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Review

REVIEW

Regulation of the *MET* oncogene: molecular mechanisms

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Abstract

The *MET* oncogene is a predictive biomarker and an attractive therapeutic target for various cancers. Its expression is regulated at multiple layers via various mechanisms. It is subject to epigenetic modifications, i.e. DNA methylation and histone acetylation. Hypomethylation and acetylation of the *MET* gene have been associated with its high expression in some cancers. Multiple transcription factors including Sp1 and Ets-1 govern its transcription. After its transcription, *MET* mRNA is spliced into multiple species in the nucleus before being transported to the cytoplasm where its translation is modulated by at least 30 microRNAs and translation initiation factors, e.g. eIF4E and eIF4B. *MET* mRNA produces a single chain pro-Met protein of 170 kDa which is cleaved into α and β chains. These two chains are bound together through disulfide bonds to form a heterodimer which undergoes either N-linked or O-linked glycosylation in the Golgi apparatus before it is properly localized in the membrane. Upon interactions with its ligand, i.e. hepatocyte growth factor (HGF), the activity of Met kinase is boosted through various phosphorylation mechanisms and the Met signal is relayed to downstream pathways. The phosphorylated Met is then internalized for subsequent degradation or recycle via proteasome, lysosome or endosome pathways. Moreover, the Met expression is subject to autoregulation and activation by other EGFRs and G-protein coupled receptors. Since deregulation of the *MET* gene leads to cancer and other pathological conditions, a better understanding of the *MET* regulation is critical for Met-targeted therapeutics.

Introduction

Copious evidence has indicated that the *MET* oncogene plays a causative role in cancer development, i.e. tumor initiation via cancer stem cell formation, tumor progression via cell proliferation and survival, drug resistance and metastasis (1). For example, the Met protein from primary melanoma cells can be packed into tumor exosomes and delivered to distal organs, i.e. bone marrows and lungs, to prime these organs for future metastases (2).

In various cancers, *MET* expression is frequently elevated. Amplification of the *MET* gene might contribute to this elevated expression in some cases. However, the majority of cancers with high Met protein expression do not have amplification of the gene (3). This discordance indicates that regulation of the *MET* expression is aberrant in these cancers. Mounting evidence has demonstrated that the expression of the *MET* gene is subject to various layers of regulation, i.e. epigenetic, transcriptional, post-transcriptional regulations.

Due to its importance in carcinogenesis and cancer progression, targeting Met has become an attractive strategy in cancer treatment (1). However, Met also displays some antitumor effects in neutrophils (4). This complicated biology means that the successful implementation of Met-targeting therapeutics requires a better understanding of this oncogene. We hereby summarize mechanisms by which *MET* expression is regulated, with an emphasis on advances in recent years.

Regulation by epigenetic modifications

Epigenetic modifications of the genome, i.e. DNA methylation and histone acetylation or methylation, are essential for regulating gene expression. These modifications change the architectures of the genome and thus alter the accessibility of transcription factors and RNA polymerases to the target genes (5).

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Abbreviations

AP-1	activated protein-1
GPCRs	G-protein couple receptors
HDAC	histone deacetylase
miRNA	microRNA
NLS	nuclear localization signal
RTK	receptor tyrosine kinase
SAHA	suberoylanilide hydroxamic acid
UTR	untranslated region

DNA methylation

DNA methylation is a process covalently modifying the genome by DNA methyltransferases. These enzymes transfer the methyl group from S-adenosyl-methionine to the 5′ position of cytosines of CpG islands. Hypermethylation of DNA may recruit some proteins e.g. methyl-CpG binding domain proteins or polycomb group proteins, to the DNA to initiate genomic silencing. Alternatively, DNA methylation may form a physical hindrance to some transcription factors and thus block transcription (6).

Methylation of the *MET* gene plays an indispensable role modulating its expression in some cancers. It has been shown that the *MET* gene is hypomethylated and thus overexpressed in pancreatic ductal adenocarcinomas (PDAC) (7). Importantly, the hypomethylation status of the *MET* gene is correlated with low overall survival and disease-free survival (7).

Of note, *MET* expression may also be regulated through methylation of L1 (Long interspersed nuclear element), a non-LTR retrotranspon widely scattered in the human genome. A copy of L1 is present in the *MET* intron between Exon 2 and Exon 3 (8). L1 contains an antisense promoter that drives expression of a fusion transcript of *MET* (L1-*MET*). This promoter is usually hypermethylated and L1-*MET* expression is relatively low. However, when the cells are treated with 5-aza-2′-deoxycytidine, a DNA methyltransferases inhibitor used for myeloid leukemia treatment, the *MET* mRNA expression is approximately 7-folds higher than the control (8). The biological roles for L1-*MET* mRNA expression seem controversial, however. In DKO and HCT16 cells, L1-*MET* mRNA expression interferes with the normal expression of *MET* and is inversely correlated with *MET* mRNA and protein (8), while in hepatic cell cancer (HCC) tissues, L1-*MET* promotes Met activity and is correlated with metastasis (9). These results suggest that the biological role of L1-*MET* is cellular context dependent.

The *MET* expression may also be regulated indirectly through methylation of its regulators, e.g. FBLN-3 (Fibulin-3), Glutathione peroxidase 3 (GPx3), Suppressor of cytokine signaling 1 (SOCS1) and MYO5B. For example, the *MET* expression is usually elevated in human sporadic insulinomas but is suppressed by 5-aza-2′-deoxycytidine in MIN6 cells. It turns out that in insulinomas, the promoter of the long non-coding RNA maternally expressed gene 3 (IncRNA Meg3) is hypermethylated, silencing the expression of IncRNA Meg3. Since IncRNA Meg3 inhibits *MET* expression, this methylation alleviates this inhibition (10). These observations may explain why treatment of lung cancer cell lines with 5-aza-2′-deoxycytidine inhibits the expression of *MET*.

Histone acetylation

Like DNA methylation, histone modifications, i.e. acetylation and methylation, also play an essential role in regulating gene expression. Histone acetylation is catalyzed by histone acetyltransferases. Acetylation of lysine residues at the N-terminus of histones neutralizes positive charges and thus decreases interactions between histones and DNA, rendering transcription factors and RNA polymerases more access to the targeted regulatory regions. Histone acetylation is a dynamic process and is constantly counteracted by the action of histone deacetylases (HDACs) (11).

Several studies have demonstrated that *MET* expression may also be subject to histone modifications. For example, siRNA depletion of HDAC 1–3 in several HCC cell lines increases histone acetylation and consequently the abundance of *MET* mRNA (12). Similarly, treatment of MDA-MB-231 cells with NaBu (sodium butyrate, a HDAC inhibitor) leads to an increase of the *MET* expression (13).

One possible mechanism for this regulation is through Death-Domain Associated Protein (Daxx) which binds to the *MET* promoter directly. Upon binding, Daxx recruits HDAC2 to the *MET* promoter and thus represses *MET* transcription. This scenario is supported by the observation that *MET* abundance is inversely correlated with Daxx in breast cancer cell lines and metastatic tissues (14).

Epigenetic regulation of *MET* expression seems to be dependent upon cellular context. For example, in the prostate cancer cell line PC3 and the non-small lung cancer cell line A549 treated with suberoylanilide hydroxamic acid (SAHA), an inhibitor of HDACs, Met protein abundance was not affected (15).

Regulation by transcription factors

An analysis of the *MET* promoter shows that the 300bp fragment upstream of the transcription start site is sufficient to drive the expression of *MET*. The promoter of the *MET* gene does not have canonic TATA or CAAT elements. Instead, several Sp1 binding motifs (G/TGGGCGGG/AG/AC/T) are required for *MET* expression, since alterations of these Sp1 motifs largely abolish the *MET* promoter activity (16,17). Sp1, together with PC4, may also bind to a region approximately 20bp upstream of the transcription start site (18). A SNP (G >C, rs1858830) in this region reduces binding of Sp1 and PC4, leading to lowered expression of *MET* in some autism patients (18).

A recent study reveals that the binding of Sp1 to the *MET* promoter may be affected by the architecture of the promoter. Between −48 to −26nt of the human *MET* promoter, a highly GC rich sequence is able to form an intramolecular parallel G-quadruplex *in vitro*. G-quadruplex is a four-stranded DNA structure and its formation may interfere with transcription factor binding and potentially regulate gene expression (19). Evidently, treatment of several cancer cell lines with TMPyP4, a cationic porphyrin that distorts G-quadruplexes and thus potentially disrupts recruitment of Sp1 to the promoter, reduces the *MET* expression in these cells (20).

Next to one of the Sp-1 binding sites (−124), an activated protein-1 (AP-1) consensus sequence (TGAGTCA) is identified at position −158 to −152. Interestingly, Sp-1 binding to this site may interfere with AP-1 binding, probably due to spatial hindrance. It seems that AP-1 binding to this site is promoted by HGF treatment, at least in Hepa 1–6 cells (21). Upon binding, AP-1 may enhance *MET* transcription. This notion is supported by the fact that ectopic expression of basic leucine zipper transcription factor, ATF-like 2 (BATF2) which binds to c-Jun and therefore blocks AP-1 DNA binding, decreases the *MET* expression in LoVo and SW620 colorectal cell lines (22).

The *MET* promoter also contains six putative Ets-1 binding sites (23). E26 transformation specific sequence 1 (Ets-1) is a transcription factor belonging to the winged helix-turn-helix (wHTH) DNA-binding protein family. Ets-1 is frequently

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overexpressed in cancer cells and regarded as a proto-oncogene. For example, Ets-1 is highly expressed in mouse mammary tumor epithelial cells, correlating with *MET* overexpression and cell invasion. Ectopic expression of Ets-1 increases the *MET* promoter activity and abundance (24). Conversely, inhibition of Ets-1 activity by peroxisome proliferator-activated receptor gamma (PPARγ) significantly attenuates the Met expression in MKN-45 cells (a human gastric cancer cell line) (25). Interestingly, Ets-2, a homolog of Ets-1, displays a different role in regulating Met activity while performing similar functions in other biological events (26). Knocking down Ets-2 in H441 lung cancer cells leads to an increase of Met phosphorylation while forced Ets-2 expression has an opposite effect (27). The mechanism for these distinct effects is currently unknown.

In epithelial tissues, some of these Ets-1 sites may also be recognized by Ese-1 and Epithelial Specific Ets factor (Ese-3). Ese-3 binds strongly to two of these consensus sites (–125 and –65) and promotes *MET* transcription, while in HEK293 cells, Ese-1 and Ese-3 transactivate *MET* expression despite the fact that they may compete for the same consensus sites of the *MET* promoter (28).

The *MET* promoter possesses several Pax (Paired Box gene) consensus sites and indeed its expression is regulated by the Pax family members, which are a group of highly conserved helix-turn-helix transcription factors. In normal adult tissues, the Pax expression is minimal. However, they are highly abundant in various cancer tissues and are strongly correlated with the *MET* expression. For example, in NSCLC tissues, there is a positive correlation between Pax8 and Met protein. Knockdown of Pax8 in A549 cells reduces the expression of Met protein and lowers cell viability (29).

Alongside with those transcription factors mentioned above, the *MET* gene may be regulated by several other transcription factors that can activate or repress its expression, dependent upon cellular context and environment. A good example of environmental changes modulating the *MET* expression is hypoxia. The primary transcription factor that regulates *MET* expression under hypoxia is hypoxia-induced factor 1 (HIF1α) (30,31), a transcription factor critical for homeostasis under hypoxic conditions. The *MET* promoter has five HIF-1α consensus sites (5′-RCGTG-3′). At least two of them, within 350 bp downstream of the transcription start site, confer hypoxia-induced Met expression *in vitro* and *in vivo* (30). Since *MET* is implicated in tumor invasion and angiogenesis, this regulation may explain why hypoxia promotes metastasis in solid tumors.

Notably, *MET* expression is inhibited by androgen receptor via different mechanisms. Androgen receptor directly competes with two distal Sp1 sites and thus inhibits *MET* transcription in DU154 cells. Indirectly, androgen receptor mediates the transcription of transmembrane protease serine 2 (TMPRSS2), an androgen-regulated cell-surface serine protease. TMPRSS2 in turn promotes HGF and Met signaling (32). Therefore, these findings may explain why androgen ablation therapy eventually becomes ineffective and why many patients have developed castration-resistant growth and even metastasis.

Regulation by alternative splicing

The *MET* gene spans approximately 120 kb on Chromosome 7q21-31 and its transcript includes 20 introns and 21 exons. Because of the two alternative splicing sites in exon 10 and exon 14, *MET* mRNA is transcribed from a single promoter and spliced to major species of 8k, 7 k, 5 k and 3 k nucleotides long in GTL16 cells (a human gastric adenocarcinoma cell line) (23,33). While the 170 kDa Met is translated from the 8k mRNA, the 7 k species does not generate any protein product, due to skipping of the 1214nt long exon 2 (23,33). Later studies have identified more *MET* mRNA species with various lengths. A list of 15 *MET* mRNA ranging from 1.2k to 2.6k is available at UCSC database: http://genome.ucsc.edu/cgi-bin/hgGene?hgg_gene=uc011kmz.1&hgg_prot=B5A933&hgg_chrom=chr7&hgg_start=116339124&hgg_end=116364901&hgg_type=knownGene&db=hg19&hgsid=442320925_VVLd0A8iEJuHuuaRA9babrYtvug6.

The importance of proper splicing of *MET* mRNA has been demonstrated in various studies. For example, in some high-grade gliomas, *MET* mRNA contains a deletion, in which exons 7 and 8 were skipped. This mRNA is translated to a protein missing extracellular IPT domain. Dubbed $Met^{\Delta7-8}$, this truncated protein primarily localizes in the cytosol and is constitutively active (34).

Another example is Δ13Met, which is an alternatively spliced version of *MET* mRNA without exon 13. The Δ13Met protein product possesses only the extracellular domain and is commonly found in human skeletal muscle. Upon binding to HGF, this truncated version fails to transduce the signal to downstream and is thus inhibitory to Met function (35). Consistently, forced expression of the Δ13Met mutant represses the downstream events, i.e. Met and ERK phosphorylation (35). In contrast, skipping exon 14 leads to the accumulation of Met protein (METex14) since this mutation loses Y1003, which is critical for Met ubiquitination and degradation by proteasomes demonstrated in various studies (36). This cancer driver mutation (METex14) has been identified in a subset of patients of lung and brain cancers (37).

The mechanism governing *MET* mRNA splicing is not clear. It is possible that miniature chromosome maintenance 7 (MCM7) plays a role in this process. MCM7 is also aberrantly expressed in multiple cancers and critical for DNA replication. In PC3 and DU145 cells, depletion of MCM7 with siRNA increases the unspliced *MET* mRNA. Although the exact mechanisms are still unknown, interaction of MCM7 with SF3B3 (splicing factor 3B, subunit 3) may be involved in regulating *MET* mRNA splicing, since depletion of SF3B3 alters *MET* mRNA splicing (38).

Regulation by microRNAs

MET translation is also regulated by more than 30 microRNAs (miRNAs) through various mechanisms (39). miRNAs are a group of non-coding RNAs of 21–25 nucleotides long that usually regulate gene expression through their specific binding to the 3′-untranslated regions (UTRs) of their target mRNAs. Their binding accelerates the degradation of target mRNAs or inhibits translation of their targets. It has been well established that aberrant expression of miRNAs is implicated in various diseases including cancers (40).

One such example is miR-1 that inhibits *MET* expression through various mechanisms. In the majority of colon cancer, lung cancer and primary HCCs, miR-1 expression is inversely correlated with the *MET* expression (41–44). miR-1 is usually methylated and its expression silenced in HCCs. miR-1 is encoded by of the intron 1 of the putative ORF166 and able to bind three consensus sites on the 3′-UTR of *MET* mRNA and inhibits its expression (44). Additionally, miR-1 may also regulate *MET* expression indirectly through inhibiting MACC1 and ETS-1, two transcription factors responsible for *MET* transcription (42). Consistently, ectopic expression of miR-1 in colon cancer cells and HCC cells reduces Met abundance and thus cell invasion (42,44).

miR-139-5p is also a tumor suppressor and frequently lost in primary lung cancer tissues. Ectopic expression of this miRNA

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in lung cancer cell lines, e.g. A549 and SKMES-1, represses cell proliferation and migration, which are dependent upon *MET* expression. There is a strong inverse correlation between miR-139-5p and *MET* expression. The 3′-UTR of *MET* mRNA has one consensus miR-139-5p binding site that confers its inhibitory effect. Indeed, ectopic expression of miR-139-5p significantly inhibits the luciferase reporter harboring the *MET* 3′-UTR. Mutation in the seed sequence of the miR-139-5p consensus site abolishes this inhibition, indicating a direct interaction of miR-139-5p with this binding site (45). Likewise, miR-206 is abundant in skeletal muscle and its expression is lost in rhabdomyosarcomas as well as a rhabdomyosarcoma RD cell line. Not surprisingly, restoration of miR-206 in RD cells decreases cell proliferation and migration, due to an inhibition of the *MET* expression by miR-206, conferred through the two miR-206 binding sites at the 3′-UTR of *MET* mRNA (46). Similar examples have also been reported in miR-181a-5p in HCC (47) and miR-7515 in lung cancer (48).

Conversely, *MET* may also modulate expression of some miRNAs. For examples, a cluster of 23 miRNAs, e.g. miR-127, miR-154 and miR-431, is upregulated in tumors isolated from the liver of the *MET* transgenic mice (49), suggesting an inhibitory effect of *MET* on the expression of these miRNAs. On the contrary, *MET* may also promote the expression of miR-221 and 222 through activation of JNK/AP-1 pathway (50), in various NSCLC and HCC cell lines. Since *MET* itself is also a target of AP-1 (21), this finding represents a complicated interactive signaling network.

Currently, it has been reported that *MET* expression can be modulated by more than 30 miRNAs (39). This list of miRNA regulators is very likely expanding. Importantly, given the importance of miRNAs in regulating expression of oncogenes like MET, some miRNAs may be potentially used for diagnostic biomarkers or therapeutic purposes. For examples, direct injection of miR-139-5p into the xenografts derived from A549 cells leads to substantial reduction in tumor growth in nude mice (45).

Regulation by protein translation

Translational regulation of oncogenes plays an important role in carcinogenesis (51). Emerging evidence indicates that *MET* expression may also be regulated at the translation level. A critical protein for translation initiation is eIF4E which binds to the 5′ ^{m7}G cap of mRNA molecules and thus facilitates ribosomal recruitment (52). In HCC822 cells, due to the elevated expression of eIF4E, these cells become erlotinib resistant. Further analysis indicates that aberrant expression of eIF4E causes upregulation of Met and thus confers this erlotinib resistance (53).

In various cancer cell lines, it has been demonstrated that Pim-1 kinase positively regulates Met translation. Pim-1 is a PIM family Ser/Thr kinase which phosphorylates S406 of the eukaryotic initiation factor 4B (eIF4B). This phosphorylation facilitates the interaction between eIF4B and eIF3 to initiate translation (54). In addition, Met translation seems to be particularly important under anchorage independent growth condition, under which global mRNA translation is down-regulated but *MET* mRNA is preferentially recruited to the polysomes to increase its translation through unknown mechanisms (55). Since anchorage independent growth is strongly correlated with tumorigenicity and invasiveness *in vivo* (56), this cap-dependent Met translation provides a novel insight on cancer metastasis.

Regulation by proteolysis

The Met protein is first synthesized as pro-Met, a single chain precursor of 170 kDa. The pro-Met undergoes extensive posttranslational modifications, e.g. glycosylation and proteolysis, to become a functionally mature protein (57). This precursor protein is cleaved into a 50 kDa α-subunit and a 145 kDa β-subunit, which form a heterodimer through disulfide bonds. After glycosylation, this heterodimer becomes a mature protein with a molecular weight of 190 kDa (57). However, the cleavage of the 170 kDa pro-Met protein may not be essential for its activity, since the uncleaved pro-Met is still glycosylated and constitutively active. Importantly, the presence of this uncleaved form in some HCC cases suggests a potential role in carcinogenesis (58).

Another type of Met proteolysis is commonly referred to as 'ectodomain shedding', in which the extracellular domain is cleaved from the cell membrane through actions of various matrix metalloproteinase and A Disintegrin and Metalloproteinase Domain (ADAM) family members, e.g. TIMP-3 sensitive metalloproteinase, ADAM10 and ADAM17 (59). The shed product may function as a decoy receptor to block HGF activity and the abundance of the shed fraction correlated with tumor progression and may thus be used a biomarker in body fluids (59).

Regulation by glycosylation

Glycosylation is essential for maturation and functions of membrane proteins, i.e. receptor tyrosine kinases (RTKs), regulating their routing, conformation and ligand binding (60). For example, inhibition of glycosylation sensitizes cancer cells that are resistant to EGFR targeted therapy to radiation (60).

The most common modification for RTKs is N-linked glycosylation, in which an oligosaccharide is transferred to an asparagine residue (60). There are 11 putative sites for N-glycosylation in the Met protein (57). When digested with neuraminidase and endoglycosidase F, two enzymes to hydrolyze N-linked oligosaccharides, the migration pattern of the Met protein is shifted on a SDS-PAGE gel, confirming that the Met protein is N-glycosylated (57).

In some other cell types, the glycosylation of the Met protein can also be O-linked. This type of glycosylation involves transferring glycans to the hydroxyl group of a serine or threonine residue. In HCC cell lines HA22T and PLC5, modification of Met with O-glycans is mediated through C1GALT1 (Core 1 b1,3-galactosyltransferase), a mucin-type O-glycosyltransferase. Depletion of G1GALT1 by siRNA decreases phosphorylation and dimerization of Met, leading to inhibition of cell migration and invasion (61).

Also in HCT116 cells, depletion of β-galactoside α2,6-sialyltransferase (ST6Gal-I) leads to reduced α2,6-sialylation of the Met protein. This decreases the expression of the mature β-subunit peptide but not *MET* mRNA, suggesting that α2,6-sialylation may be important for the proper cleavage of the pro-Met and thus its function (62).

Several lines of evidence have shown that the Met glycosylation is essentially for its functions. First, treatment of GTL-16 cells with tunicamycin blocked the Met glycosylation. As a result, the Met protein without glycosylation could not be properly cleaved and phosphorylated *in vivo* (57). Second, tunicamycin treatment of MHCC-97H (human HCC cell line) and RBE (human cholangiocarcinoma cell line) leads to the cytoplasmic retention of the Met protein. Third, tunicamycin treatment accelerates the degradation of the Met protein by proteasomes (63). Fourth, inhibition of glycosylation attenuates the Met signaling (61,62). Last, N-glycosylation is critical for the conformation, orientation and dimerization of EGFRs on the membrane (64,65).

Regulation by phosphorylation

Like other RTKs, phosphorylation plays an essential role governing the activity and the fate of Met. Upon HGF binding, Met

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undergoes dimerization and Y1234/Y1235 autophosphorylation, which stimulates its kinase activity. Subsequently, two adjacent residues (Y1349 and Y1356) are also phosphorylated, resulting in generation of a dock site for signal transduction (66).

Met may also be phosphorylated by other kinases. A major kinase to phosphorylate Met is Src, a non-RTK. When an activated Src is expressed in HC11, a non-neoplastic breast epithelial cell line, Met phosphorylation at Y1230, 1234 and 1235 is elevated. Conversely, expression of a dominant negative Src (kinase dead) or treatment with PD180970 (Src inhibitor) abolishes the constitutive Met phosphorylation in SP1 breast cancer cell line (67). In some cells, Src-dependent phosphorylation requires cell-matrix adhesion and FAK (67). Protein kinase C family also plays a critical role in Met phosphorylation to adapt to environmental changes. For example, PKCα-mediated Y1003 phosphorylation upon LPS (lipopolysaccharide) treatment, leading to the internalization of Met and lung epithelial barrier dysfunction (68), while treatment with H_2O_2 or TPA (12-O-tetradecanoylphorbol-13-acetate) decreases tyrosine phosphorylation but promotes phosphorylation of S985 by PKCδ/ε (69).

Meanwhile, the phosphorylation status of Met is also determined by the activity of phosphatases, i.e. PP2A, DEP-1, SHP2 and PTP-1B. Density-enhanced phosphatase 1 (DEP-1) is a receptor tyrosine phosphatase that dephosphorylates Met Y1349 and Y1365, which are important for Gab1 binding and morphogenesis (70). For example, Leukocyte cell-derived chemotaxin 2 (LECT2) interacts with the Met α-chain (aa 159–175) directly through its HxGxD Motif. This interaction recruits the protein tyrosine phosphatase 1B (PTP-1B). Dephosphorylation of the Met protein by PTP-1B triggers the dissociation of adaptors proteins, e.g. Gab1 and Src, leading to attenuation of the Met signaling (71).

Regulation by internalization and degradation

Upon HGF binding, Met is internalized and sorted to endosomes through endocytosis which is clathrin- and dynamin-dependent. The mechanisms have been reviewed elsewhere (72). From endosomes, Met can either be recycled back to the membrane or delivered to lysosomes for degradation. Recent studies indicate that PKC is critical in this process since depletion of PKCε blocked the Met degradation in HCC cell (73).

Met is also subject to ubiquitin-mediated proteasome degradation. Met degradation by proteasomes has been well demonstrated in various studies (74). A critical player is Cbl (Castias B-lineage lymphoma), an E3 ubiquitin ligase responsible for Met ubiquitination and degradation by proteasomes (36). Phosphorylation of Met at Y1003 creates a docking site for recruiting Cbl directly through an atypical DpYR motif in the juxtamembrane domain of Met (74). Alternatively, Cbl may be recruited to Met through its interaction with the adaptor protein Grb2 (74). Evidently, Tpr-Met, a fusion protein found in various cancers, loses Cbl binding region in the juxtamembrane domain of Met and thus escapes protein degradation (74).

Similarly, Socs1 (Suppressors of Cytokine Signaling) promotes degradation of RTKs, including Met. Socs1 is frequently repressed in human HCC and regarded as a tumor suppressor. The SH2 domain of Socs1 binds to the Met protein while its Socs box recruits the E3 ubiquitin machinery, leading to proteasome-dependent degradation. Moreover, Socs1-mediated Met degradation is not lysosome dependent, since bafilomycin and chloroquine, inhibitors of lysosomes, fail to block the Met degradation. Consistently, overexpression of Socs1 decreases the Met phosphorylation and thus attenuates the Met signaling in liver. Interestingly, modifications of lysines, e.g. K48-linked polyubiquitination, are required for Socs1-mediated degradation (75).

In contrast to Y1003, Y1313 phosphorylation plays role stabilizing the Met protein. This phosphorylation enables TNS4 (Tensin 4) binding to Met through its SH2-domain. Upon binding, TNS4 inhibits the Met internalization to lysosomes. There is a strong correlation between TNS4 and the Met expression in colon and ovarian cancers (76).

Regulation by nuclear localization

Similar to other EGFRs, the Met protein activates downstream signaling pathways in the cytoplasm. However, recent evident also indicates that the Met protein may translocate to the nucleus. For example, Met is localized in the nuclei of some breast cancer cases and cancer cell lines (77,78). This nuclear Met is a 60-kDa fragment cleaved from its C-terminus. The mechanism(s) for generating this nuclear fragment are not clear. Possibly, Met is processed sequentially by ADAM 10 and 17 to generate N-terminal fragment and a membrane-anchored C-terminal fragment (shedding). This C-terminal fragment is further cleaved by γ-secretase (79).

The nuclear presence of Met was at first puzzling since the Met protein does not have any canonical nuclear localization signal (NLSs). Serial truncations have identified that the Met juxtamembrane domain is critical for its nuclear localization (77,78). Indeed, a putative bipartite NLS (**H**VVIGPSSLIV**H**) at aa1068–1079 of the juxtamembrane domain plays a critical role in the Met nuclear localization. The two flanking histidines are essential for the NLS function since the mutation of either one to alanine abolishes the Met nuclear localization due to decreased binding with importin β, which, together with Gab1, is required for the Met nuclear localization (80). Very interestingly, this NLS serves as a pH sensor since lowered cellular pH increases the Met binding to importin β and thus the nuclear localization (81).

The biological roles for the Met nuclear localization remain elusive. However, it is reported that the nuclear Met regulates calcium signaling via activating PLC-γ (phospholipase C) and forming InsP3 (1,4,5-trisphosphate) in the nucleus (80). In addition, transcriptional activation has been reported in other RTKs. For example, EGFR is able to relocate to the nucleus and promotes transcription of Cyclin D1 in MCF10A cells (82). It is likely that the Met protein is also involved in gene transcription since Met displays transactivation activity when fused with Gal4 DNA-binding domain (78). The exact transcriptional targets of the nuclear Met require further investigation.

The nuclear presence of the 60kDa Met is correlated with tumor malignancy. This protein presents in MDA-MB231, an invasive breast cancer cell line but not in MCF-7, a non-metastatic cell line (78). This differential expression pattern of the 60kDa nuclear Met may be dependent upon the presence of Wwox (WW domain-containing oxidoreductase), a putative tumor suppressor gene. MDA-MB231 expresses low Wwox while MCF-7 has an opposite pattern. Indeed, ectopic expression of Wwox in MDA-MB231 cells prevents the Met nuclear accumulation, which leads to decreased migration of the cells, indicating that the Met nuclear localization may be implicated in metastasis/invasiveness (78).

The nuclear localization of the Met protein is cell type specific. For example, the 60kDa fragment translocates to the nucleus in HeLa cells but not Chang Liver cells (81). Additionally, Met migrates to the nucleus upon HGF stimulation in SKHep1 cells (80) whereas in MDA-MB231 cells, its nuclear localization is constitutive (78).

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Regulation by other membrane molecules

Like other RTKs, Met receptor activity may be regulated via cross-talk with other cell surface receptors, e.g. EGFR and G-protein couple receptors (GPCRs). Importantly, the Met signaling pathway can also be activated by other EGFRs and by GPCRs through a process called 'transactivation' (83,84). The cross-talk between GPCRs and Met, as well as other RTKs, reveals a complicated interaction and coordination among different signaling pathways upon external stimuli.

One such mechanism is through heterodimerization and trans-phosphorylation with other RTKs. It has been shown that in COS cells, RON (Recepteur d'Origine Nantais) kinase, a Met related RTK, forms heterodimers with Met upon stimulation with either of their ligands, i.e. HGF or Scatter Factor 2, and mutually phosphorylates each other (85). In addition to GPCRs and EGFRs, the Met signaling pathway may also be activated through interactions with other cell surface molecules, e.g. plexins, CD44 and tetraspanin (86). Interestingly, these molecules are involved in exosome secretion (87), which is important for Met-mediated metastasis (2). The interactions between Met and other cell surface molecules have been reviewed elsewhere (86).

Met activation by surface molecules is different from EGFR activation, at least in gastric cancer cells. In MKN28 and MKN74, two human gastric cancer cell lines, S1P (Sphingosine 1-phosphate) transactivates both EGFR and Met through GPCRs (84). However, they are activated through different mechanisms. For example, G_i protein and matrix metalloproteinase are required for the activation of EGFR but not for the Met receptor (84). Moreover, Met transactivation may be dependent upon EGFR activity in response to some external signals, e.g. prostaglandin E2, in colon cancer cells (88) but not in HCC cells in response to LPA treatment (83). This result suggests that Met transactivation is dependent upon the cellular context and the nature of external stimulus.

Regulation by p53

The fact that *MET* expression is regulated by p53 was first demonstrated by Rong et al. Their study indicated that *MET* is highly expressed in sarcomas from patients of Li-Fraumeni syndrome and p53-deficient mice (89). Later, several groups have illustrated that p53 regulates the *MET* expression via various mechanisms.

First, p53 may inhibit Sp1-mediated transcription of *MET*. p53 physically interacts with Sp1 and thus reduces its binding to the *MET* promoter in SKOV-3 and OVCA433 cells (90). However, under certain circumstances, p53 may also promote the *MET* transcription. At position –278 to –216 of the *MET* promoter, there is a p53 responsive element which confers p53 transactivation activity in response to UV irradiation in RKO cells (91). This transcriptional regulation may be cell type/stimulation dependent, since activation of p53 in HCT116 cells does not promote the *MET* expression (92).

Second, p53 inhibits translation of *MET* mRNA via miR-34 which is frequently lost in various cancers (93–97). In HCC tissues, there is a strong inverse correlation between miR-34a and Met abundance. It has been shown that miR-34a directly binds to the 3'-UTR of *MET* mRNA and inhibits its translation, while this miRNA itself is a target transcriptionally activated p53 (97,98).

Third, p53 may also regulate Met receptor activity post-translationally, since mutation of p53 (R175H) seems to promote Met phosphorylation and kinase activity. A common p53 mutation (R175H) activates the Met RTK to enhance tumor cell invasion. R175H, as well as another common mutation 273H, may enhance recycling of the Met receptor to the cell surface and thus promote its activity and invasive behavior of the cell (99). Similarly, ectopic expression of the mouse equivalent p53 mutation of R175H or R273H (R163H or R261H in mouse), promotes Met protein abundance in MDCK (Madin-Darby canine kidney) cells (100).

Autoregulation

In addition to being regulated by the aforementioned mechanisms, the Met pathway may also induce itself through various mechanisms. First, activation of the Met kinase can be intermolecularly autocatalytic (101) since the Met receptor interacts with other receptor species to promote receptor clustering (102). Second, the Met signal upregulates transcription factors, e.g. ETS1 (103), MACC1 (42) and HIF-1 (104), providing a positive feedback on its own transcriptional. Third, Met may downregulate some miRNAs, e.g. miR-1 to de-repress their inhibitory effects on Met (42). Fourth, the Met pathway also represses its degradation through downregulating Cbl protein and Socs1 (105,106). Fifth, the Met signaling promotes ROS production, which in turn, promotes Met phosphorylation (107). This ramification system may significantly enhance the downstream signaling pathways even with moderate increase of Met abundance.

Mutations enhancing Met activity

Various mutations of the *MET* genes have been identified in cancer patients. Some mutations may be cancer drivers given that these mutations significantly enhance Met activity via different mechanisms as discussed below.

1. Constitutive phosphorylation of the Met receptor

Activating *MET* mutations are frequently observed in sporadic papillary renal cell carcinomas (13%) (108). Mutations H1112L, H1124D and Y1248D lead to constitutive phosphorylation of the Met receptor. When ectopically expressed in NIH3T3 cells, these mutated receptors significantly promote focus formation, an *in vitro* assay determining oncogenic potentials of genes (108).

Tyrosine 1235 (Y1235) is one of the two autophosphorylation sites (Y1234 and Y1235) for activation of the Met receptor kinase. Y1235D mutation results in the replacement of tyrosine 1235 with negatively charged aspartic acid which mimics constitutive phosphorylation. This mutation is highly enriched in the metastatic but not primary head and neck squamous carcinomas (109).

2. Reduced protein degradation

As previously mentioned, skipping exon 14 leads to the loss of the juxtamembrane Cbl binding site and thus reduces ubiquitination/degradation of the Met protein (36). Interestingly, a recent study shows that in pulmonary sarcomatoid carcinomas, there is a high frequency (22%, 8/36) of this mutation (110).

A different mutation leads to the stabilization of the Met protein via a similar mechanism. The substitution of Y1003 with phenylalanine (Y1003F) or substitution of neighboring aspartate or arginine residue with alanine (D1002A or R1004A) impairs recruitment of Cbl to the Met receptor and thus decreases its ubiquitination and degradation (111).

3. Promoting kinase activity

The typical mutation of the *MET* gene is *TPR-MET*, in which *TPR* (translocated promoter region) fuses with the region coding the Met kinase domain and C-terminus, generated by exposing a human osteogenic sarcoma cell line (HOS) with the carcinogen

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N-methyl-N'-nitronitrosoguanidine (112). The fusion protein localizes in the cytoplasm due to the lack of the extracellular, transmembrane and juxtamembrane domains so. The presence of the Tpr leucine zipper promotes homodimerization of this mutated Met, leading to the constitutively activated Met receptor kinase even in the absence of the ligand, i.e. HGF (112,113). Similar mutations have been identified in clinical samples. For example, *BAIAP2L1* (BAI1-associated protein 2-like 1) or *C8orf34* (Chromosome 8 Open Reading Frame 34) translocates to and fuses with the *MET* gene in-frame in some patients with papillary renal carcinoma. *BAIAP2L1-MET* and *C8orf34-MET* encode proteins with motifs that facilitate dimerization of the fusion proteins and thus promote the Met activity (114).

The V1110I mutation has been identified in multiple papillary renal-cell carcinomas. This mutation causes a change of the valine to an isoleucine in the well conserved ATP-binding pocket of the Met receptor, enhancing its kinase activity (115). Another example is the M1268T mutation, which is similar to RET (Receptor Tyrosine Protein Kinase) M918T mutation that leads to multiple endocrine neoplasia (116,117). Indeed, this mutation is very potent at transforming cells and the resulting xenografts grow much larger than other mutations tested, e.g. D1246H or D1246N. It is postulated that the M1268T mutation causes changes within the COOH-terminal lobe of the Met kinase domain. This change somehow alleviates inhibition on the Met kinase activity (117).

Another type of the *MET* mutation promotes Met receptor activity via a distinct mechanism. The T1010I mutation is also within the Met tyrosine kinase domain. However, it may primarily block S985 phosphorylation that is inhibitory to Met activity. The presence of this type of mutation is correlated with metastasis (118,119).

4. Change in Met receptor recycling

A rare mutation P1009S has been identified from gastric cancer (120). In contrast to other mutations, P1009S is not constitutively active but its phosphorylation status persists much longer than wild type Met. It is possible that Met receptor recycling/degradation is defective in this mutation. However, the exact mechanism remains elusive (120).

Very interestingly, different mutations may possess distinct properties to activate downstream events. For example, the M1250T and D1228H mutations primarily activate the Ras pathway and promote transformation, while the L1195V and Y1230C mutation are primarily anti-apoptotic via activation of PI3K pathway. The mechanisms for these differential outcomes are not clear (121).

Summary and perspectives

MET is tightly regulated at every layer of the regulating network, from epigenetic regulation to its protein degradation (Summarized in Figure 1). Regulation of *MET* is also complicated, since different regulatory mechanisms may be interwoven. For example, the miR-34 promoter is hypermethylated in almost half the CRC cases, leading to down regulation of miR-34. Since miR-34 inhibits *MET* expression, the hypermethylation silences the miR-34 expression and thus indirectly promotes the *MET* expression (94). Similarly, miR-1 is preferentially hypermethylated in HCC tissues in comparison with their normal counterparts. Treatment of different HCC cells with 5'-aza restores miR-1 expression and thus reduces the *MET* expression (44).

Given the fact that Met is an attractive drug target for various cancers, understanding the regulation of *MET* is critical for

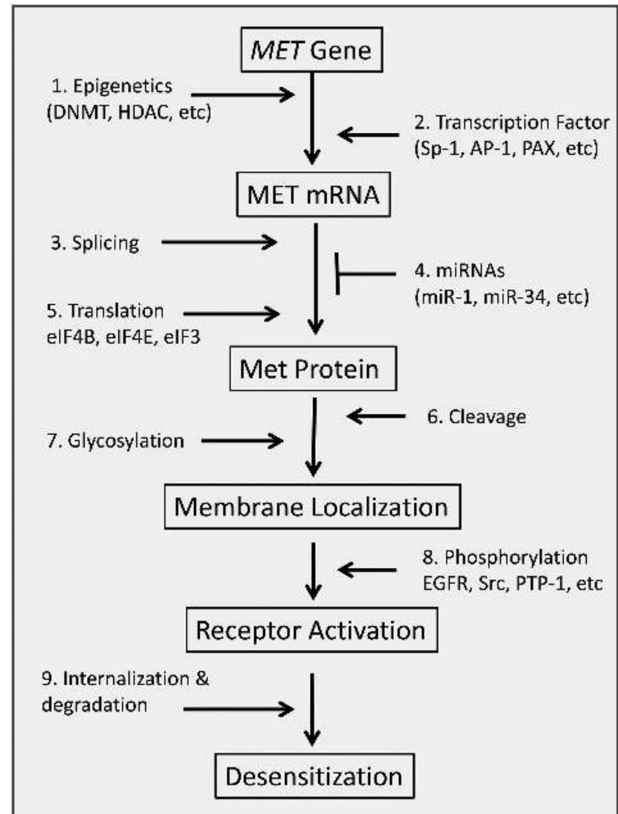

Figure 1. Simplified scheme of regulation of *MET*. 1. *MET* expression is affected by the status of DNA methylation and histone acetylation/methylation, which are modulated by DNA methyltransferases (DNMT) and histone (de)acetylases (HATs or HDACs), respectively. 2. Multiple transcription factors, e.g. Sp-1 and AP-1, modulate transcription of *MET* gene. 3. Once transcribed, *MET* mRNA is subject to alternative splicing, resulting in mature mRNAs of various lengths. 4. In the cytoplasm, the 3'-UTR of *MET* mRNA is recognized by dozens of microRNAs, e.g. miR-1 and miR-34, leading to inhibition of *MET* mRNA translation. 5. Translation of *MET* mRNA may also be regulated at translation initiation step, in which eIF4B/E and eIF3 are involved. 6. *MET* mRNA is translated to a 170kDa pro-protein which is subsequently cleaved into α and β submits. These two subunits dimerize through disulfide bonds to form a 190kDa mature protein. 7. In the Golgi apparatus, the Met protein undergoes multiple N-linked and O-linked glycosylation before translocating to the cell membrane. 8. Upon ligand binding, the Met receptor kinase is activated auto-phosphorylation or phosphorylation by other kinases, e.g. Src and EGFR, while counteracted by phosphatases like PTP-1. 9. The Met receptor is desensitized via internalization followed by either receptor recycling through endosomes or ubiquitin-mediated proteasome degradation.

targeting this oncogene in cancer therapeutics, as inappropriate approaches may accelerate Met-mediated drug resistance and cancer relapse. For example, Sorafenib is a commonly used TKI inhibitor. Treatment of cells with Sorafenib may increase Met activity, since Sorafenib inhibits DEP-1 phosphatase activity which dephosphorylates Met Y1349 (122). Similarly, treatment of human lung cancer cell line H1993 and NIH3T3 cells with SU11274 and PHA665752, two known Met inhibitors, leads to Met accumulation, because the treatment abolishes Y1003 phosphorylation and subsequent ubiquitination (123).

A recent study reveals that *MET* may also be of anti-tumor activity in neutrophils (4). *MET* is required for neutrophil infiltration to tumors since deletion of *MET* on neutrophils promoted tumor progression and compromised the efficacy of anti-Met treatment. This new finding suggests that a better tactic should be designed to target Met on tumor cells but not on neutrophils to achieve maximum therapeutic effects (4).

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Expression of Met$^{\Delta7-8}$ may have important consequences for choice of therapy. As aforementioned, Met$^{\Delta7-8}$ is a truncated but active kinase without extracellular domains (34) for which current anti-Met antibodies is not effective. Therefore, it would be beneficial for patients with this mutation to be screened out to avoid unnecessary exposure to anti-Met antibody treatment. Conversely, screening for METex14 mutation in patients with lung cancer may be beneficial in Met-targeted therapy, since a recent study has shown that creation of METex14 mutation in HEK293 cells with CRISPR (Clustered regularly-interspaced short palindromic repeats) technology sensitizes the cells to crizotinib treatment (124). Given the fact that some of the *MET* mutations are drivers for carcinogenesis, as discussed previously, CRISPR technology, may be used to correct these genetic defects with its awesome power in genome editing (124).

Other biomarkers should also be considered when screening patients for Met targeted therapeutics. For example, anti-Met treatment with SU11274 induced apoptosis and this effect was dependent on the presence of wild-type p53 gene status in lung cancer (125). Likewise, Met amplification promotes development of TNBC (triple negative breast cancer), synergistically with the loss of p53 (126). These data suggest that screening wild type p53 patients may potentiate Met-targeting therapeutics in these cancers.

DNA methylation has been implicated in carcinogenesis and targeting DNA methylation is considered a tactic for cancer treatment. SAHA (ZOLINZA®, Merck) is currently used for the treatment of cutaneous T cell lymphoma (CTCL). Some evidence has shown that SAHA enhances the Met signal which leads to the resistance to SAHA. This finding suggests that a combination of SAHA together with Met inhibitors would be more effective in the treatment CTCL and possibly other cancers (15). Furthermore, Met may also regulate expression of other genes through epigenetic mechanisms. Activation of Met stabilizes a complex between Ets-2 and MLL (mixed-lineage leukemia) which specifically methylate histone H3 on lysine 4 (H3K4) in HCC cell lines. This methylation facilitates transcription of MMP1 and MMP3 genes, which are critical for cancer metastasis (127).

Some novel strategies may also be considered targeting the Met signaling. For example, FasL interacts with Met directly and stimulates Met phosphorylation and activity. Blocking this interaction with a specific peptide significantly down-regulates the Met signaling. Thus, small molecules or peptides disrupting this interaction may be an intriguing therapeutic tool (128). Moreover, caspases cleave Met at D1000 and D1374 sites in the juxtamembrane region, creating a pro-apoptotic fragment of 40 kDa from its C-terminus (79). If this phenomenon is harnessed, a therapy may provide double effects: destroying Met and accelerating apoptosis in cancer cells.

Solid evidence has indicated that Met is an ideal drug target in cancer therapeutics. Currently, there are 122 clinical trials of various phases targeting this oncogene (c-Met) registered at www.clinicaltrials.gov. These drugs may be roughly classified into several types, based on their mechanism of action. There are selective Met kinase inhibitors, e.g. ARQ197, or nonselective ones, e.g. COMETRIQ® (cabozantinib). Alternatively, some anti-c-MET monoclonal antibodies, e.g. Onartuzumab, bind to the Met receptor specifically and promote its internalization and degradation (129).

Some Met-targeting drugs have displayed promising results in early clinical trials. For example, Onartuzumab, a.k.a. MetMAb developed by Genentech, is a Met specific monoclonal antibody, which prevents HGF from binding to Met receptor and thus blocks the downstream signals. In a Phase II clinical trial in NSCLC patients with positive Met, treatment with both erlotinib and onartuzumab provided better overall survival (OS) than that with erlotinib alone (12.6 versus 3.8 months) (130). Among these drugs, Cabozantinib has been approved for the treatment of medullary thyroid cancer in the USA in 2012. The results of some early clinical trials have been reviewed elsewhere (129,131).

The *MET* oncogene was discovered more than 30 years ago (1), but its regulation mechanisms are still being dissected. Aberrant expression of *MET* is implicated in tumor initiation, progression, drug resistance and metastasis. Thus, a better understanding of its regulatory mechanisms and downstream events will potentially assist to design novel strategies to reduce Met-mediated drug resistance and relapse.

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