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Control of mammalian G protein signaling by N-terminal acetylation and the N-end rule pathway

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Abstract

Rgs2, a regulator of G proteins, lowers blood pressure by decreasing signaling through $G\alpha_q$. Human patients expressing Met-Leu-Rgs2 (ML-Rgs2) or Met-Arg-Rgs2 (MR-Rgs2) are hypertensive relative to people expressing wild-type Met-Gln-Rgs2 (MQ-Rgs2). We found that wild-type MQ-Rgs2 and its mutant, MR-Rgs2, were destroyed by the Ac/N-end rule pathway, which recognizes N^{α}-terminally acetylated (Nt-acetylated) proteins. The shortest-lived mutant, ML-Rgs2, was targeted by both the Ac/N-end rule and Arg/N-end rule pathways. The latter pathway recognizes unacetylated N-terminal residues. Thus, the Nt-acetylated Ac-MX-Rgs2 (X = Arg, Gln, Leu) proteins are specific substrates of the mammalian Ac/N-end rule pathway. Furthermore, the Ac/N-degron of Ac-MQ-Rgs2 was conditional, and Teb4, an endoplasmic reticulum (ER) membrane-embedded ubiquitin ligase, was able to regulate G protein signaling by targeting Ac-MX-Rgs2 proteins for degradation through their N^{α}-terminal acetyl group.

Regulators of G protein signaling (RGSs) bind to specific Gα subunits of heterotrimeric G proteins (Gαβγ) and accelerate the hydrolysis of Gα-bound guanosine tri-phosphate, thereby abrogating the signaling by G proteins (1–4). The mammalian Rgs2 protein regulates stress responses, translation, circadian rhythms, Ca^{2+} channels, specific hormones, and cardiovascular homeostasis (3–10). Blood pressure–increasing vasoconstrictors such as norepinephrine and angiotensin II are up-regulated by activated $G\alpha_q$ proteins, which are

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SUPPLEMENTARY MATERIALS

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deactivated by Rgs2 (7). Both *Rgs2*$^{-/-}$ and heterozygous *Rgs2*$^{+/-}$ mice are strongly hypertensive (8, 9). Human patients with decreased Rgs2 signaling are hypertensive as well (10).

In some hypertensive patients, one of two *Rgs2* genes encodes Met-Leu-Rgs2 (ML-Rgs2), in which Gln at position 2 of wild-type Met-Gln-Rgs2 (MQ-Rgs2) is replaced by Leu. Another hypertension-associated Rgs2 mutant is Met-Arg-Rgs2 (MR-Rgs2) (10). The Gln → Leu and Gln → Arg mutations are not detected in the general population (10). All three Rgs2 proteins are up-regulated by a proteasome inhibitor, which suggests that they may be targeted by a proteasome-dependent proteolytic system (11).

The N-end rule pathway recognizes proteins containing N-terminal (Nt) degradation signals called N-degrons, polyubiquitylates these proteins, and thereby causes their degradation by the proteasome (fig. S1) (12–20). The main determinant of an N-degron is a destabilizing Nt residue of a protein. Recognition components of the N-end rule pathway, called N-recognins, are E3 ubiquitin ligases that can target N-degrons. Regulated degradation of proteins by the N-end rule pathway mediates a broad range of biological functions (fig. S1) (12–20).

The N-end rule pathway consists of two branches. One branch, the Arg/N-end rule pathway, targets unacetylated destabilizing Nt residues (12, 14, 16). The Nt residues Arg, Lys, His, Leu, Phe, Tyr, Trp, and Ile, as well as Nt-Met [if it is followed by a bulky hydrophobic (Φ) residue], are directly recognized by N-recognins (16). In contrast, the unacetylated Asn, Gln, Asp, and Glu (as well as Cys, under some conditions) Nt residues are destabilizing, owing to their preliminary enzymatic modifications (fig. S1D).

The pathway's other branch, called the Ac/N-end rule pathway, targets proteins through their N$^{\alpha}$-terminally acetylated (Nt-acetylated) residues (fig. S1, A and C) (13, 15, 16). Degrons and E3 ubiquitin ligases of the Ac/N-end rule pathway are called Ac/N-degrons and Ac/N-recognins, respectively. Approximately 90% of human proteins are cotranslationally and irreversibly Nt-acetylated by ribosome-associated Nt-acetylases (21, 22). (In contrast, acetylation of internal Lys residues is reversible and largely posttranslational.) Doa10, an ER membrane-embedded E3 ubiquitin ligase of the yeast *Saccharomyces cerevisiae* (23, 24), functions as an Ac/N-recognin (13). Not4, a cytosolic and nuclear E3, is another yeast Ac/N-recognin (15).

The Arg/N-end rule pathway is present in all examined eukaryotes, from fungi to mammals and plants (fig. S1D) (12, 18, 19). In contrast, the Ac/N-end rule pathway (fig. S1C) has been identified in *S. cerevisiae* (13, 15, 16), but its presence in mammals and other multicellular eukaryotes has been conjectural so far.

We began by subjecting wild-type human MQ-Rgs2 and its ML-Rgs2 mutant to cycloheximide (CHX) chases in yeast (13, 15, 16). The C-terminally HA (hemagglutinin)–tagged MQ-Rgs2$_{ha}$ was short-lived ($t_{1/2} \approx 30$ min) in wild-type yeast and was stabilized in both *naa20*Δ and *doa10*Δ cells, which lacked, respectively, the cognate NatB Nt-acetylase and the Doa10 Ac/N-recognin (13) (Fig. 1, A and C, and figs. S1C and S2). Thus, in yeast, wild-type MQ-Rgs2$_{ha}$ was degraded largely by the Ac/N-end rule pathway (13, 15, 16).

The mutant human ML-Rgs2$_{ha}$ was also short-lived in wild-type *S. cerevisiae* ($t_{1/2}$ < 15 min) and was partially stabilized in both *naa30*Δ and *ubr1*Δ cells, which lacked, respectively, the cognate NatC Nt-acetylase and the Ubr1 N-recognin of the Arg/N-end rule pathway (Fig. 1, B and D, and figs. S1C, S2, and S3, A and D). ML-Rgs2$_{ha}$ was nearly completely stabilized in double-mutant *naa30*Δ *ubr1*Δ cells, including strongly elevated time-zero (pre-chase) levels of ML-Rgs2$_{ha}$ (Fig. 1, B and D, and fig. S3, A and D).

Many cellular proteins are partially Nt-acetylated (21). Non–Nt-acetylated yeast MΦ-type proteins are eliminated by the Arg/N-end rule pathway, whereas the Nt-acetylated counterparts of these proteins are destroyed by the Ac/N-end rule pathway (15, 16). This dual-targeting pattern was also observed with human ML-Rgs2$_{ha}$ (Fig. 1, B and D, and fig. S3, A and D). The contribution of the Ubr1 N-recognin to the degradation of ML-Rgs2$_{ha}$ (Fig. 1B and fig. S3A) indicated its incomplete Nt-acetylation in *S. cerevisiae*, similarly to results with natural MΦ-type yeast proteins (15, 16). Both wild-type MQ-Rgs2 and the second hypertension-associated mutant MR-Rgs2 were targeted (after their Nt-acetylation) solely by the Ac/N-end rule pathway, because the unacetylated Nt-Met followed by a non-Φ residue such as Gln or Arg is not recognized by the Arg/N-end rule pathway (in contrast to an MΦ-type protein such as ML-Rgs2$_{ha}$) (fig. S1D) (16).

Wild-type MQ-Rgs2 is a predicted substrate of the NatB Nt-acetylase (fig. S2), in agreement with stabilization of MQ-Rgs2$_{ha}$ in *naa20*Δ *S. cerevisiae*, which lack NatB (Fig. 1, A and C). Using mass spectrometry (13), we analyzed the MQ-Rgs2 protein from human embryonic kidney (HEK) 293 cells, confirming its Nt-acetylation (fig. S5A).

Considerably unequal levels of transiently expressed MQ-Rgs2$_{ha}$, MR-Rgs2$_{ha}$, and ML-Rgs2$_{ha}$ in human HeLa cells suggested their different stabilities (MQ > MR > ML) in these cells—an interpretation consistent with near-equal levels of the corresponding *MX-Rgs2* mRNAs (fig. S4, C, F, and G). Indeed, CHX chases in HeLa cells showed that MR-Rgs2$_{ha}$ ($t_{1/2}$ < 8 min) and ML-Rgs2$_{ha}$ ($t_{1/2}$ < 5 min) were shorter-lived than the also unstable wild-type MQ-Rgs2$_{ha}$ ($t_{1/2}$ ≈ 15 min) (Fig. 1, E and F, and fig. S3, B and E). The same (MQ > MR > ML) order of degradation rates was observed in ^{35}S pulse chases of MQ-Rgs2$_{ha}$, MR-Rgs2$_{ha}$, and ML-Rgs2$_{ha}$ (Fig. 1J and fig. S4J).

In contrast to Nt-acetylatable (and therefore short-lived) wild-type MQ-Rgs2$_{ha}$, the otherwise identical PQ-Rgs2$_{ha}$ (Pro-Gln-Rgs2$_{ha}$), generated cotranslationally from MPQ-Rgs2$_{ha}$, was neither Nt-acetylated nor recognized by the Arg/N-end rule pathway (figs. S1 and S2). PQ-Rgs2$_{ha}$ was long-lived in HeLa cells (Fig. 1, G and H). This result was an additional, conceptually independent piece of evidence for the targeting of Ac-MQ-Rgs2$_{ha}$ by the Ac/N-end rule pathway (fig. S1C).

Remarkably, the exogenous (overexpressed) wild-type MQ-Rgs2$_{ha}$ was much shorter-lived ($t_{1/2}$ ≈ 15 min) than the endogenous MQ-Rgs2 ($t_{1/2}$ ≈ 3 hours) in HeLa cells that did not overexpress MQ-Rgs2$_{ha}$ (Fig. 1, E and F, Fig. 2B, and figs. S3, B and E, and S4, A and B). These results, with human cells, agreed with the recent demonstration of the biologically relevant conditionality of *S. cerevisiae* Ac/N-degrons (through their steric shielding in cognate protein complexes) (15). Given this understanding with natural Ac/N-end rule

substrates in yeast (15, 16, 25), the present results (fig. S4, A and B) are what one would expect if Ac-MQ-Rgs2, in human cells that do not overexpress it, can be (reversibly) shielded from the Ac/N-end rule pathway soon after Nt-acetylation of MQ-Rgs2. This shielding would occur through formation of one or more physiologically relevant protective complexes between Ac-MQ-Rgs2 and its protein ligand(s). In contrast to the long half-life of the endogenous, "stoichiometrically" expressed MQ-Rgs2, its overexpression in HeLa cells would make the resulting "unprotectable" excess of Ac-MQ-Rgs2$_{ha}$ molecules vulnerable to destruction by the Ac/N-end rule pathway, thereby accounting for the large difference between the slowly degraded endogenous MQ-Rgs2 (in cells that do not overexpress MQ-Rgs2) and the short half-life of overexpressed MQ-Rgs2$_{ha}$ (fig. S4, A and B). Indeed, the short-lived MQ-Rgs2 was strongly stabilized by co-overexpression of one of its binding partners, the Gα_q protein (Fig. 3, A and B).

A candidate Ac/N-recognin of the mammalian Ac/N-end rule pathway was Teb4, an ER membrane–embedded E3 ubiquitin ligase that polyubiquitylates proteins retrotranslocated from the ER. Teb4 is similar to the *S. cerevisiae* Doa10 Ac/N-recognin (13, 23, 24). We found that human Teb4 was indeed an Ac/N-recognin:

1) Transiently expressed MQ-Rgs2$_{ha}$ was up-regulated by a proteasome inhibitor, whereas coexpression of human Teb4 (Teb4$_{3f}$) and MQ-Rgs2$_{ha}$ in HeLa cells down-regulated MQ-Rgs2$_{ha}$ (Fig. 2, A and F). In addition, incrementally higher levels of Teb4$_{3f}$ resulted in incrementally lower levels of endogenous MQ-Rgs2 (Fig. 2G).

2) The level of MQ-Rgs2$_{ha}$ in HeLa cells was not decreased when MQ-Rgs2$_{ha}$ was coexpressed with $\mathrm{Teb4}_{3f}^{C9A}$, a missense mutant that is inactive as a ubiquitin ligase (24) (Fig. 2F). Analogous assays with MR-Rgs2$_{ha}$ and ML-Rgs2$_{ha}$ gave similar results (fig. S4, D and E).

3) The in vivo polyubiquitylation of MQ-Rgs2$_{ha}$ in HeLa cells was increased by coexpression of Teb4$_{3f}$, but not by coexpression of the mutant $\mathrm{Teb4}_{3f}^{C9A}$ (fig. S3F).

4) In CHX chases, endogenous MQ-Rgs2 was stabilized by RNA interference (RNAi)–mediated knockdowns of either endogenous Teb4 E3 or endogenous cognate NatB Nt-acetylase (Fig. 2B and fig. S4A). Similar results were obtained with exogenous (overexpressed) MQ-Rgs2$_{ha}$, confirming the targeting of MQ-Rgs2 by Teb4 and indicating that this targeting required Nt-acetylation of MQ-Rgs2 by the NatB Nt-acetylase (Fig. 2B and fig. S4, A and B). In addition, overexpressed (hypertension-associated) MR-Rgs2$_{ha}$ as well as the engineered MK-Rgs2$_{ha}$ (which has a different basic residue at position 2) were stabilized by RNAi-based knockdown of Naa60, the catalytic subunit of cognate NatF Nt-acetylase (21, 22), but these MX-Rgs2$_{ha}$ proteins were not stabilized by knockdown of the noncognate NatB (Naa20) Nt-acetylase (Fig. 1I and fig. S4, H and I).

5) Cross-linking and coimmunoprecipitation experiments indicated that Teb4 interacted with MQ-Rgs2 (more accurately, with Nt-acetylated Ac-MQ-Rgs2$_{ha}$, as shown below) (Fig. 2, H and I).

6) PQ-Rgs2$_{ha}$ was neither Nt-acetylated nor recognized by the Arg/N-end rule pathway and was a long-lived protein, in contrast to short-lived MQ-Rgs2$_{ha}$ (Fig. 1, G and H, and fig. S1C). In agreement with these in vivo results, Teb4$_{3f}$ coimmunoprecipitated with MQ-Rgs2$_{ha}$ (Ac-MQ-Rgs2$_{ha}$) but not with PQ-Rgs2$_{ha}$ (Fig. 2, H and I, and fig. S3C).

7) Glutathione *S*-transferase (GST) pull-down assays showed that Teb4$_{f}$ interacted with Ac-MQ-Rgs2$^{1-10}$-GST (containing the first 10 residues of wild-type human Rgs2) but not with MQ-Rgs2$^{1-10}$-GST or GST alone, indicating that the binding of Teb4 to Ac-MQ-Rgs2$^{1-10}$-GST required its Nt-acetyl group (Fig. 2, C to E, and fig. S5B).

By increasing the rate of deactivation of Gα_q, Rgs2 can down-regulate Gα_q-activated protein kinases, including the growth-promoting kinase Erk1/2 (3, 4). We asked whether the Teb4 Ac/N-recognin could regulate the activation of Erk1/2 through the degradation of Nt-acetylated Ac-MQ-Rgs2. The Gα_q-coupled M3 acetylcholine receptor was expressed in HeLa cells either alone or together with MQ-Rgs2$_{ha}$. By activating the M3 receptor–coupled Gα_q, the agonist carbachol strongly increased the level of activated Erk1/2 [measured by detecting its phosphorylation by the “upstream” Mek1/Mek2 kinases (3, 4)]. As predicted by the model in which the Teb4-Rgs2 circuit regulates the activation of Erk1/2, this effect of carbachol on Erk1/2 was decreased in cells that also expressed MQ-Rgs2$_{ha}$ and was further diminished upon RNAi-mediated knockdown of Teb4—a change that stabilized MQ-Rgs2$_{ha}$ and thereby further elevated its level (Fig. 3C).

Thus, wild-type human Ac-MQ-Rgs2, its hypertension-associated natural mutants Ac-ML-Rgs2 and Ac-MR-Rgs2, and its engineered mutant Ac-MK-Rgs2 are conditionally short-lived physiological substrates of the mammalian Ac/N-end rule pathway, and the Teb4 ubiquitin ligase acts as an Ac/N-recognin of this pathway. Teb4 promotes G protein signaling by destroying the Ac-MX-Rgs2 proteins (X = Arg, Gln, Leu), which are targeted for degradation through their N$^{\alpha}$-terminal acetyl group. The faster degradation of ML-Rgs2 and MR-Rgs2 (relative to wild-type MQ-Rgs2) and their consequently lower levels can account, at least in part, for the hypertensive phenotypes of these mutants. The resulting understanding of Rgs2 with respect to the N-end rule pathway is summarized in fig. S6.

Identification of Teb4 as an Ac/N-recognin of the mammalian Ac/N-end rule pathway suggests that some previously characterized substrates of Teb4, including specific transmembrane proteins [(23, 24, 26) and references therein], may be recognized by Teb4 at least in part though their Nt-acetylated Nt residues (Ac/N-degrons)—a testable proposition.

More than 30 human proteins contain an RGS-type domain (fig. S7) (3, 4). The N-terminal Cys residue, a common feature of Rgs4, Rgs5, and Rgs16, can be oxidized in vivo by nitric oxide (NO) and oxygen. The resulting Nt-arginylation of oxidized Cys and the ensuing degradation of these RGSs by the Arg/N-end rule pathway mediate its previously described function as a sensor of NO and oxygen (fig. S1D) [reviewed in (12, 18, 19)]. Our study identified Rgs2 (MQ-Rgs2) as a different kind of N-end rule substrate, an Nt-acetylated one. Given their inferred N-terminal sequences, it is possible, indeed likely, that a number of other mammalian RGS proteins (in addition to Rgs2, Rgs4, Rgs5, and Rgs16) may also

prove to be substrates of either the Ac/N-end rule pathway or the Arg/N-end rule pathway, or both of these proteolytic systems (fig. S7).

Many cellular proteins contain Ac/N-degrons, having acquired them during synthesis (13). These degradation signals, recognized by the Ac/N-end rule pathway, tend to be conditional, owing to their shielding in cognate protein complexes (15). In addition, the Ac/N-end rule pathway and the Arg/N-end rule pathway are functionally complementary (16). It could therefore be possible to control the levels (and thus the activities) of many different proteins therapeutically, including Rgs2 and other RGSs, by modulating their Ac/N-degrons or specific components of the Ac/N-end rule pathway.

Supplementary Material

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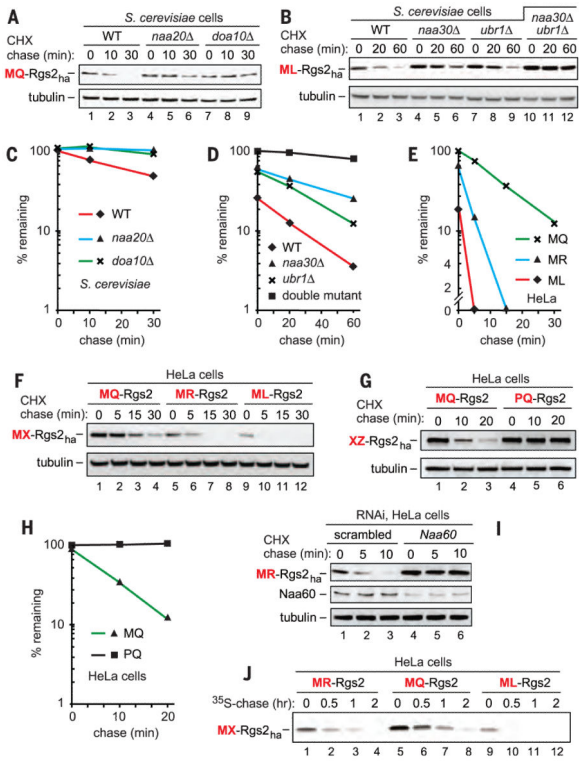

Fig. 1. Rgs2 as an N-end rule substrate

(**A**) CHX chases with wild-type human MQ-Rgs2$_{ha}$ in wild-type, *naa20*Δ, and *doa10*Δ *S. cerevisiae*. (**B**) As in (A) but with ML-Rgs2$_{ha}$ in wild-type, *naa30*Δ, *ubr1*Δ, and *naa30*Δ *ubr1*Δ strains. (**C** to **E**) Quantification of data in (A), (B), and (F), respectively. See the legend to fig. S3 for definitions of "100%" levels at zero time. (**F**) CHX chases with exogenously expressed MX-Rgs2$_{ha}$ (X = Arg, Gln, Leu) in HeLa cells. (**G**) As in (F) but with MQ-Rgs2$_{ha}$ versus PQ-Rgs2$_{ha}$. (**H**) Quantification of data in (G). (**I**) CHX chases with exogenously expressed MR-Rgs2 in HeLa cells subjected to RNAi for a either a "scrambled" target or Naa60. (**J**) ^{35}S-pulse chases with MX-Rgs2$_{ha}$ (X = Arg, Gln, Leu) in HeLa cells.

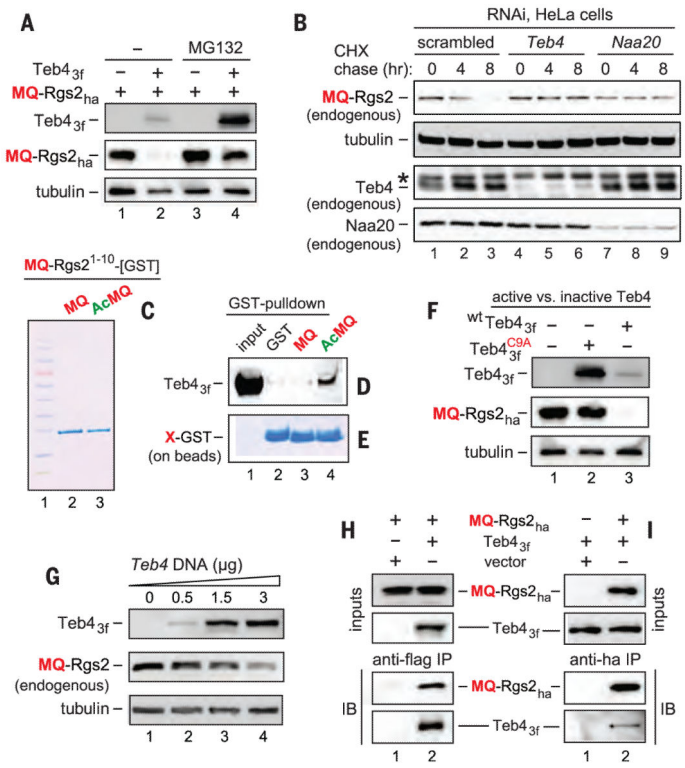

Fig. 2. Teb4 as an Ac/N-recognin

(**A**) MQ-Rgs2$_{ha}$ in HeLa cells with or without Teb4$_{3f}$ or the MG132 proteasome inhibitor. (**B**) CHX chases with endogenous MQ-Rgs2, Teb4, and Naa20 subjected to RNAi for a "scrambled" target, Teb4, or Naa20. The asterisk indicates a protein cross-reacting with anti-Teb4. (**C**) Molecular weight standards, purified MQ-Rgs2$^{1-10}$-GST, and purified Ac-MQ-Rgs2$^{1-10}$-GST, respectively. (**D**) Lane 1, Teb4$_{3f}$ input; lanes 2 to 4, GST pull-downs with GST alone, MQ-Rgs2$^{1-10}$-GST, and Ac-MQ-Rgs2$^{1-10}$-GST, respectively. (**E**) As in (D) but Coomassie-stained GST fusions released from beads. (**F**) MQ-Rgs2$_{ha}$ in HeLa cells overexpressing wild-type Teb4$_{3f}$ or $\mathrm{Teb4}_{3f}^{C9A}$. (**G**) Increases in Teb4$_{3f}$ led to decreases in MQ-Rgs2$_{ha}$. (**H**) HeLa cells expressing MQ-Rgs2$_{ha}$ alone (lane 1) or together with Teb4$_{3f}$ (lane 2) were treated with a cell-penetrating cross-linker, followed by immunoprecipitations with anti-FLAG, reversal of cross-links, SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotting with anti-HA and anti-FLAG. (**I**) As in (H) but Teb4$_{3f}$ alone in lane 1, and immunoprecipitations with anti-HA.

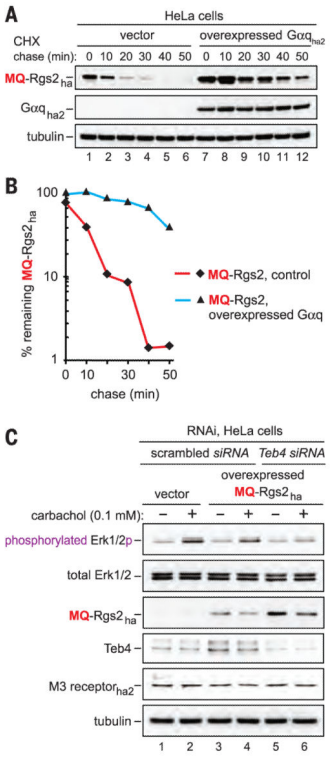

Fig. 3. Gα$_q$ stabilizes Rgs2 while the Teb4-mediated degradation of Rgs2 increases signaling by Gα$_q$

(**A**) CHX chase of MQ-Rgs2$_{ha}$ in HeLa cells that did not express or overexpressed the HA-tagged Gα$_q$. (**B**) Quantification of data in (A). See the legend to fig. S3 for definitions of "100%" levels at zero time. (**C**) HeLa cells were subjected to RNAi either for a "scrambled" target or for Teb4. The M3 receptor was transiently expressed either alone or together with MQ-Rgs2$_{ha}$. The levels of indicated proteins, including the levels of either total Erk1/2 or the activated (specifically phosphorylated) Erk1/2p, were determined by SDS-PAGE and immunoblotting of cell extracts that had been prepared 10 min after treatment of cells with carbachol.