HNF4α regulates sulfur amino acid metabolism and confers sensitivity to methionine restriction in liver cancer

Qing Xu¹, Yuanyuan Li², Xia Gao³, Kai Kang², Jason G. Williams⁴, Lingfeng Tong⁵, Juan Liu³, Ming Ji¹, Leesa J. Deterding⁴, Xuemei Tong⁵, Jason W. Locasale³, Leping Li², Igor Shats¹✉ & Xiaoling Li¹✉

Methionine restriction, a dietary regimen that protects against metabolic diseases and aging, represses cancer growth and improves cancer therapy. However, the response of different cancer cells to this nutritional manipulation is highly variable, and the molecular determinants of this heterogeneity remain poorly understood. Here we report that hepatocyte nuclear factor 4α (HNF4α) dictates the sensitivity of liver cancer to methionine restriction. We show that hepatic sulfur amino acid (SAA) metabolism is under transcriptional control of HNF4α. Knocking down HNF4α or SAA enzymes in HNF4α-positive epithelial liver cancer lines impairs SAA metabolism, increases resistance to methionine restriction or sorafenib, promotes epithelial-mesenchymal transition, and induces cell migration. Conversely, genetic or metabolic restoration of the transsulfuration pathway in SAA metabolism significantly alleviates the outcomes induced by HNF4α deficiency in liver cancer cells. Our study identifies HNF4α as a regulator of hepatic SAA metabolism that regulates the sensitivity of liver cancer to methionine restriction.
Increasing evidence indicates that availability of dietary nutrients, including amino acids and fatty acids, has profound impacts on tumor metabolism, growth, and therapeutic outcomes\(^{1-7}\). For example, restriction of dietary methionine, a sulfur-containing essential amino acid enriched in animal products, has been shown to suppress proliferation and progression of a variety of tumors, including colon, prostate, and breast cancer\(^{8-13}\). This dietary intervention can impact metabolic flux in one-carbon metabolism, inhibit tumor growth, and sensitize tumors to chemotherapy and radiotherapy in certain human cancer cells in a tumor-cell autonomous manner\(^{14}\). However, different human cancer cells have varying degrees of methionine dependence\(^{15}\), and the underlying molecular determinants of this heterogeneity are still unclear.

Systemic methionine metabolism, more broadly sulfur amino acid (SAA) metabolism, is thought to mainly take place in the liver, a central metabolic organ that metabolizes half of all dietary methionine\(^{16}\). Once transported into the liver, dietary methionine is converted to S-Adenosyl methionine (SAM) primarily through the action of MAT1A, a liver-specific methionine adenosyltransferase. S-adenosylhomocysteine (SAH) generated from SAM via the transmethylation reaction is then hydrolyzed to form homocysteine (Hcy) and remethylated back to methionine through betaine-homocysteine S-methyltransferase (BHMT). Hcy can also enter the transsulfuration pathway to form cystathionine (Ctt) and cysteine (Cys) through cystathionine-beta-synthase (CBS) and cystathionine gamma-lyase (CTH). CBS and CTH also produce hydrogen sulfide (H\(_2\)S)\(^{17,18}\), a gasotransmitter whose metabolism and biological functions in cancer, aging, and age-associated diseases are still being unraveled\(^{19,20}\). Cys can be further used to produce antioxidant glutathione (GSH), or generate taurine through cysteine dioxygenase (CDO1) (Fig. 1a). Therefore, aside from being indispensable for protein synthesis, methionine plays important roles in tissue and systemic sulfur metabolism, antioxidant defense, epigenetic regulation, and signaling\(^{21}\).

Interestingly, many enzymes involved in SAA metabolism, including MAT1A, GNMT, BHMT, and CBS, are reported to be downregulated in human liver tumors, particularly hepatocellular carcinoma (HCC), the most common and deadly form of liver cancer. Reduced expression of these enzymes is also associated with more aggressive tumors and poor prognosis\(^{22-25}\). Liver cancer is the fourth leading cause of global cancer death and its incidence is rapidly growing in the US\(^{26}\). Risks factors for liver cancer include chronic hepatitis B/C infection, cirrhosis linked to alcohol abuse, diabetes, and obesity\(^{27}\). Liver cancer is commonly diagnosed at an advanced stage when tumors are already resistant to conventional chemotherapy or radiotherapy. Development of therapeutic agents for liver cancer has been challenging. For decades, sorafenib, a multiple kinase inhibitor, has been the sole approved first-line treatment for advanced HCC despite the fact that it offers only three-month survival benefit over placebo\(^{28}\). Therefore, there is an urgent need to develop therapeutic strategies for effective treatment of liver cancer.

The dysregulated SAA metabolism in liver cancer suggests that manipulation of this metabolic pathway through dietary methionine intervention could serve as a promising treatment approach. However, currently the molecular mechanisms underlying aberrant SAA metabolism in liver tumors are poorly understood. Whether methionine restriction can lower the risk of liver cancer and/or increase the sensitivity of liver tumors to available chemotherapy also remains unexplored.

In this study, we establish a link between hepatocyte nuclear factor 4a (HNF4a) and SAA metabolism in liver cancer. HNF4a, the master regulator of hepatic genes, is a member of nuclear receptor family of transcriptional factors that is critical for maintenance of hepatocyte identity and specification of hepatic functions\(^{29,30}\). Downregulated in HCC\(^{31-33}\), HNF4a is considered as a tumor suppressor that represses the development of HCC and inhibit epithelial-mesenchymal transition (EMT), a process that promotes cancer progression and metastasis\(^{34-36}\). Although hepatic HNF4a is known to regulate genes essential for gluconeogenesis, bile acid synthesis, cholesterol and lipid metabolism\(^{38}\), whether and how HNF4a modulates SAA metabolism are not known. Through bioinformatic analyses, metabolomics, and molecular, cellular and in vivo characterizations, we demonstrate that HNF4a plays a central role in controlling hepatic SAA metabolism and dictating sensitivity to methionine restriction in liver cancer both in vivo and in vitro.

### Results

**HNF4a and SAA enzymes are positively correlated in liver cancer.** It has long been noticed that SAA metabolism is one of the major dysregulated metabolic pathways in liver tumors\(^{22,37}\). Our analysis of The Cancer Genome Atlas (TCGA) human Liver Hepatocellular Carcinoma (LIHC) dataset confirmed that key genes involved in SAA metabolism, including MAT1A, BHMT, CBS, CTH, and CDO1, are suppressed in HCC compared to normal liver (Supplementary Fig. 1a). Moreover, HCC patients with low tumor expression of these key SAA enzymes had significantly worse prognosis than those with high expression of these genes (Supplementary Fig. 1b). These data suggest that SAA metabolism is frequently disrupted in liver tumors and this disruption is correlated with patient prognosis.

Notably, HNF4a expression was also progressively reduced in tumors of the HCC patients with the advancement of the tumor stage in the TCGA LIHC dataset (Supplementary Fig. 1c), and lower expression of HNF4a was associated with shorter patient survival (Supplementary Fig. 1d). As in many other cancers, HCC progression is characterized by EMT with loss of epithelial markers (e.g., E-cadherin, ZO-1, Cytokeratin) and gain of mesenchymal markers (e.g., Vimentin, TWIST1, ZEB1, CD44, SNAI1, SNAI2)\(^{31,34}\). HNF4a has been reported to inhibit hepatocarcinogenesis by suppression of EMT\(^{31,34-36}\). To understand the molecular mechanisms underlying dysregulation of SAA metabolism during liver cancer development, we first investigated the relationship between the expression levels of five well-established hepatic HNF4a target genes, five general mesenchymal markers, and eight SAA metabolic enzymes in the TCGA LIHC dataset of 373 HCC patients. As shown in Fig. 1b, the mRNA levels of seven out of eight analyzed SAA metabolic enzymes (orange) were clustered together with those of HNF4a and other liver-specific functional genes (red), whereas mesenchymal marker genes (blue) formed a separate cluster. The only exception among eight analyzed SAA enzymes is MAT2A, a ubiquitously expressed methionine adenosyltransferase whose expression is primarily controlled by post-transcriptional RNA methylation that induces efficient splicing and mRNA stabilization in response to methionine starvation\(^{39,40}\). Consistent with their clustering patterns, the expression of five key SAA metabolic genes, MAT1A, BHMT, CBS, CTH, and CDO1, was positively correlated with that of HNF4a in both non-viral and viral HCC patients (Fig. 1c, Supplementary Fig. 2a, b). In contrast, their expression was negatively correlated with that of TWIST1, a mesenchymal marker, in these HCC patients (Fig. 1d). Notably, the negative correlation between key SAA enzymes and TWIST1 was in a comparable range as that between HNF4a and TWIST1 (Fig. 1d). These observations raise the possibility that the expression of key SAA metabolic enzymes is under control of HNF4a in human liver tumors.

To further test this possibility, we performed a cluster analysis of RNA-seq data from 25 liver cancer cell lines derived from...
human liver tumors in the Broad Institute Cancer Cell Line Encyclopedia (CCLE) database. Based on their mRNA expression levels of liver-specific markers, including HNF4α and its direct target HNF1α, and mesenchymal markers, these liver cancer cell lines can be clustered into two groups (13 epithelial vs. 12 mesenchymal) (Fig. 1e). In line with our observations in HCC patients (Fig. 1b), three key SAA metabolic enzymes, MAT1A, CBS and CTH, were significantly enriched in the epithelial group together with HNF4a and liver-specific markers (Fig. 1e, f). Additional cluster analyses using RNA-seq data from 81 human liver cancer cell lines in LIMORE database41 confirmed the significant positive correlation of MAT1A and CBS with HNF4a.
and liver-specific markers (Supplementary Fig. 2c, d). Further immuno-blotting analysis indicated that three epithelial cell lines Huh7, Hep3B, and HepG2 that express high levels of HNF4α also displayed high levels of many SAA enzymes compared to two mesenchymal cell lines SNU449 and SNU475 that are negative for HNF4α (Fig. 1g). Therefore, the expression of key SAA metabolic enzymes is positively correlated with that of HNF4α in both liver cancer patients and liver cancer cell lines.

Importantly, the positive correlation between HNF4α and SAA metabolic enzymes had functional consequences in liver cancer cells. An unbiased LC-MS-based metabolomic analysis of the small molecule metabolites in HNF4α-positive HepG2 cells and HNF4α-negative SNU449 cells, two widely used cell lines in the research community of liver cancer, revealed that SNU449 cells are significantly different from HepG2 cells in the abundance of 174 metabolites (Supplementary Table 1, p < 0.05, [FC] > 1.5). Pathway analysis demonstrated that these metabolites were enriched with metabolites from SAA metabolic pathways, particularly cysteine and methionine metabolism (Fig. 2a). Further targeted LC-MS and biochemical assays confirmed that SNU449 cells accumulated methionine and cysteine, but were depleted of SAM, SAH, Ctt, hypotaurine, GSH, and H2S (Fig. 2b, c). All of these metabolites were regulated by key SAA enzymes that were reduced in SNU449 cells compared to HepG2 cells (Fig. 2d). Taken together, our observations indicate that HNF4α, SAA metabolism genes, and SAA metabolism are positively linked in human liver tumors and cell lines, and that SAA metabolism is altered in HNF4α-negative mesenchymal liver cancer cell lines. Since mesenchymal lines typically originate from invasive and metastatic tumors, our results suggest that regulation of SAA metabolism genes and the consequent rewiring of SAA metabolism may represent hallmarks of liver cancer progression.

**HFN4α-negative liver cancer lines are resistant to MCR.** To evaluate the possible functional impacts of altered SAA metabolism in mesenchymal liver cancer cell lines, we compared the responses of two mesenchymal liver cancer lines, SNU449 and SNU475, to methionine restriction with those of three epithelial cell lines Huh7, Hep3B, and HepG2.

Our LC-MS analysis indicated that our complete DMEM cell culture medium (CM, DMEM medium plus 10% regular Fetal Bovine Serum (FBS)) contains about 130 μM methionine and 160 μM cystine (Cys-Cys), the oxidized dimer form of cysteine. The commonly used dietary methionine restriction regimen has been shown to extend life span, delay aging, prevent metabolic diseases, reduce cancer growth, and sensitize cancer cells to chemotherapy and radiation in mice. Since this regimen restricts methionine in the absence of cystine, we restricted both methionine and cystine in our cell culture DMEM medium by combining methionine/cystine-free DMEM with 10% dialyzed FBS, which resulted in a restricted DMEM medium (MCR) containing 0.12 μM methionine and undetectable levels of cystine. Further LC-MS analysis revealed that both media are able to maintain or even increase their respective methionine/cystine concentrations during a 24-h experimental timeframe, as the concentrations of methionine and cystine were 139 μM and 200 μM, respectively, in CM, and 0.15 μM and 0.11 μM in MCR after 24-h cell culture. This observation is consistent with the notion that small peptides or single amino acids can be derived from proteolysis of large serum proteins or from proteolysis of cell components via autophagy or similar processes during cell culture. Interestingly, cysteine was not detectable in these media. In contrast, the intracellular methionine and cysteine levels were dramatically reduced to ~0.3% and undetectable, respectively, after 1 h of methionine/cystine restriction in both HepG2 and SNU449 cells (Supplementary Fig. 3), methionine/cystine restriction also quickly reduced intracellular glutathione levels (Supplementary Fig. 3).

Upon amino acid deprivation, it is established that uncharged transfer RNAs (tRNAs) triggers an adaptive integrated stress response, termed amino acid response (AAR), which activates the expression of stress-responsive transcription factor ATF4. As expected, in three epithelial cell lines, restriction of both methionine and cystine for 6 h significantly increased the expression of ATF4 and its two target genes involved in the regulation of cell stress and apoptosis, CHOP and Asparagine Synthetase (CHS), as well as Met RNA synthetase (MetT), which encodes the enzyme that synthesizes Met from Cys in mammalian cells.

**HFN4α-positive liver cancer cell lines are sensitive to MCR.** To investigate the sensitivity of HNF4α-positive liver cancer cell lines to MCR, we performed long-term MCR experiments on HepG2 and SNU449 cells. We observed that both cell lines were significantly more sensitive to MCR than their mesenchymal counterparts. These observations are consistent with the notion that mesenchymal cells with HNF4α expression in liver cancers are resistant to MCR, which suggests that mesenchymal cells are more resistant to cell death caused by a 24-h methionine/cystine restriction compared to epithelial Huh7, Hep3B, and HepG2 cells. Intriguingly, this mesenchymal resistance was specific to the restriction of methionine/cystine, and not to the depletion of other non-SAA amino acids including leucine (essential), threonine (essential), or glutamine (conditionally essential). This observation suggests that differential responses...
SNU449 cells have altered SAA metabolism compared to HepG2 cells. Indicated metabolites were quantified by LC-MS (n = 3 replicates per group). The log2 ratios of the relative abundance of metabolites and enzymes in indicated pathways in SNU449/HepG2 cells were presented by color (y axis, enrichment p values) and the pathway topology analysis (x axis, pathway impact values, indicative of the centrality and enrichment of a pathway) in the Pathway Analysis module of MetaboAnalyst 4.0 (Supplementary Fig. 4e and Fig. 4e). These results confirmed that HNF4α was significantly enriched on the HNF4α binding sites of these SAA metabolism genes in HepG2 but not HNF4α-negative SNU449 cells by Chromatin Immunoprecipitation (ChIP)-qPCR assay (Fig. 4a). Consistently, knocking down HNF4α by siRNAs significantly reduced the mRNA and/or protein levels of these key SAA metabolic enzymes in HNF4α-negative HepG2, Huh7, and HepB3 cells (Fig. 4b, c and Supplementary Fig. 4d), and normal human hepatocytes (Fig. 4d). Conversely, overexpression of HNF4α in HNF4α-negative SNU449 cells strongly induced the luciferase activities from reporters containing the consensus (WT) HNF4α binding sites of MAT1A, BHMT, or CBS promoters, but this induction was significantly reduced when the binding sites were mutated (Mut) (Supplementary Fig. 4e and Fig. 4e). These results confirmed that HNF4α regulates the transcription of SAA metabolic enzymes.

of epithelial and mesenchymal liver cancer cells to methionine/cystine restriction are not simply because methionine is essential and indispensable for protein synthesis.

Intracellular cysteine depletion can also be induced by sorafenib, the sole approved first-line drug for advanced HCC that inhibits the cystine-glutamate transporter (system x_c⁻) in addition to multiple kinases.47,48 Notably, compared to epithelial liver cancer lines, HNF4α-negative mesenchymal liver cancer lines also showed increased resistance to cell death induced by sorafenib when cultured in complete medium, and methionine/cystine restriction failed to enhance the effect of sorafenib in these cells (Fig. 3e, SNU449 and SNU475). In contrast, HNF4α-positive epithelial liver cancer lines were sensitive to sorafenib treatment when cultured in complete medium, and this sensitivity was further augmented by lowering medium concentrations of methionine/cystine (Fig. 3e, Huh7, Hep3B, and HepG2). Collectively, our results suggest that the status of HNF4α and SAA metabolism may dictate the sensitivity of liver cancer cells to methionine/cystine restriction and sorafenib treatment.

HNF4α regulates the transcription of SAA metabolic enzymes.

To further understand how HNF4α regulates SAA metabolism and the resulting cellular sensitivity to methionine/cystine restriction and sorafenib, we tested whether SAA metabolic enzymes are direct transcription targets of HNF4α since the expression of HNF4α is strongly correlated with those of SAA metabolic genes in liver cancer (Fig. 1 and Supplementary Fig. 1).
HNF4α directly controls the transcription of these SAA metabolic enzymes in part through the identified consensus HNF4α binding sites on their promoters. Overexpression of HNF4α in SNU449 cells failed to rescue the expression of endogenous SAA metabolic enzymes (Fig. 4f, top), likely due to the reported epigenetic silencing of these genes in cancer.40–32 In support of this idea, co-treatment with a histone deacetylase inhibitor trichostatin A (TSA) enabled ectopic HNF4α to promote the expression of these endogenous SAA genes (Fig. 4f, bottom). Together, our data indicate that HNF4α controls SAA metabolism in liver cancer cells through transcriptional regulation of the expression of SAA enzymes.

HNF4α deficiency alters SAA metabolism and MCR resistance. To further test the importance of SAA metabolism in HNF4α-mediated metabolic program, we knocked down HNF4α in HepG2 cells and compared their metabolomic profiles with those from HepG2 cells transfected with a control siRNA. Notably, unbiased pathway analysis revealed that the two most significantly altered metabolic pathways in HNF4α-depleted HepG2 cells were two SAA metabolism pathways, taurine/hypotaurine metabolism and cysteine/methionine metabolism (Fig. 5a). Specifically, HNF4α-depleted HepG2 cells showed increased levels of methionine and cysteine but reduced Ctt, hypotaurine, taurine, and H₂S (Fig. 5b–d). This dysregulated SAA metabolism was strikingly similar to that
observed in mesenchymal SNU449 cells (Fig. 2). As HNF4α suppresses EMT\textsuperscript{31,34-36}, the shared metabolic changes between above two cell types suggest that defective SAA metabolism driven by HNF4α deficiency could be a feature of EMT, which is commonly associated with tumor metastasis and drug resistance\textsuperscript{53}.

To investigate the contribution of HNF4α-mediated SAA metabolism to metabolic remodeling during EMT, we compared the global metabolic profiles of epithelial HepG2 cells, HNF4α-depleted HepG2 cells, and mesenchymal SNU449 cells. As shown in Fig. 5c, loss of HNF4α in HepG2 cells significantly changed the
The production of H2S from indicated cells were analyzed over 24 h using the lead sulfa
depleted HepG2 cells and SNU449 cells. The 34 metabolites that were altered in the same
direction in SNU449 cell and siHNF4α
were analyzed by the Principal Component Analysis (PCA).

The log2 ratios of the relative abundance of metabolites and enzymes in the indicated pathways in siHNF4α
HepG2 cells shifted the global metabolic pro
le toward that of
SNU449 cells. All detectable metabolites in siNeg HepG2 cells (green), siHNF4α
HepG2 vs. siNeg HepG2 and siHNF4α
SNU449 cells at the PC1 axis compared to control HepG2 cells
(Fig. 5). Therefore, HNF4α deficiency in epithelial liver cancer cells results in a global metabolic shift towards mesenchymal liver cancer cells. HNF4α-depleted HepG2 cells also metabolically clustered together with SNU449 cells but not with control HepG2 in a cluster analysis of all significantly changed metabolites in HNF4α-depleted HepG2 cells (Supplementary Fig. 5 and Supplementary Table 2). Remarkably, metabolites involved in SAA metabolism were the most significantly enriched metabolites among the 34 metabolites that were significantly changed in the

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**Fig. 5** Altered SAA metabolism is a common feature of HNF4α defective and mesenchymal liver cancer cells. a Metabolite sets related to SAA metabolism are enriched in HNF4α-depleted HepG2 cells. The 65 metabolites significantly altered by HNF4α knockdown in HepG2 cells were analyzed in the Pathway Analysis module of Metabo Analyst 4.0 as described in Methods (n = 3 replicates per group, p < 0.05, |FC | > 1.5). b HNF4α depletion alters SAA metabolism. Indicated metabolites were quantified by LC-MS (n = 3 replicates per group). c HNF4α depletion reduces H2S production in HepG2 cells. The production of H2S from indicated cells were analyzed over 24 h using the lead sulfa assay as described in Methods. HepG2 cells with knockdown of CBS, a key H2S producing enzyme, were used as a negative control (n = 3 replicates per group). d SAA metabolic pathway in siNeg cells vs. siHNF4α
HepG2 cells. The log2 ratios of the relative abundance of metabolites and enzymes in the indicated pathways in siHNF4α/siNeg HepG2 cells are presented by a color scale (n = 3 replicates per group, all colored metabolites were significantly changed with p < 0.05). e HNF4α-depleted HepG2 cells and SNU449 cells have significantly overlapping metabolic profiles (hypergeometric p < 0.05). The indicated significantly altered metabolites between siNeg SNU449 vs. siNeg HepG2 and siHNF4α vs. siNeg HepG2 are visualized by a Venn-diagram (p < 0.05, |FC | > 1.5). f HNF4α depletion shifts HepG2 cells metabolically toward SNU449 cells. All detectable metabolites in siNeg HepG2 cells (green), siHNF4α HepG2 cells (red), and siNeg SNU449 cells (blue) were analyzed by the Principal Component Analysis (PCA). g SAA metabolites are enriched among the commonly altered 34 metabolites in HNF4α-depleted HepG2 cells and SNU449 cells. The 34 metabolites that were altered in the same direction in SNU449 cell and siHNF4α
HepG2 were analyzed in the Pathway Analysis module of Metabo Analyst 4.0 (n = 3 replicates per group, p < 0.05, |FC | > 1.5). The color of a circle is indicative of the level of enrichment significance, with yellow being low and red being high. The size of a circle is proportional to the pathway impact value of the pathway. For graphs in (b, c), values are expressed as mean ± s.e.m., two-tailed, unpaired Student’s t-test, *p < 0.05.
same direction in both HNF4α-depleted HepG2 cells and SNU449 cells (Fig. 5g and Supplementary Table 3), suggesting that impaired SAA metabolism is one of the major shared metabolic characteristics of HNF4-depleted epithelial cells and HNF4-negative mesenchymal liver cancer cells.

In addition to similar metabolic defects in SAA metabolism, both HNF4α-depleted HepG2 cells and SNU449 cells had significantly increased intracellular levels of reactive oxygen species (ROS) (Supplementary Fig. 6a) compared to control HepG2 cells when cultured in the complete medium. Methionine/cystine restriction increased ROS levels in control HepG2 cells but not further in HNF4α-depleted HepG2 cells and SNU449 cells (Supplementary Fig. 6a and 6b, MCR vs. CM), suggesting that HNF4α-depleted cells with dysregulated SAA metabolism already experience elevated oxidative stress under normal condition and are nonresponsive to further cellular stress induced by methionine/cystine restriction. On the other hand, although both HNF4α-depleted HepG2 cells and SNU449 cells displayed reduced proliferation, as evident by a reduced fraction of cells in S-phase compared to control HepG2 cells (Supplementary Fig. 6c, siHNF4α vs. siNeg HepG2 cells, siNeg SNU449 vs. siNeg HepG2 cells), methionine/cystine restriction was able to further reduce the fraction of S-phase cells while increasing G1-phase cells in both HNF4α-depleted HepG2 cells and SNU449 cells (Supplementary Fig. 6c). Methionine/cystine restriction increased ROS levels in control HepG2 cells, with reduced intensities of puromycin-labeled peptides based on the SUNSET assay54 (Supplementary Fig. 6d), but methionine/cystine restriction suppressed protein synthesis in all these cells regardless of their HNF4α status (Supplementary Fig. 6d).

To directly test the role of HNF4α in SAA metabolism and cellular stress resistance, we investigated the impact of HNF4α deficiency on EMT and its associated resistance to methionine/cystine restriction and sorafenib. Consistent with previous reports31,34–36, knocking down HNF4α in epithelial HepG2 cells resulted in a decreased expression of epithelial markers E-cadherin (CDH1) and CPED1 yet an increased level of a number of mesenchymal markers55 (Fig. 6a), along with enhanced mesenchymal cellular morphology (Fig. 6b) and massively increased cell migration in a transwell assay (Fig. 6c). Knocking down HNF4α also reduced the expression of epithelial markers and/or increased the levels of mesenchymal markers in epithelial Huh7 cells and in normal human hepatocytes (Supplementary Fig. 7a). Notably, knocking down HNF4α in three epithelial liver cancer cell lines increased resistance to cell death induced by methionine/cystine restriction (reduced apoptosis in Fig. 6d, and increased surviving cell number in 6e and Supplementary Fig. 7b, siHNF4α vs. siNeg) or sorafenib treatment (Fig. 6f, g, and Supplementary Fig. 7b, siHNF4α SORafenib vs. siNeg SORafenib).

To further confirm above observations, we investigated whether HNF4α-depleted HepG2 cells also display enhanced resistance to methionine/cystine restriction in vivo. Since HNF4α is required for maintenance of hepatocyte identity29,30, we chose to use siRNA-treated HepG2 cells for an in vivo xenograft experiment based on a xenograft mouse model established to generate HNF4α-deficient tumors56. In this study, the authors demonstrated that siRNA-mediated knockdown of HNF4α initiates a microRNA-inflammatory feedback loop that continuously suppresses HNF4α expression and sustains a stable phenotype of tumorigenesis56. Specifically, we injected control (siNeg) and HNF4α knockdown (siHNF4α) HepG2 cells into nude mice. When the average tumor volume reached 200 mm³, we randomized these mice into two groups and fed them either a methionine-restricted diet containing 0.172% DL-methionine and no cystine (MCR) or a control diet containing 0.86% DL-methionine with no cystine (CTR). A similar MCR diet has been recently shown to reduce plasma methionine levels by 50% within two days and alter methionine metabolism in colorectal PDX tumors and liver tissues in mice34. We confirmed that the MCR diet that contains 0.172% DL-methionine and no cystine suppresses liver cancer growth in a diethylnitrosamine (DEN)/high-fat-diet (HFD)-induced liver cancer model in mice compared to the CTR diet (Supplementary Fig. 8). Xenografted tumors from siHNF4α HepG2 cells maintained partial HNF4α knockdown during our experiment timeframe (about 5 weeks, Supplementary Fig. 9a). Feeding mice with the MCR diet significantly inhibited the growth of xenografted control HepG2 tumors with enhanced tumor damage and death in vivo (Fig. 6h and Supplementary Fig. 9b, siNeg MCR vs. siNeg CTR), whereas knocking down HNF4α significantly blunted the inhibitory impact of the MCR diet on the growth and proliferation of HepG2 tumors (Fig. 6h, i and Supplementary Fig. 6d, siHNF4α MCR vs. siNeg MCR). Therefore, the status of HNF4α affects the sensitivity of liver tumors to methionine restriction both in vitro and in vivo.

Defective transsulfuration is partially responsible for EMT. To further assess the importance of SAA metabolism in HNF4α-mediated tumor suppression and stress sensitivity in liver cancer, we knocked down key SAA metabolic enzymes individually in epithelial HepG2 cells (Supplementary Fig. 10a) and analyzed whether deficiency of any of these enzymes mimics HNF4α deficiency-induced mesenchymal characteristics. Consistent with observations in Fig. 5, knocking down HNF4α in HepG2 cells led to significantly increased resistance to stress induced by methionine/cystine restriction or sorafenib, as indicated by the reduced activation of caspase (Fig. 7a, top panels, siHNF4α vs. siNeg), reduced induction of CHOP, a proapoptotic ATF4 target gene (Fig. 7a, bottom panels, siHNF4α vs. siNeg), and enhanced cell survival (Fig. 7b, siHNF4α vs. siNeg). Similar to HNF4α knockdown, knocking individual SAA enzymes led to various degrees of resistance to methionine/cystine restriction or sorafenib treatment (Fig. 7a, b), various degrees of morphological alterations (Supplementary Fig. 10b), and increased cell migration (Fig. 7c). Particularly, depletion of CBS or CDO1 increased stress resistance, and induced cell morphological changes and cell migration to a degree comparable to those induced by HNF4α knockdown (Fig. 7a, b, and Supplementary Fig. 10b). CBS is a key enzyme in the transsulfuration pathway mediating Ctt, H2S, or taurine production, and CDO1 is a critical enzyme in taurine synthesis, as well as maintenance of the hepatic intracellular free cysteine range. These observations suggest that a defective transsulfuration pathway with reduced production of Ctt, H2S, or taurine may be sufficient to recapitulate the outcomes induced by HNF4α deficiency in epithelial liver cancer cells. In support of this idea, incubation with a H2S donor NaSH partially prevented the resistance of HepG2 cells to methionine/cystine restriction upon HNF4α knockdown (Fig. 7d, middle). Supplementation of Ctt, NaSH, or taurine also significantly alleviated the resistance of HepG2 to the sorafenib treatment in response to HNF4α knockdown (Fig. 7d, right), and attenuated the cell migration induced by HNF4α deficiency (Fig. 7e). Interestingly, addition of cystine, a major SAA that directly participates in the transsulfuration reactions (Fig. 1a), partially rescued the methionine/cystine restriction-reduced cell survival in control HepG2 cells but not in HNF4α-depleted HepG2 nor HNF4α-negative SNU449 cells (Fig. 7f), suggesting that HNF4α-regulated cysteine metabolism significantly contributes to cellular sensitivity to MCR.
Further gene expression analyses revealed that at the transcriptional level, knocking down individual SAA enzymes has distinct impacts on EMT changes that are induced by HNF4α deficiency (Fig. 6a). As shown in Fig. 8a and Supplementary Fig. 10a, knocking down CBS in HepG2 cells primarily elevated the expression of a number of mesenchymal markers (e.g., COL3A1) that were also induced in HNF4α-depleted HepG2 cells, whereas knocking down other SAA enzymes modestly reduced the expression of two epithelial markers that were repressed in HNF4α-depleted cells. Supplementation of CBS products, Ctt and particularly NaSH, also significantly repressed the expression of a number of HNF4α deficiency-induced mesenchymal markers (Fig. 8b). Furthermore, knocking down CBS resulted in a 10-fold induction of a master regulator of EMT, SNAI2 (SLUG) (Supplementary Fig. 10c). These observations, together with the finding that Ctt and NaSH strongly repressed cell migration in HNF4α-depleted cells (Fig. 7e), raised the possibility that CBS may be the major effector in HNF4α-mediated EMT suppression. In agreement with this possibility, overexpression of CBS alone significantly repressed HNF4α deficiency-induced cell migration in HepG2 cells (Fig. 8c).
Finally, genetic restoration of SAA metabolism also significantly reduced the mesenchymal characteristics of SNU449 cells. As shown in Fig. 8d and Supplementary 10d, overexpression of individual SAA enzymes, particularly BHMT, CBS, or CDO1, significantly suppressed cell migration in SNU449 cells similar to HNF4α overexpression. Again, at the transcriptional level, overexpression of individual SAA enzymes had distinct impacts on EMT changes (Fig. 8e). Consistent with observations in Fig. 8a, b, overexpressing CBS in SNU449 cells primarily suppressed the expression of a number of mesenchymal markers that were induced in HNF4α-deficient cells. Overexpression of BHMT not only repressed the expression of many mesenchymal markers but also induced the expression of two epithelial markers that were repressed in siHNF4α cells (Fig. 8e). Interestingly, overexpression of MAT1A or CDO1 induced expression of the majority of tested EMT markers despite their distinct activities on cell migration (Fig. 8e). In sum, these observations confirmed that SAA metabolism is an essential element in HNF4α-mediated stress sensitivity and EMT suppression.

Discussion
Dietary methionine restriction has been shown to extend life span, reduce body fat, and improve insulin sensitivity through
various signaling pathways in animal studies. Recent studies have also demonstrated methionine restriction as a powerful dietary intervention capable of inducing rapid and specific metabolic changes to influence cancer therapeutic outcomes. However, we and others have found that the response of different human cancer cells to this nutritional manipulation is heterogeneous (Fig. 3). In this study, we show that the heterogeneous response of liver cancer cells to methionine restriction is due to, at least partially, their distinct HNF4α status. We provide evidence that the expression of key enzymes in SAA metabolism...
is under transcriptional control by HNF4α. Consistently, HNF4α-negative mesenchymal liver cancer cell lines have rewired SAA metabolism and are more resistant to cell death induced by methionine/cystine restriction or sorafenib than HNF4α-positive liver cancer cells. Knocking down HNF4α in HNF4α-positive epithelial liver cancer lines impairs SAA metabolism, promoting epithelial-mesenchymal transition and increasing resistance to methionine restriction in vitro and in vivo (Fig. 6). We further show that overexpression of enzymes or supplementation of key metabolites in the transsulfuration pathway of SAA metabolism significantly restores the sensitivity of HNF4α-depleted liver cancer cells to methionine/cystine restriction or sorafenib treatment and inhibits cell migration (Fig. 7). Collectively, our findings not only identify a genetic master regulator of SAA metabolism, but also demonstrate that HNF4α-mediated transsulfuration is a key determinant of sensitivity to methionine/cystine restriction in liver cancer.

Development of liver cancer features mutations or aberrant expression of a number of cancer-associated genes, as well as hundreds of metabolic genes59–61. Our study identifies HNF4α-mediated SAA metabolism as a key mechanism of EMT suppression that contributes to nutritional and chemical sensitivity in liver cancer. We found that the expression levels of HNF4α and SAA metabolic genes are inversely correlated with those of the mesenchymal markers in HCC patients (Fig. 1b, d). Knockdown of HNF4α in epithelial liver cancer cells led to an impaired SAA metabolism that closely resembles mesenchymal liver cancer cells (Fig. 5). Moreover, knocking down HNF4α or SAA metabolic enzymes increased nutritional and chemical resistance (Fig. 6).

Finally, deficiency of HNF4α or SAA enzymes promoted EMT characterized by reduced expression of epithelial markers and/or increased expression of mesenchymal markers (Figs. 6a and 8a, b), as well as enhanced cell migration (Fig. 7c). Importantly, restoring the expression of SAA metabolic genes suppressed EMT markers (Fig. 8c) and inhibited cell migration in mesenchymal liver cancer cells (Fig. 8d). Therefore, our study suggests that nutritional supplementation of SAA metabolites, such as Ctt, H2S precursors, and taurine, might offer therapeutic strategies to increase the sensitivity of liver tumors to dietary intervention (e.g., methionine restriction) or chemotherapy. It will be of great importance to test this possibility in future studies.

Our study has important translational implications as it identifies HNF4α as a potential biomarker for liver cancer patient selection in prospective clinical trials of dietary interventions with methionine restriction. We propose that patients with high HNF4α levels in tumors would be good candidates for such trials. Combination of methionine restriction with sorafenib treatment in these patients could produce promising survival benefits based on our in vitro results (Fig. 3e).

Although our study uncovers a key role of HNF4α in regulation of SAA metabolism, it does not exclude the involvement of other factors in this process, particularly in human liver cancer cell lines. Human liver cancer lines are genetically highly heterogeneous. For example, considering HepG2, Huh7, and Hep3B, even though they are all epithelial liver cancer cells with high expression of HNF4α, HepG2 cells have normal expression of wild-type p53, Huh7 cells overexpress a mutant p53, while Hep3B cells are p53 null65. It will be interesting to find out whether various mutations in different liver cancer cells lines also contribute to distinct SAA metabolism features in future studies.

Methods

TCGA database mining. We downloaded the processed TCGA RNA-seq gene expression data for 50 normal and 373 tumor LIHC samples from The Cancer Genome Atlas—Data Portal (https://portal.gdc.cancer.gov/). We log-transformed the normalized read counts (per million reads mapped) for RNA-seq data (all values less than 1 were assigned value 1 before transformation) but carried out no further normalization.

For correlation analysis, we computed the pair-wise Pearson correlation coefficient and the corresponding p-value between the expression levels of two genes using MATLAB. For data shown in Fig. 2b, we removed three outlier samples.
in which HNF4A expression levels were more than 3 interquartile ranges (IQRs) below the first quartile among the 371 samples.

For survival analysis, we used the full survival data including clinical information on characteristics of both patients (e.g., demographics, vital status at the time of report, treatment regimens, and clinical follow-up) and their tumors (e.g., disease-specific diagnostic/prognostic factors). The accurate stage of disease at the time of the TCGA biospecimen procurement was often not available. The pathological data such as primary tumor staging information were referenced to the patient’s initial cancer diagnosis. Though ideally, we would like to measure survival time from initial diagnosis to death, we know that patients received their initial diagnoses before TCGA procured their biospecimens; thus, these patients have been at risk for a primary tumor prior to the TCGA procurement. In a few cases, some patients may die after diagnosis but before their samples could be procured. Because of this lag between the initial cancer diagnosis and TCGA biospecimen procurement, our analysis is based on patients’ survival from the time of TCGA biospecimen procurement to death or last follow-up. Specifically, the curated post-procurement survival is calculated as follows, post-procurement survival = days_to_last_contact – days_to_sampleProcurement. If a patient has multiple follow-ups, we used the latest lost-to-follow-up date or the earliest death date. In addition, we filtered out one patient with negative post-procurement survival.

We calculated the coefficient estimate (beta value) and p-value using Cox proportional hazards regression implemented in MATLAB, and we visualized the survival distribution with Kaplan-Meier survival curves. Note that the Kaplan-Meier survival curves were generated using samples whose expression levels were among the top and bottom 33% of expression values for corresponding genes.

For clustering, we extracted the RNA-seq expression data of the 18 genes from the 371 samples and performed principal component analysis (PCA) using MATLAB. Next, any data points with a standardized value less than the negative of the maximum standardized value (i.e., 5.5) were assigned to −5.5. Only 14 data points out of 8,026 data points were affected. This reassignment was to ensure that the colors in the heatmap were balanced. We then carried out a two-way hierarchical clustering analysis using the Euclidean distance metric for (dis)similarity measure and displayed the clustering results using a heatmap.

**Cluster analysis of liver cancer cell lines.** To analyze the association between HNF4α and SAA metabolic genes in liver cancer cells, we performed a hierarchical cluster analysis on the expression data of the 25 liver cancer cell lines downloaded from the TCGA database. For each gene, we standardized its expression levels across the 25 liver cancer cell lines. We then carried out a two-way hierarchical clustering analysis on the expression data of the 25 liver cancer cell lines downloaded from the TCGA database. For each gene, we standardized its expression levels across the 25 liver cancer cell lines. We then carried out a two-way hierarchical clustering analysis on the expression data of the 25 liver cancer cell lines downloaded from the TCGA database.

**Cell culture.** Five liver cancer cell lines, Huh7, HepG2, HepG2, and SNU449, were obtained from the Cell Repository at the Tissue Culture Facility of the UNC Lineberger Comprehensive Cancer Center, all of them were originated from ATCC. SNU475 was purchased directly from ATCC. Huh7, HepG2, and HepG2 were kept in DMEM (ThermoFisher Scientific) supplemented with 10% FBS (HyClone). SNU449 and SNU475 were maintained in RPMI-1640 (ThermoFisher Scientific) supplemented with 10% heat inactivated FBS. All liver cancer cell lines were cultured in DMEM (GIBCO) supplemented with 10% FBS (HyClone) for experiments.

Normal human primary hepatocytes (ThermoFisher Scientific) were thawed in the Cryopreserved Hepatocyte Recovery Medium, centrifuged, then resuspended and plated in the culture medium (Williams Medium E without phenol red supplemented with Hepatocyte Plating Supplement Pack). After incubation at 37 °C for 6 h, the medium was replaced with incubation medium (Williams Medium E without phenol red supplemented with Hepatocyte Maintenance Supplement Pack). After overnight incubation, the cells were used for the experiments. The regenerants for normal human primary hepatocyte were purchased from ThermoFisher Scientific.

To knock down HNF4α and SAA genes, siRNAs against HNF4α (S1HN4αa1, S1HN4αa2, S1HN4αa3, and S1HN4αa4) and SAA (Ss28455, CTH, S7711, and CD0I, S2860) were designed and purchased from GenePharma. The siRNAs were transfected into the indicated cells with Lipofectamine 3000 (ThermoFisher Scientific).

**Metabolomics analysis.** To quantitatively analyze metabolite profiles in siNeg HepG2, siHNF4α HepG2, and siNeg SNU449 cells, we cultured in regular DMEM + 10% FBS medium. Metabolites were then extracted and analyzed as described previously. Briefly, cells cultured in 6-well plates (triplicates) were extracted with 1 ml ice-cold extraction solvent (80% methanol/water) by incubation at −80°C for 10 min and centrifugation at 20,000 × g for 10 min at 4°C. The supernatant was transferred to a new Eppendorf tube and dried in vacuum concentrator. The dry pellets were stored at −80°C for liquid chromatography with high-resolution mass spectrometry analysis. Samples were reconstituted into 30–60 μl sample solvent (water:methanol:acetoneitrile, 2:1:1, v/v/v) and were centrifuged at 20,000 × g for 4 min. The supernatant was transferred to liquid chromatography–MS vials. The injection volume was 3 μl for hydrophilic interaction liquid chromatography (HILIC).

**High-performance liquid chromatography was performed essentially as described previously.** Specifically, an Ultimate 3000 UHPLC (Dionex) was coupled to the Q Exactive-Plus mass spectrometer (QE-MS, Thermo Scientific) for metabolite separation and detection. For additional polar metabolite analysis, a HILIC method was used, with an Xbridge amide column (100 × 2.1 mm internal diameter, 3.5 μm; Waters), for compound separation at room temperature.

**Mass spectrometry and data analysis:** The QE-MS was equipped with a HESI probe, and the relevant parameters were: heater temperature, 120°C; sheath gas, auxiliary gas 10; spray voltage, 3.6 kV; sweep gas, 1; capillary temperature, 325°C, and the 5-λs. A full scan range was set at 60 to 900 (m/z) when coupled with the HILIC method. The resolution was set at 70,000 (at m/z 200). The maximum injection time was 200 ms. Automated gain control was targeted at 3 × 10^3 ions. Liquid chromatography–mass spectrometry peak extraction and integration were analyzed with Xcalibur (Thermo Scientific). The integrated peak intensity was used for further data analysis.

**Metabolite pathway analysis was performed in the Pathway Analysis module of MetaBioAnalyst 4.0.** All metabolites were normalized with total analyzed cell numbers for analysis. For metabolite clustering analysis, average linkage hierarchical clustering was used in the Statistics module of MetaBioAnalyst 4.0 using Euclidian distance as a similarity metric. For PCA analysis, all metabolites were used following quantile-normalization in the Statistical Analysis module of MetaBioAnalyst 4.0.

**Targeted analysis of metabolites from the SAA metabolic pathways.** To confirm the abundance of SAA metabolites in siNeg HepG2, siHNF4α HepG2, and siNeg SNU449, we performed liquid chromatography–MS analysis with a Q Exactive Plus mass spectrometer (QE-MS, Thermo Scientific) and Vanquish (ThermoScientific) UHPLC system. Reverse-phase chromatography was performed using a CORTECS C18 guard column (5 mm × 2.1 mm i.d., 1.6 μm; Waters) and CORTECS C18 analytical column (100 × 2.1 mm i.d., 1.6 μm; Waters) with solvent A being 5 mM ammonium formate in water (pH 6.5) and solvent B being methanol. The LC gradient included a 0.5-min hold at 0% B followed by a ramp from 0 to 42% B over the next 6 min followed by a ramp to 95% over the next minute. A 3-min hold at 95% was followed by a return to 0% B over the next 0.5 min. The run was completed in a 5-min reconditioning at 0% B. For the mass spectrometry, a PRM method was employed with an included list for the masses of the metabolites of interest and their optimized normalized collision energies (Supplementary Table 4). The QE-MS was equipped with a HESI source used in the positive ion mode with the following instrument parameters: sheath gas, 10; auxiliary gas, 10; sweep gas, 1; spray voltage, 3.6 kV; capillary temperature, 325°C; 5-λ; S-lens, 50; scan range (m/z) of 70 to 1000; MS resolution 70,000; 2 m/z isolation window; MSMS resolution, 17,500; MS automated gain control (AGC), 3 × 106 ions; MSMS AGC, 2 × 105 ions; and a maximum injection time of 200 ms. Mass calibration was performed before data acquisition using the LTQ Velos Positive Ion Calibration mixture ( Pierce). PRM data were processed using the Qual Browser application in the Xcalibur software suite (Thermo Scientific). Extracted ion chromatograms for fragment ions were drawn for each compound in their respective channels and areas under the peak calculated and used to represent the relative abundance of the metabolites in the samples.

**Cell survival and caspase assays.** WST-1 (Sigma) or Caspase-Glo 3/7 assay (Promega) were used to measure proliferation and apoptosis of liver cancer cells, respectively. WST-1 and caspase assays were performed according to manufacturer’s instructions.

**ROS and cell cycle assays.** Oxidative stress was measured with CellROX Green Flow Cytometry Assay Kit (ThermoFisher Scientific) and Cell proliferation was measured with Click-iT EdU Flow Cytometry Assay Kit (ThermoFisher Scientific) according to the manufacturers’ instructions.

**Lead sulfide assay for H2S production.** H2S production of HepG2 and SNU449 cells was detected by the lead sulfide method described in Hines et al. Briefly, cells were cultured in 96 well plates in growth media supplemented with 10 mM L-cysteine and 10 μM pyridoxal 5-phosphate hydrate (Sigma). A piece of 703 style
Whatman filter paper (VWR), soaked in 20 mM lead acetate (Sigma) and dried, was placed over the culture wells and covered with the plate lid with a heavy object on the top of the membrane in a transwell insert with 8 μm pore size (Corning). The inserts were placed in the wells of a 24-well plate filled with 10% FBS-containing DMEM, cultured for 24 h and then stained with crystal violet (Sigma). A cotton-tipped applicator was used to remove the non-migrated cells on the top side of the insert. Migrated cells were imaged with a microscope and quantified with the ImageJ 2.0 ( Fiji software).

To analyze the impact of metabolites in the transsulfuration pathway on HNF4α depletion-induced cell migration, HepG2 cells were transfected with control siRNAs (siNeg) or siRNAs targeting HNF4α (siHNF4α) in the complete medium. On the next day, cells were switched to the complete medium with or without 1 mM Ctt, 1 mM NaS, or 10 mM taurine and cultured for 48 h. Cell migration was then analyzed in a transwell assay for 24 h.

Quantification statistical analysis. Values are expressed as mean ± standard error of the mean (s.e.m.) from at least three independent experiments or biological replicates, unless otherwise indicated in the figure legend. Significant differences between the means were analyzed by the two-tailed, unpaired, non-parametric Mann–Whitney test for in vivo experiments and the two-tailed, unpaired Student’s t-test for in vitro experiments, and differences were considered significant at *p < 0.05. Data were analyzed using Prism Software 8.0 (GraphPad) or Microsoft Office Excel (Version 16.16.23).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The TCGA RNA-seq gene expression data were downloaded from The Cancer Genome Atlas—Data Portal (http://gdac.broadinstitute.org/normal-rna-data/LIHC_20160128). The RNA-seq data of different human liver cancer cell lines were downloaded from Broad Institute Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle/data?name: CCLE_RNAseq_gene_expression_tpm_20181208). Liver cancer cell line CCLE data were also downloaded from Liver Cancer Model Repository (LIMORE, https://www.picb.ac.cn/limore/batch; download link: Gene expression profiles of 81 cell lines). Metabolomics data are provided in Supplementary Tables. Source data are provided with this paper.

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Author contributions
Q.X. designed the study, performed experiments, analyzed data, and wrote the manuscript. Y.L., K.K., and L.L. performed bioinformatic analysis of TCGA, CCLE, and LIMORE databases. X.G., J.L., and J.W.L. performed global metabolomic analysis and determined medium methionine and cystine concentrations by LC-MS. J.G.W. and L.J.D. performed targeted analysis of SAA metabolites by LC-MS. L.T. and X.T. analyzed the impact of MCR diet on liver tumorigenesis in the DEN/HFD model. M.J. assisted the mouse xenograft experiment. I.S. guided and designed the study, analyzed data, and wrote the manuscript. All authors critically reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to I.S. or X.L.

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