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Review Article

Redox biology in normal cells and cancer: Restoring function of the redox/Fyn/c-Cbl pathway in cancer cells offers new approaches to cancer treatment

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ABSTRACT

This review discusses a unique discovery path starting with novel findings on redox regulation of precursor cell and signaling pathway function and identification of a new mechanism by which relatively small changes in redox status can control entire signaling networks that regulate self-renewal, differentiation, and survival. The pathway central to this work, the redox/Fyn/c-Cbl (RFC) pathway, converts small increases in oxidative status to pan-activation of the c-Cbl ubiquitin ligase, which controls multiple receptors and other proteins of central importance in precursor cell and cancer cell function. Integration of work on the RFC pathway with attempts to understand how treatment with systemic chemotherapy causes neurological problems led to the discovery that glioblastomas (GBMs) and basal-like breast cancers (BLBCs) inhibit c-Cbl function through altered utilization of the cytoskeletal regulators Cool-1/βpix and Cdc42, respectively. Inhibition of these proteins to restore normal c-Cbl function suppresses cancer cell division, increases sensitivity to chemotherapy, disrupts tumor-initiating cell (TIC) activity in GBMs and BLBCs, controls multiple critical TIC regulators, and also allows targeting of non-TICs. Moreover, these manipulations do not increase chemosensitivity or suppress division of nontransformed cells. Restoration of normal c-Cbl function also allows more effective harnessing of estrogen receptor-α (ERα)-independent activities of tamoxifen to activate the RFC pathway and target ERα-negative cancer cells. Our work thus provides a discovery strategy that reveals mechanisms and therapeutic targets that cannot be deduced by standard genetics analyses, which fail to reveal the metabolic information, isoform shifts, protein activation, protein complexes, and protein degradation critical to our discoveries.

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Introduction

The development of therapies that are more effective at eliminating cancer cells without also destroying the normal cells of the body is the central goal of cancer research. There are numerous strategies employed to address this challenge, such as targeting of mutated proteins that are uniquely expressed by cancer cells, targeting enzymes or transcriptional regulators thought to be of more importance in cancer cells than in nontransformed cells, targeting of tumor-initiating cells (TICs, also known as cancer stem cells), and analysis of gene expression patterns to identify regulatory circuitry that may be unique to cancer cells. All of these strategies are frequently combined with attempts to develop "personalized" therapies targeted at the features of a particular cancer found in a particular patient.

The existing strategies to attack cancers are often stymied by the enormous complexity of these diseases. It is not simply that every patient has a different genetic background, a different starting point for the development of cancer, and a unique constellation of specific genetic changes. At least equally important is that every individual cancer cell has a unique evolutionary future associated with the generation of constantly evolving unique progeny, and studies on genetic microheterogeneity in tumors reveal that cells in the same tumor may differ greatly from each other. Although there may be a reliance of some cancer cells on particular mutated proteins, therapies directed against those proteins generally are associated with escape as a result of new mutations. Overcoming these complexities to attack cancer has proven a very difficult challenge to address.

But what if there were simple rules underlying the complexity of cancer? Would that provide a more effective path to the discovery of new therapies? And if such rules existed, what might they be?

The central premise of much of our work is that one way to discover simple rules of biology is to study the most fundamental aspects of cell regulation and then to integrate these findings with more complex aspects of this regulation. As there is no more fundamental aspect of cellular regulation than redox state (which is the outcome of the balance between agents that donate electrons and those that accept electrons), this is where we begin. Without electron flux, nothing happens in biology. Indeed, without electron flux, there would have been no initiation of even the simplest steps along the road leading to cellular life. While there are also other fundamental regulators that are worthy of equal interest (e.g., nutrient availability), redox state is one of the most fundamental of all cellular regulators.

Thus, we are addressing the challenge of developing improved cancer treatments in a somewhat different manner than many other investigators. First, our work is based on integration of multiple lines of investigation in order to discover disruptions of normal regulatory circuitry that are functionally critical in diverse cancer cell populations with different lineage origins and mutational backgrounds—but that are not shared with nontransformed cells. This work began with what initially were two distinct areas of research, one of which was focused on novel discoveries on molecular mechanisms underlying redox-mediated control of survival of primary cells and division of primary progenitor cells, and the second of which was focused on attempts to understand why normal progenitor cells of the central nervous system (CNS) were extremely sensitive to a variety of anticancer agents while most cancer cells we examined were more resistant. Integration of these lines of investigation has revealed new molecular

mechanisms that appear to be central to modulating the resistance of different types of cancers to anticancer agents. Moreover, these mechanisms appear to be critical in maintenance of TIC function. Unlike strategies that target mutated proteins, however, we are targeting nonmutated proteins that appear to be organized into cancer-specific regulatory networks. This approach enables us to attack alterations in signaling architecture that are common to multiple cancers, and thus provides approaches applicable in cancers that have different mutational backgrounds. Our findings offer new therapeutic targets that appear to enable effective targeting of transformed cells in a cancer-selective manner and in a manner that transcends the genetic uniqueness of different cancers.

Discovery of novel molecular mechanisms by which intracellular redox state controls the function of nontransformed cells

Regulation of cell survival and signaling function in normal cells by small changes in intracellular redox state

The work leading to our discoveries targeted on cancer therapy actually began with studies focused on developing new means of protecting primary cells from death induced by physiological stressors or by exposure to inadequate levels of survival factors. Our initial goals were to protect oligodendrocytes (the myelin-forming cells of the CNS) from death induced by exposure to tumor necrosis factor-α (TNFα) and glutamate [1–10]. Oligodendrocyte death is important in multiple CNS diseases and injuries, and these two stressors are thought to be important contributors to such death. For example, TNFα levels increase prior to relapse in multiple sclerosis, a disease in which oligodendrocyte destruction is of central importance (e.g., [11–14]). This cytokine is also thought to be important in oligodendrocyte death in, for example, traumatic injury to the spinal cord [15–17]. Preventing glutamate-induced death of oligodendrocytes is also thought to be of importance in multiple CNS injuries, as both inflammatory and traumatic injuries in the CNS are associated with increased exposure to glutamate, which may be derived from multiple sources (e.g., [18–24]).

We found that making oligodendrocytes more reduced by exposure to 1 mM *N*-acetyl-L-cysteine (NAC) was sufficient to prevent cell death caused by either TNFα or glutamate [2]. NAC is a potent cysteine prodrug that is readily taken up by cells and converted to cysteine, the rate-limiting precursor in synthesis of glutathione (GSH). GSH, in turn, is one of the most central components of the redox regulatory system (e.g., [25–29]). Thus, providing NAC to cells is an effective means of making them more reduced.

We also found that NAC (and other antioxidants) could be used to prevent death induced by exposure to insufficient concentrations of signaling molecules required to support cell survival [2]. Indeed, antioxidants were so effective in this regard that their inclusion with progesterone relieved oligodendrocytes of their obligate need for protein survival factors, at least for several days. In all of these experiments, however, we found that NAC was a more potent enhancer of cell survival than either vitamin C or Trolox (a water-soluble analog of vitamin E), which may reflect a central role of glutathione in modulating these processes.

Although these studies were among the first to employ antioxidants as a means of preventing cell death, the aspect of this work we considered the most critical—and that was central in launching the investigation that would lead to new potential cancer therapies—was the finding that altering redox status was sufficient to not only prevent cell death but also to dramatically enhance the efficacy of suboptimal levels of prosurvival agents. We found that the same changes in redox state that prevented death by TNFα or glutamate exposure also markedly enhanced the ability of prosurvival signaling molecules to promote cell survival [2]. For example, if we exposed spinal ganglion neurons to nerve growth factor (NGF, a well-studied promoter of their survival (see, e.g., [30] and references therein) at a dose that rescued a small number of neurons and added 1 mM NAC, we saw a $>1000\%$ increase in the number of neurons. Similar effects were seen when we combined NAC with suboptimal levels of ciliary neurotrophic factor or insulin-like growth factor-I to promote oligodendrocyte survival [2]. These concentrations of NAC had no effect on cell survival when applied on their own, however, thus suggesting that they were altering the efficacy of the signaling molecules to which cells were exposed.

Intracellular redox status as a central regulator of glial progenitor cell function

We next found [31] that redox status was also a central modulator of the balance between self-renewing division and differentiation in dividing oligodendrocyte/type 2 astrocyte progenitor cells, the immediate ancestor of oligodendrocytes (e.g., [32,33]). (These progenitor cells are also referred to as oligodendrocyte precursor cells, and are here abbreviated as O-2A/OPCs.) The balance between self-renewing division and differentiation into terminally differentiated nondividing cells is essential to the function of precursor cells, and thus represents one of the important general problems in studies on stem and progenitor cell biology. If cells stop dividing too early, then not enough cells may be generated to properly build a tissue. In contrast, if cells continue to divide then they essentially function as cancer cells. Thus, understanding the control of this balance represents one of the critical challenges in the field of precursor cell biology.

O-2A/OPCs are a particularly useful population for studying the control of self-renewal and differentiation. These cells can be readily isolated as pure cell populations from multiple regions of the developing CNS and can be grown at the clonal level in chemically defined medium, with clonal analysis rendered particularly easy by the morphological changes that occur in these cells when they differentiate into nondividing oligodendrocytes (represented diagrammatically in Fig. 1; see, e.g., [34–36]). As will be discussed, the basal mitogen for these cells is known, as are a large numbers of other cell-extrinsic signaling molecules that modulate self-renewing division and differentiation.

Several different observations demonstrated the importance of redox state as a normal regulator of the balance between self-renewing division and differentiation of O-2A/OPCs (summarized in Figs. 2 and 3). First, we found that when freshly isolated O-2A/OPCs were purified on the basis of their redox state and grown in clonal cultures in identical basal division conditions (i.e., exposure to platelet-derived growth factor (PDGF), a critical mitogen for these progenitor cells (e.g., [37–39]), the more reduced cells underwent more self-renewal, while the more oxidized cells underwent more differentiation into nondividing oligodendrocytes (Fig. 2) [31]. The propensity of the more oxidized O-2A/OPCs to differentiate did not represent a commitment to differentiation, however, as coexposure of these cells to PDGF+NAC caused them to undergo more division. In contrast, pharmacological alteration of redox state with sublethal concentrations of prooxidants induced cell-cycle exit and differentiation of progenitor cells into nondividing oligodendrocytes.

We next found that precursor cells of the developing rat brain appear to use redox state as a modulator of cell function during CNS development [36]. We had initiated these studies in attempts to understand why different regions of the CNS myelinate over different time scales, as originally summarized in [40]. We found

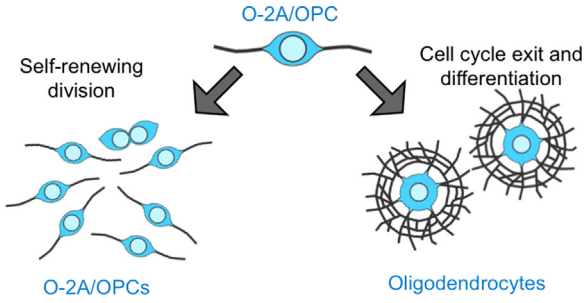

Fig. 1. The O-2A/OPC can undergo self-renewing division and produce more progenitor cells, or can exit the cell cycle and generate nondividing oligodendrocytes. This progenitor cell also can generate an antigenically distinct astrocyte, called a type 2 astrocyte, but the generation of astrocytes from O-2A/OPCs has been studied in much less detail than the generation of oligodendrocytes and therefore is not included in this diagram.

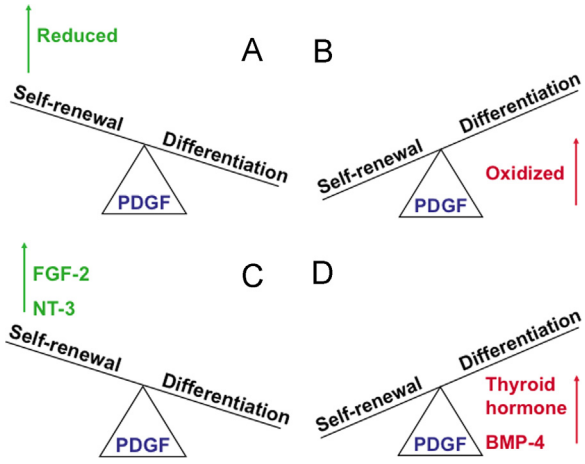

Fig. 2. In O-2A/OPCs growing under basal division conditions (i.e., chemically defined medium supplemented with PDGF), making cells more reduced (A) promotes self-renewing division and making cells more oxidized promotes cell-cycle exit and differentiation (B). These different choices can also be promoted by exposure to extracellular signaling molecules. If cells are exposed to FGF-2 or NT-3, in the presence of PDGF, they undergo more self-renewal (C). If they are exposed to TH or BMP-4, they differentiate into oligodendrocytes or astrocytes, respectively (D). These observations raise the question of whether the extracellular signaling molecules also modify redox state (see text for discussion of this question).

that progenitors from the cortex, which myelinates late and over a long time period, undergo extensive self-renewal *in vitro* under basal division conditions and are relatively resistant to inducers of oligodendrocyte generation [36]. In contrast, progenitors from the optic nerve, a region that myelinates relatively early and relatively rapidly, undergo more differentiation when grown under the identical conditions, and are very responsive to inducers of differentiation. We further found that these differences were cell intrinsic. One component of this regulation appears to be intracellular redox state. As predicted by our prior redox analyses [31], at the time of isolation from the developing rat CNS the cortex-derived O-2A/OPCs were much more reduced than optic nerve-derived O-2A/OPCs [36].

Redox modulation is necessary and sufficient in the action of cell-extrinsic signaling molecules that modulate the balance between division and differentiation in dividing O-2A/OPCs

Studies on the control of the balance between division and differentiation in O-2A/OPCs also had led to the identification of multiple cell-extrinsic signaling molecules that impinge on this balance. We first discovered that cells dividing in the presence of

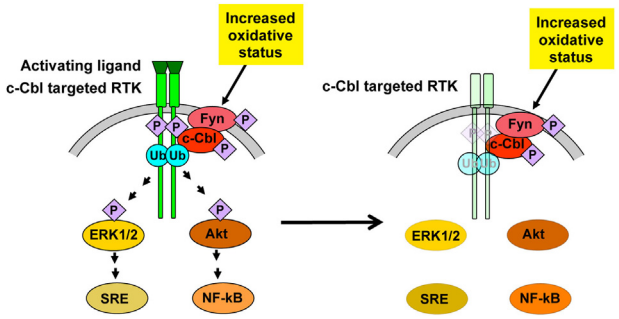

Fig. 3. Summary of the consequences of activation of the redox/Fyn/c-Cbl pathway. Left figure: In normal signaling processes, binding of ligand (dark green) to receptor (light green) causes receptor phosphorylation, leading to activation of the downstream signaling mediators, including Erk1/2, Akt, SRE, and NF-κB. Mild oxidation of cells, however, leads to sequential activation of Fyn kinase and the c-Cbl ubiquitin ligase. C-Cbl attaches ubiquitin to its target receptors. Right figure: The targets of c-Cbl are then degraded. Loss of receptor decreases or eliminates activation of Erk1/2, Akt, SRE, and NF-κB by that receptor.

PDGF will undergo continued division (in the absence of differentiation) if they are also exposed to fibroblast growth factor-2 (FGF-2) [41], and later studies showed that neurotrophin-3 (NT-3) also increased division and decreased differentiation in dividing O-2A/OPCs [35]. In contrast, thyroid hormone (TH) induces cell-cycle exit and differentiation of dividing progenitor cells into oligodendrocytes [35,42]. Exposure to bone morphogenetic proteins (BMPs), such as BMP-4, induced differentiation of O-2A/OPCs into type 2 astrocytes [43].

In contrast with standard approaches to understanding the mechanism of action of extracellular signaling molecules—which are usually focused on analysis of such events as phosphorylation cascades and changes in transcriptional regulation—we found that the key point of integration between control of division and differentiation by redox state and the control of this balance by cell-extrinsic signaling molecules was redox state itself [31]. Growth of O-2A/OPCs in the presence of [PDGF+FGF-2] or [PDGF+NT-3] made cells more reduced than when those cells were grown in PDGF alone. These changes occurred within 18 h, a period of time that preceded effects of these conditions on division and differentiation. In contrast, cells exposed to [PDGF+TH] or [PDGF+BMP-4] rapidly became more oxidized than those grown in PDGF alone, even though TH and BMP-4 induced different cell fates and work through entirely different signaling pathways.

We next found that the redox changes induced by exposure to cell-extrinsic signaling molecules were critical to understanding the effects of these agents [31]. Exposure of progenitor cells to NT-3+PDGF (which made cells more reduced and promoted self-renewing division) in the presence of buthionine sulfoximine (which inhibits glutathione production [44]), eliminated the enhancement of self-renewal [31]. We could also replace NT-3 with NAC and obtain similar enhancement of self-renewal as seen with NT-3. Conversely, if we exposed cells to TH+PDGF (which made cells more oxidized and promoted oligodendrocyte generation) in the presence of NAC, we eliminated the effects of TH. Moreover, we could replace TH with a chemical prooxidant and induce similar levels of oligodendrocyte generation.

The above studies showed that the redox changes produced by TH and NT-3 were both necessary and sufficient to explain their activities, and our collective findings essentially fulfilled the equivalent of Koch's postulates for analysis of a cellular biological problem. This is a very different understanding of how these agents work than is revealed by traditional studies on cell signaling and/or regulation of gene transcription and demonstrates that approaches that lack analysis of metabolic components of

signaling factor activity are providing an incomplete view of how this critical precursor cell function is regulated.

The redox/Fyn/c-Cbl pathway as a central integrator of redox regulation of O-2A/OPC function

How does redox state alter cell function? While we had provided evidence that the redox changes we saw were both necessary and sufficient for the effects of NT-3 and TH on O-2A/OPC function, these observations did not tell us how these redox changes were translated into differences in cell behavior.

In our next studies we discovered a new regulatory pathway, which we called the redox/Fyn/c-Cbl (RFC) pathway [45] that converts small increases in intracellular oxidative status into sequential activation of Fyn kinase and the c-Cbl E3 ubiquitin ligase (as summarized in Fig. 3). Fyn is a member of the Src family of kinases that can be activated by increases in intracellular oxidative state [46–49]. Although Fyn has multiple targets, one of these is c-Cbl [50–53]. When c-Cbl is activated it attaches ubiquitin to its target proteins (such as the activated PDGFRα [54,55]), thus enhancing their degradation (e.g., [56–58]).

The targets of c-Cbl make this perhaps one of the most interesting of regulatory proteins in stem cell biology and cancer biology, as multiple proteins that are critical in regulating precursor cell behavior and cancer cell behavior are c-Cbl targets (as summarized in Table 1). Just some of the critical proteins controlled by c-Cbl include such receptor tyrosine kinases (RTKs) as the PDGF receptor-α (PDGFRα [54,55]), the epidermal growth factor receptor (EGFR, [52,56]), the receptor for hepatocyte growth factor (c-Met) [45,59], the receptor for stem cell factor (c-Kit [60]), and the insulin-like growth factor-I receptor (IGF-IR [61]), as well as Notch-1 [62], activated β-catenin [63], and multiple other proteins of great interest in multiple fields of biological study.

Fyn kinase appears to be the key redox target in the RFC pathway

One of the major concerns in understanding how redox state controls cell function is to identify the specific proteins for which changes in redox state alter protein function. The list of proteins that are potential redox targets is already large, and continues to grow (see, e.g., [64–67] for review). The proposed targets of redox-mediated modulation of activity include multiple receptors and intermediates in cell signaling.

In contrast with the view that redox changes control multiple cell signaling molecules, our studies reveal the possibility of a much simpler redox regulatory architecture, in which increases in oxidative status activate Fyn kinase as a primary target (apparently through increases in oxidized glutathione [48], although how such increases would activate Fyn are not known). Everything else that occurs is then downstream of Fyn activation.

The reasons why we propose that increases in oxidative status may be more selective in their activity than has been otherwise suggested come from several experiments conducted in our original discovery of the RFC pathway. For example, in our original studies [45] we found that exposure to low levels of methyl mercury, lead, or paraquat (levels sufficient to cause an ~20% increase in oxidative status) caused reductions in NF-κB and serum response element (SRE)-mediated transcription, phosphorylation of Akt and ERKs1/2, and phosphorylation of the PDGF receptor-α (PDGFRα) (summarized in Fig. 4). Such an outcome gives the appearance of redox state affecting many proteins. Indeed, if Akt, ERKs1/2, etc. were examined individually in the context of increased oxidative status, one might draw the conclusion that they were targets of oxidative increases based on observations that treatment with antioxidants prevents the prooxidant-induced decreases in their activation.

Table 1
Examples of c-Cbl targets.

Cell surface receptors (ligand)	References
alpha5 integrin	[388]
c-Met receptor (hepatocyte growth factor)	[45,59]
Epidermal growth factor receptor	[52,56]
ErbB2/HER2/neu	[343]
Eph A (Eprin A1)	[389]
Fms-like tyrosine kinase 3 (Flt-3, cytokine flt 3 ligand)	[390]
Insulin receptor (via the APS adapter protein)	[391]
Insulin-like growth factor-I receptor	[61]
c-Kit (stem cell factor)	[60]
P75NTR	[392]
Platelet-derived growth factor receptor-α	[54,55]
Ron (macrophage stimulating protein)	[393]
Intracellular regulators	**References**
c-Abl	[394]
Activated β-catenin	[63]
Cystic fibrosis transmembrane receptor	[395]
Focal adhesion kinase (FAK)	[373]
Fyn kinase	[396]
Notch-1	[62]
PI3kinase	[397]
Src	[398]
Stat5	[399]

Two observations demonstrate that NF-κB and SRE-mediated transcription, as well as phosphorylation of Akt, ERKs1/2, and PDGFRα, are not the actual targets of increased oxidation. First, exposure of O-2A/OPCs to methylmercury, lead, or paraquat in combination with NT-3 did not cause any changes in the activation status of any of these intracellular signaling mediators, and no change in phosphorylation of the TrkC receptor for NT-3. This is as predicted if the changes in downstream signaling were the consequence of c-Cbl activation (via Fyn), for even though PDGFRα and TrkC are both RTKs with many similarities in structure and in components of their downstream signaling, PDGFRα is a c-Cbl target and TrkC is not [45]. In further support of this view, inhibition of c-Cbl was able to prevent changes in PDGFRα levels, as well as downstream changes in protein function, from occurring. Moreover, inhibition of Fyn was sufficient to prevent activation of c-Cbl. These results indicated that it was not SRE, NF-κB, Akt, ERKs1/2, or even the RTKs themselves that were redox targets. Instead, redox was regulating something else, which from our studies and studies of others appears to be Fyn kinase [48].

Thus, it appears that the RFC pathway provides a means by which changes in redox state can influence signaling pathways downstream of RTKs with great pathway specificity and without having to directly modulate the components of these pathways that may be utilized by multiple different receptor systems. The presence of a pathway that integrates redox regulation of cell function at the level of degradation of specific RTKs and other proteins critical in control of division and survival offers an efficient means of integrating multiple different signals within a single convergent pathway.

Target-dependent activation of c-Cbl versus pan-activation of c-Cbl

Our studies on the RFC pathway differ from other studies on c-Cbl in two important ways: First, cellular oxidation appears to trigger c-Cbl activation more broadly than the known feedback circuits between an individual receptor tyrosine kinase (RTK) and c-Cbl, as studied in investigations that have identified c-Cbl as an important negative regulator of RTK signaling (e.g., [58,68–76]). While we thus far have no reason to suggest any alterations in the requirement that the targeted RTK needs to be activated by ligand in order to be

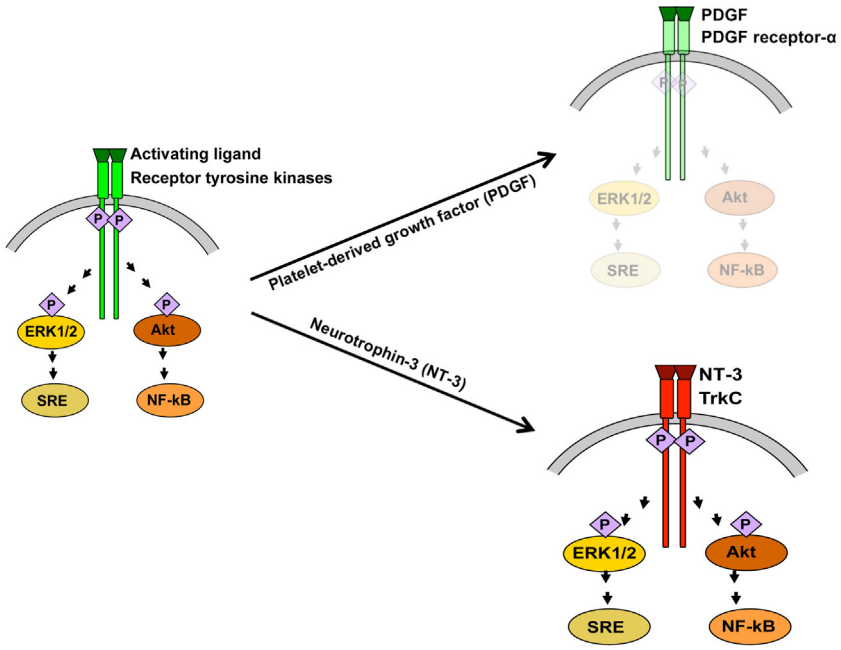

Fig. 4. Oxidation suppresses signaling mediated by the PDGF receptor-α (PDGFRα), but not by the TrkC receptor for NT-3. When O-2A/OPCs were exposed to concentrations of methylmercury, lead, or paraquat that were sublethal, and which increased oxidative status by only ~20%, signaling initiated by exposure to PDGF was suppressed at multiple levels. Promoter-reporter assays showed reductions in activity of the transcription factors NF-κB and the serum response element (SRE), in phosphorylation of Erk1/2 and Akt, and in phosphorylation of PDGFRα. In contrast, when cells were stimulated by exposure to NT-3, there were no effects of these environmental toxicants on SRE or NF-κB activity, or on Erk1/2, Akt or TrkC phosphorylation. Thus, the effects of increased oxidation on signaling are pathway specific, and indicate that the effects of oxidation (at least at this level of redox change) are not mediated by effects directly on Erk1/2, Akt, SRE, or NF-κB. As discussed in the text, the key regulatory event leading to decreased levels of Erk1/2, Akt, SRE, and NF-κB activity was enhanced degradation of PDGFRα.

ubiquitinated by c-Cbl, it also appears that activation of c-Cbl is modulated by Fyn rather than by an activated RTK.

Extending still further the differences between receptor-modulated and redox-modulated activation of c-Cbl, the cause of cellular oxidation thus far appears to be irrelevant with respect to whether or not the RFC pathway is activated. We originally discovered the RFC pathway in studies on the effects of exposure of O-2A/OPCs to low levels of environmental toxicants (methyl mercury, lead, and paraquat) [45]. We also have found that oxidative changes induced by cell-extrinsic signaling molecules (specifically TH and BMP-4) induce similar activation of the RFC pathway as a critical means of promoting cell-cycle exit in dividing O-2A/OPCs (unpublished observations; summarized in Fig. 5). At least in these progenitor cells, the RFC pathway is also modulated by other physiological stressors than environmental toxicants, as well as by genetic and epigenetic differences in redox state.

While our studies have thus far focused on O-2A/OPCs and cancer cells (as discussed later), the ubiquitous nature of cellular oxidation and the broad expression of both Fyn and c-Cbl, suggest that the RFC pathway is relevant to multiple cell types. Studies in our laboratory on neurons and endothelial cells, for example, indicate that this is the case.

The toxicity of anticancer agents for the central nervous system defines a central challenge in developing safer cancer treatments

There is no question but that our current approaches to cancer treatment will someday be regarded as barbaric, as the major tools in the current cancer treatment arsenal are administration of a large variety of poisons, treatment with radiation, or the combination of the two. Such treatments have provided only rare examples of progress in reducing the toll of malignancy and their application can cause a wide range of adverse side effects that can greatly decrease the quality of life for patients experiencing these treatments.

As we not only need to develop cancer treatments that are more effective, but also that target cancer cells in a more selective manner, we initiated studies to identify key features distinguishing normal and cancer cells and that might offer potential targets for developing improved therapies. We approached this problem by focusing first on an important toxicity reaction that had not received the attention it warranted, which was toxicity to the CNS.

Neurological side effects of treatment with a wide range of anticancer agents have been demonstrated in multiple clinical studies (e.g., [77–91]). For example, studies over two decades ago found that 18% of all breast cancer patients receiving standard-dose chemotherapy showed cognitive defects on posttreatment evaluation [92], and such problems were reported in more than 30% of patients examined 2 years after treatment with high-dose chemotherapy [93,94], a greater than eightfold increase over the frequency of such changes in control individuals. Even these numbers may be underestimates of the frequency of adverse neurological sequelae in association with aggressive chemotherapy, as two longitudinal studies on breast cancer patients treated with high-dose chemotherapy with carmustine (BCNU), cisplatin, and cyclophosphamide, and evaluated using magnetic resonance imaging and proton spectroscopy, showed that changes in white matter in the CNS induced by the treatment could occur in up to 70% of individuals, usually with a delayed onset of several months after treatment [95,96]. Delayed toxicity reactions can occur in individuals treated only with systemic chemotherapy, but also are particularly well documented in children exposed to both chemotherapy and cranial irradiation [97–107].

A wide range of anticancer agents with different mechanisms of action can have adverse neurological side effects. For example, bis-chloroethylnitrosourea (BCNU, also known as carmustine) is an alkylating agent used in treatment of gliomas, glioblastoma

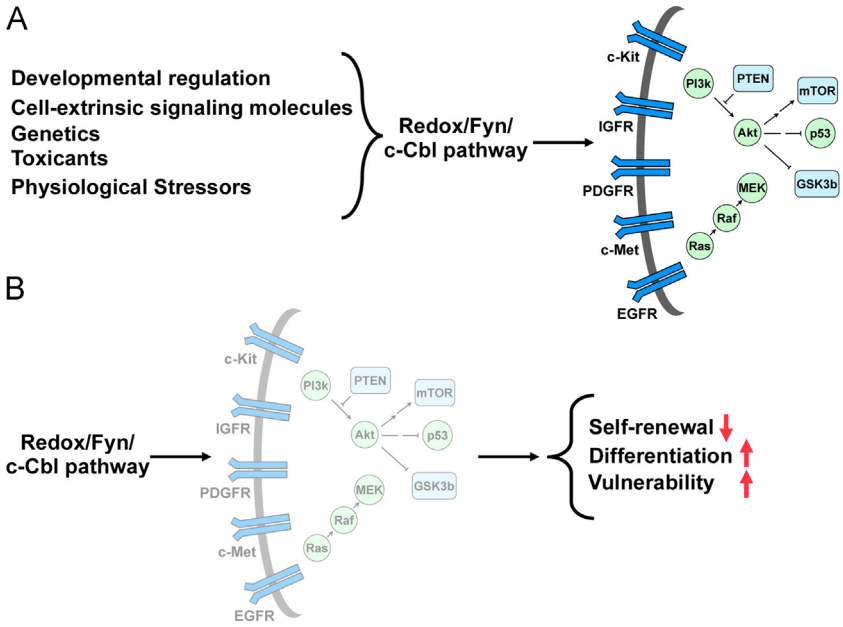

Fig. 5. Oxidative regulation via the RFC pathway can be induced by many modulators and has multiple downstream effects on O-2A/OPCs. In our studies thus far, it appears that the RFC pathway can be activated in O-2A/OPCs by certain cell signaling ligands (e.g., thyroid hormone or BMP-4), genetic inducers of an increased oxidative state, epigenetic (developmental) regulators of redox state, chemical toxicants, or other physiological stressors that cause cells to become more oxidized. This causes a pan-activation of c-Cbl, enabling it to regulate a multitude of its downstream targets (including, e.g., c-Kit, the IGF-I receptor, PDGFRα, c-Met, and EGFR). These receptors are all modulators of a network of downstream signaling mediators. (B) Degradation of the targets of c-Cbl leads to decreases in downstream signaling. The consequences for dividing O-2A/OPCs are a reduction in cell division, an increase in cell-cycle exit, and differentiation and increases in vulnerability to physiological stressors or cell death (depending upon the level of RFC pathway activation and extent of loss of c-Cbl targets).

multiforme (GBM), multiple myeloma, and lymphoma) and which also can be used as part of a preparatory (conditioning) regimen prior to bone marrow transplantation. BCNU treatment has been associated with significant changes in mental status and with white matter degeneration [108,109]. Cisplatin is a DNA cross-linking agent used in treatment of various kinds of cancers, including sarcomas, some carcinomas, lymphomas, and germ cell tumors. At high doses, it has been associated with leukoencephalopathy and destruction of CNS white matter [110]. The antimetabolite cytarabine is a nucleoside analog used mainly in treatment of white blood cell tumors, such as acute myeloid leukemia, acute lymphocytic leukemia, and lymphomas. Application of cytarabine has also been associated with acute encephalopathy, confusion, memory loss, and white matter changes [110,111]. 5-Fluorouracil (5-FU) is a pyrimidine analog that inhibits thymidylate synthase and is used alone or as an important component of adjuvant therapies in treatment of breast, colorectal, stomach, anal, pancreatic, esophageal, skin cancers, and head and neck cancers. Acute CNS toxicities associated with systemically administered 5-FU (most frequently in combination with other chemotherapeutic agents) include a pancerebellar syndrome and subacute encephalopathy with severe cognitive dysfunction, such as confusion, disorientation, headache, lethargy, and seizures. With high-dose treatment, as many as 40% of patients show severe neurological impairments that may progress to coma [112–114]. In addition, a delayed cerebral demyelinating syndrome reminiscent of multifocal leukoencephalopathy has been increasingly identified following treatment with drug regimens that include 5-FU, with diagnostic findings obtained by both magnetic resonance imaging (MRI) and analysis of tissue pathology (e.g., [115–139]).

The potential number of people affected by adverse effects of chemotherapy on the CNS is extremely sobering, emphasizing the magnitude of the need to develop cancer treatments that are more selective in their action. Even if examination of all cancers were to lower the frequency of these problems to 25% of the lower estimates in the literature (that is, to around 4.5% of patients receiving low-dose therapy and 7.5% of patients receiving high-dose chemotherapy) the prevalence of cancer in the world's populations means that the total number of individuals for whom adverse neurological changes are associated with cancer treatment is as great as for many of the more widely recognized neurological syndromes that are the subject of extensive research efforts. For example, if we consider the United States alone, there are more than 1.5 million new cases of cancer diagnosed per year. If 5% of people with cancer showed neurological consequences of treatment, this would be more than the total number of newly diagnosed cases of Parkinson's disease each year. It would be many times greater than the number of newly diagnosed individuals each year with rare genetic neurological diseases. Yet the efforts to understand—and prevent—effects of systemic chemotherapy on the CNS are very much less than for either of these examples of more widely studied neurological afflictions.

Precursor cells and oligodendrocytes of the CNS are targets of multiple chemotherapeutic agents

We found that normal CNS progenitor cells and oligodendrocytes are exceptionally vulnerable to the toxic effects of clinically relevant levels of the chemotherapeutic agents BCNU, cisplatin, cytarabine, and 5-FU [140,141]. Vulnerability to these drugs was observed for all classes of lineage-restricted progenitor cells that can be readily grown as purified cell populations. It was particularly striking that O2A/OPCs and oligodendrocytes were one-to-two orders of magnitude more sensitive to cisplatin, cytarabine, and 5-FU than has previously been observed in studies on multiple neuronal populations from both the CNS and the peripheral nervous system [142–149]. It also was striking that vulnerability was not restricted to dividing cells, as nondividing oligodendrocytes were also targets of these drugs, at exposure levels routinely achieved during treatment.

In contrast with the vulnerability of primary CNS progenitor cells, comparative analysis of multiple cancer cell lines from different tissues only identified one cell line (ES-2 ovarian cancer

cells) in which vulnerability was comparable to that observed for primary neural progenitor cells, with most such cell lines being more resistant to these agents than the normal cells. This outcome was seen despite the fact that the cancer cell lines we studied often were chosen because of their previous use in studies on the response to the drugs studied.

In vitro analyses of purified cell populations were highly predictive of effects seen following systemic treatment with any of the chemotherapeutic agents *in vivo*, in terms of both the cellular targets and the ability of exposure to these agents to cause cell death and also to suppress precursor cell division. In particular, *in vitro* studies showed that doses of BCNU, cisplatin, and cytarabine that did not kill normal precursor cells were nonetheless sufficient to suppress their division [140]. Such a loss of dividing cells would compromise the ability of dividing progenitor cells to contribute to tissue maintenance and repair processes, and could also contribute to long-term or delayed toxicity reactions. *In vivo* studies demonstrated death of both neuronal and glial precursors, as well as of oligodendrocytes and also demonstrated effects on cell division like those we had seen *in vitro* [140]. Moreover, systemic treatment with BCNU, cisplatin, cytarabine, or 5-FU all caused continued reductions in cell division in one or more CNS regions after treatment ended, suggesting a long-lasting depletion of populations required for cell replenishment.

Studies on 5-FU were also conducted to examine one of the most puzzling aspects of chemotherapy-induced damage to the CNS, which is the occurrence of toxicity reactions with a delayed onset. Although most reports of 5-FU-associated neurotoxicity indicate a relatively acute onset, a delayed demyelinating cerebral complication reminiscent of multifocal leukoencephalopathy has also been increasingly reported in patients treated with chemotherapy regimens that include 5-FU (e.g., [115–139]). Neurological symptoms may occur in some patients several months after adjuvant therapy with 5-FU and include declines in mental status, ataxia, and the appearance of prominent multifocal enhancing white matter lesions detectable by MRI.

We found that short-term systemic administration of 5-FU caused both acute CNS damage and also caused a syndrome of progressively worsening delayed damage to myelinated tracts of the CNS associated with altered transcriptional regulation in oligodendrocytes and extensive myelin pathology [141]. When we examined the status of myelination in mice given three injections of 5-FU 8 weeks previously, we found that oligodendrocytes in the corpus callosum showed a loss of expression of the Olig2 transcription factor, which is critical in oligodendrocyte generation [150–154]. At the ultrastructural level, we saw severe disruptions in myelination, and electrophysiological studies of impulse conduction in the auditory nerve showed a slowing of the speed of impulse conduction (a predicted consequence of myelin destruction).

It is also important to note that our findings most likely underestimate the actual extent of damage that may be done by systemic chemotherapy, further underscoring the need for developing better treatments. Both acute and delayed neurological side effects have been observed for many other chemotherapeutic agents [78,80,85,92,93,155–168], and Investigations on multiple other chemotherapeutic agents in the laboratory have provided results similar to ours (e.g., [169–199]). Thus, it would be incorrect to think that these problems were restricted to the agents we have studied. We also have not examined delayed degenerative changes outside of our work with 5-FU [141], nor have we examined the effects of several courses over an extended period of time despite the knowledge that current treatment protocols use exactly such regimens. In addition, there are multiple therapeutic protocols associated with higher concentrations of drugs than those we studied (for example, intraarterial administration, liposome-encapsulated drugs, or locally applied biodegradable wafers in the treatment of brain tumors). Moreover, the advances that have been made in rescuing patients from the toxicity of chemotherapeutic agents for bone marrow have been associated with a tendency to apply yet higher doses of these agents, thus potentially increasing the risk of neurotoxicity.

Even tamoxifen is toxic to cells of the CNS

There are several ways to respond to the above results, one of which is to determine whether any anticancer agents have better safety profiles. With respect to our efforts of developing better cancer treatments (and particularly ones focused on modulation of redox state, as discussed later), we were fortunate to find that one that is slightly safer is tamoxifen (TMX). TMX is used both before and after breast cancer surgery and may be applied for multiple years in women at increased genetic risk for breast cancer, thus providing a wealth of knowledge regarding its use in humans.

Although TMX is considered to be one of the safest of cancer treatments, it would be wrong to conclude that it is entirely benign, and women treated with TMX may exhibit subtle but significant changes in neuropsychological function and brain structure (e.g., [200,201]). TMX-related toxicities include cerebral and cerebellar toxicities, irritability, confusion and ocular toxicities [202], hippocampal atrophy, and widespread areas of hypometabolism in the inferior and dorsal lateral frontal lobes [203]. Moreover, several studies suggest an association between TMX exposure and cognitive dysfunction [200,204].

When we examined the potential toxicity of TMX, we first found that clinically relevant exposure levels were toxic for CNS progenitor cells *in vitro* [205]. *In vivo*, a 2-week treatment with systemic TMX caused increased death of cells in the corpus callosum and suppressed cell division in the corpus callosum, subventricular zone of the ventricles, and the dentate gyrus of the hippocampus. As for the other anticancer agents studied by ourselves and others, the increases in apoptosis in the corpus callosum and reductions in cell division in the subventricular zone and dentate gyrus of the hippocampus could present a possible cellular biological basis for the adverse neurological consequences that sometimes occur in patients receiving long-term TMX treatment.

Despite the toxicity of TMX, the degree of toxicity we observed was less than that seen with BCNU, cytarabine, cisplatin, and 5-FU. These outcomes appear to be consistent with the clinical experience.

Convergence on the redox road to possible new treatments for cancer

The findings of ourselves and others regarding the vulnerability of multiple CNS cells to a large variety of anticancer agents underscore the importance of developing therapies that are not only more effective but also are more selective in their toxicity. We therefore next addressed two questions: Why are cancers resistant to anticancer agents that are toxic for normal progenitor cells of the CNS? Can we use this information to develop new interventions that are more selective in their effects on cancer cells? Although there have been multiple mechanisms described contributing to the therapeutic resistance of cancer cells, few of these studies have been focused on a parallel analysis of the differences between normal progenitor cells and cancers.

As discussed in this section, addressing the above two questions has revealed a remarkable convergence on redox state and the RFC pathway in cancer cells. These discoveries provide new ways of targeting cancer cells in a selective manner, and with the unexpected opportunity to simultaneously attack multiple proteins known to be critical in cancer cell function.

BCNU and TMX are prooxidants—and TMX is potentially a very useful prooxidant for treatment of a wide range of cancers

Multiple standard anticancer agents appear to make cells more oxidized. It was thus a natural question for us to ask whether the effects of these agents on O-2A/OPCs might be mediated through activation of the RFC pathway.

The agents we chose for our first studies were BCNU and TMX. BCNU was of interest due to its known ability to inhibit glutathione reductase [206], thus preventing the proper recycling of oxidized glutathione back to a reduced state and hence making cells more oxidized.

TMX deserves more detailed comments both because our current studies indicate that this is an anticancer agent of particular promise in the development of redox-based approaches to cancer treatment and also because it is a widely misunderstood drug. While TMX is best known for its use in treatment of ERα$^+$ luminal breast cancers, higher dosages of TMX have also been examined in clinical trials in a broad range of other cancers (including gliomas [207], lung cancer [208], metastatic melanoma [209], desmoid tumors [210], non-Hodgkin's lymphoma [211], hormone refractory prostate cancer [212], hepatocellular carcinoma [213], cervical cancer [214], metastatic renal cell carcinoma [215], and metastatic uterine leiomyosarcoma [216]). The interest in TMX as a potential treatment for so many different cancers is quite distinct from its utility as an ERα antagonist, and stems instead from observations that different concentrations of TMX have very different effects. While the mid-nanomolar (nM) exposure levels most widely studied for TMX inhibit division of estrogen-dependent luminal breast cancer cells, induction of cell death (even in TMX-sensitive breast cancer cell lines [217], as well as other cancer cell types) requires exposure to low micromolar (μM) concentrations of this agent. Such concentrations are not supraphysiological, and instead are clinically relevant even with standard exposures to TMX in treatment of luminal breast cancer. Even with such standard treatments, TMX concentrations in tumors is ~2 μM [218–223]), and higher-dose applications will increase these levels still further.

The ability of low micromolar TMX to make cells more oxidized [224–226] is what made this agent of potential interest in our studies on redox and cancer, and has provided a means of more effectively harnessing TMX's ERα-independent effects for cancer treatment. As a result of these studies we are now able to enhance TMX's ERα-independent activities in a rational and mechanism-driven manner.

BCNU and TMX activate c-Cbl in O2A/OPCs—but not in cancer cells

One reason for focusing attention on anticancer agents (such as BCNU and TMX) that have activity as prooxidants (as contrasted with making cells more oxidized as a secondary response to damage) is because we found that cancer cells and primary CNS progenitor cells differ markedly with respect to the consequences of being more oxidized.

As predicted from our previous studies on the RFC pathway, exposure of O-2A/OPCs to BCNU caused these cells to become more oxidized, activated Fyn kinase, and activated c-Cbl [227]. Moreover, these exposures caused reduction in levels of the EGFR, a c-Cbl target that is of interest as a potential therapeutic target in a wide range of cancers. In ongoing studies, TMX also activates the RFC pathway in O-2A/OPCs and causes decreases in levels of EGFR (unpublished observations).

In contrast to effects of BCNU and TMX on O-2A/OPCs, when we examined the effects of BCNU exposure on multiple glioblastoma (GBM) cell lines, we found that even when exposed for 24 h to several-fold higher concentrations of BCNU than were effective in O-2A/OPCs, the GBM cells showed no increases in c-Cbl phosphorylation or decreases in EGFR levels [227]. (All experiments on GBM cells were conducted using cell lines grown under conditions that select for cells with the ability to generate isotypic tumors when transplanted into immune-compromised mice [228,229]. Moreover, in all studies conducted attention was focused on wild-type EGFR and not on the EGFRvIII oncogenic mutant that is intrinsically resistant to c-Cbl mediated degradation [230].)

The failure to cause c-Cbl activation and decreases in EGFR levels in GBM cells was not due to a failure of BCNU to enter cells, to render them more oxidized, or to activate Fyn kinase [227]. BCNU caused a twofold increase in Fyn kinase activity in GBM cells, which was blocked by the Src-family kinase inhibitor PP1, caused a $>50\%$ decrease in levels of total glutathione, and increased the proportion of oxidized glutathione (GSSG) from levels almost too low to detect to up 50% of total GSH. This is an extraordinarily high GSSG content, as GSSG normally represents $\leq 2\%$ of total glutathione in normal cells [231]. It is unlikely that any primary cells would remain viable with such a high GSSG content, yet GBM cells showed no impairment in survival or division.

Similar outcomes also were seen in multiple BLBC cell lines treated with TMX. Exposure to 10 μM TMX (a concentration relevant to high-dose application of this agent) or to H_2O_2 did not cause increases in c-Cbl phosphorylation or decreases in levels of EGFR. As for GBM cells, the failure to activate c-Cbl and cause decreases in EGFR levels was not due to a failure to oxidize cells or to activate Fyn kinase. Exposure to 10 μM TMX caused 15–35% increases in oxidative status, a >2-fold increase in the proportion of oxidized glutathione in the total intracellular glutathione pool (from 10% to 23%), and activation of Fyn kinase. Fyn kinase activation was prevented by pretreatment of cells with NAC, thus indicating that activation was redox dependent.

Restoring RFC pathway function, and c-Cbl activation, by targeting proteins that inhibit c-Cbl, increases sensitivity to anticancer agents in GBM and BLBC cells

As BCNU and TMX exposure caused oxidation and Fyn activation in GBM cells and BLBC cells, respectively, the lack of BCNU- or TMX-induced activation of c-Cbl and decreases in levels of EGFR must be due to changes at other points in the redox/Fyn/c-Cbl pathway. Clues on possible means of inhibiting c-Cbl activity were provided by previous reports that c-Cbl function in some breast cancer cell lines, with maintenance of high levels of EGFR, can be caused by sequestration of c-Cbl by cell division control protein 42 homolog (CDC42) [232,233], a member of the family of Rho GTPases that plays an important role in cytoskeletal regulation. In addition, studies on experimental transformation of NIH3T3 cells by v-Src revealed that the function of the Cbl family member Cbl-b could be inhibited by the PAK-interacting exchange factor β-pix/Cool-1/Rho guanine nucleotide exchange factor 7 (identified as a guanine nucleotide exchange factor for Rho family small G protein Cdc42, and herein referred to as Cool-1/β-pix) [234]. Cbl-b is frequently overexpressed in breast cancers and is thought to be functionally homologous to c-Cbl. Cdc42 is a known cytoskeletal regulator that has been of interest in multiple cancers due to its overexpression in tumor tissue compared with normal tissue [235,236]. Unlike cdc42, however, there was no published information on functional contributions of Cool-1/βpix to naturally occurring tumors, either in humans or in experimental animals.

In light of the above studies, we first focused our attention on Cool-1/βpix and Cdc42 in our studies on GBMs and BLBCs. We examined both proteins in both tumors, as previous studies indicated that Cool-1/βpix functions in concert with Cdc42 (e.g., [237–239]).

We found that Cool-1/βpix—but not Cdc42—is a critical inhibitor of c-Cbl function in GBM cells (summarized in Fig. 6). In GBM cells and GBM biopsy samples—but not in normal brain or in primary O-2A/OPCs—we found that c-Cbl was complexed with Cool-1 [227]. We also found that Cool-1/βpix in GBM cells and tumor biopsies, but not in normal brain or primary glial progenitor cells, was tyrosine-phosphorylated (and thus thought to be active). In addition, the proportions of different Cool-1/βpix isoforms were different in tumor biopsies and in normal brain tissue. While it was previously reported that there were brain-specific forms of Cool-1/β-pix [240], the isoform shift that we saw was between normal brain and transformed brain tissue and thus may represent a still different isoform divergence.

We next found that knockdown of Cool-1/βpix in GBM cells—but not knockdown of Cdc42—restored RFC pathway activity, increased the sensitivity of these cells to anticancer agents, and had other effects of interest. When we decreased levels of Cool-1/βpix with shRNAi in GBM cells, we restored the ability of BCNU exposure to cause activation of c-Cbl and reductions in EGFR levels and increased the sensitivity to BCNU as a cytotoxic agent *in vitro*. Even in the absence of BCNU exposure, Cool-1/βpix knockdown decreased division and migration of GBM cells. In contrast, Cdc42 knockdown had no effect on any of these parameters in GBM cells.

Of particular interest were the findings that Cool-1/βpix knockdown also increased sensitivity of GBM cells to temozolomide, the front-line anticancer agent used in treatment of these tumors. This increase in sensitivity occurred despite observations that temozolomide did not make GBM cells more oxidized, suggesting that allowing normal activation of c-Cbl also made cells vulnerable in other ways, a topic we will consider later in this review.

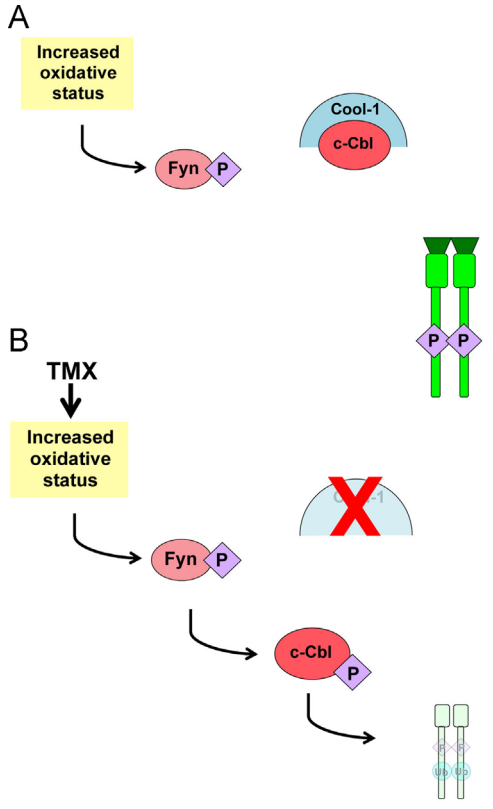

Fig. 6. Cancer cells suppress c-Cbl activation with inhibitory proteins. (A) In normal O-2A/OPCs, activation of the RFC pathway causes degradation of c-Cbl's target proteins. (B) In GBM cells, c-Cbl is sequestered by Cool-1, thus suppressing its ability to ubiquitylate its target proteins and enhance their degradations. C-Cbl activity is also inhibited in BLBC cells, although not by Cool-1. It is clear that Cdc42 plays an important role in this inhibition in BLBC cells, but thus far does not itself appear to bind to c-Cbl, as discussed in the text.

Our studies on BLBC cells revealed similar inhibition of c-Cbl as found in GBM cells, but further showed that in the breast cancer cells this inhibition was mediated by Cdc42 rather than by Cool-1/β-pix [241]. Cdc42 knockdowns—but not Cool-1/β-pix knockdowns—increased the sensitivity to TMX of multiple BLBC cell lines (including cell lines representative of Basal B, Basal A and Basal A with HER2 amplification subsets of BLBCs). One difference between our studies on GBM and BLBC cells, however, was that we found no evidence of Cdc42 complexed with c-Cbl in BLBC samples (nor did we find Cool-1/c-Cbl complexes in these cells). It is possible, however, that the interacting protein that needs to be studied in these cells is Cdc42-interacting protein 4 (CIP4), which is overexpressed in invasive breast cancer cells [242] and was previously reported to interact with c-Cbl via a Src Homology 3 domain [243].

Restoring c-Cbl function enables us to harness TMX for RFC pathway activation and tumor treatment in BLBC cells

Exposure of BLBC cells to TMX enabled us to determine that restoration of RFC pathway function was of potential utility in targeting these cells. Treatment of cells with shRNAi for Cdc42 enabled TMX-mediated activation of c-Cbl with associated decreases in levels of EGFR, and suppressed BLBC cell division and cell migration. None of the effects we saw were likely due to antiestrogenic effects of TMX as all cell lines we examined were ERα-negative. Instead, as for the effects of BCNU on Cool-1/βpix knockdown GBM cells, effects of TMX on Cdc42 knockdown BLBC cells appeared to be dependent on the RFC pathway and could be blocked by NAC or by pharmacological inhibition of Fyn.

The most effective means of moving our studies forward to potential clinical evaluation would be through development of pharmacological interventions that enable us to restore normal c-Cbl function, experiments which were possible to carry out in BLBC cells. The investment of the NIH in development of inhibitors of RhoGTPase family members (which includes Cdc42) led to discovery of a chemical inhibitor of Cdc42, which was given the name of ML141 [244]. We found that ML141 was effective both *in vitro* and *in vivo* [241].

In vitro, we found that pharmacological inhibition of Cdc42 with ML141 was similarly effective as genetic knockdown at enabling TMX-induced reductions in levels of EGFR [241]. ML141 exposure also enhanced the ability of TMX to suppress BLBC cell growth, through both induction of cell death and suppression of cell division, at TMX concentrations similar to those known to cause apoptosis (rather than inhibition of proliferation) in MCF-7 cells [217].

We were also able to demonstrate the ability of ML141 to render BLBC cells sensitive to TMX *in vivo* [241], even though the original identification of this compound was not associated with selection for favorable properties *in vivo*, and its solubility characteristics only allowed analysis over a 2-week period *in vivo* (i.e., only slightly longer than used in previous analyses of notch inhibitors on BLBC tumors [245]). In our experiments, animals were transplanted with 1×10^6 unmanipulated MDA-MB-231 cells and tumors were allowed to establish for 24 days before initiating treatment. After confirming tumor establishment by luciferase imaging, animals were treated every day for 2 weeks with ML141, TMX, or both agents together.

Our experiments demonstrated that, even without optimization for *in vivo* utilization, pharmacological inhibition of Cdc42 with ML141 enabled TMX to suppress growth of MDA-MB-231-derived tumors [241]. Remarkably, considering that tumors were generated from a TMX-resistant and ERα-negative BLBC cell line,

exposure to TMX+ML141 was associated with a marked suppression of tumor growth during the 2 weeks of treatment. In mice treated with vehicle only, 5/6 tumors increased markedly in size over these 2 weeks and one mouse showed no tumor growth. Neither TMX nor ML141 altered this outcome when applied individually. When both agents were combined, however, now only 1 out 6 animals exhibited a marked increase in tumor size, 2/6 mice showed only modest tumor growth, and 3/6 mice showed no tumor growth at all.

Restoring c-Cbl activity by Cool-1/βpix or Cdc42 knockdown suppresses the function of tumor-initiating cells in GBMs and BLBCs

We also found that restoring c-Cbl function in GBM and BLBC cells provides multiple other benefits, and in particular enables us to address one of the central goals of contemporary cancer research, which is to identify means of targeting TICs (also known as cancer stem cells). Studies on a growing variety of cancers have found that only a subset of cells in a tumor population have the ability to generate new tumors (see, e.g., [246–276] and citations therein). Multiple investigators have described means of prospectively identifying such cells and/or means of propagating cancer cell isolates so as to enrich for cells with TIC function. While there is considerable controversy as to whether the TIC phenotype can be unambiguously identified in any tumor, it nonetheless seems clear that cells with TIC-like properties tend to also be highly resistant to chemotherapy and radiation. Thus, treatment strategies that fail to eradicate cells with TIC-like properties will likely always be associated with tumor recurrence, as remaining TICs generate new cancer cells.

We found that restoration of c-Cbl function provided a potent means of suppressing critical aspects of TIC function in both BLBC cells and GBM cells [227,241]. These effects did not require coexposure to anticancer agents and instead appeared to be an intrinsic consequence of restoration of normal c-Cbl function.

In BLBC cells—using commonly used cell lines that had not been isolated or subsequently grown in a manner to enrich for TICs—the evidence for suppression of TIC function was based on examining generation of adhesion-independent spheroids (also known as mammospheres) and on generation of tumors following transplantation [241]. We observed an ~85% reduction in mammosphere generation in Cdc42 knockdown cells. In three different BLBC cell lines injected orthotopically into female mouse mammary fat pads, we also found marked suppression of tumor generation. For the MDA-MD-231 (Basal B) cell line, we found that tumors could be generated readily by transplantation of 1000 cells expressing scrambled shRNAi but were only seen in 1 out of 5 and 3 out of 8 NOD/SCID mice transplanted with 1000 or 10,000 Cdc42 knockdown cells, respectively. Cdc42 knockdown was even more effective at preventing tumor generation by HCC1954 (Basal A with HER2 amplification) and HCC70 (Basal A) cells, and no cells expressing shRNA for Cdc42 generated tumors even when 10,000 cells were transplanted. Comparable transplants with HCC1954 and HCC70 cells expressing scrambled shRNAi caused tumor generation in 60% of recipient mice.

We also found that pretreatment of cells with ML141 prior to cell transplantation allowed targeting of TIC function [241]. To determine whether pharmacological inhibition of Cdc42 with ML141 affects TIC properties of BLBCs, 10,000 MDA-MB 231 cells were preexposed for 40 h either to vehicle alone or to ML141 and then were implanted into animals. At 20 days after transplantation, a time when 4 out of 5 mice generated tumors from vehicle-treated cells, none of the mice implanted with ML141 pretreated cells formed tumors. Even when examined 40 days after implantation, only 2 of 5 mice harboring ML141 pretreated cells started to form tumors (a comparable frequency as with Cdc42 knockdown cells). Moreover, the tumor sizes resulting from ML141 pretreated cells were markedly smaller than those of the control group.

As the GBM cells we studied were isolated and grown in such a manner as to enrich for cells with TIC characteristics, and TIC biology has been particularly well studied in GBMs [227], we were able to identify a broader range of effects of Cool-1/βpix knockdown on function of TICs in these cells. As for BLBCs, we saw a marked (and almost complete) suppression of neurosphere generation *in vitro*, with the few spheres that were generated by Cool-1/βpix knockdown cells being much smaller in size than those generated by cells expressing scrambled shRNAi constructs. When we examined effects of Cool-1/βpix knockdown on the proportion of cells expressing putative antigenic markers of TICs in GBMs [227], we found a >80% reduction in prevalence of CD133+ cells, an ~50% reduction in CD15+ cells, and a >85% reduction in CD133/CD15 double-positive cells [227].

Cool-1/βpix knockdown reduced the capacity of GBM cells to establish tumors to such an extent as to raise the possibility of total inhibition of TIC function [227]. Readily detectable tumors developed in ~60% of mice within 8 weeks after transplantation of as few as 10,000 GBM cells expressing scrambled shRNAi constructs. In contrast, with Cool-1/βpix knockdown cells, no tumors were generated following transplantation of 10,000 or even 50,000 cells. The lack of tumor initiation was not due to a lack of viable cells, as transplanted Cool-1/βpix knockdown cells were readily detected 2 weeks posttransplantation by analysis of sectioned brain with antibodies to a human-specific mitochondrial antigen.

As further confirmation of the importance of Cool-1/βpix knockdown in preventing tumor generation by GBM cells, tumors arose rarely in mice (≤20% of transplants) when 200,000 or 500,000 Cool-1/βpix knockdown cells were engrafted intracranially and these rare tumors all showed reexpression of Cool-1/βpix and high levels of EGFR. Thus, generation of tumors in mice transplanted with Cool-1/βpix knockdown cells seems most likely dependent on a subset of cells in which Cool-1/βpix was expressed.

Restoring c-Cbl function enables simultaneous targeting of multiple proteins of critical importance for tumor-initiating cells and for cancer cells in general

One of the ways in which the scientific community is trying to target cancer cells is by identifying proteins that are critical for initiation and/or maintenance of tumors. This has been a particularly fruitful research activity in regard to TICs, with a growing list of proteins that appear to be important in the ability of TICs to generate tumors.

A particularly exciting potential aspect of restoration of c-Cbl function is that this ubiquitin ligase modulates the degradation of a large number of proteins that are being studied as potential targets for cancer treatment. Thus, activating c-Cbl appears to provide a means of simultaneously reducing levels of multiple targets of interest in cancer biology, including multiple regulators of TIC biology.

One group of proteins targeted by c-Cbl consists of proteins known to be of interest in a variety of different types of cancer. For example, in comparing GBMs and BLBCs, the EGFR, the c-Met receptor for hepatocyte growth factor, and Notch-1 are all of interest as potential therapeutic targets and also are all regulated by c-Cbl (e.g., [45,59,62,245,277–280,281]. Another example of a protein known to be important in multiple cancers (including GBMs) is activated β-catenin, which is also a c-Cbl target [63,282–284]. C-Met, β-catenin, and Notch-1 all have been found to regulate TIC function in a variety of cancers (e.g., [285–310]).

We demonstrated in our work on GBMs [227] that it is indeed possible to simultaneously target multiple c-Cbl targets by its

reactivation. Knockdown of Cool-1/βpix caused decreases in levels of EGFR, Notch-1, and β-catenin, the three c-Cbl targets examined in these studies. Of these, Notch-1 and β-catenin are known to be important in GBM TICs (e.g., [284,295]).

It also needs mention that there are multiple c-Cbl targets that have more restricted expression patterns but are nonetheless important cancer targets. Such proteins include HER2/neu (a potential target in a subset of BLBCs and also an important target in other forms of breast cancer) (e.g., [311–314]), Fms-like tyrosine kinase 3 (Flt-3, which is important in acute myeloid leukemia [315–318]) and the c-kit receptor for stem cell factor (which is important in, e.g., leukemia, gastrointestinal stromal tumors, and melanoma [60,319–323]). Thus, restoration of normal c-Cbl function may allow simultaneous targeting of a large number of proteins of great interest in the oncology community.

A further reason for interest in restoring c-Cbl activity was that activation of c-Cbl was also associated with reduced levels of Akt and NF-κB activity [45], a predicted outcome given the role of receptors such as EGFR, c-Met, and HER2 in activating signal transduction pathways. Such downstream targets of RTKs offer additional targets of interest in current cancer therapeutic studies (see, for example, [324–326]). As Akt and NF-κB are important contributors to cell survival, such effects may also explain why Cool-1/βpix knockdown in GBM cells made these cells more sensitive to temozolomide even though this agent had no detectable prooxidative activity [227].

Finally, we demonstrated in our work on GBM cells that restoration of c-Cbl function also caused decreases in proteins important in function of TICs that are not known c-Cbl targets [227]. In this respect our findings that levels of Sox2 (a protein of great interest in a wide range of cancers) were also reduced following Cool-1/βpix knockdown was completely unexpected. In addition, CD133 appears to be critical for TIC function in GBM cells [327] and also was reduced in Cool-1/βpix knockdown cells [227], even though it is not a known target of c-Cbl.

The observations that c-Cbl activation can target so many proteins of interest in cancer cell function may help to explain why restoration of c-Cbl function was so effective at suppressing GBM initiation. Indeed, as all tumors generated after transplantation of Cool-1/βpix knockdown cells showed Cool-1/βpix expression levels like those seen in nonmanipulated cells, our data are consistent with the hypothesis that Cool-1-mediated suppression of c-Cbl may even be essential for initiation of some forms of GBM.

Targeting of c-Cbl reactivation may be more effective than attempts to inhibit individual targets of c-Cbl. For example, EGFR expression is frequently elevated in malignant breast cancers, including BLBCs, and such increased expression is thought to contribute to chemoresistance, aggressive growth, and expression of tumor-initiating ability [277–279]. Inhibition of EGFR activity is of great interest as a treatment strategy for multiple cancers, but the reductions in EGFR levels that occur when c-Cbl function is restored—and are further decreased by exposure to TMX—may achieve similar purposes. Restoring c-Cbl activity is likely to extend, however, beyond regulation of the EGFR. For example, mammosphere generation by ductal carcinoma in situ cells *in vitro* was suppressed by about 60% by Gefitinib [328] (which inhibits EGFR but also inhibits multiple other kinases [329]), but there appears to be no evidence of suppression of tumor generation or ability to render ERα-negative cells sensitive to TMX with this agent.

Restoration of c-Cbl function appears to be the first approach to cancer treatment able to simultaneously affect so many different proteins known to be of key importance in cancer cell function. Thus, while the potential role of c-Cbl as a tumor suppressor gene is of increasing interest (for review, see, for example, [330,331]), our studies suggest that inhibition of c-Cbl may be still more important than previously suggested. If combined restoration of c-Cbl function and oxidation of cells with such agents as TMX enables even more effective targeting of these multiple cancer control nodes (as indicated by work in progress), this should enhance the value of this strategy still further.

The effects of Cool-1/βpix and Cdc42 knockdown thus far appear to be specific to cancer cells

Considering the multiple effects of targeting Cool-1/β-pix or Cdc42 on cancer cell function, these interventions thus far appear to be remarkably benign in their effects on nontransformed cells. Perhaps most importantly, Cool-1/β-pix knockdown did not alter the sensitivity of O-2A/OPCs to BCNU or temozolomide [227]. Moreover, expression of Cool-1/βpix shRNAi in O-2A/OPCs did not alter cell-cycle parameters, division, or increases in cell number over 10 days. The one parameter yielding a marked decrease was in O-2A/OPC migration, as predicted by the role of Cool-1/β-pix in modulating cytoskeletal function (e.g., [234,332]).

Similarly, despite the increased toxicity of TMX for BLBC cells in which Cdc42 function was inhibited, such inhibition did not increase TMX toxicity on nontumorigenic mammary epithelial cells [241]. When MCF10A mammary epithelial cells were exposed to TMX, ML141, or the combination, no reduction in cell number was detected. Thus, effects of Cdc42 inhibition on TMX-responsiveness appeared to be specific to BLBC cells.

Are the effects we observe really due to c-Cbl and the RFC pathway?

One question raised by our studies concerns the extent to which outcomes of Cool-1/β-pix or Cdc42 knockdown are due to restoring c-Cbl function. Secondary knockdown of c-Cbl in GBM cells with a Cool-1/β-pix knockdown restored tumor initiation, increased neurosphere formation, partially restored levels of CD15 expression, and prevented BCNU-induced decreases in EGFR levels [227]. In BLBC cells, c-Cbl knockdown in cells that also had a Cdc42 knockdown (or cells exposed to pharmacological Cdc42 inhibition) also restored tumor initiation, prevented tamoxifen-induced decreases in EGFR, and prevented response to tamoxifen *in vitro* and *in vivo* [241]. Thus, inhibition of c-Cbl activity appears to be central to understanding effects of Cool-1/β-pix and Cdc42 in GBM and BLBC cells, respectively.

The degree to which benefits obtained by Cool-1/β-pix or Cdc42 knockdown are dependent on restoring RFC pathway activity will be the subject of future research. Nonetheless, the ability of NAC and pharmacological inhibitors of Fyn (and other Src family members) to overcome at least some of the effects of Cool-1/β-pix or Cdc42 knockdown [227,241] suggests that at least some of the effects we have seen are regulated by the RFC pathway.

Next steps

Our studies present a novel general strategy for cancer treatment, in which restoration of normal c-Cbl function—by targeting proteins that inhibit c-Cbl—has enabled us to target TIC function while at the same time making cancer cells more vulnerable to at least three different anticancer agents (i.e., BCNU, temozolomide, and tamoxifen). This strategy is summarized in Fig. 7, using tamoxifen as an example of an anticancer agent useful in activating the RFC pathway.

The questions that we are investigating now are all focused on determining the general applicability of our discoveries and on other investigations required to determine if these discoveries could lead to therapeutic advances. One of the reasons for such a focus is the possibility that our results are broadly relevant. In our

studies on BLBC cells, we found identical results on 7 independent BLBC cell lines representing Basal B triple-negative cells, Basal A cells, and Basal A cells with Her2 amplification [241]. In our studies on GBMs, five independent GBM cell lines all yielded identical results, as did examination of biopsies from five different patients [227]. In addition, analysis of five c-Cbl targets in GBM samples (via the Cancer Genome Atlas) showed increased levels of at least two c-Cbl target proteins in 73% of cases and of at least three c-Cbl targets in 43% of cases. A more focused examination of tumors with increased EGFR protein expression showed increased expression of Cool-1/βpix mRNA in 65% of samples, and in 52% of all GBM samples in the database. With the caveat that these databases provide no information on isoform changes and alterations in Cool-1/β-pix phosphorylation, such information nonetheless suggests that a more extended examination of Cool-1/β-pix alterations in gliomas would be of interest.

Similarly, levels of Cdc42 in breast tumor lysates exceed by as much as 50-fold those seen in normal tissue from the same patients and elevated Cdc42 levels also are observed in lung cancer and in colorectal cancer [235,236], raising the possibility that suppression of c-Cbl function by inhibitory proteins may be of relevance in multiple categories of tumors.

It is also important to note that it thus far appears that c-Cbl inhibition fits our primary criteria of occurring in cancers with different genetic backgrounds. The GBM and BLBC cells we examined were all independently isolated from different patients, yet all shared the common property of inhibiting normal c-Cbl function as a central part of their biology.

New views on the role of c-Cbl in cancer and novel strategies: Inhibiting the inhibitors

Our approach differs from most studies on the role of c-Cbl in cancer by focusing on inhibitors of c-Cbl as potential therapeutic targets. Disruption of normal c-Cbl function is becoming increasingly recognized as important in various carcinomas [333–336] and in tumors of the hematopoietic system [337–339]. Prior studies have largely focused, however, on disruption of c-Cbl function by mutation of either c-Cbl itself or of its targets so as to become resistant to c-Cbl-mediated degradation (see, e.g., [330,331]) for review). Restoring normal regulation in such instances is far more difficult than targeting proteins that are inhibiting c-Cbl function. As shown by our studies on BLBCs and GBMs, inhibiting the inhibitors of c-Cbl appears to offer a promising path for further development.

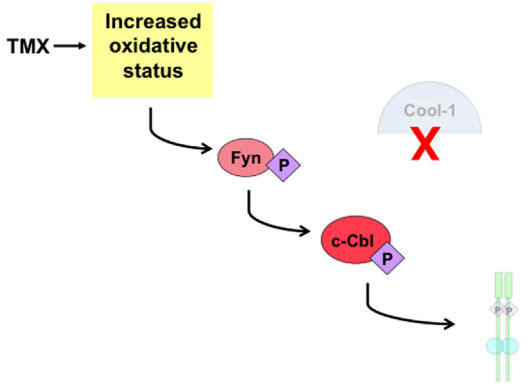

Fig. 7. Tamoxifen (TMX) exposure as an example of a cancer treatment strategy indicated by our discoveries. We found that we can use the ability of TMX to make cells more oxidized (through estrogen receptor-α-independent mechanisms) to activate Fyn kinase. If we at the same time restore normal c-Cbl function by targeting its inhibitors (here depicted as Cool-1), we can use TMX to cause c-Cbl activation and degradation of c-Cbl's targets. Our published studies demonstrate the ability to cause such activation in BLBC cells, while unpublished studies show that we can also employ the prooxidative effects of TMX to attack GBM cells.

Our work also differs from previous analyses of the contributions of Cbl to tumor biology in other ways. Although it is well recognized that c-Cbl can function as a tumor suppressor gene (e.g., [58,69,331,340–343]), prior studies have not investigated the ability of c-Cbl reactivation to target regulatory components needed for tumor initiation. In contrast, our findings that inhibition of c-Cbl function is critical in enabling tumor initiation and spheroid generation by GBM cells and BLBC cells suggest that this will be a fruitful avenue for further investigation [227,241]. In addition, our findings (in GBM cells [227]) that restoring c-Cbl function enables us to simultaneously target multiple proteins that are important cancer targets (e.g., EGFR, Notch-1, β-catenin, Sox2, CD133) should lead to increased interest in the potentially central integrating role played by c-Cbl in tumor cell function.

One of the possible implications of our studies that also appears to offer a novel view of the role of c-Cbl in cancer is the possibility that inhibition of c-Cbl function is responsible for increased protein expression of multiple RTKs and other proteins in cancer cells in the absence of gene amplification. Many c-Cbl targets are known to be overexpressed in cancer, and although the presence of gene amplification is predictive of protein overexpression, the converse is not true. Our current understanding of the RFC pathway suggests that inhibition of c-Cbl might provide an explanation for these poorly understood examples of overexpression of proteins of great interest in cancer biology, and that restoring c-Cbl function will provide a means of simultaneously decreasing levels of multiple such proteins.

Our studies also are novel in providing the first evidence of the ability of cancer cells to subvert function of c-Cbl's regulation via the RFC pathway and in providing a molecular pathway that allows for activation of c-Cbl using currently available anticancer agents. By targeting c-Cbl's inhibitors and driving activation of c-Cbl via the RFC pathway (with, e.g., BCNU or TMX), we offer a means of simultaneously reducing levels of multiple central regulators of tumor cell function and also making cancer cells more sensitive to existing anticancer agents—with the advantage of apparently not conferring such enhanced sensitivity in normal cells. Such selectivity is consistent with our findings thus far that we are simply restoring the activity of a pathway (i.e., the RFC pathway) that already exists in normal cells. Thus, the lack of change in their sensitivity to anticancer agents is thus far consistent with our current understanding of this pathway and emphasize the potential value in restoring of c-Cbl function by targeting its inhibitors.

New views on redox treatment of cancer

The approach we have taken differs in multiple ways from other strategies attempting to utilize redox state modulators as anticancer therapies (see, e.g., [344–352]), and it is important to consider these differences and their potential implications for trying to use redox modulators in cancer treatment.

The first question that must be answered if one is going to treat cancer cells via redox-modulation strategies is whether the goal is to make cells more reduced or more oxidized, each of which has significant challenges in its application. In particular, even within a single cancer, there is no single redox state that defines cancer cells. For example, there are many observations indicating that cancer cells are more oxidized (see, e.g., [344–352] and references therein), but even within a tumor from a single patient there are cells that are oxidized and other cells that are reduced. Indeed, a more reduced redox status may be critical to the biology of TICs [353,354].

If cancer cells use a more reduced state to promote and/or protect TIC function, then using antioxidants to treat cancers (or to attempt to decrease the toxic side effects of anticancer treatments, almost all of which make cells more oxidized) runs the risk of protecting the cancer cells and perhaps particularly protecting TICs. In contrast, if the goal is to make cancer cells more oxidized, then there is the question of how this would differ from the effects of current treatment approaches (which largely seem to converge on making cells more oxidized). There is the further question of how much of a difference this would make, as cancer cells have numerous mechanisms for protecting themselves against oxidative stress, and do not seem to be functionally impaired by levels of oxidative challenge that would be toxic for nontransformed cells.

There is also the further challenge that if TICs are more reduced, and changing their biology requires making them more oxidized—but bulk cancer cells are more oxidized and changing their biology requires making them more reduced—then how do you accomplish both at one time? Whichever strategy is chosen runs the risk of providing benefit to a subpopulation of cancer cells. As it seems increasingly clear that cells with non-TIC patterns of antigen expression can acquire TIC characteristics (for review, see, e.g., [268,273,276,355]), this is a potentially serious issue.

Our discoveries on the consequences of restoring normal c-Cbl function seem to provide a way out of these dilemmas. Regardless of the role of redox state in TIC function, restoring normal c-Cbl activation provides a means of attacking TICs. At the same time, our studies on BLBCs indicate that restoring c-Cbl function simultaneously provides a means of attacking non-TICSs [241], a particularly important concept as it is becoming increasingly clear that the distinction between TICs and non-TICs is a plastic one and that cells in the putative non-TIC compartment also can maintain and initiate tumors (for review, see, e.g., [268,273,276,355]). Based on our observations on the frequency of mammosphere and tumor formation from MDA-MB-231 cells, we estimate a TIC frequency in the range of ~0.1 to 1%. Yet, in cells with Cdc42 knockdown or treated with ML141 and exposed to TMX, cell death *in vitro* increased from ~3% of cells in untreated cultures to ~40% of cells, while the proportion of Ki67+ cells fell by two-thirds (from ~75% in untreated cultures to ~25% in cultures receiving both treatments) [241]. Moreover, treatment with ML141+TMX caused a suppression of further tumor growth *in vivo*. Such outcomes argue for effects that extend beyond the small fraction of cells with TIC function in these populations. When taken together with the ability of restoration of c-Cbl function via Cdc42 knockdown to inhibit mammosphere formation and tumor initiation *in vivo*, it appears that such manipulations modulate the biology of both TICs and of the bulk population of BLBC tumor cells—and have the additional benefit of thus far not disrupting function of nontransformed cells.

Our discoveries also focus attention on how tumor cells escape the consequences of increased oxidation rather than on how cancer cells suppress the redox changes themselves. This is a very different concern from whether cancer cells can protect themselves from prooxidant stress by increasing their production of glutathione (e.g., [356–360] and references therein), for example. Instead, our studies suggest that cancer cells have ways of preventing the consequences of prooxidative changes that would be lethal in normal cells. In this regard, RFC pathway inhibition appears to provide a novel means by which tumor cells can escape normal functional consequences of being oxidized. For example, as mentioned earlier, we found in BLBC cells that oxidized glutathione (GSSG) represented ~10% of the total glutathione pool, as compared with generally representing ≤2% of total glutathione in normal cells [231]. In BCNU-treated GBM cells, GSSG represents >40% of the total glutathione. Such oxidative challenges are severely toxic for primary cells, yet both BLBC cells and GBM cells are not noticeably impaired by such GSSG abundance. As GSSG has been reported to activate Fyn [48], inhibition of RFC pathway-mediated activation of c-Cbl provides a means of escaping such an oxidative challenge.

Our work raises the possibility that the ability to successfully harness redox changes as an anticancer strategy may be dependent on first overcoming the ability of cancer cells to resist the effects of redox change on c-Cbl activation. The implications of protection from redox challenges by inhibition of c-Cbl function are that attempts to attack tumor cells by increasing their oxidative status may benefit greatly if combined with strategies to restore normal RFC pathway activity—and may even require restoration of normal RFC pathway activity to be effective and selective in their outcomes.

Pharmacological restoration of c-Cbl function: Opportunities and challenges

Our work may be of particular interest with respect to therapeutic development as inhibition of proteins that themselves inhibit c-Cbl provides more tractable approaches to treatment than restoration of activity in mutated c-Cbl proteins. The extensive interest in Rho GTPase family members led to discovery of the Cdc42 inhibitor used in our studies on BLBC cells [241], and our studies on GBMs suggest there would be similar value in developing Cool-1/βpix inhibitors, perhaps targeted to isoforms that are more highly expressed in GBMs than in normal brain [227].

As developing pharmacological interventions to restore c-Cbl function are likely to benefit from an enhanced understanding of the potential complexity of c-Cbl regulation, it is important to develop a more sophisticated understanding of this regulatory circuitry. Moreover, as we already have found that GBMs and BLBCs use different means of inhibiting c-Cbl, it may be important that the therapeutic approaches employed are targeted on the inhibitors relevant for the tumor being treated or on other as yet unknown means of restoring normal c-Cbl function. That such a possibility needs to be considered is also supported by findings that expression of transglutaminase-2 in GBMs (another potential inhibitor of c-Cbl in these cells) in >60% of instances indicated by analysis of TGAP data were found in the mesenchymal GBM subtype [361]. In contrast, in our own similar analyses we found that changes in Cool-1/βpix expression were found almost entirely in proneural and classical GBM subtypes [227], raising the possibility that different GBM types use different strategies for inhibiting c-Cbl but nonetheless converge on inhibiting this enzyme.

At present there appear to be several proteins that are able to inhibit c-Cbl, including Cool-1/βpix and Cdc42 (see [75] for a review on this topic). For example, studies on internalization and ubiquitination of the EGFR raise the possibility that the apoptosis-linked gene 2-interacting protein X/apoptosis-linked gene 2-interacting protein 1 (Alix/AIP1) can inhibit c-Cbl function by negatively regulating the interaction between SETA/CIN85 and c-Cbl, as well as preventing c-Cbl phosphorylation following EGFR activation [335]. Sprouty 2 also can bind to c-Cbl and inhibit ubiquitylation and degradation of EGFR [362,363] (although the biological details of this interaction seem somewhat mysterious as Sprouty 2 is itself reported to be a target of c-Cbl-mediated ubiquitylation and subsequent degradation [364]). Another potential inhibitor of c-Cbl is Cdc42-interacting protein 4 (CIP4), which is seen at high levels in in invasive breast cancer cells [242] and which interacts with c-Cbl via a Src Homology 3 domain [243]. When we examined the effects of CIP4 knockdown in MDA-MB 231 BLBC cells (due to not finding evidence for complex formation between c-Cbl and Cdc42), we found that knockdown caused a marked decrease in EGFR levels and that exposure of the CIP4

knockdown cells to TMX caused a further decrease in EGFR levels [241]. In addition, it also was recently reported that transglutaminase-2 also can inhibit c-Cbl function in some GBM cell lines [361].

There also is a growing list of proteins known to regulate the activity of Cool-1/βpix and Cdc42, and each of these is of potential interest as a means of controlling c-Cbl activity. For example, inhibition of a particularly central activator of Cool-1/βpix might produce outcomes that were similar to knockdown of Cool-1/βpix itself. In addition, it has been reported that MEK inhibition also decreases Cool-1/β-pix phosphorylation [365], providing another possible means of regulating this inhibitor of c-Cbl.

The ways in which proteins that might regulate c-Cbl interact and whether such interactions change in cancer cells are, however, poorly understood. An excellent example of this problem is offered by studies on Cool-1/βpix and Cdc42. Prior to our studies, a clear prediction from the existing literature would be that a tumor cell sensitive to disruption of one of these proteins would also be sensitive to disruption of the other due to several publications reporting that Cool-1/βpix works in concert with Cdc42 in modulation of cytoskeletal function [237,238] and insulin release in pancreatic islet cells [239]. Despite these previous findings, however, we found no effects of Cdc42 knockdown in GBM cells and no effects of Cool-1/βpix knockdown in BLBC cells. Another example of the need to be cautious in drawing conclusions from previous studies is that studies based on overexpression experiments led to the conclusion that Cool-1/β-pix could bind to Cbl-b but showed weak-to-no binding of Cool-1/βpix to c-Cbl [366]. In contrast, we readily detected complexes containing both Cool-1/β-pix and c-Cbl in GBM cell lines and tumor biopsies [227]. Whether this was because there is another linking protein (or proteins) that forms part of this complex, or whether the existence of this complex requires expression of specific Cool-1/β-pix isoforms, is unknown. The differing results emphasize, however, that we have much to learn about the c-Cbl regulatory circuitry.

There are still other confusing aspects about interpreting existing data, including the possibility that even the overexpression of a protein in a tumor sample does not provide information about the function of that protein. An example of this problem is seen in comparing our findings on the effectiveness of Cool-1/βpix knockdown in GBM cells with the lack of effect of similar knockdowns in BLBC cells even though previous studies reported that Cool-1/βpix appears to be overexpressed in the great majority of human breast cancers [367]. Why would such overexpression occur if it was not functionally important and how could Cool-1/β-pix be overexpressed without binding to c-Cbl? With respect to the first question, overexpression of Cool-1/βpix could theoretically result from c-Cbl inhibition by Cdc42 in BLBC cells, based on observations that c-Cbl can ubiquitylate Cool-1/βpix and target it for degradation through a negative feedback loop [238]. Thus, the overexpression of Cool-1/βpix in breast cancer cells might be simply a consequence of c-Cbl inhibition by other means. Moreover, the prior studies on Cool-1/βpix overexpression in breast cancers did not examine the isoform shifts that we found occurring in brain tumors [227], and it may be that these shifts are of functional importance in enabling Cool-1-mediated inhibition of c-Cbl.

Nonetheless, despite these potential complexities, it is important to emphasize that we saw the same outcomes in 7 different BLBC cell lines of different types and in five independently derived GBM cell lines. Thus, developing means of better targeting Cdc42 and Cool-1/βpix seems to be a worthy priority.

It also is likely to be the case that targeting some c-Cbl inhibitors may be of particular value as a consequence of participation in other functions relevant to cancer cells. For example, inhibition of Cool-1 or Cdc42 might also yield benefits with respect to tumor cell migration. Cdc42 is well studied as a regulator of cell migration (for recent reviews, see, e.g., [368,369]). Cool-1 inhibition also can activate focal adhesion kinase (FAK) [370], regulates Cdc42 and Rac1 [237,238,371], and is important in integrin-mediated signaling (for review, see [372]). In addition, Cool-1 inhibition can decrease migration of transformed cells [332]. It currently appears that these functions of Cool-1 are distinct from effects with respect to cell division and vulnerability to chemotherapeutic agents, as Cool-1 knockdown in O-2A/OPCs had no effect on these parameters even though it did inhibit progenitor cell migration. Thus, it currently appears that effects of Cool-1 knockdown on migration are distinct from effects on regulation of c-Cbl activation. That said, it also needs mention that it recently has been reported that FAK itself may be a target of c-Cbl-mediated ubiquitylation and subsequent degradation [373], raising the possibility that restoring normal c-Cbl function in cancer cells may be sufficient to beneficially alter migration.

Further perspectives on TMX

If we had to choose a single agent with which to activate the RFC pathway for cancer treatment, this currently would be TMX. Although we appear to be the first to discover how to enhance the utility of ER-α-independent activities of TMX so as to render this compound effective on ER-α-negative tumors [241], it is has long been thought that such ER-α-independent activities might be useful in treatment of many cancers. As mentioned earlier, such interest has led to clinical trials examining the potential utility of TMX in over a dozen different cancers [207–210,211–216].

Although TMX appears thus far to be the safest anticancer agent in wide usage, it is also important to note that it may be possible to further improve TMX's safety profile with other substances of interest in cancer treatment. A screen of the NINDS-II library of FDA-approved drugs and agents in clinical trials revealed over 20 compounds that were able to protect O-2A/OPCs (the cell type we found to be most vulnerable to TMX) from TMX-induced cell death [374]. As predicted if it at least part of TMX's toxicity was due to its effects on ERα-mediated signaling, some protective agents were estrogen mimetics. Of greater interest as protective agents, however, were MEK1/2 inhibitors due to interest in such compounds as anticancer treatments. These findings led us to examine the compound AZD6244, which is currently in Phase II clinical trials (e.g., [375,376]). We found that AZD6244 protected O-2A/OPCs *in vitro*, and further found that administration of AZD6244 *in vivo* prevented TMX-induced increases in cell death and reductions in the number of O-2A/OPCs in the CC [374].

Our studies on AZD6244 as a potential protective agent were particularly intriguing, as this agent has been shown previously to suppress tumor progression and to sensitize cancer cells to other therapeutic agents by inhibiting MEK1/2 activation [377,378]. Similarly, we found that AZD6244 rendered MCF7 luminal breast cancer cells more sensitive to TMX [374], raising the possibility that AZD6244 could be utilized as an adjunct breast cancer therapy with TMX that offered the simultaneous possibility of protecting against at least some aspects of TMX-induced neurotoxicity. While other studies have not examined AZD6244 in the context of either breast cancer or effects on the CNS, previous studies [379] showed that the antitumor efficacy of TMX was not compromised by coexposure to MEK1/2 inhibition (by CI-1040) *in vivo*.

Our studies on the ability of AZD6244 to protect normal CNS progenitor cells but not luminal breast cancer cells provide an example of a substance that appears to make cancer cells more sensitive to anticancer agents while protecting normal cells. The property of protecting normal cells while enhancing the sensitivity

of cancer cells is a puzzling one, and understanding how this can be accomplished is likely to provide insights important for developing better anticancer therapies. How a MEK1/2 inhibitor might have such different effects on normal cells and transformed cells is not yet known. It is curious, however, that there are previous observations indicating that MEK inhibition can also decrease Cool-1/β-pix phosphorylation [365]. It will be of great interest to determine if the MEK inhibitors we discovered through investigations on protective strategies have the unexpected property of also being able—at least in some cancers—of restoring normal c-Cbl function. If so, their value in cancer treatment may be considerable, but also would benefit from a mechanism-based understanding of the best drugs with which to combine them.

Summary and conclusions

Our attempts to understand the relationship between intracellular redox state and regulation of cell function have produced several insights that shed new light on fundamental problems in precursor cell biology and cancer cell biology and on the development of therapeutic approaches that may be able to target cancer cells while sparing the normal cells of the body. The discoveries that small changes in redox state were sufficient to protect vulnerable oligodendrocytes from death induced by TNFα or glutamate, and that similarly small changes in redox state controlled the balance between self-renewing division and differentiation into nondividing oligodendrocytes and O-2A/OPCs, set the stage for novel findings regarding mechanisms by which redox state and cell-extrinsic signaling molecules converge to regulate cell function. The first of these novel findings was the discovery that the activity of such widely studied signaling molecules as TH and NT-3 in modulating O-2A/OPC differentiation was dependent on effects on redox state, and that redox changes were both necessary and sufficient to explain the activities of these agents on changing the balance between division and differentiation. Such findings provide a very different view of the mechanisms of signaling molecule action than traditional studies on, e.g., phosphorylation cascades and transcriptional regulation, by placing the redox changes as a key controlling element in the effects of these signaling molecules.

Analysis of mechanisms by which oxidation exerts its effects on cell function led to discovery of the RFC pathway, which provides a means by which redox state controls levels of many of the most critical proteins in the biology of precursor cells and cancer cells. By enabling oxidation to activate the c-Cbl ubiquitin ligase, this provides control of a large number of RTKs and other proteins (e.g., Notch-1, β-catenin) of central importance in regulating cell division, cell survival, and differentiation.

The fact that the RFC pathway is activated by oxidation creates a problem for cancer cells, which are generally more oxidized than nontransformed cells—and, as is so often the case with cancer cells, they have “responded” with an elegant solution. Instead of battling the oxidation itself (which may be a necessary consequence of transformation), or being wholly reliant on the vagaries of mutation to inactivate c-Cbl or to make its target proteins resistant to c-Cbl-mediated induction of degradation, GBM and BLBC cells (and most likely other cancers) are able to utilize nonmutated proteins to inhibit c-Cbl activation. In this way, the oxidation of cancer cells becomes markedly less relevant to their biology (because they are resistant to the effects of such changes) and enables them to maintain increased levels of multiple proteins that promote division and survival, suppress differentiation, and provide protection against chemotherapeutic agents and other physiological stressors.

The solution that at least some cancer cells employ to overcome oxidative regulation of important cancer control proteins also appears to offer a therapeutically relevant vulnerability that may offer the selectivity that is critical in the development of improved cancer therapies, and that enables exploitation of noncanonical properties of existing drugs. The ability to target both TICs and non-TICs by restoring normal c-Cbl function makes this an attractive opportunity for further investigation. In addition, combining restoration of c-Cbl function with the use of prooxidant activities of TMX to activate the RFC pathway enables us to harness and enhance ERα-independent activities of TMX that have been of wide interest in the cancer community but which have not previously been combined with mechanism-based exploitation. That the toxicities associated with TMX usage, although present, are thus far less than associated with other anticancer agents may make TMX a therapeutic agent of broad utility when combined with restoration of c-Cbl function.

Moreover, in normal cells that already use the RFC pathway for cell regulation (such as O-2A/OPCs), inhibiting a c-Cbl inhibitor should not be problematic, and our research thus far indicates an apparent selectivity for cancer cells of interventions targeted at restoration of c-Cbl function. As seen, restoration of c-Cbl function did not increase sensitivity of O-2A/OPCs to BCNU or temozolomide, or of nontransformed breast epithelial cells to TMX. It should be noted, however, that although the inhibition of c-Cbl by Cool-1/β-pix, Cdc42, or transglutaminase-2 far thus far appears to be a reconfiguration of existing proteins into a novel network utilized by transformed cells, it is important to also consider the possibility that those normal cells that respond to increased oxidation with more rapid division (e.g., [380,381]) might also employ similar circuitries. This is an important topic for future research.

One of the particularly exciting aspects of our discoveries is that they support a key hypothesis underlying our approach: specifically, although cancer arises from varied combinations of mutations and cell types, diverse cancers share multiple properties and multiple differences from normal cells. Might this mean that there is an underlying regulatory architecture that is shared by different cancers and that distinguishes transformed cells from normal cells? When we examined RFC pathway function in glioblastoma (GBM) and BLBC cells, we found that cells from both of these cancers inhibit c-Cbl function and its activation via the RFC pathway. The generality of this finding was particularly intriguing as inhibition of c-Cbl occurred in 5 independent GBM lines isolated so as to enrich for TICs [227] and in 7 different BLBC lines representing three major BLBC subcategories (Basal A, Basal A with Her-2 amplification, and Basal B) [241]. Thus, c-Cbl inhibition fits our primary criteria of occurring in cancers with different genetic backgrounds.

It is particularly striking that there appears to be no way to reach our discoveries using the widely applied tools of genomics research. We are working on nonmutated proteins that become organized in different ways in cancer cells, and none of the critical findings of redox regulation, isoform shifts in Cool-1/β-pix, complexes of Cool-1/β-pix with c-Cbl, or even of the RFC pathway itself could be reached by genomics analysis. Even the integration of genomics and proteomics research would not provide the full detail of our discoveries, which requires further integration with redox analysis—and it is this redox-based analysis that leads to mechanism-supported reasons for focusing attention on TMX as an activator of the c-Cbl pathway in cancer cells in which c-Cbl function has been restored. Moreover, as breast cancers express high levels of Cool-1 protein [367], but we find no effect of Cool-1 knockdown on BLBC cell function [241], it is clear that the integration of all the approaches we have employed is necessary to most effectively explore these novel opportunities for intervention.

It should be noted that the above comments are not meant to argue against the use of genomics-based approaches, just as our

analyses of the role of redox state in cell signaling do not argue against the importance of more standard approaches to analysis of signaling pathway function. Instead, it is the integration of all of these various approaches together that is likely to offer the greatest power going forward. Indeed, we suspect that one of the greatest opportunities going forward will be to combine our work with studies on genetic networks that also are shared by multiple cancers and that arise in specific response to the action of cooperating oncogenes. The network of cooperation response genes (CRGs) was first identified in studies by Hartmut Land and colleagues on cooperation between dominant-negative p53 and constitutively active Ras^{V12} (Ras) mutants in a model of colon cell transformation [382]. Systematic functional analysis demonstrated that CRGs are highly enriched for regulators of malignancy, and resetting expression of individual CRGs to normal cell levels suppressed growth of both experimental tumors and naturally occurring human tumors [382,383]. Restoring normal expression of even a single CRG was, in most cases, sufficient to reduce tumor growth, and similar effectiveness was seen in both experimentally induced cancers and xenograft tumor formation by human cancer cells [382–384].

Regulation of cell transformation by CRGs appears to be a general principle, as CRGs that regulate transformation of colon cells play a role in multiple epithelial tumor types, and particularly in cancers (such as BLBCs) in which p53 and Ras pathway mutations are often found. Although there are distinct CRG sets that contribute to transformation in blast crisis-chronic myelogenous leukemia (induced by BCR-Abl+Nup98-HoxA9), basal cell carcinoma (induced by Hedgehog-GLI+EGFR), and T-cell acute lymphoblastic leukemia (induced by Notch1+TLX1) [382,383,385–387], the CRG principle appears to be broadly applicable. Just as for inhibition of c-Cbl, the individual genetic nuances of different cancer cells appear to be transcended by the generality of CRG importance.

c-Cbl inhibition and CRG expression thus far appear to be independent aspects of cancer cell function (unpublished observations), which raises the question of what would happen if interventions of these two pathways were combined. Such experiments are now in progress, but the concept of simultaneously targeting two core vulnerabilities of multiple cancer cells is inherently attractive based on the value of such approaches in targeting such highly mutable pathogens as human immunodeficiency virus (HIV) and tuberculosis (tubercle bacillus).

The next necessary steps in all of the work discussed are (i) identification of pharmacological interventions that might be of clinical relevance, so that we can target c-Cbl activity—and CRG function—without requiring genetic modification of cells and (ii) discovery of the most efficient means of determining which c-Cbl inhibitor (or/and CRG set) is most critical to a particular population of tumor cells. Discovering such interventions and diagnostic criteria is currently the central goal of the ongoing research of ourselves and our colleagues.

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