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## Biotinylation of the c-Myc promoter binding protein MBP-1 decreases c-Myc and COX-2 expression in mammary carcinoma MCF-7 cells

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# Biotinylation of the c-Myc promoter binding protein MBP-1 decreases c-Myc and COX-2 expression in mammary carcinoma MCF-7 cells

by

Jie Zhou

A THESIS

Presented to the Faculty of  
The Graduate College at the University of Nebraska  
In Partial Fulfillment of the Requirements  
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Major: Nutrition

Under the Supervision of Professor Janos Zemleni

Lincoln, Nebraska

December, 2013

# Biotinylation of the c-Myc promoter binding protein MBP-1 decreases c-Myc and COX-2 expression in mammary carcinoma MCF-7 cells

Jie Zhou, M.S

University of Nebraska, 2013

Advisor: Janos Zempleni

Biotin feeding in a Balb/c mouse model of breast cancer suggest that the tumor load is ~100% greater in biotin-deficient mice compared with biotin-sufficient controls, and that the increase is associated with metastasis rather than new tumors. The oncogenes c-Myc and COX-2 are implicated in cancer metastasis, and their expressions are repressed by binding of MBP-1 to the promoter. Mass spectrometry studies, designed to discover novel biotin-containing proteins, suggest that lysine residues K10 and K12 in the c-Myc promoter binding protein (MBP-1) contains covalently bound biotin (MBP-1bio). We hypothesized that biotinylation of MBP-1 increases the binding of MBP-1 to the c-Myc and COX-2 promoter, thereby decreasing oncogene expression and metastasis. A novel antibody against MBP-1bio was used to confirm the existence of MBP-1bio in human mammary carcinoma MCF-7 cells; the abundance of MBP-1bio depended on the concentration of biotin in culture media: supplemented > sufficient > deficient. The activity of a c-Myc reporter gene was ~37% greater in biotin-deficient cells compared with sufficient and supplemented controls. Likewise, both c-Myc and COX-2 mRNA levels were significantly increased in deficient compared with sufficient cells. When K10

and K12 in MBP-1 were removed by site-directed mutagenesis, biotin supply no longer affected the expression of c-Myc and COX-2 in MCF-7 cells. Biotinylation of MBP-1 is an enzymatic process catalyzed by using recombinant holocarboxylase synthetase (HLCS), judged by using recombinant HLCS and recombinant MBP-1. Consistent with this observation, c-Myc and COX-2 are de-repressed in HLCS mutant WG2215 human fibroblasts compared with controls. We conclude that loss of MBP-1<sup>bio</sup> in biotin-depleted and HLCS-deficient organisms leads to an increased expression of oncogenes, thereby increasing cancer risk.

# Acknowledgements

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I would also like to extend my appreciation to Professor Timothy P. Carr and Professor Istvan (Steve) Ladunga, my committee, for their kind advice and assistance.

I would like to thank Samudra, Mengna, Dandan, Jing, Liz, Wei Kay, Dave, Yong, Scott, Daniel and all other lab members for their generous help of this research work as well as my life in Lincoln.

Finally, I thank my parents for everything, especially their constant encouragement and support during my Master's studies.

Jie Zhou

November 2013

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# **CHAPTER 1**

## **LITERATURE REVIEW**

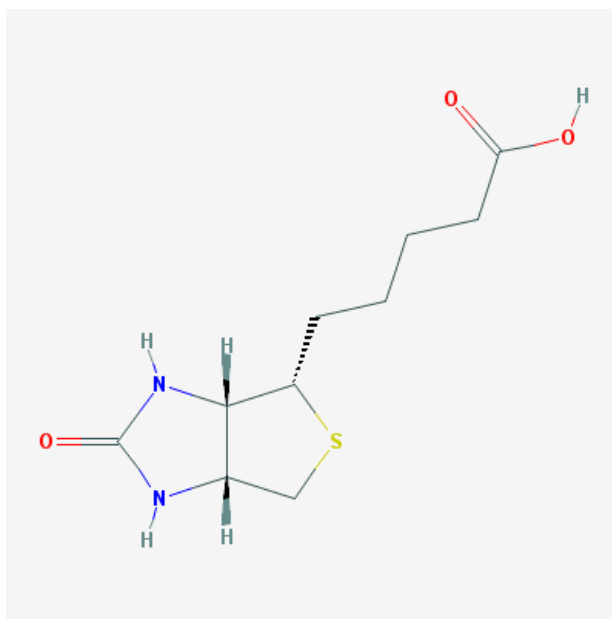
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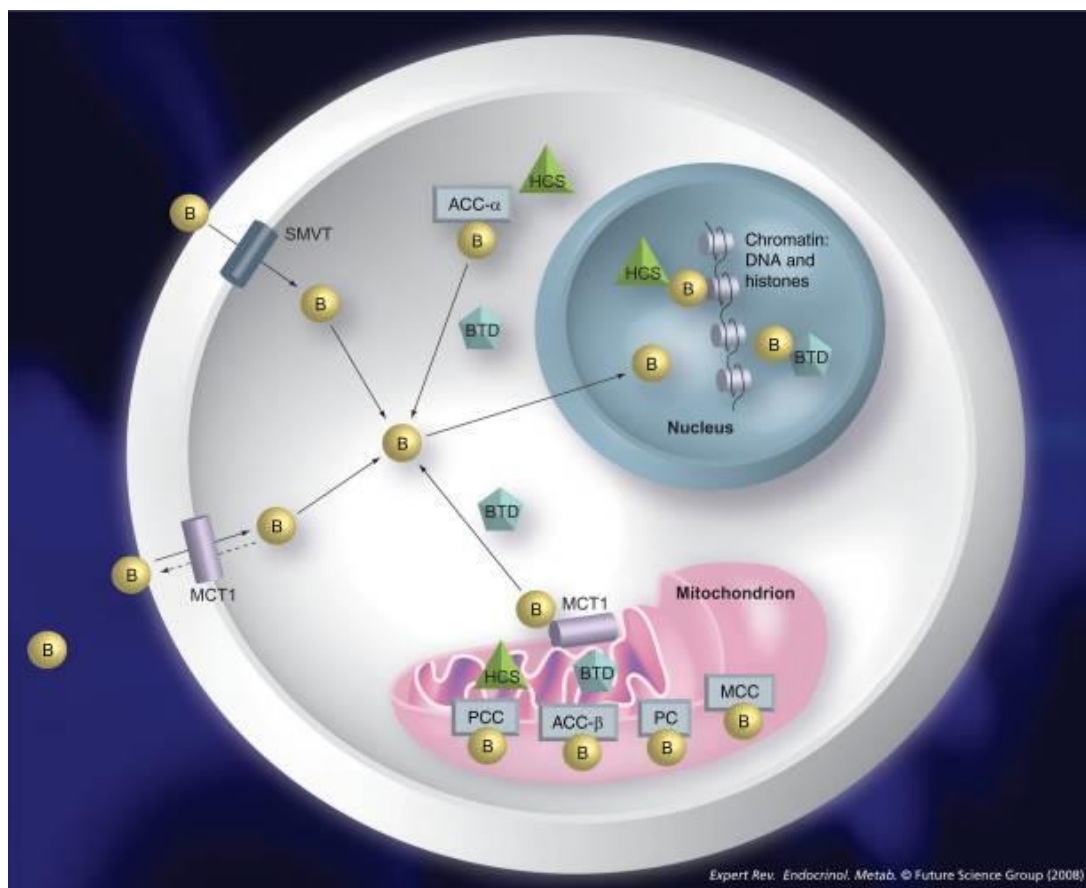
## Biotin, Holocarboxylase Synthetase (HLCS) and biotinylation

Biotin ( $C_{10}H_{16}N_2O_3S$ , MW: 244.31064), known as vitamin B7 or H, is a water-soluble vitamin involved in epigenetic regulations and many metabolic pathways in mammals (1-7). Biotin is a monocarboxylic acid contains an ureido group, thiophane heterocyclic ring, and a valeric acid side chain (7-9) (Fig. 1).



**Figure 1. 2D structure of biotin (Pubchem CID 171548).**

Biotin is found in high concentration in varieties of foods including egg yolk, cow milk, liver and some vegetables in the form of free biotin or biotin-binding protein conjugates (10). For uptake of biotin from dietary resource, the biotin-binding proteins are converted to free biotin by gastrointestinal proteases, peptidases, and biotinidase (7). The fundamental metabolic role of biotinidase (BTD) is to hydrolyze biocytin generated in the breakdown of biotin-binding carboxylases (11). Therefore, free biotin is released and recycled in holocarboxylase synthesis and used as co-factor in epigenetic and metabolic processes (9). The transportation of free biotin to the cells is depended on the sodium-dependent multi-vitamin transporter (SMVT) (12). It has been reported that monocarboxylate transporter 1 (MCT1) is also involved in cellular biotin transportation in peripheral blood mononuclear cells (11, 13, 14) (Fig. 2).



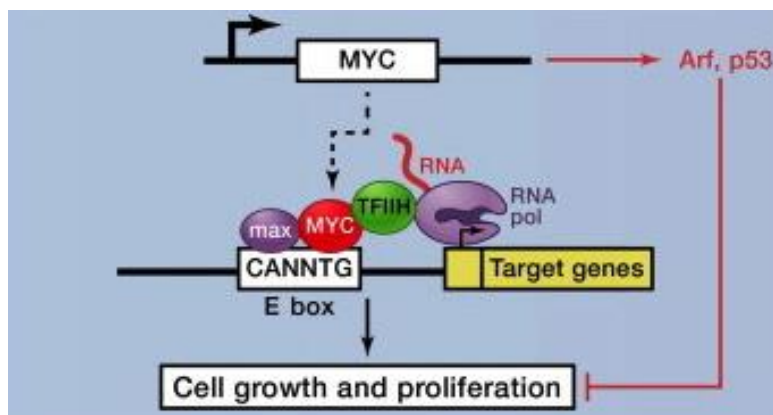
**Figure 2. Biotin transport and metabolism, taken from (15).** ACC1: cytoplasmatic acetyl-CoA carboxylase alpha, ACC2: mitochondrial acetyl-CoA carboxylase beta, B: biotin, MCC: and 3- methylcrotonyl-CoA carboxylase, MCT1: monocarboxylate transporter 1, PC: pyruvate carboxylase, PCC: propionyl- CoA carboxylase, SMVT: sodium multivitamin transporter.

Holocarboxylase synthetase (HLCS), a 726 amino acids protein, is the sole biotin protein ligase in the human proteome and is responsible for catalyzing biotinylation of acetyl-CoA carboxylase 1 and 2 (ACC1, ACC2), 3-methylcrotonyl-CoA carboxylase (MCC), pyruvate carboxylase (PCC) and propionyl-CoA carboxylase (PCC) and other proteins (16). Biotinylated carboxylases are localized to cytoplasm and mitochondria and play roles in intermediary metabolism including fatty acid synthesis, regulation of fatty acid catabolism, elimination of odd-chain fatty acids, leucine metabolism, and gluconeogenesis (1-3, 5, 6, 17, 18). HLCS also enters the nuclear compartment (2, 19-21). Nuclear HLCS orchestrates the assembly of a multiprotein gene repression complex (22, 23). The formation of the multiprotein complex depends on the availability of biotin; the complex appears to play an important role in the repression of long terminal repeats (LTRs), thereby contributing to genome stability (24). The covalent binding of biotin to histones (1) appears to be a mere side effect of the close physical proximity of HLCS and histones (22, 23)

## **c-Myc promoter binding protein (MBP-1) and target oncogenes of MBP-1 repression**

c-Myc promoter binding protein (MBP-1, NCBI Reference Sequence: NM\_001201483.1) is a transcriptional variant encoded by ENO1 enolase 1, (alpha) [*Homo sapiens*](25) (Gene ID: 2023). This MBP-1 protein consists of 341 amino acids is localized to the nucleus that functions as a novel transcriptional repressor of oncogene c-Myc through binding to the TATA box of its P2 promoter (26-28). In addition to c-Myc, MBP-1 also represses the oncogenes cyclooxygenase-2 (COX-2) and ERBB2 (26, 29, 30). The MBP-1 N-terminus is crucial for oncogene repression, which also involves accessory factors such as activated Notch1 receptor, Kelch protein NS1-BP, and histone deacetylases (31-34). C-Myc, COX-2, and ERBB2 are associated with tumorigenesis including breast and gastric cancer, and low levels of MBP-1 have been observed in invasive ductal breast carcinoma (IDC), which supporting the role of MBP-1 as a potential prognostic marker in carcinoma (29, 35, 36).

c-Myc, Cox-2, and ERBB2 are examples of oncogenes regulated by MBP-1. c-Myc dimerizes with Max; the heterodimer binds to E boxes in target genes and causes aberrations in cell cycle, growth, apoptosis and metabolism (37-39) ( Fig. 3).



**Figure 3. Mechanisms of c-Myc involved in gene transcription, cell growth and proliferation.**

COX-2 is the inducible isoform of the *COX-2/PTGS2* gene encoding prostaglandin-endoperoxide synthase 2 (PTGS2, Gene ID: 5743), which is the key enzyme in prostaglandin biosynthesis. High levels of COX-2 have been observed in tumor tissues from a breast cancer mouse model housed in standard environment (40). Strong associations between COX-2 and breast or gastric cancer have been established, COX-2 dependent activation of the p38/MAPK pathway (41), Jun N-terminal kinases mediated activation of cell invasion in MCF-7 cells (42), and IL-1beta and NFkappaB mediated induction of inflammation (43). COX-2 inhibitors such as celecoxib are considered promising agents in the treatment and prevention of breast cancer (44, 45).

ERBB2 (Gene ID: 2064) encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. High levels of ERBB2 have been observed in metastatic breast cancer. ERBB2 contributes breast cancer metastasis through increased expression of glutaminase 1(36), activation of NF-kappaB (46), and increased breast cancer cell migration and invasion (47).

In conclusion, MBP-1 is an important repressor of three oncogenes that play crucial roles in tumorigenesis in human prostate, gastric and breast cancer.

## **CHAPTER 2**

# **BIOTINYLATION OF THE C-MYC PROMOTER BINDING PROTEIN MBP-1 DECREASES C-MYC AND COX-2 EXPRESSION IN HUMAN MAMMARY CARCINOMA MCF-7 CELLS**



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## Introduction

The c-Myc promoter binding protein, MBP-1, is encoded by the *Enolase-1* gene and consists of 341 amino acids. MBP-1 localizes in the cell nucleus and functions as a transcriptional repressor of the oncogenes c-Myc, cyclooxygenase-2 (COX-2), and ERBB2 (26-30, 48, 49). The N-terminus in MBP-1 is essential for repressor activity (31). Repression may involve accessory factors such as Notch1 receptor (32), Kelch protein NS1-BP (33), histone deacetylases (34), and miR-29b (50).

Recently, our discovery-based LC/MS/MS analysis identified MBP-1 as one of 108 novel biotin-containing proteins in human embryonic kidney HEK293 cells (Li Y. et al., submitted). There is precedence that posttranslational modifications affect the activity of transcription factors. For example, biotin supplementation decreases Sp1 and NF- $\kappa$ B binding to their response elements (51); HLCS-dependent biotinylation of HSP72 increases RANTES expression(52). Importantly, at the time we had completed a biotin feeding study in a murine breast cancer model, which suggested that the tumor load increases by about 100% in biotin-deficient mice compared with biotin-sufficient and biotin-supplemented mice (see Results). Based on the roles of MBP-1 in oncogene repression, the detection of biotinylated MBP-1 by mass spectrometry, and the observations in biotin feeding studies, we hypothesized that the binding of biotin to MBP-1 increases oncogene repressor activity. We further hypothesized that biotinylation of MBP-1 is mediated by the sole biotin protein ligase in the human proteome, holocarboxylase synthetase (HLCS) (16).

## Materials and Methods

### *Cell cultures*

Human embryonic kidney HEK293 cells (American Type Culture Collection, CRL-1573) were cultured in Dulbecco's Modified Eagle Medium (DMEM) media containing 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine as described previously (53). Human mammary carcinoma MCF-7 cells were cultured in DMEM/Nutrient F-12 Ham (DMEM/F12). In select experiments, MCF-7 cells were cultured in biotin-defined DMEM, prepared as described previously (53). Biotin-defined media contained the following levels of biotin: 0.025 nmol/L, 0.25 nmol/L, and 10 nmol/L, representing biotin plasma levels observed in deficient, normal, and supplemented persons, respectively (11, 53, 54). HLCS-deficient WG2215 fibroblasts from an HLCS mutant patient (Montreal Children's Hospital Cell Repository, Montreal, Canada) and HLCS wild-type human neonatal normal dermal fibroblast (American Type Culture Collection, PCS-201-010) were cultured in Fibroblast Basal Medium supplemented with 2% fetal bovine serum, 5 ng/mL recombinant human fibroblast growth factor (rh FGF) beta, 5 µg/mL recombinant human insulin, 1 µg/mL hydrocortisone, 50 µg/mL ascorbic acid, and 7.5 mM L-glutamine.

### *Purification of biotinylated proteins and mass spectrometry analysis*

Whole protein extracts were prepared from HEK293 cells using ice-cold BugBuster<sup>®</sup> Protein Extraction Reagent (Novagen, EMD Millipore), protease inhibitor cocktail (Sigma). Biotin-containing proteins were purified using a Monomeric Avidin Agarose following the manufacturer's recommendations (Thermo Scientific; biotin-binding capacity=1.2 mg biotinylated protein/mL settled resin) (Fig. 1).

Biotinylated proteins were resolved by gel electrophoresis and cut-out bands were digested with trypsin (55). Biotinylated proteins and biotinylation sites were identified by LC/MS/MS in the mass spectrometry core facility in the Redox Biology Center at the University of Nebraska-Lincoln. The acquired spectrum was compared to the human reference proteome for sequence identification using the MASCOT database analysis software (56).

### ***Plasmids and recombinant proteins***

The c-Myc promoter luciferase reporter plasmid, Luc-SNM, includes base pairs -467 to +36 in the human *c-Myc* gene and two MBP-1 binding sites (57); the plasmid was generously provided by Dr. Mark D Minden in the Department of Cellular and Molecular Biology, Ontario Cancer Institute, Toronto, Canada.

Full-length human MBP-1 (Genbank NM\_001201483.1) was cloned using a human enolase 1 (alpha) cDNA clone (MGC: 17252; IMAGE: 3447583) using forward primer 5'- TTCGGGTAG TGGAAAACCAG-3' and reverse primer 5'- CAGCAGCTCGAATT TCTTCC - 3'; the PCR product was ligated into vector pET28a+ (Novagen) using FastDigest *Bam*HI and *Sal*I (Thermo Scientific), thereby creating plasmid pET28a+\_MBP-1 for preparing recombinant MBP-1. Recombinant his-tagged MBP-1

was expressed and purified as described previously (1). Our previous studies suggest that BirA in *E. coli* catalyzes the binding of biotin to some recombinant proteins (58); biotinylated recombinant MBP-1 was removed by avidin chromatography (not shown). Lysine residues K10 and K12 were identified as targets for biotinylation (see Results). Site-directed mutagenesis was used to create a plasmid in which arginines were substituted for K10 and K12. Mutant MBP-1 was created using MGC clone 17252, the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen), and primers ATGGAACAGAAAATAGATCTAGGTTTG GTGCGAACGC and GCGTTCGCACCAAACCTAGATCTATTTTCTGTTCCAT. The plasmid was denoted ENO\_Mut. The plasmid was digested with Fastdigest *EcoRI* and *KpnI* and ligated into the p3XFLAG-Myc-CMV™-26 expression vector (Sigma-Aldrich) to create plasmid MBP-1<sub>R10,R12</sub> coding for a Flag-tagged MBP-1 protein.

Plasmid Flag-MBP-WT and MBP-1<sub>R10,R12</sub> for expression of FLAG-tagged recombinant wild-type and mutant MBP-1, respectively, were created as described for pET28a+\_MBP-1, using forward primer 5'- ACAGGCTTCCATTGACCAG -3' and reverse primer 5'- TCACCATAGAGTGCTTCCAAC - 3'. Plasmid C7472-CMV-7-BAP codes for FLAG (Sigma-Aldrich) and was used as control.

GST-tagged recombinant full-length HLCS was prepared by using plasmid pET41a (+)-HLCS as described previously (1). The identities of all plasmids were verified by sequencing.

### ***Generation of Anti-MBP-1 Antibody and Dot-blot Assay***

The following synthetic, MBP-1 based peptide was used to raise antibodies against MBP-1, biotinylated at K10 and K12 (MBP-1bio), as described previously (3): ac-CZIEMDGTENK(bio)SK(bio)FGANA-NH<sub>2</sub> (Cocalico Biologicals, Inc., PA).

Antibody specificity was assessed using dot blot analysis. Briefly, dilution series (1.5 ug, 0.75 ug and 0.375 ug) of the following peptides were spotted onto PVDF membranes, air dried, and probed with anti-MBP-1bio and goat anti-rabbit IgG: synthetic K10, K12-biotinylated MBP-1 (MBP-1bio), synthetic unbiotinylated MBP-1, chemically biotinylated bovine serum albumin (BSAbio), synthetic peptides mimicking K18- and K8-biotinylated histones H3 (H3K18bio) and H4 (H4K8bio).

### ***Western Blot Analysis***

Proteins were extracted from MCF-7 cells as described above; equal amount of protein (~30 µg) were resolved by gel electrophoresis, and transblots were probed with the following antibodies at 4°C overnight: anti-MBP-1bio, anti-Anti-6X His tag (Origene) and anti- GAPDH (Santa Cruz Biotechnology). Secondary antibodies and visualization and quantification by infrared imaging were conducted as described previously (59). Blots were revealed and quantitated by Odyssey Infrared Imaging System (LI-COR Biosciences).

### ***In vitro biotinylation assay***

His-tagged MBP-1 was incubated with GST-tagged HLCS as described previously (1).

Biotin bound to MBP-1 was probed using streptavidin (1). Anti-His and Coomassie Blue were used to confirm equal loading.

### ***Transfection and luciferase assay***

These assays were conducted to assess whether mutation of K10 and K12 in MBP-1 disrupts the regulation of MBP-1 repressor activity by biotin supply. Briefly, MCF-7 cells were with Flag-WT-MBP1, MBP-1R10,12R, or C7472-CMV-7-BAP (control) using TurboFect (Thermo Scientific) following the manufacturer's instructions (about 2 µg plasmid/1,000,000 cells). Forty-eight hours after transfection, the expression of c-Myc and COX-2 were analyzed using qRT-PCR.

MCF-7 cells cultured in biotin-defined media were cotransfected with luciferase reporter Luc-SNM (pGL2 basic vector as control) and beta-galactosidase internal control vector using TurboFect (Thermo Scientific) following the manufacturer's instructions (about 2 µg plasmid/1,000,000 cells). Forty-eight hours after transfection, cells were harvested for the quantification of luciferase and beta-Gal activities according to standard methods (Promega). Luciferase activity was measured by subtracting machine background and normalizing via beta-galactosidase activity. The results are the average of at least three independent experiments.



### ***Real-Time PCR Analysis (qRT-PCR)***

Total RNA was isolated from MCF-7 cells using the illustra RNAspin Mini Kit (GE Healthcare Life Sciences) and reverse transcribed into cDNA using the ImProm-II™ Reverse Transcription System (Promega) following the manufacturer's protocol. The abundance of transcripts coding for human c-Myc, COX-2, HLCS and GAPDH (control) were quantified by using Power SYBR Green PCR Master Mix (Applied Biosystems) and the following primers: human c-Myc, 5'- TTCGGGTAGTGGAAAACCAG -3' (forward) and 5'-CAGCAGCTCGAATTCT TCC -3' (reverse); human COX-2, 5'- ACAGGCTTCCATTGACCAG-3' (forward), 5'- TCACCATAG AGTGCTTCCAAC-3' (reverse); HLCS, 5'- TGAGACCTGATCCTTAACTTCC -3' (forward) 5'- ATGGAAGATAGACTC CACAT -3' (reverse); GAPDH (control), 5'- TCCACTGGCGTCT TCACC -3' (forward), 5'- GGCAGAGAT GATGACCCTTT -3' (reverse). Transcript abundance was quantified using the comparative threshold cycle (Ct) method.

### ***Statistical Analysis***

Homogeneous variances were analyzed by using Bartlett's test. Significance of differences was tested by one-way ANOVA and the Fisher's Protected Least Significant Difference (PLSD). For pairwise comparisons the t-test was used. StatView5.0.1 (SAS Institute) was used to perform all calculations. Data were reported as mean  $\pm$  SD. Differences were considered significant if  $P < 0.05$ .

## Results

### *Discovery of novel biotinylated proteins in HEK293 cells*

Analysis of protein extracts by LC/MS/MS revealed the identities of 108 novel biotinylated proteins in human cells (Li Y. et al., in preparation), including biotinylation of K10 and K12 in the N-terminus of MBP-1 (Fig. 1).

We raised anti-MBP-1bio to determine whether MBP-1bio is detectable in using western blot analysis and to assess whether the abundance of MBP-1bio depends on the levels of biotin in culture media. Anti-MBP-1bio produced a strong signal with peptide MBP-1bio, but did not cross-react to a meaningful extent with unbiotinylated MBP-1, chemically biotinylated bovine serum albumin (BSAbio), synthetic peptides mimicking K18- and K8-biotinylated histones H3 (H3K18bio) and H4 (H4K8bio) (Fig. 2A). Pre-immune serum produced negligible signal with biotinylated MBP-1 and other controls (Fig. 2B). Equal loading was confirmed using Ponceau S stain as probe (not shown). The signal intensity produced by MBP-1bio was about 40 times greater than that produced by unbiotinylated MBP-1 (Fig. 2C). Pre-immune serum produced negligible signal compared with anti-MBP1bio, when MCF7 cell extracts were assayed using western blot analysis (Fig. 2D).

After 3 days of culture in biotin-defined media, the abundance of MBP-1bio in biotin sufficient MCF-7 cells was increased, compared with that of deficient and physiological cells (Fig. 3A). Compared with anti-MBP-1bio, pre-immune serum produced no signal.

The efficacy of biotin treatment was confirmed using the abundance of biotinylated carboxylases as markers for biotin status (Fig. 3B).

### ***Enzymatic biotinylation of MBP-1***

HLCS is the sole enzyme responsible for catalyzing covalent binding of biotin to proteins in the human proteome. To determine the biotin ligase activity of HLCS on MBP-1, the *in vitro* biotinylation assay was conducted. His-MBP1 was biotinylated in a time-dependent manner after incubation with GST-HLCS (Fig. 4) (52).

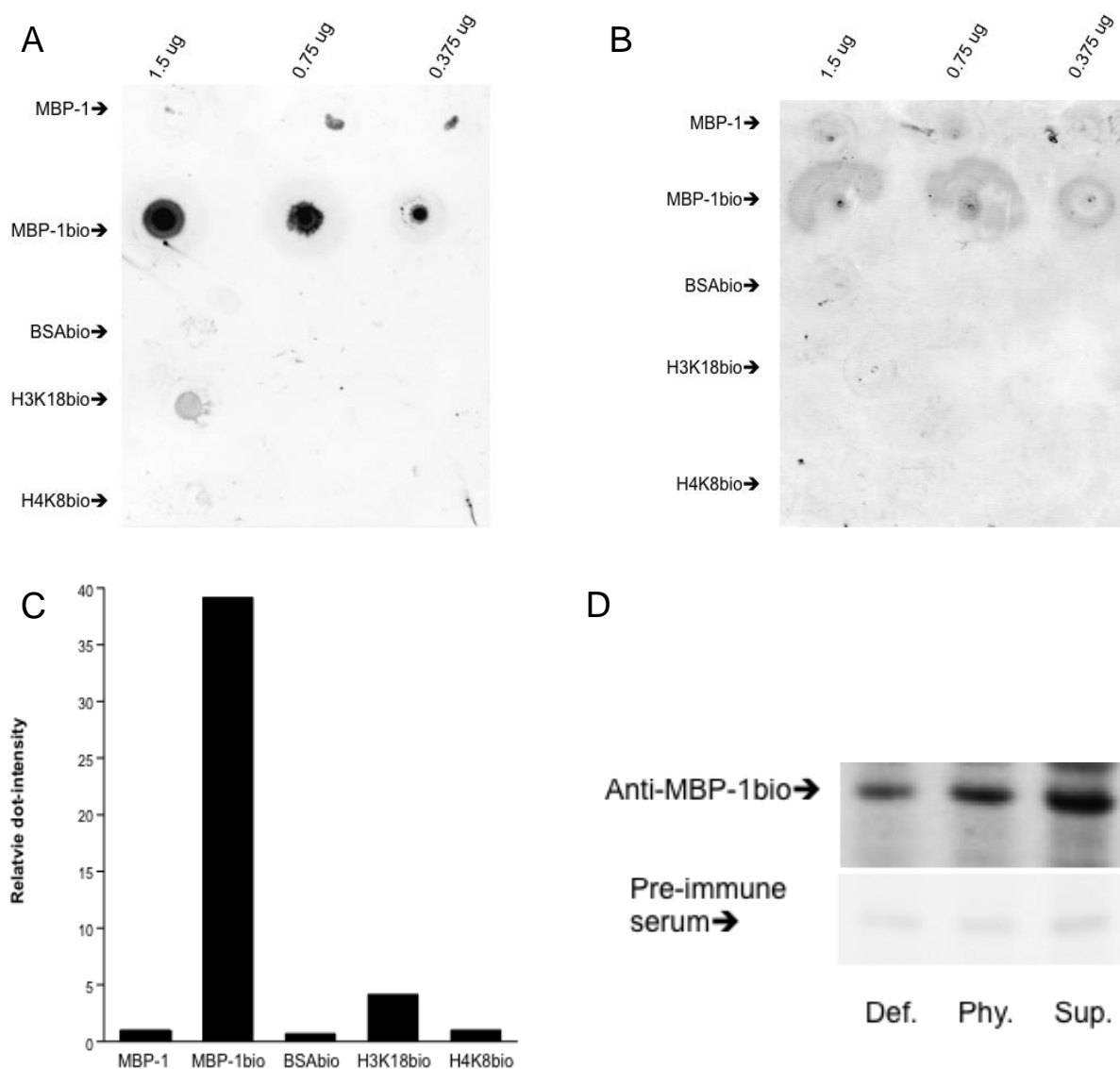
### ***c-Myc and COX-2 expression depends on biotin supply and HLCS activity in MCF-7 cells***

The expression of c-Myc and COX-2 is inversely linked with biotin concentrations in culture media. When MCF-7 cells were cultured in biotin-defined media for 3 days, the luciferase activity of c-Myc promoter reporter gene construct Luc-SNM (57) was significantly improved in deficient cells, while decreased in sufficient cells, compared with physiological cells (Fig.5). Following the treatment of biotin deficiency for 3 days, MCF-7 cells showed a strong upregulation of COX-2 (Fig.6A) and c-Myc (Fig.6B) mRNA expression compared with biotin treatment of physiological level. Treatment with biotin-supplemented media for 3 days significantly decreased c-Myc (Fig.6B) expression but had no effect on COX-2 (Fig.6A).

HLCS activity is an important factor in repressing oncogene expression, particularly that of COX-2. For example, the abundance of COX-2 mRNA was about 9-fold greater in HLCS mutant WG2215 fibroblasts compared with wild-type controls (Fig. 7A). The abundance of c-Myc mRNA followed the same trend but the increase in HLCS mutant fibroblasts amounted to only 20% and did not reach a statistically significant level ( $P = 0.071$ ) (Fig. 7B).

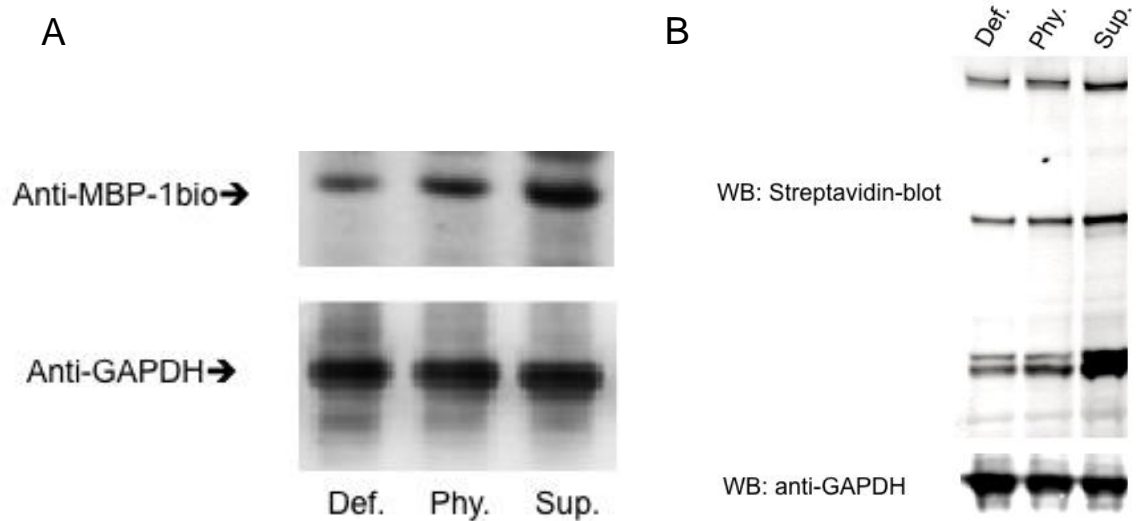
Mutagenesis studies suggest that biotinylation of K10 and K12, as opposed to biotinylation of other residues, is a crucial event in the repression of oncogenes. When MCF-7 cells were transfected with Flag constructs, the expression of COX-2 mRNA decreased by >50% in cells transfected with the wild-type plasmid Flag-WT-MBP1 compared to cells transfected with the mutant plasmid MBP-1<sub>R10,12R</sub> (Fig. 8A). Not surprisingly, COX-2 expression was intermediate in cells transfected with the Flag-control vector, because these cells presumably express wild-type MBP-1 from the two endogenous alleles; in Flag-WT-MBP1 transfectants the plasmid provides additional template, whereas in Flag-Mut-MBP1 transfectants mutant MBP-1 outcompetes the wild-type MBP-1 transcribed from the two endogenous alleles. The pattern was the same for c-Myc (Fig. 8B) but the magnitude of effect was smaller than that observed for COX-2.





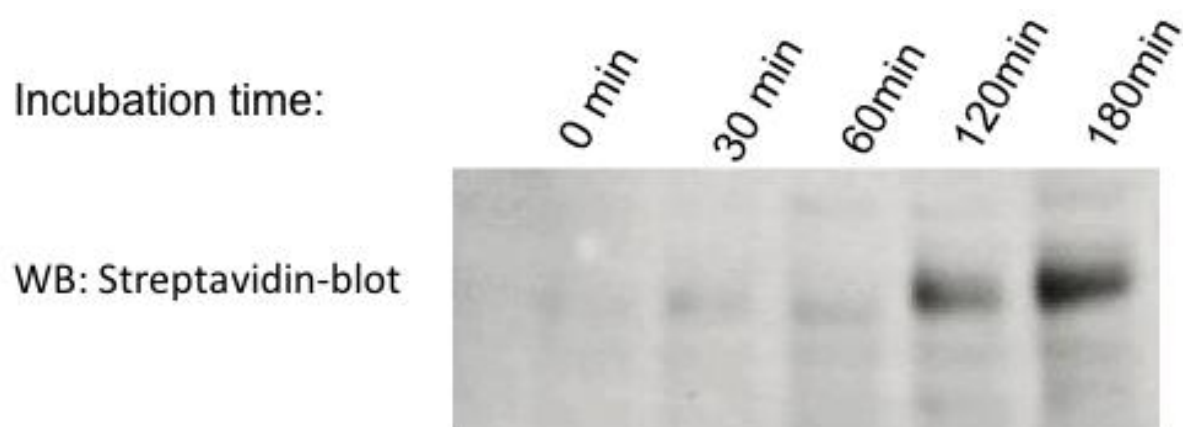
**Figure 2. Generated anti-MBP-1bio specific against biotinylated MBP-1.** (A) Anti-MBP-1bio produced a strong signal with peptide MBP-1bio in a dose-dependent manner (1.5 ug, 0.75 ug and 0.375 ug). (B) No signal produced by pre-immune serum. (C) The signal intensity produced by 1.5 ug peptides, compared with that produced by unbiotinylated MBP-1. (D) Extracts of MCF-7 cells after 3 days cultured in biotin-defined media, resolved by gel electrophoresis and probed with anti-MBP-1bio and pre-immune serum. MBP-1: synthetic unbiotinylated MBP-1 peptides; MBP-

1bio: synthetic K10,K12-biotinylated MBP-1 peptides; BSAbio: chemically biotinylated bovine serum albumin; H3K18bio: synthetic peptides mimicking K18-biotinylated histone H3; H4K8bio: synthetic peptides mimicking K8-biotinylated histones H4.

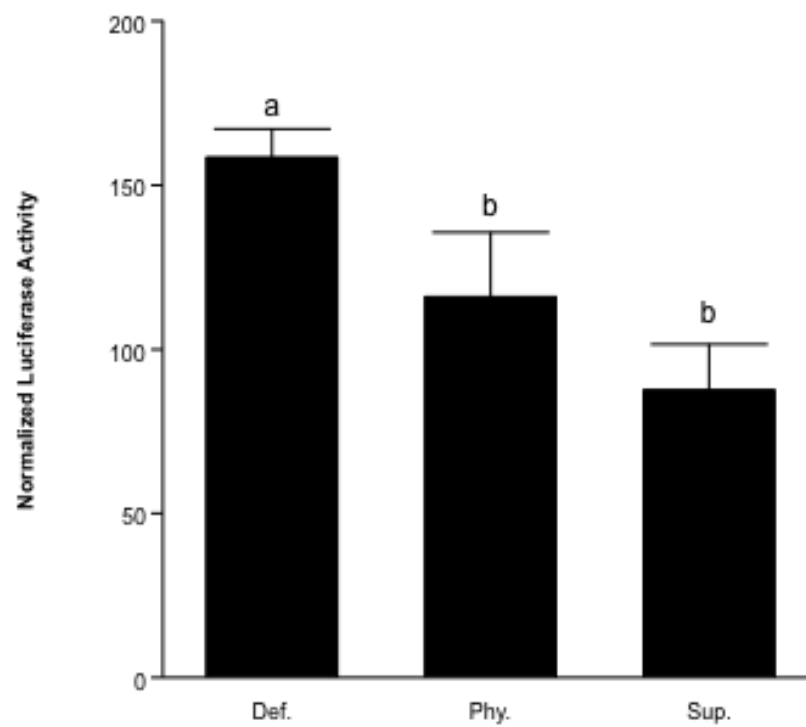


**Figure 3. The abundance of MBP-1 biotinylation was directly correlated with biotin-supply. (A)** Abundance of biotinylated MBP-1 in MCF-7 cells after 3 days of culture in biotin-defined media. **(B)** Abundance of biotinylated carboxylases in MCF-7 cells after 3 days of culture in biotin-defined media.

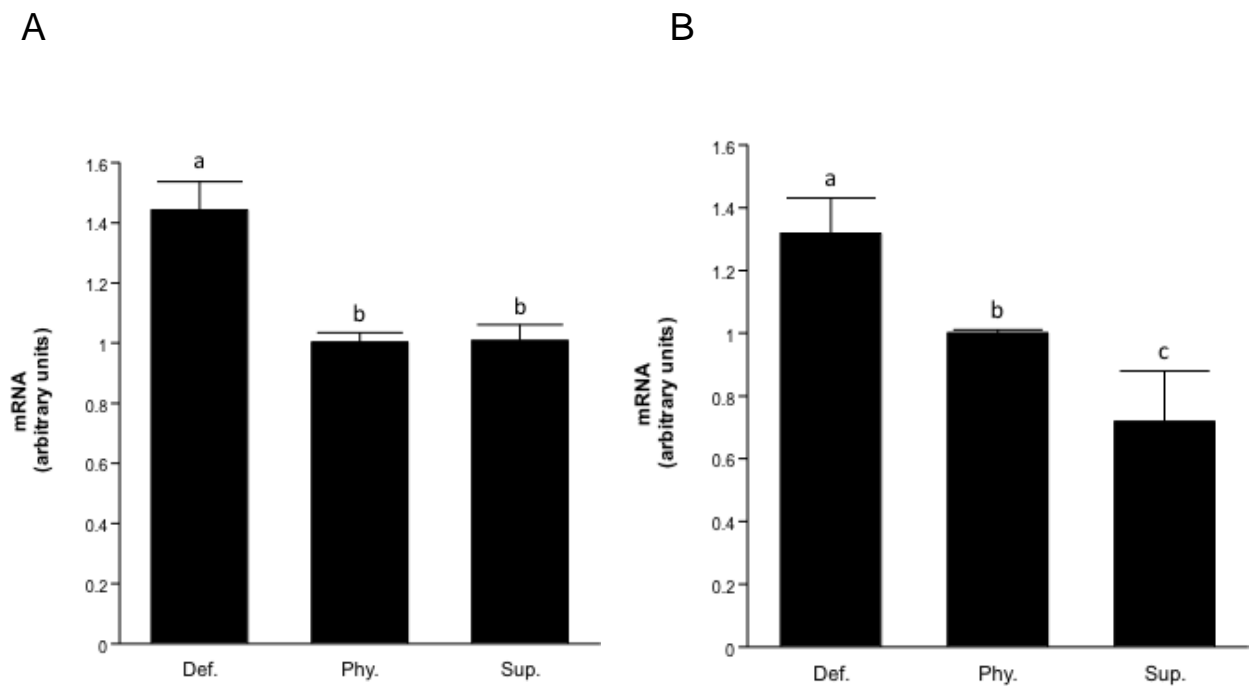




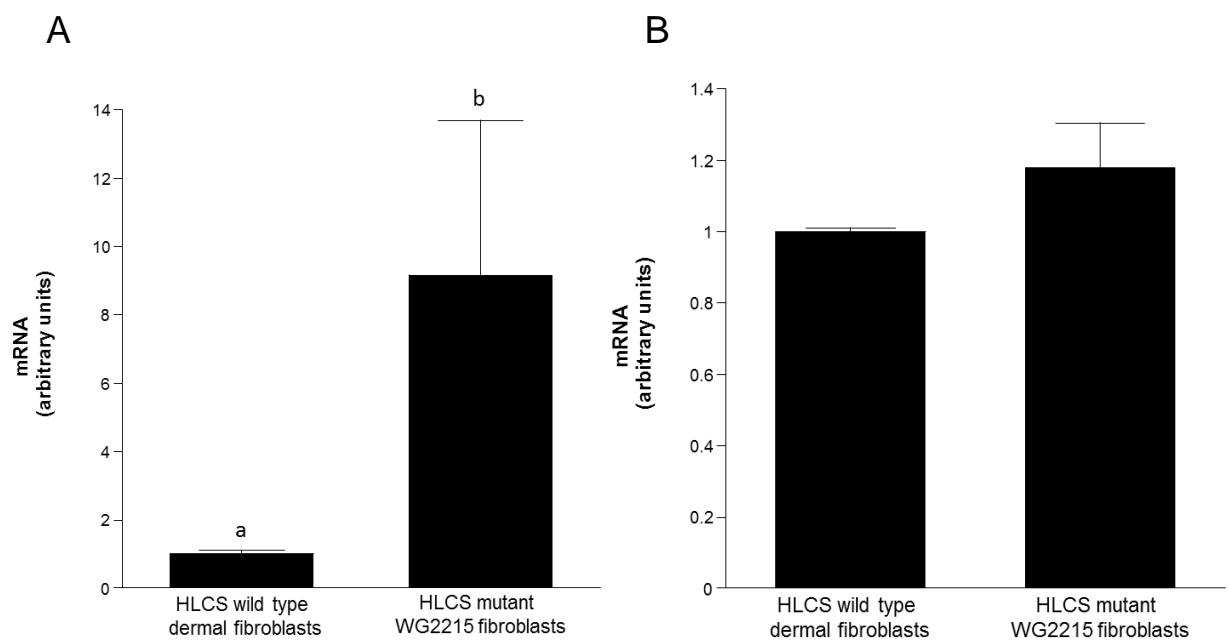
**Figure 4. MBP-1 interacts with HLCS in vitro.** Recombinant GST-HLCS catalyzed the binding of biotin to recombinant His-MBP-1, judged by streptavidin blots.



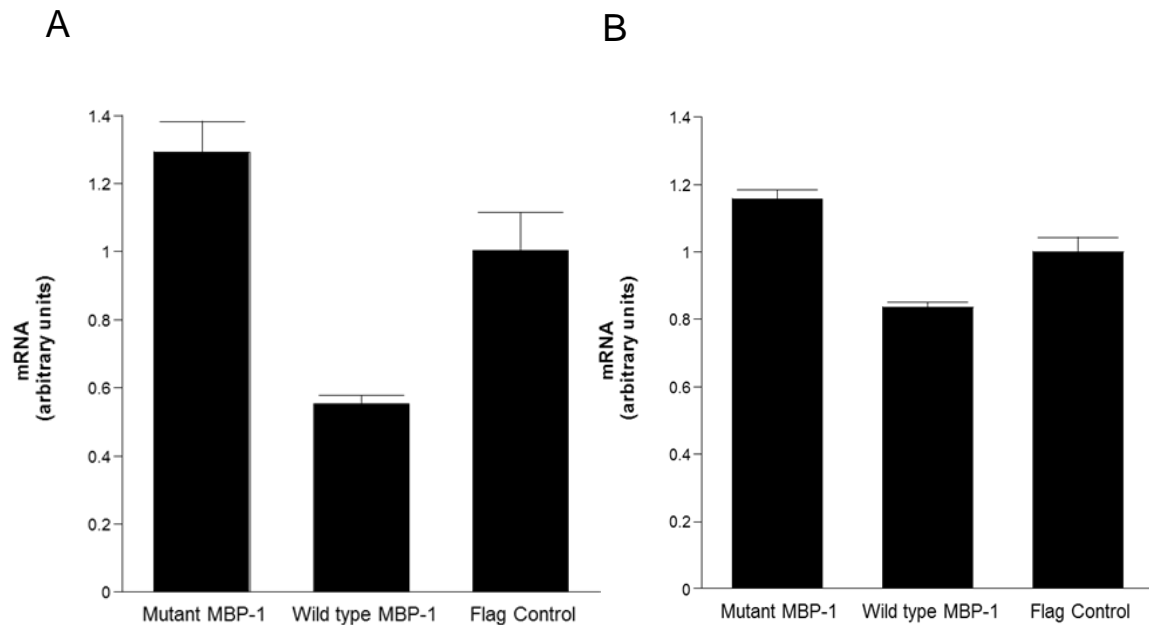
**Figure 5. Biotin-supply regulates c-Myc promoter activity in MCF-7 cells.** Luciferase activity of c-Myc promoter reporter construct in MCF-7 cells cultured in biotin-defined media, normalized by beta-galactosidase activity. <sup>a,b</sup>Bars not sharing the same letter are significantly different;  $P < 0.05$ ;  $N=3$ .



**Figure 6. Biotin-supply regulates COX-2 and c-Myc in MCF-7 cells.** Expression of COX-2 (A) and c-Myc (B) in MCF-7 cells after 3 days of culture in biotin-defined media.<sup>a,b,c</sup> Bars not sharing the same letter are significantly different;  $P < 0.05$ ;  $N=3$ .



**Figure 7. The biotin-ligase activity of HLCS is required for suppressor activity of MBP-1 on COX-2 and c-Myc.** Expression of COX-2 (A) and c-Myc (B). <sup>a,b</sup>Bars not sharing the same letter are significantly different;  $P < 0.05$ ;  $N=3$ .



**Figure 8. The repressor ability of MBP-1 was inhibited when the MBP-1 lacking biotinylation sites K10 and K12.**

Overexpression of wild type MBP-1 decreased COX-2 (A) and c-Myc (B) expression. However, the abundance of COX-2 (A) and c-Myc (B) were upregulated in MCF-7 cells overexpressing mutant MBP-1 compared with MCF-7 cells overexpressing Flag-WT-MBP1 (N=1 with three technical replicates).

## Discussion

The novelty of this study comes from the following points: 1) this is the first report of c-Myc promoter binding protein (MBP-1) biotinylation in the human proteome; 2) HLCS is the sole enzyme responsible for catalyzing covalent binding of biotin to MBP-1, in addition to HSP72, carboxylases and histones (16, 52, 60); 3) K10 and K12 residues located in the N-terminal functional domain of MBP-1 are the targets of biotinylation (31, 48).

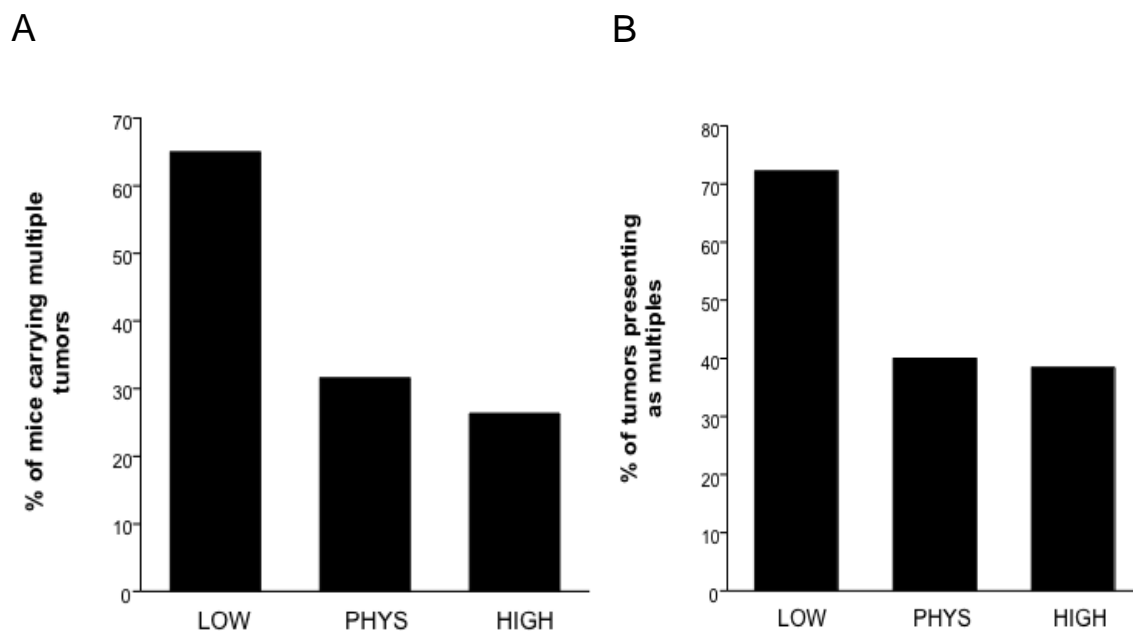
Importantly, we showed that biotinylation of MBP-1 depends on biotin supply. We also provided the evidences that the abundance of MBP-1 targets c-Myc (34) and COX-2 (29), oncogenes strongly associated with tumorigenesis and metastasis of breast cancer (41, 61, 62), can be regulated by biotin supply as well as in an HLCS-mediated manner.

Combined with these observations, this study may provide insights into epigenetic explanations of observations in our Bal b/c wild type mice biotin feeding studies, which suggests that biotin-deficiency induced a ~100% increase in percentage of mice carrying multiple tumors or the percentage of tumors presenting as multiple (Fig. 8). Given the observations of biotin deficiency increasing oncogenes c-Myc and COX-2 expression *in vitro* in this study, the increase of tumor load in biotin-deficient mice may be induced by improving expression of oncogenes, unregulated by lacking of MBP-1 biotinylation.

Several uncertainties need to be considered: 1) no human biotin requirements or food intake recommendations have been estimated; 2) currently, there is no reliable marker for quantification of biotin status in human (63). Therefore, it is uncertain whether the biotin-

dependent regulation of mechanisms contributing to tumorigenesis and is significant in western population. However, marginal biotin deficiency is common in pregnant women, which may cause human birth defects(64); biotin deficiency and low serum biotinidase activity can be induced by Valproic acid (VPA) treatment in human (65, 66). More importantly, several population-based studies reported that a substantial proportion of cancer patients treated with chemotherapy and radiation therapy have deficiencies of multiple vitamins (67-70), which suggests that biotin deficiency may be common in cancer patients with chemotherapy and radiation treatment.

Collectively, the covalent binding of biotin to MBP-1 is strongly associated with oncogenes expression. Future studies including biotin status in cancer patients, breast cancer risk in HLCS knock-out mice (conditional knock-out project in progress) will be conducted to determine whether biotin deficiency increases metastasis, development of new tumors or both in breast cancer.



**Figure 9. Tumor load of Bal b/c wild type mice in biotin-feeding study.** Both percentage of mice carry multiple tumors (A) and percentage of mice carrying tumors presenting as multiple (B) were ~100% increase in low-biotin-feeding mice compared with mice fed with physiological and high levels of biotin in diet.



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## APPENDIX

### List of Primers

**Table 1 Human primers used for qRT-PCR**

Gene		Primer Sequence (5'-3')	Ta (°C)
<i>c-Myc</i>	Forward	TTCGGGTAGTGGAAAACCAG	50
	Reverse	CAGCAGCTCGAATTTCTTCC	
<i>COX-2</i>	Forward	ACAGGCTTCCATTGACCAG	50
	Reverse	TCACCATAGAGTGCTTCCAAC	
<i>HLCS</i>	Forward	TGAGACCTGATCCTTAACCTCC	50
	Reverse	ATGGAAGATAGACTCCACAT	
<i>GAPDH</i>	Forward	TCCACTGGCGTCTTCACC	50
	Reverse	GGCAGAGATGATGACCCTTT	

*c-Myc*= v-Myc Avian Myelocytomatosis Viral Oncogene Homolog (NCBI Reference Sequence: NM\_002467.4); *COX-2*= Cyclooxygenase 2 (NCBI Reference Sequence: NM\_000963.2); *HLCS*= Holocarboxylase Synthetase (NCBI Reference Sequence: NM\_000411.6); *GAPDH*= Glyceraldehyde-3-phosphate Dehydrogenase (NCBI Reference Sequence: NM\_002046.4).

**Table 2 Human primers used for PCR amplification and Site-directed mutagenesis (SDM)**

Plasmid		Primer Sequence (5'-3')	Restriction enzyme sites	Ta (°C)
<i>His-MBP1</i>	Forward	TTCGGGTAGTGGAAAACCAG	Bamh1	58
	Reverse	CAGCAGCTCGAATTTCTTCC	Sal1	
<i>Flag-MBP-WT</i>	Forward	ACAGGCTTCCATTGACCAG	EcoRI	55.5
	Reverse	TCACCATAGAGTGCTTCCAAC	KpnI	
<i>MBP-1<sub>R10,R12</sub></i>	Forward	ACAGGCTTCCATTGACCAG	EcoRI	55.5
	Reverse	TCACCATAGAGTGCTTCCAAC	KpnI	

**Table 2 Primers used Site-directed mutagenesis (SDM)**

Plasmid		Primer Sequence (5'-3')
<i>ENO1_Mut</i>	Forward	ATG GAA CAG AAA ATA GAT CTA GGT TTG GTG CGA ACG C
	Reverse	GCG TTC GCA CCA AAC CTA GAT CTA TTT TCT GTT CCA T

Annotation: IEMDGTENKSK mutated to IEMDGTENRSR