MAPK13 stabilization via m\(^6\)A modification limits anti-cancer efficacy of rapamycin

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Abstract

\(N^6\)-adenosine methylation (m\(^6\)A) is the most abundant mRNA modification that controls gene expression through diverse mechanisms. m\(^6\)A-dependent regulation of oncogenes and tumor suppressors indeed contribute to tumor development. However, the role of m\(^6\)A-mediated gene regulation after drug treatment or resistance is poorly understood. Here, we report that m\(^6\)A modification of mitogen-activated protein kinase 13 (MAPK13) determines the sensitivity of cancer cells to the mechanistic target of rapamycin complex 1 (mTORC1)-targeting chemotherapeutic agent rapamycin. mTORC1 induces m\(^6\)A modification of MAPK13 mRNA at its 3' untranslated region (3'UTR) through methyltransferase-like 3 (METTL3)-METTL14-Wilms' tumor 1-associating protein (WTAP) methyltransferase complex, thereby stimulating its mRNA degradation via an m\(^6\)A reader protein YTH domain family protein 2 (YTHDF2). Rapamycin blunts this process and stabilizes MAPK13. Unexpectedly, MAPK13 silencing suppresses cell growth and enhances rapamycin's anti-cancer effects, suggesting that MAPK13 is an oncogenic gene activated by rapamycin via a feedback regulation. Together, our data indicate that rapamycin-mediated MAPK13 mRNA stabilization may confer drug resistance, and it can thus be a therapeutic target to sensitize cancer cells to rapamycin.
**Keywords**

m^6^A; RNA modification; MAPK13; p38; mTORC1; Rapamycin; Feedback inhibition

**Introduction**

Transcriptional regulation is a central mechanism required for controlling gene expression. In addition to this canonical process, cells modify genetic material with various chemical moieties as an additional layer of gene regulation. While epigenetic modifications of DNA and histones are well-established, chemical modifications of RNA itself (i.e., epitranscriptomic regulation) have been recently shown to also play crucial roles in gene regulation. Of the RNA modifications, m^6^A is the most abundant (1). m^6^A is deposited on mRNA by a methyltransferase complex, which is composed of three core proteins: METTL3, METTL14, and WTAP (2, 3). m^6^A is mostly enriched on the last exon of mRNA near the stop codon and 3'UTR as revealed by transcriptome-wide sequencing (4, 5). These m^6^A-modified mRNAs then recruit m^6^A-binding “reader” proteins that will determine the diverse fates of these mRNAs. For example, YTHDF destabilizes or promotes the translation of m^6^A-containing transcripts (6, 7).

m^6^A-dependent gene regulation is involved in various biological processes such as stem cell differentiation, embryo development, sex determination, and circadian rhythm; dysregulation of this process can cause various diseases including cancers (8, 9, 10). Interestingly, both increased and decreased m^6^A levels can lead to cancer development, depending on the downstream target genes. METTL3 overexpression in leukemia cells induces the expression of oncogenes such as cMyc and Bcl2 (11). On the other hand, METTL3 downregulation in endometrial cancer induces pro-survival Akt signaling by decreasing the expression of the Akt inhibitor, PHLPP2 (12). Therefore, a comprehensive examination of m^6^A target genes is necessary to better understand the impact of m^6^A modification in different biological contexts.

mTORC1 is overactivated in most human cancers as a master regulator of cell growth (13, 14, 15, 16). The mTORC1 inhibitor rapamycin faces several clinical challenges as chemotherapy, largely due to the developed resistance by tumors or regrowth of tumors after treatment (17, 18, 19). It has been suggested that mTORC1-dependent post-translational modifications of proteins underlie observed rapamycin resistance mechanisms (19). However, whether post-transcriptional RNA modifications confer rapamycin resistance is unknown.
Recent works from our and other labs revealed that activation of m⁶A processing by mTORC1 contributes to tumor progression. mTORC1 increases the expression of METTL3, METTL14, and WTAP, which downregulates cell growth-suppressing genes such as autophagy genes and cMyc suppressor (20, 21, 22). From our transcriptome-wide m⁶A sequencing, we identified additional genes that are potentially regulated by mTORC1-dependent m⁶A modification (20). In this study, we report that a MAPK/p38 isoform, MAPK13/p38δ, is a downstream target of the mTORC1-m⁶A-pathway, which likely contributes to the limiting tumor suppressive effects of rapamycin.

Results

Identification of genes regulated by mTORC1 and m⁶A writer complex

We previously performed miCLIP-seq in human embryonic kidney HEK293E cells, identifying the genes whose m⁶A level is decreased when total mRNA expression is increased by the mTOR catalytic inhibitor, torin1 (20). Since torin1 suppresses both mTORC1 and mTORC2, we used rapamycin to selectively block mTORC1 and performed a qPCR as a secondary screen (Fig. 1A). In parallel, we depleted m⁶A writer complex proteins, METTL3/14 or WTAP, to identify genes that are regulated by m⁶A modification. For these screens, we used lymphangioleiomyomatosis (LAM) patient-derived kidney angiomyolipoma cell line (LAM 621-101), which has an overactivated mTORC1 activity due to TSC2 deficiency (23, 24). Consistent with our previous findings, rapamycin reduced the protein levels of m⁶A writer proteins METTL3, METTL14, and WTAP (Fig. 1, B and C) (20, 21). We found 10 genes (BEX1, EIF4A2, EIF6, FGFR3, MAPK13, NOP56, PKD1, SLC25A37, STAT5B, and TRP) whose mRNA levels were induced by rapamycin (Fig. 1D). METTL3/14 knockdown induced mRNA levels of BEX1, EIF6, MAPK13, and SLC25A37 (Fig. 1E), and WTAP knockdown increased mRNA levels of EIF6 and MAPK13 (Fig. 1F). Thus, we decided to further study MAPK13 because its mRNA level was most dramatically and consistently induced by all three conditions (Fig. 1D-F).

m⁶A modification regulates MAPK13 expression among p38 isoforms

Next, we assessed protein levels of MAPK13 to examine whether the changes in MAPK13 mRNA levels are reflected in MAPK13 protein expression. Upon rapamycin treatment, the protein levels of MAPK13 increased by 2-fold (Fig. 2, A and B). Double knockdown of METTL3/14 also resulted in a 2-fold increase in MAPK13 protein expression
MAPK13 is a member of the p38 MAPK protein family composed of p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13). These proteins control diverse cellular signaling processes, including proliferation, differentiation, inflammation, and cell death responses (25, 26, 27, 28). Interestingly, in contrast to MAPK13, knockdown of METTL3/14 did not induce mRNA levels of MAPK11, MAPK12, or MAPK14 (Fig. 2E). In the case of MAPK11, its mRNA level was decreased (Fig. 2E). To examine the protein level of these isoforms, we used an antibody that detects amino acid sequences across three p38 isoforms, MAPK11, MAPK12, and MAPK14 (29). This antibody does not detect MAPK13. Interestingly, the protein expression of these p38 isoforms (MAPK11, MAPK12, and MAPK14) did not change regardless of METTL3/14 knockdown (Fig. 2, F and G). Thus, MAPK13 is a unique p38 isoform regulated by mTORC1-dependent m6A modification.

mTORC1-m6A-YTHDF2 destabilizes MAPK13 mRNA

Once modified with m6A, mRNAs recruit m6A reader proteins that determine the fate of target transcripts such as mRNA stability or translation efficiency (6, 7). From our previous miCLIP-seq, we found that a specific m6A modification site on 3'UTR of MAPK13 was decreased by mTOR inhibition (Fig. 3A) (20). Given that suppression of such m6A modification by METTL3/14 or WTAP knockdown increases both MAPK13 mRNA and protein levels (Fig. 1 and 2), we hypothesized that MAPK13 mRNA is degraded by YTHDF2, an m6A reader protein that destabilizes target transcripts (6, 7, 30, 31). Consistent with our hypothesis, knockdown of YTHDF2 resulted in a significant increase in MAPK13 mRNA levels (Fig. 3B). Consequently, MAPK13 protein levels also increased (Fig. 3C). Hence, YTHDF2 is the effector protein responsible for MAPK13 mRNA degradation after mTORC1-mediated m6A modification.

To further confirm whether the stability of MAPK13 mRNA is indeed regulated by mTORC1-dependent m6A modification, we sought to quantitate mRNA half-life. To this end, we treated actinomycin D to block de novo mRNA synthesis and measured the remaining transcript levels at different time points (32). In control cells, MAPK13 mRNA was degraded in a time-dependent manner with a half-life of 6 h (Fig. 3D). However, upon rapamycin treatment, the stability of MAPK13 mRNA was dramatically increased, with 90% of transcripts remaining even after 8 h (Fig. 3D). Similarly, METTL3/14 double knockdown also markedly increased the half-life of MAPK13 mRNA but not that of the other p38 isoforms (Fig.
Collectively, these results demonstrate that rapamycin increases the mRNA stability of MAPK13 via the m\(^6\)A-YTHDF2 axis.

**MAPK13 inhibition enhances rapamycin's anti-tumor effects**

Among various mitogen-activated MAPK family member proteins, p38 MAPKs are mostly responsible for reduced cell growth or increased apoptosis (25, 26, 27, 28). We therefore reasoned that mTORC1-activated cancer cells actively degrade MAPK13 mRNA to promote their cell growth. To test this hypothesis, we knocked down MAPK13 in LAM cells and measured cell proliferation (Fig. 4A). However, to our surprise, MAPK13 knockdown preferentially reduced cell growth, indicating that MAPK13 plays a role in promoting cell growth. In the absence of MAPK13, rapamycin treatment further suppressed cell growth (Fig. 4B). MAPK13 knockdown also augmented cell migration suppression by rapamycin (Fig. 4, C and D). Thus, MAPK13 induction by rapamycin treatment may limit the tumor suppressive effects of the drug. These findings also suggest that inhibition of MAPK13 coupled with rapamycin can be more effective at impairing tumor growth compared to sole rapamycin treatment (Fig. 4E).

**Discussion**

Because of its specific inhibitory activity on mTORC1, rapamycin was thought to be a ground-breaking anti-cancer therapeutic for a broad spectrum of mTORC1-overactivated human cancers. However, clinical trials revealed that rapamycin was not as efficient as expected. Some tumors even regrow into a bigger size after cessation of rapamycin treatment (17, 18, 19). One of the mechanisms for rapamycin resistance is activation of other growth-promoting signaling pathways (19). In breast cancer patients, mitogenic ERK MAPK signaling was increased in cancer tissues upon rapamycin treatment (33). This unexpected observation led to the identification of negative feedback signaling pathways downstream of mTORC1; while mTORC1 promotes anabolic pathways for cell growth, it ironically inhibits several pro-growth signals including PI3K, Ras, and MEK (19). Some of these pro-growth signals such as PI3K and RAS are upstream activators of mTORC1; therefore, when mTORC1 is suppressed by rapamycin, these negative feedbacks are released, resulting in the continued growth of cancer cells (34). On the other hand, co-treatment rapamycin with PI3K or MEK inhibitors is more effective for tumor suppression in cell culture and mouse models (33, 35). Here, we identified MAPK13 as another player that
potentially limits rapamycin’s tumor suppressive effects. MAPK13 has been shown to activate mTORC1, indicating the negative feedback loop between MAPK13 and mTORC1 (36). Indeed, suppression of MAPK13 in combination with rapamycin treatment enhanced rapamycin’s effect on cell growth and migration inhibition, suggesting MAPK13 as a potential therapeutic target for augmenting rapamycin sensitivity (Fig. 4).

MAPK13 is one of the four p38 MAPK family proteins. Among the isoforms, MAPK14/p38α is expressed in most cell types, whereas the other MAPK family genes are expressed in specific tissues; MAPK11/p38β and MAPK12/p38γ are expressed in the brain and skeletal muscle, while MAPK13/p38δ is expressed in the kidney and lung (28). Intriguingly, the kidney and lung are the two dominant organs that develop tumors in LAM patients (37). Our data indicate that, among the p38 MAPK family genes, only MAPK13/p38δ was regulated by mTORC1-dependent m6A modification (Fig. 2 and 3). Therefore, small molecule inhibitors that specifically target MAPK13/p38δ isoform can be a selective therapeutic regimen with improved efficacy and lower toxicity. While MAPK14/p38α isoform has been most extensively studied, MAPK13/p38δ has recently emerged as a potential drug target because of its roles in stress responses, cytokine production, and tumor development (27, 26, 38, 39). In this sense, our findings suggest MAPK13 as a promising target for combination chemotherapy with rapamycin to overcome the limited efficacy of rapamycin.

Experimental Procedures

Cell culture

Human kidney angiomyolipoma-derived LAM 621-101 cell line (mTORC1 overactivation due to TSC2 deficiency; RRID: CVCL_S879) was provided by Drs. Jane Yu and Elisabeth Henske (23). LAM 621-101 cells were grown in DMEM (GIBCO) with 10% FBS (Sigma-Aldrich) at 37°C with 5% CO2. 5X10^6 cells counted by Multisizer 4e Coulter Counter (Beckman) were plated on a 60 mm plate and serum starved before analysis unless otherwise indicated. Rapamycin (Calbiochem) dissolved in DMSO was treated at the final concentration of 20 nM. siRNAs (Sigma-Aldrich) dissolved in nuclease-free water were transfected using Lipofectamine RNAiMAX reagent (Invitrogen) at the final concentration of 30 nM. siRNA list is provided in Table S1.

Quantitative PCR (qPCR)

PureLink RNA isolation kit (Life Technologies) was used to isolate total RNA from cells. After
removing genomic DNA by DNase I (Sigma-Aldrich-Aldrich), RNA was reverse transcribed to cDNA using the iScript kit (Bio-rad). The resulting cDNA was analyzed by quantitative RT-PCR (qPCR) using SYBR green master mix (Life Technologies) on QuantStudio6 Real-Time PCR system (Life Technologies). For the qPCR screen in Figure 1, 17 final candidate genes from our previous miCLIP-seq performed in HEK293E cells with and without mTOR inhibitor, torin1, were used (Cho et al., 2021) (20). mRNA levels were calculated by delta-delta CT method using housekeeping genes Actin, PPIB, and TBP. The primer list is provided in Table S2.

**Immunoblot**

Cells were homogenized on ice using RIPA lysis buffer (25 mM Tris-HCl [pH 7.4], 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 2 mM DTT, 1% Sodium deoxycholate, 1% NP-40) supplemented with protease inhibitors (1 mM PMSF, 2 μg/mL pepstatin A, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and phosphatase inhibitors (10 mM NaF, 1 mM Na3VO4). Cell lysates were cleared by centrifugation at 13,000 rpm at 4°C for 30 min. Detergent compatible (DC) protein assay (Bio-rad) was used to measure protein concentration. Proteins were boiled for 10 min with Laemmli sample buffer. SDS-PAGE gels were used to separate proteins (10-30 μg) and transferred to the nitrocellulose membrane (Amersham Biosciences). Membranes were then incubated with Odyssey blocking solution (Li-COR Biosciences), followed by incubation with primary and IRDye secondary (Li-COR Biosciences) antibodies. Immunoblot signals were detected and quantified by Image Studio software with the Li-COR imaging system (Li-COR Biosciences). Immunoblot images are representative of at least two independent experiments. Antibodies against pS6(S240/S244), S6, Beta-actin, p38 (MAPK11/12/14), METTL3, and METTL14 (Cell Signaling Technology); YTHDF2 (Proteintech); WTAP (Abcam); and MAPK13 (R&D Systems) were used.

**mRNA stability assay**

Cells were treated with 5 μg/ml actinomycin D (Sigma-Aldrich) to inhibit transcription and were collected at 0, 4, and 8 h to analyze the remaining mRNA levels. Total RNA was extracted and mRNA levels were analyzed by qPCR as described above.

**Cell proliferation assay**
siRNA-transfected cells were seeded on a 60 mm plate. After 24 h, cells were treated with DMSO (control) or rapamycin without FBS. Cell numbers were measured using Multisizer 4e Coulter Counter (Beckman) at 0 h and 72 h after treatment. Cell proliferation (fold change) was calculated by dividing the cell numbers at 72 h by the cell numbers at 0 h.

**Cell migration assay**

Wound-healing assay was applied to assess cell migration. siRNA-transfected cells were seeded on a 6-well plate. After 24 h, cells were treated with DMSO (control) or rapamycin without FBS. Once cells are confluent, a clear wound line was created using a sterile 200 μL pipette tip. Cell images containing the wound area were taken at 0 h and 24 h using Eclipse Ts2-FL microscope and DS-Fi3 Camera (Nikon). Cell migration efficiency (%) was calculated by measuring the cell migration area (0 h ~ 24 h) using the ImageJ software program.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software. All values are presented as mean ± standard deviation (SD). Statistical significance was determined using a two-tailed Student’s t-test for comparison between two groups or multiple unpaired t-tests for comparison between multiple groups. Statistical significance is presented as *p < 0.05, **p < 0.01, ***p < 0.001, or ns = not significant.

**Abbreviations and nomenclature**

Bcl-2 (B-cell lymphoma 2), PHLPP2 (PH domain and leucine-rich repeat protein phosphatase 2), LAM (Lymphangioleiomyomatosis), TSC (Tuberous sclerosis complex), miCLIP-seq (m6A individual-nucleotide resolution UV crosslinking and immunoprecipitation), BEX1 (Brain expressed X-linked 1), EIF4A2 (Eukaryotic translation initiation factor 4A2), EIF6 (Eukaryotic translation initiation factor 6), FGFR (Fibroblast growth factor receptor), NABP1 (Nucleic acid binding protein 1), NOP56 (NOP56 ribonucleoprotein), OBSCN (Obscurin), PKD (Polycystic kidney disease), PRPF4B (Pre-mRNA processing factor 4B), SDF2 (Stromal cell-derived factor 2), SLC25A37 (Solute carrier family 25 member 37), STAT5B (Signal transducer and activator of transcription 5B), TRDMT1 (tRNA aspartic acid methyltransferase 1), TRP (Transient receptor potential), ERK (Extracellular signal-regulated kinase), PI3K (Phosphoinositide 3-kinase).
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References


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**Figure Legends**

Figure 1. Identification of MAPK13 as the downstream target of rapamycin and m⁶A writer complex.

(A) Schematic of the qPCR screen in LAM 621-101 (*TSC2−/−*) cells to identify target genes whose expression is regulated by rapamycin and m⁶A writer complex. The screen sets include three conditions treated with DMSO (control) vs. rapamycin for 48 h, transfected with siNTC (control) vs. siMETTL3/14, and transfected with siNTC vs. siWTAP. Candidate genes were selected from our previous miCLIP-seq in HEK293E cells treated with mTOR inhibitor, torin1 (20).

(B, C) Immunoblot analysis of LAM 621-101 cells treated with DMSO or rapamycin. (C) shows a quantification graph of immunoblot bands. N=5.
(D-F) qPCR analysis of 17 candidate genes in LAM 621-101 cells treated with DMSO vs. rapamycin (D), transfected with siNTC vs. siMETTL3/14 (E), or transfected with siNTC vs. siWTAP (F). N=5.

*p < 0.05, **p < 0.01, ***p < 0.001. Error bars show standard deviation (SD). Numbers on the immunoblot indicate the positions of molecular weight markers.

Figure 2. mTORC1 and m^6^A regulate MAPK13 expression among the other p38 MAPK isoforms.

(A, B) Immunoblot analysis of LAM 621-101 cells treated with DMSO or rapamycin. (B) shows the quantification graph of immunoblot bands. N=5.

(C, D) Immunoblot analysis of LAM 621-101 cells transfected with siNTC or siMETTL3/14. (D) shows a quantification graph of immunoblot bands. N=5.

(E) qPCR analysis of p38 MAPK family genes. LAM 621-101 cells were transfected with siNTC or siMETTL3/14. N=5.

(F, G) Immunoblot analysis of LAM 621-101 cells transfected with siNTC or siMETTL3/14. (G) shows a quantification graph of immunoblot bands. N=5.

***p < 0.001, ns = not significant. Error bars show standard deviation (SD). Numbers on the immunoblot indicate the positions of molecular weight markers.

Figure 3. MAPK13 mRNA stability is regulated by the mTORC1-m^6^A-YTHDF2 axis.

(A) Schematic of MAPK13 mRNA. From miCLIP-seq (Cho et al., 2021) (20), an mTORC1-dependent m^6^A modification site (red) was identified on the 3'UTR of MAPK13.

(B, C) qPCR and immunoblot analysis of LAM 621-101 cells transfected with siNTC or siYTHDF2. N=5.

(D) mRNA stability analysis of MAPK13 in LAM 621-101 cells treated with rapamycin. Cells were treated with actinomycin D for the indicated times. N=5.

(E-H) mRNA stability analysis of MAPK11 (E), MAPK12 (F), MAPK13 (G), and MAPK14 (H) in LAM 621-101 cells transfected with siNTC or siMETTL3/14. Cells were treated with actinomycin D for the indicated times. N=5.

***p < 0.001. Error bars show standard deviation (SD). Numbers on the immunoblot indicate
the positions of molecular weight markers.

**Figure 4. MAPK13 inhibition enhances rapamycin’s suppressive effect on cancer cell growth and migration.**

(A) Immunoblot analysis of LAM 621-101 cells transfected with siNTC or siMAPK13, followed by treatment with DMSO or rapamycin. N=5.

(B) Cell proliferation assay of LAM 621-101 cells transfected with siNTC or siMAPK13, followed by treatment with DMSO or rapamycin. The graph shows the fold increase of cell numbers 3 days after the treatment. N=6.

(C, D) Wound healing assay of LAM 621-101 cells transfected with siNTC or siMAPK13, followed by treatment with DMSO or rapamycin. After scratching the cell layer to form a wound, images were captured at 0 h and 24 h to evaluate cell migration. Black dotted lines indicate the initial wound area; red dotted lines mark the migrating front of cells (C). Cell migration efficiency was calculated by measuring the wound area at each time point by Image J software (D). Scale bar, 500 µm. N=12.

(E) A schematic diagram describing the regulation of MAPK13 expression by mTORC1-dependent m6A methylation (left, without rapamycin; middle, with rapamycin) and the potential impact of MAPK13 inhibition in tumor suppression in combination with rapamycin treatment (right).

***p < 0.001. Error bars show standard deviation (SD). Numbers on the immunoblot indicate the positions of molecular weight markers.

**Supplemental Information**

Table S1. siRNA list.

Table S2. Primer list.
Figure 1

A) QPCR screen of miCLIP-seq candidate genes

B) Rapamycin

C) Protein level (fold change)

D) mRNA level (fold change)

E) siNTC vs siMETTL3/14

F) siNTC vs siWTAP
Figure 2

Panel A: Western blot analysis of MAPK13, p-S6, and Actin expression under Rapamycin treatment.


Panel C: METTL3 and METTL14 protein levels under siMETTL3/14 treatment.

Panel D: MAPK13 protein level fold change under siNTC or siMETTL3/14 treatment.

Panel E: mRNA level fold change of MAPK11, MAPK12, MAPK13, and MAPK14 under siNTC or siMETTL3/14 treatment.


Panel G: MAPK11/12/14 protein level fold change under siNTC or siMETTL3/14 treatment.
Figure 3

A

MAPK13 mRNA

5'UTR   CDS   3'UTR

CCAGACACTGCCC

B

MAPK13 mRNA level (fold change)
siNTC

siYTHDF2

***

C

siYTHDF2

MAPK13

YTHDF2

Actin

DMSO

Rapamycin

D

MAPK13 mRNA level (fold change)

Actinomycin D (hr)

E

MAPK11

mRNA level (fold change)
siNTC

siMETTL3/14

F

MAPK12

mRNA level (fold change)
siNTC

siMETTL3/14

G

MAPK13

mRNA level (fold change)

H

MAPK14

mRNA level (fold change)
Figure 4

A

B

C

D

E

Rapamycin

siMAPK13

- - + - - - + + +

MAPK13

p-S6

S6

Actin

50 37 25 37 25 37 50

(kDa)

Cell proliferation (fold change)

Cell migration (%)

0 1 2 3 4

No treatment

Rapamycin

Rapamycin + MAPK13 inhibition

mTORC1

m6A

MAPK13

mRNA degradation

Expression

Inhibition

Tumor progression

Mild tumor suppression

Strong tumor suppression

DMSO

+ siNTC

DMSO

+ siMAPK13

Rapamycin

+ siMAPK13

0 h

24 h

0 10 20 30 40

Rapamycin

siMAPK13

- - - + + +

0 10 20 30 40