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## Holocarboxylase Synthetase Represses Long Terminal Repeats through Epigenetic Synergies between Biotin and Methyl Donors, and Activates Immune Response by Catalyzing Biotinylation of Heat Shock Protein 72

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HOLOCARBOXYLASE SYNTHETASE REPRESSES LONG TERMINAL REPEATS  
THROUGH EPIGENETIC SYNERGIES BETWEEN BIOTIN AND METHYL  
DONORS, AND ACTIVATES IMMUNE RESPONSE BY CATALYZING  
BIOTINYLATION OF HEAT SHOCK PROTEIN 72

by

Jing Xue

A DISSERTATION

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July, 2013

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Jing Xue, Ph.D.

University of Nebraska, 2013

Advisor: Janos Zempleni

Holocarboxylase synthetase (HLCS) is the sole human protein biotin ligase in the human proteome. Other than its role as a coenzyme to the five carboxylases in intermediary metabolism, HLCS and biotin are involved in epigenetic gene regulation and immune response. Previously, it was shown that (1) HLCS physically interacts with histone methyl transferase EHMT-1 which creates histone H3 lysine 9 methylation (H3K9me) marks and biotinylates lysine (K) 161 in EHMT-1, thereby strengthening the HLCS/EHMT-1 interaction; (2) biotin depletion and HLCS knockdown cause a loss of H3K9me marks leading to de-repression of long terminal repeats (LTRs) and chromosomal abnormalities; and (3) deletion of DNA methylation impairs HLCS-catalyzed biotinylation events in the epigenome. Here we demonstrate a novel paradigm that HLCS contributed to the repression of LTRs through mediating protein/protein interactions and synergies between methylation and biotinylation events. We show that (1) HLCS interacts physically with the maintenance DNA methyl transferase DNMT1 and the methyl CpG binding protein MeCP2; (2) HLCS overexpression causes a 200% increase of H3K9me marks in LTRs and a corresponding 84% decrease in mRNA coding

for LTRs; and (3) erasure of DNA methylation abrogated HLCS-dependent enrichment of H3K9me and transcriptional repression of LTRs. It was thus concluded that HLCS mediated the integration of biotin and methyl dependent gene repression signals in the epigenome, which was further supported by cell culture studies where biotin and folate compensated for each other's deficiency in the repression of LTR in human Jurkat and U937 cells, and interactively regulated the expression of proinflammatory cytokines through activation of the NF- $\kappa$ B pathway. We also demonstrate that HLCS is involved in cellular immune response by catalyzing biotinylation of the stress inducible heat shock protein 72 (HSP72). In screen for detecting novel biotinylated proteins in the proteome of the human embryonic kidney HEK293 cells, members of the heat shock superfamily of proteins, including HSP72, were overrepresented. At least five lysine residues in HSP72 are targeted for biotinylation, and HLCS-dependent biotinylation of HSP72 elicited the expression of the chemokine RANTES from HEK293 cells. In conclusion, HLCS and biotin have major impacts in epigenome and immune function.

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## INTRODUCTION

Biotin is a water-soluble vitamin that is found in a wide variety of foods in the form of free biotin or protein-bound conjugates. Biotin from dietary sources or synthesized by intestinal microflora is absorbed in the intestine through the sodium-dependent multivitamin transporter (SMVT) (H. M. Said, 2004), and distributed throughout the body, including hepatocytes mainly for storage purpose (H.M. Said, L. McAlister-Henn, R. Mohammadkhani, & Horne, 1992), central nervous system where it is able to pass the blood-brain barrier (Spector & Mock, 1987), kidney for reabsorption (Baur & Baumgartner, 1993), and fetus through placental transportation (Schenker et al., 1993).

In mammals, biotin is appreciated for its function in intermediary metabolism as it serves as the essential cofactor for the five carboxylases: acetyl CoA carboxylase 1 and 2, pyruvate carboxylase, propionyl CoA carboxylase and methylcrotonyl CoA carboxylase (McMahon, 2002). The covalent binding of biotin to protein lysine residues on the amine groups in human is catalyzed by holocarboxylase synthetase (HLCS), which is the sole human biotin ligase that is located both in the cell cytoplasm and nuclear (Bao, Pestinger, et al., 2011; Bao, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011; Suzuki et al., 1994). Functional biotinylation of proteins does not limit to carboxylases, for example, histone biotinylation has been identified on 12 lysine residues in different histones and the biological significance of histone biotinylation in gene repression has been well studied (Chew et al., 2008; Gralla, Camporeale, & Zempleni, 2008; Kuroishi, Rios-Avila, Pestinger, Wijeratne, & Zempleni, 2011; Pestinger, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011). Another chromatin protein, the euchromatic histone-lysine N-methyltransferase (EHMT), also is a target for HLCS-mediated biotinylation, which for

the first time directly linked biotinylation to histone methylation events (Li, Hassan, Moriyama, & Zempleni, 2013). Recently, a total of 108 novel biotinylation sites has been reported in the human embryonic kidney HEK293 cell proteome, using mass spectrometry assay, with members of the heat shock protein (HSP) superfamily being overrepresented among the novel biotinylated proteins (Li, et al. in preparation).

Severe biotin deficiency in human leads to a variety of health outcomes, including thinning of hair, skin rash, depression, lethargy, hallucination and paresthesias found in adults, and the effects are more profound in infants, as represented by delay in development (D. M. Mock, 2007; D.M. Mock, 1986). The deficiency of biotin is caused by insufficient intake of biotin from food, prolonged consumption of raw egg while, and genetic deficiency in biotinidase or HLCS (D. M. Mock, 2007). Sufficient biotin intake is important for maintaining healthy immune system in that biotin deficiency compromised T cell activation and proliferation through regulating the expression and secretion of interleukin-2 (IL-2) (Báez-Saldaña, Díaz, Espinoza, & Ortega, 1998; Baez-Saldana & Ortega, 2004). The mechanisms causing symptoms of biotin deficiency have yet to be fully elucidated, however, it was suggested to be related to disrupted lipid metabolism (D.M. Mock, Johnson, & Holman, 1988; Suchy & Wolf, 1986), declined pyruvate carboxylase activity accompanied brain lactic acidosis (Diamantopoulos, Painter, Wolf, Heard, & Roe, 1986), as well as epigenetic regulation of gene expression by protein biotinylation (Chew et al., 2008). Dietary intake of biotin correlates with the abundance of biotinylated proteins, based on the evidence that biotinylated proteins such as carboxylases rapidly lose the coenzyme biotin in biotin-depleted cell cultures and human (Eng et al., 2013; Kaur Mall, Chew, & Zempleni, 2010).

This introduction will focus on the biological significance of biotin from following two perspectives: one is the epigenetic synergies between biotin and methyl donors, as mediated by HLCS in the repression of repeats and the maintenance of genome integrity; the other is the biotinylation of the heat shock protein 72 and its role in activating intracellular signaling cascades for immune response.

### **HLCS interacts with DNA and histone methylation machineries.**

DNA methylation in vertebrate mammals is the covalent binding of methyl groups to the cytosine within CpG dinucleotides (Bogdanovic & Veenstra, 2009). DNA methylation is associated with repressed chromatin status and gene expression, and the genome-wide distribution of DNA methylation is dynamic during development, creating a cell-type specific pattern in differentiated cells (Brenet et al., 2011; Meissner et al., 2008). CpG islands are defined as regions with overrepresented C+G content and CpG dinucleotides compared with the background of the genome (Gardiner-Garden & Frommer, 1987). In non-embryonic cells, 60-90% of CpG dinucleotides are methylated, whereas CpG islands are usually unmethylated to ensure constitutive transcription from nearby or overlapped promoters (Bogdanovic & Veenstra, 2009; Jaenisch & Bird, 2003).

Mammalian DNA methylation is mediated by a family of DNA methyl transferases (DNMTs). The maintenance methyl transferase DNMT1 is responsible for adding methyl groups to semi-methylated DNA during cell proliferation and maintains the DNA distribution pattern through DNA replication (Robert et al., 2003). Both DNMT3A and DNMT3B are *de novo* DNA methyl transferases for difference genomic regions and are highly expressed in developing cells for establishing DNA methylation patterns (Okano,

Bell, Haber, & Li, 1999). DNMT3L lacks DNA methyl transferase activities and cooperates with DNMT3A to establish DNA imprinting (Bourc'his, Xu, Lin, Bollman, & Bestor, 2001; Hata, Okano, Lei, & Li, 2002). The functions of different DNMTs are non-redundant and in most cases independent (Robert et al., 2003).

DNA methylation changed chromatin accessibility and gene expression through direct recruitment of methyl-CpG binding proteins or blocking genomic sequence otherwise bound by proteins required for transcription (Jaenisch & Bird, 2003; Watt & Molloy, 1988). Proteins that recognize and binds to methyl-CpG generally shares a conserved methyl-CpG binding domain (MBD), and those proteins include methyl-CpG binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4 (Bogdanovic & Veenstra, 2009; Hendrich & Bird, 1998). Not all MBDs are essential for recruiting gene repression machineries or specific for binding to methylated DNA, which is determined by the presence of the transcription repression domain ( found in MeCP2, MBD1 and MBD2) or auxiliary domains for recognition of additional DNA sequences (zinc finger motif found in MBD1) (Bogdanovic & Veenstra, 2009). MeCP2 is more abundant and widely expressed compared to its counterparts, and interacts with DNMT1 through its transcriptional repressor domain to comprise a dynamic machinery for maintaining the DNA methylation patterns (Kimura & Shiota, 2003; Kolker et al., 2012). DNA methylation does not always lead to gene repression. For example, methylation of the chromatin boundary element binding protein releases the connection between an enhancer and its nearby promoter, thereby increasing the transcription from the promoter (Hark et al., 2000).

DNA methylation was demonstrated to be better correlated with the distribution of histone methylation marks than the genomic DNA sequence itself (Meissner et al., 2008). In mammals, the most well studied histone methylation refers to covalent attachment of methyl groups to  $\epsilon$ -amine group of lysine residues (Murray, 1964), in addition to arginine and histidine residues (Greer & Shi, 2012). Methylation of different lysine residues on different histone proteins has distinct functions. The active histone mark histone H3 lysine (K) 4, which is associated with transcriptional initiation, and the repressive mark H3K27 are found to be paired indicators for distinguishing among active transcribed, bivalent and stably repressed genes (Mikkelsen et al., 2007). Tri-methylated H3K36 (H3K36me3) correlates with transcriptional elongation and is enriched at transcribed coding and non-coding regions (Kim, Kiefer, & Dean, 2007; Mikkelsen et al., 2007). Repeat regions in the genome are usually marked by condensed chromatin structure and repressed transcription, and different repeat regions are selectively enriched of the repressive H3K9, H3K27 and H4K20 methylation marks (Martens et al., 2005a). CpG content in the core promoter region distinguishes two classes of promoters with distinct chromatin status and expression patterns (Saxonov, Berg, & Brutlag, 2006). Promoters with high CpG content tends to be unmethylated and transcribed, and these promoters are usually seen in housekeeping genes and highly regulated developmental genes; genes with low levels of CpG content in the core promoter region are more likely to be methylated and expressed in a tissue-specific manner (Meissner et al., 2008).

Histone biotinylation is a rare (<0.001% of histone H3 and H4) natural modification (Bailey, Ivanov, Wallace, & Polyak, 2008; Kuroishi et al., 2011; Stanley, Griffin, & Zemleni, 2001). HLCS is the only protein in the human proteome that catalyzes the

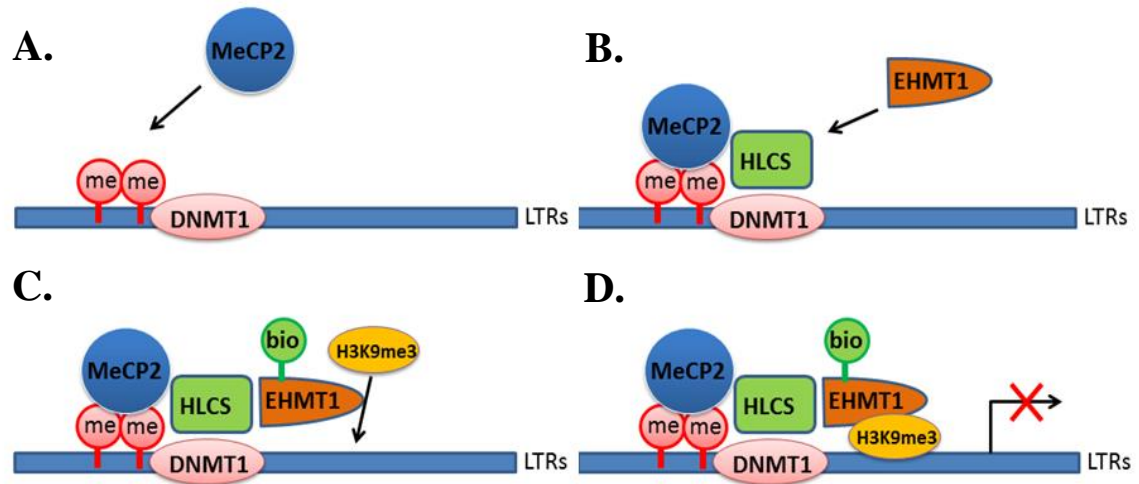
covalent binding of biotin to the lysine residues on proteins (Bao, Pestinger, et al., 2011; Bao, Wijeratne, et al., 2011; Suzuki et al., 1994). HLCS enters the nuclear and its localization in the chromatin is locus-specific despite that it lacks DNA binding domains (Camporeale, Giordano, Rendina, Zempleni, & Eissenberg, 2006; Singh, Pannier, & Zempleni, 2011). HLCS catalyzes the covalent binding of biotin to lysine residues on histone H2A, H3 and H4 and HLCS knock-down leads to apparent loss of biotinylation marks on histone H3 and H4 (Bao, Pestinger, et al., 2011; Chew et al., 2008; Pestinger et al., 2011; Rios-Avila, Pestinger, & Zempleni, 2012). HLCS is linked with epigenetic machineries in gene repression based on the findings that: (i) HLCS binds to chromatin in a punctuate manner; (ii) HLCS directly interacts with histone H3; (iii) histone biotinylation marks are found to co-localize with the repressive histone methylation marks (Bao, Pestinger, et al., 2011; Chew et al., 2008).

Biotin status and histone biotinylation marks has been linked to the chromatin status and expression of long terminal repeats (LTRs) in the human genome (Chew et al., 2008). Evidence shows that both biotin depletion in culture medium and HLCS deficiency caused by genetic deficiency correlate with increased transcription of LTRs and decreased enrichment of H3K9me2 marks in LTR (Chew et al., 2008). The binding of HLCS to chromatin at specific location as indicated by enrichment of histone biotinylation marks relies on prior DNA methylation, and biotin depletion has no effects on the level of DNA methylation (Chew et al., 2008). The association between biotin, HLCS and histone methylation events was further investigated and it was demonstrated that (i) HLCS physically interacts with the histone H3 lysine-9 methyltransferase EMHT1 and the *in vitro* biotinylation of EMHT1 by HLCS enhances the interaction (Li

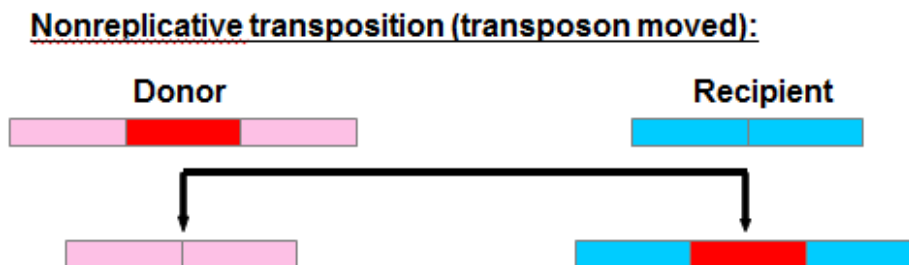


et al., 2013); (ii) HLCS physically interacts with the maintenance DNA methyl transferase DNMT1 and MeCP2, and mediates the repression of LTRs through recruiting EMHT1, thereby creating the repressive H3K9me3 marks at the locus for gene silencing (**Figure 1**) (Xue, Wijeratne, & Zemleni, 2013).

The transcriptional repression of LTRs in the genome is ensured by condensed chromatin structure, DNA methylation and enrichment of repressive histone methylation marks (Martens et al., 2005a). However, an appreciable number of LTRs have been found to be transcriptional active in the human genome (Buzdin, Kovalskaya-Alexandrova, Gogvadze, & Sverdlov, 2006; Mikkelsen et al., 2007), and mount the risk for interrupted gene expression as de-repression of LTRs enables retro-translocation of the LTR-containing retrotransposon and increases the rates for spontaneous mutations in the genome (**Figure 2**) (Kazazian, 2004; Martens et al., 2005b; Waterston et al., 2002). A great variety of human diseases have been linked to retrotranspositions in the genome, including breast cancer and colon cancer (Kazazian et al., 1988; Miki et al., 1992; Morse, Rotherg, South, Spandorfer, & Astrin, 1988).



**Figure 1. HLCS mediates the repression of LTRs.** (A) DNMT1 maintains the DNA methylation patterns by adding methyl groups on semi-methylated DNA nearby LTR genomic locus, which recruits MeCP2 to bind to methylated CpG; (B) HLCS locates LTR in the genome through direct interaction with MeCP2 and DNMT1, and further recruits EHMT1; (C) HLCS biotinylates EHMT1 which strengthens the association between HLCS and EHMT1, the latter creates H3K9me3 marks; (D) the enrichment of H3K9me3 at LTRs inhibits the transcription from their promoters.



**Figure 2. Retrotransposon translocates between different genomic loci.** The de-repression of LTRs activates retro-transcription from its promoter, allowing its associated retrotransposon to be transferred from the donor loci to recipient loci in the genome.  
(Adapted from Lewin, 2004)

**HLCS biotinylates extracellular HSP72 (eHSP72) and activates intracellular immune response.**

During a genome wide screening for biotinylated proteins in the human embryonic kidney HEK293 cells, 108 novel biotinylated sites were discovered, and a significant large proportion belongs to the HSP superfamily. HSPs are a group of highly conserved proteins that are found both in prokaryotes and eukaryotes, and different HSPs that are present in a variety of cellular organelles play fundamental roles in orchestrating cellular activities (Kiang & Tsokos, 1998). Among the mammalian HSPs, the heat shock protein 70 kDa (HSP70) family is highly conserved and thoroughly-studied (Asea, 2005). The HSP70 family is composed of four members, each with distinct cellular localization and function: (i) the 78 kDa glucose regulated protein (GRP78) exclusively localizes in the endoplasmic reticulum (ER) and regulates protein processing and secretion through the

ER; (ii) the 75 kDa GRP75 was found to localize in mitochondria, and is responsible for cell proliferation and aging; (iii) the 73 kDa GRP73 localizes in the cytoplasm and nucleus, facilitates folding of nascent peptides as a protein chaperone, and is also required for recycling of the vesicles; (iv) the stress-inducible HSP72 is broadly distributed, as it is found in cytoplasm, nucleus and extracellular space, and constitutes a cellular stress-responsive machinery (Asea, 2005; Mambula, Stevenson, Ogawa, & Calderwood, 2007).

The HSP70s share three major functional domains: an N-terminal ATPase domain of 44 kDa, immediately followed by a substrate binding domain of 15 kDa, and a little-studied carboxy-terminal variable domain weights about 10 kDa (Bukau & Horwich, 1998; Ohno, Kitabatake, & Tani, 2004). The HSP70s are most recognized as protein chaperones, as they form the cellular machineries in assisting protein folding under varieties of conditions, *e.g.*, prevention of protein aggregation and assisting refolding of misfolded proteins under stressed conditions, and guidance of protein translocation and assisting nascent protein folding and protein turnover under normal conditions (Hartl, 1996). The chaperon function of HSP70s rely on their featured common structures, as ATP binding by the N-terminal ATPase domain alters conformation of the substrate binding domain and opens the binding pocket for frequent interaction with substrates, followed by ATP hydrolysis and subsequent closing of the binding pocket, for high-affinity binding of the substrate (Bukau & Horwich, 1998; Rudiger, Buchberger, & Bukau, 1997). The interaction between substrate binding domain and substrate in return accelerates the hydrolysis of ATP, demonstrating an allosteric nature of HSP70s in its chaperon function (Vogel, Bukau, & Mayer, 2006).

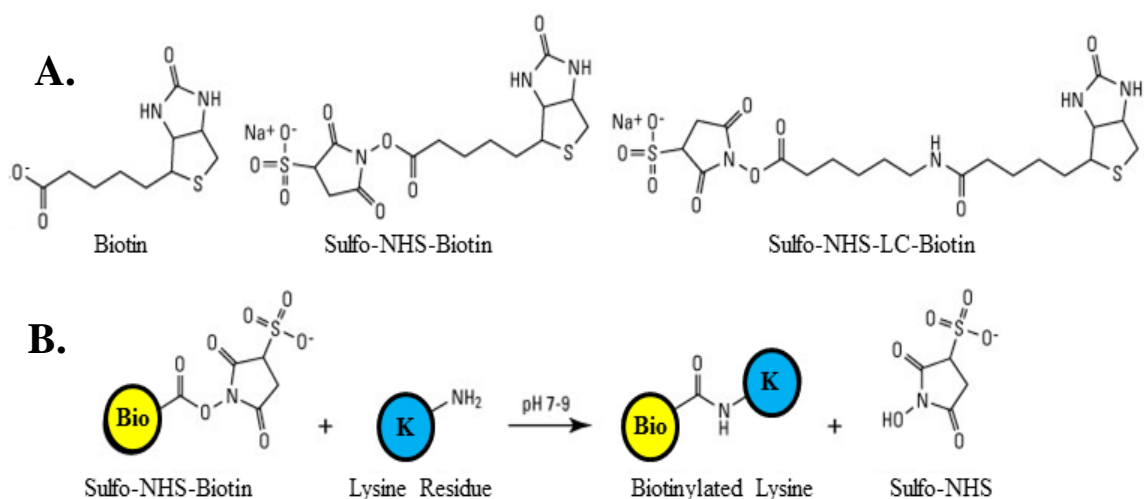
In addition to its role as a protein chaperone, HSP72 is highly stress-inducible and is involved in cell-protection mechanisms. A great variety of stress conditions have been reported to induce increased production of HSP72 protein in the cytoplasm including heat shock, heavy metals, viral or bacterial infection, inflammation, autoimmunity (Asea, 2005). Elevated intracellular HSP72 inhibits the activation of NF- $\kappa$ B pathway and in this manner represses the expression of proinflammatory cytokines (Cahill, Waterman, Xie, Auron, & Calderwood, 1996).

Of note, a marked increase in extracellular HSP72 was observed in response to bacteria challenge and brief electric shock in rats (Campisi, Leem, & Fleshner, 2003) and in response to treatment of lipopolysaccharides in THP-1 human immune cells (Abboud et al., 2008). HSP72 is encoded by the HSPA1A and HSPA1B genes, and both of them lack the ER signal sequence, which is required for the ER facilitated canonical secretion pathway. It was proposed that HSP72 is released from the cell through three types of pathways: (i) leakage by dead cells; (ii) active secretion by forming “blebbing” vesicles near the cell membrane; (iii) carriage by endolysosomes which fuse with cell membrane to release the included HSP72 into the extracellular space (Mambula et al., 2007).

eHSP72 functions as a danger signal to the innate immune system and promotes secretion of proinflammatory cytokines from immune cells through interaction with cell surface receptors, such function being referred to as “chaperokine” activity of HSP72 (Asea et al., 2000; Campisi et al., 2003; Multhoff et al., 1999).

While enzymatic biotinylation of protein is mediated by HLCS, the *in vitro* biotinylation through chemical conjugation is achieved by modified active biotin compounds, which react nono-specifically with any free amino group in proteins. In such cases, an N-

hydroxysuccinimide (NHS) or sulfo NHS group is attached to the carboxylic acid group on biotin through an ester bond, and the resulting ester is highly reactive. Both chemical and HLCS catalyzed biotinylation lead to the modification of lysine residues by a biotin molecule through a stable amide bond (**Figure 3**).



**Figure 3. Chemical conjugation between active biotin compounds.** (A) the chemical structure of unmodified biotin, sulfo-NHS conjugated biotin, and a modified sulfo-NHS conjugation with elongated chain between biotin and sulfo-NHS. (B) the active sulfo-NHS-biotin reacts with the amine group on lysine residue to form biotinylated lysine and a sulfo-NHS. (Adapted from: <http://www.piercenet.com/browse.cfm?fldID=F330F14F-EBCC-97DB-7F6E-9664D3ACE886#nhsester> )

Although it is commonly assumed that biotinylation of protein does not alter the structure or functionality of the protein, evidence suggests that biotinylated protein exhibits different properties otherwise not seen in unmodified counterparts. It was demonstrated

that chemically biotinylated HSP72 binds to HEK293 cells in a saturable manner with greater affinity than unmodified HSP72, and that the binding of biotinylated HSP72 to cell surface induce the secretion of the chemokine RANTES (Tao, Nishikawa, Nomura, Kitabatake, & Tani, 2005). RANTES is essential for the amplification of the T-cell mediated immune response due to the fact that its expression is specifically delayed in T-cells in response to immune activation (Nelson & Krensky, 2001). RANTES is implicated in various diseases, including AIDS, atherosclerosis and autoimmune diseases and, like other chemokines, it plays an important role in guiding migration of immune cells to lesions during immune response (Nelson & Krensky, 2001). In consistent with the chemical conjugated biotinylation on HSP72, enzymatically biotinylation elicited an increase in RANTES transcription in HEK293 cells in a dose-dependent manner (Xue, *et al.* submitted). In addition, when recombinant HLCS was added to HEK293 cell suspensions in the absence of HSP72, RANTES expression also increased, indicating the existence of endogenous eHSP72 in the cell culture that are targeted for biotinylation.

## **Conclusion**

Biotin, other than its classical roles as cofactor for the five carboxylases in intermediary metabolism, has been implicated in epigenetic regulation of gene expression and immune response. The following chapters will provide in-depth investigations of the underlying mechanism of the (i) HLCS-dependent epigenetic synergies between biotin and methyl donors in repression of repeat sequences; (ii) endogenous biotinylation of HSP72 and its effect in inducing RANTES production and (iii) synergies between biotin and folate in the regulation of the expression of inflammatory cytokines from the myeloid lineage human U937 cells and the lymphoid lineage human Jurkat cells.

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## CHAPTER I

### **Holocarboxylase synthetase synergizes with methyl CpG binding protein 2 and DNA methyl transferase 1 in the transcriptional repression of long terminal repeats**

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## Abstract

Holocarboxylase synthetase (HLCS) is a chromatin protein that facilitates the creation of histone H3 K9-methylation (H3K9me) gene repression marks through physical interactions with the histone methyl transferase EHMT-1. HLCS knockdown causes a depletion of H3K9me marks in mammalian cell cultures and severe phenotypes such as short lifespan and low stress resistance in *Drosophila melanogaster*. HLCS displays a punctuate distribution pattern in chromatin despite lacking a strong DNA-binding domain. Previous studies suggest that the binding of HLCS to chromatin depends on DNA methylation. We tested the hypothesis that HLCS interacts physically with the DNA methyl transferase DNMT1 and the methyl CpG binding protein MeCP2 to facilitate the binding of HLCS to chromatin, and that these interactions contribute toward the repression of long terminal repeats (LTRs) by H3K9me marks. Co-immunoprecipitation and limited proteolysis assays provided evidence suggesting that HLCS interacts physically with both DNMT1 and MeCP2. The abundance of H3K9me marks was 207% greater in the LTR15 locus in HLCS overexpression human embryonic kidney HEK293 cells compared with controls. This gain in H3K9me was inversely linked with a 87% decrease in mRNA coding for LTRs. Effects of HLCS abundance on LTR expression were abolished when DNA methylation marks were erased by treating cells with 5-azacytidine. We conclude that interactions between DNA methylation and HLCS are crucial for mediating gene repression by H3K9me, thereby providing evidence for epigenetic synergies between the protein biotin ligase HLCS and dietary methyl donors.

**Key words:** Biotin; DNA methyl transferase; holocarboxylase synthase; methyl CpG binding protein 2; methylation; repression; long terminal repeats.

## Introduction

HLCS is the sole protein biotin ligase in the human proteome (Suzuki et al., 1994) and has translational start sites in methionine-1, methionine-7, and methionine-58 (Hiratsuka et al., 1998). The classical role of HLCS is to catalyze the covalent binding of biotin to five distinct carboxylases in cytoplasm and mitochondria (Zempleni, Wijeratne, & Kuroishi, 2012). More recently, it became evident that HLCS also enters the cell nucleus (Bailey, Wallace, & Polyak, 2010; Bao, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011) where it binds to chromatin in a punctuate, locus-specific pattern (Camporeale, Giordano, Rendina, Zempleni, & Eissenberg, 2006; Singh, Pannier, & Zempleni, 2011). Unambiguous evidence suggests that recombinant HLCS catalyzes biotinylation of histone H3 *in vitro* and that HLCS knockdown causes a loss in histone H3 and H4 biotinylation marks *in vivo* (Bao, Pestinger, et al., 2011; Chew et al., 2008; Pestinger, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011; Rios-Avila, Pestinger, & Zempleni, 2012). HLCS-dependent histone biotinylation marks are enriched in repressed genomic loci such as long terminal repeats (LTRs) suggesting a role of HLCS in gene repression (Camporeale, Oommen, Griffin, Sarath, & Zempleni, 2007; Chew et al., 2008; Pestinger et al., 2011; Rios-Avila et al., 2012; Wijeratne, Camporeale, & Zempleni, 2010).

Biotinylation of histones H3 and H4 is a natural modification, but the abundance of biotinylation marks is too low (<0.001%) to account for the severe phenotypes seen in HLCS knockdown cells and flies (Bailey, Ivanov, Wallace, & Polyak, 2008; Kuroishi, Rios-Avila, Pestinger, Wijeratne, & Zempleni, 2011; Stanley, Griffin, & Zempleni, 2001). These phenotypes include a de-repression of LTRs in human and murine cell lines and in *Drosophila melanogaster*, and short life span and low stress resistance in

*Drosophila* (Camporeale et al., 2006; Chew et al., 2008). Collectively, these observations point toward a role of HLCS in gene repression at the epigenetic level that is independent of histone biotinylation.

We recently proposed that the roles of HLCS in gene repression are caused by interactions between HLCS and other chromatin proteins (Fig. 1) (Kuroishi et al., 2011). This theory integrates the discoveries described above with the following observations into a coherent model. First, HLCS binds to chromatin despite lacking a strong DNA-binding domain (Camporeale et al., 2006; Singh et al., 2011). Second, two HLCS-binding proteins have been identified, namely histone H3 and the histone H3 lysine-9 methyltransferase EHMT-1 (Bao, Pestinger, et al., 2011; Y. Li, Hassan, Moriyama, & Zempleni, 2013). Lysine-9 methylated histone H3 (H3K9me) is an abundant gene repression mark and EHMT-1 is one of the enzymes capable of creating this mark (Kouzarides & Berger, 2007). HLCS physically interacts with EHMT-1 and catalyzes the biotinylation of lysine (K) residues in EHMT-1, which strengthens the interactions between the proteins *in vitro* (Y. Li et al., 2013). Both biotin depletion and loss of HLCS cause a decrease of H3K9me marks (Chew et al., 2008; Gralla, Camporeale, & Zempleni, 2008; Pestinger et al., 2011; Rios-Avila et al., 2012). Third, when DNA methylation marks are erased by 5-azacytidine treatment in human cell cultures, the abundance of histone biotinylation marks decreases considerably in LTRs; this is an uni-directional effect and biotin depletion has no effect on the abundance of DNA methylation marks (Chew et al., 2008).

Here we tested the hypothesis that HLCS interacts with DNA methyltransferases (DNMT) and methyl-CpG-binding proteins (MeCPs), and that the binding of HLCS to



loci rich in methylated DNA increases the local abundance of H3K9me marks, thereby contributing toward the repression of LTRs. Our rationale for testing DNMT1, as opposed to DNMT3a and DNMT3b, was that the maintenance methyltransferase DNMT1 mediates DNA methylation in all somatic cells, whereas the *de novo* methyltransferases DNMT3a and DNMT3b mediate DNA methylation only in embryonic cells (E. Li & Bird, 2007; Schermelleh et al., 2007). However, DNMT3a and DNMT3b were tested as specificity controls. Our rationale for testing MeCP2, as opposed to other methylated DNA binding proteins such as MBD1, MBD2, and MBD4 (Bogdanovic & Veenstra, 2009; E. Li & Bird, 2007) was that MeCP2 binds exclusively to methylated DNA to cause gene repression, whereas MBD1 can bind to non-methylated DNA and MBD4 does not cause gene repression; also MeCP2 is more abundant and widely expressed compared to MBD1 (Bogdanovic & Veenstra, 2009; Kolker et al., 2012). Our rationale for assessing the effects of DNA methylation on HLCS-mediated and H3K9me-dependent repression of LTRs, as opposed to other loci, was that DNA methylation, HLCS activity, biotin availability, and H3K9me have been implicated in LTR repression (Brenet et al., 2011; Chew et al., 2008; Martens et al., 2005; Pestinger et al., 2011). At least 54 LTRs in the human genome are transcriptionally active (Buzdin, Kovalskaya-Alexandrova, Gogvadze, & Sverdlov, 2006), and pose a threat to genome stability, as their mobilization facilitates recombination between non-homologous loci, leading to chromosomal deletions and translocations (Kazazian, 2004; Martens et al., 2005). Drug-induced hypomethylation of DNA is associated with activation of LTRs in mice (Jaenisch, Schnieke, & Harbers, 1985; Jahner et al., 1982; Stewart, Stuhlmann, Jahner, & Jaenisch, 1982) and with chromosomal instability and tumors (Eden, Gaudet, Waghmare,

& Jaenisch, 2003; Gaudet et al., 2003). Mobilization of LTR transposons is associated with 10% of all spontaneous mutations in mice (Waterston et al., 2002). Pericentromeric alpha satellite repeats on chromosomes 1 (Chr1alpha) and 4 (Chr4alpha) were tested as specificity controls because HLCS-dependent biotinylation events are implicated in their repression (Camporeale et al., 2007).

## Results

### *HLCS interacts with DNMT1 and MeCP2*

HLCS interacts physically with both DNMT1 and MeCP2, based on the following lines of evidence. Human embryonic kidney HEK293 cells were co-transfected with pCMV-Myc-HLCS and pCMV-HA-DNMT1. When cell lysates were precipitated with anti-Myc and probed with anti-DNMT1, a distinct signal was obtained for DNMT1 (Fig. 2A, first lane). Likewise, when cell lysates were precipitated with anti-HA and probed with anti-HLCS, a distinct signal was obtained for HLCS (Fig. 2B, first lane). When empty vectors (pCMV-Myc or pCMV-HA) were substituted for pCMV-Myc-HLCS and pCMV-HA-DNMT1, respectively, no signals were obtained (negative controls, second lane in both gels). Overexpression of DNMT1 and HLCS was confirmed by probing whole cell extracts with anti-DNMT1 and anti-HLCS (input control, third and fourth lane in both gels). In contrast, no signal was obtained when HEK293 cells were co-transfected with pCMV-Myc-HLCS and pCMV-HA-DNMT3a or pCMV-HA-DNMT3b, followed by probing anti-Myc precipitates with anti-HA, or when anti-HA precipitates were probed with anti HLCS (specificity control, data not shown).

Similar experiments were conducted for MeCP2. HEK293 cells were co-transfected with pCMV-Myc-HLCS and pCMV-HA-MeCP2. When cell lysates were precipitated with anti-Myc and probed with anti-MeCP2, a distinct signal was obtained for MeCP2 (Fig. 2C, first lane). Likewise, when cell lysates were precipitated with anti-HA and probed with anti-Myc, a distinct signal was obtained for HLCS (Fig. 2D, first lane). When empty vectors (pCMV-Myc or pCMV-HA) were substituted for pCMV-Myc-HLCS and pCMV-HA-MeCP2, respectively, no signals were obtained (second lane in both gels).

Overexpression of MeCP2 and HLCS was confirmed by probing whole cell extracts with anti-MeCP2 and anti-Myc (input control, third and fourth lane in both gels).

Next, limited proteolysis assays were conducted using recombinant HLCS, DNMT1, and MeCP2. Limited proteolysis assays are based on the principle that physical interactions between proteins delay their degradation by dilute solutions of proteases such as trypsin (Chazin, 2007). When His-tagged DNMT1 was mixed with GST-tagged HLCS prior to treatment with trypsin, the proteolytic digestion of DNMT1 and HLCS was slow, as evidenced by staining gels with blue stain (Fig. 3A, left panel). Meaningful amounts of protein were detected even after 90 min of incubation with trypsin. In contrast, when DNMT1 was incubated with GST tag alone, DNMT1 was degraded within less than 5 min, and GST started to degrade within 5 min of incubation (Fig. 3A, right panel).

Likewise, when His-tagged MeCP2 was mixed with GST-tagged HLCS prior to treatment with trypsin, the proteolytic digestion of HLCS and MeCP2 was slow. Meaningful amounts of protein were detected even after 45 min of incubation with trypsin. (Fig. 3B, left panel). In contrast, when MeCP2 was incubated with GST tag

alone, both MeCP2 and GST were degraded within 10 to 30 min of incubation (Fig. 3B, right panel).

### ***Synergies between DNA methylation events and HLCS in the repression of LTRs***

Our central hypothesis is that DNMT1 and MeCP2 facilitate the binding of HLCS to distinct loci in chromatin, and that HLCS recruits the histone methyltransferase EHMT-1 to repress genes through creating H3K9me marks. This hypothesis is partly based on previous observations suggesting that HLCS knockdown causes a loss of H3K9me mark in LTRs (Chew et al., 2008). Here we substantiated these previous observations by conducting HLCS overexpression experiments. HLCS was stably overexpressed in HEK293 cells. The abundance of mRNA coding for HLCS was  $69 \pm 4.6$  times greater in overexpression cells compared with non-transfected controls; values were normalized by the abundance of mRNA coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The differences were similar when HLCS was probed with anti-HLCS or anti-Myc in western blots (Fig. 4).

Consistent with our central hypothesis, HLCS overexpression caused a 207% increase of H3K9me marks in the LTR15 locus in HEK293 cell compared with non-transfected controls (Fig. 5A). A similar HLCS-dependent increase (33%) of H3K9me marks was also seen in the LTR22 locus but the difference was not statistically different between HLCS overexpression cells and controls. The HLCS-dependent enrichment of H3K9me marks was not specific for LTRs but was also seen in Chr1alpha and Chr4alpha repeats (Fig. 5A). In contrast, HLCS overexpression had no effect on the enrichment of H3K9me

marks in promoter of *GAPDH* gene in euchromatin and the promoter of the transcriptionally repressed, colon-specific *Apobec1* gene.

The HLCS-dependent enrichment of H3K9me marks in LTRs caused an 87% decrease in the abundance of mRNA coding for LTRs (Fig. 5B). Importantly, this effect was abrogated when DNA methylation marks were erased by treating HEK-293 cells with 5-azacytidine. Note that transcripts coding for distinct LTRs are nearly identical and cannot be distinguished by regular quantitative real-time PCR. Therefore the mRNA values reported for LTRs represents the global expression of LTRs, as opposed to representing individual LTRs. Also note that LTRs may produce two distinct transcripts, U5 and R-U5, and that both transcripts depended on HLCS expression and DNA methylation. Again, effects of HLCS overexpression and 5-azacytidine treatment on transcriptional activity were not specific for LTRs, but were also seen in Chr1alpha and Chr4alpha repeats (Fig. 5B).

## Discussion

This is the first report offering a potential mechanism for recruiting HLCS to distinct loci in chromatin. Based on this report, physical interactions between HLCS and DNMT1 and MeCP2 contribute toward the positioning of HLCS in human chromatin. This observation goes beyond our previous studies, which suggest that HLCS interacts physically with the core histones H3 and H4 and the histone methyltransferase EHMT-1 (Bao, Pestinger, et al., 2011; Y. Li et al., 2013). The interactions between HLCS and histones may account for the binding of HLCS to chromatin, but does not explain the punctuate pattern of

HLCS localization that was observed in previous studies (Camporeale et al., 2006; Singh et al., 2011), simply because core histones can be found in all nucleosomes in chromatin. Likewise, the interactions between HLCS and EHMT-1 are unlikely to mediate the initial positioning of HLCS, based on previous observations that HLCS knockdown causes a loss of EHMT-1 dependent H3K9me marks (Chew et al., 2008; Gralla et al., 2008; Pestinger et al., 2011). This study provides compelling evidence that HLCS interacts physically with DNMT1 and MeCP2 and that DNA methylation marks are important for subsequent HLCS/EHMT-1 interactions leading to the creation of H3K9me marks.

This paper is also the first report to identify epigenetic synergies between dietary methyl donors such as folate, methionine and choline, which are essential one-carbon donors in DNA and histone methylation events (Stover, 2009; Wolff, Kodell, Moore, & Cooney, 1998; Zeisel, 2009), and the micronutrient biotin, which appears to be essential for the creation of H3K9me marks (Chew et al., 2008; Gralla et al., 2008; Pestinger et al., 2011). It remains to be determined whether methyl donors and biotin can compensate for each other's deficiency or whether the deficiency of one of the two can precipitate the other's deficiency symptoms. We do not propose that biotinylation of histones *per se* contributes in quantitatively meaningful ways to gene repression, because histone biotinylation marks are too rare to elicit such effects (Bailey et al., 2008; Kuroishi et al., 2011; Stanley et al., 2001).

Our model that HLCS is member of a multiprotein gene repression complex is more complicated than shown in Figure 1. For example, we have developed an algorithm to predict HLCS-interacting proteins and that algorithm identified not only EHMT-1 but also the nuclear co-repressor N-CoR2 as an HLCS interacting protein (Y. Li et al., 2013).

N-CoR participates in the recruitment of histone deacetylases to human chromatin (Wen et al., 2000). Histone deacetylases catalyze the removal of acetylation marks from histones, thereby causing gene repression (Wang et al., 2009). Ongoing studies in our laboratory suggest that HLCS interacts physically with both N-CoR and histone deacetylases (D. Liu and J. Zemleni, unpublished). Depending on the outcome of these studies, the model shown in Figure 1 will need to be amended in the future.

The chromatin HLCS/multiprotein complex constitutes a plausible machinery contributing toward transcriptional repression of LTRs. Transcriptionally competent LTRs are enriched with H3K9me3 marks and the CpGs located in LTRs are generally hypermethylated (Meissner et al., 2008; Mikkelsen et al., 2007). Evidence suggests that LTRs are also docking sites for HLCS, judged by the local enrichment of HLCS-dependent biotinylation marks (Chew et al., 2008). Both LTR15 and LTR22 are transcriptionally active and were chosen as representative examples of abundant 3' proviral and solitary LTRs respectively, in the human genome (Buzdin et al., 2006). Considering the importance of repressing LTRs for maintaining genome stability, this paper may have implications for methyl/biotin and HLCS/protein synergies in gene regulation and disease risk. Importantly, our studies suggest that these synergies are not limited to the repression of LTRs, but also play roles in the repression of other repeats including pericentromeric alpha satellite repeats.

Some uncertainties remain. One might ask why HLCS participates in the creation of repression marks in the *LTR15* locus, but to a much small extent in the *LTR22* locus, and at non-detectable levels in the *Apobec1* locus. A possible explanation could be that LTR15 is a proviral LTR, whereas LTR22 is a solitary LTR (Buzdin et al., 2006).

Redundancies among histone K9-methyltransferases might also contribute to site specificity. The human genome encodes for H3K9 methyltransferases other than EHMT-1, e.g., SETDB1 and ESET (Schultz, Ayyanathan, Negorev, Maul, & Rauscher, 2002; Yang et al., 2002). Histone methyltransferases other than EHMT-1 might not interact with HLCS. This issue will need to be addressed in future studies demonstrating co-localization of HCS and EHMT-1 in the same loci by using chromatin immunoprecipitation studies, or by using transgenic cell lines in which histone methyltransferases other than EHMT-1 have been knocked down. Likewise, methyl-CpG-binding proteins other than MeCP2 may have distinct effects in regard to recruiting HLCS to target loci. The absence of effect in the *Apobec1* locus might be due to the fact that HLCS containing multiprotein complexes specifically target repeat regions such as LTRs and pericentromeric alpha satellite repeats, but that is untested speculation.

We conclude that HLCS exerts some of its roles in gene regulation through the formation of multiprotein gene repression complexes in human chromatin. Possible members of this complex include proteins involved in DNA methylation, EHMT-1, N-CoR2, and histone deacetylases. This theory would integrate previous observations that HLCS and biotin are important for gene repression, the low abundance of biotinylated histones in chromatin, and the known roles of members of the putative multiprotein complex in mediating gene repression into a coherent model.

## **Materials and Methods**

### ***Plasmids***



Full-length human HLCS (NM\_000411) was subcloned from plasmid pET41a (+)-HLCS (Esaki, Malkaram, & Zemleni, 2012) into vector p3XFLAG-CMV-26 (Sigma-Aldrich, Cat# E7283) using *NotI* and *XbaI*, thereby creating plasmid FLAG/Myc-HLCS for studies of HLCS overexpression and its effects on H3K9me marks and LTR transcriptional activity. Plasmid pCMV-myc-HLCS was created as described previously (Y. Li et al., 2013) and was used for overexpression of Myc-tagged HLCS in co-immunoprecipitation studies. Full-length human MeCP2 (Genbank Accession:NM\_004992) was subcloned from plasmid pGADT7-MeCP2 (unpublished) into vector pCMV-HA (Clontech, Cat# 635690) using *SfiI* and *XhoI* for overexpression of HA-tagged MeCP2. Full-length DNMT1 (Genbank Accession:NM\_001130823.1) was subcloned from pBluescript SK (+)-DNMT1 (unpublished) into pCMV-HA vector using *NdeI* and *Sall* for overexpression of HA-tagged DNMT1. Plasmid pET41a (+)-HLCS was used to prepare GST-tagged recombinant full-length HLCS as described previously (Bao, Pestinger, et al., 2011). Full-length MeCP2 was subcloned from plasmid pET41a (+)-MeCP2 (unpublished) into vector pET28a+ (Novagen, Cat# 698646) using *EcoRI* and *XhoI* (creating pET28a-MeCP2), and full-length DNMT1 was subcloned from pET41a (+)-DNMT1 (unpublished) into pET28a+ vector using *EcoRI* and *Sall* (creating pET28a-DNMT1) for the preparation of recombinant proteins. As specificity controls, full-length human DNMT3a and DNMT3b were subcloned from plasmids pCMV-sport6-DNMT3A (Invitrogen, cat#6150112) and pENTR223.1-DNMT3B (Invitrogen, cat#40080762) into vector pCMV-HA using *EcoRI* and *Sall* to create plasmids pCMV-HA-DNMT3a and pCMV-HA-DNMT3b, respectively, for overexpression of HA-tagged DNMT3a and DNMT3b. The identities of all plasmids were verified by sequencing.

### *Cell lines*

Human embryonic kidney HEK293 cells (American Type Culture Collection, CRL-1573) were cultured following the vendor's recommendations. Cells were transfected with plasmid FLAG/Myc-HLCS by using electroporation and the transfectants were selected using 1 mM G418 for 14 days, and maintained in medium containing 0.4 mM G418. The expression of HLCS and GAPDH (control) was assessed by quantitative real-time PCR (qRT-PCR; see below) and by western blot analysis using anti-Myc (Abcam, cat# ab9106) and anti-HLCS serum (Chew, Camporeale, Kothapalli, Sarath, & Zemleni, 2006). For co-immunoprecipitation assays, HLCS, DNMT1 and MeCP2 were transiently overexpressed as described below.

### *Co-immunoprecipitation assays*

HEK293 cells were seeded in 25-cm<sup>2</sup> flasks ( $2.5 \times 10^6$  cells/flask) 24 h before transient transfection with 8 µg of plasmids (4 µg each for Myc- and HA-tagged proteins) in TurboFect reagent (Fermentas, Cat# R0531) and Opti-MEM I medium (Invitrogen, Cat# 31985). Cells from six flasks were collected 48 h after transfection and lysed in 20 mM Tris HCl buffer (pH 8.0), containing 137 mM NaCl, 10% glycerol, 1% CA-630, 2 mM EDTA, phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich, Cat# P2714). Samples were pre-cleared using rabbit IgG before precipitation of proteins with rabbit polyclonal anti-HA (Abcam, Cat# ab9110), anti-Myc (Abcam, Cat# ab9106), and protein A resin (Thermo Scientific, Cat#20338). Western blots were performed using anti-MeCP2 (Abcam, Cat# ab2028), anti-DNMT1 (Abcam, Cat# ab16632), anti-Myc,

anti-HLCS(Chew et al., 2006) and appropriate fluorophore conjugated secondary antibodies (Pestinger et al., 2011).

### ***Limited-proteolysis assays***

Recombinant proteins were overexpressed in ArcticExpress (DE3) E. coli and purified using a Glutathione resin (Genscript, Cat# L00206) or a HisTrap FF column (GE Healthcare, Cat# 17-5319-01) as described previously (Bao, Pestinger, et al., 2011). Limited proteolysis was performed at 37°C in 75 mM Tris acetate buffer (pH 7.5), containing 0.3 mM dithiothreitol and 45 mM MgCl<sub>2</sub>. One aliquot (time-zero sample) was collected before adding trypsin (Sigma-Aldrich, Cat# T6567), and equal volumes of aliquots were collected at timed intervals after initiation of digestion. Proteins were separated by gel electrophoresis and stained using Coomassie blue (Y. Li et al., 2013). In control experiments MeCP2 and DNMT1 were incubated with GST peptide to formally eliminate the possibility that the GST tag protected the proteins against proteolytic digestion.

### ***5-Azacytidine treatment***

5-Azacytidine solutions were prepared freshly immediately prior to use. Pilot tests suggested that 1  $\mu$ M 5-azacytidine does not affect HEK293 cell viability. HEK293 cells were seeded in T25 flasks in fresh growth medium at day 0, and 5-azacytidine was added on day 1 to produce a final concentration of 1  $\mu$ M. Media were changed daily maintaining the concentration of 5-azacytidine at 1  $\mu$ M. On day 5, the cells were washed with phosphate-buffered saline and collected for analysis of gene expression by qRT-PCR.

### ***Micro chromatin immunoprecipitation assay***

The enrichment of H3K9me in distinct loci in human chromatin was assessed by micro chromatin immunoprecipitation ( $\mu$ ChIP) assay as described before, using  $2.5 \times 10^6$  HEK293 cells (Dahl & Collas, 2008; Pestinger et al., 2011). ChIP-grade anti-H3K9me was purchased from Abcam (ab8898). Quantification of amplicons, calculation of relative enrichment of histone marks and normalization of nucleosomal occupancy using (nonmodified) C-terminus in histone H3 (Abcam, Cat# ab1791) were conducted as described previously (Chew et al., 2008; Rios-Avila et al., 2012). Data are expressed as percent of input DNA.

### ***qRT-PCR***

Power SYBR Green PCR Master Mix (Applied Biosystems, Cat# 4309155) was used to quantify the abundance of transcripts by qRT-PCR. Perfecta SYBR Green FastMix (Quanta Biosciences, Cat# 95073) was used to quantify the abundance of amplicons in immunoprecipitated chromatin (Pestinger et al., 2011). PCR primers are listed in Table 1. Pericentromeric alpha satellite repeats are repressed through HLCS- and methylation-dependent epigenetic events and were used as specificity controls (Camporeale et al., 2007; Martens et al., 2005). Apobec1 is specifically expressed in colon and its expression is silenced in kidney tissues as confirmed by searching against the BioGPS database (Wu et al., 2009).

### ***Statistical analysis***

Data from  $\mu$ ChIP assays were tested for normality of distribution and homogeneity of variances by constructing the probability plot of the residues, the stem-and-leaf plot of the residuals, and the normal probability plot and performing the Shapiro-Wilk test (SAS, 2008). using the SAS 9.2 software package (SAS Institute, USA). After log transformation, the data were still not normally distributed and, therefore, were analyzed by the Kruskal-Wallis test as a robust substitute for one-way Anova (McDonald, 2009). Calculations are based on five repeats. Differences were considered statistically significant if p-value was  $<.01$ . The data for quantification of LTR transcripts were analyzed by student's t-test. Calculations were based on three repeats. Differences were considered statistically significant if p-value was  $<.05$ . Data are expressed as mean $\pm$ S.D.

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**Table 1. PCR primers**

Target	Sequence	F/R <sup>a</sup>	Template <sup>b</sup>
Apobec1	5'-TCCCATAACTGCCTGAGATG-3'	F	DNA
	5'-TTGTTCTCCTGGACTTTGTTGC-3'	R	DNA
Chr1alpha	5'-TCATTCCCACAACTGCGTTG-3'	F	DNA/Transcript
	5'-TCCAACGAAGGCCACAAGA-3'	R	DNA/Transcript
Chr4alpha	5'-CTGCACTACCTGAAGAGGAC-3'	F	DNA/Transcript
	5'-GATGGTTCAACACTCTTACA-3'	R	DNA/Transcript
GAPDH	5'-CGTAGCTCAGGCCTCAAGA-3'	F	DNA
	5'-GCTGCGGGCTCAATTTATAG-3'	R	DNA
GAPDH	5'-TCCACTGGCGTCTTCACC-3'	F	Transcript
	5'-GGCAGAGATGATGACCCTTT-3'	R	Transcript
HLCS	5'-TGAGACCTGATCCTTAACTTCC-3'	F	Transcript
	5'-ATGGAAGATAGACTCCACAT-3'	R	Transcript
LTR15	5'-TTGTCATTGGTTCTGTGTAGGG-3'	F	DNA
	5'-CCTCCATATGCTGAACGCTG-3'	R	DNA
LTR22	5'-GACCATTTCATGGACAAATC-3'	F	DNA
	5'-CCTCCATATGCTGAACGCTG-3'	R	DNA
LTR, R-U5	5'-GCGGGCAGCAATACTGCTTTGTAA-3'	F	Transcript
	5'-ACCAGCGTTCAGCATATGGAGGAT-3'	R	Transcript
LTR, U5	5'-AACTCAGAGGCTGGCG-3'	F	Transcript
	5'-AGACACAGAGACAAAGTATAGAGA-3'	R	Transcript

Apobec1= apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (Genbank NC\_000012.11); Chr1alpha= chromosome 1 alpha satellite repeats (GenBank M26919);

Chr4alpha= chromosome 4 alpha satellite repeats (GenBank M38467); GAPDH= glyceraldehyde-3-phosphate dehydrogenase (GenBank NG\_007073.2 for genomic DNA, NM\_002046.4 for transcript); HLCS= Holocarboxylase Synthetase (GenBank NM\_000411.6); LTR15= intact LTR (GenBank AC116309) (Buzdin et al., 2006); LTR22= solitary LTR (GenBank AL451165) (Buzdin et al., 2006); LTR, R-U5= region between R and U5 of LTR of HERV-K families (Kovalskaya, Buzdin, Gogvadze, Vinogradova, & Sverdlov, 2006); LTR, U5= U5 region of LTR of HERV-K families (Kovalskaya et al., 2006).

<sup>a</sup>F= Forward; R= Reverse.

<sup>b</sup>DNA, genomic DNA for ChIP assay; Transcript, cDNA for qRT-PCR.

## Figure and legends

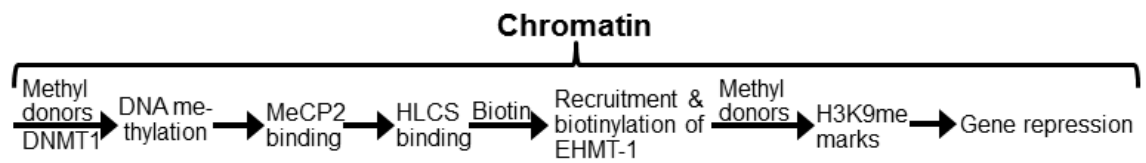


Figure 1. HLCS facilitates methylation events in the epigenome through physical interactions with DNA methyltransferase 1 (DNMT1), methyl CpG binding protein 2 (MeCP2), and eukaryotic histone methyltransferase (EHMT-1).



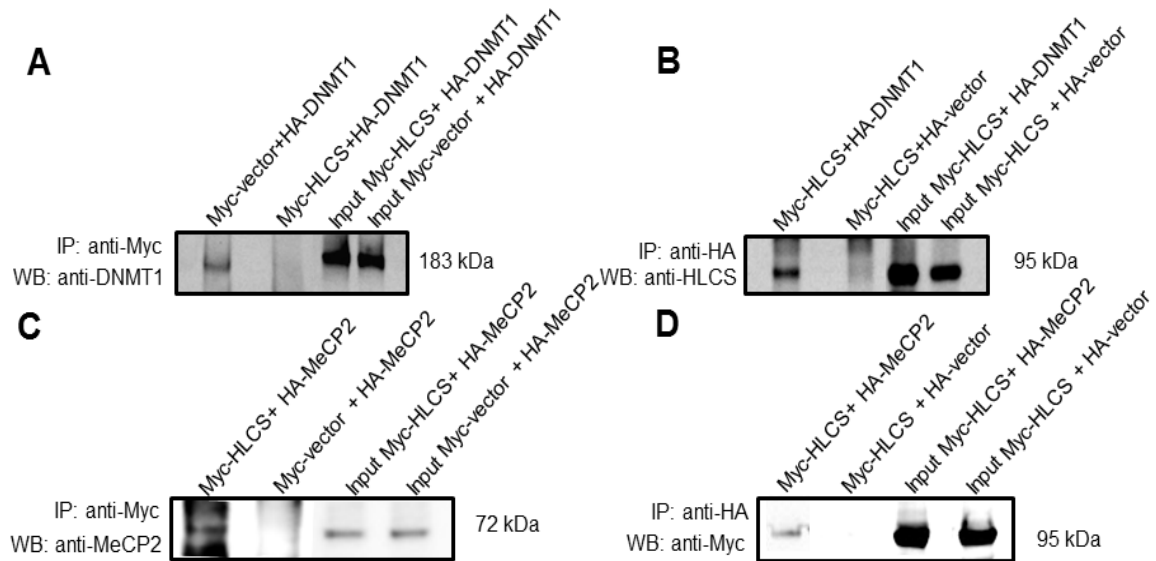


Figure 2. HLCS interacts with both MeCP2 and DNMT1 in vivo, judged by co-immunoprecipitation assay. Myc-HLCS and HA-DNMT1 were overexpressed in HEK293 cells and protein extracts were immunoprecipitated using anti-Myc (panel A) and anti-HA (panel B). Proteins were resolved by electrophoresis and probed with anti-DNMT1 (panel A) and anti-HLCS (panel B). Panels C and D are similar to A and B, but HA-MeCP2 was substituted for HA-DNMT1 in overexpression experiments and anti-MeCP2 (panel C) and anti-Myc (panel D) were substituted for anti-DNMT1 and anti-HLCS, respectively. Transfections with empty vectors were used as negative controls (second lane in each gel), and whole cell lysates were used as input controls (third and fourth lane in each gel). Gels were electronically re-arranged to facilitate comparisons.

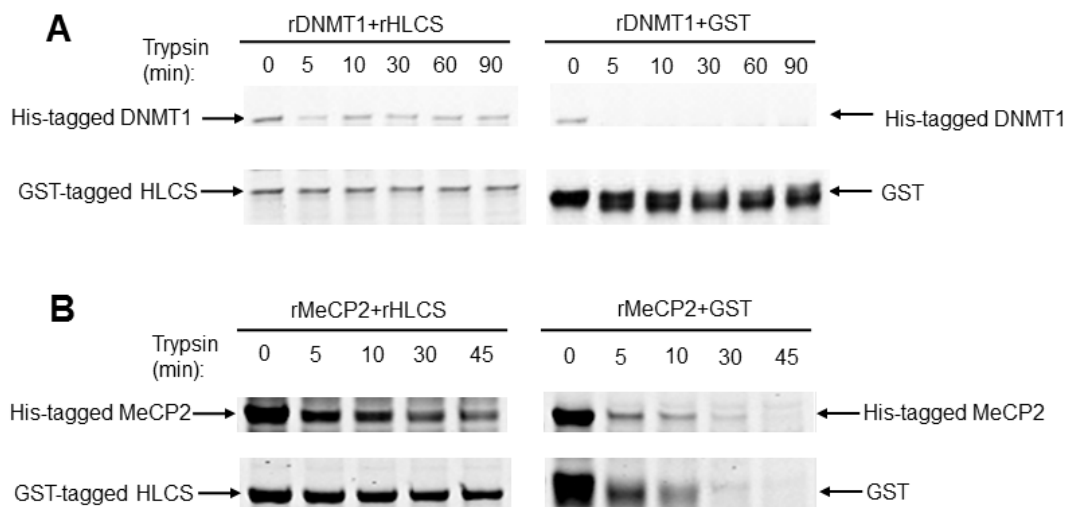


Figure 3. HLCS interacts with both MeCP2 and DNMT1 in vitro, judged by limited proteolysis assay. (A) Purified recombinant GST-HLCS protected His-DNMT1 from trypsin digestion (left panel) compared with DNMT1 that was pre-incubated with GST (right panel). (B) Purified recombinant GST-HLCS protected His-MeCP2 from trypsin digestion (left panel) compared with MeCP2 that was pre-incubated with GST (right panel).

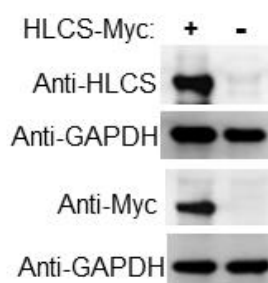


Figure 4. Transfection with plasmid FLAG/Myc-HLCS produced a stable overexpression of HLCS compared with non-transfected controls. The Myc-tagged HLCS in cell extracts was probed with anti-HLCS and anti-Myc. GAPDH was probed as loading control.

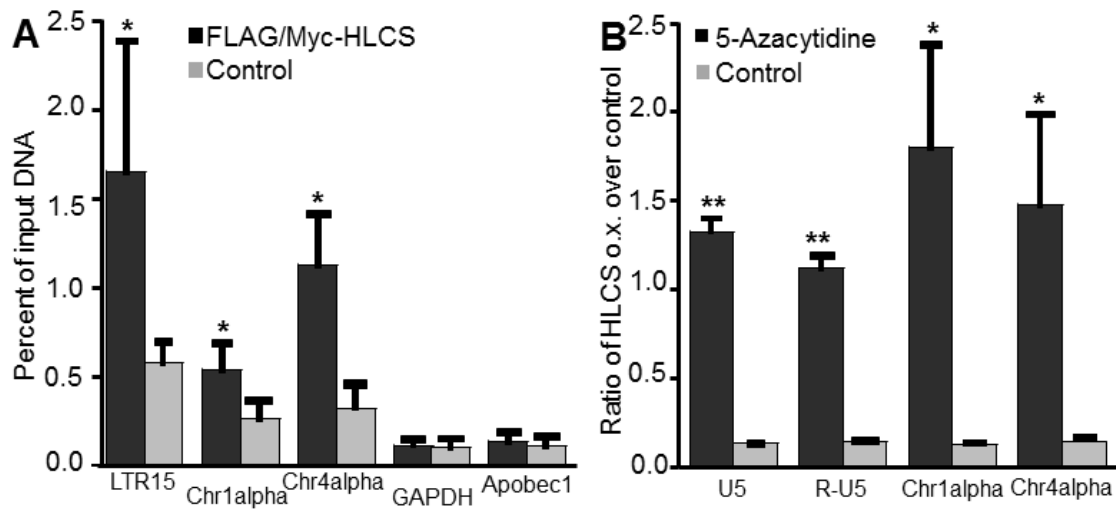


Figure 5. The effects of HLCS on LTR repression depend on DNA methylation. (A) The enrichment of H3K9me3 marks in loci coding for LTR15, Chr1alpha, Chr4alpha, GAPDH, and Apobec1 was quantified by chromatin immunoprecipitation assay/qRT-PCR in HEK293 HLCS overexpression cells and control cells ( $H=6.9018$ , 1 d.f., \* $p$ -value=0.0086 HLCS overexpression vs. control,  $H=4.8402$ , 1 d.f., \* $p$ -value=0.0278 for Chr1alpha,  $H=6.8598$ , 1 d.f.,  $p$ -value=0.0088 for Chr4alpha,  $H=0.1756$ , 1 d.f.,  $p$ -value=0.6752 for GAPDH, and  $H=0.7245$ , 1 d.f.,  $p$ -value=0.3947 for Apobec1). (B) The transcription of LTRs, Chr1alpha, and Chr4alpha was repressed in HLCS overexpression HEK293 cells compared with controls. Treatment of 5-azacytidine (1  $\mu$ M) abrogated the effects of HLCS on repeat repression (\* $p$ -value<0.0001, \* $p$ -value<0.05, 5-azacytidine treatment vs. control).

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## CHAPTER II

### **Holocboxylase synthetase catalyzes biotinylation of heat shock protein 72, thereby inducing RANTES expression in HEK293 cells**

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#### **Abstract**

In a recent mass spectrometry screen we have identified 108 new proteins that were modified endogenously by covalent binding of biotin; members of the heat shock superfamily of proteins, including heat shock protein 72 (HSP72), were overrepresented among the biotinylated proteins. Mammals respond to infections by secreting extracellular HSP72 (eHSP72) which elicits an immune response. Here, we identified five biotinylation sites in HSP72 using mass spectrometry and site-directed mutagenesis. We used co-immunoprecipitation, mass spectrometry, and limited proteolysis assays to demonstrate that HSP72 interacts physically with the protein biotin ligase,



holocarboxylase synthetase (HLCS) leading to the biotinylation of residues K112, K128 K348, K361, K415, and probably additional lysines. Finally, we demonstrated that HLCS-dependent biotinylation of eHSP72 increases the expression of the chemokine RANTES by the HEK293 human embryonic kidney cells. In conclusion, we report a novel endogenous modification of HSP72 and demonstrated that the binding of biotin to eHSP72 poises cells for a strong immune response.

**Key words:** Biotin; heat shock protein 72; holocarboxylase synthetase; posttranslational modification; RANTES.

## Introduction

The stress-inducible heat shock protein 72 kDa (HSP72) localizes in cytoplasm, nucleus and extracellular space, and is essential for the cellular response to the accumulation of unfolded protein and immune function (Asea, 2005; Mambula, Stevenson, Ogawa, & Calderwood, 2007). HSP72 is released from cell through passive and active pathways (Mambula et al., 2007, Asea, 2005 #8472). The level of extracellular HSP72 (eHSP72) increased in response to bacterial infection in rats (Campisi, Leem, & Fleshner, 2003), treatment with lipopolysaccharides in THP-1 human monocytic cells (Abboud et al., 2008), and inhibition of phospholipase C in A431 human squamous carcinoma cells (Evdonin et al., 2009). Unlike intracellular HSP72, eHSP72 promotes the secretion of proinflammatory cytokines, mediated by binding to cell surface receptors (Asea et al., 2000; Campisi et al., 2003; Multhoff et al., 1999). Covalent binding of the vitamin biotin to eHSP72 (eHSP72bio), accomplished by synthetic cross linkers and recombinant HSP72, increases the binding eHSP72 to its receptors on the cell surface, thereby enhancing the secretion of the chemokine RANTES in cell cultures (Tao, Nishikawa, Nomura, Kitabatake, & Tani, 2005).

Recently, we discovered 108 novel biotinylated proteins in a human kidney cell line (Li Y. et al., in preparation). Molecular chaperones, including HSP72, were overrepresented among biotinylated proteins and accounted for 17% of the novel targets for biotinylation. Presumably, the binding of the vitamin biotin to HSPs is catalyzed by the sole protein biotin ligase in the human genome, holocarboxylase synthetase (HLCS) (Suzuki et al., 1994). Considering the importance of eHSP72bio in RANTES signaling and the biological significance of RANTES in the chemoattraction and activation of a wide

variety of immune cells during immune response (Krensky & Ahn, 2007), we (i) determined whether HLCS interacts with HSP72 to catalyze its biotinylation, (ii) identified biotinylation sites in HSP72, and (iii) determined whether HLCS-dependent biotinylation of HSP72 increases the expression of RANTES.

## Results

### *HSP72 interacts physically with HLCS*

Whole cell lysates from HEK293 cells overexpressing HLCS were precipitated with anti-Myc and probed with anti-HSP72 and anti-Myc in western blots. Distinct bands for HSP72 and HLCS were observed in HLCS overexpression cells; only a faint HSP72 band and no HLCS band were detected in untransfected controls (Fig. 1A). The abundance of HSP72 in input samples was the same for HLCS overexpression cells and controls.

Next, whole cell lysates were precipitated with anti-Flag and resolved by gel electrophoresis. A distinct band with an apparent mass of 72 kDa was visible after blue staining (Fig. 1B) and was identified as HSP72 using LC/MS/MS and Mascot (probability score= 242) (Perkins, Pappin, Creasy, & Cottrell, 1999).

Overexpression of HLCS did not cause cell stress in Flag-Myc-HLCS cells, judged by the expression of HSP72. The abundance of HSP72 protein was not affected by HLCS overexpression (Fig. 2A). The abundance of HSP72 mRNA was only moderately increased (~50%) in HLCS overexpression cells compared with controls (Fig. 2B).

Transcripts from both *HSPA1A* and *HSPA1B* contributed to the moderate increase in HSP72 mRNA (Fig. 2C).

The physical interaction between HLCS and HSP72 was confirmed using limited proteolysis assays. Pre-incubation of His-HSP72 with GST-HLCS protected HSP72 against proteolysis when compared with GST tag as negative control (Fig. 3).

### ***HLCS-dependent biotinylation of HSP72***

HLCS has HSP72 biotin ligase activity. When GST-HLCS was incubated with His-HSP72, biotin, and co-factors at 37°C for 2 hours, covalent binding of biotin to HSP72 was evident, as judged by streptavidin gel electrophoresis; GST served as negative control (Fig. 4A).

HSP72 is also a target for biotinylation *in vivo*. Biotinylated proteins were purified from HEK293 whole cell lysates using avidin column and analyzed by LC/MS/MS. Seven independent samples were assayed in separate analytical runs, and consistently produced a biotinylation signal for HSP72. As expected for HLCS-dependent biotinylation, lysine residues were the only biotinylation sites detected. Five biotinylation sites were identified: K112, K128 K348, K361 and K415 (Fig. 4B). The Mascot probability scores for HSP72 were 1067, 808, 3848, 440, 11447, 108 and 2965.

### ***Impact of individual lysine residues on HSP72 biotinylation and interaction with HLCS***

The following mutants were created: (i) HSP72<sub>K112,128R</sub> with lysines 112 and 128 mutated to arginines; (ii) HSP72<sub>K348,361,415R</sub> with lysines 348, 361, and 415 mutated to arginines;

and (iii) HSP72<sub>K112-415R</sub> with all five lysines mutated. None of the mutated residues is highly conserved in HSP72, based on the profile Hidden Markov Model (pHMM) (Sonnhammer, Eddy, & Durbin, 1997) (Fig. 5A). In pHMM, the height of the stack of letters at each position represents its information content, which measures the conservation of a position in a profile (Schuster-Bockler, Schultz, & Rahmann, 2004). All three HSP72 mutants interacted physically with HLCS and were substrates for biotinylation by HLCS (Fig. 5B), suggesting that lysines other than the five residues identified by LC/MS/MS are also targets for biotinylation, at least *in vitro* (see Discussion and Materials and methods).

#### ***HLCS-mediated biotinylation on HSP72 stimulate RANTES production***

Biotinylated HSP elicits an increase in the expression of RANTES in HEK293 cells. Recombinant HSP72 and HSP72<sub>K112-415R</sub> were biotinylated using recombinant HLCS. When biotinylated HSP72 was added to cell culture media, the expression of RANTES mRNA increased significantly at HSP72 levels of  $\geq 0.5 \mu\text{M}$  compared with HSP72 prepared in the absence of HLCS (Fig. 6A). When HLCS was added to the cell cultures, RANTES mRNA levels increased suggesting that eHSP72 might have been biotinylated by exogenous HLCS. The addition of biotinylated HSP72<sub>K112-415R</sub> to culture media also caused an increase in RANTES expression (Fig. 6B), consistent with the theory that lysines other than the five residues identified by LC/MS/MS are also targets for biotinylation. Biotinylated HSP72<sub>K112-415R</sub> caused an increase in RANTES mRNA at concentrations as low as  $0.1 \mu\text{M}$ .

## Discussion

This is the first report of a novel biotinylated protein in the human proteome, HSP72.

This discovery is biologically important from the following points of view. First, we recently identified 108 novel proteins that are targets for biotinylation *in vivo* (Li Y. et al., in preparation). Here we show that the covalent binding of biotin is of biological significance for at least some of these proteins. Second, heat shock proteins like HSP72 play essential roles in protein processing and transport and in stress and immune response (Asea, 2005; Bukau & Horwich, 1998; Hartl, 1996; Mambula et al., 2007). One can assume with a reasonable level of confidence that posttranslational modifications that affect the activities of these proteins will have meaningful effects in cellular metabolism.

The biotinylation of HSP72 was of particular interest in our follow-up studies for the mass spectrometry screen, based on the rationale that biotinylated HSP72 was detected in every single cell extract and mass spectrometry experiment that we conducted. In addition, HSP72 is unique in that it is secreted into the extracellular space where it binds to receptors on the cell surface to increase the expression and secretion of the chemokine RANTES (Krensky & Ahn, 2007), thereby enhancing the immune response (Campisi et al., 2003). Note that a recent report suggests that chemical, not site-specific biotinylation of eHSP72 increases the secretion of RANTES by HEK293 cells (Tao et al., 2005), consistent with our observations using enzymatic, site-specific biotinylation of lysine residues. The increased binding of biotinylated HSP72 to cell surface receptors compared with non-biotinylated HSP72 may be caused by an increase in the hydrophobicity of proteins due to the biotin mark (Storm, Loos, & Kaul, 1996), but these lines of

interpretations are controversial (Tao et al., 2005) and biotin might have a more specific effect.

A few uncertainties remain and will need to be addressed in future studies. For example, it is unknown whether a person's biotin status affects the abundance of HSP72 biotinylation marks. Evidence suggests that biotinylated proteins such as carboxylases rapidly lose the coenzyme biotin in biotin-depleted cell cultures and persons (Eng et al., 2013; Kaur Mall, Chew, & Zempleni, 2010). Our studies using the HSP72<sub>K112-415R</sub> mutant suggest the existence of additional biotinylation sites, but these sites escaped detection in our mass spectrometry analysis. We speculate that biotinylation marks in these sites are rare and might be less important than the five sites identified in this study.

In conclusion, we provide evidence that the number of biotinylated proteins in the human proteome is substantially larger than previously thought, and we posit that some of these biotinylation marks affect protein function, including stress response systems.

## **Methods and Materials**

### ***Cell lines***

Human embryonic kidney HEK293 cells (American Type Culture Collection, CRL-1573) were cultured following the vendor's recommendations. Cells overexpressing Flag- and Myc-tagged HLCS were created as described previously (Xue, Wijeratne, & Zempleni, 2013).

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

Plasmid pET41a (+)-HLCS was used to prepare GST-tagged recombinant full-length HLCS as described previously (Bao et al., 2011). Full-length human HSP72 (NCBI accession NM\_005345.5) was cloned from HEK293 cell cDNA prepared using forward primer 5'-GTCCGAATTCGGGGCCAAAGCCGCGGCGATCGGCATCGA-3' and reverse primer 5'-GGACCTCGAGCATCTACCTCCTCAATGGTGGGGCCTGACCC-3'. The PCR product was digested and ligated into vector pET28a+ (Novagen) using *EcoRI* and *XhoI* to create pET28a+\_HSP72. Recombinant HSP72 was expressed in *E. coli* and purified as described previously (Bao et al., 2011), including the removal of recombinant HSP72 that was biotinylated by BirA in *E. coli* (Kobza, Sarath, & Zemleni, 2008). HSP72 mutants were created by site-directed mutagenesis, using plasmid pET28a+\_HSP72 and the Geneart Site-Directed Mutagenesis kit (Invitrogen). The identities of all plasmids were verified by sequencing.

### ***Co-immunoprecipitation assay***

Co-immunoprecipitation assays were conducted in HEK293 cells overexpressing Flag- and Myc-tagged HLCS as described previously (Li, Hassan, Moriyama, & Zemleni, 2013) except that the cells were pre-treated with 0.125 M formaldehyde at room temperature for 15 min, followed by quenching with 0.125 M glycine. Proteins were resolved using 4-12% Bis-Tris gels, using untransfected HEK293 cells as negative controls. The gels were blue stained for analysis by mass spectrometry or probed with anti-HSP72 (Enzo Life Sciences) and anti-Myc (Abcam).

### ***Limited proteolysis assay***



Limited proteolysis was performed by incubating His-tagged HSP72 or indicated mutants with GST-tagged HLCS or GST tag at 37°C as described previously (Li et al., 2013).

### ***Semi-quantitative PCR and quantitative real-time PCR (qRT-PCR)***

The HSP72 monomer is encoded by two distinct genes, *HSPA1A* and *HSPA1B* with 5nt difference in their coding sequences (Milner & Campbell, 1990). cDNA was prepared from HEK293 cells overexpressing HLCS and untransfected controls. Semi-quantitative PCR was performed using the following primers: HSPA1A transcript was amplified using the primers specified above; for HSPA1B transcripts the reverse primer was changed to 5'-GGACCTCGAGCATCCACCTCCTCAATGGTAGGGCCTGACCCAG-3'. Beta-actin (forward; 5'-GCACAGAGCCTCGCCTTTGCC-3'; reverse: 5'-CGGCCAGAGGCGTACAGGGA-3') and GAPDH (forward: 5'-GGTCGTATTGGGCGCCTGGT-3'; reverse: 5'-ACAGTTTCCCGGAGGGGCCA-3') were used as controls. Power SYBR Green PCR Master Mix (Applied Biosystems) was used to quantify the abundance of transcripts by qRT-PCR, not distinguishing between HSPA1A and HSPA1B transcripts. The following primers were used: forward primer 5'-AGGACATCAGCCAGAACAAG-3' and reverse primer 5'-CTGGTGATGGACGTGTAGAAG-3'. Primers for GAPDH were described previously (Xue et al., 2013).

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

Thirty million HEK293 cells were pelleted and lysed in 10 mL BugBuster lysis buffer (Novagen) containing protease inhibitor cocktail and Benzonase Nuclease (Novagen). After centrifugation, the supernatant was filtered through a 22-µm membrane and loaded

on a 2-mL monomeric avidin column (Thermo Scientific). Biotinylated proteins were eluted with 0.1 M glycine (pH 2.8) and concentrated using a 5,000 MWCO concentrator (Corning). Protein samples were resolved on polyacrylamide gels and stained with Coomassie Blue.

### ***Mass spectrometry***

For identification of immune-precipitated proteins in Co-IP assay, the band observed in the HLCS overexpression group rather than in the control group was cut and prepared for mass-spectrometry assay as described previously (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). All mass-spectrometry assays were performed in the proteomic and metabolomics core facility in Redox Biology Center at the University of Nebraska-Lincoln. The same protocol was used for samples purified by avidin columns. The acquired spectrum was compared to the human reference proteome using Mascot ([www.matrixscience.com](http://www.matrixscience.com)) (Perkins et al., 1999).

### ***Biotinylation assay***

His-tagged HSP72 or mutants were mixed with GST-tagged HLCS or GST tag alone and processed as previously described (Bao et al., 2011). Proteins were resolved using polyacrylamide gels and probed with streptavidin (Pestinger, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011). Equal loading was confirmed by anti-His (Fig. 4, Abcam) or Coomassie Blue (Fig. 5).

### ***RANTES stimulation***

HEK293 cells were seeded in 24-well plates ( $0.5 \times 10^6$  cells/500  $\mu$ L medium) for 24 hrs prior to stimulation with enzymatically biotinylated recombinant HSP72 and HSP72<sub>K112-415R</sub> mutant, which were prepared as described in “Biotinylation assay.” Cells were stimulated for 20 hrs before collection for analysis of RANTES transcripts.

### ***Statistical analysis***

Normality was tested by normal quantile plot, and homogeneity of variances was tested by F-test. Student’s t-test was used for pairwise comparisons. One-way ANOVA and Fisher’s least significance difference (LSD) test were used when more than two groups were compared. Means and standard deviations are reported. Differences were considered significant if  $p < 0.05$ .

### **Acknowledgments**

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## Figures and legends

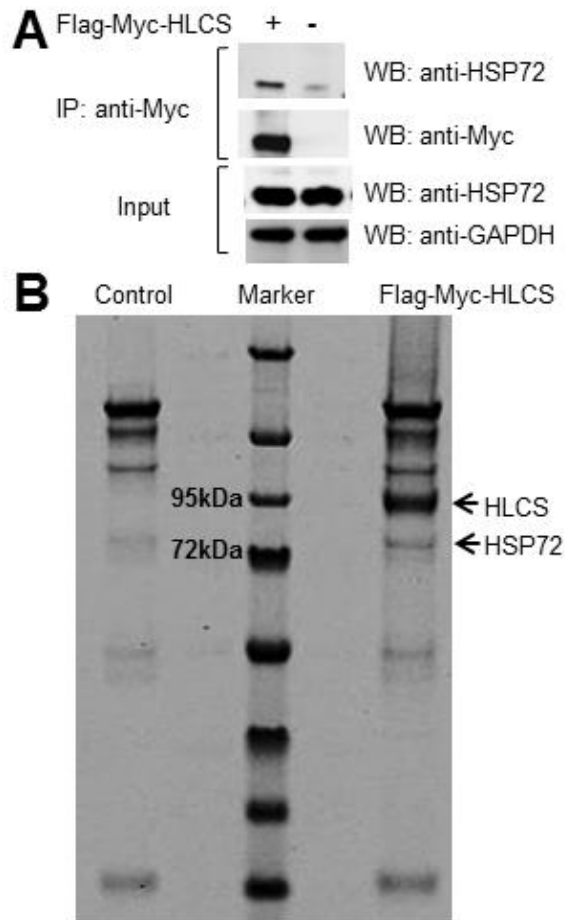


Figure 1. HLCS interacts physically with HSP72 in HEK293 cells. (A) Lysates from cells overexpressing Flag- and Myc-tagged HLCS and normal controls (-) were immunoprecipitated with anti-Myc; proteins in precipitates were resolved on polyacrylamide gels and probed with anti-HSP72 and anti-Myc. Samples prior to precipitation served as input controls and were probed with anti-HSP72 and anti-GAPDH. (B) Same as in panel A, but proteins were precipitated using anti-Flag, gels were stained with Coomassie blue, and cut-out pieces (marked by “HLCS” and “HSP72”) were analyzed by LC/MS/MS. Probability scores for protein identities were 1170 and 242 for HLCS and HSP72, respectively.

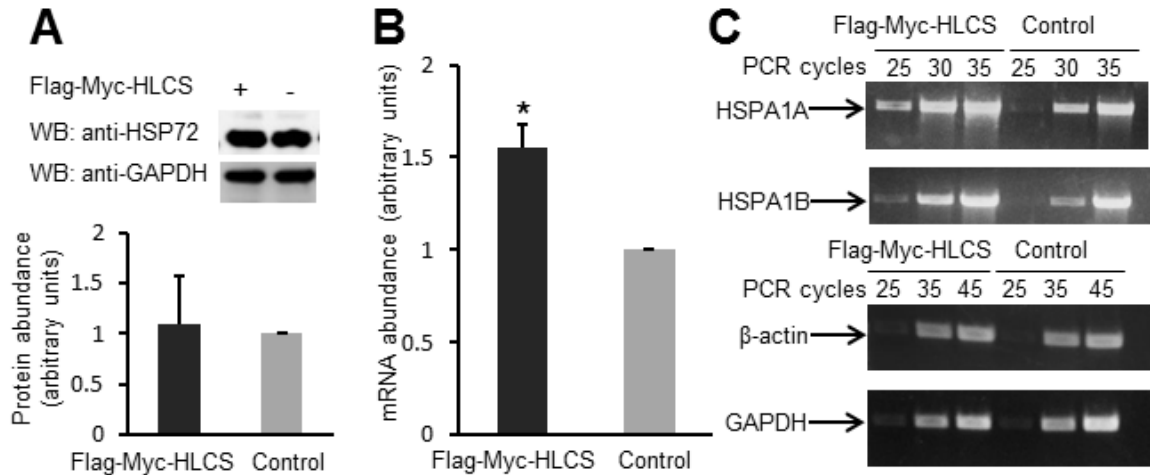


Figure 2. HLCS overexpression does not cause an increase in HSP72 expression in HEK293 cells. (A) Proteins in cell lysates from cells overexpressing Flag- and Myc-tagged HLCS and normal controls (-) were resolved using polyacrylamide gels and transblots were probed using anti-HSP72 and anti-GAPDH (control). The abundance of HSP72 was quantified by band densitometry and normalized for GAPDH (p-value=0.905; n=3 independent experiments); the insert depicts a typical gel. (B) total HSP72 mRNA abundance (HSPA1A + HSPA1B) was quantified using RT-PCR. Error bars represent the standard deviation for three independent runs (\*p-value=0.0086). (C) Semi-quantitative PCR analysis of HSPA1A and HSPA1B full-length transcripts using cDNA from cells overexpressing Flag- and Myc-tagged HLCS and normal controls.

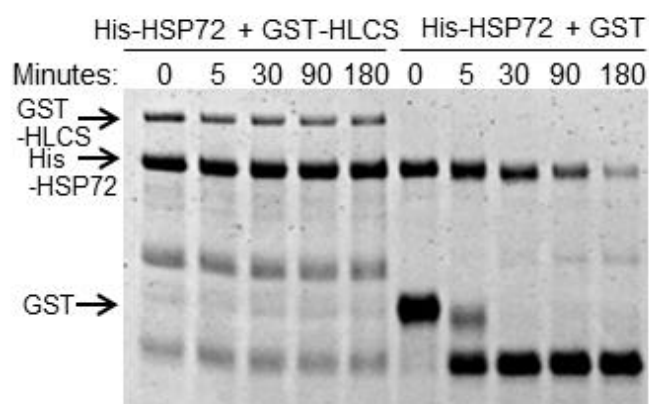


Figure 3. HSP72 interacts physically with HLCS in limited proteolysis assays.

Recombinant GST-tagged HLCS was incubated with recombinant His-tagged HSP72 prior to treatment with trypsin. At timed intervals, aliquots were collected, resolved using polyacrylamide gel electrophoresis and stained with Coomassie blue. Controls were created by incubating His-tagged HSP72 with GST alone.

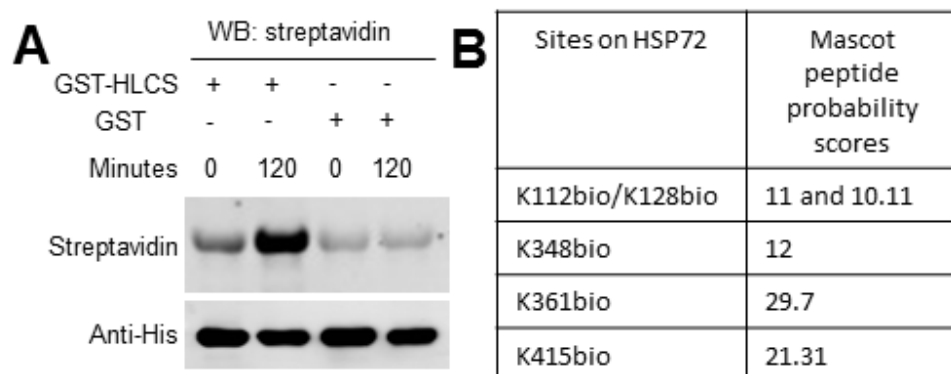


Figure 4. Lysines in HSP72 are targets for biotinylation by HLCS. (A) His-tagged HSP72 was incubated with GST-tagged HLCS, biotin, and co-factors for enzymatic biotinylation. Proteins were resolved by polyacrylamide gel electrophoresis, and HSP72-bound biotin was probed using streptavidin. Controls were prepared by omitting HLCS. Equal loading was confirmed using anti-His. (B) Biotinylated proteins from HEK293 cell lysates were enriched using avidin columns, desalted, resolved by gel electrophoresis, and cut-out pieces were analyzed by LC/MS/MS. Five biotinylation sites were identified in HSP72 and the scores represent peptide probability scores from Mascot analysis. Two distinct scores were obtained for K112 and K128 dually biotinylated HSP72 in independent experiments, and both scores are shown.

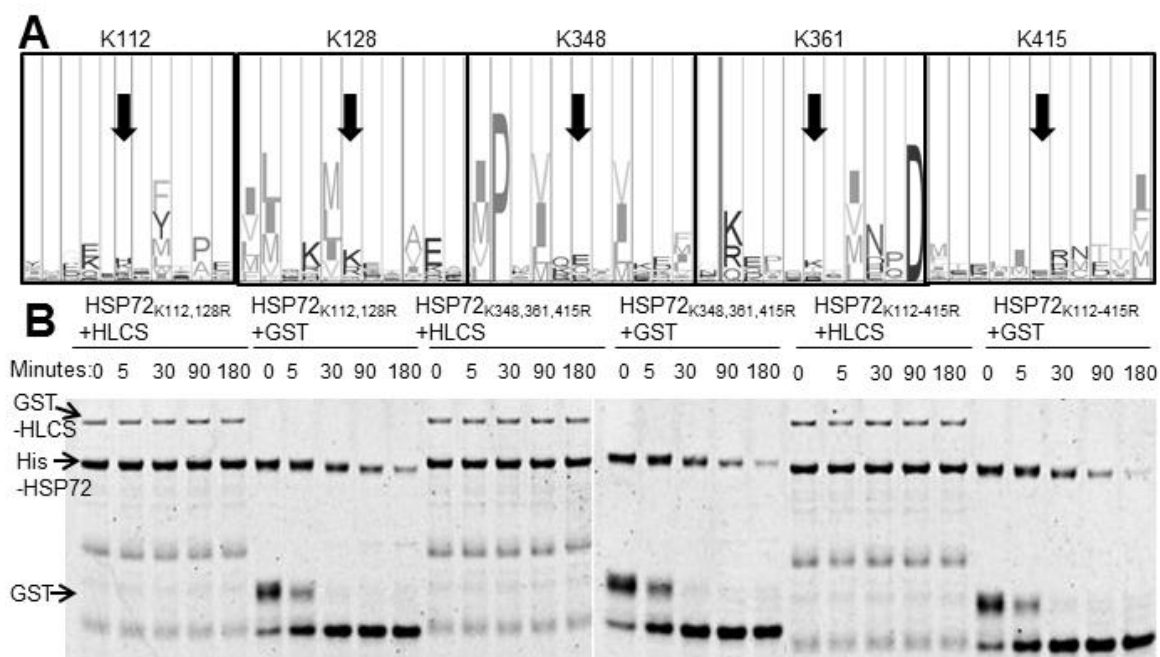


Figure 5. Mutations of biotinylation sites in HSP72 do not affect interactions with HLCS.

(A) HSP72 biotinylation sites are not evolutionary conserved. The HMM logo was retrieved by searching the Pfam database with the protein sequence of HSP72 (NCBI Accession NP\_005336.3); amino acids flanking the biotinylation site are also shown. (B) Limited proteolysis assays were conducted as described for Fig. 3, but HSP mutants were assessed.



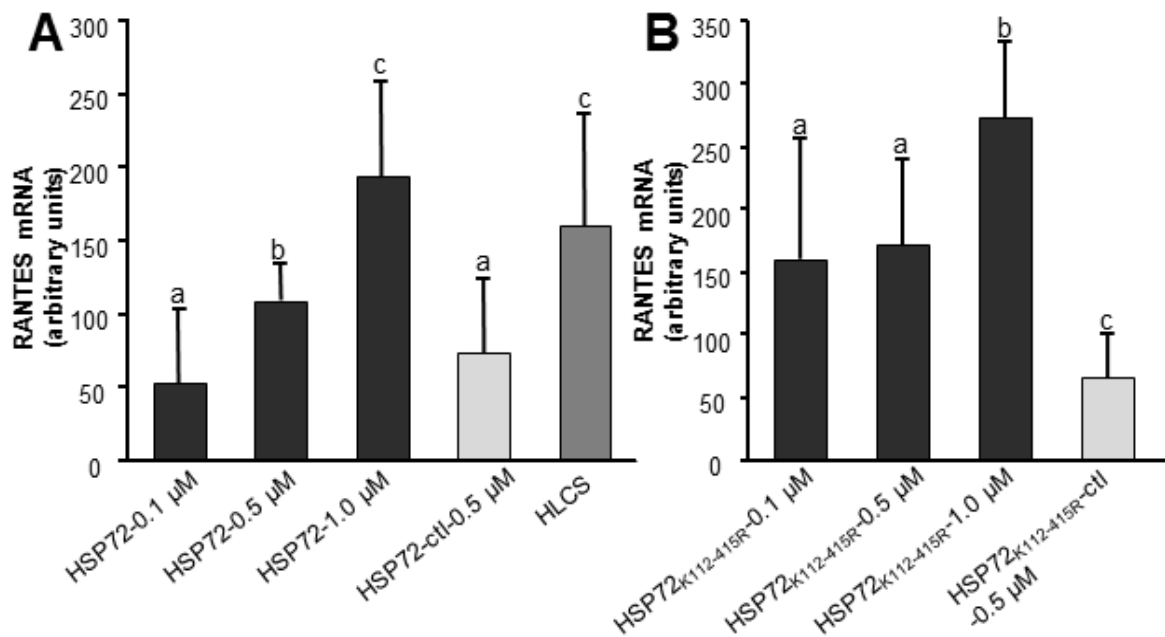


Figure 6. Biotinylation of wild-type and mutant eHSP72 causes an increase in RANTES transcription in HEK293 cells. (A) HEK293 cells were cultured in the presence of the indicated amount of biotinylated His-tagged HSP72 for 20 h. Biotinylated HSP72 was prepared by incubation with HLCS prior to cell cultures. Negative controls were prepared by omitting HLCS in the biotinylation reaction and adding 0.5  $\mu$ M HSP72 to cell cultures (HSP72-ctl-0.5  $\mu$ M). An HLCS control was created by adding an equimolar amount of recombinant HLCS without recombinant HSP72 to cell cultures. (B) As described for panel A, but the HSP72<sub>K112-415R</sub> mutant was substituted for wild-type HSP72. Means $\pm$ standard deviations are reported (<sup>a,b,c</sup> Bars not sharing the same letter are significantly different;  $p < 0.05$ ;  $n=3$ ).

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### CHAPTER III

#### **Epigenetic synergies among biotin, folate, and holocarboxylase synthetase in the regulation of pro-inflammatory cytokines and repeats**

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Running head: Epigenetic synergies in gene regulation

Scientific heading: Basic Immunology

## Abstract

The protein biotin ligase, holocarboxylase synthetase (HLCS), is a chromatin chromatin that interacts physically with the DNA methyltransferase DNMT1, the methylated cytosine binding protein MeCP2, and the histone H3 K9-methyltransferase EHMT1, all of which participate in folate-dependent gene repression. Here we tested the hypothesis that HLCS mediates epigenetic synergies between the biotin and folate in the repression of pro-inflammatory cytokines and long-terminal repeats (LTRs). Biotin and folate supplementation could compensate for each other's deficiency in the repression of LTRs in Jurkat and U937 cells. For example, when biotin-deficient Jurkat cells were supplemented with folate, the expression of LTRs decreased by >70%. Epigenetic synergies were more complex in the regulation of cytokines compared with LTRs. For example, the abundance of TNF- $\alpha$  was 100% greater in folate- and biotin-supplemented U937 cells compared with biotin-deficient and folate-supplemented cells. The NF- $\kappa$ B inhibitor curcumin abrogated the effects of folate and biotin in cytokine regulation, suggesting that transcription factor signaling adds an extra layer of complexity to the regulation of cytokine genes by epigenetic phenomena. We conclude that biotin and folate synergize in the repression of LTRs, and that these interactions are probably mediated by HLCS-dependent epigenetic mechanisms. In contrast, synergies among biotin, folate, and HLCS in the regulation of cytokines need to be interpreted in the context of transcription factor signaling.

**Key words:** biotin; holocarboxylase synthetase; folate; interleukin-6; methyl donors; synergies; tumor necrosis factor  $\alpha$ .

## Introduction

The roles of nutrients in immune function are undisputed, including the vitamins biotin and folate. For example, children with hereditary abnormalities of biotin metabolism developed candida dermatitis, had absent delayed-hypersensitivity skin-tests responses, IgA deficiency, and subnormal percentages of T lymphocytes in peripheral blood (Cowan et al., 1979). In biotin-deficient rats, the synthesis of antibodies is reduced (Kumar & Axelrod, 1978). Biotin deficiency in mice decreases the number of spleen cells and the percentage of B lymphocytes in spleen (Báez-Saldaña, Díaz, Espinoza, & Ortega, 1998), inhibits thymocyte maturation (Baez-Saldana & Ortega, 2004), and increases the production of pro-inflammatory cytokines (Kuroishi et al., 2009). Likewise, severe folate deficiency inhibits the proliferation of primary human CD8<sup>+</sup> T lymphocytes *in vitro*, may cause atopy, and impairs natural killer cell-mediated cytotoxicity in rats (Courtemanche, Elson-Schwab, Mashiyama, Kerry, & Ames, 2004; Husemoen et al., 2006; Kim, Hayek, Mason, & Meydani, 2002). However, evidence also suggests that an intake of more than 400 µg/day folate may impair natural killer cell cytotoxicity in postmenopausal women (Troen et al., 2006), i.e., both folate deficiency and supplementation can be detrimental to immune function.

The interpretation of the effects of nutrition on immune function is further complicated by the fact that recommendations for nutrient intake are largely based on considering nutrients in isolation as opposed to taking into account their synergies and interactions (National, 1998). Notable exceptions include vitamins B<sub>6</sub> and E and to some extent folate. In previous studies we laid the groundwork for establishing synergistic mechanisms between biotin and folate in gene regulation (Fig. 1). In these previous studies we

demonstrated that the folate-dependent methylation of DNA is a pre-requisite for the subsequent binding of the protein biotin ligase, holocarboxylase synthetase (HLCS), to chromatin but that DNA methylation does not depend on HLCS-dependent events (Chew et al., 2008). We further demonstrated that HLCS interacts physically with the DNA methyltransferase DNMT1 and the methylated cytosine binding protein MeCP2 (Xue, Wijeratne, & Zempleni, 2013). While histone biotinylation marks are overrepresented in repressed loci, these marks are very rare in the epigenome and, therefore, can hardly explain the robust correlation between those marks and gene repression (Chew et al., 2008; Gralla, Camporeale, & Zempleni, 2008; Kuroishi, Rios-Avila, Pestinger, Wijeratne, & Zempleni, 2011). Importantly, HLCS also interacts physically with the histone H3 K9-methyltransferase (H3K9me) EHMT1 and catalyzes the biotinylation of K161 in the HLCS-binding domain in EHMT1, thereby strengthening the interaction between the two proteins (Li, Hassan, Moriyama, & Zempleni, 2013). When the biotinylation site in EHMT1 is mutated or deleted, the physical interaction between the two proteins is reduced. Importantly, H3K9me marks are abundant in the epigenome and play an undisputed role in gene repression (Kouzarides & Berger, 2007). HLCS knockdown causes a depletion of H3K9me marks and, consequently, de-represses loci coding for the biotin transporter SMVT, long-terminal repeats (LTRs), and interleukin-2 (Chew et al., 2008; Gralla et al., 2008; Rios-Avila, Pestinger, & Zempleni, 2012).

Here we tested the hypothesis that biotin, folate, and HLCS synergize in the regulation of pro-inflammatory cytokines and LTRs, using vitamin concentrations in cell cultures that are nutritionally relevant. We assessed the regulation of the following loci and genes. (a) LTR transcripts were tested, because their regulation depends on biotin, HLCS, and

methylation events (Chew et al., 2008; Martens et al., 2005a), and de-repression of LTRs impairs genome stability (Kazazian, 2004; Waterston et al., 2002). (b) Tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) were tested because folate supplementation represses the lipopolysaccharide-induced transcription of TNF- $\alpha$  in RAW264.7 macrophages (Feng, Zhou, Xia, & Ma, 2011) whereas folate deficiency increases the expression of TNF- $\alpha$  (Kolb & Petrie, 2013); both TNF- $\alpha$  and IL-6 are inducible by NF- $\kappa$ B (Oshima & Oshima, 2012) and play a central role in the pathogenesis of Crohn's disease (Fujitake et al., 2013) and fat-induced liver inflammation (Park et al., 2010).

## Results

### *Efficacy of treatment*

The abundance of biotinylated carboxylases in Jurkat cells responded well to changes in biotin concentrations in culture media (Fig. 2). In U937 cells, the abundance of biotinylated carboxylases was not different in biotin-sufficient and biotin-supplemented cells, suggesting that carboxylases were saturated with coenzyme at physiological concentrations of biotin in this macrophage cell line. Of note, propionyl-CoA carboxylase was barely detectable in U937 cells. Likewise, acetyl-CoA carboxylases 1 and 2 were barely detectable in both cell lines, consistent with previous observations in immune cells (Manthey, Griffin, & Zemleni, 2002). Collectively, these observations are consistent with the notion that changes in biotin supply affected cellular biotin.

The levels of SAM and SAH in cell cultures were below the detection limit of the reversed-phase HPLC system (about 93.3 and 9.6 mmol/L for SAM and SAH, respectively) and were not quantified.



### ***LTR transcripts***

Supplementation with folate rescues the repression of LTRs in biotin-depleted Jurkat and U937 cells. When biotin-deficient Jurkat and U937 cells were supplemented with folate, the expression of LTRs decreased by >70% and >30%, respectively (Fig. 3A). Likewise, when folate-deficient Jurkat cells were supplemented with biotin, the expression of LTRs decreased by >50% (Fig. 3B). In contrast, biotin supplementation had no significant effect in folate-deficient U937, possibly because cellular biotin status cannot be increased in biotin-supplemented compared with biotin-sufficient macrophages. Collectively, these observations suggest that biotin can rescue folate-deficient cells and *vice versa*, consistent with the theory of synergies between the two micronutrients.

### ***Pro-inflammatory cytokines***

The effects of biotin and folate on the expression of TNF- $\alpha$  and IL-6 presented a much more complex and less coherent picture than that of LTRs; the cytokine data can be summarized as follows. The expression of TNF- $\alpha$  increased significantly in response to biotin supplementation when folate-supplemented U937 cells were cultured in biotin-defined media (Fig. 4A). In contrast, the expression of TNF- $\alpha$  decreased significantly in response to folate supplementation when biotin-supplemented U937 cells were cultured in folate-defined media (Fig. 4B). However, the expression of TNF- $\alpha$  was not affected by biotin in folate-supplemented Jurkat cells (data not shown), consistent with the notion that cells of the T-cell lineage play a minor role in TNF- $\alpha$  production. Subsequent studies of cytokines focused on the myeloid U937 cells rather than T-cells, because innate

phagocytic cells, *i.e.*, macrophages, are the primary source of TNF- $\alpha$  in immune defense (Allie et al., 2013).

Similar to the effects described for TNF- $\alpha$ , the expression of IL-6 increased in response to biotin supplementation when folate-supplemented U937 cells were cultured in biotin-defined media, although the differences between biotin-sufficient and biotin-supplemented media did not reach statistical significance (Fig. 5).

Biotin-dependent cell signals include the transcription factors Sp1/Sp3, NF- $\kappa$ B and Fos/Jun (Griffin, Rodriguez-Melendez, & Zempleni, 2003; Rodriguez-Melendez, Griffin, Sarath, & Zempleni, 2005; Rodriguez-Melendez, Schwab, & Zempleni, 2004). Curcumin blocks NF- $\kappa$ B signaling through inhibiting the I $\kappa$ B kinase alpha/beta (Meng, Yan, Deng, Gao, & Niu, 2013; Singh & Aggarwal, 1995). Curcumin (diferuloylmethane) abolished effects of biotin and folate in the regulation of TNF- $\alpha$  (data not shown) and IL-6 (Fig.5), suggesting that the activation of biotin-dependent transcription factors precede the epigenetic events that regulate cytokine expression. Jurkat cells do not express IL-6 and were not considered for analysis.

## Discussion

Here we report novel synergies between the vitamins folate and biotin in gene regulation, including genes coding for pro-inflammatory cytokines. For LTRs, the data are unambiguous and suggest that folate and biotin supplementation can compensate for each other's deficiency in mediating LTR repression to a quantitatively meaningful extent. This observation is firmly grounded in our previous reports that the binding of HLCS to

human chromatin depends on prior folate-dependent DNA methylation and that folate-dependent creation of H3K9me histone repression marks depends on HLCS and perhaps HLCS-dependent biotinylation events (Li et al., 2013; Xue et al., 2013). The unambiguous nature of the LTR data is probably due to the tight regulation of these repeats through epigenetic mechanisms including various methylation marks (Martens et al., 2005b).

Previous studies suggest that the expression of TNF- $\alpha$  increases and decreases in folate-free and folate-supplemented media, respectively, in macrophages (Feng et al., 2011; Kolb & Petrie, 2013). However, the levels of folate used in these studies lacked physiological relevance, because zero folate is not consistent with survival and millimolar concentrations greatly exceed levels observed in human plasma (George et al., 2002; Selhub, Morris, & Jacques, 2007). Here we demonstrated for the first time that biologically relevant concentrations of folate and biotin decrease and increase, respectively, the expression of TNF- $\alpha$ . We propose that cytokines, unlike LTRs, are regulated by a complex network involving numerous transcription factors, and that changes in biotin- and folate-dependent transcription factors need to be considered when investigating synergies between the two vitamin in gene regulation. For example, biotin deficiency – and nutrient deficiency cell stress in general, causes activation of NF- $\kappa$ B (Rodriguez-Melendez et al., 2004). In this study, curcumin (diferuloylmethane) abrogated the effects of biotin and folate on cytokine expression, consistent with the theory that transcription factor signaling is an important component when evaluating epigenetic synergies between vitamins.

Synergies between biotin and folate are important for human health, based on the following rationale. LTRs make up about 8% of the human genome and at least 51 LTRs are transcriptionally competent (Buzdin, Kovalskaya-Alexandrova, Gogvadze, & Sverdlov, 2006). Repetitive elements such as LTRs pose a burden to genome stability, as their mobilization facilitates recombination between non-homologous loci, leading to chromosomal deletions and translocations (Kazazian, 2004; Martens et al., 2005a). Mobilization of LTR transposons is associated with 10% of all spontaneous mutations in mice (Waterston et al., 2002). De-repression of LTRs may impair genome stability through insertional mutagenesis, recombination events that cause translocations and other rearrangements, de-regulation of genes in the host genome mediated by LTR promoter activity, and antisense effects if transcription extends into exon sequence downstream of the transposon (Yoder, Walsh, & Bestor, 1997). Likewise, the tight regulation of pro-inflammatory cytokines is important to ensure an appropriate response of the immune system to external challenges such as infection (Dhar & Ogra, 1985). An unregulated continued expression of pro-inflammatory cytokines is associated with diseases such as rheumatoid arthritis (Choy, 2012) and inflammatory bowel disease (MacDonald, Biancheri, Sarra, & Monteleone, 2012), which affect a significant portion of the population in western societies (Centers, 2010, 2011).

We conclude that biotin and folate synergize in the repression of LTRs, and that these interactions are probably mediated by HLCS-dependent epigenetic mechanisms. In contrast, synergies among biotin, folate, and HLCS in the regulation of cytokines need to be interpreted in the context of transcription factor signaling. We are currently creating a

conditional HLCS knockout mouse model, so that future studies of epigenetic synergies can be conducted in a whole animal model at a mechanistic level.

## **Materials and Methods**

### ***Cell cultures***

Human T lymphoma Jurkat cells and monocytic myeloid U937 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in biotin- and folate defined media. Culture media were prepared using customized RPMI-1640 (Hyclone, Ogden, UT, USA), which was free of biotin, folate, and the methyl donor L-methionine. RPMI-1640 was mixed with 10% of biotin-depleted fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) prepared as described previously and antibiotics (Manthey et al., 2002). Chemically pure L-methionine was added back to produce a concentration of 10  $\mu\text{mol/L}$  methionine representing the levels in normal human plasma (Shinohara, Hasegawa, Tagoku, & Hashimoto, 2001). The endogenous levels of folate (22.7 nmol/L) and methionine (2.7  $\mu\text{mol/L}$ ) in FBS were quantified in the Physicians' Laboratories (Omaha, NE, USA) and the Metabolomics core facility at the University of Nebraska-Lincoln, respectively, and taken into account when adjusting nutrient levels in culture media. Biotin was added to media to produce the following concentrations (nmol/L): 0.025 (deficient), 0.25 (sufficient) and 10 (supplemented) (Mock, Lankford, & Mock, 1995; Zempleni, Helm, & Mock, 2001). Folate levels were adjusted to 4.9, 14 and 32 nmol/L, representing deficient, physiological and supplemented serum levels, respectively, in women living in regions without mandatory

folate fortification (George et al., 2002). Cells were cultured in specified defined medium for consecutive 14 days, and were re-suspended in fresh medium every 48 hrs.

The secretion of cytokines was induced by treatment with 50 µg/L phorbol 12-myristate 13-acetate (PMA) and 2 mg/L phytohemagglutinin (PHA) (Manthey et al., 2002). At timed intervals (see Results), aliquots were collected for analysis of mRNA by quantitative real-time PCR (qRT-PCR).

In select experiments NF-κB signaling was inhibited by treatment with curcumin (diferuloylmethane), which is an inhibitor of the IκB alpha/beta kinase (Meng et al., 2013; Singh & Aggarwal, 1995). Briefly, cells were treated 5 µmol/L curcumin (final concentration; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr before addition of PMA and PHA to culture media.

#### ***Determination of biotin and folate status***

Biotinylated carboxylases are well-established markers for biotin status (Zempleni, Wijeratne, & Kuroishi, 2012) and were assessed by streptavidin gel electrophoresis as described previously (Pestinger, Wijeratne, Rodriguez-Melendez, & Zempleni, 2011). The ratio of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), two metabolites of methyl donors, represented the methylation index (Stabler & Allen, 2004) and the intracellular concentrations of SAM and SAH were measured by reversed-phase high-performance liquid chromatography (HPLC) and a photodiode array detector.

#### ***Quantitative real-time PCR***

qRT-PCR analysis of mRNA coding for LTRs and cytokines was conducted as described previously (Pestinger et al.). The following primers were used. LTR R/U5 transcripts: 5'-GCGGGCAGCAATACTGCTTTGTAA-3' (forward) and 5'-ACCAGCGTTCAGCATATGGAGGAT-3' (reverse); TNF- $\alpha$ : 5'-ACTTTGGAGTGATCGGCC-3' (forward) and 5'-GCTTGAGGGTTTGCTACAAC-3' (reverse); IL-6: 5'-CCACTCACCTCTTCAGAACG-3' (forward) and 5'-CATCTTTGGAAGGTTTCAGGTTG-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TCCACTGGCGTCTTCACC-3' (forward) and 5'-GGCAGAGATGATGACCCTTT-3' (reverse). GAPDH was used to normalize for amplification efficiency. Note that the transcript sequences are nearly identical in the 54 transcriptionally active LTRs in the human genome (Buzdin et al., 2006). Therefore, the LTR transcript abundance reported here represents the total of all transcriptionally active LTRs. Transcription in human can initiate in both the U5 region and the R/U5 boundary in LTRs. The epigenetic regulation is the same for both transcripts (Chew et al., 2008); we quantified R/U5 transcripts because they mark active retro-transcription and retro-transposition events (Buzdin et al., 2006; Kovalskaya, Buzdin, Gogvadze, Vinogradova, & Sverdlov, 2006).

### ***Statistical analysis***

Normality was tested by normal quantile plot (Thode, 2002), and homogeneity of variance was analyzed by F-test (Hand & Nagaraja, 2003). Data sets with a p-value < 0.05 in the F-test were log transformed before subsequent statistical analysis. Student's t-test was used when comparing two groups. When more than two groups were compared, one-way ANOVA and Fisher's least significant difference (LSD) posthoc test was used.

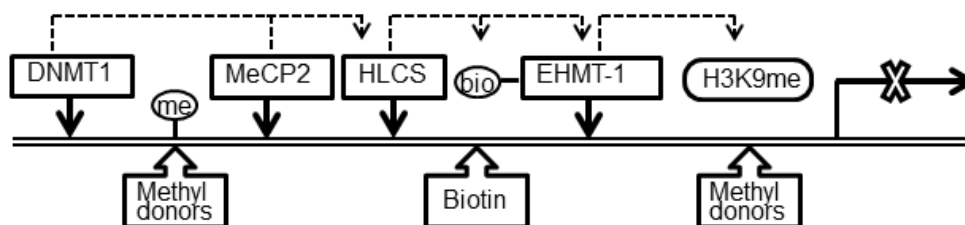
Means and standard deviations from independent samples are reported. Different letters indicated significant difference with  $p\text{-value} < 0.05$ . All calculations were based on three independent experimental repeats. Error bars represented standard deviations.

### **Acknowledgements**

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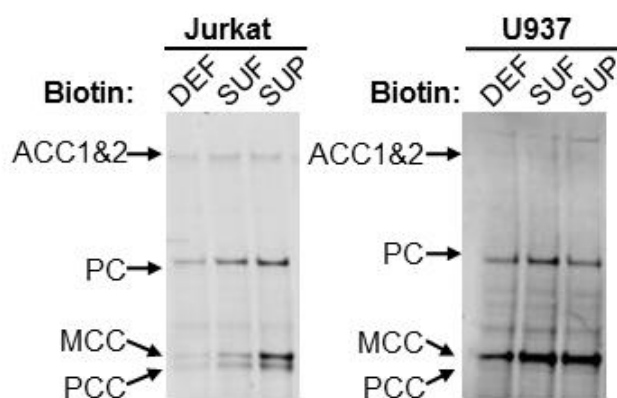
## Figures and legends



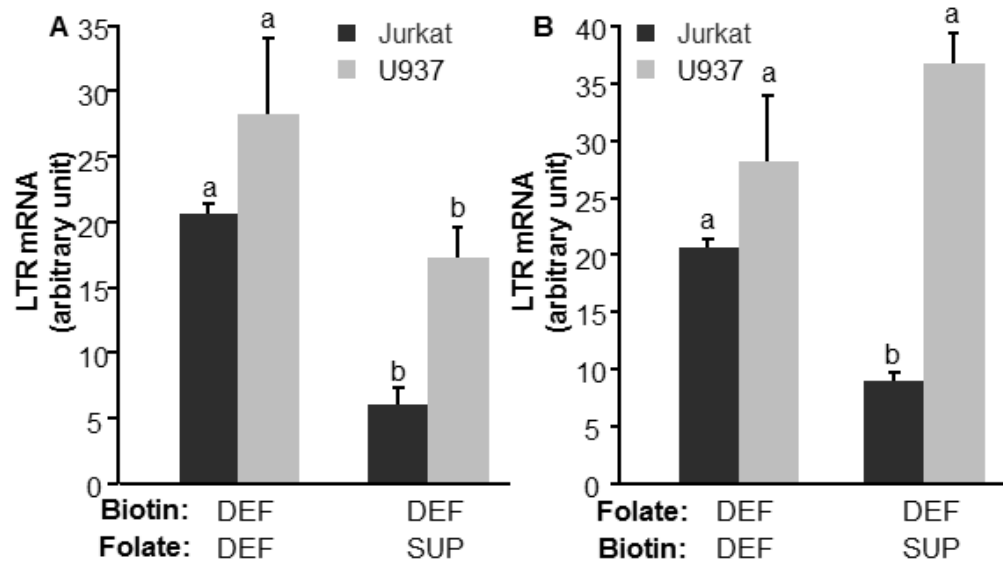
**Figure 1.** Synergies among biotin, folate, and chromatin proteins in gene repression.

Methyl donors may include folate, methione and perhaps choline and betaine.

Abbreviations: bio, biotin; me, methyl.

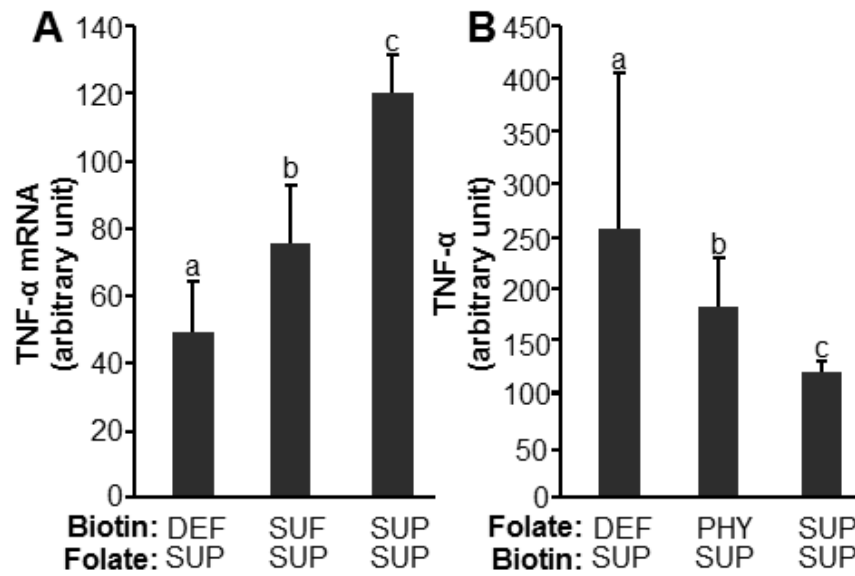


**Figure 2.** The abundance of biotinylated carboxylases depends on biotin in culture media in Jurkat and U937 cells. Abbreviations: ACC1&2, acetyl CoA carboxylases 1 and 2; PC, pyruvate carboxylase; MCC, methylcrotonyl CoA carboxylase; PCC, propionyl-CoA carboxylase.

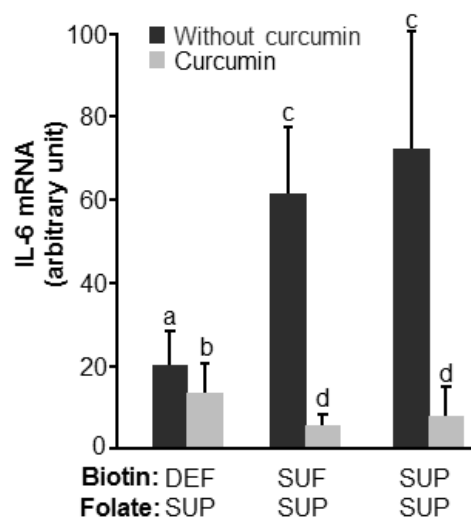


**Figure 3.** Folate and biotin can compensate for each other's deficiency in the repression of LTRs in Jurkat and U937 cells. (A) Biotin-deficient cells were supplemented with folate. (B) Folate-deficient cells were supplemented with biotin. <sup>a,b</sup>Columns not sharing the same letter are significantly different in the same cell line ( $p < 0.05$ ,  $n = 3$ ).

Abbreviations: DEF, deficient; SUP, supplemented.



**Figure 4.** Biotin and folate have opposing effects in the regulation of TNF- $\alpha$  transcription in U937 cells. (A) Folate-supplemented cells were cultured in biotin-defined medium and stimulated with PMA/PHA. (B) Biotin-supplemented cells were culture in folate-defined medium and stimulated with PMA/PHA. <sup>a,b,c</sup>Columns not sharing the same letters are significantly different in the same dataset ( $p < 0.05$ ,  $n = 3$ ). Abbreviations: DEF, deficient; SUF, sufficient; SUP, supplemented.



**Figure 5.** Curcumin abrogates the biotin-dependent expression of IL-6 to in U937 cells.

<sup>a,b,c,d</sup>Columns not sharing the same letters are significantly different ( $p < 0.05$ ,  $n = 3$ ).

Abbreviations: DEF, deficient; SUF, sufficient; SUP, supplemented.

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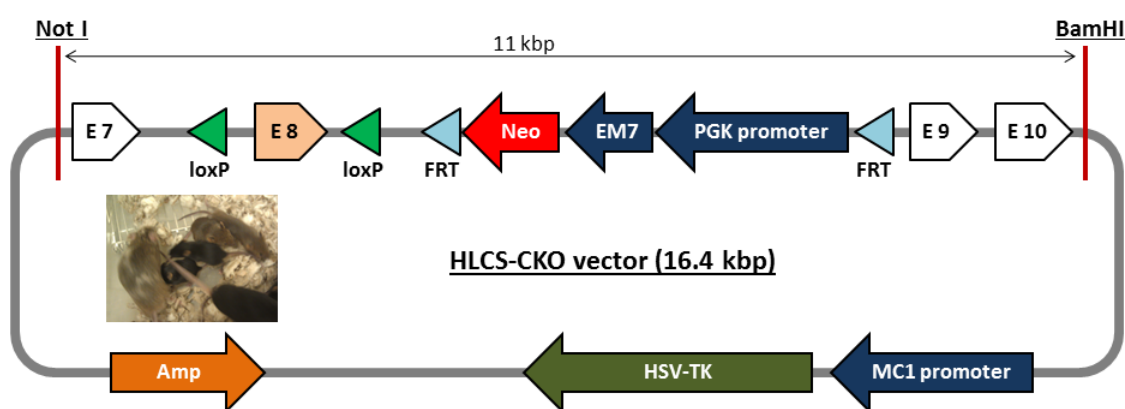
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## OUTLOOKS

Even though the HLCS mediated epigenetic regulation and protein has been well-characterized in cell cultures, creating a conditional knockout HLCS (CKO) mouse model will be of great interest to validate the cell-culture derived results and perform animal studies otherwise cannot be achieved *in vitro* or *ex vivo*. We are in the process of creating HLCS -CKO knock-out mice using the following protocol:

(i) Construct the HLCS-CKO vector composed of a vector backbone, a loxP flanked Exon (E) 8 of HLCS gene, an FRT flanked neomycin cassette, and two homology arms with sequences homologous to the genomic sequences flanking E8. The backbone vector illustrated in Figure 1 is PL253 (<http://recombineering.ncifcrf.gov/Plasmid.asp>); the backbone vector donating the neomycin cassette is PL451 (<http://recombineering.ncifcrf.gov/Plasmid.asp>).



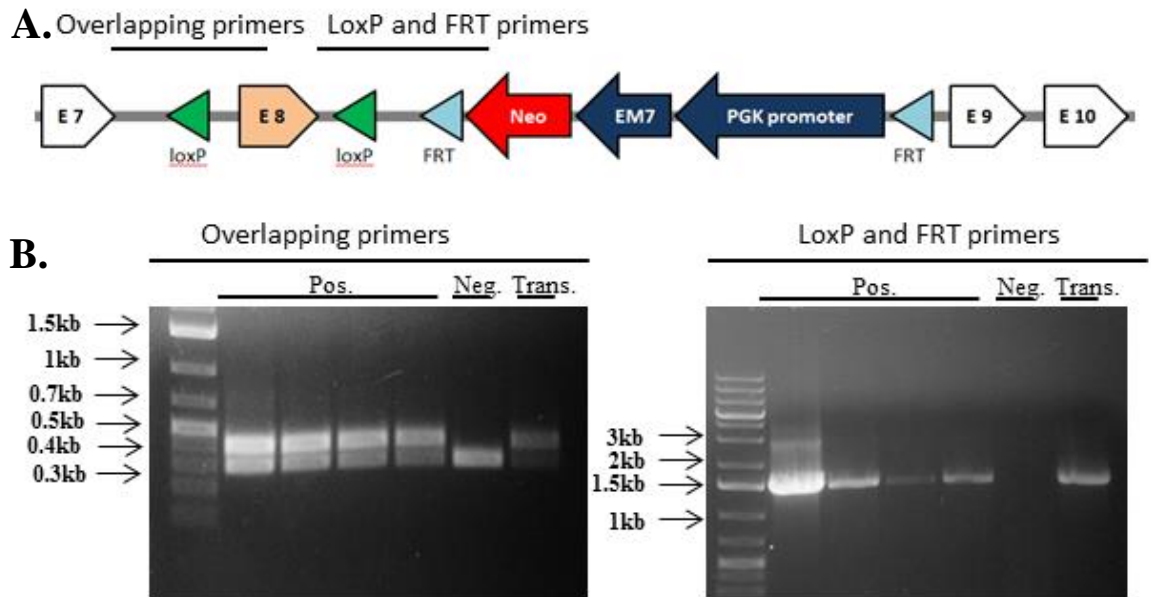
**Figure 1. Creation of HLCS conditional knock out (CKO) mouse.** The plasmid was created to add a loxP site flanking each side of the E8 sequence of HLCS. The neomycin cassette is flanked by two FRT sequences. The homology arms flanking the left loxP site

and the right FRT site facilitates chromosomal recombination for substitute the wild-type E8 sequence with the loxP flanked E8 followed by a neomycin cassette. The insert depicts the offspring pups (color in agouti or black) from the chimera breeder mice.

(ii) Linearize the HLCS-CKO construct and introduce into the A129 mouse embryonic stem (ES) cells through electroporation. Select positive clones with successful homologous recombination using the positive (G418 resistance conferred by neomycin cassette) and negative selection (Ganciclovir resistance due to loss of HSV-TK sequence as denoted in Figure 1) markers provided by the vector.

(iii) Inject the positive ES cells into the blastocysts of C57BL/6J mice for breeding of chimera mice.

(iv) Cross the chimera offsprings with C57BL/6J mice to confirm germ line transmission (Figure 2).



**Figure 2. Genotype to confirm the germline transmission of modified HLCS**

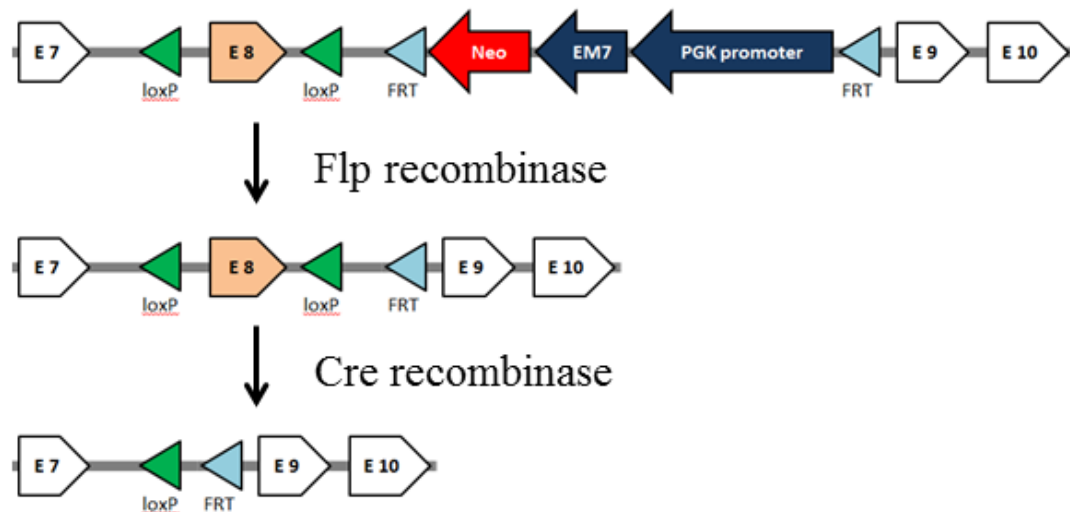
**sequence.** (A) Two pairs of primers were designed. The overlapping primers amplify a fragment overlapping the upstream loxP site, thereby creating amplicon of two sizes, one from the wild-type genomic DNA (300 bp), the other from the transgenic copy (427). The negative control is the genomic DNA prepared from the surrogate breeder A129 strain. The loxP and FRT primers amplify a fragment spanning from the middle of the flanked E8 sequence to the neomycin sequence, thereby creating an amplicon (1485 bp) only from the transgenic copy of genomic DNA. (B) DNA electrophoresis was performed to visualize the identified transgenic mice. (Pos.= positive control; Neg.= negative control; Trans.= transgenic mice)

(v) **(Current stage of the project)** CKO in the C57BL/6J background will be created by backcrossing with C57BL-6J breeders for ten generations, and genotyping will be conducted in each generation (Fig. 2).

(vi) The neomycin cassette will be removed by crossing transgenic mice with FLP transgenic mice for two generations. The FLP transgenic mice express Flp-recombinase, which will remove the neomycin cassette, leaving one FRT site (**Figure 3**).

(vii) Homozygous offspring will be created by inbreeding the transgenic mice for three generations.

(viii) The derived transgenic mice will be crossed with the Cre transgenic mice in order to remove the loxP flanked E8, leaving a loxP site, thereby creating the HLCS-CKO mice (**Figure 3**).



**Figure 3. Removal of the neomycin cassette and loxP flanked E8 by FLP recombinate and Cre recombinate, respectively.**

With the establishment of the homozygous transgenic strain, we would be able to knock out HLCS gene in desired tissues by crossing the homozygous transgenic mice derived from step (vii) with a Cre transgenic mice expressing Cre recombinase in the desired tissue. For achieving inducible knockout, a Cre transgenic mice with inducible elements in their genome could be used, where the expression of Cre recombinase is controlled by inducing reagents. As a powerful tool, the HLCS-CKO mice will enable us to investigate the function of HLCS in different tissues, as well as study the health impacts of tissue-specific HLCS knockout on the whole body.

## APPENDIX

### --- Raw data for each chapter

**Table 1.(Chapter I) The effects of HLCS on LTR repression dependent on DNA methylation.**

Panel A		RT-PCR Ct values normalized by histone H3 control and input				
Cell-line	Repeat	1	2	3	4	5
FLAG/Myc-HLCS	LTR15	1.95	1.02	1.52	2.77	1.02
Control	LTR15	0.61	0.62	0.51	0.62	0.32
FLAG/Myc-HLCS	Chr.1 alpha sat.	0.17	0.09	0.13	0.12	0.07
Control	Chr.1 alpha sat.	0.15	0.16	0.05	0.12	0.03
FLAG/Myc-HLCS	Chr.4 alpha sat.	0.19	0.11	0.14	0.19	0.07
Control	Chr.4 alpha sat.	0.14	0.14	0.07	0.18	0.03
FLAG/Myc-HLCS	GAPDH	0.66	0.33	0.66	0.64	0.41
Control	GAPDH	0.22	0.35	0.26	0.39	0.1
FLAG/Myc-HLCS	Apobec1	1.38	0.72	1.42	1.15	0.99
Control	Apobec1	0.27	0.43	0.4	0.43	0.09

Panel B		RT-PCR Ct values normalized by GAPDH control and untransfected control		
Treatment	Repeat	1	2	3
5-Azacytidine	U5	1.401	1.249	1.332
Control	U5	0.133	0.135	0.122
5-Azacytidine	R-U5	1.193	1.045	1.115
Control	R-U5	0.152	0.135	0.144
5-Azacytidine	Chr1alpha	1.712	1.26	2.429
Control	Chr1alpha	0.121	0.143	0.121
5-Azacytidine	Chr4alpha	1.969	0.935	1.525
Control	Chr4alpha	0.146	0.163	0.123

**Table 2. (Chapter II) HLCS overexpression does not cause an increase in HSP72 expression in HEK293 cells.**

Panel A		Band densitometry values normalized by GAPDH control		
Cell-line	Repeat	1	2	3
FLAG/Myc- HLCS		10.03	5.93	9.89
Control		10.49	8.5	6.11

Panel B		RT-PCR Ct values normalized by GAPDH control		
Cell-line	Repeat	1	2	3
FLAG/Myc- HLCS		1.03	0.99	0.99
Control		1.57	1.57	1.59

**Table 3. (Chapter II) Biotinylation status of HSP72 and its mutants.**

		Band densitometry values normalized by blue staining		
Proteins	Repeat	1	2	3
HSP72		0.822	0.745	0.978
HSP72 <sub>K112,128R</sub>		0.863	0.559	0.850
HSP72 <sub>K348,361,415R</sub>		0.761	0.689	1.050
HSP72 <sub>K112-415R</sub>		0.546	0.750	1.203

**Table 4. (Chapter II) Biotinylation of wild-type and mutant eHSP72 causes an increase in RANTES transcription in HEK293 cells.**

Panel A		RT-PCR Ct values normalized by GAPDH control		
Treatment	Repeat	1	2	3
HSP72-0.1 $\mu$ M		112.01	26.28	19.64
HSP72-0.5 $\mu$ M		110.91	132.70	81.32
HSP72-1.0 $\mu$ M		134.43	264.68	181.02
HSP72-ctl-0.5 $\mu$ M		25.92	127.04	65.39
HLCS		84.06	154.22	241.01

Panel B		RT-PCR Ct values normalized by GAPDH control		
Treatment	Repeat	1	2	3
HSP72 <sub>K112-415R</sub> -0.1 $\mu$ M		166.02	57.46	251.31
HSP72 <sub>K112-415R</sub> -0.5 $\mu$ M		240.02	168.91	100.66
HSP72 <sub>K112-415R</sub> -1.0 $\mu$ M		320.55	291.94	201.24
HSP72 <sub>K112-415R</sub> -ctl-0.5 $\mu$ M		78.73	22.38	91.03



**Table 5. (Chapter III) Folate and biotin can compensate for each other's deficiency in the repression of LTRs in Jurkat and U937 cells.**

				RT-PCR Ct values normalized by GAPDH control		
Cell- line	Biotin	Folate	Repeat	1	2	3
Jurkat	Deficient	Deficient		21.61	20.16	20.16
	Deficient	Supplemented		8.98	9.62	8.38
	Supplemented	Deficient		5.4	7.64	5.04
U937	Deficient	Deficient		22.11	29.18	33.51
	Deficient	Supplemented		34.3	36.76	39.4
	Supplemented	Deficient		14.59	19.25	17.96

**Table 6. (Chapter III) Biotin and folate have opposing effects in the regulation of TNF- $\alpha$  transcription in U937 cells.**

Panel A				RT-PCR Ct values normalized by GAPDH control		
Cell- line	Biotin	Folate	Repeat	1	2	3
U937	Deficient	Supplemented		64	50.5	33.1
	Sufficient			79	56.3	90.7
	Supplemented			111.5	115.6	132.8

Panel B				RT-PCR Ct values normalized by GAPDH control		
Cell- line	Biotin	Folate	Repeat	1	2	3
U937	Supplemented	Deficient		265	401.7	101.4
		Physiological		181.13	230.7	137.2
		Supplemented		111.5	115.6	132.8

**Table 7. (Chapter III) Curcumin abrogates the biotin-dependent expression of IL-6 to in U937 cells.**

					RT-PCR Ct values normalized by GAPDH control		
Cell- line	Treatment	Biotin	Folate	Repeat	1	2	3
U937	Control	Deficient	Supplem- ented		64	50.5	33.1
	Curcumin				27.9	40.8	42.6
	Control	Sufficient			79	56.3	90.7
	Curcumin				20.4	32	39.5
	Control	Supplemen- ted			111.5	115.6	132.8
	Curcumin				59.9	50.4	79