



Intracellular pH dynamics and charge-changing somatic mutations in cancer

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Abstract

An unresolved question critical for understanding cancer is how recurring somatic mutations are retained and how selective pressures drive retention. Increased intracellular pH (pHi) is common to most cancers and is an early event in cancer development. Recent work shows that recurrent somatic mutations can confer an adaptive gain in pH sensing to mutant proteins, enhancing tumorigenic phenotypes specifically at the increased pHi of cancer. Newly identified amino acid mutation signatures in cancer suggest charge-changing mutations define and shape the mutational landscape of cancer. Taken together, these results support a new perspective on the functional significance of somatic mutations in cancer. In this review, we explore existing data and new directions for better understanding how changes in dynamic pH sensing by somatic mutation might be conferring a fitness advantage to the high pH of cancer.

Keywords Intracellular pH dynamics · Oncogenes · pH sensing · Somatic mutations

1 Introduction

Most cancer cells have a constitutively higher intracellular pH (pHi) of 7.4–7.6 compared with normal cells (7.2). The increased pHi of cancer, as several previous reviews described [1–4], enables multiple cancer cell behaviors, including proliferation, metastasis, metabolic adaptation, and evasion from apoptosis. Increased pHi is also reported to be an early event in cancer development [5] and can induce dysplasia in the absence of activated oncogenes [6]. In this review, we present an emerging view on somatic charge-changing mutations altering pH sensing by proteins as an additional mechanism for how increased pHi in conjunction with somatic mutations can enable disease progression.

2 Charge-changing mutations in cancer

The incidence of recurrent somatic mutations in cancer is in part determined by fitness advantages to a dynamic

microenvironment [7–9], including changes in metabolism [10], oxygen availability [11, 12], extracellular matrix composition [13], and pH dynamics [1]. While extensive work has characterized cancer heterogeneity based on tissue origin [14], driver mutations [15], or nucleotide mutational signatures [16–18], recent analyses highlight cancer heterogeneity by amino acid mutational signatures [19–21]. Fitness advantages are generally conferred by alterations in protein function, and analysis of amino acid signatures may be more representative of selection effects from tumor or microenvironment pressures in cancer evolution. Work from Szpiech and colleagues identified six amino acid mutational signatures in a tumor-normal paired database [16], and the signatures are dominated by charge-changing amino acid substitutions (arginine (Arg)>histidine (His) and glutamate (Glu)>lysine (Lys) (Fig. 1a) [19]. These same signatures are recapitulated when the analysis is performed at the level of the individual patient (Fig. 1b). These substitution signatures are independent of both underlying nucleotide mutation signature [16] and codon bias. Importantly, the Arg>His and Glu>Lys signatures are mutually exclusive (i.e., cancers that were dominated by patients with high Glu>Lys had very few patients with an Arg>His signature and *vice versa*). Anoosha and colleagues independently identified similar charge-changing amino acid mutational signatures in an analysis of the entire COSMIC dataset [20]. Moreover, the Anoosha analyses suggested that the Arg>His mutational signature is enriched in driver genes whereas the Glu>Lys signature has no preference for driver genes over passenger genes [20]. These same amino acid signatures have not been identified in

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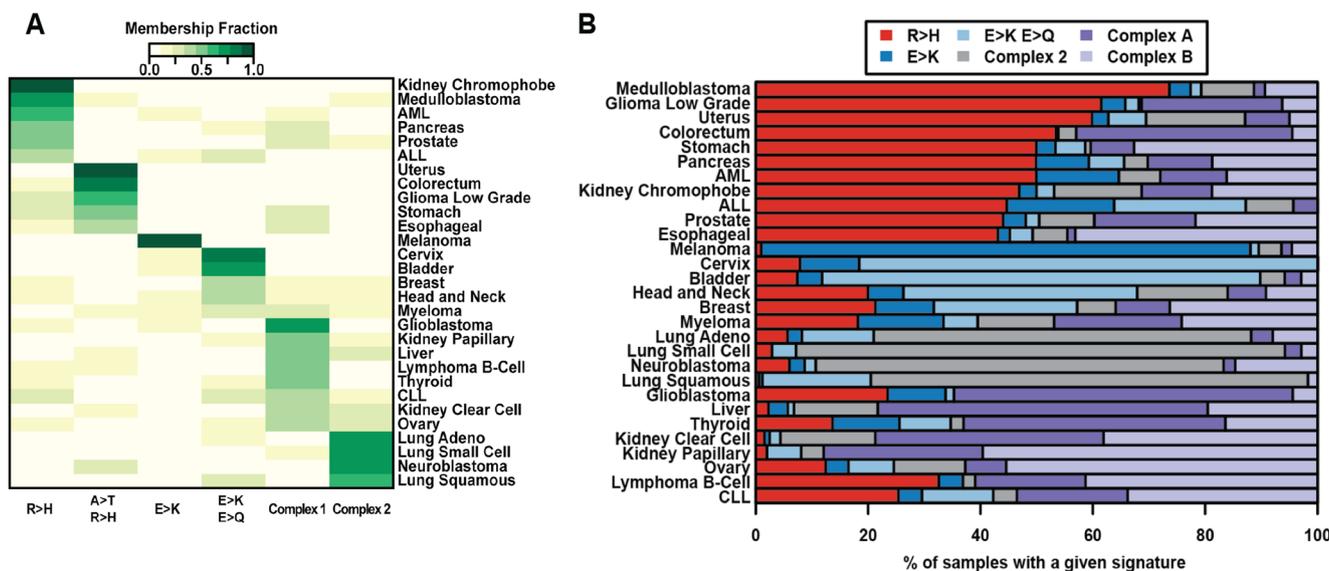


Fig. 1 Charge-changing mutations define the mutational landscape of most cancers. **a** Non-negative matrix factorization identifies six amino acid mutational signatures across cancers. Four are defined by prominent charge-changing mutations (Arg>His (R>H), Glu>Lys (E>K), and

analyses of genetic drift and evolutionary selection [22, 23], suggesting that the amino acid substitution signatures found in cancer somatic mutations might reflect selective pressures distinct to the cancer microenvironment or physiology.

Taken together, these published datasets suggest that charge-changing mutations define the amino acid mutational landscape of cancers and could reflect the unique selective pressures driving cancer selection and evolution. The predominance of charge-changing somatic mutations in cancer is perhaps unsurprising since charge-changing mutations are likely to alter protein function and this altered protein function leads to cumulative changes in signaling and tumorigenic behaviors associated with cancers. Somatic cancer mutations are commonly considered to de-regulate proteins, constitutively activating oncogenes or inactivating tumor suppressors. While it is clear that these static protein functions can enable tumor cell behaviors, cancer cells must adapt and respond to a dynamic microenvironment [1, 2]. The potential for cancer mutations to confer dynamic functions dependent on microenvironment changes is understudied but critical for understanding how cancer cells adapt and respond during disease progression.

3 Gain of pH sensing by arginine to histidine substitutions in cancers

Recent work from our lab showed that recurrent charge-changing Arg to His mutations can confer dynamic pH sensing to the mutant proteins [24]. The imidazole R-group of histidine has a pK_a in solution of 6.5, meaning that at pH 6.5, half of the molecules are protonated and half are deprotonated. However, depending on the protein landscape, the pK_a of histidine can also

Glu>Gln (E>Q)). **b** Charge-changing mutational signatures dominate when patient samples are analyzed individually. Bars show the total fraction of individual samples with a majority of a particular signature within each cancer. Reprinted with permission from [19]

be upshifted, for example, to 7.2 as determined by NMR [25, 26]. In contrast, arginine has an invariant pK_a of 12.5 in solution and remains protonated regardless of the pH. Therefore, while histidine can titrate between protonated and neutral forms within the cellular range, arginine is always protonated. We showed in several key instances that Arg>His mutations can confer pH-sensitive activity to mutant proteins.

One example of a gain in pH sensing conferred by an Arg>His mutation is the recurrent Arg776His somatic mutation in the epidermal growth factor receptor (EGFR-R776H). Overexpression and mutational activation of EGFR are linked to a number of cancers, with nearly 20% of non-small-cell-lung cancers having EGFR mutations [27]. The EGFR-R776H mutation is recurrent in lung cancers and in mesotheliomas [28]. We found that EGFR-R776H has a gain in pH sensing not seen with the wild-type receptor, causing increased kinase activity and signaling at higher pH [24]. In wild-type EGFR, Arg776 stabilizes the inactive conformation of EGFR through a hydrogen bonding network with residues in the αC helix. Molecular dynamics simulations suggest that the protonation state of His776 alters conformation of the αC helix (Fig. 2a) [24]. In these simulations, a deprotonated histidine promotes the active conformation of EGFR. Consistent with these molecular dynamics simulations, EGFR-R776H induces increased signaling, cell proliferation, and cellular transformation specifically at the higher pH of cancer in the absence as well as presence of EGF [24]. Hence, EGFR-R776H confers dynamic pH-sensing activity and is an example of pH-sensitive activation of an oncogene. The mutation only becomes fully penetrant when it has the cancer cue of increased pH. In cancers where increased pH is an early event, this somatic mutation may confer an adaptive advantage leading to selection and recurrence in the patient population.

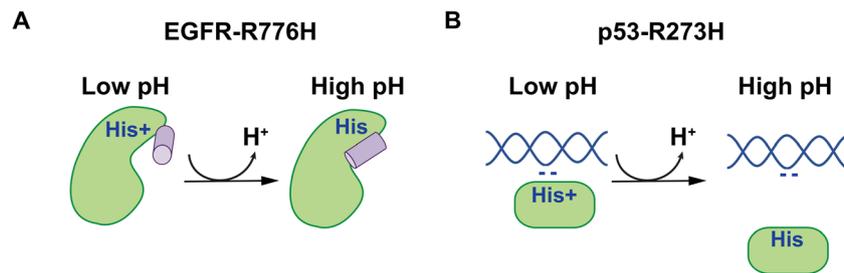


Fig. 2 Known pH-sensing mechanisms of Arg>His mutations. **a** EGFR-R776H confers pH-sensitive activity. Protonation state of His776 alters orientation of the α C helix. Increased pH (deprotonated histidine) promotes the active “swung-in” conformation of the α C helix, increasing activity of EGFR specifically at high pH. **b** p53-R273H confers

pH-sensitive DNA binding. Protonation state of His273 mediates binding and transcriptional activity of p53-R273H. At low pH, protonated His273 can bind to the negatively charged phosphate backbone of DNA. At high pH, His273 is deprotonated and decreased DNA binding, transcription, and cell death in response to double-strand breaks is observed [24]

Another example of Arg>His somatic mutation conferring a gain in pH sensing is p53-R273H. p53 is mutated in nearly 50% of all cancers, with Arg273His being one of the most frequent mutations [29]. In wild-type p53, Arg273 forms an electrostatic interaction with the negatively charged phosphate backbone of DNA. We observed a gain in pH sensing conferred by the highly recurrent p53-R273H, with decreased DNA binding, decreased transcriptional activity, and decreased cell death at higher pH [24]. We had predicted that this is mediated by direct binding to DNA, whereby a deprotonated His273 is unable to maintain an electrostatic interaction with the phosphate backbone of DNA, resulting in decreased DNA binding and p53 activation at higher pH (Fig. 2b). While p53-R273H was traditionally thought to be a loss-of-function mutation, we showed that simply lowering pH recovered p53-R273H DNA binding, transcriptional, and cell death responses [24]. These data strongly suggest that dynamic pH-sensing function conferred by p53-R273H can provide an adaptive advantage to cancer cells having an increased pH. This is an example of pH-sensitive inactivation of a tumor suppressor and highlights the potential therapeutic effects of lowering pH in cells that are adapted to high pH.

At the normal pH of cancer cells, the Arg>His mutant proteins are able to somewhat recapitulate normal wild-type activity. Because there is no strong deleterious effect for these mutations at normal pH, the mutations avoid negative selection. It is only with the “cancer-like” cue of increased pH (driven by dysregulation of ion transport proteins) that these mutations then confer an adaptive advantage by either activating EGFR signaling or inactivating p53-driven apoptotic responses. These mutations are likely recurrent because they avoid purifying selection in normal cells and yet provide a strong fitness advantage at the increased pH of cancer.

4 Potential molecular mechanisms for other arginine to histidine substitutions

In addition to the previously reported gain in pH-sensing mutations, there are many recurrent somatic mutations in cancers that

may also manifest their tumorigenic qualities in a similar manner. As examples, we briefly propose molecular mechanisms for predicted gain in pH sensing by Smad4-R361H in TGF- β signaling, RNA helicase activity of DDX3X-R534H, and substrate recognition by a mutant ubiquitin ligase FBXW7-R465H.

Smad4 functions in TGF- β signaling as the central binding partner for all receptor-activated R-Smads. The R-Smad/Smad4 heterotrimeric complex translocates from the cytosol to the nucleus where it activates tumor-suppressive transcriptional programs [30]. Mutations in SMAD4 are common in Juvenile Polyposis Syndrome that is associated with increased risk of gastrointestinal cancers, and mutations or deletions in SMAD4 are found in 30–60% of pancreatic cancers and linked to lower survival [31, 32]. Smad4 is also thought to be a major suppressor of colorectal cancer progression where it is inactivated later in the stage of malignancy [32]. SMAD4-R361H is a recurrent mutation in colorectal and pancreatic cancers [28]. Arg361 is located at the heterotrimerization interface and forms a salt bridge with an evolutionarily-conserved aspartate residue found in all R-Smads (D408 of Smad3 and D450 of Smad2) [33]. This salt bridge is necessary for Smad4 binding to the R-Smads; a prediction is that Smad4-R361H binding to R-Smads is pH sensitive with a protonated histidine being required for electrostatic preservation of the salt bridge (Fig. 3a). Based on this prediction, Smad4-R361H would enable normal Smad4-Smad2/3 heterotrimerization and TGF- β signaling at normal pH, but heterotrimerization and TGF- β signaling would be decreased specifically at the higher pH of cancer cells (Fig. 3b). Decreased TGF- β signaling is linked to tumorigenesis and inactivation of the Smad pathway *via* deletion of Smad4 is a driver of many cancers. Therefore, we predict Smad4-R361H would specifically enable cancer phenotypes at the higher pH of cancer.

DDX3X is an ATP-dependent RNA helicase that regulates RNA metabolism, including pre-mRNA splicing, ribosome biogenesis, RNA export, translation initiation, and stress granule formation [34]. DDX3X is the second most common mutated gene in medulloblastomas, found in up to 8% of cases [28, 35]. DDX3X-R534H is a common mutation in medulloblastoma, and the substitution is at the catalytic core where wild-type Arg534 makes contacts with the phosphate of ATP and is critical for ATP

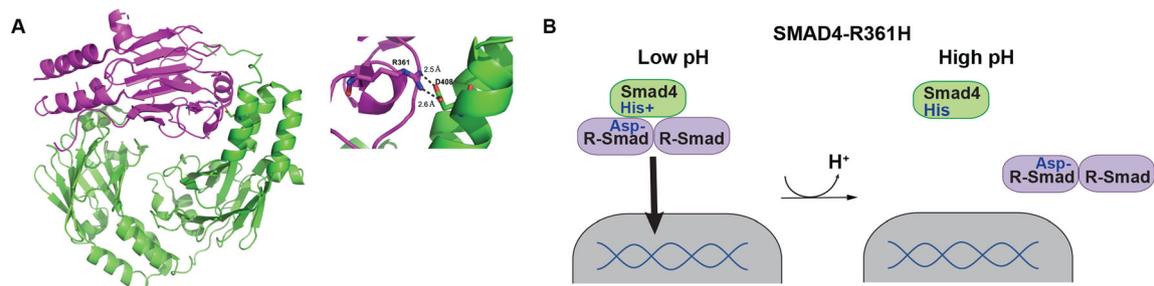


Fig. 3 Proposed pH-sensing mechanism for SMAD4-R361H. **a** SMAD4-R361H. Arg361 in Smad4 (magenta) forms a salt bridge with a conserved aspartate residue in Smad3 (R-Smad, green) (PDB: 1U7F). **b** We predict that at low pH, protonated His361 is more likely to form an electrostatic

interaction with Asp, allowing formation of the heterotrimer, translocation to the nucleus, and transcription of target genes. At high pH, when His361 is likely to be deprotonated, the heterotrimer cannot form, blocking heterotrimer formation, translocation, and target gene transcription

binding and hydrolysis (Fig. 4a) [36, 37]. The DDX3X-R534H mutation was previously described as inactivating with RNA duplex unwinding activity decreased 1000-fold *in vitro* compared with wild type [36]. However, this lower activity was determined at pH 8.0, where equilibrium is shifted toward neutral His534. Based on the pH-dependent titration of histidines, at the lower pH of untransformed cells, His534 might be protonated and enable higher helicase activity with DDX3X-R534H than was previously observed at high buffer pH. We predict that in DDX3X-R534H, the substituted histidine confers pH-dependent helicase activity, with decreased activity at the higher pH of cancer cells (Fig. 4b). Because DDX3X helicase activity is critical for translation initiation of several mRNAs with structured 5' UTRs, inactivating mutations in DDX3X have previously been shown to drive stress granule assembly [38]. This results in untranslated mRNA being sequestered and ultimately impairing translation of several DDX3X targets [38]. This stress response is believed to provide a survival advantage for tumor cells under hypoxic stress resulting in reduced protein synthesis [39].

An additional prediction is a gain in pH sensing by FBXW7-R465H. FBXW7 is the substrate recognition component of SCF-type ubiquitin ligases and mediates the ubiquitin-dependent proteolysis of various oncoproteins such as myc and CyclinE [40]. FBXW7, an E3 ubiquitin ligase, is the substrate recognition protein of a multiprotein complex. Suspected to be a potent tumor suppressor, mutations in

FBXW7 are found in almost a third of all T cell acute lymphoblastic leukemias and over a third of cholangiocarcinomas [41]. Arg465His is the most common somatic mutation in FBXW7 and is located in the substrate recognition site [41]. FBXW7 has two requirements for substrate recognition, the first is phosphorylation of the Ser or Thr residue at the P-0 position and the second is presence of a proline at the P+1 position. Arg465 is the only residue of FBXW7 that interacts *via* hydrogen bonding to residues in both of these positions [41] (Fig. 5). For that reason, Arg465 is proposed to be critical for substrate recognition. We predict the Arg465His mutation may confer pH-dependent substrate binding, with less binding at higher pH.

5 Loss of pH sensing by histidine to arginine substitutions in cancer

While Arg>His mutations can confer a gain in pH sensing, the reverse effect of a His>Arg somatic mutation conferring a loss in wild-type pH sensing can also occur. We recently identified the adherens junction and signaling protein β -catenin as a previously unrecognized pH sensor, with decreased stability at higher pH [42]. An evolutionarily conserved histidine in the N-terminal region of β -catenin (His36 in human β -catenin) is responsible for mediating its pH-sensitive stability

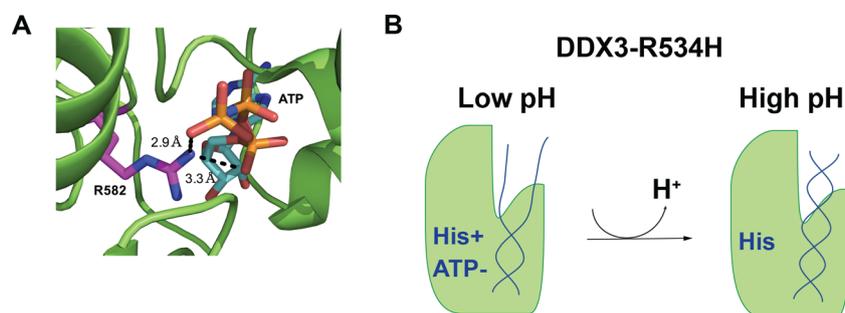


Fig. 4 Proposed pH-sensing mechanism for DDX3X-R534H. **a** DDX3X *Drosophila* homolog Vasa in the closed and active state (PDB: 2DB3). Vasa Arg582 (magenta stick) corresponds to Arg534 in human DDX3X. Arg582 is important for proper coordination of ATP (cyan stick). **b** We

predict that a protonated His534 might be able to properly bind ATP and enable helicase activity at low pH. However, at high pH, when His534 is more likely to be deprotonated, helicase activity will be decreased

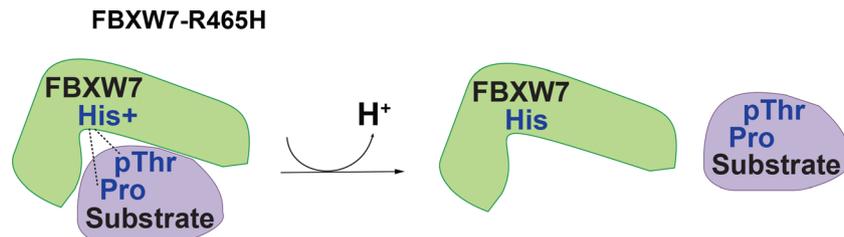


Fig. 5 Proposed pH-sensing mechanism for FBXW7-R465H. FBXW7 is an E3 ligase responsible for recognizing and ubiquitinating proteins for proteasome-mediated degradation. Arg465 is in the substrate recognition pocket of FBXW7 and coordinates two residues at the P0 and P+1 position (pThr and Pro, respectively) *via* hydrogen bonding networks.

We predict that a protonated His465 at low pH might be able to form this hydrogen bonding network and stabilize binding to the substrate target. However, at high pH, when His465 is more likely to be neutral, we predict that substrate binding and ubiquitination will be decreased

[42]. Stability of β -catenin is regulated by proteasome-mediated degradation. Phosphorylation of serine and threonine residues in the N-terminal region of β -catenin mediates binding to the E3 ligase β -TrCP which ubiquitinates β -catenin, targeting the protein for degradation. We found that while obligate phosphorylation of serines in the β -catenin destruction motif (DSGIHS) was unchanged by pH, a deprotonated His36 increases β -catenin binding to β -TrCP, promoting the ubiquitination and degradation of β -catenin at high pH (Fig. 6). The pH-sensitive regulation of β -catenin functions in coincidence with obligate phosphorylation events to regulate β -catenin levels in cells [42]. We found that a recurrent mutation in β -catenin (H36R) abrogates pH-sensitive binding

to β -TrCP, stabilizing β -catenin and producing differentiated, ectopic tumors in the fly eye [42]. This is the first demonstration that a somatic cancer mutation can disrupt dynamic pH-sensing function of the wild-type protein. Because wild-type β -catenin is destabilized at higher pH, the β -catenin-H36R mutation provides a way for the cancer cells to activate and stabilize β -catenin for signaling or adhesion purposes at high pH.

6 Potential pH sensing by networks of ionizable residues

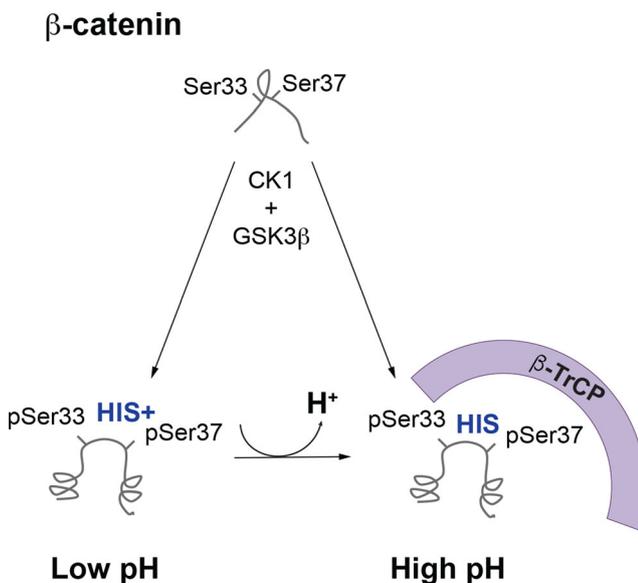


Fig. 6 pH sensing mechanism of β -catenin. β -Catenin is pH sensitive, with decreased stability at increased pH [42]. β -Catenin stability is regulated by obligatory phosphorylation by the kinases CK1 and GSK3 β . Phosphorylated β -catenin is recognized by the E3 ligase β -TrCP, ubiquitinated, and targeted for proteasome-mediated degradation. Binding of wild-type β -catenin to β -TrCP is pH sensitive, with increased binding at high pH, and is mediated by an evolutionarily conserved histidine residue (His36 in human β -catenin)

While previous works identifying pH-sensitive proteins and mutants have predominantly focused on histidine switches, depending on the protein environment, some charged residues can have pK_a s that are up- or down-shifted, enabling titration within the cellular pH range. Experimental work has confirmed that glutamates and aspartates can have pK_a s that are significantly up-shifted into the physiological range [43, 44] and buried lysine residues can have a significantly down-shifted pK_a . In several instances, series of glutamate residues (pK_a 4.5) have been shown to be critical for mediating pH-sensitive protein functions, either alone [45] or in cooperation with histidine residues [26, 46]. Because of the effects of upshifted pK_a s, Glu>Lys mutations, which are enriched in melanoma and myeloma as well as cervical, bladder, head and neck, and breast cancers [24] (Fig. 1a), may in some instances function similar to His>Arg mutations to confer a loss of pH sensing.

One potential example of this effect is the recurrent PIK3CA-E545K somatic mutation. PIK3CA is the p110 α regulatory subunit of phosphatidylinositol 3 kinase (PI3K), which phosphorylates PI(4,5)P2 in the plasma membrane to generate PI(3,4,5)P3 and activate downstream signaling to promote cell growth and transformation. PIK3CA is mutated in $\sim 27\%$ of breast cancers with almost half being E545K [47]. Wild-type PI3K was

shown to be pH-sensitive using *in vitro* assays with increased activity at lower pH, but a mechanism of pH sensing has not been reported. Glu545 is located in the p110 α subunit of PI3K at the interface with the inhibitory nSH2 domain of the p85 α subunit (Fig. 7). The E545K and E542K mutations are predicted to relieve autoinhibition by nSH2 and allow signaling in the absence of phosphotyrosine binding [48, 49]. PIP2 binding by E545K is not different than wild type [48], which suggests that this mutation does not confer constitutive membrane association, a known mechanism for the highly recurrent PIK3CA-H1047R mutation [50]. In wild-type PI3K, Glu545 is in a network of charged residues at the protein–protein interaction interface, ten in p110 α and three in p85 α (Fig. 5b). Three residues in this network have significantly upshifted pK_as in predictive PropKa software: Glu542, pK_a 5.80; Glu579, pK_a 6.99; and Glu503, pK_a 6.30. Moreover, charge reversals on p85 α (K379E) at the interface with p110 α -E545K abrogate transformation with E545K [51], and activity and oncogenicity of E545K were shown to be independent of p85 α [52]. We predict that pH sensing by wild-type PI3K is conferred through this network of residues at the p110 α /p85 α interface, and that this is disrupted by E545K, resulting in loss of pH sensing and high PI3K activity at the higher pH_i of cancer cells. The resulting effect, similar to that observed with β -catenin-H36R, would be to stabilize and enhance PI3K signaling effects of the protein at the high pH_i of cancer cells, where the wild-type protein has decreased activity.

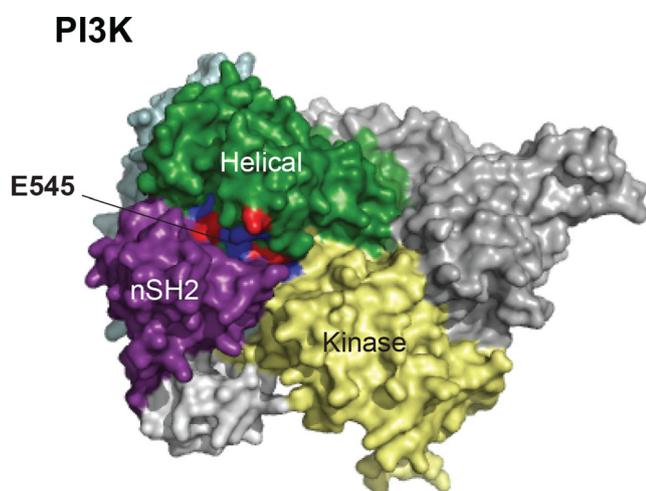


Fig. 7 Network of ionizable residues at the helical/nSH2 interface of PI3K. PI3K-E545K is a highly recurrent mutation in cancer. PI3K is a multisubunit protein whose activity is predominantly regulated by protein–protein interactions between the helical domain of the catalytic p110 α subunit (green) and regulatory subunit of p85 α (purple). Glu545 is located at the interaction interface in a cleft that is rich in charged amino acids. Negatively charged residues (Glu and Asp) in red, positively charged residues (Lys and Arg) in blue

7 Discussion

This review highlights a new idea on charge-changing somatic mutations relative to the established higher pH_i of cancer. We describe a confirmed gain in pH sensing that at higher pH_i increases oncogenic activity of EGFR-R776H and decreases tumor suppressor activity of p53-R273H [24]. Also confirmed is a loss of pH sensing by β -catenin-H36R [42]. We also propose molecular mechanisms for possible changes in pH sensing conferred by additional Arg>His as well as a recurrent Glu>Lys mutation. With more experimental evidence for a role for pH_i dynamics in regulating the function of proteins with charge-changing mutations, we anticipate these predictions will be tested.

Moreover, the concept of charge-changing mutations being sensitive to changes in protonation state induced by pH_i dynamics is broadly applicable beyond cancer. Examples for non-cancer effects of Arg>His somatic mutations in other diseases include an Arg206His mutation in the activin receptor 1 that causes fibrodysplasia ossificans progressive [53, 54] and occurs in pontine gliomas [55], and an Arg290His mutation in the neuronal guanine nucleotide exchange factor collybistin that impairs membrane phospholipid binding and causes epilepsy and intellectual disability [56].

Somatic mutations in cancer are generally recurrent only if the mutant protein confers a fitness advantage to the cancer cell, allowing it to proliferate and outcompete less fit clones. This positive selection is mediated by distinct selective pressures of both the tumor microenvironment and tumor physiology. Standard of care for many solid cancers now includes biopsy and sequencing analyses. Unfortunately, much of the collected sequencing data is not used because we lack either the appropriate drugs to target certain mutations or we lack experimental understanding of how selective pressures shape the mutational landscape of cancer. Our work on a handful of charge-changing mutations has demonstrated that investigating the dynamic functions of somatic mutations might be an important avenue for connecting mutations and selective pressures. Future work strengthening the link between distinct selective pressures and the retention of somatic mutations could be transformative for how we diagnose, stage, and treat cancer.

Another future direction is to explore further the classification of cancer subtypes based on amino acid mutation signatures proposed by Szpiech and colleagues [19]. Using principal component analysis to score for genetic distance and relatedness between cancer subtypes, they identified cancers sharing high arginine to histidine substitutions, including medulloblastoma and acute myeloid leukemia, and those sharing high glutamic acid to lysine substitutions, including melanoma, cervix, and bladder. Further investigating relatedness and physiological links between these seemingly disparate cancer subtypes, which is not identified in previous

classification analyses, could reveal new insights toward our understanding of the retention of recurring somatic mutations as well as cancer evolution and heterogeneity.

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