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Rag Ubiquitination Recruits a GATOR1: Attenuation of Amino Acid-Induced mTORC1 Signaling

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In recent issues of *Molecular Cell*, two reports identify that K63-linked RagA polyubiquitination and subsequent recruitment of GATOR1, a complex with GAP activity toward RagA/B GTPases, can attenuate amino acid-induced mTORC1 signaling.

Cells respond rapidly to fluctuations in availability of nutrients (i.e., amino acids, energy) to coordinate anabolic versus catabolic metabolism. Multicellular organisms respond additionally to long-range systemic signals (i.e., growth factors, hormones, cytokines) for communication between tissues. The mechanistic target of rapamycin (mTOR), an evolutionarily conserved kinase, integrates these diverse signals to maintain cellular homeostasis (Huang and Fingar, 2014). mTOR complex 1 (mTORC1) phosphorylates substrates that promote anabolic processes (i.e., S6K1) and suppress catabolic processes (i.e., ULK1) such as autophagy. mTORC1 signaling absolutely requires sufficient levels of amino acids, and mTORC1 responds rapidly to fluctuations in amino acids. While great effort has focused on mechanisms for activation of mTORC1, significantly less attention has focused on the important mechanisms for suppression of mTORC1. In this and the next issue of *Molecular Cell*, Deng et al. (2015) and Jin et al. (2015) propose similar mechanisms for inactivation of mTORC1 within different cellular contexts—upon acute amino acid withdrawal or after prolonged amino acid exposure. The combined work demonstrates that K63-linked polyubiquitination of RagA recruits GATOR1, a complex with GTP-hydrolyzing GTPase-activating protein (GAP) activity toward the Rag GTPases. Interestingly, two different E3 ligases—RNF152 and Skp2-SCF–were found to mediate RagA polyubiquitination on different sites. GATOR1 recruitment converts active GTP-bound RagA into inactive GDP-bound RagA, an event that releases mTORC1 from lysosomal membranes, resulting in mTORC1 inactivation.

How cells sense and respond to fluctuating amino acid levels remained a long-standing mystery until relatively recently. We now know that heterodimeric Rag GTPases (guanosine triphosphatases) composed of either RagA or RagB together with RagC/D link amino acid sensing to mTORC1 (Kim et al., 2008; Sancak et al., 2008) (Figure 1A). Amino acid stimulation loads RagA/B GTPases with GTP, which recruits mTORC1 to lysosomal membranes via its partner protein raptor (Sancak et al., 2010) and dissociates the TSC1/2 complex (a GAP for the mTORC1 activator Rheb, another GTPase) from lysosomal membranes (Demetriades et al., 2014). As Rheb resides on lysosomal membranes, mTORC1 recruitment to lysosomes promotes mTORC1 activation. The multimeric ragulator complex, which tethers Rags to lysosomal membranes, functions as a guanine nucleotide exchange factor (GEF) for RagA/B (Bar-Peled et al., 2012) and thus promotes mTORC1 activation. The heterotrimeric GATOR1 complex opposes the action of the ragulator complex by functioning as a GAP for RagA/B (Bar-Peled et al., 2013).

Deng et al. (2015) asked whether ubiquitination regulates Rag GTPases. They found that amino acid withdrawal increased while acute amino acid stimulation decreased RagA polyubiquitination. By screening a panel of E3 ubiquitin ligases, they discovered that RNF152, a lysosomally anchored E3 ligase, mediates K63-linked RagA polyubiquitination on four lysine residues (K142, K220, K230, and K244). Consistently, amino acid withdrawal promoted an interaction between RNF152 and RagA, while amino addition reduced this interaction. Interestingly, RNF152 interacted more strongly with GDP-loaded than GTP-loaded RagA. By knocking down and overexpressing RNF152, they showed that RNF152 promotes TSC2 binding to RagA and TSC2 localization to lysosomes while it decreases mTOR localization to lysosomes as well as amino acid-induced mTORC1 signaling. Consistent with suppression of mTORC1 signaling, RNF152 reduced cell size and augmented autophagy. These data indicate that amino acid withdrawal recruits RNF152 to RagA to mediate RagA polyubiquitination, resulting in reduced mTORC1 signaling by decreasing mTORC1 localization to lysosomes. By studying an ubiquitin-deficient RagA-4KR mutant, the authors confirmed that RNF152-mediated RagA polyubiquitination reduced RagA GTP-loading and mTORC1 signaling. The authors next investigated the mechanism by which RNF152-mediated RagA polyubiquitination suppresses mTORC1 localization and function. They demonstrated that RNF152 promotes binding between RagA and components of the GATOR1 complex (DEPDC5, Nprl2, and Nprl3) and confirmed that GATOR1 is required for RNF152 to suppress mTORC1 signaling. As the grand finale, Deng et al. employed the CRISPR-Cas9 system to generate RNF152 knockout mice, which were born at the expected Mendelian ratios. Analysis of knockout MEFs from these mice confirmed key points of the data described above. Deng et al. suggest a model (Figure 1A) whereby amino acid withdrawal leads to RNF152-mediated polyubiquitination of

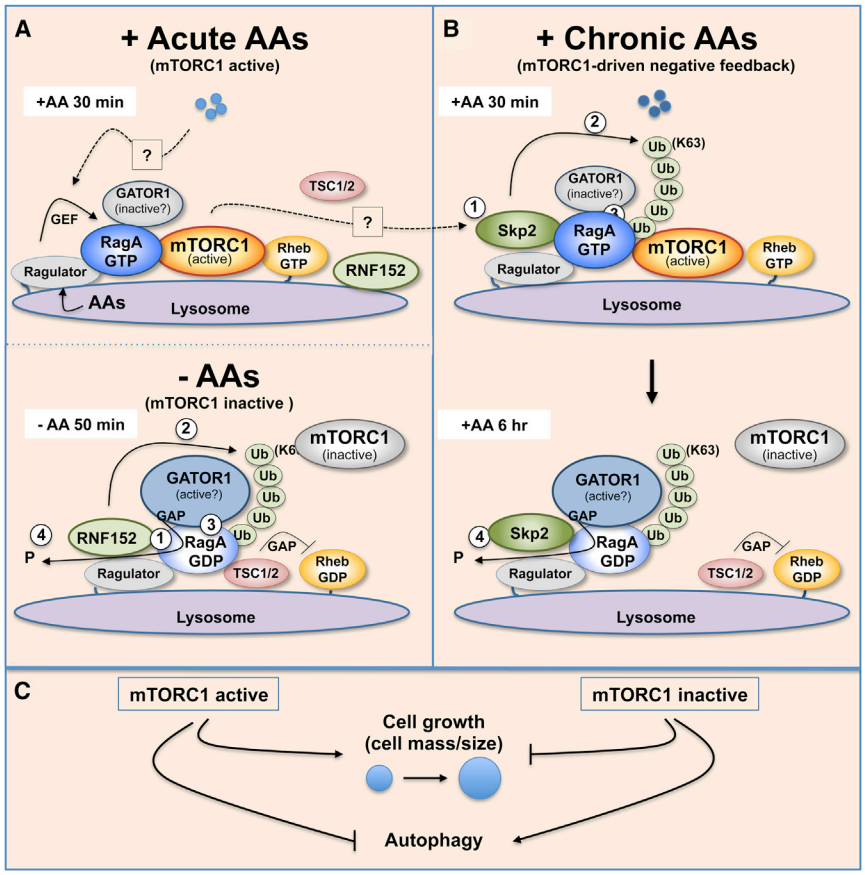

Figure 1. Attenuation of mTORC1 Signaling upon Amino Acid Withdrawal or after Prolonged Amino Acid Exposure by K63-Linked RagA Polyubiquitination and GATOR1 Recruitment

(A) Deng et al. (2015) propose a mechanism by which insufficient amino acid levels attenuate mTORC1 signaling. Amino acid withdrawal recruits RNF152 to GDP-loaded RagA (step 1). RNF152 then polyubiquitinates RagA (step 2), which augments interactions between RagA and GATOR1 and between RagA and TSC (step 3). GATOR1 engagement maintains RagA in an inactivation through GTP hydrolysis (step 4). These events reduce mTOR but increase TSC localization to lysosomes and reduce mTORC1 function, which results in reduced cell growth and increased autophagy (Figure 1C).

(B) Jin et al. (2015) propose that mTORC1 mediates negative feedback to suppress its hyperactivation. mTORC1 signaling recruits Skp2 to GTP-loaded Rag A (step 1). Skp2 then polyubiquitinates RagA (step 2), which augments an interaction between RagA and GATOR1 (step 3), leading to hydrolysis of RagA-GTP to RagA-GDP (step 4). These events reduce mTOR localization to lysosomes and reduce mTORC1 function, which reduces cell growth and increases autophagy.

(C) Active mTORC1 promotes cell growth and suppresses autophagy while inactive mTORC1 promotes autophagy.

RagA, which promotes RagA association with both TSC1/2 and GATOR1. GATOR1 action inactivates Rag heterodimers, causing mTORC1 dissociation from lysosomes and mTORC1 inactivation. These events in turn reduce cell growth and augment autophagy (Figure 1C).

Jin et al. (2015) investigated the mechanism by which GATOR1 inhibits Rag GTPases; in addition, they were interested in understanding how prolonged exposure of cells to amino acids (6 hr) attenuates mTORC1 signaling. They found that acute amino acid stimulation increased RagA polyubiquitination and augmented an interaction between RagA and GATOR1. By screening a panel of E3 ubiquitin ligases, they discovered that Skp2, the F box protein component of the Skp1/Cullen/F box (SCF) E3 ligase complex, associates with RagA and drives K63-linked RagA polyubiquitination on lysine 15 upon amino acid stimulation. Similar to Deng et al.'s work studying RNF152, Skp2 reduced amino acid-dependent localization of mTOR to lysosomes and promoted an interaction between RagA and GATOR1. Intriguingly, the mTORC1 inhibitor rapamycin suppressed the amino acid-induced interaction of RagA with both Skp2 and GATOR1. These data reveal that Skp2 is required for the recruitment of GATOR1 to RagA in a manner dependent on mTORC1 signaling. An ubiquitination-deficient RagA-K15R mutant displayed reduced interaction with GATOR1 relative to wild-type RagA and displayed increased GTP loading. Consistently, expression of K15R RagA, but not wild-type, blunted the ability of Skp2 to reduce GTP loading, revealing that of K15 ubiquitination suppresses RagA GTP loading. In *Skp2*$^{-/-}$ MEFs, amino acid-stimulated mTORC1 signaling to S6K1 was enhanced at early time points and more prolonged at later time points (3 hr). In addition, Skp2 knockdown increased cell size and reduced autophagy in cultured cells. In *Skp2*$^{-/-}$ mice, hepatocyte cell size was reduced and autophagy was increased in certain tissues. Based on these data, Jin et al. suggest that mTORC1 mediates negative feedback to suppress its hyperactivation. In this model (Figure 1B), mTORC1 signaling recruits Skp2 to GTP-loaded RagA. Skp2 then polyubiquitinates RagA, which augments an interaction between RagA and GATOR1, leading to hydrolysis of RagA-GTP to RagA-GDP. These events culminate in reduced mTOR localization to lysosomes and reduced mTORC1 function, which reduces cell growth and increases autophagy (Figure 1C).

As usual, important research findings beget a host of further research questions. How does amino acid withdrawal recruit RNF152 to RagA? Due to the strong interaction of RNF152 with inactive GDP-bound RagA, Deng et al. (2015) suggest that an unknown GAP may act on RagA prior to GATOR1, which recruits RNF152. Alternatively, it seems possible that some other signal may recruit RNF152 to RagA, and the strong RNF152-RagA-GDP interaction may function to anchor RNF152 to the Rag complex to maintain Rag inactivation through GATOR1 action. How does amino acid-stimulated mTORC1 signaling recruit Skp2 to RagA? A theoretical possibility includes Skp2 phosphorylation mediated by mTORC1 or a downstream kinase. As acute stimulation of cells with amino acids (30 min) promotes

association of GATOR1 with RagA (Jin et al., 2015), what mechanism provides the brake to restrict GATOR1 activity at early time points of amino acid stimulation, and how is this brake released at later time points to attenuate mTORC1 signaling? As GATOR2 inhibits GATOR1 (Bar-Peled et al., 2013), perhaps GATOR2 represents the brake? It is important to note that a curious discrepancy exists in the results from the two groups: Deng et al. show that amino acid withdrawal increases while amino acid stimulation decreases RagA polyubiquitination. Lin et al., on the other hand, show that amino acid stimulation increases RagA polyubiquitination. How can amino acid stimulation both decrease and increase RagA polyubiquitination? In addition, it is curious that both RNF152-mediated (Deng et al., 2015) and Skp2-mediated (Jin et al., 2015) RagA polyubiquitination enhance binding between RagA and GATOR1, events that occur upon amino acid withdrawal and amino acid stimulation, respectively. While Jin et al. indeed demonstrated that amino acid stimulation enhances the RagA-GATOR1 interaction, Deng et al. did not show similar data with amino acid withdrawal. Lastly, how are polyubiquitin chains removed from RagA? Which deubiquitylases (DUBs) are responsible?

The finding that two different E3 ligases regulate RagA function suggests that polyubiquitination may represent an underappreciated posttranslational modification (PTM) for regulation of diverse mTORC1 pathway components. Indeed, TRAF6 was shown to mediate K63-linked polyubiquitination of mTOR to promote mTORC1 function (Linares et al., 2013). As ubiquitination regulates target protein function in myriad ways, this PTM may modulate the mTORC1 pathway by a broad array of mechanisms, an exciting prospect for future work.

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Gear Up in Circles

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Two studies published in this issue of *Molecular Cell* (Rybak-Wolf et al., 2015) and in the April issue of *Nature Neuroscience* (You et al., 2015) independently report the upregulated expression of back-spliced circular RNAs (circRNAs) in brains and suggest that they have a potential to regulate synaptic function.

Non-polyadenylated transcriptomic analyses with specific biochemical and bioinformatic strategies have uncovered widespread expression of circular RNAs from back-spliced exons (circRNAs), further expanding the fast growing families of enlisted noncoding RNAs (ncRNAs). Accumulated lines of evidence have revealed that the biogenesis of circRNAs is catalyzed by canonical spliceosomal machinery (Starke et al., 2015) and can be facilitated by both *cis*-elements (Zhang et al., 2014) and *trans*-factors (Ashwal-Fluss et al., 2014; Conn et al., 2015). Although expressed at a low level in general and having mostly unknown functions, some circRNAs were identified to be more abundant than their linear transcripts in different cell types (Salzman et al., 2013). Additionally, circRNAs are preferentially back-spliced from neural genes (Ashwal-Fluss et al., 2014) and upregulated in neural tissues in an age-dependent manner in *Drosophila* (Westholm et al., 2014), implying a potential role of circRNAs in fly brain function. However, a more complete analysis and functional characterization of circRNA expression in mammalian brain has been lacking. In this issue of *Molecular Cell* and in the April