B cell linker protein (BLNK) is a regulator of Met receptor signaling and trafficking in non-small cell lung cancer

Highlights

- A validated MaMTH-compatible PPI mapping system for Met is established
- MaMTH screens for Met detects 16 previously unreported Met-interacting proteins
- BLNK is an interactor of Met
- BLNK regulates Met signaling, localization, and metastatic functions in NSCLC

Shivanthy Pathmanathan, Zhong Yao, Paula Coelho, ..., Igor Jurisica, Morag Park, Igor Stagljar

igor.stagljar@utoronto.ca

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B cell linker protein (BLNK) is a regulator of Met receptor signaling and trafficking in non-small cell lung cancer

Shivanthy Pathmanathan,1,2 Zhong Yao,1,3 Paula Coelho,4,5 Robert Valla,1,6 Luka Drecun,1,2 Caroline Benz,1,7 Jamie Snider,1,3 Punit Saraon,1 Ingrid Grozavu,1,3 Max Kotlyar,8 Igor Jurisica,8,9,10 Morag Park,4,5,11,12 and Igor Stagljar1,2,3,13,14,15,*

SUMMARY
Met is an oncogene aberrantly activated in multiple cancers. Therefore, to better understand Met biology and its role in disease we applied the Mammalian Membrane Two-Hybrid (MaMTH) to generate a targeted interactome map of its interactions with human SH2/PTB-domain-containing proteins. We identified thirty interaction partners, including sixteen that were previously unreported. Non-small cell lung cancer (NSCLC)-focused functional characterization of a Met-interacting protein, BLNK, revealed that BLNK is a positive regulator of Met signaling, and modulates localization, including ligand-dependent trafficking of Met in NSCLC cell lines. Furthermore, the interaction between Met and GRB2 is increased in the presence of BLNK, and the constitutive interaction between BLNK and GRB2 is increased in the presence of active Met. Tumor phenotypical assays uncovered roles for BLNK in anchorage-independent growth and chemotaxis of NSCLC cell lines. Cumulatively, this study provides a Met-interactome and delineates a role for BLNK in regulating Met biology in NSCLC context.

INTRODUCTION
Met is a growth factor receptor and proto-oncogene of the receptor tyrosine kinase (RTK) family of integral membrane proteins.2 It is activated by its cognate ligand hepatocyte growth factor (HGF), which induces trans and autophosphorylation of tyrosine residues in the kinase domain and the C-terminal multi substrate docking site, which in turn recruit downstream effectors through protein-protein interactions (PPIs), including GRB2, GAB1, SHC, PI3K and STAT3. This in turn initiates several signaling cascades including, Ras/MAPK, PI3K/AKT, and STAT3 pathways, cumulating in Met-dependent biological functions.3,4 Met is a potent mediator of migration and proliferation during embryogenesis, and this inherent ability is exploited in multiple cancers, including colorectal, kidney, liver, lung, breast, pancreatic and ovary, where aberrantly activated Met promotes metastasis, enhanced proliferation, angiogenesis, and survival of tumor cells.5,6 In addition, Met is associated with the evolution of cancer drug resistance whereby the Met/HGF axis facilitates resistance to EGFR inhibitors in NSCLC and colorectal cancers,7,8 to BRAF inhibitors in melanoma,9 and to T cell immunotherapy.10 Recent studies have defined more complex roles for Met, whereby Met present in melanoma-derived exosomes can “educate” progenitor cells toward metastasis11 whereas high glucose can activate Met in a ligand-independent manner in HCC increasing tumor burden,12 as well as its role as a tumor suppressor has been uncovered, whereby Met is necessary to recruit anti-tumour neutrophils to tumor microenvironment, lack of which promoted tumor growth.13 Met is therefore a key target in cancer drug discovery.

Despite its widespread role in cancer, therapeutic targeting of Met has been challenging. This is mainly due to the paucity of biomarkers for Met-mediated cancer states14,15 and is further underscored by the role of Met in cancers, which is not only pleiotropic, but also dynamic and system specific, all of which are only partially understood. Therefore, to begin to effectively target Met, we need to understand the biology of Met thoroughly.
Protein-Protein interactions (PPI) facilitate majority of functions of any given protein, including that of Met, and form the basis of molecular mechanisms such as signaling pathways and regulatory cascades. Consequently, disruptions in these PPI networks often lead to drastic alterations in cellular systems and produce disease phenotypes. Thus, PPI mapping of any given protein can be an invaluable tool to understand protein-associated molecular events and functions in both physiology and pathophysiology. Notably, PPI networks have also recently been identified to be druggable and in the case of Met, have proven to be potential biomarkers for cancer-states and drug sensitivity.

Thus far PPI mapping of Met has been limited. Most of the known interactors of Met have been identified through classical approaches such as co-immunoprecipitation and GST pull-down, and although these approaches are informative, they are low throughput, involve harsh experimental conditions and are difficult to apply toward integral membrane proteins because of their hydrophobicity. To the best of our knowledge, the only comprehensive full-length Met-targeted PPI study to date has been by Xie et al. who used affinity-purification coupled with mass spectrometry (AP-MS) in mouse neocortex synaptosomes to identify synaptic interactors of Met. Conversely, other AP-MS studies have instead identified Met as an interactor of various query proteins, including SARS-CoV-2 proteins, and although these studies have uncovered novel functional significance of Met in their native environment, they do not take a targeted approach toward Met PPI mapping per se. On the other spectrum, yeast two-hybrid studies have targeted Met, although because of assay constraints used truncated Met, and in vitro fluorescence polarization-based microarray approaches have used phosphopeptides from Met for PPI mapping. Although these approaches improve coverage and ease of use, the lack of full-length Met cannot account for any regulation provided by other domains of the protein. Furthermore, these approaches do not utilize the native environment of Met and can miss context-specific interactions. Therefore, a systematic approach that can be amenable to high-throughput technologies and comprehensive library coverage, and queries full-length Met in its native environment, to PPI mapping of Met remains a necessity in the interaction proteomics field.

One of the current PPI technologies that can address some of the concerns above is Mammalian Membrane Two-Hybrid (MaMTH) technology. MaMTH is a split ubiquitin-based live cell PPI assay that enables the study of full-length integral membrane proteins. Here, an integral membrane and/or membrane-associated “bait” protein tagged with C-terminal portion of a split ubiquitin and a transcription factor is tested against candidate “prey” proteins that are tagged with the N-terminal portion of a split ubiquitin. During an interaction, the ubiquitin molecules reconstitute to form a “pseudo-ubiquitin” molecule, which is cleaved by cellular deubiquitinases, freeing the transcription factor to translocate to the nucleus and activate a MaMTH reporter system within the cellular context of the assay (Figure 1A). PPIs can then be proxied via the reporter signal. MaMTH is sensitive, scalable, easy to use, and adaptable to condition-dependent PPIs as needed.

Here, to enable study of Met, we have generated and characterized a MaMTH-compatible PPI mapping system for Met, and using this system, have screened a targeted MaMTH ORF library of eighty-two human Src Homology 2 (SH2) and Phosphotyrosine-binding (PTB) domain-containing proteins against Met, and identified sixteen previously unreported interactors of Met. In addition, we report the functional characterization of one such Met-interacting protein, B cell linker (BLNK), which regulates signaling and trafficking of Met in NSCLC.

**RESULTS**

**Generation and characterization of MaMTH reporter cell lines expressing full-length Met**

To map the interactome of Met, we developed a MaMTH-based platform with full-length Met as “bait”, here on referred to as the Met-MaMTH system. Full-length Met cDNA was cloned into a MaMTH bait vector with Cub-GAL4 at the C-terminus of the open reading frame (ORF) and Flip-In TREx was used to generate tetracycline-inducible stable cell lines expressing Met bait in Flip-compatible HEK293 cells expressing Gaussia princeps luciferase reporter gene. The expression of Met bait was verified with western blotting, using the associated V5 epitope of Met bait construct (Figure 1B). Under physiological conditions, Met is both expressed as a single chain precursor localized to vesicles in the cytosol (Pre-Met) and as a mature heterodimer at the plasma membrane (Met). Our results indicate that in the Met-MaMTH system, both mature and precursor Met bait were expressed, with the apparent molecular weight of Met bait in line with expected value, indicating expression of full-length Met and this expression was only observed in the presence of tetracycline, confirming the inducibility of Met-MaMTH system. We further tested the
Figure 1. Validation of Met “bait” in the Met-MaMTH system

(A) Schematic of MaMTH is shown. Created with Biorender.com.

(B) Expression of tetracycline-inducible Met bait tagged to V5 epitope was assessed in the presence and absence of tetracycline using α-V5 antibody. Tubulin (α-Tubulin) was used as a loading control.

(C) Expression and activation of Met bait to increasing concentrations of tetracycline (24h) is shown. Total Met bait (α-V5), phosphorylation of Met (α-pMet Y1234/1235), phosphorylation of ERK (α-pERK), and total ERK (α-ERK) are represented. Tubulin (α-Tubulin) was used as loading control.

(D) Surface biotinylation assay examining for Met bait (α-V5) in whole cell lysate (WCL), affinity-purified and unbound fractions are shown. GAPDH (α-GAPDH) was used as loading control.
activation status of the expressed Met bait, using α-phospho-Met (Figure 1C), and α-phospho-tyrosine (Figure S1) antibodies. Met bait and downstream signaling pathways were active correlated with its protein expression, indicating overexpression-mediated activation of Met.

We then examined whether known Met subcellular localization could be recapitulated by Met-MaMTH system, using surface biotinylation assay. Both affinity purified fraction (biotinylated) and the unbound fraction following affinity purification (non-biotinylated) were blotted for Met bait using α-V5 (Figure 1D). In agreement with previous studies, mature Met was localized to the plasma membrane, as seen by its presence in the affinity purified fraction, and its consequent depletion in the unbound fraction (non-biotinylated), whereas precursor Met was only found in the unbound fraction (non-biotinylated).

Next, we tested the ability of Met-MaMTH system to detect previously known interactors of Met. We performed a pilot MaMTH screen with nine known interactors of Met from BioGrid database, namely, SHC1, GRB2, HSP90AA1, HSP90AB1, STUB1, SPSB4, GAB2, HSPA4, and GNG12. All known interactors were cloned into N terminally Nub-tagged MaMTH “prey” expression vector with a P2A-based mCherry at the 3’ end of the ORF, to detect “prey” expression. mCherry fluorescence was used to determine “prey” expression and the resultant luminescence signal was normalized to the mCherry fluorescence to examine relative PPI signal. Based on a PPI scoring cut-off of p<0.01, the Met-MaMTH system detected five known interactors of Met, SHC1, GRB2, HSP90AA1, HSP90AB1 and STUB1 (Figure 1E). The lack of detection of the remaining known Met PPIs may be due to assay-specific or context-specific differences amongst dynamic interactomes.

Targeted interactome mapping of Met in Met-MaMTH system

As with known molecular mechanisms of all RTKs, Met on activation presents several phosphorylated tyrosine motifs which serve to recruit downstream signaling effectors.3,53 SH2/PTB domains on proteins preferentially bind to phosphorylated tyrosine motifs53,54 and are one of the recruited binders to an activated Met. Therefore, we screened an SH2/PTB-domain-containing protein collection in N terminally Nub-tagged MaMTH “prey” vector fused to P2A-based mCherry against Met using Met-MaMTH system (Figure S2). We identified thirty Met interactors out of eighty-two SH2/PTB-domain containing proteins screened (Figure 2A), and annotation analysis using Integrated Interactions Database (IID) ver. 2021-05 revealed that fourteen were previously known interactors of Met confirming the robustness of the Met-MaMTH approach to identify Met PPIs, and sixteen previously unreported Met PPIs for further exploration (Figure 2B).

We further placed our targeted interactome map of Met in the larger Met PPI network using NAViGaTOR ver. 3.0.16, to further highlight both known and predicted Met PPIs. Our targeted interactome map comprises interactors of varied functions, including kinases, signaling adaptors and phosphatases. We noted highly-interconnected proteins within our targeted interactome with either >199 interacting partners which included SHC1, PTPN6, and NCK1, or >299 interacting partners such as GRB2, FYN and ABL1 (Figure 2B).

BLNK is a protein-protein interactor of Met

From our MaMTH screens, we identified a previously unreported interactor of Met, B cell linker (BLNK) also named SLP65.66 BLNK is mainly expressed in B cells and macrophages and is a major downstream effector of the B cell receptor signaling. On phosphorylation, it recruits multiple effectors modulating several signaling pathways, including MAPKs ERK1/2, JNK, p38 and PLCγ2 signaling, contributing to B cell development, maturation, and differentiation.59–61 Of interest, Met is also expressed in B cells and is implicated in B cell physiology and neoplasia, although precise molecular mechanisms are unknown.

We began by orthogonally validating the Met-BLNK interaction using co-immunoprecipitation (Figure 3A, top panel). In HEK293 cells, V5-tagged Met was immunoprecipitated and blotted for either GFP-tagged BLNK, or GFP-tagged Lymphocyte Cytosolic Protein 2 (LCP2) and GFP-tagged B lymphoid tyrosine kinase
(BLK), both SH2-domain-containing lymphocytic adaptor proteins and were not scored as Met PPIs in our MaMTH. In concordance with our MaMTH screens, only BLNK immunoprecipitated with Met as compared to LCP2 or BLK (Figure 3A, bottom panel).

To further validate Met-BLNK interaction, we utilized SIMPL assay, an emerging PPI mapping assay for both integral membrane and cytosolic proteins that relies on split-intein protein complementation approach. We developed a SIMPL-compatible Met PPI mapping platform, here-on referred to as Met-SIMPL. We generated tetracycline-inducible HEK293 cell lines stably expressing Met-IN “bait” using Flp-In TREx, and verified the inducibility (Figure S3A), and the plasma membrane localization (Figure S3B) of Met-IN bait in Met-SIMPL. We then utilized Met-SIMPL to validate Met-BLNK interaction; IC-BLNK, or IC-SHC1 and IC-GRB2 (positive controls) and IC-PEX7 (negative control) were tested against Met-IN using Met-SIMPL. In this format of SIMPL, if an interaction occurs, the corresponding tagged split-inteins reconstitute and self-excise to form a spliced protein of both bait and prey, which is of higher molecular weight and can be detected via western blotting (Figure 3B, top panel). Interaction between Met-BLNK, as well as positive controls, Met-SHC1 and Met-GRB2 were observed in both α-V5 and α-FLAG blots, and no interaction could be detected in the negative interaction pair, Met-PEX7 (Figure 3B, bottom panel). These results cumulatively confirm the interaction between Met and BLNK, initially identified by MaMTH.

Because BLNK is an SH2-domain-containing protein, we investigated whether the interaction between Met and BLNK is interfaced through the BLNK SH2 domain. We generated BLNKΔSH2 using Gibson Assembly (Figure 3C, top panel), and examined the interaction between BLNK, BLNKΔSH2 and Met using SIMPL and MaMTH. PEX7 was used as negative control. No detectable interaction between Met and ΔSH2 BLNK was observed in SIMPL (Figure 3C, bottom panel), whereas in MaMTH, a marked reduction of interaction was observed, as compared to Met-BLNK interaction (Figure S3C). Therefore, the interaction between Met and BLNK is facilitated through the SH2 domain of BLNK.

BLNK is tyrosine phosphorylated in a Met-dependent manner
BLNK is one of the most abundantly and dynamically phosphorylated proteins in all human proteome. The tyrosine phosphorylation of BLNK has also been shown to be critical for BLNK-mediated signaling cascades and functions, including BCR-induced ERK1/2 activation in B cells. Because Met is an RTK and is capable of phosphorylating downstream adaptor proteins in their tyrosine residues, we investigated whether BLNK could be phosphorylated in a Met-dependent manner. To that end, FLAG-BLNK was transfected into Met-MaMTH cells, and was immunoprecipitated using α-FLAG, either in the absence or presence of tetracycline and blotted for tyrosine phosphorylation using α-phosphotyrosine antibody. To further corroborate Met-dependency, a condition where cells were induced tetracycline and treated with Met tyrosine kinase inhibitor Crizotinib was included. BLNK phosphorylation was detected only in the presence of tetracycline, and this phosphorylation was attenuated in the presence of Crizotinib, indicating BLNK can be phosphorylated in a Met-dependent manner (Figure 3D).

BLNK is a regulator of Met signaling
Even though BLNK is predominantly expressed in B cells and macrophages, surprisingly, data mining with CCLE gene expression database revealed a subset of epithelial cancers that express BLNK mRNA with Met. We examined BLNK protein expression ourselves in three of the listed nonsmall cell lung cancer (NSCLC) cell lines, namely, NCIH2122, NCIH1568 and NCIH2126, using α-BLNK antibody. NCIH1568 robustly expressed BLNK protein, and NCIH2122 to a lesser extent, also expressed detectable BLNK protein and no detectable protein was observed in NCIH2126 (Figure S4). Thus, given the well-characterized oncogenic role of Met in epithelial cancers, and the observed interaction between Met and BLNK, we reasoned that BLNK could play a role in Met-mediated functions in epithelial cancers.

Figure 2. Targeted interactome mapping of Met
(A) A MaMTH screen of eighty-two SH2/PTB-domain containing proteins against Met using Met-MaMTH system is shown. Data normalized to mCherry signal (prey expression) and expressed as a % of transcription factor (maximal reporter system activation). Interactors scored based on a p<0.01 and 1% transcription factor (TF) or above signal intensity. Significance against negative control PEX7 was assessed using unpaired two-tailed Welch’s t test. Results are shown as mean ± SD, with n = 3 technical replicates. The shaded box represents the interactors identified.

(B) PPI Network map of the targeted interactome of Met mapped by Met-MaMTH system is shown. Data is further integrated with known and predicted Met PPIs from Integrated Interactions Database (IID) ver. 2021-05.
Figure 3. BLNK is a protein-protein interactor of Met

(A) (Top panel) Schematic of Met-BLNK PPI validation using co-immunoprecipitation. Created with Biorender.com. (Bottom panel) Orthogonal validation of Met-BLNK PPI using co-immunoprecipitation is shown.

(B) (Top panel) Schematic of Met-BLNK PPI using SIMPL. Created with Biorender.com. (Bottom panel) Orthogonal validation of Met-BLNK PPI using SIMPL is shown. SIMPL assay examining interaction between Met and BLNK, SHC1 and GRB2 (positive controls), and PEX7 (negative control) is shown. Parental prey proteins are highlighted by white triangles and spliced proteins are highlighted by white asterisks.

(C) (Top panel) Schematic of the domain structure of BLNK and BLNKΔSH2 is shown. Tyr-Tyrosine residues. Created with Biorender.com. (Bottom panel) SIMPL assay examining interaction of Met and BLNK, BLNKΔSH2, SHC1 and GRB2
We first investigated whether BLNK could regulate Met signaling by determining HGF-mediated ERK1/2 phosphorylation, a culmination of the Ras/MAPK pathway downstream of Met, either in the presence of exogenously expressed FLAG-tagged BLNK or empty vector-transfected cells in HEK293 cells. Phosphorylation of ERK1/2 in response to HGF was increased at all time points in BLNK-transfected cells, as compared to empty vector-transfected cells (Figure 4A). We then performed siRNA-mediated knockdown of BLNK (siBLNK) in NSCLC cell lines that expressed BLNK and Met above, NCIH2122 (Figure 4B) and NCIH1568 (Figure 4C) and examined ERK1/2 and AKT (of the PI3K/AKT pathway downstream of Met) phosphorylation, in response to HGF. Consistent with the results observed in HEK293 cells, both cell lines showed reduced HGF-mediated ERK1/2 phosphorylation at all time points in siBLNK-transfected cells, as compared to siControl-transfected cells. HGF-mediated phosphorylation of AKT was affected at later time points, where in the absence of BLNK protein, it was reduced at 10 min in NCIH2122 and 30 min for NCIH1568 but remained comparable at 5 min after HGF stimulation, indicating a deficiency in sustained AKT activation in response to HGF.

Because the observed effect of BLNK on Met signaling is visualized as early as 5 min, we examined whether BLNK affected Met kinase activity. In this experiment design for Met signaling assays however, we were unable to detect phosphorylated Met in response to HGF because of the reduced sensitivity of α-phospho-Met antibody. Instead, we immunoprecipitated Met, after siRNA-mediated knockdown of BLNK in both NCIH2122 (Figure 4D) and NCIH1568 (Figure 4E) and examined phosphorylation in response to HGF using α-phospho-Met antibody. No changes in Met phosphorylation at tyrosine residues Y1234/Y1235 (located within the kinase domain) were detected in NCIH2122 and NCIH1568 suggesting that the role of BLNK does not affect Met kinase activity at 5 min after HGF stimulation.

Collectively, these results suggest that BLNK positively regulates Met signaling in NSCLC cell lines.

**BLNK affects HGF-independent surface localization and HGF-dependent trafficking of Met**

Next, we sought to investigate the molecular mechanisms behind the observed effects of BLNK in Met signaling pathway. Signaling cascades initiated by RTKs are regulated on multiple levels, including the initial concentration of surface RTK to receive their ligand, the endocytic trafficking, positive and negative feedback loops, and the efficacy of degradation of RTKs. Trafficking endosomes have recently emerged as distinct platforms to initiate signaling cascades by spatially and temporally regulating its constituents. Likewise, endosomal trafficking of Met has been shown to be critical to its signaling, including HGF-mediated ERK1/2 activation. Hence, we examined whether BLNK affects HGF-independent or HGF-dependent localization of Met.

Using surface biotinylation coupled with siRNA-mediated knockdown of BLNK, we looked at the initial surface presentation of Met protein in NCIH2122 (Figure 5A) and NCIH1568 (Figure 5B). Cells were transfected with either siBLNK or siControl and surface Met levels were analyzed with surface biotinylation assay. Both NCIH2122 and NCIH1568 displayed a reduced amount of Met protein at the surface in siBLNK-transfected cells, as compared to siControl-transfected cells, and this was observed in an HGF-independent manner.

On activation by HGF, Met is internalized and trafficked through the endocytic pathway, eventually culminating in its degradation. Using immunofluorescence combined with siRNA-mediated knockdown of BLNK, we additionally visualized HGF-dependent trafficking of Met in NCIH2122 (Figure 5C left panel) and NCIH1568 (Figure 5D left panel). Met subcellular localization was mapped by calculating either the average distance for NCIH2122 because of its uniform cellular morphology or the relative distance for NCIH1568 due to its irregular morphology, between Met puncta and plasma membrane after indicated times of HGF stimulation. Knockdown of BLNK was verified with western blotting (Figure 5C, right panel, Figure 5D, right panel). Post HGF stimulation, in both NCIH2122 (Figure 5C, middle panel) and
NCIH1568 (Figure 5D, middle panel), distance (average or relative as indicated) of Met puncta to the plasma membrane was significantly less in siBLNK-transfected cells, as compared to siControl-transfected cells.

Figure 4. BLNK is a regulator of Met signaling
(A) Time course signaling assay in response to HGF in FLAG-BLNK- and empty vector-transfected HEK293 cells is shown. Total Met (α-Met), BLNK (α-FLAG), phosphorylation of ERK (α-pERK) and total ERK (α-ERK) are represented. GAPDH (α-GAPDH) was used as loading control.
(B) Time course signaling assay in response to HGF in NCIH2122 cells with siRNA-mediated knockdown of BLNK is shown. Total Met (α-Met), BLNK (α-BLNK), phosphorylation of ERK (α-pERK), total ERK (α-ERK), phosphorylation of AKT (α-pAKT) and total AKT (α-AKT) are represented. GAPDH (α-GAPDH) was used as loading control.
(C) Time course signaling assay in response to HGF in NCIH1568 cells with siRNA-mediated knockdown of BLNK is shown. Total Met (α-Met), BLNK (α-BLNK), phosphorylation of ERK (α-pERK), total ERK (α-ERK), phosphorylation of AKT (α-pAKT) and total AKT (α-AKT) are represented. GAPDH (α-GAPDH) was used as loading control.
(D) and (E) Phosphorylation of Met in response to HGF post siRNA-mediated knockdown of BLNK is assessed using immunoprecipitation of Met in NCIH2122 (D) and NCIH1568 (E). Total Met (α-Met), phosphorylated Met (α-pMet Y1234/Y1235) and BLNK (α-BLNK) are shown. GAPDH (α-GAPDH) was used as loading control.

NCIH1568 (Figure 5D, middle panel), distance (average or relative as indicated) of Met puncta to the plasma membrane was significantly less in siBLNK-transfected cells, as compared to siControl-transfected cells.
Figure 5. BLNK is a regulator of HGF-independent and-dependent subcellular localization of Met

(A) Surface Biotinylation assay detecting surface Met levels of NCIH2122 in response to siRNA-mediated knockdown of BLNK is shown. Whole cell lysate (WCL) and surface Met (α-Met) and BLNK (α-BLNK) are represented. Tubulin (α-Tubulin) was used as loading control.

(B) Surface Biotinylation assay detecting surface Met levels of NCIH1568 in response to siRNA-mediated knockdown of BLNK is shown. Whole cell lysate (WCL) and surface Met (α-Met) and BLNK (α-BLNK) are represented. Tubulin (α-Tubulin) was used as loading control.

(C) (Left panel) Immunofluorescence to assess localization of Met at 0- and 30 min of HGF stimulation in response to siRNA-mediated knockdown of BLNK in NCIH2122 is shown. Scale bar = 10μM (Middle panel) Average distance of Met puncta to the plasma membrane is plotted. Individual dots represent one cell. Significance was assessed with one way ANOVA. Data is represented as mean ± SD for n = 45 cells; ns = not significant, ****p<0.0001. (Right panel) Knockdown of BLNK was verified using western blotting. Met (α-Met) and BLNK (α-BLNK) are shown. Actin (α-Actin) was used as loading control.

(D) (Left panel) Immunofluorescence to assess localization of Met at 0-, 15- and 30 min of HGF stimulation in response to siRNA-mediated knockdown of BLNK in NCIH1568 is shown. Scale bar = 10μM (Middle panel) Relative distance of Met puncta to the plasma membrane is plotted. Individual dots represent one cell. Significance was assessed with one-way ANOVA. Data is represented as mean ± SD for n = 45 cells; ns = not significant, ****p<0.0001. (Right panel) Knockdown of BLNK was verified using western blotting. Met (α-Met) and BLNK (α-BLNK) are shown. Actin (α-Actin) was used as loading control.
Cumulatively, these results illustrate that BLNK regulates HGF-dependent trafficking of Met, where in the absence of BLNK protein, Met is localized peripheral to plasma membrane, and does not traffic to the perinuclear region of the cell, away from the plasma membrane.

**Interaction between Met and GRB2, and BLNK and GRB2 are modulated by BLNK and active Met, respectively**

BLNK is a scaffold with no intrinsic enzymatic activity and its functions are determined by the effectors it recruits. Therefore, any functions exerted by BLNK, like that of observed effects of BLNK in signaling, subcellular localization and HGF-dependent trafficking of Met is mediated by some thus far unknown binder(s) to BLNK.

Several BCR-dependent interactors of BLNK have been reported in the literature, including GRB2, NCK2, VAV, BTK, and PLCγ2. To this end, we studied GRB2, which is a known constitutive binder of BLNK and, because of this constitutive complex, we hypothesized that Met-BLNK interaction complex will likely contain associated GRB2 and this GRB2 may have a function in observed effects of BLNK on Met biology. We ourselves verified the constitutive association of BLNK and GRB2 via co-immunoprecipitation in HEK293 cells (Figure S5A).

To investigate whether a recruitment of GRB2 to Met could be possible, we examined, albeit in an indirect manner, whether the interaction between Met and GRB2 can be modulated by BLNK. We used a modified MaMTH assay using the Met-MaMTH system (Figure 6A, Left panel), where we tested the interaction between Met and GRB2 in the presence of either GFP-tagged BLNK or GFP-tagged LCP2 as negative control (Figure 6A, Right panel). LCP2 was used as the negative control because it is a paralog of BLNK in T cells and was found to not interact with Met in MaMTH. Interaction between Met and GRB2 was increased in the presence of BLNK, as compared to LCP2. Comparable protein expression of all constructs was verified using western blotting (Figure 6A, Right panel). We further confirmed these results using co-immunoprecipitation (Figure S5B).

To further understand the binding dynamics between Met, BLNK and GRB2, we focused on the interaction between BLNK and GB2. The constitutive interaction between BLNK and GB2 is augmented after BCR activation in B cells. Thus, we investigated if a similar mechanism could exist for Met, that is whether activated Met could modulate the interaction between BLNK and GB2, using co-immunoprecipitation. FLAG-tagged GB2 was immunoprecipitated and probed for the associated GFP-tagged BLNK, in the presence of V5-tagged Met. We examined three conditions: (1) untreated, (2) treated with Met TKI Crizotinib as overexpressed Met displays ligand-independent constitutive activation, and therefore inhibition of this activity would allow us to determine Met-activity dependent modulations of BLNK-GRB2 interaction, and (iii) stimulated with HGF as it is not fully understood whether HGF can influence Met’s protein-protein interactome and subsequently could exert differential effects on BLNK-GRB2 interaction (Figure 6B). Crizotinib inhibited Met kinase activity as visualized by the lack phosphorylation of Met, whereby HGF had no additional effects on the phosphorylation of Met. The interaction between BLNK and GB2 was reduced in the presence of Crizotinib, while remaining unaffected in the presence of HGF, as compared to no treatment. Met as a co-immunoprecipitated species was not detected in GB2 (α-FLAG) immunoprecipitates, likely because of the difficulty in applying co-immunoprecipitation to full-length membrane proteins, such as Met.

These results indicate that the interaction between Met and GB2 is increased in the presence of BLNK, and the interaction between BLNK and GB2 is increased in the presence of active Met.

**BLNK (GRB2 binding deficient) mutant reduces BLNK-dependent effects on HGF-mediated ERK1/2 activation**

To investigate whether the BLNK-associated GB2 contributes to any of the observed effects of BLNK on Met function, such as signaling, we undertook a structure-function approach to uncouple the constitutive interaction between BLNK and GB2.

GB2 interacts with BLNK through its SH3 domain binding to the atypical SH3 binding motif bordered by proline residues 204 (P204) and 212 (P212) on BLNK and its SH2 domain, independent of detectable tyrosine phosphorylation. Though positional residue information of this binding is not known, we examined a
Figure 6. Met-GRB2 interaction is increased in the presence of BLNK, and BLNK-GRB2 interaction is increased in the presence of active Met

(A) (Left panel) Schematic of modified MaMTH assay is shown. Created with Biorender.com. (Right panel) MaMTH assay examining the interaction between Met and GRB2 or PEX7 (negative control) in the presence of GFP-tagged BLNK or GFP-tagged LCP2 is shown. Significance was assessed using unpaired two-tailed Welch’s t test. Results are shown as mean ± SD, with n = 4 technical replicates. ***p<0.001. Protein expression of all constructs was detected using western blotting. Met bait (α-V5), GRB2/NCK2 preys (α-FLAG) and BLNK/LCP2 (α-GFP) are shown. GAPDH (α-GAPDH) was used as a loading control.

(B) Co-immunoprecipitation investigating interaction between GRB2 and BLNK in the presence of Met under different conditions is shown. Phosphorylated Met was detected using α-pY antibody. Tubulin (α-Tubulin) was used as a loading control. IP - Immunoprecipitated fraction; WCL – Whole cell lysate.
canonical GRB2 SH2 binding motif, YXN91 (https://www.hprd.org/) at tyrosine 72 (Y72) of BLNK, with tyrosine 84 (Y84) as an unrelated control. Single, double, and triple mutants of BLNK were generated by mutating proline residues to alanine (P204A, P212A and P204AP212A) and tyrosine residues to phenylalanine (F) (Y72F, Y84F, P204AP212AY72F) using site-directed mutagenesis.

Interaction between NIN-GRB2 (bait) and IC-BLNK or IC-BLNK mutants (preys) were examined using SIMPL-ELISA,71 which is a quantitative approach to SIMPL that further allows us to quantify bait and prey expression to account for expression-related anomalies between BLNK mutants. The resultant spliced protein product expression was normalized to both bait and prey expression to determine relative luminescence. Only BLNK P204A, BLNK P212A and BLNK P204AP212A significantly reduced interaction between BLNK and GRB2, whilst BLNK P204AP212AY72F showed the most marked decrease in interaction between BLNK and GRB2 (Figures 6C and S6A) and was used in downstream functional analysis. We also orthogonally confirmed the observed reduced interaction between BLNK P204AP212AY72F and GRB2 using co-immunoprecipitation (Figure S6B).

To investigate whether GRB2 was part of the molecular mechanism of BLNK-mediated increase in HGF-mediated ERK1/2 phosphorylation, in the presence of exogenously transfected BLNK or BLNK P204AP212AY72F or empty vector in HEK293 cells. ERK1/2 phosphorylation was reduced in BLNK P204AP212AY72F-transfected cells as compared to BLNK-transfected cells but was increased as compared to empty vector-transfected cells (Figure 6D). These results illustrate that GRB2 is at least partly responsible for the observed BLNK-mediated positive regulation of Met signaling in HEK293 cells.

BLNK affects anchorage-independent growth in NCIH2122 and HGF-induced migration of NCIH1568

Both NCIH2122 and NCIH1568 were isolated from metastatic secondary sites,92 indicating that metastasis was one of the biological functions in the cells. Given the well-characterized role of Met in metastasis, including anchorage-independent growth and chemotaxis,3,93 we investigated whether BLNK played a role in either of these functions in NCIH2122 and NICH1568 non-small cell lung cancer cells.

For NCIH2122, we examined whether BLNK played a role in anchorage-independent growth by using soft agar colony formation assays. We targeted BLNK via CRISPR-Cas9-mediated gene editing. We examined two isogenic BLNK knockout cell lines (BLNK-targeting clone 1 and clone 2), and a control cell line where antibiotic resistance cassette was targeted to the AAAS1 locus. As compared to AAAS1-targeted and parental cells, clone 1 expressed no detectable BLNK protein, and clone 2 expressed minimal BLNK protein, as verified using western blotting. Both clones expressed reduced total Met, with clone 1 showing the most marked decrease. Clone 1 also displayed reduced basal ERK1/2 phosphorylation as compared to clone 2, parental and AAAS1-targeted cells. These differences between clones may be because of minimal BLNK protein levels found in clone 2 that exerts a partial rescue effect (Figure 7A). In soft agar colony formation assays, both BLNK knockout cell lines formed significantly less colonies than that of their respective parental and AAAS1-targeted controls, which were similar in their ability to form colonies (Figure 7B, left and right panels). Therefore, BLNK may play a role in anchorage-independent growth of NCIH2122.

In NCIH1568, we examined whether BLNK affected HGF-directed chemotaxis using transwell migration assay, coupled with siRNA-mediated knockdown of BLNK (Figure 7C, left panel). Basal migrations of siBLNK- and siControl-transfected cells were similar; however, in the presence of HGF, siBLNK-transfected cells migrated significantly less toward HGF, as compared to siControl-transfected cells (Figure 7C middle panel). siRNA-mediated knockdown was verified via western blotting (Figure 7C, right panel). Therefore, BLNK plays a role in the HGF-directed chemotaxis of NCIH1568.
Figure 7. BLNK affects anchorage-independent growth of NCIH2122 and HGF-induced chemotaxis of NCIH1568

(A) Validation of NCIH2122 BLNK CRISPR knockout cell lines via western blotting is shown. Total Met (α-Met), BLNK (α-BLNK), phosphorylation of ERK (α-pERK) and total ERK (α-ERK) are represented. GAPDH (α-GAPDH) was used as loading control.

(B) (Left panel) Soft agar colony formation assay of NCIH2122 BLNK CRISPR knockout clonal cell lines, with parental NCIH2122 and NCIH2122 AAVS1 targeting control shown. Wells with no cells were used as the negative control. Results are representative of n = 3 technical replicates. (Right panel) Number of colonies counted using ImageJ. Significance was assessed using unpaired two-tailed Welch’s t test. Results are shown as mean ± SD, with n = 3 technical replicates. *p<0.05; **p<0.01.

(C) (Right panel) Transwell migration assay investigating the migration of NCIH1568 (NSCLC) cells toward either no HGF or 50 ng/mL HGF in response to siRNA-mediated knockdown of BLNK is shown. (Middle panel) Quantification of Crystal violet staining is shown. Significance was assessed using unpaired two-tailed Welch’s t test. Results are shown as mean ± SD, with n = 2 technical replicates *p<0.05, (Right panel) Knockdown of BLNK was verified using western blotting. Met (α-Met) and BLNK (α-BLNK) are shown. GAPDH (α-GAPDH) was used as loading control.
DISCUSSION

Despite its multifaceted role in cancer, Met is considerably understudied with limited therapeutic targeting options. PPI networks underlie biological functions elicited by any protein, including that of Met, and delineating these networks can uncover novel biology and consequently therapeutic avenues. However, to date, comprehensive interactome analysis of full-length Met has been insufficient, and therefore there is a need in the Met community to both generate a systematic platform for Met PPI mapping and to generate Met interactomes to enable further study of Met.

Here, we present a MaMTH-based PPI mapping platform for full-length Met, namely, Met-MaMTH system. Met-MaMTH system expresses tetracycline-inducible Met “bait”, that is both activated and signaling competent, both of which are dependent on Met “bait” expression. The system expresses both mature and precursor Met “bait”, where mature Met “bait” is predominantly localized to the plasma membrane and precursor Met within the cell, as expected. Met-MaMTH system can detect known interactors of Met.

Met-MaMTH system allows us to examine Met PPIs in a targeted manner, with prey libraries of both small and large complexities, either enriched in their binding capacity toward Met (biased) or unrelated (unbiased), provided that the “prey” libraries can be generated. This is useful for future research, as any putative interactor that is hypothesized to interact with Met including PPIs that occur under selective or narrow endogenous conditions or PPIs that aren’t easily accessible to existing PPI technologies, can be tested against Met in this system. Notably, with the recent studies showing Met as an interactor of SARS-CoV-2 proteins and the well-characterized role of Met in Listeria Monocytogenes infection, the Met-MaMTH system can also be used to investigate PPIs of Met if the virus/bacterial proteins could be expressed and are MaMTH-prey compatible. The inducibility of the system allows for greater regulation of bait expression, and thus can be used to map PPIs under different expression levels of Met. This in hypothesis can be translated to disease conditions, where we can fine-tune Met expression to mimic disease environments. Therefore, the Met-MaMTH system can be a useful tool for the Met community to generate critical knowledge on Met biology and to ease existing therapeutic limitations.

Using the Met-MaMTH system, targeted interactome mapping of Met against eighty-two SH2/PTB domain-containing proteins identified thirty Met interactors, of which sixteen were previously unreported. Annotation of the Met PPI network shows that proteins with varied activities were detected, including signaling adaptors, kinases, and a phosphatase, providing new information on both Met function and regulation. Of interest, of the sixteen PPIs identified, for which there was no experimental evidence, ten Met PPIs were also predicted by IID, lending more evidence to their interaction and the benefits of using bioinformatics-based prediction toward PPI mapping. The remaining six were not predicted by IID; however, were captured by MaMTH, highlighting the role of experimental methods in PPI mapping, and how they can work together with predictive PPI mapping. Furthermore, when our targeted interactome map of Met was placed within the larger cellular PPI network, a few interactors of Met were found to coordinate multiple networks of their own, indicating the intricate wiring of Met PPI networks within a cell. Both the targeted and the additional predicted PPI networks presented in this study will be apt starting points for future functional studies and targeted screens using the Met-MaMTH system, respectively.

We further characterized one of the PPIs identified through our MaMTH screens, BLNK, for its functional significance in Met biology. Our results indicate that BLNK is a positive regulator of Met signaling in NSCLC cell lines that co-express BLNK and Met, and BLNK increases surface Met levels and mediates HGF-dependent endocytic trafficking of Met, whereby in the absence of BLNK, on HGF stimulation, Met does not traffic to perinuclear regions, and instead is localized proximal to the plasma membrane.

The observed role of BLNK in regulating HGF-dependent endocytic trafficking of Met suggests that BLNK may be an endocytic adaptor of Met. Notably, adaptor proteins that facilitate Met endocytic trafficking, and recycling such as Gamma ear-containing Arf-binding protein 3 (GGA3) which increases HGF-dependent entry of Met into recycling compartments and FCH/F-BAR and Double SH3 Domain-Containing Protein (FCHSD2) which mediates Met recycling or subcellular localization, such as phosphatase 1B (PTP1B) which regulates Met subcellular localization by mediating early endosomal fusion in Met endocytic trafficking have been previously reported. Thus, delineating the molecular details of how BLNK regulates Met subcellular localization will provide insight.
Although we conclude that BLNK regulates HGF-dependent Met trafficking, whether this function of BLNK, or the HGF-independent increase surface localization of Met, is directly responsible for the observed positive regulation of Met signaling by BLNK is unknown. Because the impact of spatial regulation and endosomal trafficking of Met on Met signaling is well established,82,85,98,99 and that increased surface RTK levels, like that of observed for Met in this study, can increase sensitivity to HGF or promote clustering and ligand-independent activation, both of which can positive regulate downstream signaling,80 we hypothesize that BLNK-mediated regulation of Met subcellular localization and BLNK-mediated positive regulation Met signaling are connected, and future studies can address this.

We find that interaction between Met and GRB2 is increased in the presence of BLNK and the constitutive interaction between BLNK and GRB2 is increased in the presence of active Met. This is analogous to B cells, where interaction between BLNK and GRB2 is increased in B cell receptor (BCR)-activation-dependent manner.56 Because BCR activation is functionally coupled to phosphorylation of BLNK,73 and we observe that BLNK is phosphorylated in a Met-activity-dependent manner, a similar mechanism may underlie the observed increase in binding between BLNK and GRB2 in the presence of active Met. Furthermore, the Met-dependent phosphorylation of BLNK could participate in recruitment of additional Met-dependent effectors that mediate additional Met-associated functions, and thus merits further exploration.

Furthermore, our structure-function approaches indicate that the GRB2 bound to BLNK is a mediator, likely one of many, of BLNK-dependent Met signaling. Because GRB2 is a well-established downstream effector of Met signaling, including Ras/MAPK pathway,27,100 and a critical mediator of Met endocytosis,85,101–103 the BLNK-associated GRB2 may also potentiate any existing functions in Met signaling or may have distinct mechanism. Future GRB2-focused studies can determine the precise molecular details.

Finally, our results establish a common function for BLNK in metastasis of two NSCLC cell lines, where the loss of BLNK impairs anchorage-independent growth, and HGF-induced chemotaxis. This is complemented by the origin of both NCIH2122 and NCIH1568 being secondary metastatic sites,92 and together we propose that BLNK plays a role in invasive growth of both NCIH2122 and NCIH1568.

Although a role for Met/HGF axis in BLNK-induced migration toward HGF is clear in NCIH1568, despite the known function of Met in anchorage-independent growth,3,93 whether Met/HGF axis is necessary for BLNK-induced anchorage-independent growth in NCIH2122 remains to be investigated. Of interest, we observed reduced total Met levels in both NCIH2122 BLNK knockout cell lines suggesting BLNK could regulate Met expression, and this could in turn affect their ability to display anchorage-independent growth. In addition, with increasing evidence establishing the role of Met trafficking in metastatic functions of Met82–85,104,105, delineating whether the observed regulation of Met trafficking by BLNK underlies the anchorage-independent growth and HGF-directed chemotaxis in NCIH2122 and NCIH1568 respectively, present interesting avenues for future research.

Although this manuscript was in preparation, recent studies have emerged implicating BLNK in epithelial tumors. Lee et al. identified BLNK to be an independent risk factor for colorectal cancer recurrence.106 Zhang et al. reported BLNK as a key regulator in autophagy-induced thyroid cancer (TC), where BLNK is hypomethylated and consequently abnormally expressed which led to poor prognosis. Of interest, BLNK expression was enriched in distant metastasis, as compared to no recurrence or locoregional recurrence of TC cells.107 These studies consolidate our model that BLNK when aberrantly expressed in epithelial cancers can mediate tumor functions, and our study further establishes a detailed mechanistic groundwork for the role of BLNK in Met-mediated oncogenicity in the context of NSCLC using two NSCLC cancer cell lines. Further studies directed toward multiple NSCLC cancer cell lines, and additional tissue-specific cancers can determine whether BLNK has a core or tissue-specific function in regulating Met tumor biology across all epithelial cancers.

Taken together, our study introduces a Mammalian Met PPI mapping platform for full-length Met and a targeted interactome map that can be leveraged to further understand novel facets of Met biology and describes a role for BLNK as a mediator of Met-associated functions, including Met signaling and trafficking in two NSCLC cell lines. We aim for the tools and findings generated through our study to be a resource to the Met community, and through our characterization of BLNK as a Met PPI in NSCLC context, our findings may potentially be applied toward Met-targeting therapeutics and biomarkers, and the
mechanistic details identified through this study can be utilized toward thoroughly understanding known roles of Met in B cell cancers.

Limitations of the study
A limitation of the Met-MaMTH system is that although both mature and pre-Met bait are expressed, because the MaMTH readout is indirect, the system as it exists cannot differentiate whether an interactor interacts exclusively or preferentially with mature or pre-Met, and further focused mutagenesis-based MaMTH screens can address this going forward. Moreover, the Met-MaMTH system cannot demonstrate location information for PPIs or kinetic information of the interaction as once interaction occurs the reporter signal (secreted Gaussia luciferase) accumulates. Further location-based orthogonal PPI mapping studies can provide insight.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell lines and cell culture
- **METHOD DETAILS**
  - Stable cell line generation
  - Constructs and transfections
  - MaMTH assays
  - Cell lysis and western blotting
  - Signaling assays
  - Surface biotinylation assays
  - SIMPL and SIMPL-ELISA
  - Co-immunoprecipitation
  - Immunoprecipitation
  - Immunofluorescent staining
  - Met trafficking assays
  - Puncta distribution analysis
  - Soft agar colony formation assay
  - Transwell migration assay
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105419.

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AUTHOR CONTRIBUTIONS
I.S. conceived the project; S.P. designed and performed all experiments; I.S. and Z.Y. supervised the project; P.C., R.V. performed experiments as requested, P.C. performed all immunofluorescence imaging; R.V. performed all SIMPL experiments; L.D. and C.B. generated SH2/PTB library; J.S. designed MaMTH vector and reporter cell lines used for Met-MaMTH assay; Z.Y. invented SIMPL assay and designed SIMPL bait/prey vector constructs. P.S. and I.G. were involved in validation experiments; M.P. supervised P.C.’s
experiments. M.K. and I.J. performed annotation and network analysis. All authors edited and approved the final manuscript.

DECLARATION OF INTERESTS
Authors declare no competing financial interests.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-V5             | Cell Signaling Technologies | Cat #132025, RRID:AB_2687461 |
| Anti-FLAG           | Sigma Aldrich | Cat #F1804-1MG, RRID: AB_262044 |
| Anti-Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) | Cell Signaling Technologies | Cat #9101S, RRID:AB_331646 |
| Anti-p44/42 MAPK (ERK1/2) | Cell Signaling Technologies | Cat #9102S, RRID:AB_330744 |
| Anti-Met            | Cell Signaling Technologies | Cat #8198S, RRID:AB_10858224 |
| Anti-phospho-Met (Y1234/1235) | Cell Signaling Technologies | Cat #3077S, RRID:AB_2143884 |
| Anti-pY             | Santa Cruz Biotechnology | Cat #SC-7020, RRID:AB_628123 |
| Anti-BLNK           | Cell Signaling Technologies | Cat #36438S, RRID:AB_2799101 |
| Anti-pAKT (Thr 308) | Cell Signaling Technologies | Cat #4056S, RRID:AB_331163 |
| Anti-AKT            | Cell Signaling Technologies | Cat #9272S, RRID:AB_329827 |
| Anti-Tubulin        | Cell Signaling Technologies | Cat #3873S, RRID:AB_1904178 |
| Anti-GAPDH          | Cell Signaling Technologies | Cat #97166S, RRID:AB_2756824 |
| Anti-Actin          | Sigma Aldrich | Cat #A5441, RRID:AB_476744 |
| Goat anti-Met (hHGFR) | R & D Biosystems | Cat #AF276, RRID:AB_355289 |
| Anti-mouse IgG-HRP  | Cell Signaling Technologies | Cat #7076S, RRID:AB_330924 |
| Anti-rabbit IgG-HRP | Cell Signaling Technologies | Cat #7074S, RRID:AB_2099233 |
| Anti-HA (ELISA)     | GeneTex | Cat #115044-01, RRID:AB_11169023 |
| Anti-MYC (ELISA)    | Santa Cruz Biotechnology | Cat #sc40, RRID:AB_627268 |
| Anti-V5 (ELISA)     | BioRad | MCA1360G, RRID:AB_1172162 |

| Chemicals, peptides and recombinant proteins | | |
| EZ-link Sulfo-NHS-SS-Biotin | Pierce Thermo Scientific | Cat#21331 |
| Human HGF | Sigma Aldrich | Cat# H9661 |
| Tetraacycline | BioShop | Cat #TET701.25 |
| Hygromycin B | BioShop | Cat #HYG003.10 |
| Crizotinib | Sigma Aldrich | Cat #877399-52-5 |
| Puromycin dihydrochloride | BioShop | Cat #PFR333.100 |
| Protein G agarose conjugated beads | EMD Millipore | Cat #16-266 |
| Streptavidin Agarose Beads | Pierce Thermo Scientific | Cat #20347 |
| Polyethyleneimine Max® (PEI) | Polysciences | Cat #02371 |
| Gibson Assembly® Master Mix | New England Biolabs | Cat #E26115 |
| KAPA HiFi Hot Start Ready Mix | KAPA Biosystems | Cat #KM2605 |
| X-tremeGENE™ HP DNA transfection reagent | Sigma Aldrich | Cat #6366244001 |
| DharmaFECT Reagent 1 | Horizon Discovery | Cat #T-2001-03 |

| Deposited data | | |
| Uncropped western blot images | Mendeley | https://doi.org/10.17632/yrdmcm8fw4j.1 |

| Experimental models: Cell lines | | |
| HEK293 | Laboratory of Dr. Jason Moffat | N/A |
| Flp-In™ T-REx™ 293 | Thermo Scientific | Cat #R78007 |
| NCIH2122 | Laboratory of Dr. Ming Tsaao | N/A |
| NCIH1568 | ATCC | NCI-H1568 [H1568] (ATCC®CRL-S876) |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Igor Stagljar (igor.stagljar@utoronto.ca).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact upon request.

Data and code availability
- Original western blot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table. Known Met interactors depicted in the Met-interactome map (Figure 2) analyzes existing, publicly available data from Integrated Interactions Database1 ver. 2021-05. The database information is listed in the key resources table.

Softwares and algorithms
- ImageJ National Institute of Health, USA N/A
- MetaMorph Molecular Devices N/A
- GraphPad Prism N/A
- NaViGaTOR ver. 3.0.16 Laboratory of Dr. Igor Jurisica https://www.cs.toronto.edu/~juris/jlab/tools.html
- Integrated interactions database (IID) ver. 2021-05 Laboratory of Dr. Igor Jurisica http://ophid.utoronto.ca/iid

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell culture
Cells were maintained in DMEM (WISENT) (Met-MaMTH, Met-SIMPL, HEK293) or RPMI (NCIH2122, NCIH1568, NCIH2122 BLNK KO, NCIH2122 AAVS1-targeted) with 10% fetal bovine serum (WISENT or Gibco) and 1% penicillin-streptomycin cocktail (WISENT) under standard condition at 37°C and 5% CO2.

METHOD DETAILS

Stable cell line generation
MaMTH reporter stable cell lines with Met bait was created using Flp-In TREx system (Thermo Fisher). FLP-compatible HEK293 MaMTH reporter cells were seeded in 6 well plates (150,000 cells/well) and grown for 16 h. Cells were transfected using XtremeGENE9 DNA transfection reagent (Roche) as per manufacturer’s protocols. Cells were grown for 48 h and were split into DMEM/10%FBS/1%PS containing 100 μg/mL hygromycin for selection. GFP-transfected wells were used as negative control for selection. Cells were grown until individual foci appeared and foci from one well were pooled and expanded into tetracycline-inducible MaMTH-Met stable cell lines for MaMTH assay.
To generate BLNK knockout cell lines using CRISPR-Cas9-mediated gene editing, protocols provided at [https://pharm.ucsf.edu/sites/pharm.ucsf.edu/files/xinchen/media-browser/PX330%20cloning%20protocol.docx](https://pharm.ucsf.edu/sites/pharm.ucsf.edu/files/xinchen/media-browser/PX330%20cloning%20protocol.docx) (Last accessed on May 11 2017) was used.

BLNK-specific guide sequence (ACCTCAACTTCTGACTGGCG) was cloned into PX330 vector (Addgene). NCIH2122 cells were seeded in 6 well plates (200,000 cells/well) and co-transfected constructs using XtremeGene9 HP DNA transfection reagent (Roche). 48 h after transfection, cells were maintained in media with puromycin (0.5 μg/mL) for 2 weeks until individual foci appeared. Foci were then picked and propagated into isogenic clonal cell lines. Knockout was confirmed with western blotting.

**Constructs and transfections**

All destination constructs were generated using gateway cloning as per manufacturer’s instructions.

For MaMTH assays, Met (OpenFreezer V9936) and candidate prey entry plasmids (Human ORFeome v8.1) were cloned into C-terminally tagged MaMTH bait and N-terminally-tagged prey vectors. MaMTH bait vectors contained GAL4TF which consists of amino acids 1-147 of GAL4 fused to amino acids 364-550 of mouse NF-κB (GAL4 (1-147)-mNFκB (364-550). For SIMPL assays, Met and candidate prey entry plasmids were cloned into appropriately tagged SIMPL bait and prey vectors. For signaling and immunoprecipitation of BLNK in HEK293 cells, N terminally FLAG-tagged BLNK was generated by cloning BLNK entry plasmid (Source: Human ORFeome V8.1) into pCMV5-based N terminal FLAG tag vector (Addgene). For co-immunoprecipitation assays in HEK293 cells, C terminally V5-tagged Met and N terminally GFP-tagged BLNK, LCP2 and BLK, N terminally FLAG-tagged GRB2 were generated by cloning entry plasmids (OpenFreezer/Human ORFeome V8.1) into pCMV5-based V5, GFP and FLAG-tagged vectors.

For site-directed mutagenesis, KAPA quick-change system was used as per manufacturer’s protocols. To generate BLNK without STOP codon and BLNK ΔSH2, Gibson Assembly (NEB) was used as per manufacturer’s protocols.

All transient transfections were performed using polyethylenimine (PEI) 5 h after seeding. For siRNA transfections, cells were transfected with siRNA 24 h after seeding using DharmaFECT Reagent 1 (Horizon Discovery) according to manufacturer’s instructions.

**MaMTH assays**

Protocols for MaMTH assays have been described elsewhere. Met-MaMTH cells were seeded in 96-well plates at 20,000 cells per well and grown at 37°C/5% CO2. 120ng prey construct/well was transfected and 24 h after, cells were induced with tetracycline (0.5 μg/mL) for another 24 h. Luciferase activity was measured via chemiluminescence using 20 μL coelenterazine per well. At least three technical replicates were assayed in every experiment, and the results are representative of at least three independent experiments.

To account for changes in prey expression levels between different genes, MaMTH prey constructs were tagged with red fluorescent protein (mCherry) via a 2A self-cleaving peptide. The mCherry fluorescent signal was measured and assumed to be representative of the prey expression in each well, and therefore luciferase activity was normalized to their corresponding mCherry fluorescence signal. Preys were considered to be expressed if the mCherry fluorescent signal was higher than that of mock-transfected cells, which were transfected with an unrelated transcription factor LEXA (non-mCherry tagged).

Transcription factor GAL4-NFκB was included to represent the theoretical maximum of the luciferase signal in the system in each plate. This enables standardization between plates where large library screening is concerned. Mock transfection represented the theoretical minimum of both mCherry and luciferase signal and was subtracted from the readings for each well prior to analysis. The luciferase signal from each well was then expressed as a percentage of transcription factor signal, after which the average and SD were calculated.

**Cell lysis and western blotting**

Cells were washed 1X with ice-cold PBS (Thermo Scientific) and lysed with lysis buffer (50 mM-β-Glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM Dithiothreitol, 0.1 mM sodium orthovanadate, 1%...
(v/v) Triton X-100, 1mM Benzaminidine, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, 1 μg/mL Pepstatin A (solvent: ethanol) on ice. Lysates were spun at 14,000 rpm for 10 min at 4°C, and 3 parts of supernatant added to 1-part 4X Laemmli buffer (40% (v/v) glycerol, 0.2 M tris pH 6.8, 0.4% (w/v) bromophenol blue, 8% (v/v) SDS, 0.1 M diethrothreitol) and boiled at 95°C for 5 min. Protein quantitation of lysates was performed using Pierce BCA protein assay kit (Thermo Scientific).

Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (BioTrace). Following transfer, membranes were blocked for 1 h at room temperature with 2% (w/v) BSA (BioShop) in TBS-T. After blocking, membranes were incubated with primary antibody in TBS-T at 4°C for 16 h, washed 3X with TBS-T and incubated with secondary antibody in TBS-T for 1 h at room temperature. Membranes were washed 3X with TBS-T and chemiluminescence was detected using either Ultra-Scence western substrate (FroggaBio) or for lower abundant epitopes, Amersham ECL selected western blotting detection reagent (GE Healthcare). Unless otherwise stated, all western blots are representative of three independent experiments.

**Signaling assays**

For signaling assays with Met-MaMTH cells, cells were seeded in 12 well plates (100,000 cells/well) and grown for 16 h at 37°C/5% CO2. Cells were serum starved with DMEM/0.1%FBS with appropriate concentrations of tetracycline (BioShop) as needed. For ligand stimulation, 25 ng/mL HGF was added for indicated times, 16 h after starvation, proceeded by western blotting.

For signaling assays in HEK293 cells, cells were seeded in 24 well plates (50,000 cells/well) and transfected with either 125 ng of FLAG-BLNK and 125ng of empty vector or 250 ng of empty vector (mock). 24 h after transfection, cells were serum starved with DMEM/0.1% FBS for 16 h. Cells were then stimulated with 25 ng/mL HGF for indicated time points, proceeded by western blotting. For BLNK mutant signaling assays, data is representative of two independent experiments.

For signaling assays in NCIH2122 and NCIH1568 the following protocol was performed; Cells were seeded in 24 well plates (50,000 cells/well) and siRNA was transfected at a final concentration of 50 nM 24 h after seeding. Knockdown was allowed to proceed for 72 h. For NCIH1568, cells were serum starved with RPMI/0.1% FBS for 16 h, and for NCIH2122 cells were serum starved with RPMI/0.1% FBS for 2 h, prior to ligand stimulation. For ligand stimulation, 25 ng/mL HGF was added for indicated time points, proceeded by western blotting.

**Surface biotinylation assays**

For surface biotinylation of Met-MaMTH cells, cells were seeded at in 6 well plates (200000/well) and incubated for 24 h. Met bait expression was induced with tetracycline (0.5 μg/mL) for 24 h. Cells were then washed 1X with ice-cold PBS with MgCl2 and CaCl2 (Sigma Aldrich) and incubated with 0.5 mg/mL Sulfo-NHS-SS-Biotin in PBS with MgCl2 and CaCl2 for 30min at 4°C. Excess biotin was quenched by washing 3X with ice-cold DMEM containing 0.1% (w/v) BSA and 1X with PBS++. Cells were lysed in lysis buffer (50 mM Tris pH 7.5, 1.5 mM EGTA, 1 mM EDTA, 50 mM NaCl, 0.1 mM sodium orthovanadate, 1% (v/v) Triton X-100, 1mM Benzaminidine, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, 1 μg/mL Pepstatin A (solvent: ethanol)) on ice. Lysates were spun at 14,000 rpm for 10 min at 4°C. Protein quantitation of the supernatant was performed using BCA assay. Equal protein was nutated with 50μL of 50% slurry streptavidin-agarose conjugated beads for 2 h at 4°C. Input fractions were collected from lysates prior to incubation with beads, mixed with 4X Laemmli buffer at 3 parts lysate to 1 part at boiled at 95°C for 5 min. After nutation, beads were washed 2X with LiCl-Tris (0.1 M Tris pH 8.0, 0.5 M LiCl) and 2X with wash buffer (50 mM tris pH 7.5, 1M EDTA, 1.5 mM EDTA, 0.1 mM Sodium Orthovandate, 50 mM NaCl), boiled in 2X Laemmli sample buffer and run on 10% SDS-PAGE gel, followed by western blotting.

For surface biotinylation of NCIH1568 and NCIH2122, cells were seeded in 6 well plates (200,000 cells/well) and siRNA transfection (final concentration of 50 nM siRNA) was performed 24 h after seeding. Knockdown was allowed to proceed for 72 h. For NCIH1568, cells were serum starved with RPMI/0.1% FBS for 16 h, and for NCIH2122 cells were serum starved with RPMI/0.1% FBS for 2 h, prior to assay using protocol described above.

**SIMPL and SIMPL-ELISA**

For both SIMPL and SIMPL-ELISA protocols described elsewhere were followed.71
For SIMPL, HEK293 TRex-compatible cells were seeded in 12 well plates (60% confluence at the time of transfection) and transfected with appropriate bait and prey plasmids. 500ng total DNA/1mL media/well at a 1:1 ratio was used for transfection. 24 h after transfection, expression was induced with 1 µg/mL tetracycline for 24 h, proceeded by western blotting.

For SIMPL-ELISA, HEK293 TRex-compatible cells were seeded in 96 well plates (15,000 cells/well) and transfected with bait and prey plasmids as indicated above. 24 h after transfection, expression was induced with 1 µg/mL tetracycline for 24 h. Cells were then lysed with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% v/v Triton X-100, 1 mM Benzenimidine, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, 1 µg/mL Pepstatin A (solvent: ethanol)) via sonication and subjected to sandwich ELISA. 384 well-ELISA plate was coated for 1 h at 37°C with primary antibodies (α-FLAG, α-V5) diluted 1:100 in PBS (20µL/well), and blocked with blocking buffer (80µL/well) (2% BSA in PBS-T) overnight at 4°C. Lysates were added to coated ELISA wells (20µL/well) and incubated with gentle rocking for 3 h at 4°C. Wells were then washed 3X with PBS-T (60µL/well) and incubated with secondary antibodies (α-HA, 1:2000, α-MYC, 1:5000) in blocking buffer (30µL/well) for 1 h at room temperature. Wells were then washed 3X with PBS-T (60µL/well). Chemiluminescence was detected with SuperSignal ELISA Pico Reagent (Thermo Scientific) using ClarioStar (BMG Labtech) with an integration time of 0.2 s/well. The spliced was normalized to bait and prey expression using the following formula:

\[
\frac{\text{Spliced Signal (Bait exp)} - \text{Spliced Signal (Prey exp)}}{\text{Bait exp}}
\]

**Co-immunoprecipitation**

For orthogonal validation of Met-BLNK interactions, HEK293 cells were seeded in 6 cm plates (to 50% confluence at transfection) and transfected with necessary constructs. 2µg total DNA/2 mL media/plate at 1:1 ratio was used for transfection. 48 h after transfection, cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 1.5 mM EGTA, 1.5mM MgCl2, 10% v/v Glycerol, 1% v/v Triton X-100, 0.1 mM Sodium Orthovanadate, 150 mM NaCl, protease inhibitor cocktail (Roche)) and lysates were spun at 14,000 rpm for 10 min at 4°C. Protein quantitation of the supernatant was performed using BCA (Pierce) assay. Equal protein was nutated with primary antibody at appropriate concentrations for 2 h. Then 50µL of 50% slurry Protein G agarose conjugated beads were added and nutated for 2 h at 4°C. Whole cell lysate fraction was collected prior to incubation with beads, mixed with 4X Lamelli buffer at 3 parts lysate to 1 part at boiled at 95°C for 5 min. After nutation, beads were washed 2X with Co-IP wash buffer (50 mM HEPES pH 7.5, 1.5 mM EGTA, 1.5mM MgCl2, 10% v/v Glycerol, 0.1 mM Sodium Orthovanadate, 150 mM NaCl), boiled in 40 µL of 2X Lamelli sample buffer and run on 10% SDS-PAGE gel, followed by western blotting.

**Immunoprecipitation**

For BLNK immunoprecipitation to detect Met-dependent phosphorylation, Met-MaMTH cells were seeded in 6 cm plates (to 50% confluence at transfection) and transfected with N terminally FLAG-tagged BLNK or empty FLAG vector at 1µg total DNA/1 mL media/well.

24 h after transfection, expression Met bait was induced with tetracycline (1 µg/mL). 48 h after transfection, cells were lysed in lysis buffer (50 mMβ-Glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM Dithiothreitol, 0.1 mM sodium orthovanadate, 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche)) and lysates were spun at 14,000 rpm for 10 min at 4°C. Protein quantitation of the supernatant was performed using BCA (Pierce) assay. Equal protein was nutated with primary antibody at appropriate concentrations for 2 h. Then 50µL of 50% slurry Protein G agarose conjugated beads was added and nutated for 2 h at 4°C. Whole cell lysate fraction was collected prior to incubation with beads, mixed with 4X Lamelli buffer at 3 parts lysate to 1 part at boiled at 95°C for 5 min. After nutation, beads were washed 2X with LiCl-Tris (0.1 M Tris pH 8.0, 0.5 M LiCl) and 2X with IP wash buffer (50 mM β-Glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM Dithiothreitol, 0.1 mM sodium orthovanadate), boiled in 40 µL of 2X Lamelli sample buffer and run on 10% SDS-PAGE gel, followed by western blotting.
For Met immunoprecipitation to assay phosphorylation of Met, NCIH2122 and NCIH1568 cells were seeded in 6 cm plates (5 x 10^6 cells/plate) and siRNA was transfected at final concentration of 50 nM 24 h after seeding. Knockdown was allowed to proceed for 72 h. As with signaling assays, for NCIH1568, cells were serum starved with RPMI/0.1% FBS for 16 h, and for NCIH2122 cells were serum starved with RPMI/0.1% FBS for 2 h, prior to ligand stimulation. For ligand stimulation, 25 ng/mL HGF was added for indicated time points, proceeded by immunoprecipitation.

NCIH2122 cells were lysed in lysis buffer (50 mM Tris pH 7.5, 1.5 mM EGTA, 1 mM EDTA, 50 mM NaCl, 0.1 mM sodium orthovanadate, 1% (v/v) Triton X-100, 1 mM Benzaminidine, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, 1 µg/mL Pepstatin A (solvent: ethanol)) on ice. Lysates were spun at 14,000 rpm for 10 min at 4°C. NCIH1568 cells were lysed in RIPA lysis buffer (Cell Signaling Technologies) with Protease inhibitor cocktail (Roche) and sonicated 2 rounds for 7 s each. The lysates were then kept on ice for 30 min, and spun at 14,000 rpm for 10 min at 4°C. For both cell lines, protein quantitation of the supernatant was performed using BCA assay. Equal protein was nutated with primary antibody for 2 h at 4°C followed by nutation with 50 µL of 50% slurry protein G agarose conjugated beads for 2 h at 4°C. Input fractions were collected from lysates prior to incubation with beads, mixed with 4X Laemmli buffer at 3 parts lysate to 1 part oil at 95°C for 5 min. After nutation, beads were washed 2X with LiCl-Tris (0.1 M Tris pH 8.0, 0.5 M LiCl) and 2X with wash buffer (50 mM tris pH 7.5, 1mM EDTA, 1.5 mM EGTA, 0.1 mM Sodium Orthovanadate, 50 mM NaCl), boiled in 2X Laemmli sample buffer and run on 10% SDS-PAGE gel, followed by western blotting.

**Immunofluorescent staining**

For immunofluorescent staining, cells were plated on coverslips (Belco Glass Inc) for 48 h, and siRNA transfections were done 24h following plating. Cells were washed in 100% Methanol for 10 min at -20°C. For staining blocking was done in 2% BSA in washing buffer (PBS with 0.2% Triton X-100 and 0.05% Tween 20) for 30 min. Primary antibodies (1:100) and secondary antibodies (1:500) treatments were in blocking solution for 60 and 45 min, respectively. Where applicable, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) prior to mounting using Immu-Mount (Thermo Shandon Inc). Confocal images were acquired on a Zeiss LSM800 laser scanning confocal microscope (Carl Zeiss) with a 63X objective and analyzed using Zen software (Carl Zeiss) and MetaMorph software (Molecular Devices).

**Met trafficking assays**

Met trafficking and immunofluorescence-based recycling assays have been described elsewhere. Briefly, cells were incubated in 0.1%FBS in RPMI with 100 µg/ml cycloheximide (Sigma) for 2 h, and cold loaded with 0.5nM HGF the presence of cycloheximide for another 1h at 4°C. HGF-media was removed by washing 3 times with 0.2% BSA in Leibovitz-15 medium at 4°C, prior to a return to 37°C for 15-30 min to allow for Met to recycle or proceed through the degradative pathway at the end of which cells were fixed in 100% Methanol for 10 min at -20°C and then stained.

**Puncta distribution analysis**

Puncta distribution analysis was performed using MetaMorph software. Individual cells were traced manually. The nucleus was detected in the DAPI-channel, and the plasma membrane (PM) was detected using either phalloidin or another stain with enough cytoplasmic presence to delineate the cell limits. A distance map was created where values indicate the Euclidean distance of any point to the PM-mask. Using the ‘granularity’ application, puncta were identified using an intensity and size threshold and a binary mask of the puncta was created. The puncta-mask was then transferred to the ‘PM-Euclidian Distance Map’ so that the distance of each individual puncta to PM (dPM) could be obtained. The average dPM per cell was calculated and plotted using a custom R script. For each condition analyzed a minimum of 45 cells were used from samples collected across a minimum of 2 experimental replicates. This method utilized object-based quantification to analyze Met puncta distribution. Refer to Figure S7A for a pictorial representation for calculation of the average puncta distance to PM. Refer to Figure S7B for a pictorial representation of calculation of the relative puncta distance to PM.

**Soft agar colony formation assay**

12 well plates were prepared with base layer with 0.5% noble Agar in RPMI +10% FBS and top layer with 0.35% noble agar in RPMI media with 10% FBS, respectively. 11,250 cells/well were plated in the top
agar layer. The feeder layer contained RPMI media with 10% FBS. The assay was incubated for 21 days and was subsequently stained with 0.2% nitroblue tetrazolium in PBS for 3 h and imaged using an Epson scanner. ImageJ colony counter plug-in with pixel range 30–600 and circularity range 0.85–1.0 were used to count colonies.

Transwell migration assay
Cells were transfected with siRNA for a final concentration of 50 nM for 72 h and serum starved with RPMI +0.1% FBS for 24 h (48 h after transfection) prior to assay. Cells were then seeded (90,000 cells/insert) in 6.5 mm Corning Costar transwell chambers with 8 μm pore size in duplicates and incubated at 37°C for 24 h. Migration media in both top and the bottom chamber contained RPMI +5% FBS and in conditions measuring migration toward HGF, the bottom chamber was additionally supplemented with 50 ng/mL HGF. 24 h later, cells in the transwell insert were fixed with 10% formalin, followed by staining in 0.5% (w/v) crystal violet in double distilled water for 2 h. Transwell inserts were rinsed with water to remove excess crystal violet and cells on the inside of the transwell membrane were removed using a Q-tip. 100,000 cells per condition were collected at the transwell stage and was verified for the siRNA-mediated knockdown of BLNK, using western blotting.

QUANTIFICATION AND STATISTICAL ANALYSIS
Quantitative data analysis was performed using Prism software (GraphPad Software v9, San Diego, CA). Each experiment was independently replicated a minimum of two times, and in quantitative experiments, each time with at least three technical replicates (different wells in the same multi-well plate, n indicated in figure legends), unless otherwise stated. More information can be found in method details. Data are represented as mean ± SD. Unpaired two-tailed Welch’s t test, unpaired two-tailed Student’s t test or one-way ANOVA was conducted as needed. Asterisks are as follows: *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001. p< 0.01 was considered significant for MaMTH assays and p< 0.05 was considered significant for all other assays. Grubbs test was used to remove any outliers in quantitative ELISA.