung	DL	36	5; 33	34; 36	325; 38	1; 80	17; 70	93; 55
HCT116	Colon	WT	19	2; 35	18; 38	297; 51	2; 100	2; 98	32; 100
HCT15	Colon	MU	21	2; 43	39; 49	918; 75	149; 34	532; 66	1243; 84
DLD1	Colon	MU	23	2; 41	17; 43	248; 58	17; 100	8; 92	194; 100
LoVo	Colon	WT	23	1; 33	7; 45	18; 55	172; 75	75; 78	367; 100
SW620	Colon	MU	20	1; 44	17; 50	143; 66	ND	ND	ND

Abbreviations: MetAP2, methionine aminopeptidase-2; ND, not determined. The EC₅₀ and maximum inhibition values were obtained from dose-response curves as shown in Figure 1. Data represent averaged value of at least two independent assays. ^aThe cellular IC₅₀ values of inhibiting MetAP2 activity as measured by unprocessed N-terminal methionine in cellular proteins (Wang *et al.*, 2003a; Sheppard *et al.*, 2006) for TNP-470, A-800141 and A357300 were 3, 20 and 150 nM, respectively.

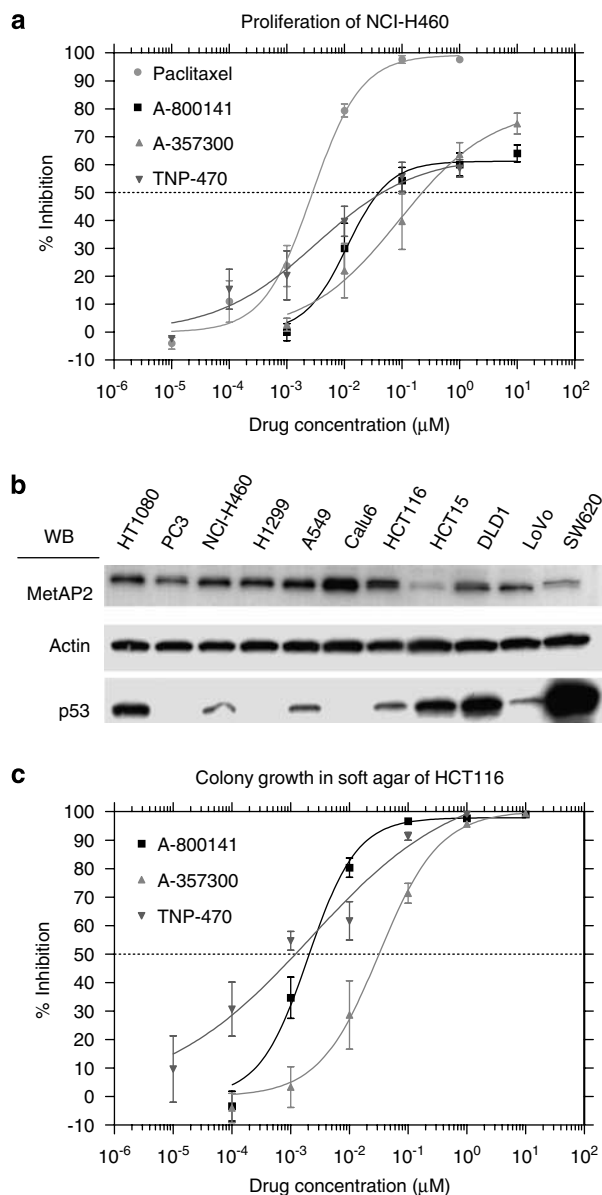


Figure 1 MetAP2 inhibitors directly inhibit the growth of tumor lines. (a) Dose–response of NCI-H460 cells in proliferation assay. Cells were grown in 96-well plates for 3 days in the presence of compounds at concentrations as shown, and MTS assay was used to quantify live cells at the end of study. Data were from experiments that were repeated at least twice. EC₅₀ and maximal inhibition shown in Table 1 were obtained from such prism graphs. (b) Western blot analysis of MetAP2 and p53 in tumor cells. Total cell lysates were made from cells in log phase growth in 6-well plates. (c) Dose–response of HCT116 cells in soft-agar assay. Cells were grown in 12-well plates for 14–28 days in the presence of compounds at concentrations as shown, and imaging analysis of Iodonitrotetrazolium Violet stained cells was used to quantify total colonies at the end of study. Data were from one of two independent experiments. EC₅₀ and maximal inhibition shown in Table 1 were obtained from such analyses.

mediate the effect of MetAP2 inhibitors in endothelial cells (Yeh *et al.*, 2000; Zhang *et al.*, 2000), but these studies were carried out only in cells with wild-type p53. Our observation that the MetAP2 inhibitors suppressed

proliferation also in p53-null cells suggests that there exist multiple pathways downstream of MetAP2 blockade leading to cell-cycle arrest.

We further examined the effect of MetAP2 inhibitors on the growth of tumor cells in soft agar. In comparison with the anchorage-dependent culture, tumor cells grown in soft agar were more sensitive to MetAP2 inhibitors. The majority of tumor lines examined were inhibited more than 90%, including lines such as HCT116 that were only partially inhibited in monolayer culture (Table 1, Figure 1c). The EC₅₀ of each compound was similar to that for monolayer assays, with a few exceptions. For example, A-800141 showed EC₅₀ range of 1 to 75 nM (Table 1), with the exception of the HCT15 cell line, which is known to express multidrug-resistance genes and exhibits resistance to drug therapies. In fact, this tumor line was less sensitive to all MetAP2 inhibitors. Taken together, these data suggest that MetAP2 plays an important role in tumor growth as all three distinct MetAP2 inhibitors showed a similar effect on a variety of tumor cells, especially the suppression of cancer cell growth in soft agar. We were encouraged by these results to examine possible roles of MetAP2 in carcinogenesis.

Ectopically overexpressed MetAP2 promotes cell growth

To determine the effect of exogenously expressed MetAP2, we made a retrovirus that expresses full-length human MetAP2. NIH-3T3 cells were infected with MetAP2 retrovirus and a stable transfectant clone (pLPCX-MetAP2) was established after selection in puromycin-containing medium. The pLPCX-MetAP2 cells showed overexpression of MetAP2 by approximately fourfold over a vector control clone (pLPCX-control), as determined by western blot analysis (Figure 2a). We compared the growth rate of the MetAP2 overexpressing cells with that of vector control in a 3-day proliferation assay using total cellular ATP as the final readout. The MetAP2 overexpressing cells grew twice as fast as the control cells (Figure 2b). We also tested MetAP2 overexpression in an immortalized normal human bronchial epithelial cell line NL-20. A similar overexpression was observed in the MetAP2 retrovirus-infected NL-20 cells (Figure 2c), and these cells also showed an increased proliferation as compared with the vector control cells (Figure 2d). These data demonstrated that ectopically overexpressed MetAP2 could promote cell proliferation, indicating a role of MetAP2 in cell growth.

Overexpression of MetAP2 causes cell transformation

We continued to examine the growth of MetAP2-overexpressing NIH-3T3 clones on plastic and in soft agar. The MetAP2-overexpressing NIH-3T3 cells grew initially as a monolayer in culture flasks after plating. These cells formed foci as they became confluent, in contrast to the vector control cells (Figure 3a). When grown in soft agar, the MetAP2-overexpressing NIH-3T3 cells formed large colonies, whereas the control cells only formed small and sparse cell clusters (Figure 3b).

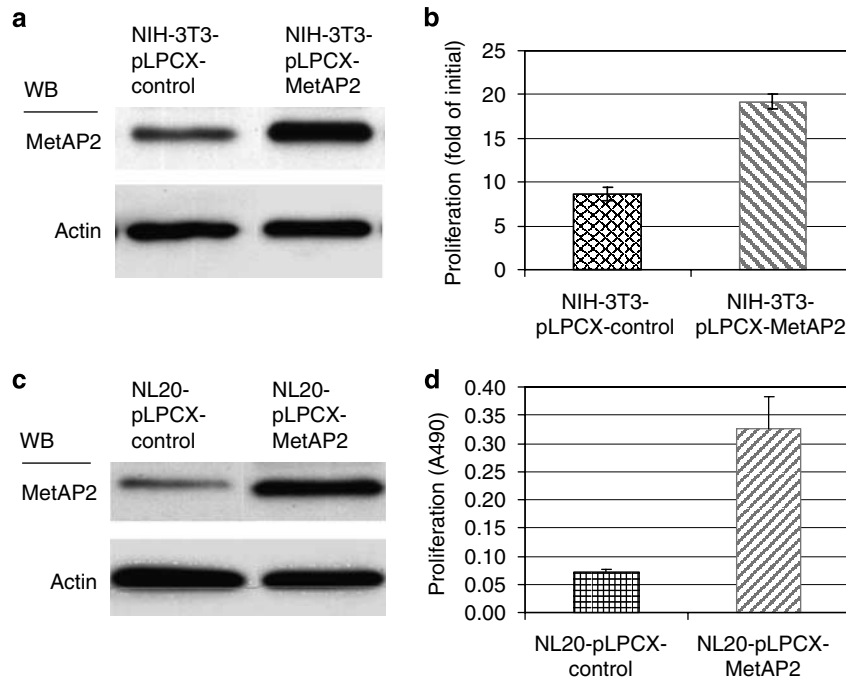


Figure 2 Ectopic expression of MetAP2 promotes cell proliferation. (a) Western blot analysis of MetAP2 in NIH-3T3 cells. The vector control cells (NIH-3T3-pLPCX-control) and a clone of MetAP2 overexpressing cells (NIH-3T3-pLPCX-MetAP2) were grown in complete growth medium. Total cell lysates of cells in log phase growth were used. (b) Proliferation of NIH-3T3 cells. The cells as in (a) were plated in 96-well plate in quadruplicate and incubated for 3 days. Proliferation of these cells was determined as fold of the initial plated cells. (c) Western blot analysis of MetAP2 in NL20 cells. The vector control cells (NL20-pLPCX-control) and a clone of MetAP2 overexpressing cells (NL20-pLPCX-MetAP2) were grown in complete growth medium. Total cell lysates of cells in log phase growth were used. (d) Proliferation of NL20 cells. The cells as in (c) were seeded in 96-well plate in quadruplicate and incubated for 3 days. Proliferation of these cells was determined by MTS assay with absorbance at 490 nm.

Cells infected with the vector control virus were never observed to form foci and grew only as small cell clusters in soft agar, ruling out the possibility of spontaneous transformation as a cause of our observations. The large colonies isolated from soft agar continued to display anchorage-independent growth in a subsequent soft agar assay (data not shown). Several independent clones of NIH-3T3 cells infected with MetAP2 virus also showed this phenotype and were found to have similar levels of MetAP2 overexpression (~4-fold). A number of other stable clones isolated after the retroviral transfection did not show this transformed phenotype and did not overexpress MetAP2 (data not shown), indicating the requirement of a threshold level of expression to achieve a transformed phenotype. The foci formation on plastic and colonies growth in soft agar manifested by the overexpression of MetAP2 in NIH-3T3 cells suggest a transforming activity of MetAP2.

MetAP2 enzyme activity is required for the transformed phenotype

To test whether MetAP2 catalytic activity was essential for the foci and colony formation by the MetAP2-overexpressing NIH-3T3 cells, we examined the effect of the MetAP2 enzyme inhibitors A-357300 and TNP-470. In monolayer culture, the reversible MetAP2 inhibitor

A-357300 at 10 μ M completely prevented foci formation (Figure 3c). When A-357300 or TNP-470 (100 nM) was included in the soft agar culture, no large colonies were observed, whereas visual colonies were formed in the culture with dimethylsulfoxide controls (Figure 3d). It is known that MetAP2 inhibition causes upregulation of MetAP2 protein in endothelial cells (Wang *et al.*, 2000). To better understand how MetAP2 inhibitors block cellular transformation in these transfected cells, we used western blot to analyse cellular MetAP2 protein level in cells treated with or without inhibitors. However, we did not see any significant changes of MetAP2 under these treatments (data not shown). Although the mechanism for the cellular transforming activity of MetAP2 remains unknown, it is clear from these experiments that the catalytic activity of MetAP2 is required for transformation of NIH-3T3 cells by the ectopically expressed MetAP2.

Dominant-negative effect of a catalytically inactive MetAP2 mutant

MetAP2 is a metalloprotease and the histidine residue 231 in the enzyme active center is involved in catalysis (Griffith *et al.*, 1998; Liu *et al.*, 1998). TNP-470 inactivates MetAP2 enzyme activity by covalently modifying this histidine residue, and a MetAP2 mutant (His231Asn) has been shown to lack catalytic activity

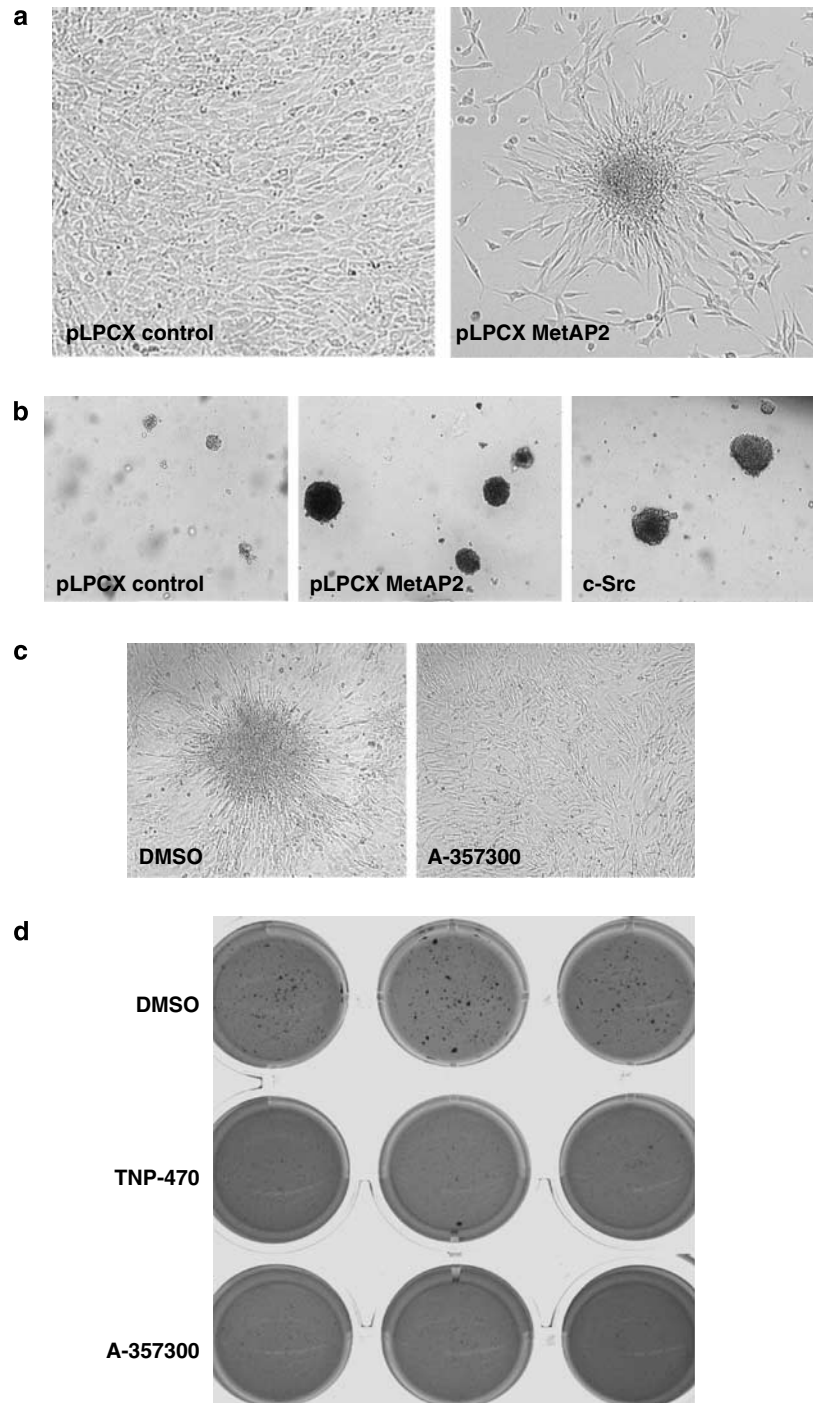


Figure 3 Ectopic expression of MetAP2 in NIH-3T3 cells causes transformation. NIH-3T3 cells overexpressing MetAP2 form foci on plastic (a) and grow colonies in soft agar (b). c-Src-transformed NIH-3T3 cells were used as a positive control. (c) MetAP2 inhibitor A-357300 blocks foci formation of NIH-3T3-pLPCX-MetAP2 cells. The cells were grown in the presence of 10 μ M A-357300 or dimethylsulfoxide (DMSO) control. Images ($\times 100$) represented consistent observations of three independent experiments. (d) TNP-470 and A-357300 inhibit the growth of NIH-3T3-pLPCX-MetAP2 cells in soft agar. The cells seeded in soft agar were treated with a top layer of medium with compounds as shown (TNP-470 at 0.1 μ M and A-357300 at 10 μ M, in triplicate) for 14 days. The images were representatives from two independent experiments.

(Griffith *et al.*, 1998). To study the effect of this catalytically inactive MetAP2 mutant (MetAP2-H231N) on cell growth, we attempted to overexpress this mutant in HT1080 human fibrosarcoma cells, which

are sensitive to MetAP2 inhibitors (Table 1). We initially transfected HT1080 cells with pCDNA3.1-MetAP2-H231N to produce stable clones. G418-resistant colonies of cells appeared after the first round of selection,

but they failed to grow after being subcloned, suggesting a possible toxic effect of this MetAP2 mutant. We then used a TET-OFF system (pTRE2) to express MetAP2-H231N under the control of doxycycline in HT1080 cells. Stable transfectant clones of MetAP2-H231N-HT1080 TET-OFF cells that grew in medium containing doxycycline were established. When the cells were cultured in the presence of $1 \mu\text{g ml}^{-1}$ doxycycline, the expression of the MetAP2-H231N in the pTRE2 vector was suppressed. After trypsinization and passage three times in the absence of doxycycline to wash out this compound, these cells expressed the mutant MetAP2, indicated by the elevated signal of MetAP2 on western blot (Figure 4a, left). An HT1080 clone expressing luciferase under the same TET-OFF system was used as the control (Figure 4a, right). We then compared the growth of the cells in the presence of doxycycline (no MetAP2-H231N expression) with that of cells after doxycycline washout (MetAP2-H231N expression).

Using a real-time cell electronic sensing device, we were able to record cell growth in real time over a period of 3 days. Expression of the catalytically inactive MetAP2 resulted in retardation of HT1080 cell growth (Figure 4b, left); luciferase expression in the control cells did not show growth alteration (Figure 4b, right). In separate experiments, we counted the cells grown in media with and without doxycycline. Induction of MetAP2-H231N overexpression decreased cell number by 50% in 3 days as compared with that without doxycycline removal (Figure 4c). The luciferase control cells did not show growth alteration by doxycycline (Figure 4c). The MetAP2-H231N-HT1080 TET-OFF cells remained in a slower growth rate and eventually could not survive in the absence of doxycycline. These data demonstrated that the catalytically inactive MetAP2 mutant was dominant negative for HT1080 tumor cell growth; further supporting that MetAP2 may play an important role in tumor cell proliferation.

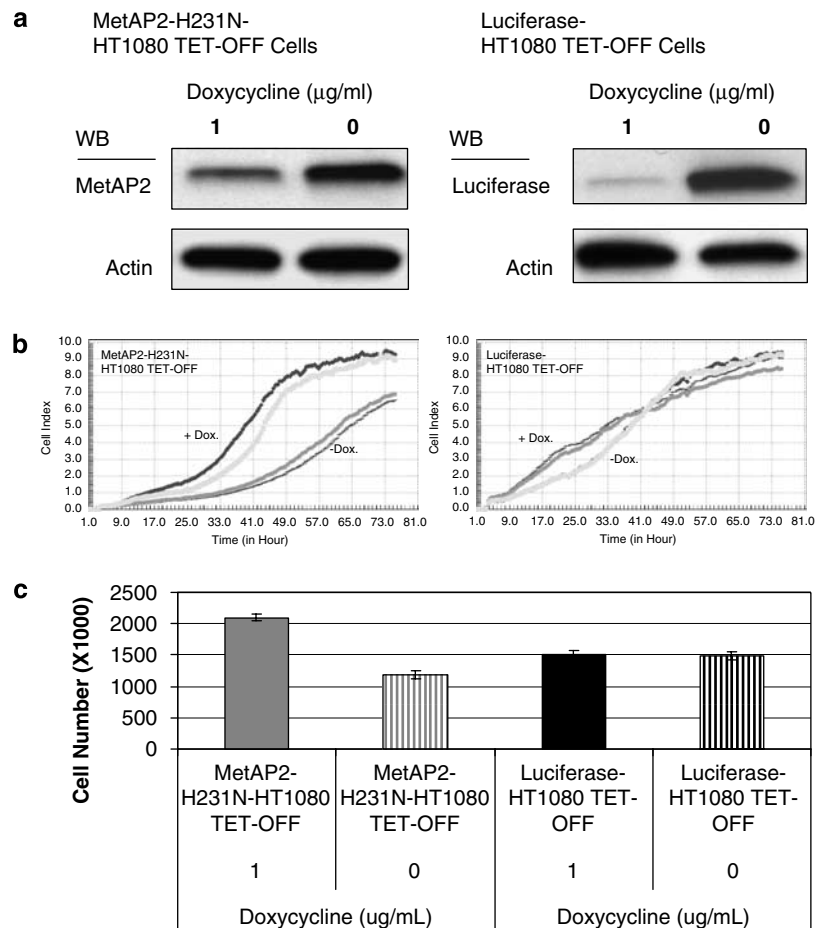


Figure 4 Catalytically inactive MetAP2 mutant is dominant negative for tumor cell growth. **(a)** Western blot analysis of MetAP2 or control luciferase expression. The MetAP2-H231N-HT1080 TET-OFF and the Luciferase-HT1080 TET-OFF cells were grown in the presence and absence of $1 \mu\text{g ml}^{-1}$ doxycycline. Total cell lysates were made from cells in log phase growth in 6-well plates. **(b)** Cell growth curves as monitored by RT-CES (real-time cell electric sensing). The MetAP2-H231N-HT1080 TET-OFF and the Luciferase-HT1080 TET-OFF cells were plated in the RT-CES 96-well plates in duplicate at 2000 cells per well in medium with or without $1 \mu\text{g ml}^{-1}$ doxycycline. Cell growth was monitored over a period of 3 days. Data were from one representative of two experiments. **(c)** Comparison of growth of MetAP2-H231N-HT1080 TET-OFF and the Luciferase-HT1080 TET-OFF cells. Identical numbers of cells (250 000) of various HT1080 transfectants as shown were plated in T25 flasks, and they were incubated in the presence or absence of $1 \mu\text{g ml}^{-1}$ doxycycline for 3 days. Total numbers of cells in each flask were counted at the end of the study. Data were from one representative of two experiments.

MetAP2 is highly expressed in clinical cancer samples

To evaluate the MetAP2 protein expression in human cancers, we utilized rabbit polyclonal antibodies raised against recombinant MetAP2 protein (Wang *et al.*, 2000) in several immunohistochemistry studies. First, we chose to examine MetAP2 expression in human adenocarcinomas, including 10 samples each of breast, colon, lung, ovarian, pancreas and prostate. Moderate to strong cytoplasmic staining was identified in a majority of these samples (Figure 5a). The staining was not only seen in adenocarcinoma cells but also in hyperplastic endothelial cells and/or desmoplastic fibroblasts. In addition to colon carcinoma that showed the highest percentage of moderate-to-strong MetAP2 staining, prostate, pancreatic, ovarian, lung (non-small cell) and breast carcinoma were shown to have high levels of MetAP2 expression. In a large-scale analysis of MetAP2 protein expression in tissue microarrays carried out in the laboratory of Dr Guido Sauter (Cantonal Hospital Basel, Switzerland), highest expression of MetAP2 was identified among gastrointestinal tumors such as severe dysplasia of colon adenoma, where 66% exhibited moderate or strong MetAP2 staining. Finally, we carried out a more detailed comparison of MetAP2 protein levels in paired human colon cancer and normal tissues. Again, MetAP2 staining appeared stronger in neoplasia and desmoplasia than in normal crypts and submucosa (Figure 5b). Using an automated cellular image system, we quantitatively determined staining intensity as well as percentage of staining in 18 pairs of tumor and normal tissues from the same patients (Figure 5c). MetAP2 expression level was significantly higher in cancer neoplasia and desmoplasia. These data are consistent with the contention that MetAP2 plays a role in human cancer.

Discussion

We have presented a new line of evidence to support a role of MetAP2 in cancer. Ectopic expression of MetAP2 causes cell transformation and promotes cell proliferation. The catalytic activity of MetAP2 is responsible for this transforming activity because MetAP2 inhibitors can block this effect. Cell transformation induced by oncogenes is typically determined by foci formation on two-dimensional culture and by growth of colonies in soft agar. These cellular responses were reproducibly observed in transfected NIH-3T3 cells with MetAP2 overexpression. Furthermore, the MetAP2-overexpressing NIH-3T3 cells did form tumors when inoculated in the flank of nude mice. However, the vector control cells also grew tumors, an observation that is not uncommon with DNA-transfected NIH-3T3 cells. Nevertheless, the comparison of these vector control or MetAP2-transfected cells *in vitro* consistently showed dramatic differences. In soft-agar assay, only transfected cells with significant MetAP2 overexpression (>4-fold) formed colonies; there were never visible colonies with vector control cells or with transfected clones without MetAP2 overexpression.

On two-dimensional culture, foci formation was seen with pLPCX-MetAP2 but not with the pLPCX vector control. In addition, cells with MetAP2 overexpression showed an accelerated growth rate. For the first time in our knowledge, these data established that MetAP2 could function as an oncogene.

The transforming activity of MetAP2 overexpression is consistent with previous findings. Several studies have indicated that MetAP2 expression is controlled by growth factors and mitogens. This expression of MetAP2 in rat hepatoma cells was dramatically decreased by serum starvation; addition of growth medium or a mitogen (PMA)-induced MetAP2 expression as well as cell growth (Datta, 2000). We have reported a similar effect of serum starvation in several endothelial cell lines and HT 1080 tumor cells (Wang *et al.*, 2000). More recently, Menssen and Hermeking (2002) found that adenovirus transfer of c-Myc increased the expression of MetAP2 in human umbilical vein endothelial cells, suggesting that cellular proliferation and transformation induced by c-Myc could involve MetAP2 overexpression.

The dominant-negative effect of the catalytically inactive MetAP2 mutant on tumor cell growth further supports a role for MetAP2 in carcinogenesis. The dominant-negative function of an inactive MetAP2 in yeast has previously been reported (Vetro *et al.*, 2005). In the current studies, we observed that constitutive expression of a catalytically inactive MetAP2 mutant was toxic to HT1080 fibrosarcoma cells, whereas induced expression of this mutant resulted in growth inhibition. The inactive MetAP2 mutant may compete with native MetAP2 for the ribosome site where processing of initiator methionine occurs, thus blocking normal MetAP2 function. In fact, it was shown that the N-terminal region of yeast MetAP2 (residues 2–57) was essential for the inactive MetAP2 mutant to fully interfere with wild-type MetAP2 function and be dominant-negative (Vetro *et al.*, 2005).

The molecular mechanism for transformation induced by MetAP2 overexpression is not clear. Eukaryotic proteins are synthesized on the ribosome with an N-terminal methionine. In the majority of cellular proteins, the methionine is removed co-translationally (Bradshaw *et al.*, 1998), and this removal of the initiator methionine is required for proper function of these proteins, that is, activity, localization and stability. MetAP2 is a protein with dual functions: MetAP activity and the stabilization of eIF-2 α as p67 (Datta, 2000). Truncation of the highly charged N-terminal domain, speculated to be involved in the binding of eIF-2 α and preventing its inactivation (Datta, 2000), does not affect MetAP2 enzyme activity *in vitro* (Yang *et al.*, 2001; Wang *et al.*, 2003b). Similarly, MetAP2 covalently inactivated by TNP-470 is still able to stabilize eIF-2 α (Griffith *et al.*, 1997). The catalytic activity of MetAP2 is required for the transformation effect because specific inhibitors blocked the phenotype; it is not clear whether the stabilization of eIF-2 α by MetAP2 protein plays a role. It would be of interest to test the overexpression of MetAP2 in a cell line or mouse embryonic fibroblasts

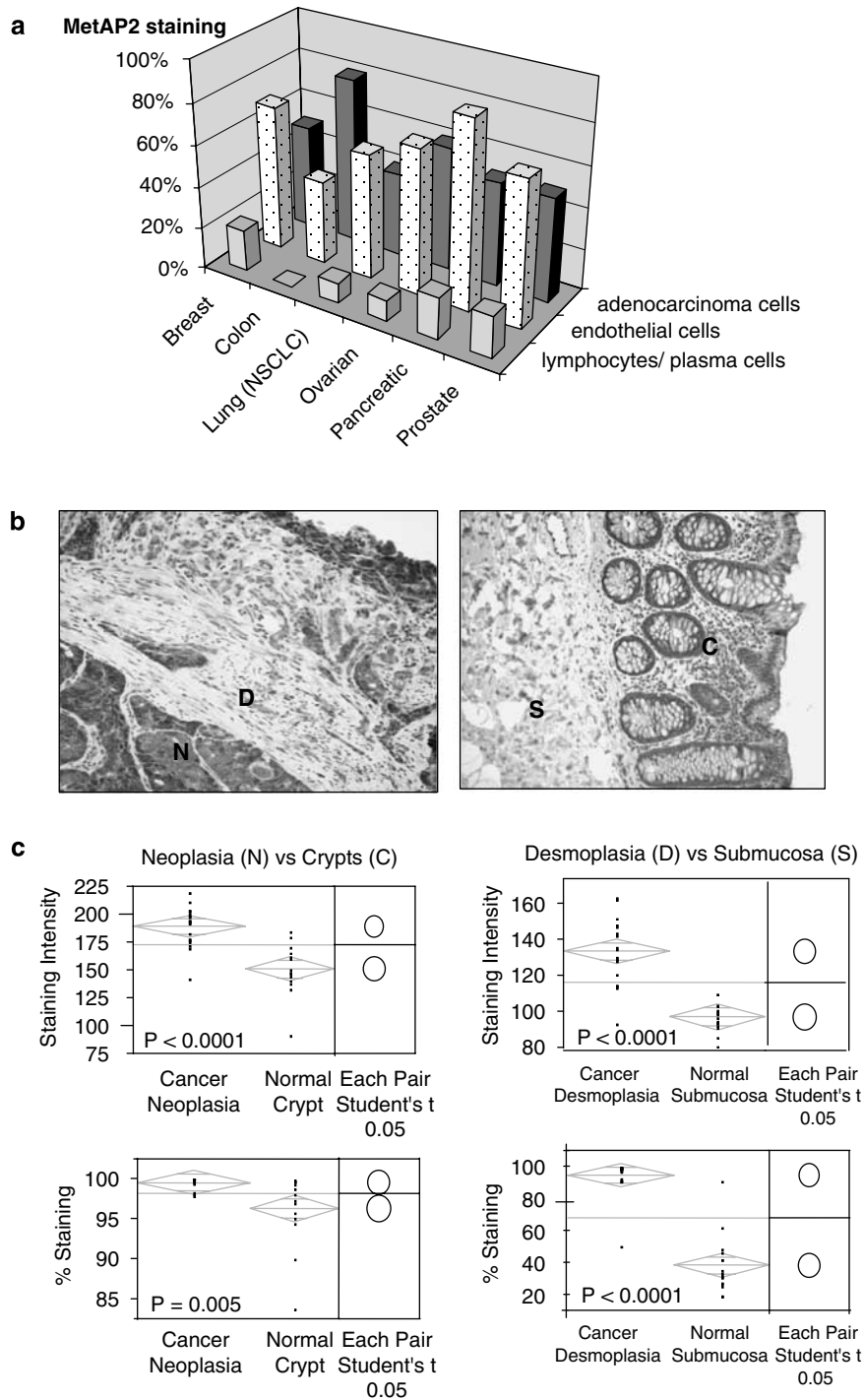


Figure 5 Immunohistochemistry analysis of MetAP2 expression in cancers. (a) MetAP2 staining in human breast, colon, lung, ovarian, pancreas and prostate cancers. Moderate-to-high staining in adenocarcinoma cells, endothelial cells and lymphocyte/plasma cells in cancer samples were shown as percentage of total samples ($n = 10$). (b) MetAP2 staining in human colon carcinoma and normal tissues. MetAP2 protein was stained in neoplasia (N), desmoplasia (D), crypts (C) and submucosa (S) in colon carcinoma or normal tissues. (c) Summary of MetAP2 staining of sections of 18 matched pairs of human normal/carcinoma tissues. Significant differences in mean staining intensity ($P < 0.0001$, neoplasia vs crypts and desmoplasia vs submucosa) and in mean percentage staining ($P = 0.005$, neoplasia vs crypts and $P < 0.0001$, desmoplasia vs submucosa) were observed for all matched pairs.

that express a phosphorylation-resistant mutant of eIF-2 α to determine whether MetAP2 upregulation can still lead to cell transformation. Nevertheless, it is conceivable that elevated cellular concentration of MetAP2 promoted translation and processing of

substrate proteins that led to aberrant cell growth and transformation. In our attempts to identify MetAP2-specific substrates, we noticed that Src family tyrosine kinases such as Fyn, Yes, Hck, Yrk and Blk; ADP-ribosylation factors such as Arf6, Arf5 and Arf4; and

thioredoxin and eukaryotic translation elongation factor-2 were preferred substrates for MetAP2 (unpublished data). These proteins are either known oncogenes or play a role in cancer cell growth, apoptosis and autophagy (Hashimoto *et al.*, 2004; Arner and Holmgren, 2006; Homsy *et al.*, 2007; Mieulet *et al.*, 2007). MetAP2 overexpression may promote and facilitate the action of these oncoproteins.

Higher expression of MetAP2 protein in human cancers further supports the contention that MetAP2 plays a role in cancer development. We saw moderate-to-strong staining of MetAP2 in all adenocarcinomas examined. This observation extends previously published findings. Marked increases in MetAP2 expression were seen in B-cell germinal centers compared with other B cells (Kanno *et al.*, 2002). High expression was also found in follicular lymphomas, 50% of the large B-cell lymphomas and all of the Burkitt's lymphomas examined. Elevated expression of MetAP2 was reported in human colorectal adenocarcinomas (Selvakumar *et al.*, 2004).

Recent data from mouse genetic studies (Yeh *et al.*, 2006) confirmed a role of MetAP2 in angiogenesis, which had been suggested by the results of small-molecule inhibitors. The natural product fumagillin and synthetic analogs such as TNP-470 (Ingber *et al.*, 1990) were potent irreversible MetAP2 inhibitors. These compounds covalently modify MetAP2 with a high selectivity, being over three orders of magnitude less potent against MetAP1 (Griffith *et al.*, 1997; Sin *et al.*, 1997). TNP-470 has been shown to inhibit tumor growth in a large number of syngeneic and human xenograft flank models. In clinical trials, it showed anecdotal tumor regression and disease stabilization in a few cases of lung and liver metastases (Castronovo and Belotti, 1996; Kruger and Figg, 2000). PPI-2458 is a novel, orally active agent of the fumagillin class of irreversible MetAP2 inhibitors and currently being evaluated in the clinic for cancer treatment (Hannig *et al.*, 2006). More recently, two classes of reversible MetAP2 inhibitors were reported to have antiangiogenic and antitumor effect in preclinical models (Wang *et al.*, 2003a; Sheppard *et al.*, 2006). A correlation of cellular MetAP2 inhibition and antiproliferative activity *in vitro* and antitumor efficacy *in vivo* has also been observed for these inhibitors (Wang *et al.*, 2003a; Sheppard *et al.*, 2006). The anticancer effect of these MetAP2 inhibitors may be a result of the combined effect of MetAP2 inhibition in endothelial cells (antiangiogenesis) and in tumor cells directly. The oncogenic activity of MetAP2 further indicates that MetAP2 inhibitors may be developed as novel agents for cancer therapy.

Materials and methods

Vector, virus and cell transfection

Full-length human MetAP2 was cloned into the retroviral vector pLPCX (Clontech, Mountain View, CA, USA). Stable virus was made by transfecting a PT67-packaging cell line (Clontech) with the pLPCX control vector and the MetAP2 pLPCX construct by calcium phosphate precipitation

(Mammalian Protection kit, Promega, Madison, WI, USA). NIH-3T3 mouse fibroblast cells and NL20 cells were both purchased from American Type Culture Collection (Manassas, VA, USA). Both cell lines were infected with control pLPCX virus and MetAP2 pLPCX virus. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Stable cell clones were isolated by growing in the same medium with $1 \mu\text{g ml}^{-1}$ puromycin. Src cDNA (Millipore, Billerica, MA, USA) was used as a positive control.

A full-length MetAP2-H231N-mutant sequence, constructed by using the QuickChange-II Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA), was cloned into the pTRE2-pur vector (Clontech) to be used with Clontech's TET-OFF system. HT1080 TET-OFF cells (Clontech) were transfected with the MetAP2-H231N mutant pTRE2-pur construct. pTRE2-Luc was also transfected as a control. Stable clones were tested for overexpression and proliferation upon the tetracycline switch. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% dialysed fetal bovine serum (Clontech), $100 \mu\text{g ml}^{-1}$ G418 and $1 \mu\text{g ml}^{-1}$ puromycin. Doxycycline (a derivative of tetracycline) was used at $1 \mu\text{g ml}^{-1}$ in all studies to suppress the expression of the gene in the pTRE2-pur vector.

Proliferation, soft agar and foci assays

Proliferation assay Cells were grown in 96-well plate in the presence of compounds for 3 days. MTS reagents or CellTiter-Glo (Promega) were used to quantify live cells at the end of study. Alternatively, cells grown in special microelectrodes imbedded 96-well plates were monitored real time with the cell electric sensing system (ACEA Bioscience, San Diego, CA, USA).

Soft agar assay Cells were plated at 10 000 cells per well in 24-well plate with of a bottom layer of 0.7% noble agar and top layer of 0.3% agar with cells. Medium was added over the top layer of agar. When treated with MetAP2 inhibitors A-357300 or TNP-470, the compounds were included in the top medium. Cells were grown at 37°C for 2-4 weeks until colonies were visible, stained with 0.5 mg ml^{-1} Iodonitrotetrazolium Violet (Sigma, St Louis, MO, USA) at 37°C overnight, and counted using a Sony DXC-970MD 3CCD color video camera and Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Foci assay Cells were plated in T25 flasks at 1×10^5 cells per flask, treated with inhibitors and observed for foci formation over the span of 1 week.

Immunohistochemistry

Rabbit polyclonal antibodies were raised against recombinant human MetAP2 as previously described (Wang *et al.*, 2000). Initial immunohistochemistry validation of this antibody and screen on human tissues were carried out at Lifespan Biosciences. The principal detection system consisted of a Vector anti-rabbit secondary (BA-1000) and a Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100), which produces a fuchsia-colored deposit. In the follow-up studies, 18 matched pairs of human colon normal/carcinoma-paraffin blocks were purchased from Cooperative Human Tissue Network. After antigen retrieval with BORG delecting system (Biocare Medical, Walnut Creek, CA, USA), $5\text{-}\mu\text{m}$ sections were incubated with polyclonal rabbit antihuman MetAP2 antibody followed by Polymer HRP/DAB detection system (Biocare). Mosaic pictures of whole tissue section stained for MetAP2 were taken with Automated Cellular

Image System (Dakocytomation, Carpinteria, CA, USA). The intensity and percentage of cells stained were analysed and scored by the same system using the software model 'Detection and Measurement of IHC staining.' A Student's *t*-test was used for statistical analysis.

References

- Arner ES, Holmgren A. (2006). The thioredoxin system in cancer. *Semin Cancer Biol* **16**: 420–426.
- Bernier SG, Taghizadeh N, Thompson CD, Westlin WF, Hannig G. (2005). Methionine aminopeptidases type I and type II are essential to control cell proliferation. *J Cell Biochem* **95**: 1191–1203.
- Bradshaw RA, Brickey WW, Walker KW. (1998). N-terminal processing: the methionine aminopeptidase and n alpha-acetyl transferase families. *Trends Biochem Sci* **23**: 263–267.
- Bradshaw RA, Yi E. (2002). Methionine aminopeptidases and angiogenesis. *Essays Biochem* **38**: 65–78.
- Castronovo V, Belotti D. (1996). Tnp-470 (agm-1470): mechanisms of action and early clinical development. *Eur J Cancer* **32A**: 2520–2527.
- Catalano A, Romano M, Robuffo I, Strizzi L, Procopio A. (2001). Methionine aminopeptidase-2 regulates human mesothelioma cell survival: role of bcl-2 expression and telomerase activity. *Am J Pathol* **159**: 721–731.
- Datta B. (2000). MAPs and POEP of the roads from prokaryotic to eukaryotic kingdoms. *Biochimie* **82**: 95–107.
- Datta B, Datta R. (1999). Induction of apoptosis due to lowering the level of eukaryotic initiation factor 2-associated protein, p67, from mammalian cells by antisense approach. *Exp Cell Res* **246**: 376–383.
- Griffith EC, Su Z, Niwayama S, Ramsay CA, Chang YH, Liu JO. (1998). Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc Natl Acad Sci USA* **95**: 15183–15188.
- Griffith EC, Su Z, Turk BE, Chen S, Chang YH, Wu Z *et al.* (1997). Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors agm-1470 and ovalicin. *Chem Biol* **4**: 461–471.
- Hannig G, Lazarus DD, Bernier SG, Karp RM, Lorusso J, Qiu D *et al.* (2006). Inhibition of melanoma tumor growth by a pharmacological inhibitor of metap-2, ppi-2458. *Int J Oncol* **28**: 955–963.
- Hashimoto S, Onodera Y, Hashimoto A, Tanaka M, Hamaguchi M, Yamada A *et al.* (2004). Requirement for arf6 in breast cancer invasive activities. *Proc Natl Acad Sci USA* **101**: 6647–6652.
- Homsy J, Cubitt C, Daud A. (2007). The src signaling pathway: a potential target in melanoma and other malignancies. *Expert Opin Ther Targets* **11**: 91–100.
- Ingbre D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H *et al.* (1990). Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* **348**: 555–557.
- Kanno T, Endo H, Takeuchi K, Morishita Y, Fukayama M, Mori S. (2002). High expression of methionine aminopeptidase type 2 in germinal center B cells and their neoplastic counterparts. *Lab Invest* **82**: 893–901.
- Kruger EA, Figg WD. (2000). Tnp-470: an angiogenesis inhibitor in clinical development for cancer. *Expert Opin Investig Drugs* **9**: 1383–1396.
- Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T *et al.* (1994). Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor tnp-470 (agm-1470). *Br J Cancer* **69**: 212–216.
- Kwon YT, Kashina AS, Davydov IV, Hu RG, An JY, Seo JW *et al.* (2002). An essential role of N-terminal arginylation in cardiovascular development. *Science* **297**: 96–99.
- Leszczyniecka M, Bhatia U, Cueto M, Nirmala NR, Towbin H, Vattay A *et al.* (2006). MAPID, a novel methionine aminopeptidase family member is overexpressed in colon cancer. *Oncogene* **25**: 3471–3478.
- Liu S, Widom J, Kemp CW, Crews CM, Clardy J. (1998). Structure of human methionine aminopeptidase-2 complexed with fumagillin. *Science* **282**: 1324–1327.
- Lowther WT, Matthews BW. (2000). Structure and function of the methionine aminopeptidases. *Biochim Biophys Acta* **1477**: 157–167.
- Menssen A, Hermeking H. (2002). Characterization of the c-myc-regulated transcriptome by sage: identification and analysis of c-myc target genes. *Proc Natl Acad Sci USA* **99**: 6274–6279.
- Mieulet V, Roceri M, Espeillac C, Sotiropoulos A, Ohanna M, Oorschot V *et al.* (2007). S6 kinase inactivation impairs growth and translational target phosphorylation in muscle cells maintaining proper regulation of protein turnover. *Am J Physiol Cell Physiol* **293**: C712–C722.
- Resh MD. (2004). Membrane targeting of lipid modified signal transduction proteins. *Subcell Biochem* **37**: 217–232.
- Selvakumar P, Lakshmikuttyamma A, Kanthan R, Kanthan SC, Dimmock JR, Sharma RK. (2004). High expression of methionine aminopeptidase 2 in human colorectal adenocarcinomas. *Clin Cancer Res* **10**: 2771–2775.
- Sheppard GS, Wang J, Kawai M, Fidanze SD, Bamaung NY, Erickson SA *et al.* (2006). Discovery and optimization of anthranilic acid sulfonamides as inhibitors of methionine aminopeptidase-2: a structural basis for the reduction of albumin binding. *J Med Chem* **49**: 3832–3849.
- Sin N, Meng L, Wang MQ, Wen JJ, Bornmann WG, Crews CM. (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, metap-2. *Proc Natl Acad Sci USA* **94**: 6099–6103.
- Varshavsky A. (2005). Regulated protein degradation. *Trends Biochem Sci* **30**: 283–286.
- Vetro JA, Dummitt B, Micka WS, Chang YH. (2005). Evidence of a dominant negative mutant of yeast methionine aminopeptidase type 2 in *Saccharomyces cerevisiae*. *J Cell Biochem* **94**: 656–668.
- Wang J, Lou P, Henkin J. (2000). Selective inhibition of endothelial cell proliferation by fumagillin is not due to differential expression of methionine aminopeptidases. *J Cell Biochem* **77**: 465–473.
- Wang J, Sheppard GS, Lou P, Kawai M, BaMaung N, Erickson SA *et al.* (2003a). Tumor suppression by a rationally designed reversible inhibitor of methionine aminopeptidase-2. *Cancer Res* **63**: 7861–7869.
- Wang J, Sheppard GS, Lou P, Kawai M, Park C, Egan DA *et al.* (2003b). Physiologically relevant metal cofactor for methionine aminopeptidase-2 is manganese. *Biochemistry* **42**: 5035–5042.
- Wu S, Gupta S, Chatterjee N, Hileman RE, Kinzy TG, Denslow ND *et al.* (1993). Cloning and characterization of complementary DNA encoding the eukaryotic initiation factor 2-associated 67-kDa protein (p67). *J Biol Chem* **268**: 10796–10801.
- Yang G, Kirkpatrick RB, Ho T, Zhang GF, Liang PH, Johanson KO *et al.* (2001). Steady-state kinetic characterization of substrates and metal-ion specificities of the full-length and N-terminally truncated recombinant human methionine aminopeptidases (type 2). *Biochemistry* **40**: 10645–10654.
- Yeh JR, Ju R, Brdlik CM, Zhang W, Zhang Y, Matyskiela ME *et al.* (2006). Targeted gene disruption of methionine aminopeptidase 2 results in an embryonic gastrulation defect and endothelial cell growth arrest. *Proc Natl Acad Sci USA* **103**: 10379–10384.
- Yeh JR, Mohan R, Crews CM. (2000). The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. *Proc Natl Acad Sci USA* **97**: 12782–12787.
- Zhang Y, Griffith EC, Sage J, Jacks T, Liu JO. (2000). Cell cycle inhibition by the anti-angiogenic agent TNP-470 is mediated by p53 and p21WAF1/CIP1. *Proc Natl Acad Sci USA* **97**: 6427–6432.

Acknowledgements

We thank Drs Milagros Colon-Lopez and Guido Sauter for tissue microarray studies on MetAP2 expression, and Dr Jack Henkin for critical reading of this paper.