RESEARCH HIGHLIGHT

ATP6AP1 was *Phast*-ID'ed as a long-sought GEF for Rheb

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A new study from Feng et al. developed an improved proximity labeling technology called PhastID and successfully identified the lysosomal v-ATPase subunit ATP6AP1 as an unconventional guanine nucleotide exchange factor for Rheb, a key positive regulator of mTORC1. ATP6AP1 binds directly to Rheb via a highly conserved C-terminal segment to promote its GTP loading and mTORC1 activation.

Signal transduction is a dynamic and complex process involving weak and transient protein–protein interactions (PPIs). In the past decade, proximity labeling technology was developed to achieve specific labeling of proteins in living cells based on their proximity to a target. Represented by the APEX and BioID systems, these technologies utilize enzymes that generate and release small and unstable reagents, which covalently tag the neighboring proteins promiscuously. When fused to a protein of interest, the enzyme can specifically label the bait's surrounding proteins in a proximity-dependent way, thus enabling the identification of potential PPIs. APEX was developed based on the characteristic of Ascorbate peroxidase from *Pisum sativum* by Alice Ting's team.[1] The mutant enzyme can oxidize numerous phenol derivatives to phenoxyl radicals that have short half-life (< 1 ms) and can thus covalently modify the electron-rich amino acids such as Tyr, Trp, His, and Cys within a small radius (< 20 nm)[2]. When biotin-phenol was employed as the substrate of APEX, the surrounding proteins could be labeled by biotin in the presence of H_2O_2 and subsequently purified by streptavidin-conjugated solid materials.[2] This system was further expanded to APEX2.[3] Although APEX-based labeling can be accomplished within 1 min, high background and cytotoxicity of H_2O_2 limit its application.

Different from APEX, the BioID technique employs mutant bacterial biotin protein ligase (BPL) to achieve promiscuous biotin labeling. BPL catalyzes the synthesis of biotinyl-5′-adenylate (bio-5′-AMP) from biotin and ATP, and transfers biotin from the adenylate to the receptor protein. Studies of the *Escherichia coli* Group II BPL, BirA (Biotin-induced repressor A), showed that the GRGRXG motif is highly conserved among different species, and while it was mutated to GRGGXG (BirA R118G, hereafter referred to as BirA*), the receptor specificity of *Ec*BirA was lost and bio-5′-AMP was released freely.[4] The highly active bio-5′-AMP prevents it from diffusing over a long distance (10 nm), which forms the basis of proximity-dependent labeling. This strategy was subsequently applied to the identification of numerous complexes.[5] Several efforts to improve the characteristics of BioID system include BioID2,[6] TurboID[7] and miniTurboID, employing Group I or II BPL from *Aquifex aeolicus*, *Bacillus subtilis*, and molecularly evolved *Ec*BirA*, respectively. However, cytotoxicity and high-level off-target labeling of TurboID were reported likely due to the incorrect folding or location of TurboID-fusion protein.

Feng et al.[8] identified *Pyrococcus horikoshii* biotin protein ligase (*Ph*BPL), which naturally lacks the DNA-binding domain, for a new proximity labeling platform called PhastID, short for *Ph*BPL-assisted biotin identification, with excellent labeling capacity in short-term biotin treatment. Compared to TurboID, PhastID exhibits modest activity but a much higher signal-to-noise ratio. This makes it possible to capture the weak and transient PPIs in the process of signal transduction in living cells.[8]

Mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) integrates environmental cues such as growth factors and amino acid concentration fluctuation to govern cell growth and metabolism. Biochemical studies over the past decades have shown that mTORC1 is recruited onto the lysosomal surface by the activated Rag GTPases, where it is activated by another GTP-bound GTPase Rheb.[9] The nucleotide-loading states of the Rag and Rheb GTPases are two independent arms that converge to regulate the mTORC1 activity. The Rag GTPases are anchored to the lysosome and essential for the amino acid sensing machinery, which also includes the vacuolar H^+-adenosine triphosphatase ATPase (v-ATPase) and Ragulator (comprising p18, p14, MP1, C7orf59 and HBXIP). The Rags are heterodimers comprising a RagA or RagB binding to a RagC or RagD, respectively, which exist in two states: an active state with GTP-bound RagA/B and GDP-bound RagC/D, and an inactive state with GDP-bound RagA/B and GTP-bound RagC/D.[9,10] The GTPase-activating protein (GAP) for Rag A/B has been well studied as GATOR complex which is known to be inhibited by the presence of nutrients such as amino acids; interestingly, upon amino acid insufficiency, mTORC1 could also be suppressed through GCN2-FBXO22-mediated mTOR ubiquitination, a process activated by uncharged tRNAs.[11] Ample evidence shows that Ragulator could serve as the guanine nucleotide exchange factor (GEF) for the Rag GTPases for its GTP-bound state. On the other hand, growth factors activate mTORC1 through inhibiting the tuberous sclerosis complex (TSC), composed of TSC1, TSC2 and TBC1D7, which acts as the GAP for Rheb, by the Akt-mediated phosphorylation of TSC2.[12] However, the GEF for activating Rheb remains unknown for decades.

Feng et al. applied the PhastID system to interrogate a Rheb interactome following insulin stimulation, and identified ATP6AP1, a

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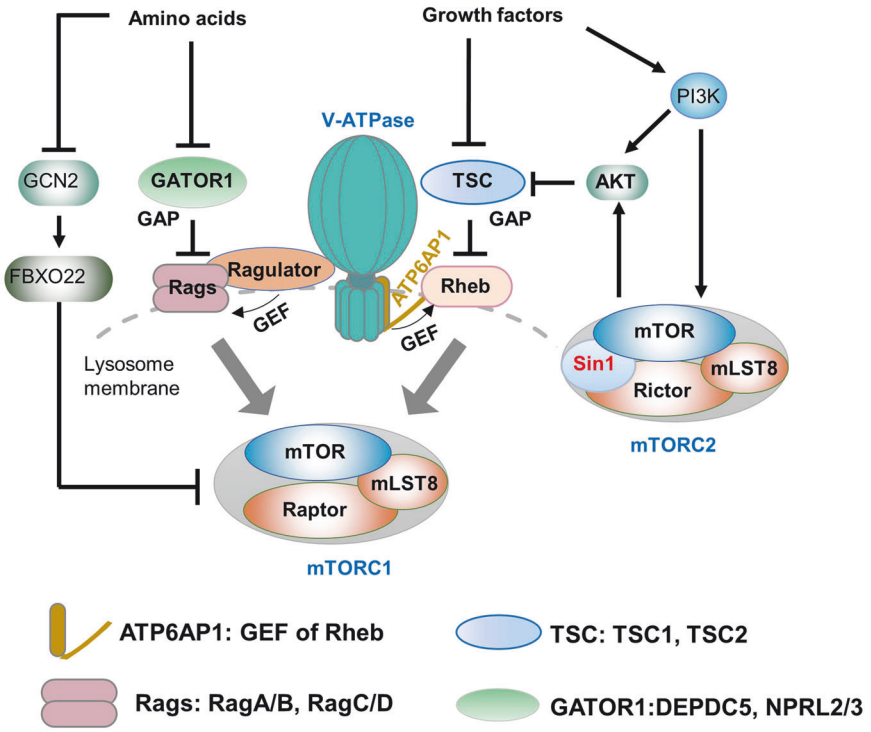

Fig. 1 A scheme showing that the lysosomal v-ATPase is involved in Rag and Rheb activation for mTORC1 signaling. Amino acid shortage activates GCN2-FBXO22 axis to suppress the activity of mTORC1. In the presence of amino acids, Rags recruit mTORC1 to the lysosome, where it could be activated by lysosome-anchored Rheb. Rag activity is regulated by its GEF, Ragulator, who is associated with v-ATPase on the lysosomal surface. Rheb is activated on the lysosomal surface by its non-canonical GEF, ATP6AP1 (the C-tail of ATP6AP1 functions as the GEF), a subunit of lysosomal v-ATPase. GATOR1 is the GAP for RagA/B, whereas TSC is the GAP for Rheb. TSC complex is regulated by the PI3K-mTORC2-AKT axis downstream of growth factors. This study suggests a critical regulatory role of v-ATPase, which concurrently hubs and regulates two types of small GTPases on the cytosolic surface of lysosome for mTORC1 activation.

known component of v-ATPase, as the potential GEF for Rheb (Fig. 1). ATP6AP1 interacts with Rheb on the lysosomal surface in an insulin-dependent manner. Interestingly, the expression level of ATP6AP1 positively correlated with mTORC1 activity, and like Rheb, its overexpression led to strong mTORC1 activation even in the absence of insulin. Furthermore, the authors found that ATP6AP1 binds to Rheb via a small C-terminal tail (C-tail) with a disordered loop facing into the cytoplasm[8] (Fig. 1). Interestingly, the amino acid sequences of the C-terminal segment are extremely conserved. The authors demonstrated that the last 12 amino acids of ATP6AP1 mediate the specific interaction with Rheb, while the 30-amino acid C-tail promotes the GTP loading with an essential role of the tri-aspartate motif. Finally, the authors demonstrated that mutation of the C-tail of ATP6AP1 inhibited the proliferation and migration of tumor cells, highlighting the importance of the ATP6AP1-Rheb axis in mTORC1 pathway. The findings described in this study filled the gap of long-sought GEF for Rheb in the current lysosomal model of mTORC1 activation (Fig. 1).

In summary, the authors have demonstrated the potential of the PhastID system in studying PPIs in signal transduction with the success of identifying ATP6AP1 as the long-sought GEF for Rheb. The current study also brings many more interesting questions that are worth pursuing. For instance, whether there exist other additional GEFs for Rheb remains to be answered. In addition, it remains to be investigated whether ATP6AP1 itself is subjected to regulation following growth factors' stimulation, and whether it could serve as the anchor for Rheb's recruitment to lysosome is also unknown. In this regard, it would be interesting to test whether ATP6AP1 and the TSC complex may competitively regulate the GTP/GDP loading on Rheb dynamically in the absence of growth factor stimulation.

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ADDITIONAL INFORMATION

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