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mTOR senses intracellular pH through lysosome dispersion from RHEB

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Abstract

Acidity, generated in hypoxia or hypermetabolic states, perturbs homeostasis and is a feature of solid tumors. That acid peripherally disperses lysosomes is a three-decade-old observation, yet one little understood or appreciated. However, recent work has recognized the inhibitory impact this spatial redistribution has on the key regulator of metabolism mTORC1. This mechanism argues for a paradigm shift in localization of mTORC1 activator RHEB, a conclusion multiple others have now independently reached. Thus, mTORC1, known to sense amino acids, mitogens, and energy to restrict biosynthesis to times of adequate resources, also senses pH and, via dampened mTOR-governed synthesis of clock proteins, regulates the circadian clock to achieve concerted responses to metabolic stress. While this may allow cancer to endure metabolic deprivation, immune cell mTOR signaling likewise exhibits pH sensitivity, suggesting suppression of antitumor immune function by solid tumor acidity may additionally fuel cancers, an obstacle potentially reversible through therapeutic pH manipulation.

Graphical Abstract

Multiple studies now indicate mTORC1 senses intracellular pH. Acid drives lysosome redistribution separating bound mTORC1 from its activator RHEB. This suggests RHEB resides on non-lysosomal endomembranes in contrast to prevailing models. We review emerging reports regarding RHEB localization and discuss ways to translate mTOR's pH sensing into cancer immunotherapy.

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Keywords

acidity; cancer immunity; circadian clock; lysosome trafficking; mTOR; pH; RHEB

1. Introduction:

Acidity of cells and tissues manifests as yellowed cell culture media in the laboratory, decreased hyperpolarized carbon-13 bicarbonate magnetic resonance signal within animal tumors,^[1] and the altered mentation of patients in the clinic. This acidity reflects the generation of protons by metabolism. The level of acidity is quantified as pH, the negative $\log_{10}$ of the proton concentration. The major extracellular buffering system in the body involves interchange between carbon dioxide (CO_2), carbonic acid (H_2CO_3), and bicarbonate (CO_3^-). The relationship of pH to this bicarbonate buffering system is expressed by the Henderson-Hasselbach equation: $\text{pH} = \text{p}K_a + \log_{10}([\text{HCO}_3^-]/[\text{H}_2\text{CO}_3])$, where $\text{p}K_a$ is the dissociation constant for carbonic acid and $[\text{H}_2\text{CO}_3]$ can be readily calculated from the partial pressure of CO_2 using Henry's law. As taught in basic chemistry, a pH of 7 is defined as neutral; greater than this is alkaline and less is acidic. Human blood—and, through compartment exchange, interstitial fluid—is normally maintained in tight range centered at pH 7.4.

2. Solid tumors are acidic

The generation of acid relevant to cancer biology is attributable largely to glycolysis. Respiration's production of carbon dioxide, which in water hydrates to carbonic acid that dissociates to release a proton, accounts for a smaller acid load (Figure 1).^[2] Solid tumors tend to outstrip their vasculature rendering regions hypoxic.^[3–5] Lack of oxygen—the final electron acceptor in oxidative phosphorylation—precludes respiration. Therefore, in hypoxia, cells upregulate glycolysis, metabolizing glucose to lactic acid as an oxygen-independent means to generate ATP. Indeed, Louis Pasteur noted this ability to use oxygen levels to toggle metabolism between respiration and anaerobic glycolysis when he studied ethanol fermentation in yeast, a pathway analogous to lactic acid production in mammalian cells.^[6] Notably, glycolysis is considerably more acidifying than oxidative phosphorylation to generate the same number of ATP.^[2] In hypoxic mammalian cells, the upregulation of glycolysis is orchestrated by hypoxia-inducible factor (HIF) transcription factors that are stabilized under low oxygen tensions. HIFs transcriptionally activate rate-limiting glycolytic enzymes as well as pyruvate dehydrogenase kinase which deflects pyruvate away from the tricarboxylic acid cycle toward lactic acid (Figure 1).^[7–9]

Oncogenes also drive acid-generating glycolytic flux independent of oxygen tensions. Most notably, the commonly activated oncogenic transcription factor MYC also transcriptionally induces glycolysis under non-hypoxic conditions. This contributes to the propensity for cancers to produce lactic acid from glucose even in the presence of oxygen—the so-termed Warburg effect.^[10] Activation of other oncogenes, such as AKT, PI3K, BCR-ABL, KRAS, and EGFR, or loss of tumor suppressors, such as TSC2 or LKB1, also has the potential to enhance aerobic glycolysis either post-translationally or through signaling that converges on MYC and HIF (Figure 1).^[11, 12] Both anaerobic and aerobic glycolytic flux thus contribute to diminished tumor pH. Hindered perfusion also contributes by slowing the efflux of acid out of the tumor microenvironment for its ultimate elimination by the lungs and kidneys.^[5]

Intracellular cytoplasmic pH (pH_i) in normal cells is roughly pH 7.2 and protected from further acidification by multiple reinforcing proton-extruding pathways.^[13] As HIFs orchestrate the switch from respiration to anaerobic glycolysis in hypoxia, they simultaneously induce monocarboxylate transporters (e.g. MCT4, a symporter for lactate and H^+) and carbonic anhydrases (e.g. CA9 and CA12) to permit extracellular venting of the anticipated additional proton load. Other ion channels, carriers, and pumps important to homeostatic pH_i maintenance include Na^+/H^+ exchangers (NHEs), H^+ -ATPases, and bicarbonate transporters.^[5]

Through the use of pH probes and spectroscopy, solid tumors have been documented *in vivo* to be acidic with pH_e as low as pH 5.6 with mean values generally between 6.6 to 7.0.^[4, 14] For example, ^{31}P NMR spectroscopy permits measurements of pH_i via chemical shifts of energetic phosphorus species (phosphate, phosphocreatine, nucleotide phosphates) in response to protonation. Other approaches, including magnetic resonance imaging of infused hyperpolarized carbon-13 bicarbonate, have revealed tumor pH_e as low as 6.5 in experimental tumors.^[1] The pH_i in human cancers tends to be on average slightly less than pH 7.2 (typically in the range 7.0–7.2), indicating that the redundant pathways for extruding

protons successfully allow for maintenance of homeostatic pH_i at the expense of acidifying pH_e in most cells.^[14] The main NHE isoform in most cells, NHE1, becomes more active in response to mitogens.^[15, 16] Classic experiments revealed cells expressing mutant NHE isoforms incapable of alkalizing the cytoplasm in response to mitogens failed to reenter the cell cycle until pH_i was elevated with alkaline or bicarbonate-containing media.^[15] As such, the prevailing paradigm is that cancers resist pH_i acidification to sustain cellular function and pro-growth pathways. This avid extrusion of H^+ is thought to generate a more acidic pH_e and more alkaline pH_i , thereby inverting the normal directionality of the H^+ gradient across the plasma membrane.^[17] However, a careful study of 31 canine cancers *in vivo* revealed a wide range of pH_i and pH_e and significant tumor to tumor variations.^[18] Contrary to dogma, pH_e was found to be greater than pH_i in over 22% of cases, with pH_i noted as low as 6.7.

These *in vivo* data suggest that sudden, sustained, or substantial acid-generating stresses (i.e. hypoxia or oncogene-driven glycolysis) or confrontation by acidic pH_e can overwhelm proton extrusion pathways and intracellular buffering capacity. Indeed, such cytoplasmic acidification in response to acidic media exposure is particularly well documented *in vitro*.^[15, 19, 20] Moreover, reported near normal average tumor pH_i values do not preclude intratumoral pH_i spatial and temporal heterogeneity—potential features which remain relatively underexplored. Additionally, the subcellular topography of proton concentrations within the cytoplasm or across cell or organelle membranes, particularly after stresses such as hypoxia that introduce a proton load, remain unexplored.

3. Sensors of intracellular acidification include both rectifiers and responders

A change in environmental or intracellular pH has myriad effects on cells and tissues, and multiple sensors and signaling cascades have been variably ascribed a contributory role in these effects.^[13] Protonation of biological molecules and changes in transmembrane proton gradients are two conceivable means of sensing pH changes. Histidine residues appear to play a dominant role in pH sensing in many different proteins owing to their near neutral pK_a .^[21] However, in addition to histidine ($\text{pK}_a = 6.5$), other functional groups with pK_a close to physiological range, such as terminal amino groups ($\text{pK}_a \sim 8$), and cysteine ($\text{pK}_a = 8.5$), have the potential to “sense” pH through protonation when their pK_a values are nudged closer to physiologic pH in the context of protein structure.^[22] Perhaps the most illustrative example of such histidine-mediated pH-sensing is the Bohr effect. Under low pH, a salt bridge is formed between a protonated histidine residue and an aspartate residue that allosterically lowers the affinity of hemoglobin for oxygen, enhancing oxygen delivery to hypoxic acidic tissues.

pH_i and pH_e are believed to be sensed independently. Sensors of acidic pH_e , including G-protein coupled receptors (GPCRs), acid-sensitive ion channels (ASICs), and transient receptor potential vanilloid (TRPV) family members, signal to diverse downstream pathways and are reviewed elsewhere.^[13, 23–25] Here we discuss pH_i sensing.

Because changes in pH_i threaten to affect protein folding, macromolecule function or localization, and transmembrane proton gradients (such as those across mitochondrial membranes), several redundant means of H^+ efflux act to protect pH_i , including bicarbonate exchangers, H^+ -ATPases, carbonic anhydrases, and NHEs. Although also activated by mitogens as discussed above, the primary stimulus for NHE1 is acidic pH_i , which allosterically activates the exchanger through a C-terminal cytoplasmic cluster of histidine residues.^[26] In this manner, NHE1 serves as a rectifier of pH_i , becoming more active in response to lowering of pH_i from the physiologic set point.

Given that glycolysis is the main acid source in tumors, it makes teleological sense that a similar feedback loop might exist to downregulate glycolysis in response to dropping pH_i . To such an end, acidification rapidly induces thioredoxin-interacting protein (*TXNIP*) transcription, largely due to activation of the MondoA/Mlx heterodimeric transcription factor, a member of the extended MYC protein family.^[27, 28] *TXNIP*, in turn, strongly suppresses glycolysis by suppressing transcription of GLUT1 and promoting GLUT1 internalization.^[29] Failure of this feedback loop would be predicted to unbridle acid-generating glycolytic flux. Indeed, lactic acidosis was the presenting symptom for a recently characterized family with homozygous *TXNIP* mutation.^[30] Others have described a concomitant increase in glutamine metabolism and associated ammonia production when glycolysis is inhibited by acid.^[31] These metabolic responses indicate that cellular efforts to maintain pH_i can profoundly influence the metabolic state of cells and contribute to tumor spatial and temporal metabolic heterogeneity. Intra- and intertumoral heterogeneity in pH_e can potentially promote this pH_i -driven heterogeneity by influencing the proton gradient that H^+ venting pathways must confront.^[5]

In addition to molecules that serve as rectifiers of pH_i , several other molecules sense pH_i changes and respond by signaling to a diverse array of downstream outputs to alter cell biology without necessarily acting to restore pH homeostasis. Perhaps the best characterized effects are those on the actin cytoskeleton, which varying degrees of evidence suggest influence cell migration, invasion, and metastasis.^[32] For example, the actin filament disassembly activity of cofilins is suppressed through association with phosphatidyl inositol at acidic pH.^[13] Likewise, protonation of residues in talin increases its association with focal adhesions, slowing focal adhesion turnover and thereby motility in low pH.^[21] Similarly, histidine protonation plays an important role in regulating additional cancer-relevant proteins involved in cytoskeleton regulation and signaling, such as β -catenin and RasGRP1.^[33, 34] Metabolites themselves can also be influenced by acidification. Recent work describes how protonation of alpha-ketoglutarate in hypoxia allows it to serve as a substrate for lactate dehydrogenase and malate dehydrogenase to generate the oncometabolite L-2-hydroxyglutarate.^[35]

In addition to affecting the cytoskeleton and metabolism, low pH_i has been reported to directly affect the physicochemical properties of the cytoplasm of yeast by triggering a fluid-to solid-like state transition.^[36] This phase transition is chiefly a result of pH_i effects on formation of higher order protein structures, but macromolecular crowding and reduced metabolic activity may make contributions.^[36] In fact, metabolic activity in bacteria is thought to trigger local molecular agitation and transition the cytoplasm from a glassy

diffusion-limiting substance to a more fluid state.^[37] In yeast, energy depletion by metabolic inhibitors can render pH_i acidic and induce the solid-like state required for survival (dormancy) under nutrient-deprived conditions.^[36] Macromolecular assembly that drives this phase transition appears to be a result of global changes in protein charges. The distribution in charges of cytoplasmic proteins is multimodal but with a significant peak at about $-4e$ (negatively charged) at pH 7.0. At pH 6, average protein charge becomes $0e$ (neutral), which portends increased protein aggregation due to reduction in repulsive forces.^[38] While further work is needed to explore the effect pH_i has on the cytoplasmic properties of mammalian cells, these observations are an additional example of how organisms sense environmental conditions through pH_i allowing them to respond with appropriate alterations of their metabolism to endure stresses.

4. mTOR is a sensor of intracellular pH through lysosome positioning

In most cultured cells, lysosomes tend to cluster around the perinuclear microtubule organizing center (MTOC).^[39] Reversible dispersion of lysosomes to the periphery of mouse macrophages and chick fibroblasts in low pH_e was first observed by Heuser in 1989.^[40] Although subsequently documented in many other cell types *in vitro*,^[41–43] a functional significance of this phenomenon remained lacking.^[44] However, in the course of our recent studies of the circadian clock (Box 1), we unexpectedly found ourselves amidst this biology.^[45]

Given the central role of the circadian clock in metabolism, we initiated our studies seeking to understand if tumor hypoxia impacted the circadian clock of cancer cells. Indeed, we observed that circadian oscillation of the molecular clock was markedly suppressed in hypoxia. We came to understand that this suppression was mediated by intracellular acidification, a consequence of HIF-mediated upregulation of anaerobic glycolysis in hypoxia. Correspondingly, exposure to acidic media was sufficient to decrease pH_i and thereby disrupt the clock, while buffering against HIF-mediated acidification or inhibiting HIF-driven glycolytic flux with inhibitors of lactate dehydrogenase (LDH) was sufficient to rescue the clock. Importantly, we found acidification inhibited translation of clock proteins through potent inhibition of mechanistic target of rapamycin complex 1 (mTORC1) signaling, which governs biosynthetic pathways in part through positive regulation of cap-dependent translation (Box 2).^[20] Immunofluorescence and live cell imaging approaches further indicated that mTOR retained proper lysosome localization despite the remarkable redistribution of lysosomes to the periphery of cells in response to pH_i acidification (Figure 2). We surmised that this spatial redistribution of lysosomes might be sufficient to inhibit mTOR. Indeed, further experiments using inhibitors or viral activators of motor proteins that traffic lysosomes supported that mTORC1 senses pH_i through lysosome positioning.^[45]

mTORC1 integrates environmental signals including levels of amino acids and growth factors to limit translation and other biosynthetic activities to periods of replete resources.^[46] It therefore seems appropriate that mTOR activity is similarly gated by pH_i to restrict protein synthesis to times when pH is conducive to proper protein folding. Additionally, as acid stress is often coupled to hypoxic stress, mTOR sensitivity to pH_i may have evolved to curb energy costly protein synthesis during periods of energy stress. Furthermore, as

mTORC1 positively regulates glycolysis, in part through driving *HIF* translation,^[47, 48] the ability of mTORC1 to sense glycolysis-generated acid may permit a feedback loop to tune glycolytic flux to maintain homeostatic pH_i.

Peripheral redistribution of lysosomes in acidic pH_i may additionally help rectify pH_i through fusion of lysosomes with the plasma membrane. As cytoplasmic protons are pumped into lysosome lumens through the action of the lysosomal H⁺ pump V-ATPase, “lysosome exocytosis” could permit dumping of protons into the extracellular space. Additionally, this would place V-ATPase on the plasma membrane where it could continue to extrude cytoplasmic protons. Indeed, cells chronically adapted to acidic (pH 6.7) media display strong plasma membrane staining for the lysosomal transmembrane protein LAMP2 consistent with lysosome exocytosis.^[17]

Trafficking of lysosomes on microtubules in cells involves opposing activities of two classes of ATP-dependent motor proteins. Dynein traffics lysosomes toward the nucleus of cells, while members of the large family of kinesins largely participate in centrifugal movement toward the plus ends of microtubules (Figure 2).^[44] The mechanism by which acid drives lysosomes to the periphery remains unsettled but is generally speculated to be an upsetting of the normal balance between centripetal and centrifugal forces. Indeed, small molecule inhibition of dynein dispersed lysosomes and diminished mTORC1 activity in neutral pH conditions.^[45] Correspondingly, knockdown of *KIF5B*, an abundant cellular kinesin and component of kinesin-1, reduced centrifugal lysosome movement in acid.^[42] and partially increased mTORC1 activity under low pH_e.^[45] Intriguingly, kinesin has been shown *in vitro* to be inactive in neutral pH through inhibitory interaction of its light chain (LC) with its heavy chain (HC) in a manner that blocks HC microtubule binding. Lowering the pH *in vitro* below 7.2 protonates a negatively charged domain within the LC and permits microtubule binding.^[49, 50] Alternatively, axonemal (flagellar) dynein is appreciated to be inhibited by intracellular acidification, which is believed to keep spermatozoa flagella inactive during storage in the epididymis,^[51] and pH-dependence of cytoplasmic dynein has been likewise proposed and attributed to histidines at dynein LC’s dimer interface.^[52] These observations suggest that a rapid response to low pH through kinesin activation or dynein inhibition may disperse lysosomes to cellular peripheries and thereby rapidly inhibit mTORC1 signaling. However, further work is necessary to elucidate the exact mechanism underlying this three-decade-old phenomenon.^[40]

5. Where is RHEB?

In acidic conditions, lysosomes dispersed peripherally and such a spatial change appeared necessary and sufficient for mTORC1 inhibition by acid.^[45] However, why this change in localization of lysosome-bound mTOR should be inhibitory to mTORC1 signaling was not clear initially. In the widely accepted model of mTORC1 signaling, mTORC1 is activated when amino acid sufficiency is sensed via RAG proteins resulting in recruitment of mTORC1 to the surface of lysosomes where it can be activated by lysosome-localized RHEB.^[53] In acidic conditions, mTOR retained its proper lysosome localization yet mTOR signaling was strongly inhibited. Even deleting TSC2 (the upstream inhibitory GTPase activating protein (GAP) for RHEB) to ensure active RHEB (Box 2) failed to rescue mTOR

signaling in acid.^[45] Given this perplexing ability of acid to strongly inhibit mTORC1 signaling without hindering mTOR lysosomal localization or activating TSC2, we surmised that perhaps RHEB's contact with mTOR was hindered. In surprising agreement with this prediction, RHEB immunostaining revealed that RHEB maintained a perinuclear distribution in acid despite centrifugal dispersion of lysosomes. This rapid separation of lysosome-bound mTORC1 from its activator RHEB appeared to underpin inhibition of mTORC1, as strongly overexpressing RHEB to intentionally cause its mislocalization throughout the cytoplasm rescued mTORC1 signaling and the circadian clock.^[45]

Retention of RHEB in a perinuclear position in acid suggested RHEB might be localized to a non-lysosomal endomembrane compartment. Indeed, high resolution images of immunofluorescence co-staining for endogenous RHEB, lysosomal protein LAMP1, and mTOR at neutral conditions revealed high coincidence in spatial distribution of LAMP1 and mTOR but distinct patterning of RHEB suggestive of RHEB's occupancy of a spatially adjacent but separate compartment.^[45] This led us to propose that RHEB normally contacts and activates lysosome-localized mTOR from a distinct endomembrane compartment (Figure 2) (a relationship which we termed “transendomembrane” to contrast to the prevailing “cis-” interaction in which both RHEB and mTOR are proposed to both be lysosomal). In other words, amino acids recruit mTOR to lysosomes enriched at the MTOC allowing mTOR to contact RHEB localized to other endomembranes likewise scaffolded by the MTOC.

Our model (Figure 2) differs from the prevailing model of mTORC1 signaling in which RHEB is asserted to be constitutively anchored to lysosomal membranes through its farnesylated tail.^[53–55] The ability of farnesylation inhibitors that dislodge RHEB from membranes to inhibit mTOR signaling^[56, 57] suggests active RHEB resides on endomembranes. But which membrane? The perinuclear location of RHEB suggests several candidate membranes including Golgi, endoplasmic reticulum (ER), and mitochondria.

Notably, despite the dominant model localizing RHEB to lysosomes,^[53] several groups have reported non-lysosomal perinuclear RHEB localization in agreement with our inferences. Using GFP-tagged RHEB and RHEB2 (*RHEBL1*) constructs, Hanker and colleagues concluded RHEB colocalized with Golgi and ER markers,^[56] Moreover, these authors failed to observe colocalization of either RHEB construct with markers of late endosomes or lysosomes, including LAMP2 and LysoTracker. An earlier report by Buerger and colleagues likewise concluded exogenous RHEB constructs localized to the ER and Golgi.^[57] Moreover, noted abolition of mTORC1 signaling after treatment with brefeldin A, an ER to Golgi trafficking inhibitor disruptive of Golgi integrity, supported the proposition that RHEB needed to reach Golgi membranes to activate mTORC1. Correspondingly, substitution of RHEB's endogenous membrane anchoring tail with one targeted to the Golgi, but not the ER, was sufficient to fully activate mTORC1. Notably, these early authors interpreted Golgi localization of RHEB as indicative of mTOR recruitment to the Golgi as lysosome-localization of mTOR had not yet been described.^[53] Shortly after publication of our work, we became aware that an independent group had concurrently converged on a similar model of “inter-organelle communication” between Golgi-localized RHEB and lysosome-localized mTOR after recapitulating the findings of Buerger et al.^[58] However, all three of these

studies relied on exogenously expressed RHEB constructs which run the risk of mislocalization. Indeed, loss of mTOR's amino acid dependence in experiments by Hao and colleagues seem to betray this confounding issue.

Fortunately, Manifava and colleagues not only independently corroborated this ER/Golgi distribution of exogenous RHEB constructs but also characterized the location of endogenous RHEB, noting very weak lysosomal localization but substantial colocalization with golgi protein giantin.^[59] Recent unpublished work by Angarola and Gerguson likewise notes lack of lysosomal enrichment of endogenous RHEB both when assessed with an endogenous antibody or with an anti-HA antibody after CRISPR-mediated introduction of a tag into the endogenous locus.^[60] When they turned to exogenous constructs, they, like Buerger and Hao, observed ER localization but perplexing absence of mTOR signaling when RHEB was constitutively ER targeted. Thus, where the active pool of RHEB resides and how it makes activating contact with lysosome-localized mTOR remains an unanswered question. Although RHEB localization to ribosome-studded ER would be a logical place for mTOR contact to be made given mTOR's regulation of translation, the above evidence seems to argue against statically ER-localized RHEB being the active pool. One possibility given the typical ER to Golgi flow of membrane is that Golgi-localized RHEB is the truly mTOR-activating pool whereas ER-localized RHEB represents more immature RHEB protein en route to the Golgi (Figure 2). Alternatively, the ER may be the site of RHEB-mTOR contact but static targeting of RHEB to the ER may be problematic perhaps due to a need for exchange with the prominent cytosolic pool of RHEB to somehow maintain RHEB in an activated state.^[61]

6. Conflicting reports argue peripheral mTOR is active

Our finding that centrifugal lysosome redistribution is both necessary and sufficient for acid's suppression of mTOR is consistent with prior work indicating dynein^[62] and perinuclear clustering of lysosomes^[63, 64] support mTORC1 signaling. However, it is notable that Korolchuk and colleagues reported a seemingly inverted spatial dynamic from our findings with starvation (and therefore inhibited mTOR) associated with perinuclear clustering of lysosomes and refeeding (and therefore active mTOR) associated with peripheral redistribution.^[41] As starvation-induced mTOR inhibition induces autophagy, this perinuclear clustering of lysosomes may reflect the movement of lysosomes and autophagosomes to the MTOC to facilitate fusion. Indeed, pharmacologic inhibition of mTORC1 and starvation appear to induce tight perinuclear lysosome aggregation.^[55, 65] As centrifugal lysosome redistribution is a cause rather than consequence of mTORC1 inhibition in our model, acid-induced silencing of mTORC1 through peripheral redistribution of lysosomes is not incompatible with autophagy-induced retrograde trafficking of lysosomes.

However, further data presented by Korolchuk and colleagues are more difficult to reconcile with our findings. These authors reported that peripheral margination of lysosomes in HeLa cells through overexpression of several kinesins and associated adaptors caused augmented mTOR signaling with the reverse true upon knockdown.^[41] However, a more recent report in HeLa cells did not observe hindered mTORC1 signaling when lysosomes clustered at the

MTOC in response to knockout of a different kinesin-associated factor.^[66] The reasons for these contradictory results in the same cell line remains unclear. Nonetheless, we cannot exclude the existence of cell-type specific differences as HeLa cells appear distinctly wired in several ways. For instance, Korolchuk and colleagues noted that starvation (including serum starvation) alkalinized HeLa cell pH_i, which they suspected mediated lysosome perinuclear aggregation. Given that it is classically noted that growth factor stimulation causes alkalization of pH_i, owing to the growth-factor responsiveness of H⁺ exchangers such as NHE1,^[15, 16] this is surprising. It is also possible that human papillomavirus 18 (HPV 18) infection of HeLa cells endows a distinct biology. Indeed, cytomegalovirus (CMV), another DNA virus, alters trafficking of mTOR to achieve amino acid independent activation that supports biosynthesis of viral progeny.^[62, 64] Intriguingly, HPV E6 has been reported to activate mTORC1 and also interact with a component of endosomal cycling.^[67, 68] Moreover, knockdown of E6 was noted to result in tighter perinuclear endosome clustering,^[67] suggesting E6 may perhaps underlie peripheral spread of mTOR-bound endomembranes in HeLa cells.

Recent work studying a panel of cell lines, including HeLa cells, has demonstrated that cells display two pools of lysosomes, a larger perinuclear pool and a smaller more dynamic peripheral pool.^[39] mTOR localization appears to mirror this distribution in the presence of amino acids.^[45, 54, 55] We suspect future investigations will clarify that perinuclear lysosome-bound mTORC1 is an active pool and that this location is critical for spatial contact with RHEB. That CMV has evolved a mechanism to actively bring mTOR to the perinuclear region and that doing so allows the virus to maintain mTORC1 activity in the face of inhibitory signals underscore, we believe, the importance of this spatial location to mTORC1 activation.^[62, 64] However, in our studies at neutral pH, the periphery of the cell was not devoid of lysosome-bound mTOR and our model need not imply that subpopulations of mTORC1 do not translocate to various niches in the cell to regionally tailor biosynthetic activities. Indeed, in other studies exploring the spatial localization of mTOR in HeLa cells—as well as in MEFs, A549, and PANC-1 cells—the majority of mTORC1 localized perinuclearly in serum-stimulated (mTORC1-activated) cells with a much smaller portion localized to plasma membranes.^[69] Intriguingly, Hong and colleagues have recently proposed a model in which an ER localized protein, protrudin, makes contact with lysosomes in response to their reception of mTORC1-activating and phosphatidylinositol-3-phosphate (PI(3)P)-generating stimuli, activates kinesin-1 in the process, and thus sends lysosomes peripherally.^[70] Such a model might reconcile a portion of discrepant findings, underscoring the critical importance of multi-organelle contact at the MTOC to activation of mTORC1 without disallowing for peripheral translocation of activated mTORC1.

7. Leukocyte mTOR inhibition by tumor acidity may suppress anticancer immunity

Tumor acidity affects not only cancer cell intrinsic biology but also the immune system.^[71] Lymphocytes have long been recognized to alkalinize pH_i upon stimulation.^[72] More recently, mTORC1 has been revealed to play a critical role in the differentiation and

activation of CD4⁺ and CD8⁺ T cells.^[73–75] For instance, pharmacologic or genetic inhibition of mTORC1 signaling prevents differentiation of CD8⁺ T cells into effector cells^[74, 76] and CD4⁺ T cells into Th1 and Th17 subtypes.^[77] Several reports have noted without mechanistic explanation that acidic media reversibly suppresses T cell cytokine production and cytolytic abilities *in vitro*.^[71, 78–81] Correspondingly, the potent suppression of mTORC1 signaling by acid observed by us in CD8⁺ and CD4⁺ cells might be the mechanism behind acid's suppressive effects on T cells.^[45] Consistent with this, suppression of IFN γ production in acid was noted to be reversible and posttranscriptional in a manner suggestive of a translational block.^[78, 81] Indeed, rapamycin has been used for decades for its immunosuppressive function.

Peripheral redistribution of lysosomes observed in acidic cancer cells suggests microtubule-based vesicle trafficking events critical to T cell function might also be disturbed in the acidic tumor microenvironment.^[45] After engaging an antigen presenting cell, the MTOC of the naïve T cell moves beneath the immune synapse (point of T cell receptor (TCR) engagement) and additional TCR receptors are delivered in a dynein-dependent manner. Lytic granules are similarly delivered to the MTOC when a cytolytic T cell encounters a target cell.^[82] Moreover, asymmetric partitioning (i.e. microtubule-directed trafficking) of fate-determining proteins amongst daughter cells during antigen-stimulated cell divisions gives rise to memory and effector lineages of CD8⁺ T cells.^[74] Therefore, it is conceivable that acid-mediated antegrade redistribution of vesicles could hinder activation, differentiation, and effector abilities of T cells in both mTOR-dependent and independent ways. Consistent with this, in previous studies of T cell cultured in acid, perforin content was lower^[81] and degranulation was also reversibly suppressed.^[79] Acid neutralizing tactics might thus be helpful adjuvants to traditional cancer therapeutics and newer anticancer immunotherapies through restoration of mTORC1 signaling and proper vesicle trafficking in immune cells.

8. Restoring mTORC1 signaling through pH neutralization may have therapeutic potential

Administration of HCO₃[−]-supplemented drinking water to tumor-bearing mice has been shown by hyperpolarized H¹³CO₃[−] magnetic resonance imaging,^[1] intravital pH indicator dye,^[83] and microelectrode measurements^[84] to increase tumor pH_e *in vivo*.^[1] Two therapeutic strategies can be envisioned. One, restoring tumor cell pH_i and thereby mTOR signaling could drive cells into a state susceptible to metabolic inhibitors, anti-proliferative agents, or other therapies. While restoring mTORC1 signaling in quiescent cancer cells potentially restores their proliferative capacity, the idea is to create an opportunity to then target this awakened metabolic state. Illustrative of this strategy, we and others demonstrated that HCO₃[−]-supplemented drinking water increased mTORC1 signaling within multiple xenograft and allograft tumors.^[45, 85] Moreover, Faes and colleagues further demonstrated that this strategy restored rapamycin sensitivity.^[85] Two, neutralizing pH_e could enhance antitumor immune function. Indeed, HCO₃[−] administration or treatment with a proton pump inhibitor elevated the pH_e of B16 melanoma allografts and enhanced response to immune checkpoint inhibitor therapy and adoptive lymphocyte transfer, respectively.^[78, 79] These

studies suggest that therapeutic strategies to normalize tumor pH_e could have clinical promise.^[25]

Large unresectable hepatocellular carcinomas (HCCs) are treated with chemoembolization, a tactic intended to deliver concentrated chemotherapy and starve cancer cells through embolization but that is of only questionable benefit.^[86] Intriguingly, however, a clinical study of unresectable HCC in 57 non-randomized Chinese patients revealed that the addition of bicarbonate to chemoembolization markedly improved objective response rate (ORR) (100% vs 44%) and reduced residual disease volume (RDV) (7% vs. 46%).^[87] Importantly, this was subsequently born out in a smaller randomized controlled trial of 20 patients (ORR 100% vs. 64%, RD 4% vs 28%). While the mechanism for this effect can only be speculated at current, the authors favor the hypothesis based on their prior *in vitro* data that tumor lactic acidosis exacerbated by embolization pushes cancer cells into a quiescent state of low metabolic demand protective against embolization-induced nutrient deprivation.^[88–90] Therefore, it's intriguing to speculate that bicarbonate-induced reactivation of mTOR may sustain untenable metabolic demand that mediate these remarkable findings. Furthermore, as available survival data also point to sustained benefit (41- vs 14-month nonrandomized mean survival), it's likewise alluring to speculate that bicarbonate-mediated enhanced immune cell mTOR signaling also contributes. Given these remarkable outcomes, we eagerly await future study revealing the mechanism of this bicarbonate-driven effect and indications for extended use.

9. Conclusions and Outlook

Setting out to assess the state of the molecular circadian clock in hypoxia, we have come away learning principles of fundamental pH sensing and mTOR activation that have ignited our inquiry into diverse biology from molecular motors and endomembrane trafficking to antitumor immunity. We have learned mTOR is a sensor of pH_i through lysosome positioning relative to RHEB. As such, mTOR appears to complement other pH sensing mechanisms while also offering an ability to appropriately broaden the metabolic response. But how acid-driven lysosome dispersion occurs at a molecular level remains unanswered. Separation of mTOR and its activator RHEB in acid have revealed RHEB to occupy a non-lysosomal membrane, the identity of which remains under active debate. We surmise that mTOR-coordinated downregulation of the circadian clock and biosynthetic processes represents a powerful stress response that cancers may rely on to confront the exceptional acidity of solid tumors. Solid tumor acidity may thus contribute to tumor dormancy and blunted antitumor immune responses. As such, additional studies are warranted to determine whether relatively straightforward pH correction could enhance a broad range of cancer therapeutic approaches.

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Abbreviations:

HIF	hypoxia-inducible factor
pH_e	extracellular pH
pH_i	intracellular cytoplasmic pH
mTORC1	mechanistic target of rapamycin complex 1
RHEB	Ras homolog enriched in brain
RAG	Ras-related GTP-binding
NHE	Na ⁺ /H ⁺ exchanger
TXNIP	thioredoxin-interacting protein
MTOC	microtubule organizing center

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Box 1 –**The molecular circadian clock governs homeostatic metabolism**

Circadian clocks allow organisms to anticipate cyclical (daily) events and coordinate optimal timing of physiologic processes.^[93] In mammals, clocks in the suprachiasmatic nucleus of the hypothalamus constitute a central pacemaker tuned to the appropriate phase by retina-sensed light. Through neuronal and hormonal (e.g. glucocorticoid) outputs, rhythmic control of behaviors such as eating, and other signaling pathways, the central clock synchronizes the oscillation of autonomous peripheral clocks found in nearly every cell.^[94, 95]

The mechanistic basis for both central and peripheral clocks is an interlocking network of feedback loops centered on the heterodimer CLOCK-BMAL1.^[96] CLOCK-BMAL1 drives the expression of many genes containing E-box consensus sequences in their regulatory regions. Among these target genes are those encoding period (PER) and cryptochrome (CRY) proteins, which feedback to inhibit CLOCK-BMAL1 posttranslationally, forming the core negative feedback loop. A second negative feedback loop arises from CLOCK-BMAL1-mediated transcription of genes encoding REV-ERB proteins, which bind the RORE motif in the promoter for BMAL1 and negatively regulate its transcription. Together these feedback loops cause circadian oscillation of CLOCK-BMAL1 activity and, consequently, directly or indirectly, the circadian expression of thousands of downstream clock-controlled genes (CCGs) in a given tissue type. Circadian rhythmicity in protein levels, enzymatic activities, metabolites, and cellular processes follow, particularly those with metabolic roles.^[97–104]

Our recent work has revealed hypoxia to suppress the circadian clock and circadian transcriptome.^[45] This is mediated by a decrease in translation of clock proteins due to mTOR inhibition by intracellular acidification, a consequence of hypoxia-inducible factor (HIF)-driven anaerobic glycolysis. (See text.) Work in flies and mice demonstrating connections between both central and peripheral clocks and mTOR^[105–109] indicate this ability of mTOR to modulate the clock in response to sensed environmental stresses may be well-conserved.

Box 2 –**mTOR senses stress signals to appropriately limit anabolic processes**

mTOR complex 1 (mTORC1), a multi-protein assembly composed in part by the kinase mTOR, regulates cell growth, matching anabolic processes to periods of substrate sufficiency and growth-affirmative humoral signals.^[46] Such coordination emerges from multiple parameters indicative of suitability for growth converging on two populations of GTP-binding proteins immediately upstream of mTORC1, Ras-related GTP-binding (RAG) and Ras homolog enriched in brain (RHEB) proteins. Sensors for arginine, leucine, and methionine signal amino acid sufficiency to lysosome-localized RAG heterodimers through a series of protein interactions that ultimately control RAG nucleotide binding states.^[110–114] Correct GTP/GDP loading of RAG proteins recruits mTORC1 to the lysosomal surface. In the widely accepted model, RHEB is anchored to the lysosomal surface thus allowing activation of amino-acid-recruited mTOR by RHEB.^[53] RHEB is GTP-bound and thus capable of activating recruited mTOR so long as the tuberous sclerosis complex (TSC), which contains TSC2, the GTPase-activating protein (GAP) for RHEB, is spatially disengaged from RHEB in response to growth factors (e.g. signals relayed through phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling)^[55] and kept inactive through absence of TSC2-activating stress signals (e.g. energy insufficiency sensed by adenosine monophosphate (AMP)-sensitive AMP kinase (AMPK)).^[115]

Active mTORC1 positively regulates translation through mTOR phosphorylation of downstream substrates.^[116, 117] Phosphorylation of ribosomal S6 kinase (S6K) stimulates S6K phosphorylation of ribosomal protein S6, a component of the 40S ribosomal subunit, driving cell growth through debated mechanisms.^[118] mTOR also phosphorylates inhibitory binding partners of the eukaryotic initiation factor 4E (eIF4E), the 4EBP proteins, preventing their engagement with eIF4E and thereby releasing suppression of cap-dependent translation. mTORC1 thus senses stress and reserves energy-costly translation and downstream biosynthetic processes for periods in which reserves of

energy and building blocks can support these demands. In addition to driving biosynthesis through activation of cap-dependent translation, mTOR also positively regulates lipid and nucleotide biosynthesis to support growth, as well as metabolic pathways such as glycolysis and production of reducing equivalents that support these anabolic processes^[115].

Figure 1. Metabolic Acid Production.

Acid-generating glycolytic flux^[2] is elevated in tumors due to both anaerobic and aerobic transcriptional induction of key enzymes by hypoxia inducible factor 1 alpha (HIF1α, HIF1α targets: blue)^[91] and oncogenic MYC (MYC targets: red)^[92] respectively. (Shared targets of MYC and HIF1α: purple). Further, MYC stimulates mitochondrial biogenesis and function, increasing glutamine oxidation and oxidative phosphorylation,^[10] resulting in the production of carbonic acid (H₂CO₃) from carbon dioxide (CO₂) and water. GLUT1 (Glucose transporter 1); HK1/2 (Hexokinase 1/2); GPI (Phosphoglucose isomerase); PFK (Phosphofructokinase); ALDA (Fructose-1,6-bisphosphate aldolase); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); PGK1 (Phosphoglycerate kinase); ENO1 (Enolase); PKM (Pyruvate kinase); PDK (Pyruvate dehydrogenase kinase); LDHA (Lactate dehydrogenase); ATP (Adenosine triphosphate); ADP (Adenosine diphosphate); NAD (Nicotinamide adenine dinucleotide, oxidized); NADH (Nicotinamide adenine dinucleotide, reduced); H⁺ (proton), HCO₃ⁱ⁺ (bicarbonate).

Figure 2. Acid-mediated dispersion of lysosomes inhibits mTORC1 signaling.

Amino acids through RAG proteins recruit mTOR to the surface of lysosomes allowing mTOR to contact RHEB localized to a non-lysosomal endomembrane. RHEB's activation of mTOR is licensed by growth factors and replete energy stores. When intracellular pH falls, lysosomes disperse to the periphery of cells which inhibits mTOR through separation from RHEB. Lysosome dispersion likely results from an imbalance between dynein-mediated centripetal forces and kinesin-mediated centrifugal forces in acid. Inhibition of mTOR decreases phosphorylation (p-) of downstream S6 and 4EBP1 proteins, which suppresses synthesis of circadian clock proteins through inhibition of cap-dependent translation. (See also Box 2.)