MARCH-mediated Post-translational regulation of Hepatocyte Growth Factor Receptor at the cell surface.

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Abstract

The hepatocyte growth factor receptor (MET, c-MET) plays a key role in both tumorigenesis and metastasis. Furthermore, increased MET protein expression correlates with poor prognosis in a variety of cancer types. Our preliminary data indicate that MET is a substrate for MARCH1, MARCH4 and/or MARCH8 E3 ubiquitin ligases. We hypothesize that MARCH1/4/8 can limit MET function through modification of membrane proximal lysine residues with ubiquitin, targeting MET for lysosomal degradation. To test this hypothesis, we will reconstitute MET knockout cell lines with either wild-type MET (control) or a mutant of MET where all four putative (membrane proximal) ubiquitination acceptor sites are mutated to Arginine (4KR). Using these reconstituted cell lines, we will first validate that MET is a MARCH1/4/8 substrate and determine if MET is regulated through ubiquitination of these four membrane proximal lysine residues. If so, the biological role of MET ubiquitination at these residues will also be examined. Taken together, these studies will investigate a potential new regulation pathway of MET that could be exploited to generate new therapeutics to limit MET function in cancer cells.

BACKGROUND

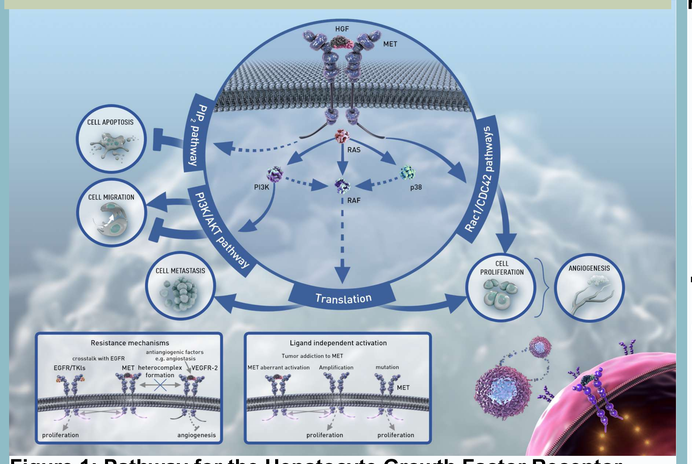

Figure 1: Pathway for the Hepatocyte Growth Factor Receptor, MET.

MET is a receptor tyrosine kinase with high binding affinity for the ligand Hepatocyte Growth Factor. Shown above HGF binds to MET to induce proliferation and activate other ligand dependent post-translational pathways. Adapted from Lilly Oncology Pipeline.

- The Hepatocyte Growth Factor Receptor (MET) is amplified in many cancers and a key therapeutic target to limit tumor cell growth
.
- The Membrane Associated RING-CH (MARCH) proteins are E3 ubiquitin ligases that modify transmembrane proteins to regulate their surface expression.

- **<u>Hypothesis: MARCH1/4/8 can limit MET function by modification of membrane proximal lysine residues, targeting MET for degradation.</u>**

- This study will characterize a novel mechanism of regulating MET surface expression and function through MARCH-mediated ubiquitination.

- A better understanding of MARCH-mediated MET expression could impact new therapeutics designed limit MET function in cancer cells.

SPECIFIC AIMS

Aim 1: Generate tools to study MARCH –Mediated regulation of MET. ✓

i. Design and validate sgRNAs targeting human MET *(completed)*.
ii. Generate MET KO Flp-In™ T-REx™ HeLa cells *(completed)*.
iii. Clone *wt* and 4KR MET with c-terminal HA tags into pcDAN5 FRT-TO vectors *(completed)*.
iv. Rescue MET KO HeLa FlpIn TREX cells with either:
  - empty vector
  - MET_wt-HA
  - MET_4KR-HA

Aim 2: Characterize MET_4KR mutant ✓

i. Downregulation of *wt* vs. 4KR MET upon transient transfection of MARCH1, MARCH4 or MARCH8.

METHODS

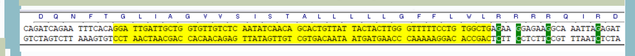

Figure 2: Generation of MET_4KR.

The nucleotide sequence of MET coding for the transmembrane domain (yellow) and flanking membrane proximal residues are depicted with the MET reading frame is shown above. The mutations changing the membrane proximal Lysine residues to Arginine are highlighted in green. Note the full ORF for *wt* MET isoform2 (NM_000245.3) and the 4KR mutant were cloned into pcDNA5_FRT-TO with a C-terminal HA epitope tag (YPYDVDPYA).

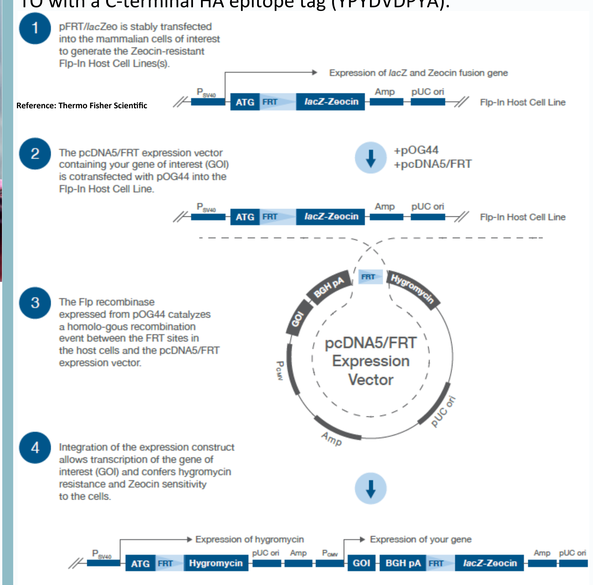

Figure 3: Workflow to generate Inducible HeLa Flp-In™ T-REx™ cell lines to rescue MET KO.

This system permits generation of stable isogenic HeLa cell lines where MET expression is Doxycycline inducible. (1) HeLa Flp-In™ T-REx™ cells were described previously (Kean et al 2013) (2 and 3) Co-transfection with pOG44 and pcDNA5/FRT-MET-wt or 4KR expression vectors allows for insertion of MET via Flp recombinase-mediated DNA recombination. (4) MET ; wt or 4KR is expressed thru Doxycycline treatment. Adapted from Thermo Fisher.

RESULTS

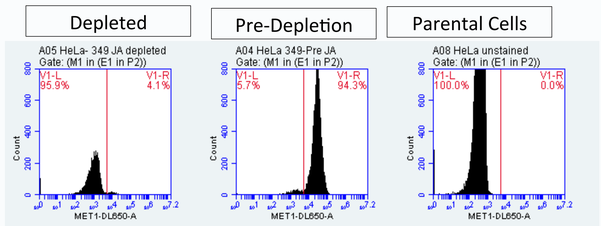

Figure 4: Generation of MET KO Hela Flp-In™ T-REx™ cells

Hela Flp-In™ T-REx™ cells were transfected with pSpCas9-2A-Puro encoding a MET specific sgRNA(#349). Cells were transiently selected with Puromycin and cultured for 10 days. After 10 days a sub-population of MET KO cells were purified by magnetic depletion with anti-human MET. Depletion of MET positive cells was verified by flow cytometry with a non-cross competing MET antibody.

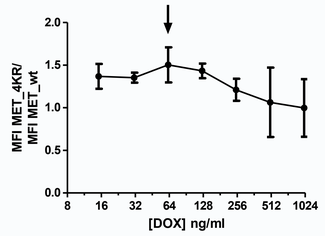

Figure 5: Doxycycline Induction of MET_wt and MET-4KR.

Rescued MET1 KO Hela Flp-In™ T-REx™ cells were treated with indicated doses of Doxycycline for 40 hours and surface MET expression determined by flow cytometry. The MFI of MET1 KO cells was subtracted and the ratio of the 4KR: wt MET is shown as a function of [Dox]. The arrow indicates the dose used for subsequent experiments.

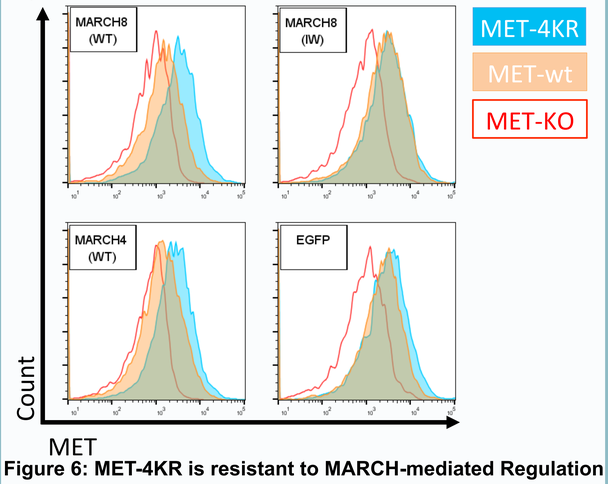

Figure 6: MET-4KR is resistant to MARCH-mediated Regulation

MET KO HeLa FlpIn TREX cells rescued with either empty vector (MET-KO), MET-wt or MET-4KR were induced with 62.5ng/ml of Doxycycline for 24hours and then transfected with the indicted MARCH-EGFP expression vectors. 24 hours post transfection, single cells suspensions of cells were surface stained for MET and viability with PI. Histograms of MET expression of viable (PI negative)/GFP+ cells are depicted.

CONCLUSIONS

- MET is a Substrate of MARCH1, MARCH4 and MARCH8, and the catalytic activity of MARCH is required to down regulate MET from the cell surface.

- MET_4KR is expressed at a higher level at the cell surface. Since expression of both wt and 4KR MET are under control of the same transcriptional system (DOX) and both cell lines are isogenic clones with a single integration, this suggests that MET is regulated post-translationally, via ubiquitination of membrane proximal lysine residues on the cytoplasmic tail.

- MET 4KR is resistant to the effects of MARCH1, MARCH4 and MARCH8 suggesting that MARCH proteins regulate MET surface expression through ubiquitination of membrane proximal lysine residues.

Future Directions

- Biochemically characterize MARCH-mediated Ubiquitination of *wt* vs. 4KR MET upon over expression of MARCH1, MARCH4 or MARCH8 in HeLa FlpIN T-REX MET rescue lines.
- Compare signaling of *wt* MET vs. 4KR MET upon HGF stimulation in HeLa FlpIN T-REX MET rescue cell lines.
- Compare signaling of *wt* MET with and without MARCH1, MARCH4 or MARCH8 over expression upon HGF stimulation in HeLa FlpIN T-REX MET1 rescue and HeLa MET1 rescue cell lines.

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