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Diverse Functional Roles of Reactive Cysteines

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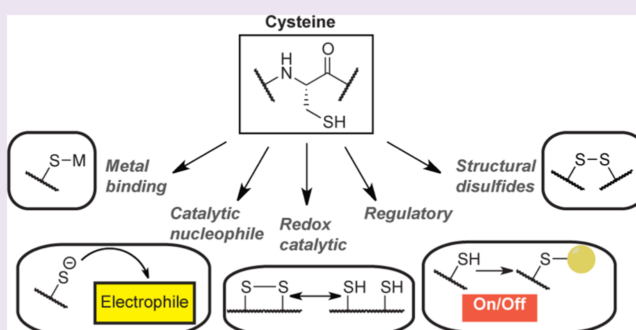
ABSTRACT: Cysteine residues on proteins play key roles in catalysis and regulation. These functional cysteines serve as active sites for nucleophilic and redox catalysis, sites of allosteric regulation, and metal-binding ligands on proteins from diverse classes including proteases, kinases, metabolic enzymes, and transcription factors. In this review, we focus on a few select examples that serve to highlight the multiple functions performed by cysteines, with an emphasis on cysteine-mediated protein activities implicated in cancer. The enhanced reactivity of functional cysteines renders them susceptible to modification by electrophilic species. Toward this end, we discuss recent advancements and future prospects for utilizing cysteine-reactive small molecules as drugs and imaging agents for the treatment and diagnosis of cancer.



Cysteine is one of the least abundant amino acids incorporated into proteins in many organisms (1.9% abundance); however, it is found to concentrate at functionally important locations within protein scaffolds.[1,2] Although a late evolutionary addition to the genetic code, cysteine has accrued with higher frequency, alluding to the preferential incorporation of cysteine residues at functional loci.[3] Furthermore, quantifying the frequency of amino acid mutations in genetic disease showed that cysteine contributes to these significantly more than other amino acids.[4] This functional importance is derived from a number of physicochemical properties that are unique to cysteine. The thiol group of cysteine, due to the large atomic radius of sulfur and the low dissociation energy of the S−H bond, renders the ability to perform both nucleophilic and redox-active functions that are unfeasible for the other natural amino acids. The pK_a of the thiol group in cysteine is close to physiological pH,[5] and the ionization state of cysteine is therefore highly sensitive to minute changes in the surrounding protein environment.[6] The thiol ionization state governs cysteine nucleophilicity and redox susceptibility, thereby facilitating the unique functions of cysteine: nucleophilic and redox catalysis, allosteric regulation, metal binding, and structural stabilization, on proteins drawn from diverse functional classes (Figure 1A).[7]

Cysteine residues are highly susceptible to a myriad of posttranslational modifications (PTMs) that serve to regulate protein activity *in vivo*. These PTMs include oxidation,[8−10] S-nitrosylation,[11] palmitoylation,[12] prenylation,[13] and Michael additions to oxidized lipids such as 4-hydroxy-2-nonenal (HNE).[14] These diverse cysteine PTMs have been extensively described recently and will not be the subject of this review. Here, we will focus on methods to identify functional cysteines in proteomes and the diverse roles fulfilled by these residues in catalysis and regulation.

METHODS TO IDENTIFY FUNCTIONAL CYSTEINES

Despite the frequency and prevalence of cysteines at functional loci, there are no consensus motifs to identify functional cysteines in proteomes; therefore, developing computational and experimental platforms to predict functional cysteines is an ongoing area of focus. Functional cysteines are often subject to the numerous posttranslational modifications described above. Thus, proteomic methods that identify cysteines susceptible to each of these modifications facilitates the identification of uncharacterized functional residues.[15] These methods include OxiCAT to quantify reversible oxidation,[16] the biotin-switch method to identify S-nitrosylated proteins,[17] derivatives of dimedone to enrich and identify sulfenic acids,[10,18,19] and alkyne-functionalized HNE and palmitic acid derivatives for lipid-modified cysteines.[20−22] Recent reviews provide a thorough overview of the numerous proteomic methods that have been developed to study these modifications.[15,23]

Another approach to identify functional cysteines has relied on the correlation between function and reduced pK_a. Even though the average pK_a of a surface-exposed thiol is ∼8.5, this value can be highly perturbed in enzyme active sites, resulting in pK_a values as low as 2.5 for some catalytic cysteines, such as the active site residues of cysteine proteases and protein tyrosine phosphatases.[6,24,25] Many computational methods aim to annotate functional cysteines using pK_a prediction algorithms to identify ionizable cysteine residues based on characteristics of the protein microenvironment that stabilize the thiolate anion. These and other computational methods to predict functional cysteines have been recently reviewed.[2,26−28] The perturbed pK_a of functional cysteines generally renders the cysteine more nucleophilic, and this increased "reactivity" forms

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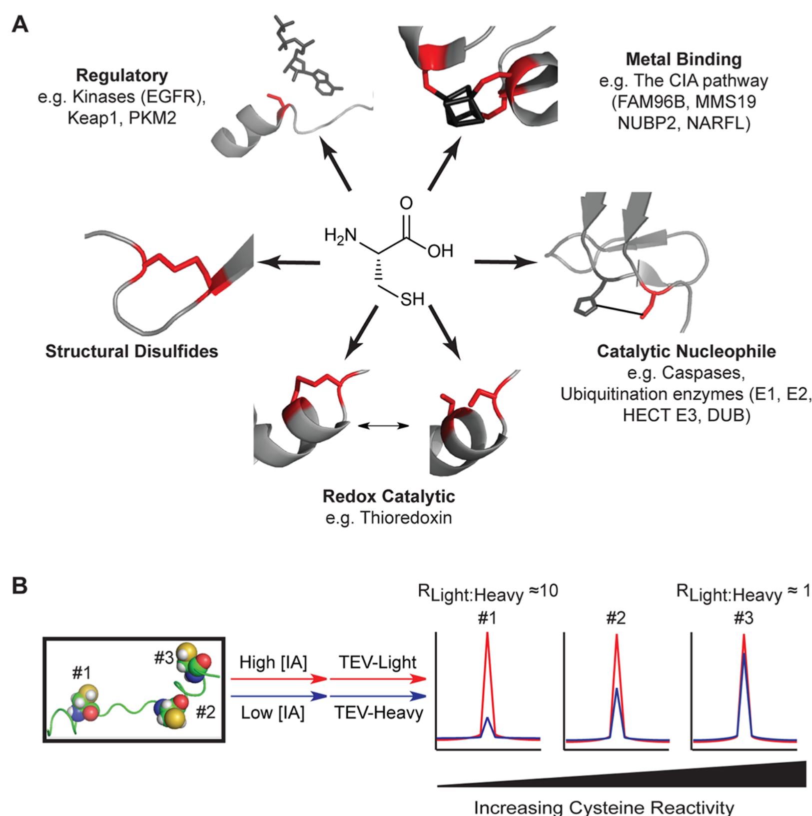

Figure 1. Functional roles of reactive cysteines and proteomic methods to quantify cysteine reactivity. (A) The known functional roles of cysteines: catalytic nucleophiles, redox-catalytic, metal-binding, regulatory, and structural disulfides. The proteins and protein families that will be discussed in this review are shown below each functional class. These select examples serve to highlight the diverse functional roles of reactive cysteines in cancer. (B) The isoTOP-ABPP platform allows for the quantification of cysteine reactivity within a complex proteome. Cysteine labeling by an alkyne-functionalized iodoacetamide probe (IA) was monitored in a concentration (or time)-dependent manner using isotopically tagged linkers to quantify the relative abundance of labeled peptides. Cysteines considered "hyperreactive" demonstrate complete IA labeling at low concentrations (or short time points), whereas less reactive cysteines display a concentration (or time)-dependent increase in IA labeling. The isoTOP-ABPP ratio, *R*, reflects the difference in signal intensity between the light- and heavy-tagged proteomes, whereby low *R* values indicate highly reactive cysteine residues. Figure adapted from Marino and Gladyshev.[134]

the basis of experimental approaches to identify functional cysteines. Toward this end, thiol alkylating agents such as biotinylated *N*-ethylmaleimide (NEM) and iodoacetamide (IA) have been used to enrich and identify reactive cysteines in complex proteomes.[29−31] These studies were effective in identifying reactive cysteines but were unable to quantitatively assess the relationship between reactivity and functionality. To fully interrogate this relationship, a quantitative proteomics platform (isotopic tandem orthogonal proteolysis-activity-based protein profiling; isoTOP-ABPP) was recently developed to evaluate the reactivity of cysteines.[32] In this study, labeling of cysteines with an alkyne-functionalized IA probe was monitored in a concentration-dependent (or time-dependent) manner. Click chemistry-based incorporation of an isotopically tagged (light or heavy) biotinylated linker[32,33] facilitated the enrichment and quantitative assessment of relative IA-labeling from samples treated at high and low IA concentrations (or short and long time points) (Figure 1B). A subset of cysteines, categorized as "hyperreactive", demonstrated complete IA-labeling at low concentrations (or short time points), whereas less reactive cysteines displayed concentration (or time)-dependent increases in IA-labeling. An isoTOP-ABPP ratio, *R*, is generated for each identified cysteine that reflects the difference in signal intensity between light and heavy tag-conjugated proteomes and hence provides a quantitative measure of cysteine reactivity. A lower isoTOP-ABPP ratio signifies a more reactive cysteine. This isoTOP-ABPP strategy was used to rank ∼1000 cysteines in a variety of cancer-cell proteomes by order of their reactivity.[32] This study demonstrated that the subset of hyperreactive cysteines was highly enriched in functional cysteines that play critical roles in nucleophilic and redox catalysis and allosteric regulation, thereby confirming the correlation between reactivity and functionality. However, not all functional cysteines were identified as hyperreactive. Interestingly, numerous active-site nucleophiles, such as those on several cysteine proteases and ubiquitin conjugating enzymes, demonstrated moderate reactivity, which could be reflective of the high degree of substrate selectivity inherent in the mechanisms of these enzymes. Therefore, hyperreactivity is a good predictor but is not necessarily a defining feature of functionality.

In this review, we will focus on a small subset of the cysteines identified in the isoTOP-ABPP studies, in order to exemplify the diverse functional roles accomplished by reactive cysteine residues (Figure 1A). These diverse roles include redox and nucleophilic catalysis, metal binding, and allosteric regulation. Although cysteine residues play an important role in structure stabilization through disulfide bonds,[34,35] this function will not be discussed here because the majority of cysteines participating in structural disulfides are generally static and

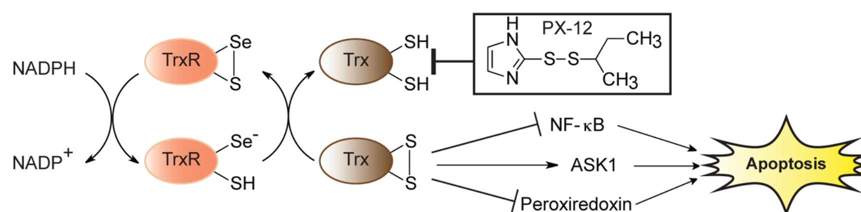

Figure 2. Thioredoxin (Trx) system. Trx relies on two conserved redox-catalytic cysteines (C32/C35) that reduce disulfide bonds within protein substrates but are consequently oxidized to form an intramolecular disulfide. TrxR shuttles reducing equivalents from NADPH to Trx to recycle the enzyme back to its active, reduced form. This system acts on numerous substrates essential to inducing apoptosis; therefore, Trx is an attractive drug target in cancer. PX-12 (inset) is a cysteine-reactive covalent inhibitor of Trx that has recently passed phase II clinical trials and is awaiting further development.

unreactive. We focus instead on reactive cysteines involved in catalysis and regulation with select examples of proteins from diverse functional classes, including oxidoreductases, proteases, kinases, transcription factors, and metabolic enzymes that are known players in cancer pathogenesis. These proteins share a highly reactive cysteine residue; therefore, targeting these functional cysteines with cysteine-reactive small molecules provides a common strategy to modulate the activity of these diverse proteins. Toward that end, we will discuss recent advances in the application of cysteine-reactive small molecules as diagnostic and therapeutic tools for cancer.

■ FUNCTIONAL ROLES OF CYSTEINE IN CANCER

Redox Catalysis. The majority of hyperreactive cysteines identified in the isoTOP-ABPP analysis belong to members of the thiol oxidoreductase family that utilize redox-active cysteine residues to catalyze thiol/disulfide exchange reactions.[36] These proteins include isoforms of thioredoxin, glutaredoxin, peroxiredoxin, and protein disulfide isomerase and envelop numerous redox functions such as substrate oxidation/reduction, disulfide bond isomerization, and detoxification of reactive oxygen species. Many of these proteins contain a conserved CxxC motif,[37] and approximately half of the members contain thioredoxin folds.[38] One of the most successful strategies for predicting redox-catalytic cysteines has been bioinformatic methods that search for cysteine/selenocysteine (Cys/Sec) pairs in homologous sequences.[28] This method relies on the observation that Sec is predominately located in the active sites of redox proteins; therefore, mining for Cys-containing homologues can reveal novel redox-catalytic cysteines. Here, we will focus on the function of thioredoxin, a prototypical member of the thiol oxidoreductase family.

Thioredoxin. The thioredoxin system is composed of thioredoxin (Trx) together with thioredoxin reductase (TrxR) and NADPH[39] and constitutes one of the major cellular redox-control systems. Trx1 and TrxR1 constitute the cytoplasmic system, whereas Trx2 and TrxR2 are localized in the mitochondria. The active site of human Trx contains two cysteine residues at positions 32 and 35, which act as the center for redox catalysis. Upon reduction of a disulfide bond in a substrate protein, the two redox-active cysteines in Trx undergo reversible oxidation to form an intramolecular disulfide bond. This oxidized Trx protein is then recycled to the reduced state through the concomitant action of TrxR and NADPH (Figure 2). TrxR is a selenoprotein, which utilizes a Cys/Sec sequence in the active site to shuttle reducing equivalents from NADPH to Trx.[40] The thioredoxin system was originally discovered as the essential reducing mechanism for the regeneration of ribonucleotide reductase (RNR) activity, but since then the functions of Trx have expanded to numerous other cellular pathways. Among the multitude of functions attributed to the Trx/TrxR system are defense against oxidative stress, scavenging of reactive oxygen species, and regulation of redox signaling by agents such as hydrogen peroxide and nitric oxide.[41,42]

Most cancer cells have a high level of expression of Trx/TrxR;[43−45] therefore, this system is thought to play numerous roles in promoting cancer pathogenesis, including the inhibition of apoptosis, the promotion of cell growth and resistance to chemotherapy.[45−47] These oncogenic roles of Trx are mediated through numerous mechanisms. First, increased Trx activity is essential for sustained RNR activity, a critical player for DNA synthesis that is key to cell proliferation.[48] Furthermore, Trx is crucial for maintaining cellular redox balance in cancer cells, and this is achieved both directly and indirectly via the regulation of other reductases including peroxiredoxins and methionine sulfoxide reductases.[49,50] Trx is also implicated in transcriptional regulation, specifically by reducing a key cysteine residue that is essential for DNA binding in the NF-κB transcription factor.[51] Interestingly, the Trx system inhibits apoptosis through direct interactions with apoptotic signaling pathways. Key to these Trx-mediated effects on apoptosis is the interaction with the apoptosis signal regulating kinase 1 (ASK1), a member of the MAPKKK family. The TRX-ASK1 interaction sequesters ASK1, inhibits kinase activity, and therefore prevents ASK1 from contributing to signaling cascades essential to apoptosis.[52] Furthermore, inhibiting the Trx-ASK1 interaction results in release of ASK1 that then proceeds to form a complex with the TNF receptor associated factor (TRAF) to trigger apoptosis.[53] More recently, it was demonstrated that oxidation of a cysteine residue (Cys250) in ASK1 is essential for the induction of apoptosis, and Trx inhibits this process by reducing the oxidized form of ASK1.[54,55] In addition to regulation of ASK1, Trx was found to catalyze the transnitrosylation of Cys163 of procaspase 3.[56] Nitrosylation of this active-site caspase residue inhibits protease activity, resulting in the deceleration of apoptosis.[56,57] Inhibition of Trx therefore stimulates apoptosis through numerous mechanisms, and targeting the reactive redox-catalytic cysteines of Trx with covalent inhibitors could provide a potential therapeutic strategy to target cancer.[58]

Toward that end, many inhibitors of Trx have been developed in recent years. One promising candidate is the compound 1-methyl-propyl-2-imidazolyl disulfide (IV-2), better known as PX-12 (Figure 2).[59,60] PX-12 is a covalent inhibitor that acts by binding to a cysteine proximal to the active site (Cys73) of Trx1.[59] PX-12-modified Trx is no longer

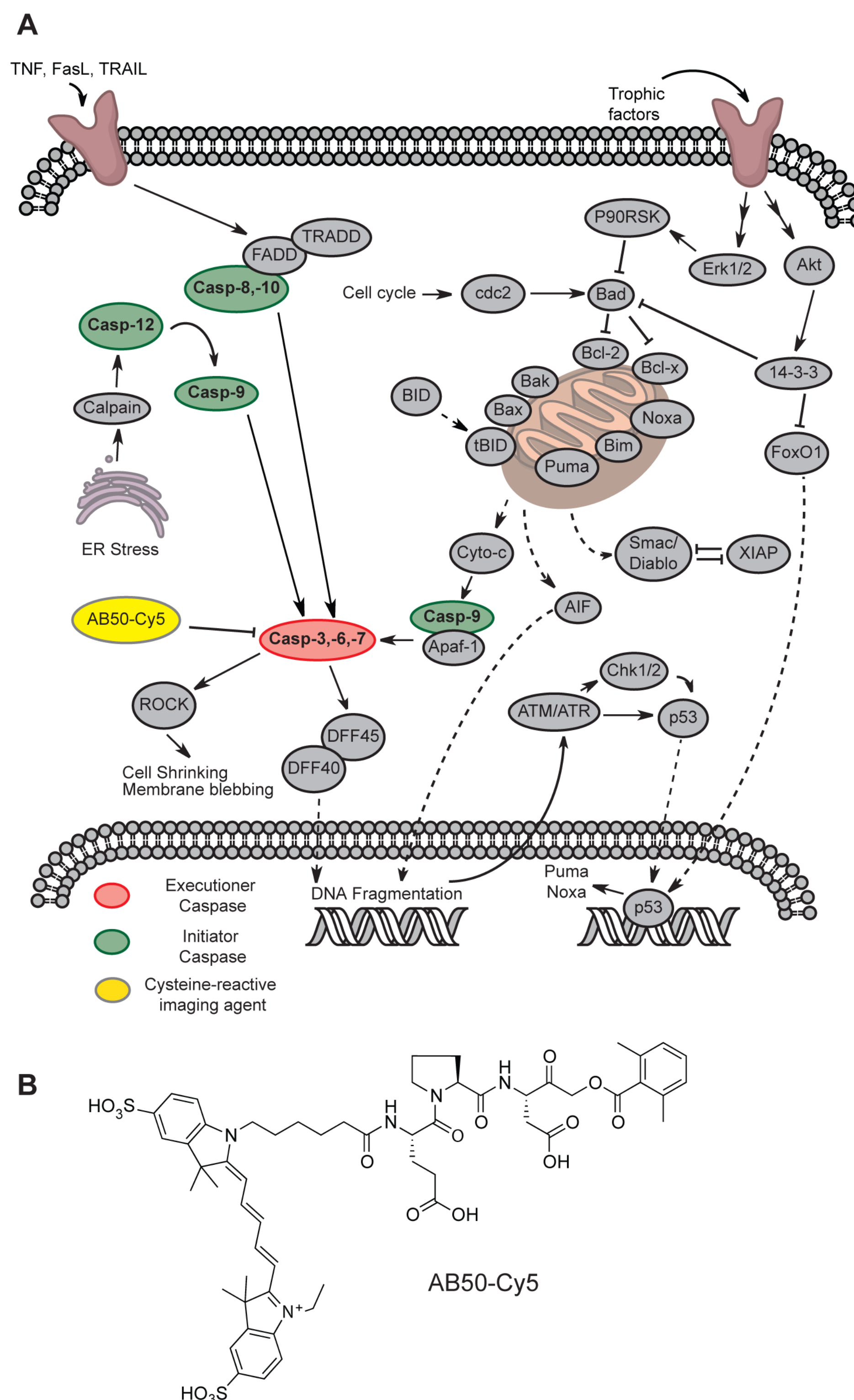

Figure 3. Cysteine proteases in apoptosis. (A) Extrinsic and intrinsic cellular apoptotic signaling pathways rely on proteolytic events catalyzed by caspases. Both initiator caspases (Casp-2,9,8,10; in green) and executioner caspases (Casp-3,6,7; in red) function through conserved catalytic dyads consisting of a nucleophilic cysteine and an adjacent histidine. Figure adapted from Cell Signaling Technology, Inc. (www.cellsignal.com). (B) AB50-Cy5 is a cysteine-reactive probe for imaging apoptosis *in vivo*.

regenerated to its active state by TrxR, thereby leading to suppression of the entire thioredoxin system resulting in the induction of apoptosis. This compound went through phase II clinical trials but is awaiting further development beyond this stage.[60,61] PX-12 was shown to be a potent inducer of apoptosis in HL-60 cells, and patients treated with PX-12 demonstrated decreased expression of VEGF, an essential mediator of angiogenesis and cancer metastasis.[62] The development and clinical evaluation of PX-12 holds promise for inhibitors directed at Trx and furthermore supports the future exploration

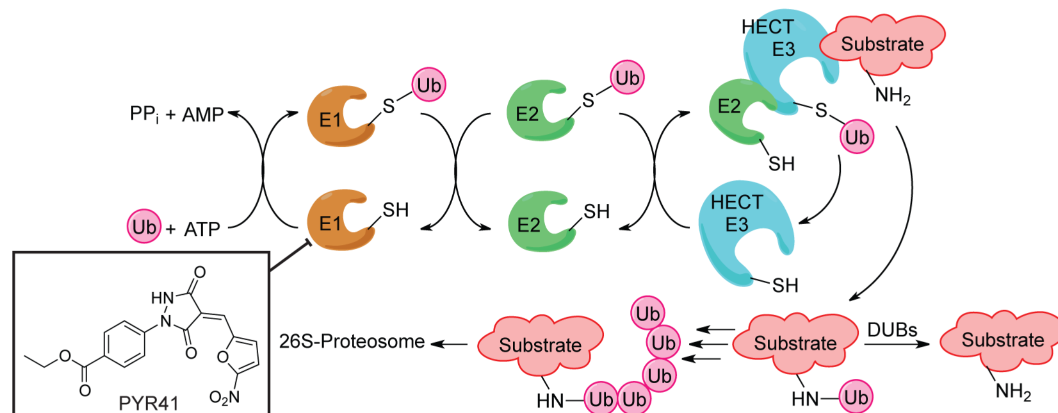

Figure 4. Ubiquitination pathway. Ubiquitin-mediated protein degradation relies on a series of ubiquitin ligases (E1s, E2s, and HECT E3s) and deubiquitinating enzymes (DUBs) that possess nucleophilic cysteine residues to catalyze the transfer and removal of ubiquitin to and from their corresponding substrates. Inset shows the structure of PYR41, a cysteine-reactive covalent inhibitor of the ubiquitin-activating E1 enzyme.

of covalent inhibitors targeting the redox catalytic cysteines of other thiol oxidoreductases for cancer therapy.

Catalytic Nucleophiles. Cysteine residues that serve the role of catalytic nucleophiles do not undergo a change in oxidation state during the catalytic cycle. Most of these cysteine residues tend to be highly conserved across related species and are found on proteins from diverse enzyme classes. Examples include the active-site cysteine residues of cysteine proteases (e.g., caspases and cysteine cathepsins),[63] ubiquitin ligases and hydrolases,[64] phosphatases (e.g., protein tyrosine phosphatase 1B),[65] metabolic enzymes (e.g., glyceraldehyde-3-phosphate dehydrogenase),[66] and protein arginine deiminases.[67] Many, if not all, of these proteins play known roles in cancer pathogenesis, but here we focus on the caspase family of cysteine proteases, as well as enzymes involved in ubiquitin-mediated protein degradation.

Caspases. Cysteine proteases catalyze the degradation of amide bonds within polypeptide chains. These enzymatic reactions typically rely on the presence of a thiolate anion at the active site resulting from ion-pair formation between a cysteine and most commonly a neighboring histidine to form a catalytic dyad. All cysteine proteases share a common mechanism involving nucleophilic attack of the thiolate on the carbonyl-carbon of an amide bond, followed by hydrolytic cleavage of the resulting thioester acyl-enzyme intermediate to regenerate the free enzyme.[63] Cysteine proteases encompass two classes including the interleukin 1b converting enzyme (ICE) class and the papain superfamily.[63] Here we will focus on the caspases, which are members of the ICE class of cysteine proteases.

Caspases rely on a highly conserved QACRG sequence and a typical cysteine−histidine dyad for catalysis. In order to moderate their activity, caspases are synthesized as an inactive zymogen but form a constitutively active heterotetramer upon proteolytic cleavage into a large and small subunit. Currently 11 caspases have been identified in humans and are classified by their function: cytokine activators (Casp-1,4,5), apoptosis initiator caspases (Casp-2,9,8,10), apoptosis executioner caspases (Casp-3,6,7), and Casp-14, which is involved in keratinocyte differentiation.[68] Here we will focus on the initiator and executioner caspases and their role in the regulation of apoptosis.

Almost 40 years ago, apoptosis was discovered to be an essential defense mechanism against tumorigenesis,[69] and the capacity to evade apoptosis has been defined as a hallmark of cancer.[70] Dysregulation of caspase activity plays a key role in evading apoptosis, as cells lacking active caspases subsist and become predisposed to tumorigenesis,[71] and mutations to all the apoptosis-regulating caspases have been documented in diverse tissue carcinomas with varying frequencies.[72] Both an extrinsic receptor-mediated pathway and an intrinsic mitochondrial pathway are central mechanisms that contribute to caspase-dependent apoptosis (Figure 3A).[68,73] The initiator caspases are responsible for converting external and internal apoptotic signals into proteolytic activity. These initiator caspases form multicomponent complexes, such as the death-inducing signaling complex (DISC) or the apoptosome, to induce their autoactivation and subsequent procession through signaling cascades that result in the cleavage of the executioner caspases.[74] Upon proteolytic activation by initiator caspases, executioner caspases then cleave a broad range of protein substrates. These include the activation of proteins such as caspase activated DNase (CAD)[75] that leads to nuclear DNA fragmentation and ultimately cell death. The entire apoptotic cascade is tightly regulated by the expression of these caspases as inactive zymogens and by endogenous inhibitors such as the inhibitor of apoptosis (IAP) proteins.[76] Furthermore, the reactive cysteines on the caspases are subject to oxidation by reactive oxygen species, resulting in the inhibition of activity and the suppression of apoptosis during tumorigenesis.[56,77]

The reactive-cysteine nucleophile in the caspases has been exploited in the development of imaging agents for tumor diagnostics. Monitoring the level of apoptosis in cancerous tissues would allow for better patient management and facilitates the rapid assessment of anticancer drugs, the majority of which function by inducing apoptosis.[78] Caspase-targeted cysteine-reactive peptides constitute an important subclass of existing imaging agents for visualizing cell death. One notable example is AB50-Cy5 (Figure 3B), which is an activity-based probe (ABP) with high specificity toward caspase-3. This ABP contains an acyloxymethylketone (AOMK) electrophile to covalently react with the catalytic cysteine, a peptide-scaffold to direct probe reactivity toward caspase active sites, and a Cy5 fluorophore for imaging.[79] This cysteine-reactive peptide was evaluated in a mouse xenograft model, whereby the monoclonal antibody Apomab was used to induce apoptosis and activate caspases. The AB50-Cy5 probe specifically labeled the Apomab-treated tumors, illustrating its selectivity for apoptotic cell populations.[79] Similarly, other cysteine-reactive covalent

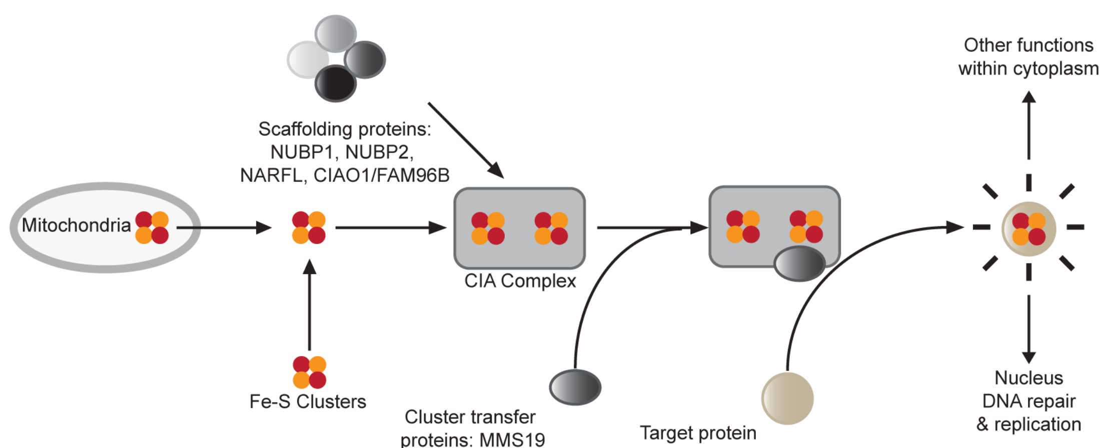

Figure 5. Cytosolic iron−sulfur cluster biogenesis (CIA pathway). The CIA pathway facilitates the maturation of Fe−S clusters that are essential cofactors within hundreds of cellular proteins. Scaffolding proteins come together and bind Fe−S clusters produced in the cytosol or mitochondria, to form the CIA complex. Once the CIA complex is properly assembled, cluster transfer proteins are able to bind to the complex and assist in the transfer of the Fe−S clusters to their corresponding protein targets.

inhibitors of caspases such as an ^{18}F-labeled isatin derivative,[80] and a fluorescent peptide fluoromethyl ketone,[81] have been adapted for imaging and diagnostic applications. These studies illustrate the utility of targeting active-site cysteine nucleophiles in cancer-relevant proteins for diagnostics.

Ubiquitinating/Deubiquitinating Enzymes. The ubiquitin-mediated protein degradation system governs many cellular processes. This system consists of the conserved 76-amino-acid protein ubiquitin, a series of ubiquitin ligases (E1, E2, E3), deubiquitinases (DUBs), and the 26S-proteosome (Figure 4). The ubiquitin ligases function to conjugate the C-terminus of ubiquitin to the ε-amino group of lysine residues on target proteins. Substrates tagged with a polyubiquitin chain are directed to the proteosome for degradation. Ubiquitination thereby modulates numerous cellular process and is implicated in the regulation of cell proliferation and apoptosis.[82,83] Many of the enzymes in the ubiquitin-mediated proteolysis pathway rely on functional cysteine residues that act as catalytic nucleophiles. Here, we will highlight the pervasive role of cysteine in the ubiquitin pathway.

Ubiquitin Ligases. The process of conjugating ubiquitin to protein targets begins with the ubiquitin-activating enzyme (E1). A molecule of ubiquitin is first coupled to E1 through an ATP-dependent coupling reaction. MgATP binds first, followed by ubiquitin, resulting in an ubiquitin adenylate intermediate that is susceptible to nucleophilic attack by the proximal catalytic cysteine to generate a thioester.[84,85] After activation, the ubiquitin is transferred to the cysteine nucleophile of an ubiquitin-conjugating enzyme (E2) to form another thioester intermediate.[84] Interestingly, unlike other cysteine nucleophiles, the catalytic cysteine in the E2 active site does not contain a general base within 6 Å of the reactive cysteine necessary to generate the essential nucleophilic thiolate.[86] It is postulated that the binding of E1 or E3 provides the necessary charged residues to facilitate ubiquitin transfer.[84] The organization of this ubiquitin ligation system is hierarchical: a single E1 couples with a limited number of E2s that interacts with a larger subset of E3s specific for a diverse panel of substrates.[84] The E3s are grouped into 4 classes based on common structural and biological features, but only the HECT (Homologue of E6-AP C Terminus) E3s rely on a cysteine nucleophile for its function. The HECT E3s form a complex with both an E2 and a specific substrate, after which the ubiquitin is transferred to the cysteine in the HECT E3 before the subsequent transfer to a lysine side chain on the target protein (Figure 4).[84] Ubiquitination is a tightly regulated process mediated primarily by an intricate network of protein−protein interactions between the E1, E2, and E3 proteins and their substrates.

Deubiquitinases (DUBs). In order to further modulate ubiquitin-mediated protein degradation, cells contain a diverse array of DUBs. Of the 5 known classes of DUBs, 4 are papain-like cysteine proteases and contain a canonical catalytic triad consisting of a nucleophilic cysteine residue adjacent to two histidines.[64] These proteases perform a variety of functions, including the activation of ubiquitin proproteins, the rescue of ubiquitin trapped by endogenous electrophiles, and the removal of ubiquitin modifications from tagged proteins (Figure 4).[87] Since ubiquitin-mediated protein degradation governs many essential cellular functions, the activity of DUBs must be tightly regulated to ensure these processes are carried out definitively. Known mechanisms of regulation include substrate-induced conformational changes, interactions with scaffolding proteins to ensure proper cellular localization, transcriptional regulation, and posttranslational modifications.[87] Like ubiquitination, deubiquitination is essential for numerous cellular functions including cell cycle regulation, gene expression, DNA repair, and modulation of kinase signaling cascades, among others.[87]

Ubiquitin-mediated protein degradation and signaling is critical for maintaining protein homeostasis and controls numerous cellular processes including protein localization, transcriptional regulation, and cell cycle progression. Therefore, many of these enzymes and pathways are dysregulated in cancer. In fact, E3 ligases are second only to protein kinases in an inventory of cancer-related genes[88] and have been shown to act as either tumor suppressors or oncogenes, depending on the specific subset of target proteins. Targeting the ubiquitination pathway with small-molecule inhibitors holds great promise for cancer therapy.[89] In fact, Bortezomib, a boronic acid-based inhibitor of the 26S proteosome, has demonstrated success as a therapeutic for multiple myeloma.[90] Another strategy to target the proteosomal degradation pathway is to develop inhibitors for the ubiquitinating and deubiquitinating enzymes.[91] Exploiting the cysteine nucleophiles in the active sites of E1, E2, HECT E3s, and DUBs is one potential approach toward

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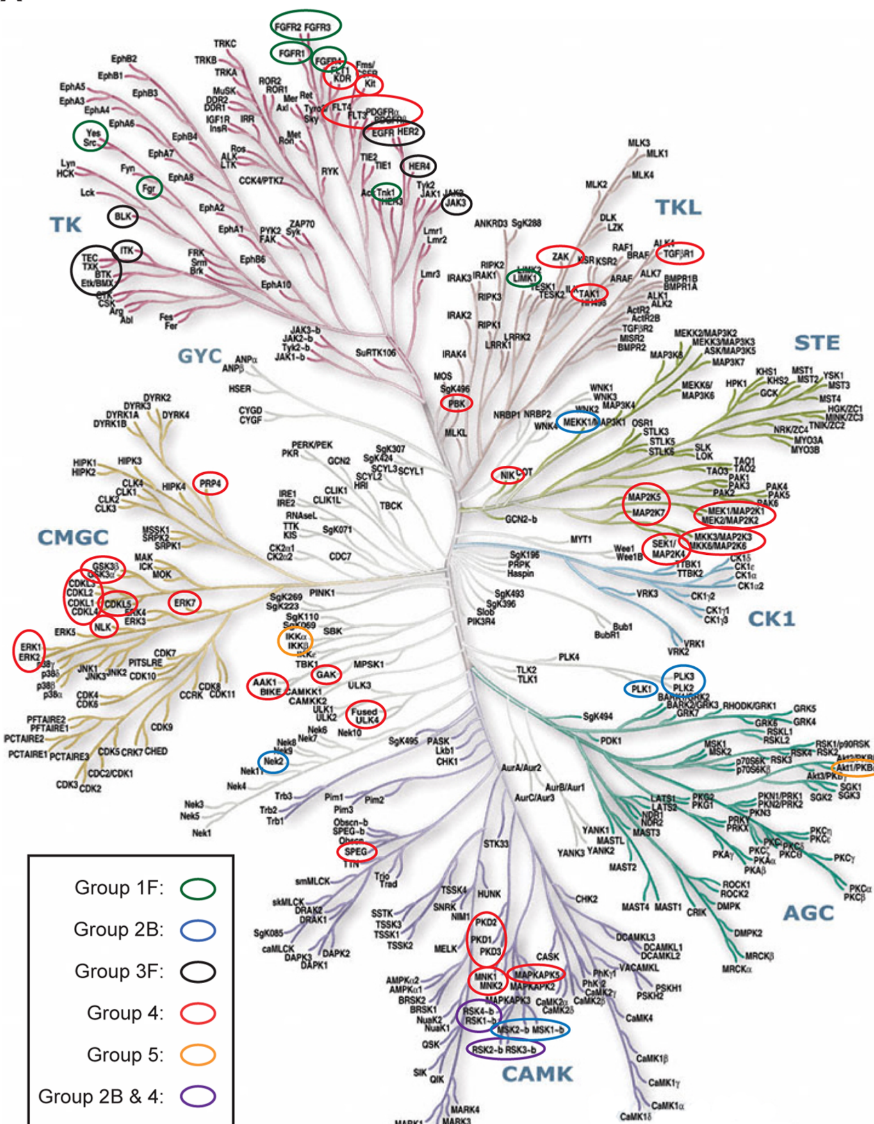

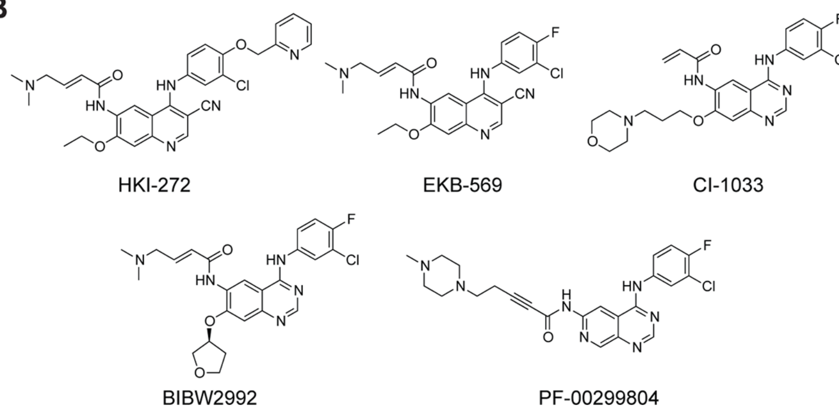

Figure 6. Human protein kinases. (A) Sequencing and structural efforts have revealed cysteine residues within the ATP-binding pocket of approximately 200 protein kinases. These cysteines typically fall into four classes, and subsets of these cysteines have been highlighted within the kinase tree: Group 1B (green), Group 2B (blue), Group 3F (black), Group 4 (red), and Group 5 (orange). RSK-1,2,3,4 contain both Group 2B and Group 4 cysteines and have appropriately been labeled as purple. (B) A reactive regulatory cysteine on EGFR had been exploited in the development of covalent inhibitors, five of which are currently undergoing clinical trials: HKI-272, EKB-569, CI-1033, BIBW2992, and PF-00299804. Kinase tree adapted from Cell Signaling Technology, Inc. (www.cellsignal.com) and Barf and Kaptein.[101]

potent inhibitors for these enzymes. A successful example has been a cysteine-reactive covalent inhibitor of the ubiquitin-activating E1 enzyme.[92] This pyrazone derivative, PYR41 (Figure 4), is shown to be thiol-reactive, although the structure of the resulting covalent adduct is poorly characterized. This compound was shown to stabilize p53 in cells, and a related compound demonstrated anti-leukemic activity in a mouse cancer model.[93] Although these data are still preliminary, it provides promising support for the application of cysteine-reactive small molecules to target other proteins in the ubiquitination pathway.

Metal Binding. A large number of proteins bind metal ions as cofactors that serve diverse functional roles including catalysis and structure stabilization.[94] Cysteine is one of the most common metal-binding residues in proteins, together with histidine, aspartate, and glutamate. The cysteine thiolate ligand binds strongly to a variety of metal ions including $Fe^{2+/3+}$, Zn^{2+}, Cd^{2+}, and Cu^{+}. Due to the multiple oxidation states available to sulfur, cysteine is able to accommodate a large number of bonds and geometries resulting in very diverse metal complexes.[95] These metal-bound cysteines are generally unreactive, since the cysteine is tightly coordinated to the metal, but are often reactive in the unbound (apo)-form of the protein. One family of proteins that contains highly reactive metal-binding cysteines are those in the cytoplasmic iron−sulfur assembly (CIA) pathway. These proteins contain cysteines that transiently bind to iron and serve to preassemble the iron−sulfur (Fe−S) cluster on a scaffold protein prior to transfer to the host. Due to the transient nature of the metal-binding event at these cysteines, they are highly nucleophilic and susceptible to modification by cysteine-reactive electrophiles.

CIA Pathway. Fe−S clusters are critical cofactors found in hundreds of proteins and primarily perform redox catalysis facilitated by the excellent electron donor/acceptor properties of these metal centers. The maturation of Fe−S clusters on cytosolic and nuclear proteins in humans is dependent on the essential CIA machinery (Figure 5), which is composed of four cytosolic proteins, NUBP1, NUBP2, NARFL, and CIAO1 (the corresponding yeast homologues are Nbp35, Cfd1, Nar1, and Cia1).[96,97] Recently, two other proteins, FAM96B[32] and MMS19,[98,99] have been shown to be essential components of this cytosolic Fe−S biogenesis pathway. Although the exact role of each of these proteins in the transfer of Fe−S clusters to client proteins is poorly understood, it is postulated that NUBP1 and NUBP2 form a heterotetrameric complex that serves as a scaffold for the nascent Fe−S cluster. This cluster is then transferred to client apoproteins via NARFL and CIAO1.[96,97,100] More recently, FAM96B was discovered to interact with members of the CIA complex and shown to be essential for Fe−S incorporation into client proteins.[32] Also, MMS19 was shown to be a downstream component in this pathway that is responsible for substrate determination. Loss of MMS19 ablated Fe−S cluster formation in a subset of client proteins, specifically those involved in DNA replication and repair.[98,99] In the isoTOP-ABPP study that identified highly reactive cysteines in proteomes, numerous members of this CIA pathway were found to contain hyperreactive cysteines. These included NUBP2, NARFL, MMS19, and FAM96B, and the hyperreactive cysteine in FAM96B was demonstrated to be essential for function.[32] This suggests that proteins involved in the CIA pathway contain hyperreactive cysteines that serve to transiently bind the newly synthesized Fe−S cluster, en route to the client proteins.

Fe−S clusters are ubiquitous and play critical roles in proteins associated with cancer pathogenesis. In particular, numerous DNA and RNA helicases and DNA and RNA polymerases are essential for DNA replication and repair in cancer and rely on key metal-bound cysteines for function. The recent observation that MMS19 is responsible for assembling Fe−S clusters on only a select subset of protein targets[98,99] suggests the intriguing possibility of inhibiting cancer-associated proteins by targeting components of the CIA pathway. For example, selective targeting of MMS19 with a cysteine-reactive small molecule would exclusively affect DNA replication and repair machinery with dramatic effects on cancer-cell proliferation. The presence of highly reactive and essential cysteines in most members of the CIA machinery lends promise to the idea that cysteine-reactive small molecules could serve as covalent inhibitors of Fe−S assembly on a select subset of protein substrates, thereby acting solely on cellular functions that are key to tumorigenesis.

Regulatory. Regulatory cysteines are not directly involved in catalysis; however, due to their proximity to either the active site or surfaces involved in essential protein−protein interactions, these cysteines are key regulators of protein activity. Modification of these regulatory cysteine residues by either reactive oxygen species or endogenous or exogenous electrophiles results in the modulation of protein activity. Here, we discuss three classes of regulatory cysteines from three diverse protein families: the protein kinases, transcription factors (e.g., Keap1/Nrf2), and metabolic enzymes (e.g., PKM2), where the reactive cysteines are found either in close proximity to the active site (protein kinases) or at protein interfaces (Keap1 and PKM2). Similar to the previous examples, we also highlight how these regulatory cysteines can be exploited by cysteine-reactive small molecules for cancer therapy.

Protein Kinases. Sequencing of the human genome, coupled with detailed structural information, has provided significant insight into structural and functional homology between the 518 human protein kinases. Numerous bioinformatic and inhibitor screening efforts revealed the presence of cysteine residues within the ATP-binding pocket of a large number (∼200) of protein kinases. A recent review classifies the kinases with cysteines within or near the ATP-binding pocket into five groups based on structural location of the cysteine in question.[101] Group 1 kinases contain a cysteine in the glycine-rich or P-loop (e.g., FGFR); group 2 kinases are those with cysteines positioned at the roof of the ATP-binding pocket (e.g., RSK); group 3 kinases present a cysteine in the hinge region and front pocket (e.g., EGFR); group 4 kinases are the most common and contains a cysteine adjacent to the DFG-motif (e.g., ERK2); and group 5 kinases have a cysteine located in the activation loop (e.g., IKKα) (Figure 6A).[102−105] Several of these kinases were identified in the isoTOP-ABPP study,[32] thereby attesting to the reactive nature of these ATP-binding site cysteines.

Numerous kinases containing these reactive cysteines play important roles in cancer.[102] Of particular interest is the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase. EGFR plays a critical role in cell growth and is overexpressed in several types of cancer, including breast, lung, esophageal, and head and neck.[106] Through phosphorylation-mediated signaling cascades, EGFR and its family members

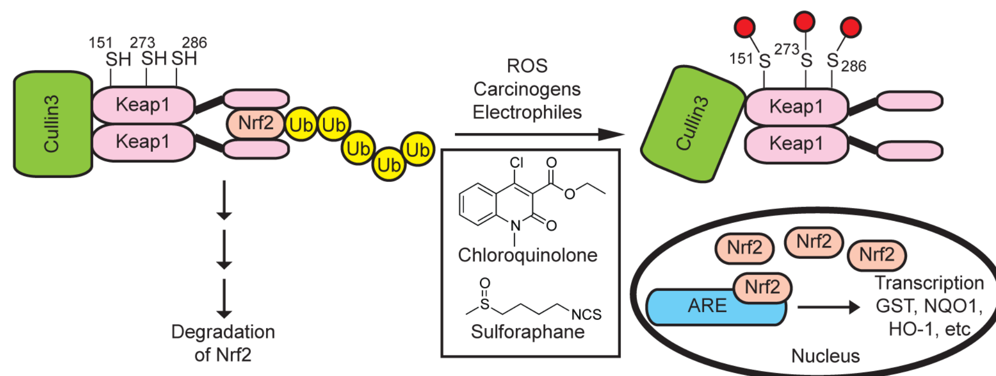

Figure 7. Nrf2/Keap1 system. Keap1 forms a complex with Cul3-dependent E3 ubiquitin ligase. Under basal conditions, Keap1 binds to Nrf2 and sequesters it to the cytosol, allowing for its ubiquitination by Cul3 and subsequent degradation. Under conditions of cellular stress, Keap1 cysteines are modified and lose their ability to bind Nrf2 and restrict it to the cytosol. Upon its translocation to the nucleus, Nrf2 binds ARE and stimulates the transcription of various phase 2 detoxifying enzymes with chemopreventive effects. Inset shows the structure of two electrophilic compounds (a sulforaphane and chloroquinolone) that act as Nrf2 inducers.

modulate growth, signaling, differentiation, adhesion, migration, and survival of cancer cells.[106,107] Recently, it was demonstrated that binding of EGF to EGFR results in the assembly and activation of NADPH oxidase (Nox) complexes, which generate hydrogen peroxide and subsequently modulate signaling cascades through oxidation of specific protein targets.[108] EGFR contains a cysteine close to the hinge region (Cys797), and this cysteine was found to be sulfenylated in EGF-stimulated cells. Oxidation of Cys797 enhances tyrosine kinase activity, thereby exemplifying the role of this cysteine as a regulatory residue that is not directly involved in catalysis yet modulates protein activity.[108] Furthermore, this cysteine has provided a functional handle for the development of covalent EGFR inhibitors, five of which (HKI-272, CI-1033, EKB-569, BIBW2992, and PF-00299804) (Figure 6B) are currently undergoing clinical trials for lung cancer.[109] These inhibitors all incorporate an acrylamide reactive group that undergoes Michael addition with the reactive cysteine to form a covalent adduct.[110]

In addition to EGFR, both RSK and ERK have been targeted by cysteine-reactive small-molecule inhibitors, although these have not been advanced to the stage of clinical trials. Selective RSK inhibitors have been developed via the incorporation of a cysteine-reactive fluoromethyl ketone electrophile into the scaffold of a pan-kinase inhibitor.[104] These compounds have been recently developed into slow dissociating, covalent inhibitors that have the potential to overcome possible toxicity issues related to irreversible inhibition.[111] Numerous natural products of the resorcylic acid lactone family contain a *cis*-enone that has been shown to form a Michael adduct with the reactive cysteine in the ERK family.[103] These studies demonstrate that reactive cysteines located at diverse positions within the ATP-binding pocket of kinases can be exploited in the development of covalent inhibitors. These inhibitors might overcome resistance issues associated with existing kinase inhibitors in the clinic, and toward this end, the covalent EGFR inhibitors have been shown to overcome drug resistance attributed to mutation of the gatekeeper threonine (T790M).[112]

Transcription Factors. The role of cysteine oxidation in regulating the activity of transcription factors was first elucidated with the bacterial transcription factor OxyR. Oxidation of Cys199 in OxyR triggers further oxidation of this cysteine-rich protein, resulting in the fully oxidized and active transcription factor.[113] Since then, numerous human transcription factors, including NF-κB,[51,114] AP-1,[115] p53,[116] and Nrf2,[117] have been shown to be modulated by cysteine oxidation events.[118,119] Of these, we will focus our attention on the NF-E2-related factor (Nrf2), which is regulated by cysteine-modification of the Kelch-like ECH-associated protein 1 (Keap1).

Phase 2 detoxifying enzymes are critical in the elimination and inactivation of carcinogens. These enzymes are regulated by the Nrf2 transcription factor, a basic leucine zipper protein that binds to the antioxidant response element (ARE) to stimulate the transcription of antioxidant and detoxifying genes.[120] Under basal conditions, Nrf2 is sequestered in the cytosol through interaction with Keap1, which acts as an adaptor protein for a Cullin3 (Cul3)-dependent E3 ubiquitin ligase. This interaction drives the ubiquitination of Nrf2 resulting in proteosomal degradation. Keap1 is a cysteine-rich protein, and Cys273 and Cys288 have been shown to be critical for Nrf2 ubiquitination.[121] It is postulated that under oxidative and electrophilic stress, Cys273, Cys288, or Cys151 can get modified, resulting in the disruption of the Keap1-Cul3 complex, which allows Nrf2 translocation to the nucleus where it binds to and activates transcription of ARE genes (Figure 7).[118,122]

The activation of Nrf2 can be exploited as a strategy for chemoprevention, by upregulating target genes to counteract oxidative and electrophilic assault.[123] Toward this end, numerous chemopreventive agents have been developed that function through covalent modification of Keap1 cysteines.[124] Some of these include small molecules identified from natural sources, including flavonoids and phenolic antioxidants such as curcumin and quercetin.[124] Efforts to mimic the action of these natural antioxidants with small molecules such as sulforaphanes[125] and chloroquinolinones[126] (Figure 7) have demonstrated that cysteine-reactive small molecules can be used as a pharmacological strategy to prime cells and build a stronger defense system against carcinogens.

Metabolic Enzymes. Numerous enzymes involved in energy metabolism possess functional cysteines. Key among these is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that contains a cysteine that serves the role of a catalytic nucleophile.[66] Recently, it was discovered that one of the enzymes involved in glycolysis, pyruvate kinase M2 (PKM2), contains a regulatory cysteine that modulates enzyme activity

and, by extension, flux through glycolysis.[127] These two enzymes exemplify the role of functional cysteines in regulating cell metabolism. Here we will describe the role of PKM2 in cancer metabolism and the key regulatory function of a cysteine located at the oligomerization interface of this enzyme.

Cancer cells are subject to increased levels of oxidative stress, and reactive-oxygen species (ROS) detoxification is dependent on the availability of reduced glutathione.[128] The reduced cellular glutathione pool is maintained through NADPH produced in the pentose phosphate pathway (PPP) and flux through the PPP is in turn controlled by the activity of glycolytic enzymes.[129] Cancer cells have been shown to express PKM2, in contrast to many healthy adult tissues that express the splice variant PKM1.[130] Unlike PKM1, PKM2 is allosterically activated by fructose-1,6-bisphosphate (FPB), and this activation is disrupted by phosphotyrosine-containing proteins.[131] Due to this allosteric regulation, PKM2 shows reduced cellular activity, relative to the constitutively active PKM1. Recently, it was found that PKM2 is inhibited by oxidants, and this inhibition is reversible by the addition of DTT. It was determined that a reactive cysteine (Cys358) in PKM2 is oxidized in the presence of cellular stress and results in disruption of the active PKM2 tetramer.[127] Cys358 was identified in the isoTOP-ABPP study as the most reactive cysteine in PKM2.[32] Inhibition of PKM2 via cysteine oxidation slows down glycolytic flux and diverts intermediates into the pentose phosphate pathway resulting in the production of NADPH for ROS detoxification. Cells expressing the cysteine mutant demonstrated lower levels of glutathione to combat oxidative stress, and as a result showed impaired growth in a mouse tumor xenograft model.[127] This exemplifies the role of reactive cysteines in regulating cell metabolism. Unlike the previous examples in which modification of a reactive cysteine would have therapeutic advantages, in the case of PKM2, cysteine modification is an adaptive strategy used by cancer cells to survive under conditions of oxidative stress. In this case, cysteine-reactive small molecules would have unfavorable therapeutic properties, unless they were able to stabilize tetramer formation.

■ SUMMARY AND FUTURE PERSPECTIVE

Recent developments in proteomic methods to identify reactive cysteines and posttranslationally modified cysteines have revealed the ubiquitous role of cysteines in cell biology. Continued development of such methods will aid in identifying novel functional cysteines in both annotated and unannotated enzymes and facilitate the assignment of function to the large portion of the proteome that remains uncharacterized. Identification of a reactive cysteine in the completely unannotated gene product FAM96B facilitated the functional annotation of this protein as a critical component of the cytosolic Fe−S cluster assembly pathway,[32] exemplifying the translation of proteomic studies to gain insight into the activity of uncharacterized proteins. Further advancements in chemical probes, enrichment strategies, and mass spectrometry methods to identify these functional cysteines will facilitate the complete characterization of this intriguing subsection of the proteome.

As described herein, many cysteine-mediated protein activities are implicated in cancer pathogenesis. These proteins provide resistance to oxidative stress, dysregulation of apoptosis, oncogenic signal transduction, and the rewiring of metabolic flux. By focusing on this single amino acid with unique properties, we can gain insight into proteins with diverse functions in numerous essential cellular processes. Targeting these reactive cysteines with electrophilic small molecules provides a potentially transformative strategy for regulating the activity of a plethora of proteins. The development of covalent inhibitors often suffers from fears related to the toxicity associated with nonspecific covalent adducts, but the success of numerous covalent drugs in the clinic suggests that these fears are overstated.[132] Furthermore, emerging proteomic techniques allow for discriminating selective covalent inhibitors from promiscuous inhibitors, further facilitating the development of selective and safe drugs based on irreversible inactivation.[133] The progression of the covalent EGFR inhibitors through clinical trials and FDA approval will lay the groundwork to utilize cysteine-targeted strategies as a generalized drug discovery platform for cancer and other diseases.

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Notes

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■ GLOSSARY

Isotopic tandem orthogonal proteolysis-activity-based protein profiling (isoTOP-ABPP): A quantitative mass-spectrometry technique that facilitates the ranking of cysteine residues in a proteome by order of reactivity with a cysteine-reactive iodoacetamide electrophile. Cysteine residues are characterized by an isoTOP-ABPP ratio, R, which signifies the degree of reactivity. Lower R values denote more reactive cysteines, and a subset of "hyperreactive" cysteines ($R \leq 2$) were identified and shown to be highly enriched in functional residues.; Redox-catalytic cysteines: A cysteine that is essential for catalytic function, and is characterized by a change in oxidation state during the course of the catalytic cycle. Typical of this functional class are cysteine residues in thiol oxidoreductases from the thioredoxin and peroxiredoxin families.; Catalytic-nucleophile cysteines: A cysteine that is essential for catalytic function, where the enzyme mechanism relies on the nucleophilic addition of the thiolate anion to an electrophilic center in the substrate. Numerous enzymes from diverse functional classes include catalytic nucleophiles. Prototypical members of this family include the cysteine proteases and ubiquitinating/deubiquitinating enzymes.; Metal-binding cysteines: A cysteine that coordinates to a metal to form a metal-protein complex. For example, proteins in the cytosolic iron−sulfur cluster assembly pathway contain highly reactive cysteines that transiently bind to iron while serving as a template for the nascent iron−sulfur cluster prior to transfer to the host protein.; Regulatory cysteines: A cysteine that is not essential to the catalytic mechanism, yet modification of this residue by oxidative or electrophilic species results in modulation of protein activity. Proteins with regulatory

cysteines span diverse classes ranging from kinases to transcription factors and metabolic enzymes. These regulatory cysteines lie proximal to the active-site or a protein–protein interface and thereby influence protein function.

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