HGF-MET signals via the MLL-ETS2 complex in hepatocellular carcinoma

Shugaku Takeda,1 Han Liu,1 Satoru Sasagawa,2 Yiyu Dong,1 Paul A. Trainor,3,4 Emily H. Cheng,1,5 and James J. Hsieh1,6,7

1Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. 2Department of Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan. 3Stowers Institute for Medical Research, Kansas City, Missouri, USA. 4Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas, USA. 5Department of Pathology and 6Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York, USA. 7Department of Medicine, Weill Medical College of Cornell University, New York, New York, USA.

HGF signals through its cognate receptor, MET, to orchestrate diverse biological processes, including cell proliferation, cell fate specification, organogenesis, and epithelial-mesenchymal transition. Mixed-lineage leukemia (MLL), an epigenetic regulator, plays critical roles in cell fate, stem cell, and cell cycle decisions. Here, we describe a role for MLL in the HGF-MET signaling pathway. We found a shared phenotype among Mll+/−, Hgf+/−, and Met+/− mice with common cranial nerve XII (CNXII) outgrowth and myoblast migration defects. Phenotypic analysis demonstrated that MLL was required for HGF-induced invasion and metastatic growth of hepatocellular carcinoma cell lines. HGF-MET signaling resulted in the accumulation of ETS2, which interacted with MLL to transactivate MMP1 and MMP3. ChIP assays demonstrated that activation of the HGF-MET pathway resulted in increased occupancy of the MLL-ETS2 complex on promoters, where MLL trimethylated histone H3 lysine 4 (H3K4), activating transcription. Our results present an epigenetic link between MLL and the HGF-MET signaling pathway, which may suggest new strategies for therapeutic intervention.

Introduction

Binding of individual growth factors to their respective receptor tyrosine kinases (RTKs) initiates signaling cascades that coordinate key cellular processes, such as proliferation, differentiation, survival, death, migration, and invasion (1). Hence, their activity needs to be tightly controlled, and aberrations commonly contribute to the pathogenesis of human cancers. HGF (also known as scatter factor, SF) encodes a highly versatile growth factor that was initially discovered based on activity in dispersing Madin-Darby canine kidney (MDCK) cells and promotion of hepatocyte proliferation (2, 3). The cognate cell surface receptor for HGF was subsequently demonstrated to be the proto-oncogene MET, which regulates cell fate, tissue growth, organogenesis, and neurite outgrowth (4). The HGF-MET signaling cascade channels through a myriad of intermediate signal transduction molecules to transcription factors, which ultimately activates expression of effectors that execute the respective cellular processes. Furthermore, excessive HGF-MET signals render invasive and metastatic phenotypes of affected human cancers (4). Although upstream signaling events and several key downstream effectors of the HGF-MET pathway have been characterized, the signaling relay from the plasma membrane–bound MET to DNA sequence–specific transcription factors remains unclear.

Regulated extracellular proteolysis is essential in maintaining tissue homeostasis. In cancer, deregulated extracellular proteolysis, which is mainly mediated by MMPs, contributes to the tumor invasion and metastasis phenotype (5, 6). At steady state, most tissues express low levels of MMPs, which can be induced and activated by inflammatory cytokines, hormones, growth factors, and cellular transformation. HGF is known to upregulate MMP1, MMP3, MMP7, and MMP9 in a range of epithelial and carcinoma cells, strongly correlating with induced invasiveness (7–11). Interestingly, shared regulatory DNA elements have been identified in the promoters of these inducible MMPs, including binding sites for E-twenty six (ETS) transcription factors, which are required for Ras-induced overexpression of the respective MMPs (12–14).

The mixed-lineage leukemia (MLL) gene, a human analog of trithorax in Drosophila, encodes an epigenetic transcriptional regulator belonging to the trithorax group family. The best-known developmental role of MLL is to maintain proper expression of Hox genes and thus orchestrate the segmental body plan (15–17). Consequentially, mice deficient for Mll (Mll−/− and Mll+/−) display homeotic transformation (18). In addition to patterning axial skeletons, MLL also regulates hematopoiesis and cell cycle (18–21). MLL possesses histone H3 lysine 4–specific (H3K4-specific) histone methyl transferase (HMT) activity (17, 22–25). H3K4 trimethylation (H3K4me3) catalyzed by MLL activates transcription, leading to orchestrated upregulation of key developmental genes, such as Hox genes (23, 24, 26, 27). Notably, the activity of MLL is modulated by posttranslational modifications, such as phosphorylation, ubiquitination, and proteolysis (20, 21, 28, 29). MLL undergoes site-specific proteolytic cleavage by taspase-1 (encoded by TASP1), which gives rise to a mature 500-kDa MLL320/C180 heterodimer (28). In the absence of taspase-1, MLL exists as a noncleaved precursor with reduced HMT activity. Hence, taspase–1–mediated MLL proteolysis is required to generate a fully functional MLL (26). Subsequent studies identified additional bona fide taspase-1 substrates, including MLL2 (also known as MLL4), TFIIA, TFIID-like factor (ALF), and host cell factor 1 (HCF-1) (26, 30, 31).

To investigate what biological processes are controlled by taspase-1–mediated MLL proteolysis, we created Mll+/− mice, in which gives rise to a mature 500-kDa MLL320/C180 heterodimer (28). In the absence of taspase-1, MLL exists as a noncleaved precursor with reduced HMT activity. Hence, taspase–1–mediated MLL proteolysis is required to generate a fully functional MLL (26). Subsequent studies identified additional bona fide taspase-1 substrates, including MLL2 (also known as MLL4), TFIIA, TFIID-like factor (ALF), and host cell factor 1 (HCF-1) (26, 30, 31).

To investigate what biological processes are controlled by taspase-1–mediated MLL proteolysis, we created Mll+/− mice, in
which noncleavable (nc) mutations were engineered into the endogenous Mll locus. As expected, Mll<sup>nc/nc</sup> mice exhibited homeotic defects consistent with the role of taspase-1–mediated MLL proteolysis in regulating Hox gene expression. Unexpectedly, these embryos also displayed impaired outgrowth of cranial nerve XII (CNXII; also known as the hypoglossal nerve), a process regulated by the HGF-MET signaling pathway, but not by Hox genes (32, 33). Genetic study of Tasp1<sup>−/−</sup> and Mll<sup>−/−</sup> embryos identified the same CNXII outgrowth defect, indicating the requirement of a fully functional MLL for the proper outgrowth of CNXII. Furthermore, defects in myoblast migration, another process controlled by the HGF-MET signaling pathway, were also observed in Mll<sup>−/−</sup> embryos.

Results
Mll<sup>nc/nc</sup> mice exhibit classical homeotic developmental defects. To investigate how taspase-1–mediated MLL proteolysis regulates biological pathways in vivo, we generated Mll<sup>nc/nc</sup> mice, which carry homozygous noncleavable alleles of Mll in which the genomic sequences corresponding to the taspase-1 recognition D/GX motif of cleavage sites 1 and 2 were replaced with A/AA (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65566DS1). Western blots showed that the 500-kDa full-length precursor MLL remained unprocessed in Mll<sup>nc/nc</sup> mouse embryos (Supplemental Figure 1B).

Together, our novel findings suggest Hox-independent engagement of MLL in the HGF-MET signaling pathway, elucidate downstream molecular details by which HGF-MET potentiates cellular invasion, and present a signaling cascade originated from a cell surface–anchored receptor through transcription complexes to extracellular matrix remodeling enzymes.
Examinations of the axial skeleton of Mll<sup>nc/nc</sup> newborns revealed increased incidence of homeotic defects, including incomplete segmentation between sternebra 3 and 4 and deformed anterior arch of atlas (C1 vertebra; Supplemental Figure 3, A and B). Neurofilament staining of E10.5 embryos also revealed homeotic defects of CNIX (also known as the glossopharyngeal nerve) in Mll<sup>nc/nc</sup> mice (Supplemental Figure 3C). The homeotic defects we observed in Mll<sup>nc/nc</sup> mice were in accordance with the fact that unprocessed precursor MLL exhibits impaired H3K4 HMT activity (26) and thus functions as a hypomorphic allele.

CNXII outgrowth and myoblast migration defects connect MLL with the HGF-MET signaling pathway. Besides the aforementioned homeotic transformation, a surprising Hox-independent CNXII outgrowth defect was discovered in Mll<sup>nc/nc</sup> embryos (Figure 1A). CNXII innervates and thus controls the movement of tongue muscles. Importantly, such defects were also present in Tasp1<sup>−/−</sup> and Mll<sup>−/−</sup> embryos (Figure 1A), indicating the prerequisite of a fully functional MLL in ensuring the proper outgrowth of CNXII. Remarkably, this phenotype has been observed in both Hgf<sup>−/−</sup> and Met<sup>−/−</sup> mice, but not in any reported Hox gene–knockout mice (33). In addition to CNXII outgrowth defects, Hgf<sup>−/−</sup> and Met<sup>−/−</sup> mice show profound defects in the migration of skeletal myoblasts to limbs, diaphragm, and tongue (34). Accordingly, we investigated whether myoblast migration was affected in Mll<sup>−/−</sup> embryos by in situ hybridization using a Pax3 probe, which marks migratory myoblasts (35). Interestingly, although migratory myoblasts were present at the forelimbs of Mll<sup>−/−</sup> embryos, they were fewer in number and appeared less organized (Figure 1B). Therefore, substantial overlap of phenotypes between MLL-deficient and HGF-MET–deficient mice was identified, connecting MLL and the HGF-MET signaling pathway. Consequently, we sought to determine whether MLL functions upstream and/or downstream of the HGF-MET pathway.

We first examined whether MLL is required to maintain expression of Hgf and Met in mouse hindbrain. Whole-mount in situ hybridization and quantitative RT-PCR assays demonstrated equal competence of CM derived from WT and Mll<sup>−/−</sup> E10.5 primary MEFs. MDCK cells were allowed to form colonies for 24 hours and incubated in DMEM (Control) or the indicated CM for an additional 24 hours before imaging. (D) Ex vivo axon outgrowth assays. Bilateral hindbrain explants of rhombomeres 7 and 8 were cultured in collagen gel for 48 hours in the presence of embedded HGF-soaked and control mock-treated heparin acrylic beads. Explants from Mll<sup>−/−</sup> embryos showed impaired axon outgrowth toward HGF-soaked beads. Axons were immunostained using anti-neurofilament Ab, quantified in binary images using NIH ImageJ, and presented as the ratio of axon density toward HGF-treated versus control beads. Data are mean ± SD. *P < 0.05. Scale bars: 0.5 mm.
activity in dispersing MDCK cells (2, 3). Accordingly, the potency of HGF derived from Mll–/– mouse embryonic fibroblasts (MEFs) was assessed by MDCK scatter assay. Conditioned media (CM) derived from WT and Mll–/– MEFs displayed comparable capability in scattering MDCK cells (Figure 2C). In summary, MLL deficiency did not affect Hgf or Met expression, nor HGF activity.

Because HGF is known to promote the axon outgrowth of cranial nerves (32), we therefore investigated the requirement of MLL in HGF-induced axon outgrowth using hindbrain explants. Rhombomeres 7 and 8 were dissected from the hindbrain and embedded in collagen gel along with presoaked HGF or control beads. WT explants showed preferential axon outgrowth toward HGF-soaked beads, whereas Mll–/– explants failed to exhibit a notably differential response toward HGF (Figure 2D). Thus, there is an intrinsic requirement of MLL for neurons to respond to HGF-dependent axon outgrowth, indicative of a permissive role of MLL in HGF-MET signal transduction. Collectively, the results of our genetic and functional studies support the notion that MLL functions downstream of the HGF-MET pathway.

**MLL is required for HGF-MET to induce cell invasion.** To examine the molecular connection between MLL and the HGF-MET signaling pathway, we resorted to an HGF-induced cell invasion assay, using 2 human hepatocellular carcinoma cell lines that express MET and respond to HGF: HepG2 and HLE cells (11, 36). Both HepG2 and HLE cells invaded through Matrigel in response to HGF (Supplemental Figure 4). In line with our genetic studies showing that Met expression was not reduced in Mll–/– embryos (Figure 2, A and B), knockdown of MLL in hepatocellular carcinoma cells had no apparent effect on protein expression of MET (Figure 3A). Notably, MLL deficiency did not significantly affect cell proliferation within our experimental time frame (Figure 3B). On the other hand, HGF-induced invasion of HepG2 and HLE cells was severely compromised when MLL was depleted (Figure 3C). Taken together, the results of our cell-based invasion assays further support a critical involvement of MLL in the HGF-MET signaling pathway.

**MLL deficiency impairs the transcriptional induction of MMP1 and MMP3 upon HGF-MET signaling.** Studies over the past 2 decades have outlined the basic signaling framework pertaining to the HGF-MET pathway. It involves an upstream growth factor/RTK pair, HGF-MET; a myriad of intermediary adaptor/signal transduction molecules, such as GAB1, GRB2, and MAPK; several transcription factors, such as AP1 and ETS families; and multiple...
downstream effectors, such as uPA and MMPs (4, 37). As MLL is best known as a transcriptional coactivator that enhances transcription, we envisioned that it may directly or indirectly affect the transactivation of certain key HGF-MET target genes. We first focused on MMPs and uPA, which function in remodeling extracellular matrix and breaking down adhesion molecules for cell invasion. Upon HGF treatment, expression of MMP1, MMP3, MMP7, and UPA was induced in HepG2 cells, whereas no induction of MMP2 and MMP9 was detected (Figure 4A), in agreement with prior reports (11, 38). Among these HGF-inducible genes, induction of MMP1 and MMP3, but not MMP7 or UPA, was significantly blunted in MLL-deficient cells (Figure 4A). Accordingly, expression of Mmp3 was also decreased in the hindbrain of Mll–/– embryos (Figure 4B). We subsequently sought to determine whether MLL-dependent induction of MMP1 and MMP3 is necessary for HGF-triggered cell invasion. Indeed, knockdown of MMP1 or MMP3, but not of MMP7, significantly impairs the invasion of HepG2 cells (Figure 4C and Supplemental Figure 5A). Furthermore, MLL-depleted HepG2 cells reconstituted with MMP1 or MMP3 significantly restored their capacity to invade (Figure 4D and Supplemental Figure 5B). Together, our data demonstrated that MLL is required for the proper induction of MMP1 and MMP3 by HGF-MET for cell invasion.

HGF-MET signals through ETS2 to transactivate MMP1 and MMP3. Since MLL does not encompass a sequence-specific DNA-binding domain (DB), MLL likely licenses HGF-MET–induced transcription of MMP1 and MMP3 through transcription factors. Prior studies of the HGF-MET pathway have recognized ETS1 and ETS2 as key transcription factors that target MMP genes (39). Accordingly, ETS1 and/or ETS2 are prime candidates that collaborate with MLL. We therefore investigated whether ETS1 and/or ETS2 are required for the transactivation of MMP1 and/or MMP3. HepG2 cells with knockdown of ETS1 or ETS2 were treated with HGF, and the transcript levels of MMP1 and MMP3 were examined. Deficiency in ETS2 severely compromised induction of MMP1 and MMP3 by HGF, whereas deficiency in ETS1 had minor effects (Figure 4E and Supplemental Figure 5, C and D). Notably, endogenous expression of ETS1 in hepatocellular carcinoma cells was low and could not be detectable by commonly used anti-ETS1 Ab. In fact, HepG2 cells deficient in ETS2, but not ETS1, exhibited a marked invasion defect in response to HGF (Figure 4C). These data support the notion that ETS2 functions as the key downstream transcription factor of the HGF-MET signaling pathway to activate MMP1 and MMP3 for the invasion of hepatocellular carcinoma cells.

The HGF-MET signal induces protein expression of ETS2. Thus far, our data demonstrated that both ETS2 and MLL are integral in
the transcriptional induction of MMP1 and MMP3 upon HGF-MET signals. However, how HGF-MET signals through MLL and ETS2, and whether MLL and ETS2 function in concert, in parallel, or in sequence to activate MMP1 and MMP3, have yet to be determined. First, we examined whether MLL and/or ETS2 can be induced upon HGF-MET engagement. Interestingly, ETS2 protein was induced approximately 4-fold upon HGF treatment, whereas MLL remained basically unchanged (Figure 5, A and B). We further investigated how HGF-MET activation results in ETS2 accumulation. ETS2 was induced within 30 minutes upon HGF treatment and continued to accumulate over the following 6 hours (Figure 5B). The relatively rapid induction of ETS2 protein (30 minutes) most likely results from blocked degradation, consistent with our finding that cells pretreated with the proteasome inhibitor MG132 exhibited increased baseline expression of ETS2 protein (3.1-fold; Figure 5B). However, additional mechanisms such as increased transcription must be in place to account for the continuous accumulation of ETS2 observed in the presence of MG132 (Figure 5B). We therefore assessed whether ETS2 transcript levels increase upon HGF treatment. A approximately 2-fold induction of ETS2 mRNA was observed at 2 hours after HGF treatment, preceding the peak expression of ETS2 protein at 3 hours (Figure 5, B and C). In summary, HGF-MET enlists ETS2 by disrupting degradation and enhancing transcription. Conversely, no change in MLL protein level was noted in HGF-treated HepG2 cells. We consequently sought to determine whether MLL functions to directly induce ETS2 protein upon HGF-MET activation. No impairment of ETS2 accumulation was observed in MLL-deficient HepG2 cells upon HGF treatment (Figure 5D). Hence, MLL must employ other mechanisms to participate in the HGF-MET pathway.

**Figure 5**

ETS2 protein accumulates upon HGF-MET signaling through blocked degradation and enhanced transcription. (A) MLL protein remained constant upon HGF treatment. HepG2 cells were incubated with 20 ng/ml HGF for the indicated times and subjected to anti-MLL IB analysis. (B) ETS2 protein accumulated upon HGF treatment. HepG2 cells treated with HGF for the indicated times without or with MG132 pretreatment (10 μM for 4 hours) were harvested and subjected to anti-ETS2 IB analysis. Numbers below lanes indicate relative protein levels of ETS2, measured by densitometry and normalized against nonspecific cross-reactive bands (asterisks). (C) ETS2 was induced within 30 minutes upon HGF treatment, ETS2 protein accumulated upon HGF treatment. HepG2 cells treated with HGF for the indicated times showed an induction peak at 2 hours. Data are mean ± SD from 3 independent experiments. (D) MLL knockdown had no effect on HGF-induced ETS2 accumulation. HepG2 cells transfected with the indicated siRNA oligos were treated with HGF for 3 hours and subjected to anti-ETS2 IB analysis. Asterisk denotes nonspecific cross-reactive band.

**MLL interacts with the activation domain 2 of ETS2.** Our results thus far favored a model in which HGF-MET induces ETS2, which readily complexes with MLL to activate the transcription of MMP1 and MMP3. To provide mechanistic insights as how MLL interacts with ETS2, we performed deletion mapping to identify critical regions of ETS2 required for its association with MLL. Domain compositions have been characterized within the ETS family proteins, including the conserved activation domain 1 (AD1), the pointed domain (PD), the DB, and the diverged activation domain 2 (AD2) (Figure 6C and ref. 40). Co-IP assays using N-terminal deletion mutants of ETS2 demonstrated that deletion of AD1 and PD did not affect MLL-ETS2 interaction, whereas deletion of AD2 in addition to AD1 and PD completely abrogated it (Figure 6C). The importance of AD2 in mediating the MLL-ETS2 interaction was further corroborated when C-terminal deletion mutants were analyzed: deletion of DB had no effect, whereas deletion of DB plus AD2 completely disrupted the interaction (Figure 6C). Since AD2 is the least conserved domain among ETS family proteins (40), it is tempting to speculate that this divergence may contribute to the differential selection of interaction partners by individual ETS transcription factors in response to specific signaling relays.

**HGF-MET signals the accumulation of MLL-ETS2 complex at MMP1 and MMP3 promoters.** To determine whether the HGF-MET-
induced MLL-ETS2 complex directly targets promoters of MMP1 and MMP3 for gene activation, we performed ChIP assays. Both MMP1 and MMP3 promoters contain ETS-binding sites (EBSs) (12, 13). The chromatin association of MLL and ETS2, as well as the H3K4me3 status, at EBSs (probes #2 and #4) and upstream non-regulatory regions (probes #1 and #3) were examined before and after addition of HGF. Prior to treatment, binding of ETS2 to EBSs was barely detectable, whereas HGF markedly increased the occupancy of ETS2 at EBSs (Figure 6D). HGF treatment also induced chromatin association of MLL at EBSs and increased H3K4me3 (Figure 6D). We next sought to determine whether MLL targets EBSs mainly through ETS2. Indeed, MLL failed to accumulate at EBSs upon HGF signaling in ETS2-deficient cells, which concurred with the failed induction of H3K4me3 at MMP1 and MMP3 promoters (Figure 6D). In agreement with a model in which MLL targets promoters through accumulated ETS2 upon HGF-MET signals, knockdown of MLL had no effect on HGF-induced ETS2 accumulation at EBSs of MMP1 and MMP3 promoters (Figure 6D).

MLL deficiency reduces metastasis of hepatocellular carcinoma cells. Our data thus far demonstrated a critical role for MLL in HGF-
MET–orchestrated cell invasion. Because HGF-MET dysregulation contributes to metastatic phenotypes in various cancers, we investigated whether MLL-deficient liver cancer cells exhibit compromised capacity in metastasis. HepG2 cells stably expressing firefly luciferase were subjected to shRNA-mediated stable knockdown of MLL before xenografting in immunocompromised NOD-SCID Il2rg−/− (NSG) mice via tail vein injection. Mice were monitored for tumor metastasis by bioluminescence imaging (BLI) for 6 weeks and then sacrificed for necropsy (Figure 7A). Control HepG2 cells exhibited prevalent metastatic cancer cell growth compared with MLL-deficient HepG2 cells (86% vs. 25%; $P = 0.0195$; Table 1), which confirmed the role of MLL in invasive tumor growth.

**Discussion**

Homeotic genes of invertebrates and Hox genes of vertebrates encode a family of related transcription-regulatory proteins that play essential roles in implementing developmental patterns and conferring critical positional information (15). Loss of *trithorax* in *Drosophila* and *Mll* in mice incurred global loss of homeotic/Hox gene expression, resulting in profound homeotic defects (41, 42). Hox genes located at the 3′ end of the clusters are expressed earlier and more rostrally during development than those at the 5′ end. Accordingly, deficiency in 3′ Hox genes leads to homeotic defects in the anterior part of the body (43–46). *Mll*−/− and *Tasp1*−/− mice exhibit homeotic defects in skeletons and nervous systems (26) that are also observed in mice deficient for *Hoxa3*, *Hoxa4*, or *Hoxa5* (44, 47, 48). This demonstrates the importance of MLL cleavage in regulating the 3′ early Hox genes, resonating with our prior in vitro data showing that knockdown of taspase-1 specifically disrupts the expression of early but not late Hoxa genes (28). Analogous to a noncleaved MLL in mammals, *trx*−/− *Drosophila*, in which the taspase-1 cleavage site was genetically deleted, exhibited selectively reduced expression of the 3′ anterior antennapedia (*ANT-C*) homeotic gene, but not the 5′ posterior bithorax (*BX-C*) homeotic gene (41). Interestingly, homeotic defects observed in *Mll*−/− mice were less penetrant and less conspicuous than those in *Tasp1*−/− mice, suggestive of potential genetic complementation from other taspase-1 substrates, such as *MLL2*, TFIIA, and ALF.

Our generation of *Mll*−/− animals uncovered a previously unrecognized role of MLL in the HGF-MET signaling pathway. It has been shown that proper outgrowth of CNXII in mouse embryos relies on an intact HGF-MET pathway. HGF is expressed in branchial arches, whereas MET is expressed in subpopulations of cranial motor neurons. HGF has been shown to promote neuronal induction and axon outgrowth and to stimulate Schwann cell proliferation (49). Genetic loss of *Hgf* and *Met* specifically disrupts axonal outgrowth from rhombomeres 7 and 8, therefore impairing the innervation of CNXII (32). Our findings of specific CNXII defects in *Mll*−/−, *Mll*−/−, and *Tasp1*−/− mice support the notion of specialized involvement of MLL in the HGF-MET signaling pathway. Indeed, our axon outgrowth assay using hindbrain explants of rhombomeres 7 and 8 demonstrated a role of MLL in HGF-MET–induced axonal growth. In addition to controlling CNXII outgrowth, HGF and MET also regulate the migration of skeletal...
myoblasts to limbs, diaphragm, and tongue (34). When the migration of myoblasts to forelimbs was examined, we observed migration defects in Mll−/− embryos. Although genetic defects associated with Mll−/− embryos were less profound than those of Hgf−/− and Met−/− embryos, the substantial phenotypic overlap between Mll−/− mice and Hgf−/− and Met−/− mice underscores the underlying genetic linkage between MLL and the HGF-MET signaling pathway.

Our study using hepatocellular carcinoma cell lines demonstrated that MMP1 and MMP3 functioned as key downstream effectors mediating HGF-MET-induced cell invasion, the transcriptional activation of which required MLL and ETS2, but not ETS1. As MLL and ETS2 formed a complex through the less-conserved AD2, this evolved divergence could contribute to the observed specific requirement of ETS2, but not ETS1, for HGF-induced transcription of MMP1 and MMP3. Therefore, ETS1 and ETS2 are more likely functionally distinct than redundant, a notion supported by prior studies in mice deficient in individual ETS genes. Ets2−/− mice die at E8.5 due to defects in trophoblast migration, whereas Ets1−/− mice are viable and grossly normal, except for reduced T cell numbers (50). Along the same line, it has been proposed that ETS2, but not ETS1, functions as the critical downstream transcription factor that signals downstream to PTEN loss in facilitating mammary epithelial tumorigenesis (51).

HGF-MET induced the activity of ETS2 via deterred protein degradation and enhanced transcription. This HGF-MET–induced ETS2 accumulation ultimately led to assembly of the ETS2-MLL complex on MMP1 and MMP3 promoters. ChIP assays demonstrated minimal EBS association of ETS2 and MLL before treatment with HGF, consistent with the low level of ETS2 protein present in hepatocellular carcinoma cells prior to HGF-MET signal activation. Knockdown of ETS2 affected the targeting of MLL onto the EBSs of MMP1 and MMP3, whereas knockdown of MLL had no effects on ETS2 binding. Hence, ETS2 is primarily responsible for directing the ETS2-MLL complex to the respective promoters. Although detailed molecular mechanisms concerning stability, transcription, and DNA binding of ETS2 remain to be determined, our results uncovered an ETS2-centered molecular switch whereby HGF-MET signals the increase of ETS2 protein, which results in accumulation of the ETS2-MLL complex that targets EBSs through ETS2 and methylates H3K4 through its MLL moiety, ultimately leading to activation of MMP1 and MMP3 for cell invasion (Figure 7B).

The HGF-MET signaling pathway plays an essential role in diverse developmental processes, and its dysregulation contributes to invasion and metastasis phenotypes of human cancers (4, 52). Like the other RTKs, MET has been targeted for anticancer therapies using chemical inhibitors and antagonizing peptides and/or Abs (25). However, human malignancies with dysregulated RTK eventually become resistant to targeted therapies due to acquired mutations in the receptor itself or in downstream signaling components. Therefore, further understanding of the molecular details downstream of the HGF-MET pathway may present additional anticancer therapeutic strategies. Here, we revealed a novel HGF-MET–enhanced ETS2-MLL complex that directly activated MMP1 and MMP3 transcription for the invasion of hepatocellular carcinoma cells. Since MLL mainly enhances transcription through its HMT activity, inhibitors of its HMT may offer specific therapeutic options downstream of HGF-MET.

Besides the HGF-MET pathway, EGF, VEGF, and bFGF have been reported to function upstream of ETS1 and ETS2 transcription factors (53–55). These signaling pathways promote cell invasion in a remarkably similar fashion, in which MMP expression is activated via ETS transcription factors. For example, EGF transiently induces protein levels of ETS1 and ETS2 and the ensuing upregulation of MMP1, MMP9, and uPA in SK-BR3 breast cancer cells (53). Given the critical role of MLL in HGF-MET signaling and the sharing of some downstream signaling components among different growth factor signaling pathways, MLL family proteins may participate in other growth factor signaling pathways beyond the present study. Future investigation of the potential involvement of MLL and its family members in individual growth factor signaling pathways may shed additional light on the epigenetic control of RTK signaling beyond HGF-MET.

Methods

Mice. The genomic DNA coding sequences for cleavage sites 1 and 2 of MLL were replaced by PCR-mediated site-directed mutagenesis. The targeting construct was fully sequenced and introduced into Rw4 ES cells, a male 129/sv ES line, through homologous recombination. Individual clones carrying the noncleavable allele were verified by Southern and Western analyses. Transient expression of Cre recombinase was performed to delete the Neo cassette. Clones with correct recombination and normal karyotype were injected into C57BL/6 blastocysts. Males with high chimerism were mated with C57BL/6 females. Successful germline transmission of the mutant allele was confirmed by PCR. Primer sequences were as follows: forward, GACACGGTAAGTGATGTCGACG; reverse, CTGCGATTCCCGGTCAACTGTG. Mll−/− mice were backcrossed with C57BL/6 mice for 10 generations before intercrossing to generate Mll−/− mice for analysis. Mll−/− and Tasp1−/− mice in pure C57BL/6 background exhibited sterility issues. Mll−/− mice have been described previously (18) and were maintained on the CD1 genetic background. Tasp1−/− mice were backcrossed with C57BL/6 mice for 6 generations and maintained by intercross (26).

Skeletal studies and whole-mount immunohistochemistry. Skeletal studies were performed as described (26). Whole-mount immunohistochemistry of neurofilament was performed on E10.5 mouse embryos; data presented were based on analysis at somite #36. Embryos were fixed with 4% PFA in PBS overnight, bleached using 6% H2O2 in methanol, and dehydrated in 100% methanol. Samples were blocked in 5% FCS and 1% Triton-X100 in PBS, then incubated with neurofilament-specific 2H3 mouse mAb (Developmental Studies Hybridoma Bank). Following incubation with peroxidase-conjugated anti-mouse IgG, staining was visualized with 4-chloro-1-napthol. Stained embryos were photographed under a stereoscopic microscope (Zeiss), and the distance from the end of CNXII to the intersection point between CNX and CNXII was measured.

In situ hybridization. For in situ hybridization, embryos were fixed with 4% PFA in PBS, cryoprotected in 20% sucrose in PBS, frozen in OCT Compound (Tissue-Tek), and sectioned. The template for Pax3 RNA probe was constructed based on NCBI probe database (P196191.1). Serial sections were thaw mounted on Super frost Plus slides (Thermo Fisher Scientific). Slides were fixed using 4% PFA in PBS, permeabilized with proteinase K, postfixed using 4% PFA in PBS, and washed in 0.1M triethanolamine-HCl with 0.25% acetic anhydride. Hybridization was performed overnight at 65°C with digoxigenin-labeled (DIG-labeled) cRNA probes in hybridization buffer. Slides were washed in 2× SSC at 61°C, washed in 0.2× SSC at 65°C, blocked with 10% sheep serum in PBS, and incubated with alkaline phosphatase-labeled (AP-labeled) anti-DIG Fab fragments (Roche) overnight. After sec-
tions were washed, staining was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche). Sections were counterstained with 90% glycerol with Vectashield Mounting Medium (Vector Laboratories).

Whole-mount in situ hybridization was performed on E10.0 mouse embryos (somites #28–#32). The templates for Hgf and Met RNA probes were provided by C. Birchmeier (Max Delbrück Center for Molecular Medicine, Berlin, Germany). Embryos were fixed with 4% PFA in PBS, permeabilized with proteinase K, and then postfixed with 4% PFA and 0.2% glutaraldehyde in PBS. Hybridization was performed overnight at 65°C with DIG-labeled RNA probes in hybridization buffer (50% formamide, 5x SSC, 0.3 mg/ml yeast RNA, 0.1 mg/ml heparin, 1x Denhardt’s, 0.1% Tween 20, and 5 mM EDTA). Embryos were washed, blocked in 10% sheep serum in TBST (250 mM Tris-HCl, 750 mM NaCl, 50 mM KCl, and 1% Tween 20), and incubated overnight at 4°C with AP-conjugated anti-DIG Fab fragments. After extensive washes, color reaction was carried out using BM purple (Roche).

Hindbrain explant culture and axon outgrowth assay. Hindbrain explants at rhombomeres 7 and 8 were obtained from E10.0 embryos. Bilateral hindbrain tissues containing the whole floor plate and the ventral half of the neural epithelium were dissected in ice-cold HBSS (Invitrogen). Collagen gel was prepared as a mixture of equal volumes rat tail collagen (Fisher), Matrigel matrix (BD), and DMEM (Invitrogen). Heparin-acrylic beads (Sigma-Aldrich) were incubated in HBSS containing 200 ng/ml mouse recombinant HGF (R&D Systems) or HBSS alone for 3 hours at room temperature. Hindbrain tissues were embedded in the collagen gel with laterally placed clusters of 10–20 beads at a distance of 500–750 μm and cultured in medium consisting of 75% OptiMEM (Invitrogen) and 25% F12 supplemented with 5% FBS, 40 mM glucose, and 1x penicillin/streptomycin. After 48 hours of culture, explants were fixed with 4% PFA and immunostained using anti-neurofilament 2H3 Ab. Stained axons were visualized with 4-chloro-1-naphtol and photographed under a stereoscopic microscope (Zeiss). For quantification, images of stained axons were processed to binary images using NIH ImageJ, as described previously (56). The proportion of the area covered by axons in lateral square areas adjacent to hindbrain explants was quantified by ImageJ.

Plasmid construction. The MLL1(210) cDNA was cloned in-frame into the expression vector CMV-MYC (Clontech). cDNAs expressing MMP1 and MMP3 were cloned into CMV-HA (Clontech). cDNAs encoding full-length and deletion mutants of ETS2 were cloned into an pCMV-3xFLAG expression vector CMV-MYC (Clontech). cDNAs expressing MMP1 and MMP3 were cloned into CMV-HA (Clontech). cDNAs expressing full-length and deletion mutants of ETS2 were cloned into an pCMV-3xFLAG expression vector CMV-MYC (Clontech). cDNAs expressing MMP1 and MMP3 were cloned into CMV-HA (Clontech).

MDCK cell scatter assay. 1 x 10^5 MDCK cells were plated on a 12-well plate and cultured for 24 hours to allow for the formation of discrete colonies. MDCK cells were then cultured for an additional 24 hours in the presence of CM harvested from primary MEF culture.

Cell invasion assay. 5 x 10^4 HepG2 or HLE cells were plated on a BioCoat Matrigel-coated transwell with 8-μm pores (BD Biosciences). Medium in the lower compartment was supplemented with 20 ng/ml HepG2) or 50 ng/ml (HLE) human recombinant HGF (Sigma-Aldrich). Following 24 hours of culture, cells attached to the upper side of the transwell were mechanically removed with cotton swabs. Cells that invaded to the lower side were fixed with formalin and stained with crystal violet (Fisher) and Hoechst 33342 (Invitrogen) for quantification. 5 independent fields per transwell were photographed under a microscope, and the number of invaded cells in every field was counted. Each assay was independently performed at least 3 times.

Abs, IB analysis, and IP. The anti-MLL(210) Ab has been described previously (28). The anti-ETS2 Ab (MO526) was generated by immunizing rabbits against the peptide encompassing aa 1-170 of human ETS2. Commercially available Abs used in this study were as follows: anti-MET (sc161; Santa Cruz), anti-β-actin (AC-15; Sigma-Aldrich), anti-MYC (9E10; Santa Cruz), anti-FLAG (M2; Sigma-Aldrich), anti-HA (12CA5), anti-ETS1 (sc330; Santa Cruz), anti-ETS2 (sc22803; Santa Cruz), anti-GAL4DB (sc510; Santa Cruz), anti-H3K4me3 (ab8850; Abcam). Cells or embryos were lysed in RIPA buffer supplemented with Complete protease inhibitors (Roche). Lysates were loaded to NuPAGE gels (Invitrogen) and transferred onto PVDF (Immobilon-P; Millipore). Proteins of interest were detected by the indicated Abs and enhanced chemiluminescence reagents (Western Lightening; Perkin Elmer) using LAS-300 Imaging system (FUJIFILM Life Science). IP assays were performed as previously described (26).

 Luciferase reporter assay. 293T cells were transiently transfected with 100 ng pGL2-GAL4-Luciferase vector (Promega), 100 ng IacZ-expressing CH110 (Pharmacia), 25 ng GAL4DB-ETS2 construct, and 1,500 ng MYC-MLL(210) construct using Lipofectamine 2000 (Invitrogen). Cells were harvested and lysed in cell lysis buffer (BD Biosciences) at 24 hours after transfection. Cell lysates were assayed for luciferase activity using Enhanced Luciferase Assay Kit (BD Biosciences). Data were acquired using a luminometer and normalized for transfection efficiency based on β-galactosidase activity.

CMP assay. ChiP assays were performed as described previously (21). In brief, HepG2 cells treated with HGF or mock treated for 1 hour were fixed, lysed, and sonicated. IP was performed using specific Abs against ETS2, MLL(210), and H3K4me3. Associated DNA was amplified by PCR with primers recognizing the upstream sequences of the MMP1 locus.

Primer sequences for probe #1 (human MMP1 15 kb upstream) were CCACATGGGCGACATTGGA (forward) and CACACAAAGTTATAGGACATCGACTA (reverse). Primer sequences for probe #2 (human MMP1 EBS) were GCTAGGAGTCACATTCTCTATG (forward) and GGAAGCTCTCCCTGCTATATAGT (reverse). Primer sequences for probe #3 (human MMP3 15 kb upstream) were GAGATTACATAGGGCGTAGGAG (forward) and GGTGGTTGGATAGTCAAGGT (reverse). Primer sequences for probe #4 (human MMP3 EBS) were TCCAGGTTCCTCTCACAAGC (forward) and GCTTTCACCAATTGGAGC (reverse).

Quantitative RT-qPCR. Total RNA from mouse tissues or HepG2 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For analyses of gene expression in mouse embryos, E10.0 embryo tissues containing the hindbrain, the third branchial arch, and the fourth branchial arch were isolated by excising from the bottom end of the second branchial arch to the top edge of the forelimb bud. RNA was reverse transcribed by Superscript II (Invitrogen) using oligo-dT (Invitrogen) and random decamer primers (Ambion). Quantitative RT-qPCR was performed using SYBR green master mix (Applied Biosystems) and gene-specific primer sequences for probe #1 (human MMP1 15 kb upstream) were CCACATGGGCGACATTGGA (forward) and CACACAAAGTTATAGGACATCGACTA (reverse). Primer sequences for probe #2 (human MMP1 EBS) were GCTAGGAGTCACATTCTCTATG (forward) and GGAAGCTCTCCCTGCTATATAGT (reverse). Primer sequences for probe #3 (human MMP3 15 kb upstream) were GAGATTACATAGGGCGTAGGAG (forward) and GGTGGTTGGATAGTCAAGGT (reverse). Primer sequences for probe #4 (human MMP3 EBS) were TCCAGGTTCCTCTCACAAGC (forward) and GCTTTCACCAATTGGAGC (reverse).
primers by ABI Prism 7300 sequence detection system (Applied Biosystems). Gene expression data were normalized against GAPDH, detected using GAPDH Taqman probe (Applied Biosystems).

Mouse primer sequences were as follows: Hgf forward, GGTGTG-GCCATGAAATTGACTCTATG; Hgf reverse, CTGGAGAAATGTCACAGACTCTCAGTGC; Met forward, TACCTGCACCACTGAGCACAGC; Met reverse, CCTCTGACCTGTGCAGAATTAGT; Mmp1a forward, TGCGATATGCGGCTGACATCAAAGAAC; Mmp1a reverse, CTTCAAGCCCATTTGGCAGTTGTG; Mmp3 forward, CTGCCATGAATTTGACCTCTATG; Mmp3 reverse, GAGGGTGCTGACTGCATCAAAGAAC.

Human primer sequences were as follows: MLL forward, CAGCTTGTCTACGCTACCC; MLL reverse, TCAGTAAGAAGCCTGAGTATTTG; MMP7 forward, GCTGACATCATGATTGGCTTTGCG; MMP7 reverse, CTGCATTAGGATCAGAGGAATGTCCC; MMP2 forward, GCTCC; MMP2 reverse, GAGGTTGCTGACTGTCGACAAAGAC.

Received for publication July 5, 2012, and accepted in revised form March 29, 2013.

Address correspondence to: James J. Hsieh, Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, New York 10065, USA. Phone: 646.888.3263; Fax: 646.888.3266; E-mail: hsiehj@mskcc.org.

16. Liu H, Cheng EH, Hsieh JJ. Birnbaum and intraperitoneally injected with 1.5 mg of luciferin (Perkin Elmer). Imaging was performed with Xenogen imaging system IVIS200 and ana- lyzed with Living Image software (Xenogen); 6 weeks after xenografting, mice were sacrificed, and tissues were procured and photographed. 

Statistics. Results are presented as mean ± SD unless otherwise specified. Significance of differences was determined by unpaired tailed Student’s t test, except for mouse metastasis incidence (Fisher exact test). A P value less than 0.05 was considered significant.

Study approval. All animal work was performed in accordance with a protocol approved by the IACUC of Memorial Sloan-Kettering Cancer Center.

Acknowledgments

We apologize to the investigators whose research could not be appropriately cited because of space limitations. We thank Carmen Birchmeier for providing the probes for Hgf and Met; Paul Gray for technical assistance and invaluable suggestions; and Raphael Kopan, Gregory Longmore, and David Ornitz for insightful discus- sions. P.A. Trainer is supported by the Stowers Institute. This work was supported by NIH grants CA119008 and CA138505 and by a Scholar Award of the American Cancer Society to J.J. Hsieh.


