Chapter 4
ATF4, Hypoxia and Treatment Resistance in Cancer

Dean C. Singleton and Adrian L. Harris

Abstract Oxygen deprivation (hypoxia) is a common feature of tumors that is associated with treatment resistance and poor patient survival. Hypoxia perturbs the oxidative environment within the endoplasmic reticulum (EnR), limiting protein folding capacity. This restriction causes an accumulation of unfolded proteins in the EnR and activation of a stress response pathway, termed the unfolded protein response (UPR). Signals from the UPR culminate in repression of general protein translation. Paradoxically, a small number of transcripts are selectively translated under these conditions. One of these transcripts encodes Activating Transcription Factor 4 (ATF4). In tumors, ATF4 expression is detected in hypoxic and nutrient-deprived regions. ATF4 promotes metabolic homeostasis and cancer cell survival by transcriptionally regulating numerous processes including amino acid uptake, antioxidant biosynthesis, and autophagy. These changes confer ATF4-expressing cells with a multidrug resistance phenotype and the ability to tolerate adverse stresses of the tumor microenvironment. However, under conditions of persistent and unresolved stress, ATF4 transcriptional reprogramming becomes pro-apoptotic. Therapeutic modulators of ATF4 signaling have the potential to modify these properties by diminishing adaptive phenotypes in cancer cells. Reprogramming cancer cells in this way can improve tumor sensitivity to anticancer treatments including chemotherapy, immunotherapy, and radiotherapy.

Keywords Autophagy · ATF4 · EnR stress · Unfolded protein response · Hypoxia · PERK · GCN2

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Tumor Hypoxia

The tumor microenvironment is characterized by transient fluctuations in oxygen concentration [1]. Areas of oxygen deprivation (hypoxia) develop because the immature and disorganized tumor microvasculature is unable to deliver sufficient oxygen to meet the metabolic demands of the tumor. Hypoxia is commonly observed in solid human tumors. For example, hypoxic regions with $pO_2 < 2.5$ mmHg (equivalent to ~0.3% O_2) are frequent in locally advanced breast tumors but are not detected in normal breast tissue [2, 3]. However, the proportion of hypoxic cells ($pO_2 < 2.5$ mmHg) in tumors is highly variable between patients and can range from 0 to 97% [4]. Hypoxic cancer cells stimulate tumor growth by secreting pro-angiogenic factors, particularly Vascular Endothelial Growth Factor A (VEGFA). In addition, hypoxic cancer cells display enhanced malignant phenotypes including tumor initiation and invasion. Critically, these cells are resistant to killing by radiotherapy and some forms of chemotherapy [3, 5]. Consequently, patients with hypoxic tumors have an increased risk of metastasis and reduced overall survival probability [6–9].

Early reports described hypoxic cells in tumors in perinecrotic regions at distances >150 μm from vessels [10, 11]. These *chronically* hypoxic cells occur because oxygen is consumed as it diffuses away from the blood vessels, resulting in a steep oxygen gradient between oxygenated cells adjacent to the tumor vasculature and severely hypoxic cells in perinecrotic regions (Fig. 4.1a). Hypoxic cells can also exist in closer proximity to the tumor microvasculature following a transient vessel occlusion [12]. These *acutely* hypoxic cells are hypothesized to pose a greater clinical problem than cells that are chronically hypoxic. This is because cells that are transiently hypoxic are likely to be temporarily chemotherapy and radiotherapy resistant, allowing them to survive treatment, and then continue proliferating following re-oxygenation. Furthermore, transcriptionally reprogrammed hypoxic cells that are near tumor vessels pose a higher risk of hematogenous metastasis than chronically hypoxic cells that are confined to perinecrotic regions that are distal to the vasculature [13, 14].

The spatial heterogeneity in acute hypoxia is also subject to fluctuations over time [15]. Animal models have demonstrated that acute hypoxia occurs in a cyclic manner with rapid changes in the subpopulation of acutely hypoxic cells within the tumor [16]. However, despite the common classification used to divide hypoxic tumor cells into two distinct subtypes, the true pathophysiology of tumor hypoxia is likely to reflect both transient and sustained episodes that range from mild oxygen deprivation to complete anoxia, resulting in heterogeneous biological responses that depend on both extrinsic and intrinsic factors.

The adverse effect of tumor hypoxia on patient survival has motivated the development of strategies to target hypoxic cells in tumors [17]. These include prodrugs designed to undergo selective activation in hypoxic cells and molecularly targeted agents developed to interfere with cellular mechanisms of hypoxic adaptation.

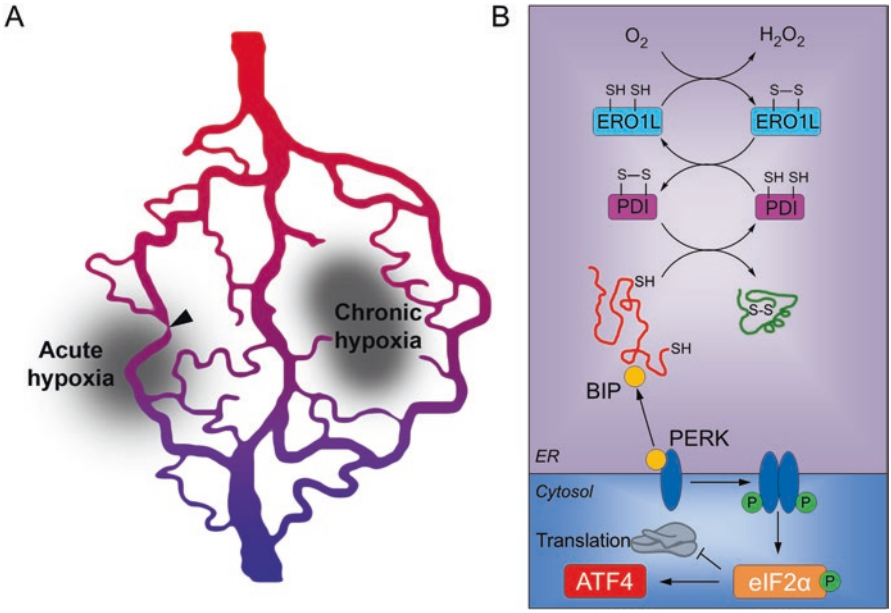

Fig. 4.1 (**a**) Schematic representation of the tumor vasculature demonstrating areas of chronic and acute hypoxia. Regions of the tumor that are beyond the diffusion limits of oxygen are chronically hypoxic. Transient vessel occlusion (arrowhead) results in an acute hypoxic episode in close proximity to the tumor vasculature. (**b**) Oxygen is required for protein folding in the EnR. In this system, protein folding and disulfide bond formation is driven by the enzymes PDI and ERO1L with oxygen acting as the final electron acceptor. Hypoxia limits this reaction resulting in the accumulation of unfolded proteins (red polypeptide). Unfolded proteins sequester the EnR chaperone BIP resulting in the autophosphorylation and activation of PERK. PERK then phosphorylates eIF2α to repress the global rate of protein translation. However, under these conditions ATF4 mRNA is selectively translated

Therapeutic Targeting of Tumor Hypoxia

Hypoxia-activated prodrugs (HAPs) represent a promising, clinically advanced strategy to eliminate hypoxic cells. HAPs are designed to be selectively activated in hypoxic cells. This is achieved via an oxygen-sensitive mechanism of activation that relies on one-electron reductases, particularly cytochrome P450 oxidoreductase (POR) [17]. In hypoxia, HAPs undergo metabolism to species that are more potent than the HAP itself. Whereas, in normoxic cells HAPs undergo minimal conversion to cytotoxic species, resulting in negligible cell killing. This difference in cytotoxicity provides therapeutic selectivity for hypoxic tumor cells. A number of promising HAPs have been developed, with some undergoing Phase II/III clinical investigation including Tirapazamine, Evofosfamide, PR-104, and EO9 [17–20].

Confining HAP activation to conditions of low (pathogenic) oxygen (<0.2% O_2) provides an opportunity to exploit hypoxia as a tumor-specific property because the lower ranges of physiological O_2 tension are approximately 3–9% in normal tissues

[21]. This feature of tumor-selective activation may be particularly useful if cytotoxic metabolites released from HAPs are designed with considerable "bystander" properties so that they can diffuse into nearby oxygenated cells to extend the level of tumor killing beyond the hypoxic region and increase the anticancer activity of the HAP [22]. Development of companion methods, including hypoxia imaging using Positron Emission Tomography (PET) will enable prediction of patients with hypoxic, POR-expressing tumors that are most likely to respond to treatment with HAPs [23–25].

Molecular Targeting of Tumor Hypoxia

An alternative strategy to eliminate hypoxic tumor cells is to exploit the molecular vulnerabilities that occur within this subpopulation. This concept has been motivated by a growing understanding of the biological changes that underpin the pro-survival adaptations to hypoxia [26]. Much of this work has focused on inhibiting the Hypoxia Inducible Factor (HIF) family of transcription factors or targeting phenotypic changes that are dependent on expression of HIF-target genes [27]. Although HIF is a challenging protein to inhibit directly, several drugs that prevent HIF transcriptional function have advanced to clinical evaluation, including PX-478 and PT2385 [28]. Indirect strategies have relied on targeting HIF-dependent genes, for example, *CA9*, *LOX*, *GLUT1,* and *RIOK3* or antagonizing phenotypes that are associated with HIF-target genes, for example, reactivation of mitochondrial respiration using dichloroacetate [29–34].

A number of HIF-independent pathways of hypoxic adaptation have been described including AMP-activated protein kinase (AMPK) signaling and the unfolded protein response (UPR) [35]. The UPR has emerged as an important mechanism that promotes tolerance to cell stress resulting from nutrient deprivation, hypoxia, or exposure to pharmacological agents. The UPR acts as a key link between oxygen availability and the rate of protein translation. Notably, several features of the UPR can be targeted with drugs, providing new strategies to eliminate or modify hypoxic cell behavior in tumors, with the potential to complement or enhance the efficacy of current cancer treatment regimens.

The Unfolded Protein Response Is Activated by Severe Hypoxia

Secreted and cell surface proteins undergo folding, glycosylation, disulfide bond formation, and structural maturation in the EnR. To accomplish these processes, EnR function is strictly dependent on the maintenance of a distinct oxidative environment [36, 37]. Severe hypoxia perturbs the redox potential of the EnR resulting

in accumulation of unfolded client proteins in the ER lumen [37]. During disulfide bond formation, an oxidoreductase (ERO1L) and isomerase (PDI) act as a relay system to transfer electrons from the EnR client protein to molecular oxygen (Fig. 4.1b). Disulfide bond formation is crucial for correct protein folding [38], highlighting the obligatory role of oxygen in EnR function. Recent studies have demonstrated that the initial phase of disulfide bond formation that occurs during protein translation can proceed independently of oxygen, whereas post-translational disulfide bond formation and isomerase steps are oxygen dependent [39].

Accumulation of misfolded proteins in the EnR activates the UPR. The UPR acts to resolve EnR stress by increasing the folding capacity of the EnR, suppressing protein translation and by increasing the rate of degradation of misfolded proteins (by EnR-associated degradation or autophagy). The UPR is initiated by three EnR transmembrane proteins: inositol-requiring enzyme 1α (IRE1α; *ERN1*), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK; *EIF2AK3*) and Activating Transcription Factor 6 (ATF6) [40, 41]. Mechanistically, the accumulated misfolded proteins in the EnR lumen displace BIP (GRP78, *HSPA5*), a molecular chaperone, from the luminal domains of IRE1α and PERK, triggering their dimerization, autophosphorylation, and activation [42].

PERK-eIF2α-ATF4 Signaling

Recent structural, biophysical, and cellular analysis suggest that EnR stress causes a transient tetrameric arrangement of PERK luminal domains, and this organization is required for PERK activity [43]. Activated PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) on serine residue 51 [44]. This post-translational modification results in reversible repression of protein translational initiation. Three additional kinases (HRI, PKR, and GCN2) can phosphorylate eIF2α in response to distinct cellular stress events.

During translation initiation the eIF2 complex (composed of α, β, and γ subunits) binds to GTP and the initiator methionyl-tRNA. This ternary complex associates with eIF1, eIF1A, eIF3, and the 40S ribosomal subunit to form the 43S preinitiation complex (PIC). The PIC scans along mRNA that has been unwound by eIF4F and recognizes the AUG start codon, prompting eIF5-dependent GTP hydrolysis [45]. The GDP-bound eIF2 is then released allowing recruitment of the 60S ribosomal subunit and initiation of translation. The guanine nucleotide exchange factor (GEF) eIF2B then catalyzes the exchange of GDP for GTP to recover eIF2-GTP, enabling another round of translation initiation. This replenishing step is a key regulatory stage in the rate of translational initiation. Importantly, the GEF activity of eIF2B is controlled by the phosphorylation state of eIF2α at serine 51. Phosphorylation at this site causes an increase in affinity of eIF2B for eIF2-GDP, reducing the exchange of eIF2-GDP to eIF2-GTP and limiting the rate of translation initiation [46, 47].

Although the global rate of mRNA translation is repressed during conditions of elevated eIF2α phosphorylation, paradoxically, the translation of a number of

mRNA transcripts, including ATF4, is increased [48]. The translational control of ATF4 expression is dependent on two upstream open reading frames (uORFs) in the 5′ region of the *ATF4* mRNA. When eIF2α phosphorylation is low, eIF2-GTP is abundant. Ribosomes translate the 5′ uORF1 and then scan along the *ATF4* mRNA to reinitiate translation efficiently at the downstream uORF2 [49, 50]. The second (inhibitory) uORF2 overlaps with the start codon of the ATF4 ORF and, therefore, translation of the ATF4 ORF is prevented and cellular levels of ATF4 protein remain low. During conditions where eIF2α phosphorylation is elevated, the availability of eIF2-GTP is reduced and the scanning ribosomes take longer to become competent. This delay allows the ribosome to bypass the inhibitory uORF2 and instead reinitiate translation at the ATF4 ORF, resulting in increased levels of ATF4 translation. Once expressed, the ATF4 protein can translocate into the nucleus and transcriptionally regulate a number of genes required for amino acid synthesis and import, autophagy, redox balance, and angiogenesis [51].

This prevailing model of uORF-based *ATF4* translational regulation has recently been refined to include a role for N^6-Methyladenosine (m6A) [52]. Zhou et al. demonstrated an increased enrichment of m6A within the inhibitory uORF2. Cellular stress resulting in phosphorylation of eIF2α reduced the abundance of this modification, in a process catalyzed by ALKBH5-dependent demethylation. Thus, m6A within uORF2 impedes the scanning ribosomes resulting in increased translation of inhibitory uORF2 and reduced translation of the *ATF4* ORF.

DDX3, an RNA-binding protein, was shown to increase phospho-eIF2α-dependent ATF4 translation via interaction with the eIF4F complex, identifying another factor that contributes to the control of ATF4 expression levels [53]. In addition, mTORC1 promotes ATF4 translation through its uORFs, but acts independently of eIF2α phosphorylation status [54]. These recent findings highlight the important work still needed to fully elucidate the mechanism of ATF4 translational control.

In addition to *ATF4*, several other transcripts undergo preferential translation during episodes of increased eIF2α phosphorylation [55]. For example, CHOP *(DDIT3)* and *GADD34*, target genes that are transcriptionally up-regulated by ATF4, are also regulated by a translational mechanism that depends on 5′ inhibitory uORFs [56, 57].

Activity of ATF4 in Hypoxic Cells

ATF4 is a basic region-leucine zipper transcription factor [58, 59]. In vitro exposure to severe hypoxia results in elevated expression of ATF4 [60, 61]. In agreement, ATF4 expression is observed in hypoxic regions of human tumors and levels of ATF4 are elevated in breast cancer, cervical cancer, melanoma, and glioblastoma compared with corresponding normal tissue [62].

Pro-Survival Versus Pro-death Roles of ATF4

ATF4 promotes adaptation and survival during periods of cellular stress and loss of ATF4 results in hypersensitivity to EnR stress generated by hypoxia [62]. However, in situations where the stress is excessive and cannot be resolved, ATF4 acts to promote cell death, with many reports implicating CHOP in this process. Detailed transcriptomic studies have shed more light on this dichotomy of function [63]. Han et al. found that overexpression of ATF4 resulted in transcription of several known ATF4 responsive genes (*ATF3, GADD34, TRIB3*). The level of transcription was markedly increased by co-expression of CHOP, demonstrating that CHOP co-operates with ATF4. In contrast, CHOP overexpression alone had negligible effect on gene expression. ATF4/CHOP responsive genes were largely involved in stimulating protein synthesis, for example, aminoacyl-tRNA synthetase genes. Notably, ATF4 and CHOP did not induce genes that promote apoptosis, suggesting that they do not directly participate in the transcriptional induction of apoptosis.

Chromatin immunoprecipitation sequencing (ChIP-seq) studies showed that ATF4 and CHOP bind at a common site in the proximal promoter region of these genes at a consensus sequence [63]. Furthermore, direct interaction between ATF4 and CHOP was confirmed by co-immunoprecipitation studies. These findings need to be carefully interpreted and considered alongside the temporal kinetics of EnR stress events. Following treatment with pharmacological inducers of EnR stress, cells display a rapid increase in eIF2α phosphorylation, proceeded sequentially by ATF4 expression, then CHOP expression and then apoptosis. The evidence suggests that early events (translational repression and ATF4 expression) are *pro-survival* responses that are directed towards alleviation of EnR stress. In support of this idea, ATF4 alone (in the absence of CHOP) transcriptionally up-regulates target genes involved in amino acid transport and biosynthesis (e.g., *SLC6A9* and *PSAT1*) [63]. In contrast, later events that depend on CHOP are directed towards re-establishment of protein synthesis. ATF4 and CHOP co-operatively induce expression of genes involved in protein translation including aminoacyl-tRNA synthetases and initiation factors (e.g., *WARS* and *EIF5*). In addition, the late up-regulation of GADD34 leads to dephosphorylation of eIF2α and a resumption of protein translation. Where the EnR stress has been resolved, for example, following re-oxygenation of tumor cells, the activity of ATF4/CHOP would promote a return to basal or even elevated levels of protein synthesis leading to enhanced tumor growth, having carried out earlier transient pro-survival functions. In contrast, during conditions of unresolved EnR stress, for example, chronic hypoxia, ATF4/CHOP would transcriptionally induce protein synthesis resulting in further oxidative stress, ATP depletion, and apoptosis. Thus, the microenvironmental heterogeneity in oxygen tension, nutrient availability, and temporal kinetics of stress within the tumor determine whether ATF4 acts in a pro-survival or pro-death capacity.

Phenotypic Reprogramming by ATF4

ATF4 promotes adaptation to cellular stress by transcriptionally up-regulating genes required for autophagy, redox maintenance, amino acid homeostasis, angiogenesis, and metastasis (Fig. 4.2).

Regulation of Autophagy by ATF4

Macroautophagy (hereafter termed autophagy) is a process whereby cytoplasmic macromolecules, protein aggregates, and organelles, including the EnR and mitochondria, are degraded by the lysosome and recycled [64]. All cells maintain a basal level of autophagy to remove damaged and long-lived proteins that are not degraded via the proteasome. During hypoxia the rate of autophagy is increased to promote cell survival. This increase in autophagy enables recycling of amino acids during episodes of stress, but, perhaps most critically, allows for detoxification and removal of proteins and organelles that have been damaged by reactive oxygen species (ROS) [65, 66]. High levels of ROS are generated during cyclic hypoxic exposures

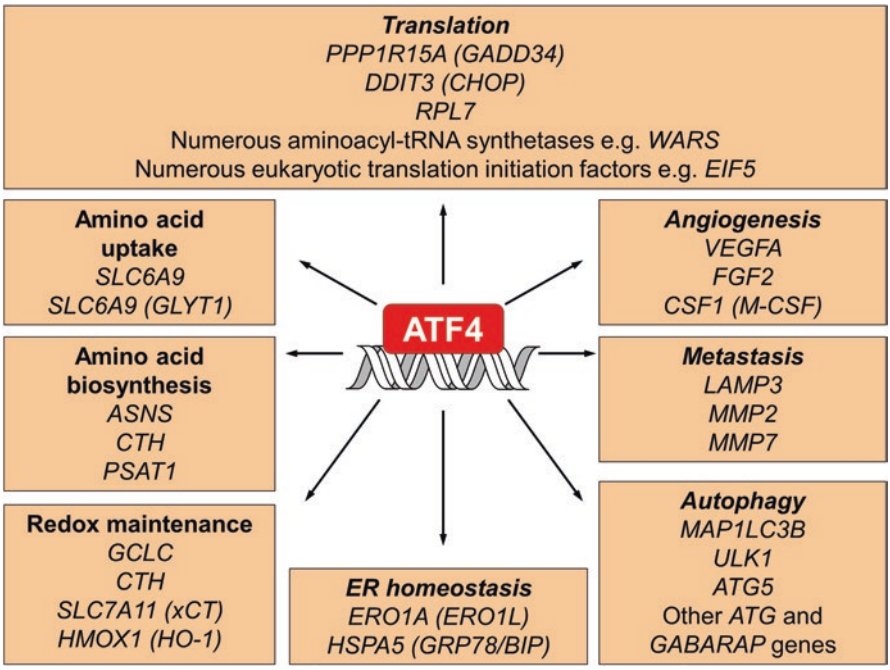

Fig. 4.2 ATF4-dependent processes and examples of target genes that are associated with these processes

[67]. Consequently, inhibitors of autophagy (e.g., choloroquine) sensitize cells to hypoxic stress [66, 68].

ATF4 is an important promoter of autophagy by transcriptionally up-regulating several autophagy-related genes including *MAP1LC3B* (commonly referred to as LC3B) [68, 69]. LC3B is incorporated into autophagosomes during autophagy. During high rates of autophagy LC3B becomes depleted, eventually limiting the rate of autophagy. ATF4-dependent transcription of *MAP1LC3B* helps to maintain LC3B concentrations and sustain high rates of autophagic flux during cellular stress. ATG5, a protein involved in autophagosome elongation, is also induced during EnR stress in an ATF4- and CHOP-dependent manner [69]. Notably, ATF4 also up-regulates ULK1, an inducer of autophagy, in certain cancer cell lines, demonstrating that ATF4 can also transcriptionally promote autophagy initiation [70]. Studies in mouse embryonic fibroblasts (MEFs) have extended the list of autophagy genes that are known to be regulated by Atf4 to include *Atg16l1*, *Atg12*, *Atg3*, *Becn1*, and *Gabarapl2* [71]. Furthermore, Atf4, in combination with Chop, has also been demonstrated to transcriptionally up-regulate *p62* (*Sqstm1*), *Nbr1* and *Atg7* [71].

These studies, and others, underline the important role of ATF4 as a master regulator of autophagy gene transcription [72]. Correspondingly, ATF4 is necessary for autophagy during various states of cell stress, including transformation. Transformation with c-Myc increases cellular rates of protein translation but can also cause apoptosis. Myc-driven protein translation results in higher levels of EnR stress [73]. The PERK-eIF2α-ATF4 pathway supports cyto-protective autophagy in this context. PERK-deficient cells undergo apoptosis following transformation with Myc and PERK is required for the growth of Myc-driven tumors. Similarly, ATF4 is required during transformation of MEFs with H-ras^{V12} and SV40 large T antigen [74]. This work establishes an essential role for the PERK-eIF2α-ATF4 pathway in the transforming activity of oncogenes. Notably, ATF4 can play multiple roles in Myc-driven oncogenesis. Depriving Myc-amplified neuroblastoma cells of glutamine activates the GCN2-eIF2α-ATF4 signaling pathway. In this context, ATF4 promotes cell death by transcriptionally up-regulating *PUMA*, *NOXA*, and *TRIB3* [75].

Role of ATF4 in Redox Maintenance

During hypoxia and other tumor microenvironmental stresses cancer cells can experience supraphysiological levels of ROS [67, 76]. These species cause damage to proteins and organelles resulting in cytotoxicity. ATF4 helps to protect cells from excessive oxidative stress by increasing levels of glutathione (GSH), a key cellular antioxidant. Increased GSH is achieved by transcriptionally up-regulating glycine import (e.g., *GLYT1*) [51], cysteine import (e.g., *SLC7A11* and *SLC1A4*) [77, 78], cysteine availability (e.g., *CTH*) [51, 79], and GSH biosynthesis (e.g., *GCLC*) [80]. Cells that lack ATF4 have lower levels of GSH and greater sensitivity to oxidative stress [79].

Role of ATF4 in Cell Metabolism and Amino Acid Homeostasis

Initial studies using ATF4 knockout MEFs demonstrated impaired cell growth kinetics compared with wild-type counterparts [51]. However, this proliferative defect was rescued by addition of non-essential amino acids (NEAA) and reducing agents, such as β-mercaptoethanol, into the culture medium. Similar effects are observed in cancer cells; ATF4 knockdown reduced cell survival and proliferation and increased apoptosis [81]. These defects were rescued by addition of either NEAA or by asparagine alone or by overexpression of asparagine synthetase (*ASNS*). Furthermore, ATF4 expression was necessary for growth of tumor xenografts [81].

Further work has confirmed the importance of the GCN2-eIF2α-ATF4 pathway as a key sensor of amino acid depletion. Upon activation, ATF4 triggers an adaptive response involving up-regulation of several genes encoding amino acid uptake and synthesis proteins. The in vitro induction of this program has been reported following depletion of several individual amino acids including methionine, asparagine, leucine, tryptophan, serine, glutamine, and cysteine, although the sensitivity to depletion of individual amino acids likely depends on the plasticity of the cellular metabolic environment and is thus cell line- and cell type dependent [82–86]. Induction of the amino acid stress response was particularly notable in conditions where autophagy was impaired and glutamine concentrations were constrained [87].

When serine concentrations are reduced, the GCN2-eIF2α-ATF4 pathway is activated, and this results in the transcription of *PHGDH*, *PSAT1,* and *PSPH* and increased serine biosynthesis [85]. Serine acts to positively regulate Pyruvate Kinase M2 (PKM2) enzyme activity. When serine levels are low, PKM2 activity is repressed leading to an accumulation of glycolytic intermediates that can feed into the serine biosynthetic pathway [88]. Thus, control of serine biosynthesis depends on the concerted activity of PKM2 and ATF4. Maintenance of intracellular serine levels is required to support mTORC1 activity and sustain cell proliferation.

In vivo, fluctuating concentrations of amino acids, particularly glutamine, can occur in the tumor microenvironment, potentially in concurrence with hypoxia and glucose limitation (i.e., ischemia) [89–91]. Activation of the GCN2-eIF2α-ATF4 pathway in response to glutamine deprivation provides an adaptive feedback mechanism to increase amino acid uptake [92]. ATF4 expression also occurs in response to pharmacological glutamine deprivation following treatment with glutaminase inhibitors [93].

A more complex picture of this biological pathway has recently emerged. In *KRAS*-mutant non-small cell lung carcinoma (NSCLC) ATF4 plays a key role in disease biology through regulation of amino acid metabolism [94]. Gwinn et al. demonstrated that KRAS-PI3K-AKT-NRF2 signaling in this context was required for expression of ATF4 in response to cell growth in conditions of physiological glutamine abundance (0.5 mM). Upstream GCN2-phospho-eIF2α signaling was implicated in the elevated expression of ATF4 observed in these conditions. However, further work highlighted

the importance of genetic context in these processes; *KRAS* mutant cells with additional *KEAP1* mutation, leading to NRF2 activation, demonstrated improved survival in conditions of glutamine deprivation when ATF4 was knocked down. Thus, the abundant ATF4 in these cells acts in a pro-apoptotic manner during conditions of amino acid starvation. In contrast, *KRAS* mutant/*KEAP1* WT or *KRAS* WT/*KEAP1* WT cells did not benefit from ATF4 knockdown during conditions of nutrient stress. However, the pro-survival effect of ATF4 loss in the *KRAS* mutant/*KEAP1* mutant context was not seen in xenograft models, where ATF4 knockout caused impaired tumor growth, suggesting that nutrient stress in tumors is not sufficiently strong to activate the pro-apoptotic effects of ATF4 observed in glutamine-starved cell culture conditions. Thus in vitro models replace do not always correlate with in vivo microenvironmental stresses.

ATF4 activity can also contribute to amino acid homeostasis in the tumor microenvironment via metabolic effects in the stromal cells. In particular, cancer-associated fibroblasts display increased stability of ATF4 due to reduced p62-dependent ubiquitination (p62 levels are commonly reduced in cancer-associated fibroblasts compared with normal fibroblasts) [95]. The associated metabolic reprogramming in these cells provides a de novo source of glucose-derived asparagine that can support cancer cell growth, particularly during episodes of glutamine deprivation.

Role of ATF4 in Cell Invasion and Metastasis

Hypoxic activation of the PERK-eIF2α-ATF4 pathway in tumors has been linked to an increased risk of metastasis [96]. ATF4 can stimulate metastasis by promoting cellular tolerance to hypoxia, as discussed earlier, and by enhancing the metastatic properties of cancer cells. Hypoxia is a recognized enhancer of metastasis [97]. Hypoxia promotes numerous steps in the metastatic cascade; remodeling of the extracellular matrix (ECM), intravasation and extravasation of cancer cells, and by promoting a less differentiated cellular state with enhanced motility and invasive properties. Many of these biological changes depend on HIF-1 transcriptional activity, yet ATF4 also contributes in distinct roles. For example, when detached from the ECM, cells experience increased levels of oxidative stress, predisposing them to apoptosis (anoikis). During this process, ATF4 expression is increased and it supports cell survival by transcriptionally up-regulating autophagy via *ATG5*, *ATG7*, and *ULK1* [65]. ATF4, in co-operation with NRF2, induced the transcription of cellular antioxidant responses, particularly the antioxidant enzyme hemeoxygenase 1 (*HO-1*). HO-1 acts to reduce ROS levels following matrix detachment and prevent anoikis. Importantly, these steps are necessary for colonization of cells in the lungs of mice, demonstrating that ATF4 is required for metastasis in this context [65]. PERK has also been shown to promote survival following ECM detachment by inducing autophagy through LKB1-AMPK signaling [98]. Consistently, circulating tumor cells in the bloodstream have increased ATF4 expression [99].

The epithelial-mesenchymal transition (EMT) is a process of transcriptional reprogramming that causes carcinoma cells to reversibly shift into a less differentiated state [100]. During EMT, cells lose their epithelial characteristics including cell-cell adhesion and cellular polarity and acquire mesenchymal attributes including motility, invasiveness, and stem cell-like characteristics. These changes in phenotype promote cancer cell dissemination and metastasis [101, 102]. Tumor hypoxia stimulates cells to undergo EMT, and HIF-1 participates in this process [103]. During EMT, cells increase the expression of secreted ECM proteins, and this results in an increase in EnR stress and a reliance on PERK-eIF2α-ATF4 signaling for maintaining proteostasis and cell survival [104]. Exacerbating this EMT-induced stress using pharmacological EnR stressors including tunicamycin and thapsigargin results in cell death. Similarly, cells that have undergone EMT display increased sensitivity to PERK inhibition, further demonstrating that PERK-eIF2α-ATF4 signaling is required to sustain EMT biology [104].

Independent reports showed that knockdown of ATF4 prevented EMT in gastric cancer cells exposed to severe hypoxia [105]. ATF4 knockdown reduced cell migration, invasion, and metastasis, whereas ATF4 overexpression enhanced these processes [106]. Consistent with these findings, analysis of clinical transcriptomic datasets demonstrated that EMT and ATF4 gene signatures were strongly correlated in their expression across several tumor types including breast, colon, gastric, and lung cancer [104].

ATF4 has also been implicated in non-canonical (β-catenin-independent) Wnt signaling [107]. In this report, non-canonical Wnt ligands (Wnt5a/b) signal via ROR2, DVL2, ATF2, and ATF4 to promote proliferation of colon cancer cells in a β-catenin-independent manner.

Metastasis depends on remodeling of the ECM. ATF4 can stimulate ECM remodeling by transcriptionally up-regulating the matrix degrading enzymes MMP2 and MMP7 [106]. ATF4 also promotes metastasis by transcriptionally up-regulating lysosomal associated membrane protein 3 (*LAMP3*) [96]. LAMP3 is required for cell migration and invasion during hypoxia and for metastasis in animal models although the exact mechanism has not been fully elucidated [108–110].

Recently, co-recruitment of ATF4 with steroid receptor coactivator-3 (SRC-3) was demonstrated in highly glycolytic breast cancers [111]. In this study, the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) was shown to phosphorylate SRC-3 at serine 857 resulting in increased transcriptional activity. Activated SRC-3 supported purine biosynthesis via transcriptional up-regulation of transketolase (*TKT*), adenosine monophosphate deaminase-1 (*AMPD1*), and xanthine dehydrogenase (*XDH*). This transcriptional program and recruitment of SRC-3 to gene promoters was dependent on direct interaction with ATF4, in conditions that favored elevated glycolysis (high glucose, PFKFB4 expression). SRC-3 expression promoted both the growth and metastatic dissemination of breast tumor xenograft models. Furthermore, phosphorylated SRC-3 was associated with worse survival outcomes in breast cancer patients. Thus, the metabolic re-orchestration mediated by ATF4 is co-opted in aggressive cancers and results in poorer outcome.

These studies highlight the important role that ATF4 biology plays at multiple stages of the metastatic cascade including maintenance of the EMT phenotype, resistance to anoikis, and promotion of ECM remodeling.

ATF4 Promotes Angiogenesis

Cells respond to hypoxia by secreting pro-angiogenic factors in an attempt to increase tissue oxygenation. HIF-1 plays a particularly important role in this response. In addition, several studies have demonstrated that the PERK-eIF2α-ATF4 pathway also contributes to tumor angiogenesis [112]. For example, tumors comprised of PERK-deficient cells grow slower than control tumors and have reduced microvessel density [113]. Similarly, potent and selective ATP-competitive small molecule inhibitors of PERK also display anti-angiogenic effects in tumor xenograft models, supporting the notion that PERK-eIF2α-ATF4 signaling is pro-angiogenic in tumors [114].

These observations are supported by the demonstration that ATF4 promotes transcriptional up-regulation of several pro-angiogenic factors during episodes of glucose deprivation [115]. Glucose deprivation or treatment with pharmacological EnR stressors activated the UPR resulting in PERK- and ATF4-dependent induction of *VEGFA*, *FGF2,* and *IL6*. ChIP studies confirmed ATF4 binding at the *VEGFA* gene promoter region. Amino acid deprivation also results in up-regulation of VEGFA secretion [116]. In this situation, both GCN2 and ATF4 are required for *VEGFA* induction. Tumor xenografts consisting of GCN2-deficient cancer cells grow with slower kinetics and have reduced microvessel density compared with controls [116]. Consistently, ATF4 was required for hypoxic transcription of *VEGFA* in osteoblasts. However, in this situation ATF4 was demonstrated to act by promoting the stability of HIF-1α [117]. This interesting finding highlights the potential for crosstalk between transcriptional responses during hypoxia.

ATF4 overexpression has been demonstrated to promote tumor growth by increasing microvessel density [118]. In this study, the effect was due to recruitment of pro-angiogenic macrophages to the tumor via ATF4-dependent secretion of macrophage colony stimulating factor (M-CSF). However, in contrast to previous findings, Liu et al. did not observe ATF4-dependent transcription of *VEGFA*, highlighting the potential for cell-type specific differences in these pathways. A recent report highlighted the importance of ATF4 activity in the disease progression of endometrial cancer [119]. In this work, the transcriptional up-regulation of *CCL2* by ATF4 was responsible for stimulation of tumor growth by increasing tumor recruitment and infiltration of macrophages.

GCN2-eIF2α-ATF4 signaling also promotes angiogenesis in conditions of sulfur amino acid (methionine and cysteine) deprivation [120]. Dietary restriction of sulfur amino acids results in GCN2/ATF4-dependent angiogenesis in mouse skeletal muscle. In this context, ATF4 is required for transcriptional up-regulation of cystathionine-γ-lyase (in addition to induction of VEGFA), leading to increased production of H_2S. H_2S acts by repressing mitochondrial electron transport and oxidative phosphorylation in endothelial cells resulting in activation of AMPK signaling and increased glucose uptake and glycolytic ATP production. These pro-angiogenic effects occur independently of hypoxia or HIF-1α.

Thus, ATF4 directs diverse biological functions that support several pro-angiogenic mechanisms.

ATF4 Activity in Tumor Infiltrating Immune Cells

While ATF4 confers pro-survival characteristics to cancer cells allowing them to resist stresses of the tumor microenvironment, this same activity can provide metabolic resilience to immune cells, possibly allowing for stronger antitumor T cell responses. For example, ATF4 expression is increased in $CD4^+$ T cells in response to oxidizing environments and amino acid deprivation [121]. ATF4 stimulates amino acid synthesis and uptake to restore mTORC1 activity, drives metabolic reprogramming to induce glycolysis, glutaminolysis, and oxidative phosphorylation, and provides nutrients for both anabolic and energetic needs. Ultimately, ATF4 activity is required for proper differentiation of T helper cell (Th) subsets. The implications of this finding on tumor biology have not yet been widely investigated. However, it is likely that ATF4 activity has key roles in maturation and resilience of subsets of antitumor immune cells, and these roles need to be considered in the development of ATF4-targeted therapeutics.

Intercellular Transmission of the Unfolded Protein Response

Cells experiencing elevated levels of EnR stress have been found to secrete signals that result in increased UPR signaling in neighboring cells [122]. This transmissible form of EnR stress, termed TERS, and the increased PERK-ATF4 signal in recipient cells, resulted in improved cellular fitness and impaired responsiveness to either proteasome inhibition or taxane treatment. This newly appreciated phenomenon provides an additional mechanism that may "prime" cancer cells within the tumor with adaptive pro-survival properties before they experience episodes of microenvironmental or pharmacologic stress.

Role of ATF4 in Therapy Resistance

ATF4 Activation in Resistance to Chemotherapy

Acquired drug resistance is a major reason for failure of chemotherapeutic agents. ATF4 has been implicated in cellular resistance to several anticancer agents including cisplatin, doxorubicin, etoposide, gemcitabine, SN-38, and vincristine [80, 123, 124]. Several reports have demonstrated that ATF4 knockdown sensitized cells to these agents, whereas ATF4 overexpression conferred drug resistance [80, 125]. This multidrug resistance phenotype has been attributed to drug efflux via ATF4-dependent transcription of ATP-binding cassette (ABC) membrane transporters ABCC2 and ABCG2 (Fig. 4.3a) [80]. ATF4-dependent transcription of STAT3 (and its target genes BCL2, Survivin, and MRP1) caused a multidrug resistance

phenotype in esophageal cancer cells [125]. Furthermore, ATF4-dependent GSH biosynthesis enhanced drug resistance, and this was abolished by an inhibitor of GSH biosynthesis (Fig. 4.3b) [80, 126]. Up-regulation of sirtuin 1 (SIRT1) has also been reported as an ATF4-induced mechanism of multidrug resistance [127]. SIRT1 plays multiple roles in drug resistance including inhibition of pro-apoptotic responses, promotion of DNA damage repair, and acquisition of cancer stem cell properties [128]. Recent work also demonstrated a role for ATF4 activity in conferring resistance to cell death by ferroptosis, via up-regulation of the xCT glutamate/cystine antiporter subunit *SLC7A11* [112].

ATF4 is induced in response to certain drugs, where it acts as a resistance mechanism. For example, proteasome inhibition by Bortezomib causes an accumulation of misfolded proteins in the EnR. These proteins can be degraded by autophagy to support restoration of EnR homeostasis (Fig. 4.3c). ATF4 acts to transcriptionally up-regulate LC3B and enhance autophagy during Bortezomib treatment [129]. Knockdown of ATF4 suppressed this activation of autophagy and sensitized breast cancer cells to Bortezomib, highlighting an important role for ATF4 in cellular resistance to proteasome inhibitors. ATF4 also promotes cyto-protective autophagy in head and neck cancer cells treated with the next generation proteasome inhibitors, Carfilzomib and Oprozomib [130] and *MYCN*-amplified neuroblastoma cells treated with the GLI1/2 inhibitor, GANT-61 [131]. Import of cystine via ATF4-dependent up-regulation of xCT has also been implicated in cellular resistance to proteasome inhibition [78].

In addition to killing cells, many chemotherapeutic agents also cause therapy-induced senescence (TIS) [132]. TIS can have beneficial effects including inhibition of tumor growth. However, retention of senescent cells within tumors can have adverse effects if these cells acquire a senescence-associated secretory phenotype that results in the increased secretion of pro-inflammatory cytokines. This change in phenotype to a highly secretory state places an increased load on the EnR resulting in reliance on ATF4 and CHOP and increased dependence on autophagy to maintain proteostasis (Fig. 4.3d) [133]. This work highlights the potential utility of targeting PERK-eIF2α-ATF4 signaling to eliminate or modify TIS secretory biology and improve the efficacy of conventional chemotherapeutic drugs.

A link between sorafenib pharmacology and ATF4 biology was recently uncovered [134]. In acute myeloid leukemia (AML) with an internal tandem duplication (ITD) in Fms-related tyrosine kinase 3 (FLT3) sorafenib treatment resulting in inhibition of FLT3 signaling causes downregulation of ATF4. Reduction in ATF4 levels de-represses the negative regulation of interferon regulatory factor 7 (IRF7) leading to elevated transcription and production of IL-15. The increased IL-15 production by FLT3-ITD AML cells generated metabolically capable leukemia-reactive $CD8^+$ T cells. This finding has significant clinical implications for the treatment of AML using allogeneic hematopoietic cell transplantation, highlighting the utility of sorafenib, and negative impact of ATF4, in this setting.

ATF4 also contributes to cellular resistance to ferroptosis, a recently described iron-dependent mechanism of cell killing, with significant potential to be exploited

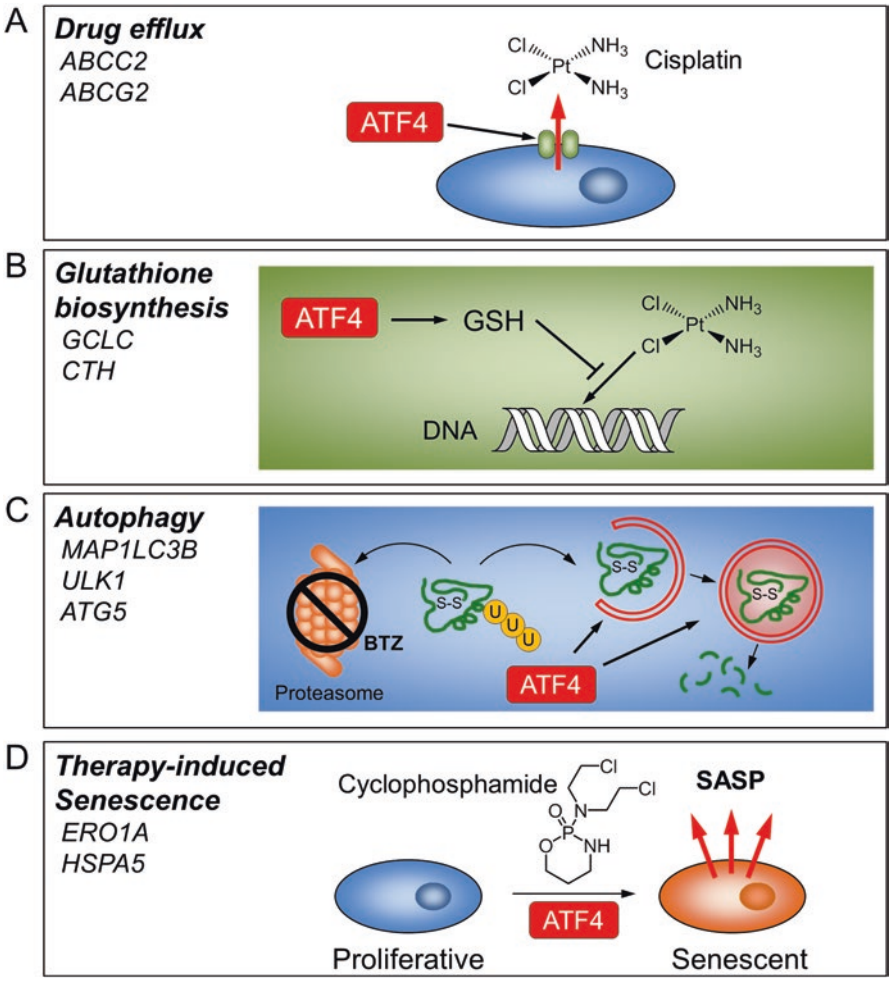

Fig. 4.3 Mechanisms of ATF4-dependent drug resistance. (**a**) ATF4 transcriptionally up-regulates drug efflux transporters including *ABCC2* and *ABCG2*. (**b**) ATF4 transcribes genes that control glutathione biosynthesis including *GCLC* and *CTH*. Glutathione can inactivate cisplatin by producing cisplatin-thiol conjugates, antagonizing its cytotoxic properties. (**c**) ATF4-dependent induction of autophagy can degrade toxic protein aggregates that occur during proteasomal inhibition with Bortezomib (BTZ) to promote drug resistance. (**d**) Treatment with cytotoxic chemotherapeutic agents, including cyclophosphamide, can result in cellular senescence leading to a senescence-associated secretory phenotype (SASP). Acquisition of this state places increased load on the EnR and ATF4 is required to maintain EnR homeostasis during this stress

for clinical benefit [135]. In addition to promoting glutathione synthesis, ATF4 has also been demonstrated to promote GPX4 stability via transcriptional activation of GRP78 [136]. This resistance to ferroptosis mediated by GRP78–GPX4 interactions reduced the sensitivity of pancreatic cancer cells to gemcitabine.

ATF4 Activation in Sensitivity to Chemotherapy

Although ATF4 acts in a pro-survival manner in some situations, it can also contribute to cell death especially when metabolic and EnR stress are excessive or prolonged (as described earlier). ATF4 activity has also been demonstrated as a requirement for drug responsiveness. Notable examples include apoptosis induced by the BRAF-inhibitor vemurafenib [137], combination treatment with BRAF/MEK inhibitor in *NRAS* mutant malignant melanoma [138], apoptosis induction by the NEDD8-activating enzyme inhibitor, MLN4924 [139], p53-independent killing by ONC201 [140, 141], and transcriptional activation of the pro-apoptotic BCL-2 family protein, *NOXA*, in response to cisplatin treatment in p53-null head and neck squamous cell carcinoma cells [142]. ATF4 induction using salubrinal, a selective eIF2α phosphatase inhibitor, also resulted in NOXA up-regulation that sensitized glioma cell lines to temozolomide. Yet, conflicting reports highlight a role for ATF4 in glioma resistance to temozolomide, largely due to transcriptional control of xCT [143].

In some cases, the pro-apoptotic increase in cytosolic Ca^{2+} is a likely trigger of EnR stress, presumably due to depletion of EnR Ca^{2+} stores [137]. Knockdown of ATF4 in this scenario modestly *reduced* the induction of apoptosis, implying that the PERK-eIF2α-ATF4 pathway plays a pro-apoptotic role in drug response. Leukemic cells treated with another RAF inhibitor, sorafenib, also experience increased cytoplasmic Ca^{2+} and induction of EnR stress resulting in activation of all three branches of the UPR [144]. However, in this context, disruption of PERK activity or inhibition of eIF2α phosphorylation *increased* sorafenib-mediated lethality, suggesting that the PERK-eIF2α-ATF4 pathway was anti-apoptotic. Notably, in both reports the observed effects were independent of MEK inhibition. These findings implicate EnR stress in the response to RAF inhibition, but present conflicting outcomes with respect to whether the UPR is pro- or anti-apoptotic.

PERK-eIF2α Signaling in Resistance to Radiotherapy

Hypoxic cells are resistant to killing by radiation [145, 146]. This occurs because molecular oxygen is required to fix DNA free radicals produced by radiation to generate DNA damage and cytotoxicity. Hypoxic cells that survive radiotherapy (RT) can re-populate the tumor and compromise the efficacy of treatment. Selective targeting of hypoxic cells is an effective strategy to overcome this problem [147, 148].

Studies have investigated the potential of targeting molecular changes in hypoxic cells to enhance the response to radiation therapy. Cellular signaling that depends on phosphorylation of eIF2α can be inhibited by expression of a c-terminal fragment of GADD34 (GADD34c) or by a dominant negative eIF2α mutant (S51A). These models have been used to compare the radiation responsiveness of phospho-

eIF2α signaling defective vs. HIF-1-deficient tumor xenografts (shHIF-1α) [149]. Tumors consisting of either phospho-eIF2α signaling defective or HIF-1-deficient cell types had approximately half the number of viable hypoxic cells, confirming that both pathways are important for maintaining hypoxic cells in tumors. Radiation treatment caused a significantly longer growth delay in tumors with phospho-eIF2α signaling defects compared with control tumors. In contrast, knockdown of HIF-1 prior to and during RT (*induced 4 days before to 3 days after initiation of RT*) did not increase the tumor radiosensitivity. This suggests that although both phospho-eIF2α and HIF-1 support hypoxia tolerance in tumors, only phospho-eIF2α is required for maintenance of the radiotherapy-resistant hypoxic subpopulation of cells. Although HIF-1 is not required for cellular tolerance to radiation per se, it is important for tumor regrowth after irradiation, presumably by acting to induce vasculogenesis [149–151]. These findings highlight important differences in the hypoxia tolerance phenotypes mediated by phospho-eIF2α and HIF-1.

Targeting ATF4

Targeting transcription factors with small molecules is challenging due to the large protein–protein and protein–DNA interactions that are implicated in transcription factor activity, although recent successes support the utility of this strategy, for example, HIF-2α-targeting using PT2385 [152]. Rather than targeting ATF4 directly, an alternative approach is either to reduce ATF4 translation by inhibiting upstream eIF2α kinases, or to target phospho-eIF2α signaling itself (Fig. 4.4).

Targeting eIF2α Phosphorylation-Dependent Signaling

A large cell-based screening effort resulted in the discovery of ISRIB, an inhibitor of eIF2α phosphorylation-dependent signaling [153]. ISRIB is a symmetric bis-glycolamide small molecule that inhibits ATF4 activity by interfering with signaling downstream of eIF2α phosphorylation (Fig. 4.4) [153, 154]. ISRIB prevented endogenous ATF4 expression following EnR stress but did not inhibit PERK activation (autophosphorylation) or the IRE1α-XBP1 and ATF6 branches of the UPR. Consequently, the transcriptional up-regulation of ATF4 target genes, *DDIT3* and *GADD34*, was prevented in ISRIB-treated cells. Treatment of cells with ISRIB alone had minimal effect on cell viability. However, ISRIB increased cell death when combined with EnR stress compared with cells treated with EnR stress alone.

The molecular mechanism of action for ISRIB was recently elucidated [155, 156]. These reports describe the structural basis for ISRIB binding to eIF2B, which prevents translation repression and ATF4 signaling in response to eIF2α phosphorylation. ISRIB represents a promising new small molecule for blocking ATF4 expression and further studies investigating its antitumor activity are warranted.

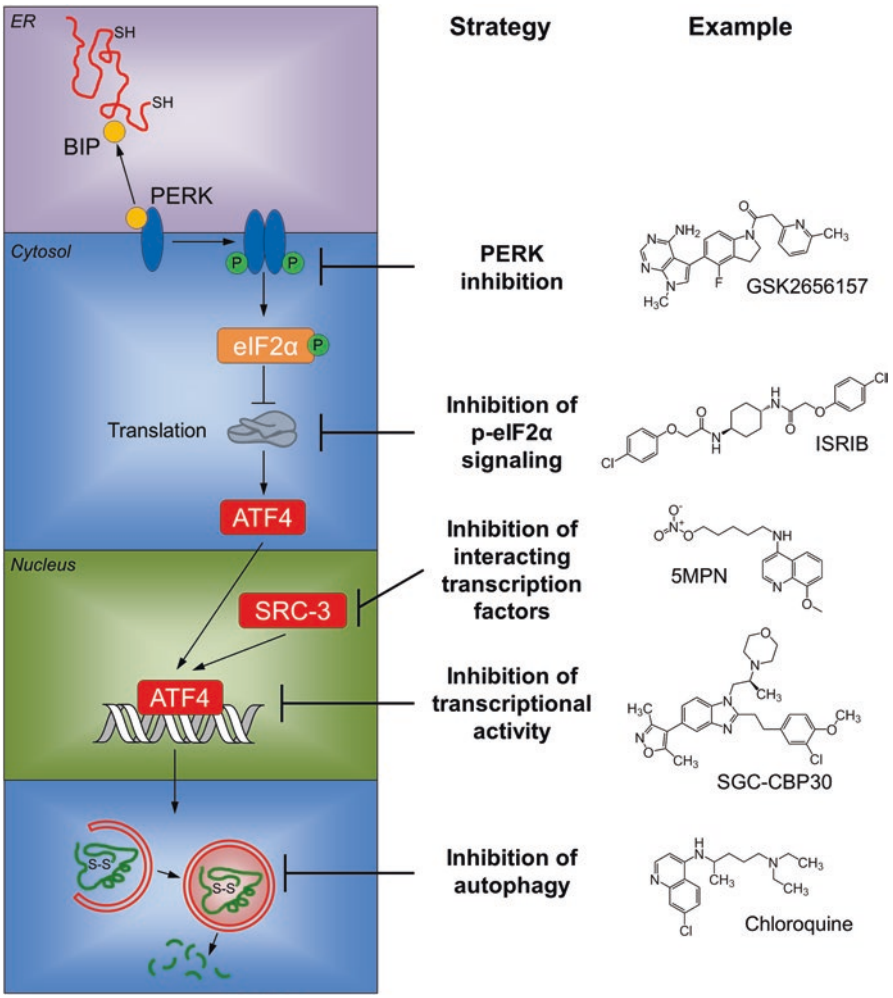

Fig. 4.4 Stages of the PERK-eIF2α-ATF4 pathway that can be targeted with drugs. Inhibition of PERK kinase activity with ATP-competitive inhibitors, e.g., GSK2656157. Inhibition of phospho-eIF2α signaling to ATF4 can be achieved using ISRIB. Interfering with the activation of interacting transcription factors, e.g., SRC-3 using 5MPN to inhibit PFKFB4 activity. Inhibition of ATF4 transcriptional activity may be possible by targeting p300 although this is yet to be demonstrated. Inhibition of downstream processes that depend on ATF4-dependent transcription, e.g., autophagy, is an alternative strategy to target hypoxic ATF4-expressing cancer cells

Targeting PERK

A potent and highly selective PERK inhibitor (GSK2606414) was developed by GlaxoSmithKline [157]. Further optimization of this series resulted in GSK2656157, an orally active ATP-competitive inhibitor of PERK with an IC_{50} of 0.9 nM [114]

(Fig. 4.4). In cells, GSK2656157 inhibited EnR stress-induced phosphorylation of eIF2α and prevented ATF4 expression at concentrations of 10–30 nM. Consequently, the transcriptional up-regulation of ATF4 target genes (*DDIT3*, *HERPUD1*, and *DNAJB9*) was reduced by GSK2656157 in cells treated with tunicamycin to induce EnR stress. GSK2656157 reduced growth of three pancreatic xenograft models and one myeloma xenograft model confirming the anticancer potential of small molecule inhibitors of this pathway. Dynamic contrast-enhanced MRI imaging of tumor xenografts demonstrated that GSK2656157 caused a reduction in vascular perfusion. Furthermore, immunohistochemistry of treated tumors confirmed reduced blood vessel density, emphasizing the importance of PERK in promoting tumor angiogenesis. However, GSK2656157 caused reversible dose-dependent on-target pancreatic toxicity in mice, consistent with previous findings from studies using an inducible PERK knockout system in adult mice [158]. These findings highlight the importance of PERK in pancreatic physiology and emphasize the caution required with clinical development of PERK-targeted therapeutics.

Studies using GSK2606414 demonstrated that pharmacologic inhibition of PERK can sensitize cells to severe or moderate hypoxia (0.2% O_2) [159]. Consistently, cells exposed to PERK inhibitor were highly sensitized to thapsigargin-induced EnR stress. In contrast, a small molecule inhibitor of IRE1α did not reduce cellular tolerance to hypoxia, despite effectively inhibiting IRE1α-dependent splicing of XBP-1. These findings suggest that PERK inhibitors represent a unique approach for preventing UPR-dependent hypoxia tolerance. Further work is needed to define why IRE1α inhibition failed to sensitize cells to severe hypoxia, while shRNA-mediated knockdown of IRE1α did. A second class of orally active PERK inhibitors has recently been reported, providing an independent chemical class to confirm these findings [160].

Although IRE1/XBP1 inhibition was unable to prevent hypoxia tolerance, recent studies have highlighted important opportunities for using IRE1/XBP1-targeted therapies in cancer treatment [161]. In particular, IRE1-XBP1 signaling was found to be induced by MYC in MYC-hyperactivated breast cancers [162]. In this work, pharmacological targeting of IRE1 RNase activity, using the small molecule inhibitor 8866, impaired tumor growth, and enhanced tumor response to docetaxel. Further studies in Triple receptor Negative Breast Cancer (TNBC) cells showed that IRE1 inhibition was effective in reducing the secretion of pro-inflammatory cytokines (particularly IL-6, IL-8, and CXCL1), even after their induction by paclitaxel [163].

A potential mechanism that may permit tumor resistance to eIF2α kinase inhibitors has been proposed [164]. GCN2 deficiency reduces growth of tumor xenografts [81], however, loss of GCN2 in an autochthonous tumor model had no effect on tumor growth [164]. Further investigation revealed that these GCN2-deficient tumors activated PERK as a compensatory mechanism to maintain up-regulation of ATF4, apparently in an eIF2α phosphorylation-independent manner, although this finding needs further clarification. This work highlights the potential for compensatory signaling by other eIF2α kinases when a single eIF2α kinase is selectively

inhibited. Studies to determine whether similar compensatory mechanisms occur in response to PERK pharmacological inhibition are warranted.

Treatment of EnR-stressed cells with an IRE1-XBP1 inhibitor resulted in reduced PERK-ATF4 signaling via a reduction in total eIF2α levels [165]. Notably, the degradation in eIF2α observed relied on PERK-dependent promotion of autophagy. This work highlights how layers of crosstalk between the UPR pathways can be exploited through selective inhibition of individual branches of the UPR to modify the activity of the other branches.

Targeting ATF4 Transcriptional Activity

Targeting post-translational modifications that are required for transcription factor function represents another promising strategy to modulate transcriptional activity. ATF4 is modified by phosphorylation, ubiquitination, and acetylation events. Details of ways to target these changes are reported elsewhere [59].

Recently, much focus has turned to the role of chromatin organization in the regulation of transcription factor activity and definition of cell type identity [166]. This interest in epigenetic control has stimulated the development of potent and selective small molecule inhibitors of epigenetic writers, erasers, and reader proteins [167]. Of interest is the transcriptional co-activator, p300 which is reported to promote ATF4 transcriptional activity by preventing its degradation [168]. Selective inhibitors of both the p300 bromodomain (SGC-CBP30, I-CBP112) and acetyltransferase domain (A-485) have been developed [169–171]. These compounds may provide an opportunity to antagonize the interaction between ATF4 and p300 leading to ATF4 deacetylation, displace the ATF4-p300 complex from acetylated chromatin and/or prevent the establishment of histone acetylation at ATF4-regulated sites in the epigenome. Each of these outcomes would be expected to reduce the level of ATF4 transcriptional activity (Fig. 4.4).

The newly recognized interaction of ATF4 with (PFKFB4-dependent) phosphorylated SRC-3, highlights another opportunity for therapeutic modulation [111]. In cancer cells, PFKFB4 is an important metabolic orchestrator of glycolytic and pentose phosphate pathway activity. 5-(*n*-(8-methoxy-4-quinolyl)amino) pentyl nitrate (5MPN) was reported as a pharmacological inhibitor of PFKFB4 [172]. Inhibition of PFKFB4 activity may provide a strategy to suppress SRC-3 phosphorylation, destabilize ATF4, and prevent the downstream pro-survival adaptive measures.

In certain conditions, it may be beneficial to increase ATF4 activity to enhance or provoke tumor cell death. This may be a desirable outcome in scenarios where ATF4 has a well-characterized pro-apoptotic role, for example, in V600E mutant BRAF melanoma cells following treatment with vemurafenib [137]. Reducing eIF2α dephosphorylation to sustain ATF4 expression can be achieved using the GADD34 inhibitors Sephin1, guanabenz, and salubrinal [173–175].

Targeting Downstream Processes That Depend on ATF4 Transcriptional Activity

ATF4 promotes adaptation to hypoxia, nutrient stress, and ROS by transcriptionally initiating and sustaining autophagic flux. Therefore, inhibiting autophagy is a promising strategy for targeting cancer cells that depend on ATF4 for survival (Fig. 4.4). Cells treated with chloroquine, an inhibitor of lysosomal acidification that prevents degradation of autophagosomal contents, are sensitized to hypoxic exposure [69]. However, chloroquine and related analogues lack potency and improved small molecule inhibitors of autophagy are needed. Additional strategies to impair the adaptive processes that depend on ATF4 target genes could include targeting of angiogenesis, redox balance, or amino acid metabolism.

Conclusions and Future Directions

Activation of the eIF2α-ATF4 pathway provides cancer cells with a key mechanism to tolerate hypoxia and nutrient stress by supporting metabolic homeostasis particularly via increased GSH biosynthesis, replenishment of amino acid pools, and by promoting high rates of autophagy. Important studies have implicated PERK-eIF2α-ATF4 signaling in hypoxic resistance to radiation, providing a clinical setting for the therapeutic development of PERK inhibitors, providing that concerns with pancreatic toxicity can be overcome. The discovery of an eIF2α phosphorylation-dependent signaling inhibitor (ISRIB) and p300 bromodomain/acetyltransferase inhibitors provide new strategies for inhibiting ATF4 activity in tumors and will be useful tools for preclinical therapeutic investigations of this process.

A particularly interesting finding has been the strong anti-angiogenic effects of PERK inhibitors, which suggests an additional important mechanism of antitumor activity [114]. This effect is consistent with reported roles of ATF4 in promoting angiogenesis by transcriptionally activating pro-angiogenic factors [115]. However, genetic models of phospho-eIF2α signaling inhibition do not always display reductions in vascular density [149]. Further investigation is needed to reconcile these findings and to elucidate the potential for direct anti-endothelial effects on tumor vasculature that may be nutritionally deprived. Similarly, much of our understanding is based on in vitro studies which do not accurately model the microenvironmental stresses in tumors. Follow-up studies need to place more emphasis on in vivo characterization of ATF4 biology.

Recent work has highlighted the influence of post-translational modifications in modulating pro-survival versus pro-apoptotic activity of ATF4 [176]. For example, methylation of ATF4 at arginine residue 239 by PRMT1 appears to confer pro-apoptotic activity [177]. Similarly, an understanding of the epigenetic co-factors required for ATF4 activity and epigenomic specificity have only recently emerged and much is yet to be uncovered in this field [178]. A greater understanding of these

modifications, including phosphorylation, methylation, acetylation, and ubiquitination may provide new therapeutic opportunities to fine-tune ATF4 stability and transcriptional activity in efforts to improve cancer therapy.

Drugs designed to selectively eliminate hypoxic/nutrient-deprived cells that depend on ATF4 have the potential to improve patient survival, especially when combined with multimodality treatment regimens containing cytotoxics, molecular targeted agents, and radiotherapy that preferentially kill non-hypoxic/unstressed cells within the tumor. The recognition that secretory phenotypes acquired during therapy-induced senescence are maintained by PERK-eIF2α-ATF4 signaling suggests that inhibiting this pathway may have synergistic effects with conventional chemotherapeutics, including doxorubicin and cyclophosphamide. In addition, the finding that EMT causes high levels of constitutive EnR stress highlights another setting where PERK-eIF2α-ATF4 inhibitors may be beneficial. Treating tumors with high numbers of cells that have undergone EMT, for example, TNBC cancers of the basal-B subtype, may be particularly beneficial. However, these inhibitors are unlikely to be used on their own and there are many potential combination therapies with a strong rationale. Careful clinical trial design and use of biomarkers is needed to predict target patient populations and to validate clinical activity.

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