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Exaptive Evolution of Target of Rapamycin Signaling in Multicellular Eukaryotes

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Target of rapamycin (TOR) is a protein kinase that coordinates metabolism with nutrient and energy availability in eukaryotes. TOR and its primary interactors, RAPTOR and LST8, have been remarkably evolutionarily static since they arose in the unicellular last common ancestor of plants, fungi, and animals, but the upstream regulatory mechanisms and downstream effectors of TOR signaling have evolved considerable diversity in these separate lineages. Here, I focus on the roles of exaptation and adaptation in the evolution of novel signaling axes in the TOR network in multicellular eukaryotes, concentrating especially on amino acid sensing, cell-cell signaling, and cell differentiation.

Target of Rapamycin: IntegraTOR and RegulaTOR

Target of rapamycin (TOR) is a central regulator of eukaryotic metabolism, monitoring cellular physiology and nutrient levels to promote anabolism and limit catabolism when conditions are favorable for growth (Liu and Sabatini, 2020). Multiple signal transduction pathways converge to tune TOR activity, and in turn, TOR phosphorylates a number of critical regulatory proteins to modulate cellular metabolism (Valvezan and Manning, 2019). Dysregulation of TOR signaling can cause or contribute to human diseases, including cancers, developmental disorders, and various age-related diseases (Kapahi et al., 2010; Saxton and Sabatini, 2017). Moreover, some viruses hijack the TOR signaling network to drive their replication (Chuluunbaatar et al., 2010; Meade et al., 2018; Ouibrahim et al., 2015; Rubio and Mohr, 2019; Schepetilnikov et al., 2011), possibly including SARS-CoV-2 and other coronaviruses (Gordon et al., 2020; Kindrachuk et al., 2015; Zhou et al., 2020). Accordingly, the TOR signaling network is being comprehensively investigated by biomedical researchers, who have discovered that drugs targeting TOR kinase activity or closely linked components of the TOR signaling network may be effective treatments for many medical conditions (Kato et al., 2019; Ní Bhaoighill and Dunlop, 2019; Saxton and Sabatini, 2017; Sengupta et al., 2019). The TOR signaling network has been thoroughly reviewed elsewhere, including excellent reviews on TOR signaling in humans (Liu and Sabatini, 2020), fungi (Eltschinger and Loewith, 2016; Shimobayashi and Hall, 2014), and plants (Fu et al., 2020; Wu et al., 2019). Rather than attempt to be comprehensive, here, I will emphasize a handful of signaling axes upstream and downstream from TOR in an effort to elucidate the evolution of TOR signaling in eukaryotes.

Across eukaryotic lineages, TOR plays a consistent role in promoting cytosolic ribosome biogenesis. Ribosomes are ~3,200 kDa megacomplexes composed of ~80 ribosomal proteins (r-proteins), which comprise ~40% of ribosomal mass, and 4 ribosomal RNAs (rRNAs), which comprise ~60% of ribosomal mass. The number of ribosomes per cell greatly varies with cell type, species, and physiological conditions: yeast cells have ~200,000 ribosomes per cell (Woolford and Baserga, 2013), mammalian cells can have up to ~10 million ribosomes per cell, and some plant cells can have 75 million ribosomes or more per cell (Lin and Gifford, 1976). Much of the cytosolic space is filled with ribosomes, such that small perturbations in ribosome abundance can shift the biophysical properties (e.g., diffusion rates, phase separation, etc.) in the cytosol (Delarue et al., 2018). Nutritionally, ribosomes can account for up to ~25% of organic nitrogen and ~50% of organic phosphorus in plants, depending on species, physiology, and environment (Veneklaas et al., 2012). These considerable resources are devoted to ribosomes because ribosome abundance is, except under extreme nutrient deprivation, rate limiting for protein synthesis and organismal growth (Shah et al., 2013). Based on current evidence across diverse taxa, TOR evolved an early role in eukaryotes to maintain cellular homeostasis by coordinating ribosome biogenesis: when amino acids, nucleotides, and energy are available, TOR is active and promotes allocating resources to synthesize new ribosomes; when amino acids, nucleotides, or energy stores are scarce, TOR activity decreases and slows the rate of ribosome biogenesis to avoid starvation (Figure 1).

Crucially, TOR must integrate multiple upstream cues, including concentrations of the monomeric metabolites required for ribosome synthesis (amino acids and nucleotides) and the energy stores required to transcribe, translate, and assemble ribosomes, to ensure that ribosome production does not deplete available resources (Figure 1) (Iadevaia et al., 2014; Pelletier et al., 2018). It is well-established that TOR activity is stimulated by amino acid availability in mammals and yeast, especially by the "essential" amino acids that cannot be synthesized *de novo* in mammalian cells, including leucine (which is strictly essential) and arginine (which is conditionally essential during fetal development and some physiological stresses). The precise relationship between amino acids and TOR activity in plants remains to be elucidated, but it is clear that amino acid metabolism also influences TOR activity in *Arabidopsis thaliana* (Cao et al.,

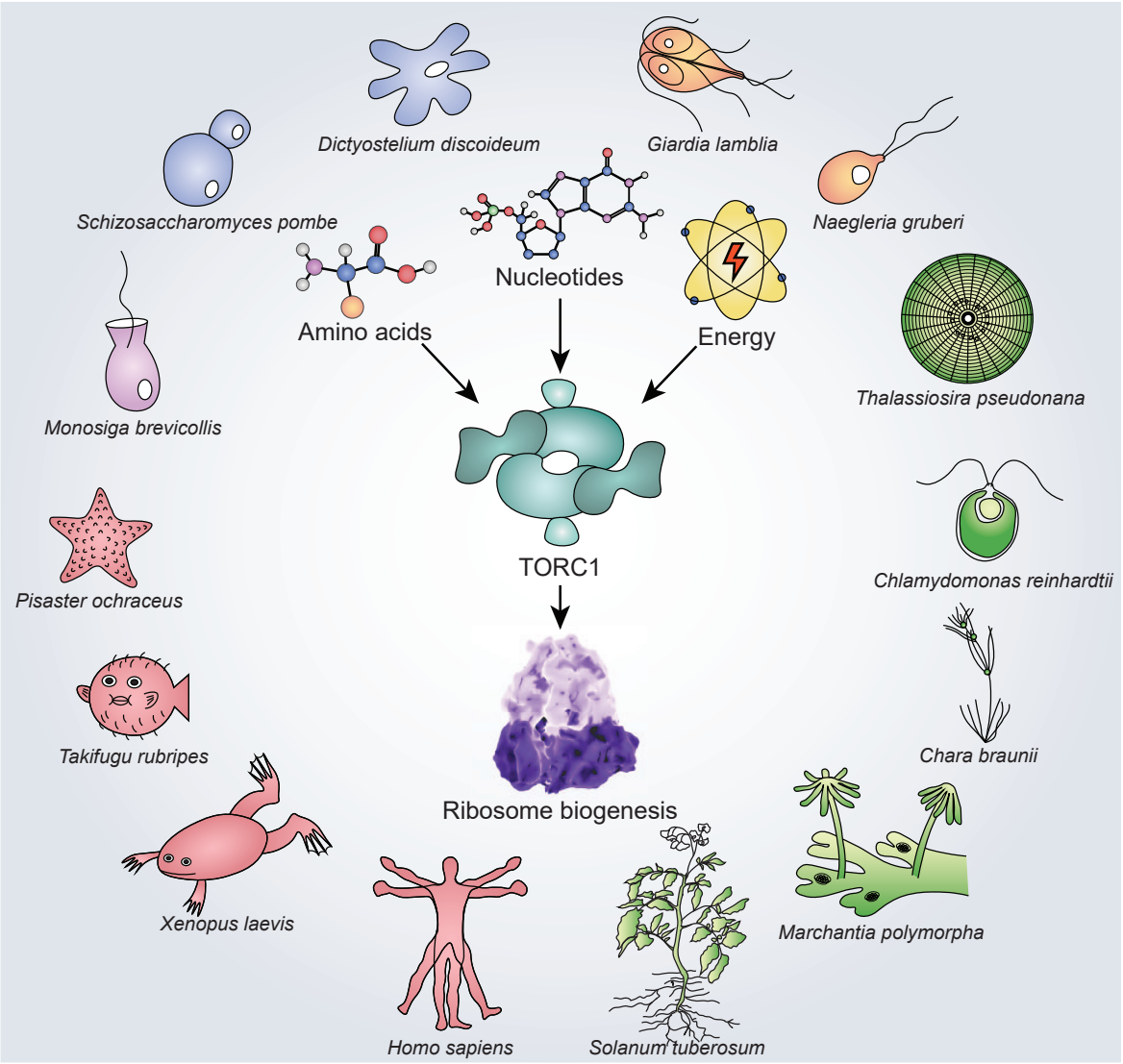

Figure 1. TOR Evolved an Early Role to Coordinate Ribosome Biogenesis with Metabolic Status in Eukaryotes
TOR complex 1 (TORC1) is an atypical protein kinase that evolved in the last common ancestor of present-day eukaryotes. TORC1 integrates diverse upstream cues to coordinate cellular metabolism, broadly promoting anabolism when growth can be supported by available nutrients and environmental conditions. There is considerable variation in the upstream regulators and downstream effectors of TOR signaling across eukaryotic lineages, but consistently, TOR senses the levels of monomeric constituents of ribosomes (amino acids and nucleotides) and energy stores to stimulate ribosome biogenesis and growth.

2019; Dong et al., 2017; Schaufelberger et al., 2019). By balancing the rate of ribosome biogenesis with free amino acid levels, TOR signaling maintains metabolic homeostasis by promoting amino acid incorporation into ribosomes and, as a consequence of increased ribosome abundance, inducing global protein synthesis rates only when cells have access to sufficient amino acid levels (Hara et al., 1998).

More recently, several labs discovered that TOR also senses nucleotide availability. In humans, depleting cellular nucleotide levels, especially purine levels, decreases TOR activity (Emmanuel et al., 2017; Hoxhaj et al., 2017). Independently, we found from a forward genetic screen that nucleotide biosynthesis is crucial for TOR activity in plants, although our results suggested that plant TOR is sensitive to disruptions of *de novo* synthesis of either purines or pyrimidines (Busche et al., 2020). The molecular mechanisms of nucleotide sensing upstream from TOR will require further investigation, but it should not be surprising that TOR activity is sensitive to nucleotide availability. The vast majority of nucleic acids are devoted to rRNA (more than all other DNA and RNA combined), such that human cells with mutations that hyperactivate TOR and thus drive excess ribosome biogenesis can be lethally depleted of nucleotides (Valvezan et al., 2017). With nucleotide pools largely exhausted to support hyperproduction of ribosomes, these cells do not have enough nucleotides to support DNA replication during S phase, which leads to replication stress, DNA damage, and apoptosis (Valvezan et al., 2017). To support increased ribosome biogenesis and the G1-S phase transition driven by elevated TOR activity, in healthy cells, TOR increases *de novo* nucleotide biosynthesis. In human cells, TOR upregulates nucleotide biosynthesis through multiple

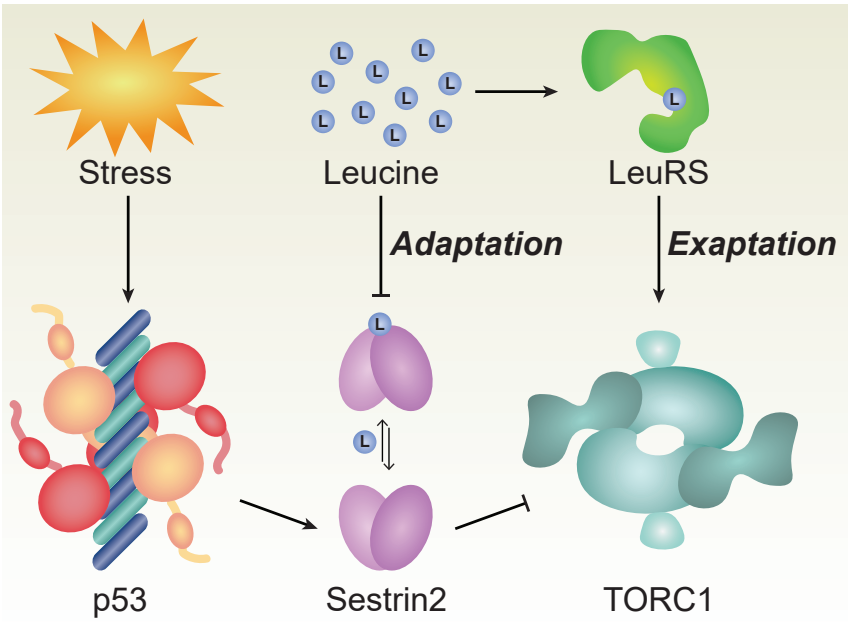

Figure 2. Exaptation, Adaptation, and Integration of Upstream Metabolic Sensors for TOR

In humans, cellular stress is sensed by the DNA-binding tetrameric transcription factor p53 (p53, orange and red; DNA, blue and teal). p53 stimulates *Sestrin2* expression, which encodes a protein that represses TORC1 activity. Sestrins first evolved in an ancestor of animals, fungi, and Amoebozoa. Sestrin2 later evolved a new role as a leucine sensor: leucine directly interacts with Sestrin2, inducing a reversible conformational change that prevents Sestrin2 from repressing TORC1. This is an example of adaptation: an existing mechanism for regulating TORC1 evolved a new, presumably fitness-enhancing trait to respond not only to the stress-p53 signaling axis but also to nutrient availability. TORC1 can also sense leucine through the cytosolic leucyl tRNA synthetase (LeuRS), which is proposed to stimulate TORC1 only when leucine is available. This is an example of exaptation: LeuRS evolved to catalyze synthesis of leucyl-tRNA^{Leu} and was later coopted to sense leucine availability for TORC1.

mechanisms, including transcriptional induction and activating phosphorylation of enzymes in the *de novo* nucleotide biosynthetic pathways (Ben-Sahra et al., 2013, 2016; Benjamin and Hall, 2017; Robitaille et al., 2013). Similarly, in plants, transcription of virtually every nucleotide biosynthesis enzyme increases when TOR is activated (Busche et al., 2020; Scarpin et al., 2020; Xiong et al., 2013). Whether nucleotides are sensed directly, as has been proposed in human cells, or indirectly through more complicated signaling pathways, TOR activity is clearly coupled to nucleotide metabolism, providing an additional regulatory layer to coordinate ribosome biogenesis with the availability of ribosome precursor molecules.

Lastly, across eukaryotes, TOR is sensitive to cellular energy levels, especially the cytosolic concentration of ATP. In mammals, TOR activity is restricted by the AMP-activated kinase (AMPK), a kinase that is directly activated by physical interaction with AMP when ATP/AMP ratios are low (Gwinn et al., 2008; Mihaylova and Shaw, 2011). Even in *ampk* knockout cells, mammalian TOR activity remains acutely sensitive to ATP/AMP levels, which is likely sensed by the Pontin and Reptin ATPase complex (Kim et al., 2013). Pontin and Reptin are universally conserved AAA+ ATPases that typically assemble in heterohexamers or heterododecamers with each other and participate in diverse protein complexes (Rosenbaum et al., 2013). Most of the roles of Pontin and Reptin do not rely on their ATPase activity, which is relatively weak and sensitive to physiologically relevant changes in cytosolic ATP concentrations (Kim et al., 2013). Pontin and Reptin ATPase activity is required, however, for its role as a co-chaperone during TOR complex assembly, and decreases in ATP levels lower TOR complex stability, suggesting that Pontin and Reptin could act as direct sensors of cellular energy status and stabilize TOR complexes only when ATP levels are high (Kim et al., 2013). Plant TOR is also sensitive to disruption of ATP synthesis (Zhang et al., 2019); the activation of TOR by glucose in quiescent Arabidopsis seedlings, for instance, is entirely dependent on glucose metabolism via glycolysis and oxidative phosphorylation (i.e., glucose activates TOR by driving mitochondrial ATP synthesis) (Xiong et al., 2013). The plant ortholog of AMPK does not directly sense ATP/AMP levels (Emanuelle et al., 2015), but the Pontin and Reptin ATPase complex is, indeed, required for TOR activity in plants (Brunkard et al., 2020; Garcia et al., 2017), suggesting that ATP-Pontin/Reptin-TOR signaling may be a conserved mechanism for energy sensing. Depending on cell type, the majority of cellular ATP may be consumed to support translation, so maintaining a balance between ATP and ribosome levels is crucial to avoid energy starvation.

A TORtoise Surrounded by Hares: Evolution, Stasis, and Innovation in the TOR Signaling Network

Ongoing mechanistic studies of the signal transduction pathways upstream from TOR are beginning to illuminate how TOR integrates multiple cues to coordinate downstream metabolism. The well-studied p53-Sestrin2-mTORC1 signaling axis in humans is an excellent example of multiple cues converging to control TOR activity (Figure 2). p53 is a transcription factor that is stabilized and/or activated by multiple stresses, including DNA damage, ribosome biogenesis stress, and hypoxia (Horn and Vousden, 2007), and then directly promotes or represses the expression of a suite of stress-response genes, including *Sestrin2*. Sestrin2 is a proposed leucine sensor for TOR in humans: at physiologically relevant levels, leucine directly binds to Sestrin2 and disrupts the interaction between Sestrin2 and a protein complex, called GATOR2, that must dissociate from Sestrin2 to activate TOR (Chantranupong et al., 2014; Saxton et al., 2016; Wolfson et al., 2016). GATOR2 then represses GATOR1, which otherwise inactivates TOR (Bar-Peled et al., 2013). The p53-Sestrin2-mTORC1 pathway allows multiple signals to integrate before the transduction pathway reaches TOR, balancing possibly opposing cues. In unstressed cells, p53 is inactive, Sestrin2 levels are relatively low, and thus relatively low

concentrations of leucine are sufficient to free GATOR2 to activate TOR. If stresses have activated p53, however, Sestrin2 levels rapidly increase, and higher concentrations of leucine are required to dissociate Sestrin2 from GATOR2 (Wolfson and Sabatini, 2017).

The steps of this signaling axis evolved at different times in the ancestors of humans, however. Sestrins and the GATOR complex components evolved in the last common ancestor of animals, fungi, and Amoebozoa (ameboid protists and slime molds), although Sestrins were later lost in an ancestor of baker's yeast. p53 evolved much more recently in an ancestor of modern animals, and many of its stress-sensing roles are specific to vertebrates (Belyi et al., 2010). The signals sensed by Sestrin2 and p53 in human cells nonetheless regulate TOR activity in other eukaryotes: plant TOR is sensitive to branched chain amino acid metabolism (Cao et al., 2019; Schaufelberger et al., 2019) and various physiological stresses (Fu et al., 2020), for example, although plants do not have homologous sensor proteins. Thus, this example should not be confused to indicate that TOR signaling has evolved more complexity in vertebrates than other lineages; rather, because the TOR signaling network has been most thoroughly studied in humans, we have a better understanding of the complexity of TOR signaling in this lineage.

Although the upstream regulators and downstream effectors of TOR signaling have evolved in different eukaryotic lineages, TOR itself has remained evolutionarily static in eukaryotes. TOR is an atypical protein kinase that is more closely related to lipid kinases (specifically, phosphatidylinositol-3-kinase, or PI3K) than to the diverse family of eukaryotic protein kinases. TOR was the first defined member of the PI3K-like kinase (PIKK) family, physically large serine/threonine kinases that are broadly conserved in eukaryotes. In humans, the PIKKs include ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3 related), DNA-PKcs (DNA-dependent protein kinase catalytic subunit), and SMG1 (suppressor of morphogenesis in genitalia 1). TRRAP (transformation/transcription domain-associated protein) is sometimes included in this family, but due to conserved mutations in its catalytic sites, it has no kinase activity. ATM, ATR, and DNA-PKcs all contribute to DNA damage responses; SMG1 participates in nonsense-mediated decay; and TRRAP is involved in chromatin remodeling. TRRAP is conserved in most animals, fungi, plants, and related lineages but is apparently absent from earlier-diverging eukaryotes. The remaining PIKKs probably evolved in the last common ancestor of most modern eukaryotes, but *PIKK* genes were sometimes lost in specific lineages. For example, the *Giardia lamblia* genome only encodes an ortholog of TOR (Manning et al., 2011), the *Plasmodium falciparum* genome does not encode any PIKKs (Ward et al., 2004), flowering plant and fungal genomes do not encode DNA-PKcs, and *A. thaliana* recently lost the *SMG1* gene (which is present in all other plants studied to date, including the closely related *Arabidopsis lyrata*) (Causier et al., 2017; Lloyd and Davies, 2013).

There are two pertinent conclusions to draw from the evolutionary history of PIKKs in eukaryotes. First, the *PIKK* gene family has not undergone notable gene expansion or contraction: with the exception of the noncatalytic PIKK-like protein TRRAP, which may have evolved sometime after the divergence of extant eukaryotic lineages, all of the extant PIKKs were most likely present in the last eukaryotic common ancestor, and no new PIKKs have evolved since. This hypothesis can be more thoroughly tested as our knowledge of protist genomes and cell biology expands; to support biomedical research, databases are currently biased toward parasites that infect humans, which have tended to lose *PIKK* genes during the genome contraction often associated with parasitism (Figure 3). Even where *PIKK* genes have duplicated and evolved separate functions, as in *S. cerevisiae*, which encodes two TOR proteins, the paralogs are nearly identical and have simply subfunctionalized pre-existing roles, rather than gaining entirely new roles. The second critical insight is that PIKK proteins have been remarkably static during eukaryotic evolution. The human and *Naegleria* TOR proteins are 43% identical and 56% similar across their entire length, with no significant gaps in their alignment, despite ~1.5 to 2 billion years since these lineages diverged. This degree of evolutionary stasis is comparable to cytosolic ribosomal protein conservation (for example, human and excavate protist *Naegleria* eS6 proteins are 43% identical and 57% similar). The critical components of TOR complex 1 (TORC1), RAPTOR and LST8, are also conserved across eukaryotes with very similar protein sequences. Thus, the many innovations in TOR signaling networks that have evolved in distinct eukaryotic lineages are not due to adaptive evolution of the TOR protein itself but instead due to the addition, removal, or modification of signaling axes upstream and downstream from TOR. In this sense, TOR is the proverbial tortoise, slow-evolving but persistent, whereas much of its surrounding signaling network are proverbial hares, fast-evolving new functions but not profoundly essential.

Adaptation and Exaptation in the Evolution of Amino Acid MoniTORs

The conceptual differences among adaptation, exaptation, and nonaptation can help to clarify the evolutionary history of TOR signaling in eukaryotes. The term "exaptation" (Gould and Vrba, 1982) arose from an effort to distinguish traits that evolved via natural selection to their current functional relevance for fitness (adaptations) from traits that currently enhance fitness but first arose with some other fitness-enhancing role or, indeed, with no selective advantage at all (exaptations). In other words, exaptations coopt existing adaptations or nonaptations (traits that do not confer any fitness advantage) to serve a new function. The classical example of exaptation is the evolution of feathers: currently, feathers provide fitness benefits for birds in flight and thermoregulation, but feathers were present in the flightless dinosaur ancestors of birds, where they contributed to thermoregulation, and were only later coopted as an exaptation for flight in avian descendants (Gould and Vrba, 1982). Thus, feathers first evolved and adapted for one role unrelated to flight and then were exapted for flight later in the evolutionary history of birds. Molecular biologists may be more familiar with the example of transposable elements (TEs): many genomes are composed of apparently functionless transposable elements that, when transposed, can gain fitness-enhancing roles in gene regulation. During maize evolution, for example, a *Hopscotch* retrotransposon inserted upstream of the *Teosinte branched 1* gene (Studer et al., 2011), enhancing expression of *Tb1* (Clark et al., 2006) and thus reducing axillary branching (Dong et al., 2019), an important agronomic trait. The *Hopscotch* TE had no function in the

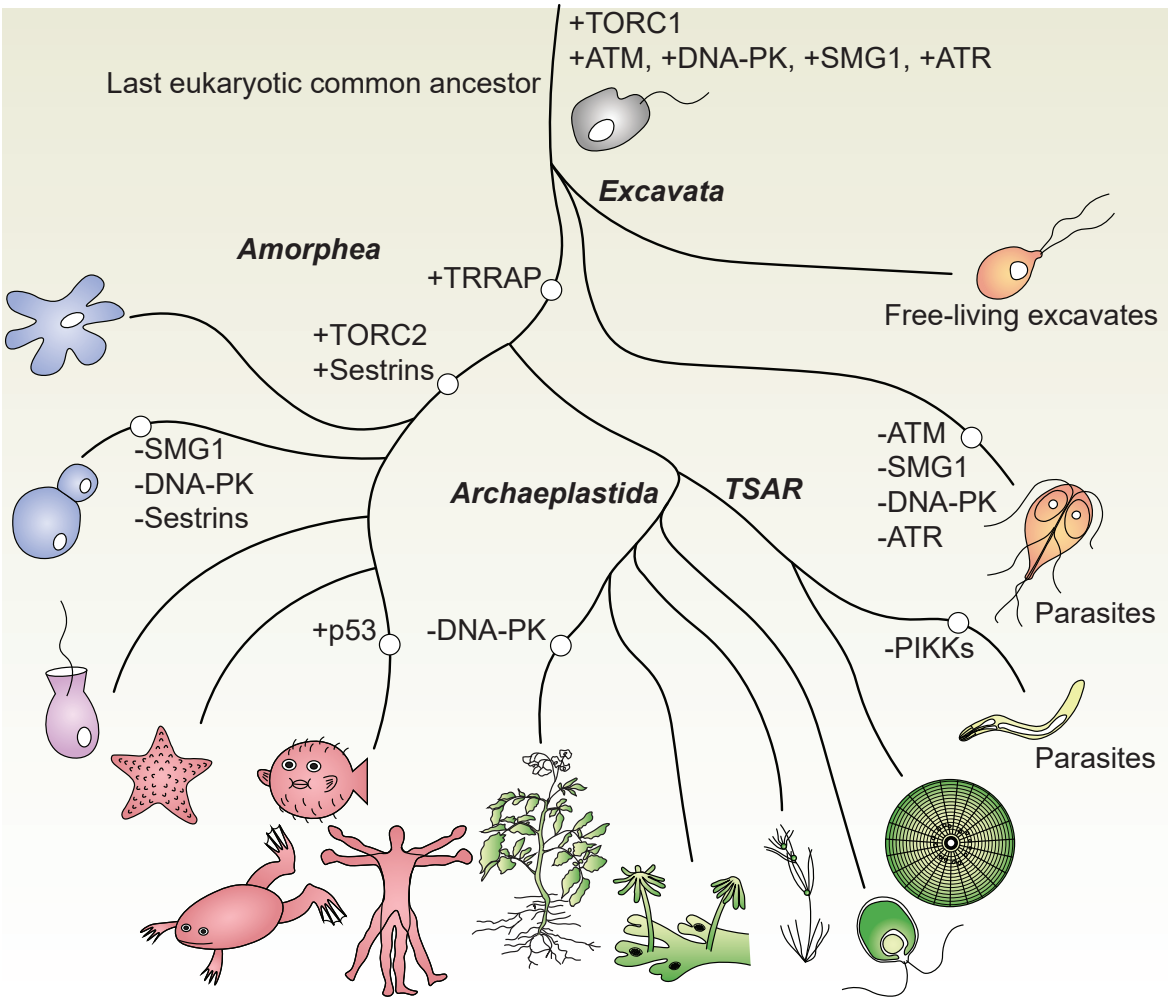

Figure 3. PIKKs Evolved before the Diversification of Eukaryotes and Remain Conserved in Most Lineages
A representative sampling of eukaryotic lineages, including species in the supergroups Amorpheae (Amoebozoa + fungi + animals), Archaeplastida (green algae + land plants), TSAR (Telonemia + stramenopiles + alveolates + Rhizaria), and Excavata, is shown to illustrate the evolution of PIKKs and select associated proteins discussed in the text. All five major PIKKs (TOR, ATM, DNA-PK, SMG1, and ATR) likely evolved before the unicellular last eukaryotic common ancestor. RAPTOR and LST8, key components of TORC1, also evolved early in eukaryotes. TRRAP, a noncatalytic PIKK-like protein, probably evolved later but before the divergence of Amorpheae and Archaeplastida + TSAR. TORC2 and Sestrins evolved in Amorpheae, and p53 evolved from p53-like genes to sense cellular stress in animals. PIKKs are conserved in most lineages, although some branches have lost specific PIKKs, as shown here. Parasitic lineages are an exception that have lost multiple PIKKs, such as *Plasmodium* (in the TSAR supergroup), which does not encode any PIKKs, and *Giardia* (in the Excavata supergroup), which only encodes TOR.

teosinte ancestor of maize before its transposition (i.e., was non-aptive) but was exapted to a new role in plant architecture (Doebley et al., 1997).

The distinction between adaptation and exaptation is especially useful for understanding the evolution of amino acid sensors for TOR. As discussed above, Sestrin2 is an established leucine sensor for TOR in human cells. There is less consensus about whether Sestrins act as leucine sensors in fungi, Amoebozoa, and other animals, however (Lee et al., 2016), and this is an active area of research in model organisms. Some have proposed that Sestrins evolved an early role to suppress TOR activity when cells experience various stresses (such as those sensed by p53), independent of any role in amino acid sensing (Lee et al., 2016). This model suggests that Sestrins "adapted" leucine sensitivity in a more recent ancestor of humans, binding to leucine as an "off switch" to integrate and balance stress and nutrient availability in determining TOR activity. Earlier studies had discovered another leucine sensor that was proposed to activate TOR in mammals and in yeast: leucyl tRNA synthetase (LeuRS) (Bonfils et al., 2012; Han et al., 2012). In the original studies, LeuRS was apparently demonstrated to act as a guanosine triphosphate (GTP)ase-activating protein (GAP) for small GTPases called Rags that stimulate TOR (Bonfils et al., 2012; Han et al., 2012). Subsequent studies in humans could not replicate the proposed GAP activity assigned to human LeuRS *in vitro*, however (Bar-Peled et al., 2013). Furthermore, since Sestrins are not conserved in yeast, LeuRS remains the only proposed leucine sensor for TOR in *S. cerevisiae* (Bonfils et al., 2012). More recently, human LeuRS was shown to directly aminoacylate lysine residues in Rags, which impacts their GTPase

activity and thereby upregulates TOR, providing a possible mechanism to reconcile the earlier conflicting results (He et al., 2018). LeuRS is conserved in all domains of life, and clearly did not evolve as a leucine sensor or regulator of TOR activity, but instead as an enzyme that catalyzes the precise ligation of leucine to $tRNA^{Leu}$ for mRNA translation. Thus, the proposed role of LeuRS in leucine sensing for TOR is a clear example of exaptation: an existing mechanism that already was sensitive to changes in leucine concentrations was coopted to regulate TOR.

Regulatory TORchbearer after Divisions: Roles for TOR in Nonproliferating Cells

Investigations of TOR signaling using unicellular models, especially yeast and immortalized mammalian cell lines, have emphasized the critical role of TOR in promoting cell-cycle progression, especially as a critical regulator of the G1 to S phase transition. In *Arabidopsis*, TOR activity is required for the G1 to S phase transition in proliferating cells in the root meristem (Xiong et al., 2013) and shoot meristem (Li et al., 2017; Pfeiffer et al., 2016), supporting the model that metabolic regulation of cell-cycle progression is an ancestral function of TOR in eukaryotes. Much less is known in plants about the role of TOR outside of meristems. In fact, early studies of *TOR* expression patterns in *Arabidopsis* using an unfortunately misleading *TOR* β-glucuronidase reporter transgenic line erroneously indicated that *TOR* is expressed almost exclusively in meristems, i.e., proliferating cells (Menand et al., 2002). Subsequent transcriptomes and proteomes have conclusively demonstrated that *TOR* mRNA and TOR protein are expressed at moderate to high levels (consistently in the top 50% of all expressed genes) in virtually every organ, tissue, and cell type studied to date, as are *RAPTOR* and *LST8* (Mergner et al., 2020; Schmid et al., 2005). Moreover, we recently showed that TOR activity increases in leaves as they mature during the sink-to-source transition, although cells in mature leaves are fully differentiated and nonproliferating (Brunkard et al., 2020).

Studying the roles of TOR in plant development and in differentiated cells can be challenging, however, because TOR is essential for so many cellular processes (Henriques et al., 2014). As a result, RNAi lines that constitutively reduce TOR levels in *Arabidopsis thaliana* are, as a rule, either lethal early during embryogenesis or so inefficient at silencing *TOR* that they have relatively mild effects on plant development and physiology (Deprost et al., 2007). To circumvent this, Deprost et al. generated an ethanol-inducible line that partially suppressed *TOR* expression (Deprost et al., 2007) and Xiong et al. generated a highly efficient estradiol-inducible TOR RNAi transgenic line that survives embryogenesis and can then be treated with estradiol during germination to effectively eliminate TOR from seedlings (Xiong et al., 2013). Coupled with precise environmental conditions to limit photosynthesis, this genetic embryo-rescue approach was deployed to demonstrate that TOR is required to sense glucose metabolism and exit quiescence during seedling establishment. Alternatively, plants can be treated with chemical TOR inhibitors, such as rapamycin or highly selective ATP-competitive TOR inhibitors (Montané and Menand, 2013; Xiong and Sheen, 2012), to attenuate TOR activity at a given developmental stage, which, for example, was used to phenocopy the effects of silencing *TOR* in germinating seedlings (Xiong et al., 2013). Neither of these methods can elucidate cell-type-specific roles for TOR signaling, however.

Biomedical researchers are beginning to address this challenge as part of ongoing efforts to translate knowledge of TOR signaling from cell culture models to multicellular organisms. For example, Kosillo et al. (2019) took an elegant genetic approach to dissect the post-mitotic roles of TOR signaling in mouse dopamine neurons (Kosillo et al., 2019). In mammalian cells, tuberous sclerosis complex 1 (Tsc1) is part of the Tsc1-Tsc2 GTPase-activating complex that stimulates a small GTPase, Rheb. Rheb-GTP interacts with TORC1 at the lysosome, where it induces conformational changes that increase TORC1 activity. The tuberous sclerosis complex Tsc1-Tsc2 restrains TOR activity by promoting GTP hydrolysis by Rheb; unlike Rheb-GTP, Rheb-GDP does not activate TORC1. In clinical settings, mutations in *Tsc1* or *Tsc2* are associated with complicated neurodevelopmental disorders, but the broad induction of TORC1 activity in these genetic backgrounds previously limited understanding of which cell types are actually responsible for the pleiotropic neuropsychiatric disorders associated with *Tsc1-Tsc2*. To address this, Kosillo et al. crossed a mouse strain that expresses the Cre recombinase only in dopamine neurons from a bicistronic mRNA that encodes the dopamine transporter to a mouse strain carrying a *Tsc1* allele that is flanked by the Cre recognition lox sites. Thus, after dopamine neurons differentiate and begin to express dopamine transporters, Cre is expressed in these cells and mediates recombination at the lox sites that deletes the *Tsc1* locus, de-represses Rheb, and ectopically activates TORC1. Post-mitotic TORC1 hyperactivation in these cells caused hypertrophy, increased dendrite complexity and length, reduced intrinsic excitability, impaired dopamine release but elevated dopamine synthesis, and reduced cognitive flexibility (Figure 4A).

One straightforward but important insight illustrated by this study is that increasing TOR activity is not sufficient to trigger all cellular processes that require TOR activity. TOR is required for cell proliferation, but TOR hyperactivity in dopamine neurons is not sufficient to trigger cell-cycle progression and cell division. Similarly, although the cell-cycle regulatory transcription factor E2FA must be phosphorylated by TOR to permit the G1 to S phase transition in *Arabidopsis* root meristems (Xiong et al., 2013) (Figure 4B), TOR activity in mature leaves is not sufficient to trigger entry into the cell cycle. Mutations in the *TOR* gene or regulatory genes that increase TOR activity are strongly associated with various human cancers (Saxton and Sabatini, 2017), however, which implies that hyperactivating TOR can trigger ectopic cell proliferation, at least under some circumstances. A recent study of TOR activity during metaplasia in the stomach and pancreas may provide a model for the conditional induction of cell proliferation by hyperactivated TOR (Willet et al., 2018). Epithelial cells in the stomach and pancreas secrete large quantities of enzymes, which is supported by significant TOR activity that drives ribosome biogenesis needed to synthesize those enzymes (Figure 4C). Damage to these cells inactivates TOR and induces autophagy to clear cellular contents, allowing the cells to de-differentiate as the first step of repair (Figure 4C). TOR later reactivates and serves as a checkpoint for cell-cycle re-entry to proliferate and regenerate damaged tissues (Figure 4C). In these metaplastic cells that are newly competent for cell division,

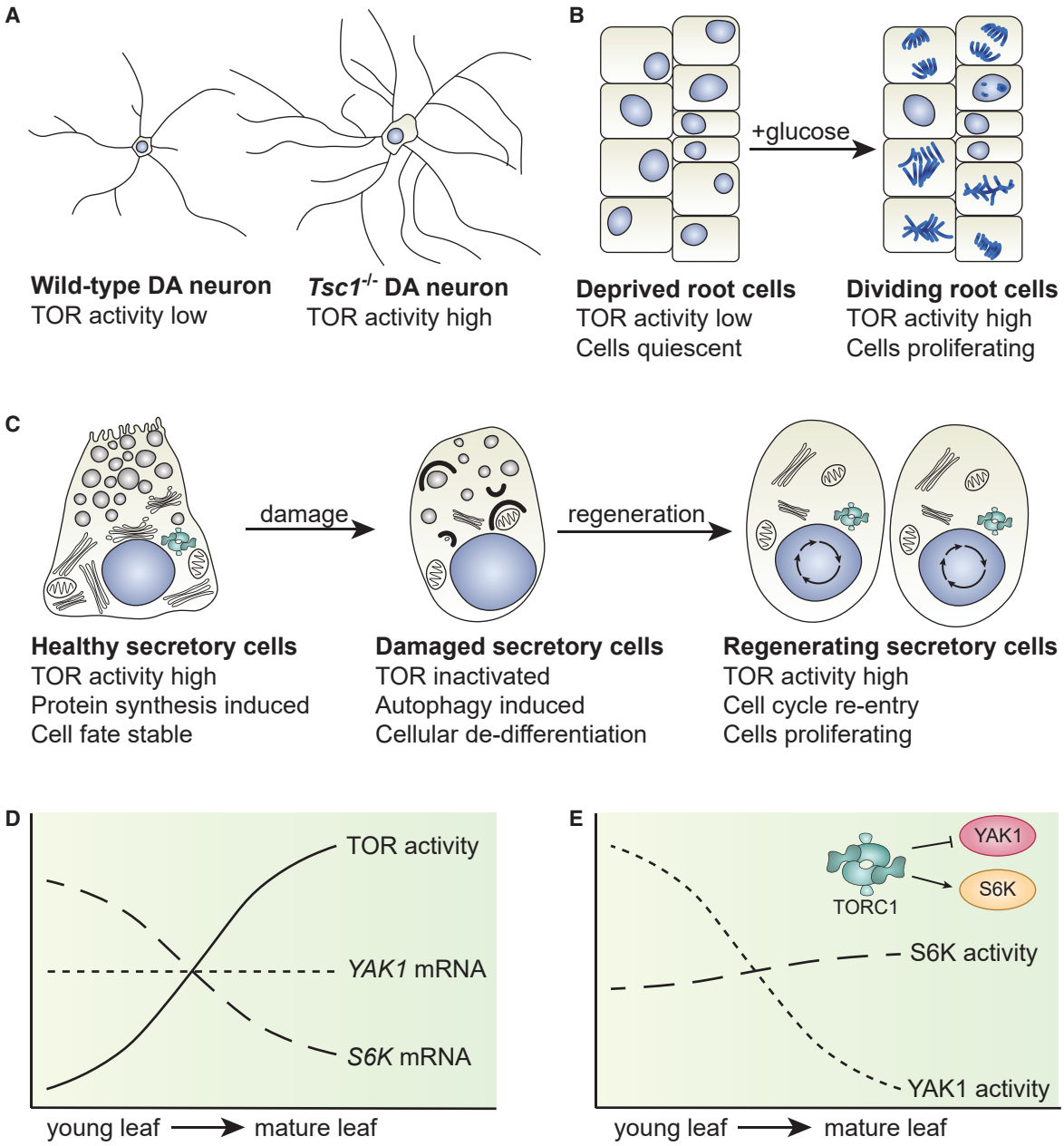

Figure 4. Developmental Context Modulates the Outcomes of TOR Activation

(A) Hyperactivating TOR in differentiated dopamine (DA) neurons by deleting *Tsc1* causes hypertrophy and physiological defects without stimulating cell proliferation.

(B) Carbohydrate-deprived root meristem cells are quiescent until they are supplied with carbohydrates (e.g., glucose) that activate TOR and permit cell-cycle progression and cell division.

(C) High TOR activity in zymogenic chief cells in the human stomach drive high ribosome concentrations to support synthesis of large quantities of proenzymes (e.g., pepsinogen) for secretion to the gut. When these cells are damaged, TOR is rapidly inactivated, permitting autophagy to clear cellular contents and the cells de-differentiate. TOR activity must be restored for de-differentiated cells to re-enter the cell cycle and proliferate to regenerate the secretory cells. If somatic mutations deregulate TOR in regenerating secretory cells, hyperactive TOR can instead promote spurious proliferation and tumorigenesis.

(D) Empirical measurements of TOR activity and mRNA levels of two TOR substrates, *S6K* and *YAK1*, show that, when comparing leaves of different ages in *Arabidopsis thaliana* (ranging from young growing leaves to mature expanded leaves), TOR activity increases with leaf age, *S6K* mRNA levels decrease with leaf age, and *YAK1* mRNA levels remain constant.

(E) S6K activity is predicted to be similar in leaves of different ages, despite overall changes in TOR activity, but YAK1 activity is predicted to negatively correlate with leaf age.

somatic mutations that hyperactivate TOR could cause tumorigenesis instead of healthy tissue regeneration (Willet et al., 2018). This hypothesis will require further testing but provides one experimental model for how equivalent levels of TOR activation can control distinct processes depending on cell type (in this case, mRNA translation in differentiated secretory epithelial cells versus cell-cycle progression in metaplastic regenerating cells).

In our investigation of TOR signaling during leaf maturation (discussed at length below), we showed that TOR activity is higher in mature leaves, where cells have finished proliferating and expanding, than in young expanding leaves, where cells have mostly finished proliferating but continue to expand (Brunkard et al., 2020). We measured TOR activity in these leaves using a *35S$_{pro}$:S6K1* line (kindly shared with us by the Sheen Lab; Xiong and Sheen, 2012) that expresses consistent levels of S6K1 protein in each leaf. In wild-type plants, however, *S6K* transcription is instead much higher in young sink leaves than in mature source leaves (Figure 4D) As a result, in preliminary studies using wild-type plants, we observed only a subtle difference in S6K phosphorylation between young and mature leaves but a very large difference in total S6K abundance. While this observation will require further experimental support before drawing any larger conclusions, I postulate that changes in *S6K* transcription may "compensate" for changes in overall TOR activity, such that the TOR-S6K signaling axis remains in a homeostatic balance (Figure 4E). By contrast, transcript levels of another TOR effector, *YAK1* (*Yet Another Kinase 1*), are effectively constant across leaves of different ages (Figure 4D). YAK1 is a dual-specificity tyrosine-regulated kinase (related to human DYRK1A) that antagonizes TOR signaling (Martin et al., 2004; Schmelzle et al., 2004). YAK1 is directly phosphorylated by TOR, which inhibits YAK1 activity (Forzani et al., 2019); when YAK1 is not phosphorylated, it strongly represses anabolism and growth. Illustrating this relationship, when plants are treated with TOR inhibitors at sufficient concentrations to drastically decelerate wild-type plant growth, loss-of-function *yak1* mutants can continue to grow and thrive (Barrada et al., 2019; Forzani et al., 2019). Since *YAK1* mRNA levels are not significantly different among leaves of different ages, post-translational regulation by TOR likely strongly suppresses YAK1 in mature leaves but lower TOR activity in young leaves allows YAK1 partial activity. These examples illustrate how cell type and physiological status could reconfigure the TOR signaling network, such that shifts in TOR activity might have different outcomes in different contexts.

A hallmark of complex multicellularity is cellular differentiation into distinct cell types with specialized physiological or structural roles, which current investigations have demonstrated can modulate the TOR signaling network. TOR can also regulate cellular differentiation and cell fate *per se*; for example, changes in TOR activity can switch the fate of differentiating T cells in the human immune system. It is tempting to speculate that TOR evolved roles in cellular differentiation late in multicellular lineages, since cell differentiation is most often discussed in the context of multicellular organisms, but it should be noted that unicellular organisms are also capable of cellular differentiation. For instance, the unicellular excavate protist, *Naegleria*, transitions among three forms in response to environmental conditions: the trophozoite stage is phagocytic, metabolically active, and actively undergoes cell division (i.e., reproduction); under nutrient starvation (or some other stresses), the trophozoite transitions to a flagellate stage, which does not feed or divide but is highly motile; and under prolonged nutrient deprivation or extreme stress, *Naegleria* differentiates into a dormant cyst stage that is effectively inert (Marciano-Cabral, 1988). As illustrated by this example, cellular differentiation in single-celled eukaryotes is often regulated by metabolic status, hinting that TOR could be involved. Indeed, in the best-studied case, TOR is crucial for sexual differentiation of *Schizosaccharomyces pombe*. When nutrients are available and TOR is active, *S. pombe* maintains an asexual life cycle, reproducing by mitosis. When nutrients are limiting, however, TOR becomes inactive and *S. pombe* cells of opposing mating types fuse and undergo meiosis to generate spores, which remain dormant until they are supplied with nutrients. Sexual development is triggered in *S. pombe* with temperature-sensitive loss-of-function alleles of *TOR* at non-permissive temperatures, demonstrating that disrupting TOR is sufficient to trigger differentiation (Matsuo et al., 2007). This transition is mediated, at least in part, by an RNA-binding protein called Mei2, which is phosphorylated by TOR and thus marked for proteasomal degradation (Otsubo et al., 2014). When TOR is inactive, Mei2 accumulates and triggers exit from the G1 phase of the cell cycle and promotes sexual differentiation. Thus, although multicellular organisms certainly adapted and fine-tuned new pathways for TOR to control cellular differentiation programs, TOR likely first gained roles in cellular differentiation early in eukaryotic evolution.

Evolutionary TORsion: Exaptation of TOR Signaling to Direct the Leaf Sink-to-Source Transition

The TOR signaling network evolved in heterotrophic eukaryotic ancestors to sense metabolic status, a very different metabolic context than later-evolving photoautotrophic lineages, such as the Viridiplantae (green algae + land plants). Several recent studies have focused on understanding new roles that TOR has evolved to coordinate photosynthetic pathways. These exaptations take advantage of TOR's ancestral role in sensing the metabolism of externally supplied resources to instead sense the metabolism of autotrophically synthesized metabolites, including carbohydrates and amino acids. For example, as mentioned briefly above, TOR is critical during seedling establishment (Xiong et al., 2013). Germinating seedlings are functionally heterotrophs that rely on maternally supplied metabolic stores to develop until they can sustain themselves as autotrophs that photosynthesize carbohydrates to support growth. TOR becomes inactive if establishing seedlings cannot complete this transition, however, and seedlings remain developmentally quiescent until environmental conditions change and they can photosynthesize sufficient carbohydrates to continue growing (Xiong et al., 2013). More broadly, TOR controls chloroplast biogenesis and photosynthetic metabolism in plants (Dobrenel et al., 2016; Scarpin et al., 2020; Zhang et al., 2018) and green algae (Upadhyaya and Rao, 2019), although the details of the relationships among TOR, chloroplasts, and photosynthesis remain largely unexplored.

Within the Viridiplantae, land plants evolved ~500 million years ago from freshwater algal ancestors that were either unicellular or formed simple, unbranched filaments. Unlike their closest

algal relatives, all land plants are complex multicellular organisms—that is, rather than forming filaments or two-dimensional thalli, some cells are interior and not in direct contact with the external environment (Niklas and Newman, 2013). Algal and plant cells are separated from each other by cellulosic cell walls, but plant cells evolved plasmodesmata (PD), narrow, membrane-lined channels in the cell wall that allow cytosol to flow between neighboring cells. Indeed, the capacity to transfer molecules between cells was most likely essential for the evolution of complex multicellularity in land plants. Complex multicellularity evolved repeatedly in separate lineages of green algae and in brown algae, and in each of these lineages (which all have cellulosic cell walls), structures analogous to PD evolved alongside multicellularity (Brunkard and Zambryski, 2017; Raven, 2005). The homoplasy of PD-like structures in every complex multicellular lineage with cellulosic cell walls suggests that PD are critical adaptations that enabled the evolution of multicellular land plants (Brunkard and Zambryski, 2017).

PD are conduits for diverse cytosolic molecules, including ions, metabolites, small RNAs, and cytosolic proteins up to ~80 kDa (Figure 5A), depending on cell type and physiological conditions (Brunkard et al., 2015). Plant viruses hijack PD to spread from infected cells, and other pathogens may use PD as conduits to spread pathogenic effectors (Figure 5A). Nearly every cell is connected to its neighbors via PD (with a few highly specialized exceptions, such as guard cells), no mutants lacking PD have ever been isolated, and any severe disruption of PD transport is lethal (Kim et al., 2002). PD transport is dynamically regulated during plant development and in response to environmental and physiological cues, including abiotic stress, biotic infection (Cui and Lee, 2016; Huang et al., 2019; Lee et al., 2011), light and the circadian clock (Brunkard and Zambryski, 2019), redox status (Benitez-Alfonso et al., 2009; Stonebloom et al., 2012), chloroplast biogenesis (Brunkard and Burch-Smith, 2018; Burch-Smith et al., 2011), and metabolic status (Brunkard et al., 2020), but the mechanisms controlling PD transport are mostly poorly defined. The best-established mechanisms are reversible callose deposition and regulated PD biogenesis (Levy et al., 2007; Thomas et al., 2008; Vatén et al., 2011). In response to various stresses and some developmental signals, callose (β-1,3-glucan) is deposited in the cell wall surrounding PD, which apparently restricts movement through the PD cytosolic sleeve. This process is reversible: callose synthases deposit callose near PD, whereas β-1,3-glucanases remove callose from the cell walls surrounding PD. The number of PD can also apparently influence PD trafficking: increased PD biogenesis is associated with increased flux through PD in several mutants and some environmental conditions. These mechanisms are not sufficient to explain all changes in PD transport, however, and several labs are now focused on understanding alternative pathways that regulate trafficking through PD (Brunkard and Zambryski, 2019; Huang et al., 2019).

In all plants, PD play an especially important role in the redistribution of sugars from photosynthetic "sources" to actively growing "sinks" (Figure 5B). Here, I will focus on the source-to-sink route of sugars photosynthesized in the leaves of apoplastic phloem-loading plants, which is a common route for sugars in the herbaceous vascular plants that are studied by most plant biologists, although it should be noted that this is not the only means of long-distance sugar transport (Rennie and Turgeon, 2009; Turgeon and Wolf, 2009). In mature leaves that are photosynthesizing more sugars than they need to consume, sugars are loaded against a concentration gradient into specialized vascular tissue called the phloem. Sugars are first exported from photosynthetic cells out to the extracellular space (the "apoplast") by active transporters in the SWEET family (Chen et al., 2012). Extracellular sugars are then imported into phloem cells via another family of active transporters, the SUT sugar importers (Bürkle et al., 1998; Riesmeier et al., 1992; Sauer and Stolz, 1994; Stadler and Sauer, 1996). PD transport between the phloem and surrounding cells is tightly restricted to prevent passive backflow of sugars along the concentration gradient. Concentrating sugars in the phloem reduces free sugar concentrations in photosynthetic cells, which maximizes photosynthetic rates and the efficiency of sugar export from source leaves (Turgeon, 2010). High solute concentrations in the phloem also create hydrostatic pressure that can accelerate phloem transport. High concentrations of sugars in the phloem are next rapidly transported to sink tissues where they freely unload from along a concentration gradient from the phloem into neighboring cells via PD. Sink tissues, which include root tips, shoot apices, and young leaves, are actively growing, so they rapidly catabolize the sugars they import from the phloem, maintaining low intracellular sugar concentrations. Unlike root tips and shoot apices, which continue to grow throughout the plant's vegetative life cycle, leaves eventually stop growing and transition from phloem-importing sinks to phloem-exporting sources, a "sink-to-source transition." A hallmark of the sink-to-source transition is the restriction of PD transport between the phloem and the rest of the leaf, which allows for efficient apoplastic loading.

Recently, we showed that the leaf sink-to-source transition is regulated by TOR (Figure 5C) (Brunkard et al., 2020). In a forward genetic screen for increased PD transport in *Arabidopsis thaliana* embryos, we found two mutants that directly impinge on TOR activity: *reptin-1* and *ise3*. As part of the Pontin and Reptin ATPase complex, Reptin promotes TOR complex assembly and stability. *reptin-1* is a weak recessive allele of *reptin* caused by a missense mutation at a residue directly adjacent to the ATP-binding Walker A motif that likely interferes with ATP binding or hydrolysis by Reptin. ISE3 is a mitochondrial Sel1-like repeat protein that co-fractionates with the mitochondrial complex III and ATP synthase and is presumably required for efficient ATP synthesis. Since Reptin is a proposed ATP sensor for TOR, we postulated that these two mutations could both impact PD transport by lowering ATP availability and/or sensing that is required to fully activate TOR (Figure 1). Indeed, directly attenuating TOR activity using genetic and pharmacological approaches was sufficient to increase PD transport in embryos and leaves, indicating that TOR activity restricts PD transport. Moreover, we showed that inhibiting TOR activity in a leaf undergoing the sink-to-source transition caused that leaf to remain a sink, whereas mock-treated leaves at the equivalent stage stopped importing molecules from the phloem. The molecular mechanism linking TOR to PD transport remains unresolved, but ongoing efforts to define plant responses to TOR activity may elucidate how TOR affects trafficking through PD.

We proposed that TOR could act as a metabolic rheostat during leaf development, detecting the shift in energetic balance

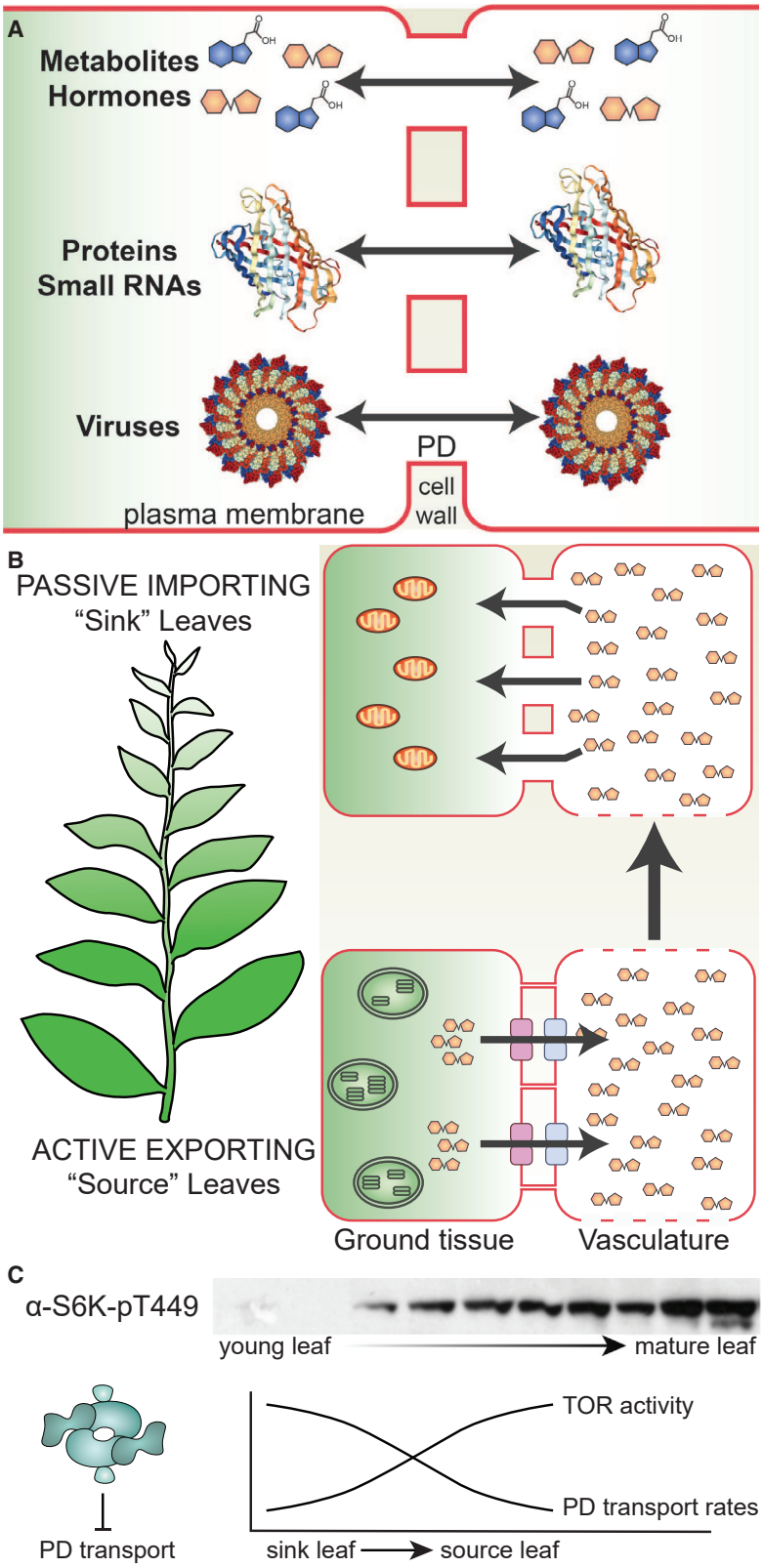

Figure 5. Vascular Plants Exapted TOR Signaling to Coordinate Cell-Cell Transport and Carbohydrate Allocation

(A) PD are narrow, membrane-lined channels that connect the cytosols of adjacent plant cells. Depending on developmental and physiological context, diverse molecules can freely traffic through PD, including ions, metabolites, phytohormones, proteins, and small RNAs. Plant viruses also travel through PD, modifying the PD to spread viruses to uninfected cells.

(B) PD transport is tightly regulated during the sink-to-source transition in leaves. In young, rapidly growing leaves ("sinks"), PD are unrestricted and allow sugars in the vasculature to freely unload into the leaf, where the sugars are rapidly catabolized to support growth. In mature, fully expanded leaves ("sources"), photosynthesized sugars are loaded into the vasculature by active transporters at the plasma membranes (shown here in pink and blue). PD transport in these leaves must be tightly restricted to prevent passive backflow of sugars after they are loaded at high concentrations in the vasculature. As leaves mature, they transition from "sinks" to "sources."

(C) We recently discovered that TOR restricts PD transport in plants using multiple experimental approaches in *Arabidopsis thaliana* embryos and *Nicotiana benthamiana* leaves. Moreover, we found that TOR activity gradually increases during the sink-to-source transition as leaves mature, that TOR activity negatively correlates with PD transport rates during the sink-to-source transition, and that inhibiting TOR can delay the sink-to-source transition in vascular transport. These new roles for TOR are an example of exaptation of the TOR metabolic sensing network to coordinate a newly evolved process, the redistribution of photosynthesized carbohydrates via leaf vascular tissues, in plants.

from "heterotrophic" consumption of imported sugars to "autotrophic" photosynthesis of excess sugars (Brunkard et al., 2020). Supporting this hypothesis, we found that TOR activity positively correlates with leaf age, with lowest TOR activity in young sink leaves and highest TOR activity in mature source leaves, as measured by the ratio of phosphorylated S6K-pT449 to total S6K (Figure 5C). This gradual elevation in TOR activity from young to mature leaves correlates with other canonical signatures of TOR activity, such as rising r-protein mRNA levels and declining autophagy-related mRNA levels. Supported by transcriptional signatures, we speculate that TOR activity is even higher in meristems, where cells are rapidly growing and proliferating, and that TOR activity decreases when mature leaves senesce, since senescent leaves induce autophagy to catabolize nutrients for redistribution to growing or storage tissues. Future experiments will be needed, however, to fully appreciate how TOR activity is modulated during plant development.

The role of TOR in the sink-to-source transition is a superlative example of exaptation in the evolution of multicellular metabolism. TOR arose in the unicellular ancestor of eukaryotes to sense the availability of nutrients, metabolites, and energy and promote anabolism only when all of these resources are available. Plants then coopted the TOR signaling network to sense when a leaf begins photosynthesizing more carbohydrates than it is consuming for growth and can start exporting sugars to growing sinks. The fitness advantages of this exaptation are clear for herbaceous plants: plants that most efficiently sense leaf metabolic status to redistribute photosynthates to growing tissues can outcompete neighbors for soil nutrients, water, and sunlight (Turgeon, 2010). TOR did not adaptively evolve to confer this fitness advantage but instead gained a role in the regulation of PD trafficking and the phloem sink-to-source transition through exaptation of this pre-existing metabolic sensor.

Conclusions

Nearly 30 years after the discovery of the TOR kinase in humans and yeast, the TOR signaling network continues to inspire creative and innovative biological studies with strong potential for clinical applications. Despite significant scientific investment, many critical components of the TOR signaling network, including the roles of putative amino acid sensors that regulate TOR activity, remain poorly resolved with conflicting interpretations. Studies of mammalian model systems alone will clearly not be sufficient to understand the range of mechanisms sensing metabolic status and responding to TOR activation. Rather, an evolutionary perspective that proposes to explain not only how the TOR signaling network operates in specific contexts, but that also seeks to understand the evolution of variation in the TOR signaling network, will broaden and enhance models of TOR signaling in human cells. For example, I suggest that considering the evolutionary processes that gave rise to two proposed leucine sensors—sestrins and leucyl tRNA synthetase—offers a means of reconciling otherwise conflicting models. Sestrins first evolved in an ancestor of animals, fungi, and Amoebozoa to repress TOR in response to cellular stress and later adapted another regulatory role as a leucine sensor to integrate and balance metabolic and environmental cues. Leucyl tRNA synthetases, which interact with leucine in all domains of life for their critical role in mRNA decoding and translation, were exapted later in eukaryotic evolution to sense leucine concentrations for TOR. Variation in the relative contributions of these amino acid sensors across lineages and, even within a species, across cell types and contexts, may reflect the distinct evolutionary origin of these leucine sensors. Growing interest in TOR signaling among biologists outside of the biomedical sciences, especially biologists studying distantly related eukaryotes, including plants, algae, and both free-living and parasitic protists, will continue to enrich and deepen mechanistic models of TOR signaling in humans.

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