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Review Article

Inhibitor of DNA binding proteins: implications in human cancer progression and metastasis

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Abstract: Inhibitor of DNA binding (ID) proteins are a class of helix-loop-helix (HLH) transcription regulatory factors that act as dominant-negative antagonists of other basic HLH proteins through the formation of non-functional heterodimers. These proteins have been shown to play critical roles in a wide range of tumor-associated processes, including cell differentiation, cell cycle progression, migration and invasion, epithelial-mesenchymal transition, angiogenesis, stemness, chemoresistance, tumorigenesis, and metastasis. The aberrant expression of ID proteins has not only been detected in many types of human cancers, but is also associated with advanced tumor stages and poor clinical outcome. In this review, we provide an overview of the key biological functions of ID proteins including affiliated signaling pathways. We also describe the regulation of ID proteins in cancer progression and metastasis, and elaborate on expression profiles in cancer and the implications for prognosis. Lastly, we outline strategies for the therapeutic targeting of ID proteins as a promising and effective approach for anticancer therapy.

Keywords: Inhibitor of DNA binding protein, cancer progression, metastasis, cancer stem cells, cancer therapy

Introduction

Inhibitor of DNA binding (ID/Id) proteins, a subgroup of helix-loop-helix (HLH) transcriptional factors are important regulators of cell fate and differentiation [1]. To date, four members (ID1-ID4) have been identified in mammals, of which Id1 is the most intensively studied. Id proteins share a highly homologous HLH dimerization domain that enables them to form heterodimers with the basic HLH (bHLH) proteins. Id1 and Id2 are encoded by unlinked genes but they share a similar HLH motif with 79% homology and most of the changes are conserved, however these two proteins differ markedly in the rest of their sequence [2]. Id1 and Id3 are well-known to compensate for each other. It has been shown that Id1 and Id3 exhibit overlapping expression patterns during early gestation through birth in mouse development, suggesting that they are evolutionally closely related and have similar biological functions [3]. In comparison to other Id proteins, Id4 has a unique polyalanine domain at the N terminus and a polyproline domain at the C terminus, and these unique domains of Id4 protein might convey specificity for certain interaction [4].

The primary binding partners of Id proteins are the class I bHLH proteins (E proteins), such as E2A/TCF3, E2-2/TCF4, and HEB/TCF12. The highly conserved bHLH domain is tripartite and comprises a basic DNA-binding region and two amphipathic α helices [5]. These two α helices, each 15-20 residues long, are separated by a shorter intervening loop with a more variable length and sequence [6]. bHLH proteins form homo- or hetero-dimers through the HLH domain. Binding occurs through the basic region adjacent to the HLH motif, to target genes containing the consensus (CANNTG) DNA sequence in their promoters, thereby activating

the transcription of target genes [7]. Id proteins can dimerize with bHLH proteins, and the resulting Id-bHLH heterodimers are not able to bind to DNA because Id proteins lack the basic DNA-binding domain [7]. Id proteins thus act as dominant-negative antagonists of bHLH proteins. Since most of bHLH proteins activate genes involved in cell differentiation, Id proteins are considered as inhibitors of cell differentiation. In addition to bHLH proteins, Id proteins can also interact with many non-bHLH proteins in complex networks [8].

The turnover of Id proteins is rapid and they are usually degraded by the ubiquitin-proteasome system within the nucleus, which is modulated by the interaction with myogenic differentiation (MyoD) protein [9, 10]. Recent studies in neural precursor cells and human glioblastoma-derived stem cells show that phosphorylation is involved in the regulation of ubiquitin-mediated degradation of Id proteins [10]. For instance, Id2 can be phosphorylated at serine 5, serine 14 and threonine 27, resulting in the enhancement of its proteasomal degradation [10], and protein phosphatase 2A, which is able to dephosphorylate Id2 protein, and contributes to the high levels of Id2 protein [10].

Notably, since all Id proteins lack a nuclear localisation signal, their localization in different cells, especially in the nucleus has been found to be dependent on the heterodimerization with proteins that carry nuclear localization signals such as E proteins [11]. E proteins can act as nuclear chaperones for the translocation of Id proteins into the nucleus, which tightly regulates the cytoplasmic reservoir and nuclear pool of Id proteins [11]. The N- and C-terminal domains of Id proteins are distinct for each member, and may account for the tissue specificity and protein-specific interactions, possibly resulting in differential functions of Id proteins [12].

Id proteins are known to participate in a broad range of biological processes in human cancers, such as cell differentiation and proliferation, cell cycle progression and apoptosis, migration and invasion, epithelial-mesenchymal transition, angiogenesis, cancer stem cell properties, chemoresistance, tumorigenesis, and metastasis. Although the roles of Id proteins in human cancer have been extensively investigated in the past years, their signaling interactions as well as downstream effectors are still poorly understood. This understanding is critical to the appreciation of its role in cancer progression and metastasis and subsequently the development of more effective approaches for cancer treatment.

Expression profiles and prognostic value of Id proteins

Although the four Id-protein members belong to the same family, their chromosomal localization, pattern of expression and functions are markedly different. Id1 and Id3 proteins are almost ubiquitously expressed, whereas Id2 and Id4 protein have a more restricted pattern of expression (predominantly in testis, brain and kidney) [13]. An overview of the mRNA expression profiles of the individual Id genes in cell lines of the cancer cell line encyclopedia is presented in **Figure 1**. All Id genes are expressed in all human cancer cell lines, however, with significant variations in abundance. For instance, while esophagus, upper aerodigestive tract, urinary tract and liver cancer cell lines have relatively high expression levels of Id1 mRNA, levels are relatively low in B-cell acute lymphoblastic leukaemia cell line.

Id proteins are not typically found in adult tissues, nonetheless they have been reported to be aberrantly expressed in numerous human cancers, such as breast, prostate, ovarian and cervical cancer [1]. Aberrant expression of Id proteins, especially Id1, Id2, and Id3 has been associated with advanced tumor stage and poor prognosis in many types of human cancers [14]. Accordingly, Id1, Id2 and Id3 are considered as potential oncogenes in many human cancers. Interestingly, the silenced expression of Id4 (primarily by hypermethylation) in breast and prostate cancer, as well as glioblastoma and leukemia indicate that Id4 is a tumor suppressor [15-17]. Furthermore, Id4 has also been demonstrated to antagonize Id1, Id2 and Id3 by forming heterodimers, promoting the DNA binding and transcriptional activity of E47 proteins [8].

Regulation of Id proteins expression

Growth factors: The expression of Id proteins is known to be regulated via different mechanisms at both the transcriptional and translation levels. In particular, Id proteins can be

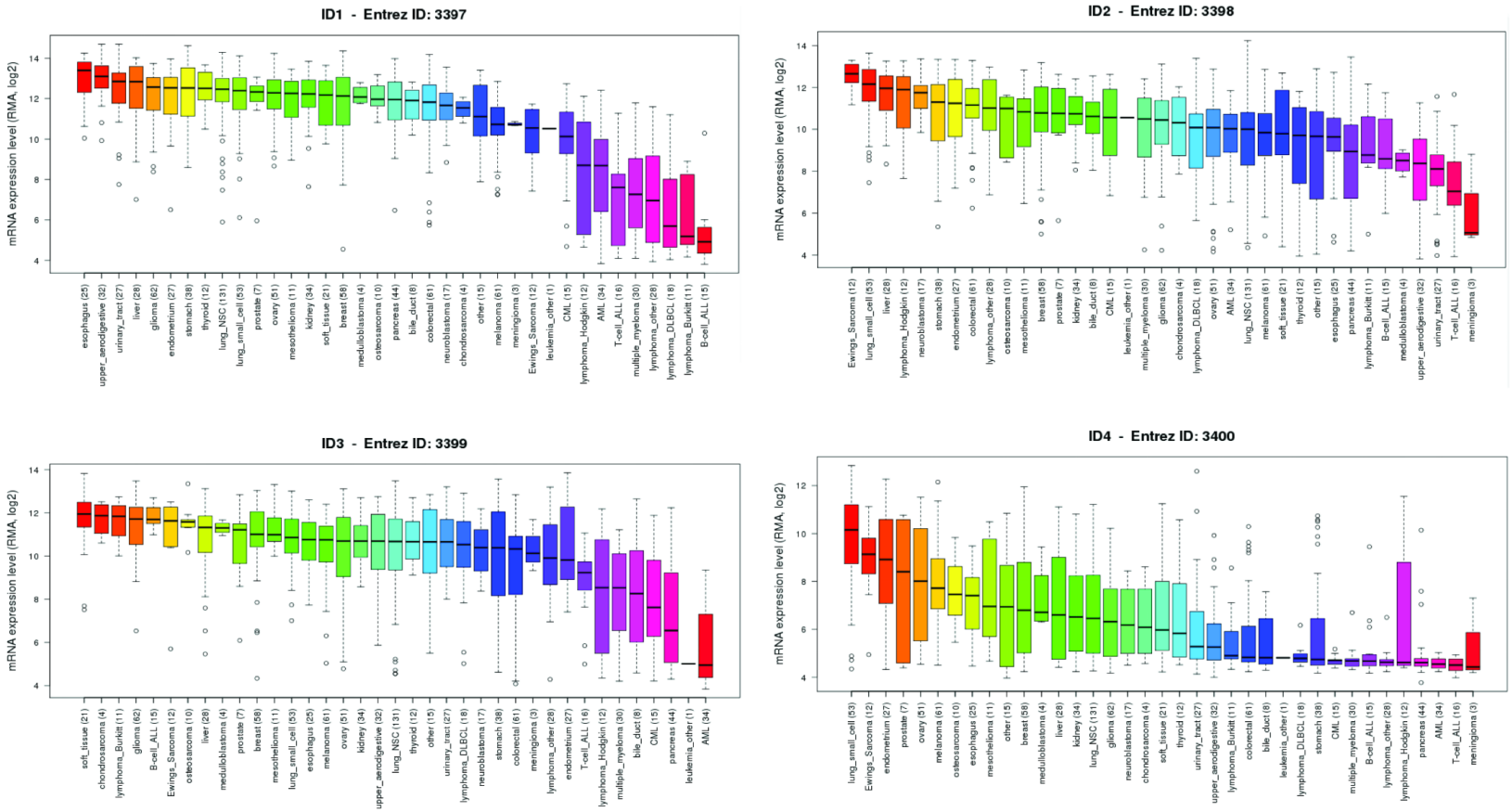

Figure 1. The relative mRNA expression of Id genes in different human cancer cell lines. Normalized mRNA expression data of different human cancer cell lines were obtained from the CCLE dataset (http://www.broadinstitute.org/ccle/home). Id mRNA expression data were extracted from this reference.

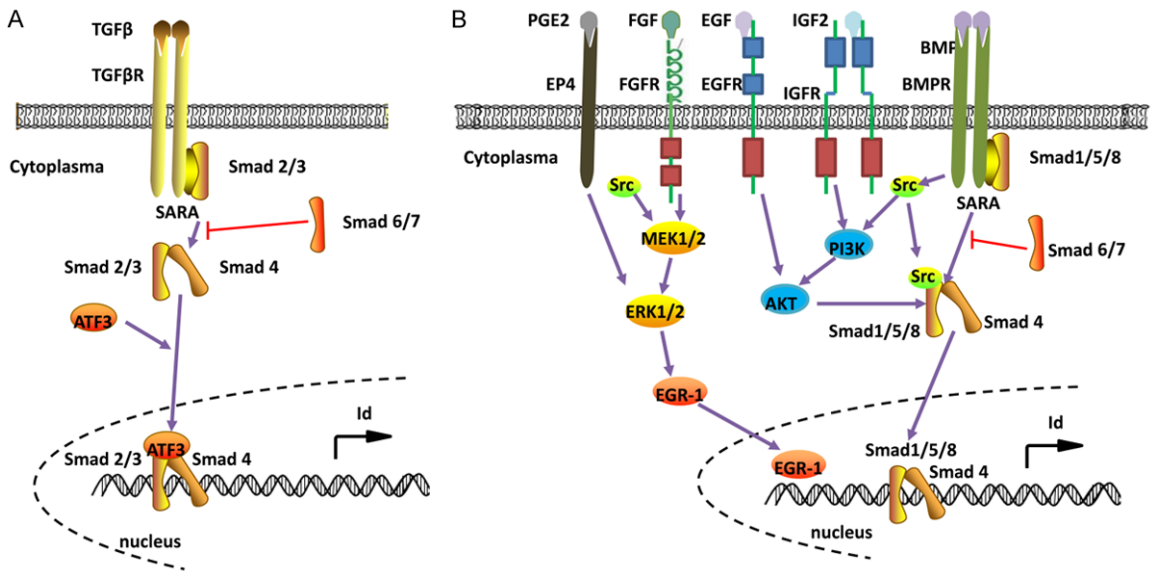

Figure 2. Transcriptional regulation of Id gene expression. A. TGFβ activates type I and II serine/threonine receptors, and the activated receptors form complex and phosphylate the receptor-associated Smads (R-Smads) including Smad2 and Smad3. These activated R-Smads form heteromeric complexes with common mediator Smad4, and then the complex ranslocates into the nucleus. TGFβ specific Smad2/3 activates ATF3, and ATF3 mediates TGFβ-repressed the transcription of Id genes by binding to the ATF/CREB site on the promoter. B. BMPs bind to type I and II serine/threonine receptors, the activated type I receptor phosphorylated R-Smads, including Smad1, Smad5, Smad8. These activated Smads form heteromeric complexes with common-mediator Smad4, and then the complex translocates into nucleus, which regulates the transcription of Id genes through binding to the regulatory region on the promoter. EGF or IGF2 binds to its receptor, and activates PI3K/Akt signaling, which leads to phosphorylation of Smad1/5/8 and subsequent transcription of Id genes. After binding to its receptor, FGF or PGE2 activates MEK signaling and then induces EGR-1 expression. EGR-1 triggers the transcription of Id genes through binding to the promoter. Src activated by BMPs can form complex with Smad1/5, which regulates the transcription of Id genes through binding to the Src-responsive region on the promoter. Abbreviations: TGFβ, transforming growth factor β; TGFβR, TGFβ receptor; ATF3, activating transcription factor 3; Id, inhibitor of DNA-binding/differentiation; BMP, bone morphogenetic protein; BMPR, BMP receptor; PEG2, prostaglandin 2; EP4, PEG receptor 4; FGF, fibroblast growth factor; FGFR, FGF receptor; EGF, epidermal growth factor; EGFR, EGF receptor; IGF2, insulin-like growth factor 2; IGFR, IGF receptor; MEK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; EGR-1, early growth response protein-1; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.

induced by a variety of growth factors, especially transforming growth factor (TGF)-β and bone morphogenetic proteins (BMPs) (**Figure 2**). In normal epithelial cells, TGF-β suppresses the transcription of Id genes [18]. Activating transcription factor (ATF3) interacts with Smad3 and then binds to CRE/ATF consensus sequence within the Id1 promoter, mediating the transcriptional repression of Id1 [19]. Both Smad3 and Smad4 can directly bind to the Id1 promoter, and Smad3 signaling has been shown to be responsible for TGF-β-mediated transcriptional repression of Id1 in mouse embryonic fibroblasts and various human epithelial cells [20]. However, in prostate and ovarian cancer cells, as well as endothelial cells, TGF-β was found to induce the expression of Id proteins [21, 22]. The regulation of Id proteins expression by TGF-β appears to be a complicated event.

The transcriptional co-activators CREB-binding protein (CBP) and p300 protein have been shown to be required for TGF-β-induced transcription of Id1 in mouse embryonic fibroblasts [23]. Protein tyrosine kinase 7 has recently been found to regulate Id1 gene and protein expression through modulation of TGF-β/Smad signaling in CD44-high glioma cells [24]. Beside TGF-β, the expression of Id proteins can also be induced in response to BMP stimulation. For example, BMP2 treatment induced the expression of Id1, Id2, and Id3 in MCF-7 human breast carcinoma cells [25]. Furthermore, treatment of mouse neuroblastoma Neuro2a cells with BMP2 induced a transient upregulation of Id1-3 proteins at the early phase, but this expression decreased when the cells differentiated into neurons [26]. The induction of Id1 transcription in response to serum is partly mediated by the early response gene (Egr)-1 [27]. Egr-1 binds

directly to the Id1 promoter and activates its transcription.

Other growth factors which have been found to induce Id expression include vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), insulin-like growth factor 2 (IGF2). VEGF was able to induce Id1 and Id3 expression in human umbilical vein endothelial cells (HUVECs), and Egr-1 was identified as the major mediator of this transcriptional activation [28]. FGF-2 was reported to upregulate Id1 mRNA and protein expression in human neuroblatoma SK-N-MC cells through activation of ERK1/2 and its downstream effectors including Egr-1 [29]. Similarly, EGF was reported to be one of the upstream regulators of Id1 gene transcription in hepatocellular carcinoma [30].

Recently, IGF2 was found to induce ID1 expression in human breast cancer cells via phosphatidyl-inositide-3-kinase (PI3K)/Akt pathway. IGF2 itself was found to be induced by NF-kB through the activation of HER2/HER3 and subsequently PI3K [31]. The PI3K/Akt signaling pathway was also been found to enhance the translation of ID1 protein by decreasing protein phosphatise, Mg2+/Mn2+ dependent 1G (PPM1G) activity and increasing 4E-binding protein 1 (4E-BP1) phosphorylation in glioma cells [32]. The IGF2 receptor, type 1 insulin-like growth factor receptor (IGF-1R) was also able to induce the protein expression of Id1 and Id2 in mouse embryonic fibroblasts [33]. The treatment of HepG2 cells with hepatocyte growth factor (HGF) reduced ID1 expression at both the mRNA and protein level through ERK-dependent and independent signaling pathways [34] (**Figure 2B**).

Oncogenes and inflammatory mediators: The expression of Id proteins can also be regulated by several oncogenes and inflammatory mediators. For example, the crosstalk between Src (a non-receptor tyrosine-protein kinase) signaling and the BMP-Smad signaling has been shown to be critical for Id1 gene expression. Following activation by BMP2, Src complexes with Smad1/5, regulating Id1 transcription by binding directly to a Src-responsive region within the Id1 promoter [35]. The small-molecule Src kinase inhibitor, AZD0530 could block Smad1/5 nuclear translocation and binding to the Src-responsive region in the Id1 promoter [35]. Consequently, Id genes were found to be among the most highly down-regulated genes following treatment of human A549 lung carcinoma cells with AZD0530 [35]. Sun et al [36] reported that activation of Src was essential for the regulation of ID1 expression and formation of invadopodia in human MDA-MB-231 breast cancer cells. In the human colon cancer HCT116 cell line, AZD0530 reduced the phosphorylation of Smad1/5/8 and the expression of Id1 gene by directly binding to BMP receptor type 1 (BMPR1). Likewise, the stimulation of pancreatic cancer cells with nicotine was found to activate Src, and subsequently induce Id1 gene and protein expression [37].

The proto-oncogene protein Myc has also been reported to regulate the expression of Id proteins. Id2 gene is a direct transcriptional target of Myc, which directly binds to, and activates its promoter [38]. In breast epithelial cells, ectopic expression of Myc rapidly resulted in the up-regulation of Id1, whereas knockdown of Myc exerted the opposite effect [39]. A positive feedback-loop regulatory loop between Id1 and Myc has also been proposed. Sharma and colleagues [40] reported that knockdown of Id1 resulted in down-regulation of c-Myc, whereas overexpression of Id1 strongly induced Myc expression in hepatocellular carcinoma cells.

In response to interleukin-6 stimulation, B-cell leukemia 3, a member of the inhibitor of κB family and a known activator of NF-kB was shown to trigger the gene expression of Id1 and Id2 in prostate cancer cells by binding to the promoters of these two genes [41]. Similarly, prostaglandin E2 (PGE2) was also shown to induce Id1 transcription through different mechanisms. For example, in breast cancer and glioma cells, cyclooxygenase-2-derived PGE2 can activate EGFR-ERK1/2-mitogen-activated protein kinase (MAPK)-Egr1 signaling by binding to its receptor 4 (EP4), leading to the upregulation of Id1 [42-44]. Likewise, in human coloncytes and colon cancer cells, PGE2 was shown to bind EP4, and enhance pCREB-mediated Id1 transcription through activation of protein kinase A [45]. The MAPK/ERK mitogenic pathway has also been implicated in the activation of Id1 protein expression in hepatocellular carcinoma cells [40].

MicroRNAs: MicroRNAs (miRNAs) have also been found to play a role in the regulation of Id expression. miRNAs are a class of non-coding RNAs, which regulate gene expression at the

posttranscriptional level by inhibiting translation or increasing the degradation of the target messenger RNA (mRNA). For example, by targeting BMPR1A, miR-885-3p was found to inhibit Smad1/5/8 phosphorylation and downregulate Id1 expression [46]. As a result, miR-885-3p was able to inhibit the xenografts growth of human colonrectal adenocarcinoma HT-29 cells in nude mice [46]. Another miRNA, miR-29b was found to be able to suppress Id1 expression levels by directly interacting with its 3'-untranslated region (UTR) [47]. Downregulation of miR-29b leading to the upregulation of Id1 was shown to play an important role in TGF-β1-induced epithelial-mesenchymal transition in human ovarian cancer cells [22]. Similarly, Id1 was demonstrated to be a direct target of miR-381 by 3'-UTR luciferase reporter assays in human lung cancer cells [48]. Ectopic expression of miR-381 led to reduced transcription, significantly decreasing cell migratory and invasive capacities of these cells [48].

Other factors: Apart from the factors mentioned above, the expression of Id proteins has been found to be regulated by other diverse molecules. For instance, a report by Fontemaggi and colleagues showed that gain-of-function p53 mutants, R175H, R273H and R280K complexed to E2F1 could assemble on specific regions of the Id4 promoter and positively control its transcription [49]. In a different study, Xu and colleagues observed that Id1 expression decreased immediately after androgen deprivation in androgen-dependent prostate cancer cells, however, this pattern was reversed after extended periods of deprivation [50]. Ao et al., on the other hand found that activation of androgen receptor induced Id1 expression in hepatocellular carcinoma cells [51]. Laminin has also been reported to be the crucial component responsible for the upregulation of Id2 in murine mammary epithelial SCp2 cells [52]. Most recently, Id1 expression was reported to be induced by etoposide in human esophageal squamous cell carcinoma cells, and the induction was mediated through the transcription factor activator protein-1 binding sites within the Id1 promoter [53].

Biological functions of Id proteins in human cancer progression and metastasis

Id proteins and tumorigenesis: A large body of research has investigated the roles of Id proteins in tumorigenesis and found these roles to be often cell-type and context-dependent. The upregulation of Id1 was proposed to be a relatively early event during tumorigenesis, which is required but not sufficient for tumor initiation in primary tumor formation and for metastatic colonization in the lymph node microenvironment [54]. Other studies seem to indicate otherwise, as the stable transfection of Id1 could convert non-tumorigenic Rhek-1A keratinocytes into tumorigenic cells in nude mice [55]. Similarly, the ectopic expression of Id1 in human papillomavirus-immortalized cervical epithelial cells was able to potentiate N-nitrosopyrrolidine-induced cell transformation, and this effect was suppressed by silencing of Id1 [56]. Recently, studies from Jin et al [57] showed that enhanced expression of Id1 in non-transformed murine astrocytes with targeted disruption of P16Ink14a and P19Arf conferred tumorigenic ability with enhanced cell growth. In a further example, the ectopic expression of Id1 in esophageal squamous cell carcinoma cells was able to enhance both primary tumor formation and metastatic potential compared with control cells [58]. Similarly, the overexpression of Id2 in primary mouse neural precursor cells was sufficient for glioma formation in the brain of mice with forced expression of platelet-derived growth factor [59]. Experiments with colorectal cancer cells also further confirmed that Id2 expression was critical for promoting primary tumor formation and metastasis *in vivo* [60]. Conversely, knocking out one, or both alleles of Id1 was found to reduce small intestinal tumor multiplicity and increase the lifespan in ApcMin/+ mice [45]. The loss of Id1 was also able to delay the initiation of MLL-AF9-driven leukemogenesis in the fetal liver transplantation model [61].

In addition to contributing malignant transformation, Id proteins are also able to foster tumor progression. For example, Hui et al [62] reported that ectopic expression of Id1 was able to increase serum-independent cell growth and G1/S phase transition in esophageal squamous cell carcinoma cells. Conversely, in an immortalized prostate epithelial cell line, inhibition of Id1 expression suppressed cell proliferation and induced cellular senescence and G2/M cell-cycle arrest [63]. Along similar lines, knockdown of Id1 in hepatocellular carcinoma cells was shown to suppress cell proliferation and reduce colony formation [40].

Similarly, the inhibition of Id2 expression was shown to reduce cell proliferation in human pancreatic cancer cells [64] as well as increase apoptosis in human prostate cancer cells [65]. Earlier studies showed that loss of Id2 induced premature differentiation and cell cycle arrest in Rb+/- melanotrophs and inhibited both cell proliferation and tumor initiation [66]. In colorectal cancer, the knockdown of Id2 decreased cyclin D1 expression while increasing p21 expression, resulting in the inhibition of cell proliferation [60]. Incidentally, the knockdown of Id2 was shown to increase the expression of pro-apoptotic Bcl-2 family members Bim/Bad and enhance the cleavage of anti-apoptotic proteins caspase-7 and poly (ADP-ribose) polymerase, leading to decreased cell survival [60].

The knockdown of Id3 also decreased proliferation and increased apoptosis in D283 medulloblastoma cells *in vitro* [67]. Furthermore, the knockdown of either Id2 or Id3 was found to reduce survival in B-cell chronic lymphocytic leukemia cells [68]. In human malignant squamous cell carcinoma, Id3 expression was reported to induce cell apoptosis through the E-twenty-six (ETS) domain transcription factor Elk-1-caspase-8-dependent pathway *in vitro* and also reduce tumor growth via apoptosis in a mouse xenograft model [69]. Furthermore, in a seeding model of medulloblastoma, knockdown of Id3 inhibited primary tumor growth and the development of leptomeningeal seeding and prolonged animal survival [67].

Id1 and Id3 exhibit overlapping expression patterns during early gestation through birth in mouse development and a double knockout of Id1 and Id3 in mice resulted in larger tumors [70]. Double knockdown of Id1 and Id3 expression has been shown to also inhibit cell proliferation in human prostate cancer cells [65]. Moreover, Id1 and Id3 expression has been shown to be required for tumor re-initiation by promoting sustained proliferative activity of metastatic tumor cells during the early stages of lung metastatic colonization of breast cancer cells [71]. Furthermore, double knockdown of Id1 and Id3 in small cell lung cancer cells does not only inhibit cell proliferation, anchorage-independent growth, invasion and angiogenesis, and increase cell apoptosis *in vitro*, but also reduce tumor growth *in vivo* [72]. Double knockdown of Id1 and Id3 in human gastric and pancreatic cancer cells was shown to reduce cell proliferation and migration, and inhibit adhesion *in vitro*, and reduce the peritoneal metastasis formation *in vivo* [73, 74].

Similarly, Id4 ectopic expression in human prostate cancer DU145 cells was found to decrease cell proliferation and increase cell apoptosis partly due to a S-phase arrest, that was linked to the increased expression of p21, p27 and p53 [15]. A recent report showed that biodegradable polycaprolactone/maltodextrin nano-carrier encapsulating human recombinant Id4 reduced cell proliferation, invasion and colony formation and increased apoptosis *in vitro*, while suppressing tumor growth of subcutaneous xenografts *in vivo* [75].

As target genes for Id proteins have been identified largely based on the knowledge of promoters activated by bHLH transcription factors, the equilibrium between Id proteins and bHLH transcription factors is important for the determination cell fate and growth. Immunoglobulin transcription factor-2 (ITF-2), a bHLH transcription factor, was identified as an Id1-interacting protein [76] and its overexpression reduced Id1-stimulated proliferation and apoptosis in mammary epithelial cells. Also, altering the balance of bHLH/Id proteins by knocking down of Id3 or overexpressing of E47 was able to induce growth arrest in pancreatic adenocarcinoma cells [77]. It would thus appear that a functional excess of Id proteins is required for supporting tumor cell proliferation.

Signaling cascades

A number of key signal transduction pathways are involved in the mediation of the tumorigenic activities of Id proteins (**Figure 3**). For instance, Id1 induction of serum-independent proliferation in prostate and hepatocellular carcinoma cells was found to be mediated through the p16INK4a/retinoblastoma pathway [78, 79]. Likewise, activation of Raf/MEK1/2 signaling was also involved in Id1-induced serum independent prostate cancer cell growth [80]. Moreover, the tumor-promotion effect of Id1 in esophageal squamous cell carcinoma cells could be attenuated by a PI3K inhibitor, indicating that these effects were mediated by the PI3K/Akt signaling pathway [59], as was the case in lung cancer [81] and gastric cancer

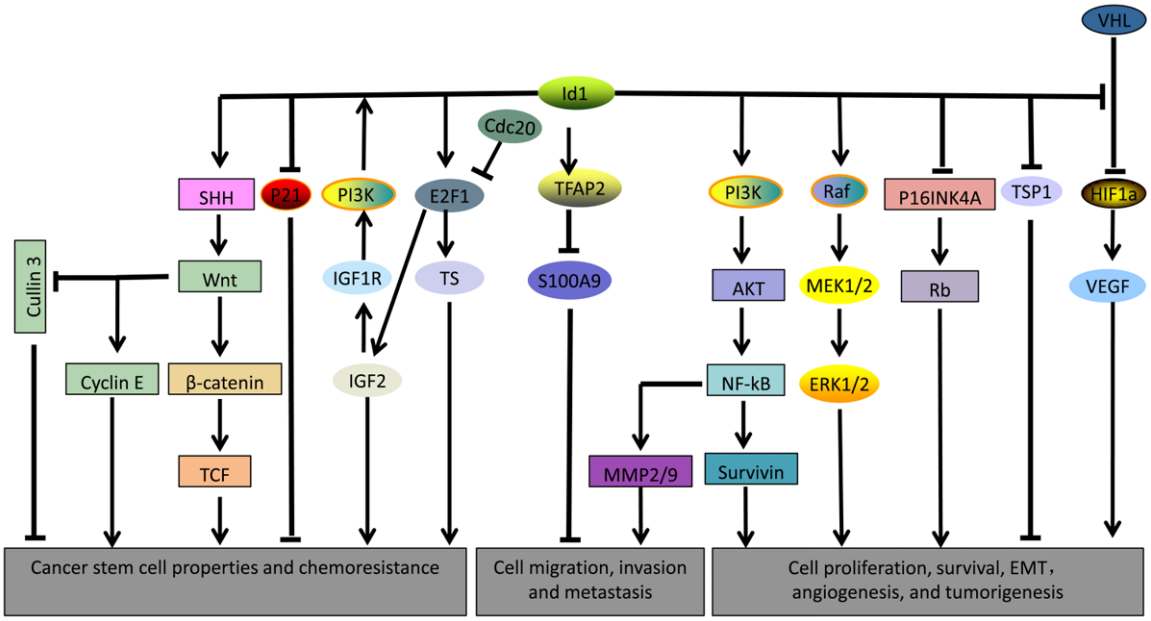

Figure 3. Biological effects of Id1 proteins on human carcinogenesis and metastasis. Id1 interacts with different signaling molecules and pathways such as PI3K/AKT signaing, MAPK/ERK signaling, $P16^{INK4A}$/Rb signaling, VEGF signaling, Wnt/β-Catenin signaling, and IGF2 signaling, which regulates the indicated tumor biological effects. Abbreviations: Id1, inhibitor of DNA-binding/differentiation protein 1; TCF, T cell factor; E2F1, E2F transcription factor 1; Cdc20, cell-division cycle protein 20; TS, thymidine synthase; IGF2, insulin-like growth factor 2; IGF1R, type I IGF receptor; TFAP2, transcription factor AP-2; S100A9, S100 calcium-binding protein A9; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MMP2/9, matrix metalloproteinase 2/9; MEK1/2, mitogen-activated protein kinase 1/2; ERK1/2, extracellular signal-regulated kinase; Rb, retinoblastoma protein; TSP-1, Thrombospondin 1; VHL, Von Hippel-Lindau; HIF1α, hypoxia-inducible factor 1-alpha; VEGF, vascular endothelial growth factor.

[82]. Similarly, in human head and neck squamous cell carcinoma cells, overexpression of Id1 induced the phosphorylation of Akt via PI3K and increased the expression of survivin via NF-kB both *in vitro* and *in vivo* [55]. In contrast, blockade of both pathways by specific inhibitors abrogated Id1-induced cell survival of keratinocytes *in vitro* [55]. Id1 has also been proposed to be a determining factor for switching TGF-β1 from a growth inhibitor to a tumor promoter during prostate carcinogenesis. In a non-malignant prostate epithelial cell line, inhibition of Id1 increased the expression of p21WAF1/Cip1 and p27KIP1, two downstream effectors of the TGF-β1 pathway, thereby making the cells more vulnerable to TGF-β1-induced growth arrest [63]. Conversely, Id1 expression could promote F-actin stress fibre formation and increase cell-substrate adhesion and cell migration in response to TGF-β1 simulation through activation of MEK-ERK signaling and subsequent phosphorylation of heat shock protein 27 [83]. Recent studies have found that platelet-derived growth factor-E2F-ubiquitin-specific peptidase 1-Id2 signaling pathway plays critical roles in the survival of murine proneural glioma cells [84]. BMP9 was shown to induce G0/G1 cell cycle arrest in human hepatocellular carcinoma cells through p38/Id3/p21 signaling pathway, which was independent of Smad signaling [85]. In colon cancer cells Id3 induction via the inflammation-induced molecule, S100A8 was able to regulate cell cycle and proliferation by altering p21 expression through Akt-Smad5 signaling [86].

Id proteins and metastasis: A substantial body of evidence suggests Id proteins play important roles in cancer metastasis. The ectopic expression of Id1 could convert a non-aggressive breast cancer cell line to one with a more aggressive phenotype with enhanced invasion capacities [87]. Conversely, systemically knocking down Id1 using a nonviral systemic gene transfer technique was able to reduce the metastatic spread of 4T1 mouse breast cancer

cells in immunocompetent mice [88]. Coincidentally, Id1 was identified as the only transcriptional regulator in the lung metastasis signature that mediated breast cancer metastasis to the lung [89]. Breast cancer cells overexpressing Id1 were found to be more active in metastasizing to the lung than the vector control cells, and the knockdown of Id1 resulted in a significant reduction of metastatic ability to the lung. The in vitro ectopic expression of Id1 promoted cell proliferation, migration and invasion in several types of human cancers including bladder, ovarian, and gastric cancer, whereas the knockdown of Id1 had the opposite effect [22, 90-92]. Urinary bladder tumors that developed in the Id1 knockout mice showed areas of extensive hemorrhage and decreased invasiveness compared to wild type mice [54]. Furthermore, knockdown of Id1 in human bladder cancer cells led to decreased cell growth *in vitro* and reduced lung colonization *in vivo* [54]. Recently, the inhibition of Id1 in human aggressive salivary cancer cells by using three different approaches including genetic knockdown, progesterone treatment and cannabidiol treatment all resulted in the reduced formation of lung metastatic foci *in vivo* [93].

Id2 has been documented to play opposing roles in different types of tumor cells. The reason for this discrepancy is not clear but is thought to be associated with intrinsic differences between the tumor cell lines, extracellular activating signals and the microenvironment. Id2 was shown to maintain breast cancer cells in a more differentiated, non-invasive phenotype and even inhibit the aggressive phenotype of human metastatic breast cancer cells [52]. Id2 was found to be a downstream target of peroxisome proliferator-activated receptor-gamma coactivator-1a (PGC1a) that suppresses pro-metastatic transcriptional programs in human melanoma cells [94]. PGC1a directly promoted the transcription of the Id2 gene, which then downregulated the expression of metastasis-related genes including integrins through binding and inactivating the transcription factor TCF4 [95]. Interestingly, the constitutive expression of Id2 could convert nonaggressive prostate cancer cells into more proliferative and invasive cells, and its downregulation was able to reduce cell growth and invasion in the highly metastatic PC3 prostate cancer cells [96].

A number of diverse molecules have also been implicated as effector molecules of Id protein activity. Several studies have demonstrated the critical roles that matrix metalloproteinase (MMPs) play in the regulation of Id protein facilitated tumor invasion and metastasis (**Figure 3**). Id1, and to a lesser extent Id2, has been reported to increase the secretion of MMPs , thereby promoting cell invasion in the nonaggressive LNCaP prostate cancer cell line [97]. Conversely, the inhibition of Id1 expression in human breast cancer cells decreased the expression of membrane-type 1 (MT1)-MMP, resulting in the inhibition of cell invasion *in vitro*, and metastatic spread *in vivo* [88]. Similarly, in glioblastoma cells, the knockdown of Id1 also reduced the expression of MT1-MMP, MMP-2, phospho-focal adhesion kinase (p-FAK) and some epithelial-mesenchymal transition markers, dramatically reducing cell invasion *in vitro* [96]. A recent study found that Id4 could also decrease MMP-2 expression by a direct inhibitory interaction with Twist1, thereby inhibiting cell invasion in glioma cells [4]. MMP-9 is also another common target of Id1, Id2 and Id3 [65]. The signal transducer and activator of transcription 5 (STAT5) has been shown to contribute to BCR/ABL-mediated leukemogenesis. The resultant MMP-9 transactivation that enhanced invasiveness was found to be dependent on Id1 [98]. A recent study by Murase et al., [93] showed that inhibition of Id1 expression in an aggressive salivary cancer cell line led to decreased MMP-9 expression and reduced cell invasion. Knockdown of Id2 almost completely suppressed MMP-9 expression and reduced cell invasion in aggressive salivary cancer cells, suggesting that Id2 was also involved in regulating the expression of MMP-9 [99]. Moreover, Dong et al [100] also reported that knockdown of Id2 in colorectal cancer cells reduced MMP-2 and MMP-9 expression and decreased cell migration. Furthermore, overexpression of Id2 in Id2-null oral squamous cell carcinoma cells increased the activity and expression of MMP-2 and the secretion of MMP-9, which was paralleled by enhanced cell invasion [101]. Notably, these findings were contrary to those of two earlier studies in which overexpression of Id2 decreased MMP-9 secretion including cell proliferation and invasion in aggressive breast cancer cells [52, 102]. Likewise, Id3 was also able to downregulate MMP-2 expression by suppressing Ets-1-induced MMP2 promoter

activity in human squamous cell carcinoma [103].

Id1 has been shown to promote lung metastasis of breast cancer through suppression of S100A9 expression [104] (**Figure 3**). S100A9 is a calcium binding protein that plays important roles in inflammation and cancer progression [105]. Id1 suppresses S100A9 expression by interacting with transcription factor AP-2 alpha (TFAP2A). Since S100A9 is able to suppress Id1-induced Rhoc activation, the suppression of S100A9 was found to promote cell migration and invasion *in vitro* and lung metastasis *in vivo* [104]. Overexpression of another calcium binding protein S100A8 promoted cell proliferation *in vitro* and metastasis *in vivo* in colorectal cancer cells through activation of the Akt1-Smad5-Id3 signaling [86].

Enhanced metastasis resulting from immune response modulation has also been attributed to Id1. Overexpression of Id1 in bone marrow-derived myeloid cells led to systemic immunosuppression by downregulating key molecules involved in dendritic cell differentiation including CD83, CD86, MHCII, FSCN1, STAT4 and IRF8. CD8+ T-cell proliferation was also suppressed leading to metastatic progression [106] Tenascin C (TNC) and connective tissue growth factor (CTGF) were identified as two candidate target genes whose expression might contribute to the biological role of Id3 in tumor metastasis [67], as was integrin α6 which demonstrated to act downstream of both Id1 and Id3 in different human cancer cell types including gastric, colorectal and pancreatic cancers [73, 74, 107]. Other molecules include Semaphorin 3F (SEMA3F), a potent inhibitor of tumor cell growth and metastasis, whose expression was suppressed by Id2 in human low-metastatic glioma cells and tumor necrosis factor (TNF)-beta [108], whose levels were suppressed by Id1 in prostate cancer cells. Id2 was able to abrogate E47-induced SEMA3F expression and consequent activities including F-actin depolymerisation, loss of stress fibers, inactivation of RhoA and Rac1 and inhibition of migration [109]. Overall, these data strongly show that Id proteins are essential for metastatic dissemination in human cancers.

Id proteins and EMT: Epithelial-mesenchymal transition (EMT) is a key process by which epithelial cells lose polarity and acquire mesenchymal-like properties, and it has been shown to play critical roles in both tumor invasion and metastasis [110]. The hallmarks of EMT include loss of epithelial markers such as E-cadherin and catenins and gain of mesenchymal markers such as N-cadherin and Vimentin [110]. Id proteins are also involved in the regulation of EMT. Inmunohistochemistry studies on human bladder cancer showed that high levels of Id1 protein were associated with a decreased expression of membranous E-cadherin and β-catenin [91]. Furthermore, overexpression of Id1 triggered the typical morphological changes of EMT in human bladder urothelial cancer cells, which was accompanied by decreased expression of epithelial markers and increased expression of mesenchymal markers, whereas knockdown of Id1 reversed these morphological and molecular changes [91]. A similar observation has also been reported in human primary glioblastoma cells after knockdown of Id1 where it was shown to reverse the mesenchymal phenotype with profound changes on morphology associated with decreased expression of mesenchymal markers such as Vimentin, alpha-tubulin and Snail [96]. In human aggressive salivary cancer cells, knockdown of Id2 reduced the expression of N-cadherin, Vimentin and Snail, and induced E-cadherin expression, resulting in a more differentiated phenotype [99]. Overexpression of Id2 in Id2-null oral squamous cell carcinoma cells has been shown to trigger the occurrence of EMT with decreased expression of E-cadherin and increased expression of N-cadherin and Vimentin [101]. Together, these results suggest that Id proteins play important roles in EMT.

A variety of mechanisms have been proposed to explain how Id proteins regulate EMT in human cancers. For example, caveolin-1, an integral cell membrane protein, has been identified as an Id1 interacting protein [111]. In prostate cancer cells, the physical interaction between Id1 and caveolin-1 was shown to be essential for Id1-induced EMT and cell survival, which was mediated through the activation of Akt signaling and promoted the binding activity between caveolin-1 and protein phosphatise 2A [111]. Krüppel-like transcription factor 17, a negative regulator of metastasis, has been demonstrated to inhibit EMT and cell invasion by directly suppressing the transcription of Id1 gene by binding to its promoter region in breast

cancer cells [95]. Tissue microarray analysis of tumor samples from patients with esophageal squamous cell carcinoma revealed that strong Id1 expression correlated with high levels of phosphorylated Akt [58]. Moreover, ectopic expression of Id1 has been shown to induce the typical molecular changes of EMT markers through activation of PI3K/Akt signaling pathway [58]. Id proteins also regulate the EMT process through their dominant negative effect on the E2A transcription factor. Downregulation of Id2 expression by TGF-β treatment led to the accumulation of E2A, which subsequently repressed E-cadherin and induced EMT in mouse mammary NMuMG epithelial cells [112]. E47 is known to be a strong E-cadherin repressor and EMT inducer, and continued expression of Id1 is required for maintaining the mesenchymal phenotype after E47-induced EMT and cell viability in madin darby canine kidney cells [113]. However, the overexpression of Id1 protein alone could not overcome E47-dependent repression of E-cadherin, suggesting that the role of Id1 in regulating EMT may also be independent of its dominant negative effect on E47 [113].

To date, several lines of evidence show that Id proteins are involved in the regulation of EMT induced by TGF-β or other growth factors in human cancers. For example, overexpression of Id1 protein led to the upregulation of N-cadherin, downregulation of E-cadherin and redistribution of β-catenin in a non-malignant prostate epithelial cell line, thereby disrupting the adherens junction complex in TGF-β1-treated cells [83]. Conversely, knockdown of Id1 with small interfering RNAs could block the morphological and gene expression changes of TGF-β1-induced EMT in ovarian cancer cells [22]. Id1-mediated phenotypic switching has been proven to play important roles in breast cancer metastasis. In breast cancer cells that had undergone Twist-induced EMT, forced expression of Id1 by TGF-β stimulation promoted lung metastasis by antagonizing Twist and inducing a mesenchymal-to-epithelial transition [23].

The HLH motif of Id2 is similar to that of Id1, but the remainder of the sequence is considerably different. Id2 has been advocated as an EMT antagonist. Overexpression of Id2 prevented TGF-β1 induced downregulation of E-cadherin and induction of smooth muscle actin in mouse mammary NMuMG epithelial cells, whereas knockdown of Id2 enhanced TGF-β1-induced EMT and permited BMP7-induced EMT in lensα-TN4 murine epithelial cells [18]. Although Id2 is well-known to interact with bHLH, little is known about its role in the regulation of EMT via its interaction with zinc finger proteins. Co-immunoprecipitation experiments showed that Id2 formed a complex with the snag domain of Snail, a zinc finger transcriptional repressor, and limited the Snail-dependent repression of epithelial genes such as integrin β4 [114]. These data were also consistent with two recent studies on oral squamous cell carcinoma that showed that Id2 could inhibit the Snail regulation of E-cadherin by physically interacting with Snail [101, 115].

Id proteins and angiogenesis: Angiogenesis is critical for tumor growth and metastasis. A key role of Id proteins in cancer is through the promotion of angiogenesis. Double knockout of Id1 and Id3 in mice led to embryonic lethality due to premature neural differentiation and vascular abnormalities in the forebrain [116]. Id1+/- Id3-/- mice failed to support tumor xenograft growth because of poor vascularization and extensive necrosis [117]. The overexpression of Id1 has been associated with high intratumoral vessel density and tumor angiogenesis in a variety of human cancers, such as breast, pancreatic, oral squamous cell, ovarian and uterine cervical cancer [118-122]. Gain-of function and loss-of function experiments have further confirmed that Id1 plays critical roles in the migration and proliferation of endothelial progenitor cells *in vitro* [117].

The downstream targets of Id proteins through which angiogenesis is regulated have also been widely investigated (**Figure 3**). For example, Id1 was reported to regulate tumor neovascularization via the transcriptional repression of thrombospondin-1, an angiogenesis inhibitor [123]. Loss of Id1 in tumor endothelial cells resulted in the downregulation several pro-angiogenic genes, such as α6 and β4 integrins, MMP-2, FGFR-1, and members of the ephrin and IGF2 families [124]. The overexpression of Id1/Id3 in human umbilical vein endothelial cells has been reported to induce ICAM-1, E-selectin, MMP-2 and MMP-9 expression, and promote angiogenic processes such as transmigration

and tube formation [125]. An earlier study showed that the acute ablation of bone marrow-derived endothelial progenitor cells using Id1-directed delivery of a suicide gene decreased circulating endothelial progenitor cells, and caused significant defects in angiogenesis-mediated tumor growth [126]. In human ovarian cancer, PI3K/Akt-Id1-integrin α4 signaling was shown to induce proliferation, migration and adhesion of human endothelial progenitor cells [127]. Moreover, knockdown of Id1 suppressed PI3K/Akt/NF-kB/surviving signalling, and inhibited cell proliferation in endothelial progenitor cells [128]. Inhibition of nuclear factor kappa B (NF-kB)/MMP-2 signaling by a specific inhibitor blocked Id1-enhanced angiogenesis in human endothelial progenitor cells signifying the importance of this axis in angiogenesis [129].

Vascular endothelial growth factor (VEGF) is major mediator of angiogenesis. Previous studies have shown that not only can VEGF induce Id protein expression, but Id proteins can also modulate VEGF activity. VEGF has been reported to induce Id1 and Id3 gene expression in human umbilical vein endothelial cells [125]. Id1 expression correlated with VEGF expression in human colorectal and hepatocellular cancers [130, 131]. Furthermore, overexpression of Id1 in human prostate adenocarcinoma cells was shown to increase VEGF transcription and secretion, and promote cell proliferation and blood vessel formation in human umbilical vein endothelial cells [132]. Knockdown of Id1 by siRNAs reduced VEGF expression in human aggressive salivary cancer cells [93], while double knockdown of Id1 and Id3 was reported to abolish proliferation, activation and angiogenic processes induced by VEGF in human umbilical vein endothelial cells [125]. Similarly, double knockdown of Id1 and Id3 in small cell lung cancer reduced VEGF expression, resulting in the inhibition of endothelial tube formation [72]. A high level of Id1 expression was demonstrated to increase VEGF expression by stabilizing hypoxia-inducible factor (HIF)-1α, a well-known transcriptional regulator of VEGF, subsequently promoting angiogenesis and metastatic potential in hepatocellular carcinoma cells [131]. Similar molecular mechanisms have also been described in both human umbilical endothelial cells and MCF7 breast cancer cells [133]. Activation of ERK signaling has been shown to be important for these processes [133]. Id1 decreases the association between HIF-1α and VHL, thereby maintaining the stability and activity of HIF-1α [133]. Von Hippel-Lindau (VHL) is a subunit of ubiquitin ligase complex directing HIF-1α degradation. Recent studies in human hepatocellular carcinoma HepG2 cells have found that HIF-1α can also suppress Id1 and c-Myc expression under anaerobic conditions via its downstream target MAX interactor 1 (Mxi1) [40]. Id1 has also been implicated in lymphangiogenesis [134]. Silencing of Id1 by lentivirus-mediated RNA interference in oral squamous cell carcinoma cells was shown to inhibit lymphangiogenesis through down-regulation of VEGF-C both *in vitro* and *in vivo* [134].

In line with its often contrasting effects, Id2 was found to exert an opposite effect on the stability of HIF-1α in human hepatocellular carcinoma cells. Overexpression of Id2 decreased the expression of VEGF by destabilizing HIF-1α [135]. Nonetheless, in human pituitary tumor and neuroblastoma cells, N-Myc-induced Id2 acted as a master regulator of VEGF expression, and promoted angiogenesis and tumor growth [66]. These studies indicate that Id2 has distinct roles in different cancer types which warrant further investigation.

The expression of Id4 has also been correlated with microvessel density in breast cancer patients [49]. Id4 induced the expression of pro-angiogenic factors including IL8 and growth related oncogene-alpha (GRO-α) by binding to, and stabilizing their mRNA transcripts [49]. Moreover, the epigenetic silencing of Id4 by promoter hypermethylation significantly reduced VEGF expression and microvessel density in glioblastoma cells [16].

Id proteins and cancer stem cells properties: Id proteins are also involved in the negative regulation of cell differentiation and the maintenance of stemness. This is achieved mostly by binding to other bHLH transcription factors. For example, the downregulation of Id1 and Id3 expression with a type I TGF-β receptor inhibitor in patient-derived primary glioblastoma cells led not only to a reduction in the glioma-initiating cell population characterized by high levels of CD44 and Id1, but also to a decrease in the tumor-initiating capacity of this population [136]. The knockdown of Id1 could inhibit self-renewal potential, and downregulate the

expression of glioma stem cell markers in human glioblastoma cells, suggesting that Id1 supports a stem-like phenotype in human glioblastoma [96]. Moreover, Id1 was found to promote cell proliferation, self-renewal and tumorigeneicity by activating sonic hedgehog (SHH)/WNT signaling and enhancing Cyclin E protein stability in glioblastoma [57]. Sonic hedgehog activation was achieved through the suppression of cullin3 ubiquitin ligase-mediated glioma-associated oncogene 2 protein (GLI2) and dishevelled segment polarity protein 2 (DVL2). Moreover, the overexpression of Id1 or the knockdown of cullin3 was able to confer stem-cell like features on murine ink4a/Arf-deficient astrocytes [57]. Barrett et al., [137] reported that high Id1 expression characterized tumor cells with high self-renewal and tumorsphere formation capacities in PDGF- and KRAS-driven murine models of gliomagenesis, while low Id1 expression characterized tumor cells with proliferative potential and limited self-renewal potential. Interestingly, the tumor cells with low Id1 expression were more tumorigenic than the tumor cells with high Id1 expression, suggesting that self-renewal properties regulated by Id1 do not predict tumor growth potential [137]. Similarly, ovexpression of Id4 conferred stemness features on primary murine Ink4a/Arf-/- astrocytes by activation of Jagged-Notch signaling, and cyclin E was found to be responsible for enhanced tumorigenicity in Id4-transduced Ink4a/Arf-/- astrocytes [138]. Furthermore, EGFR mediated activation of Akt-Smad5-Id3 signaling and subsequent activation of Id3-regulated cytokines GRO1, interleukins (IL)-6 and IL-8 was found to enrich cancer stem cell properties in glioblastoma cells [31]. Id2 and Id4 are upregulated by inducing the differentiation of glioblastoma multiforme stem-like cells with histone deacetylase inhibitors [139]. The overexpression of Id2 and Id4 depleted the cancer stem cell pool in glioblastoma cells by reducing stem cell marker expression and neurosphere formation ability. This promoted neuronal/astroglial differentiation and suppressed oligo-dendroglial differentiation in culture [139]. These effects were likely mediated by the downregulation of the oligodendroglial lineage-associated transcription factors 1 and 2 [139]. The concurrent genetic deletion of Id1, Id2 and Id3 in a mouse model of human mesenchymal high-grade glioma de-repressed Rap1gap and inhibited the TERF2 interacting protein, RAP1, resulting in the rapid release of glioma initiating cells from the perivascular niche and tumor regression [140]. In breast cancer, Id1 increased cancer stem cell activity by activating Wnt/β-catenin/TCF signalling [141]. Most recently, a IGF2-IGF1R-PI3K-Id1-IGF2 positive feedback circuit was shown to be crucial for the maintenance of stem-like properties in patient-derived breast cancer cells [31] (**Figure 3**).

Still, Id1 has been identified as a functional marker for intestinal stem and progenitor cells, which is required for the normal response to injury [142]. Double knockdown of Id1 and Id3 reduced the self-renewal capacity of human colon cancer stem/initiating cells through the downregulation of p21 [143]. Id1b, an isoform of Id1 generated by alternative splicing, inhibited proliferation, maintained cell quiescence and promoted self-renewal and an undifferentiated cancer stem cell-like phenotype in murine colon carcinoma cells [144]. The MLLT3, super elongation complex subunit (MLL-AF9) was found to downregulate Id2 and upregulate transcription factor-4 (TCF4) expression in MLL-rearranged acute myeloid leukemia [145]. Id2 has also been found to promote the self-renewal potential of cancer stem cells by acting downstream of hypoxia-induced Wnt/β-catenin signaling during colorectal cancer development [100]. The bone morphogenetic proteins (BMPs) also contribute to the cancer stem cell phenotype by acting as transcriptional regulators of Id proteins. As evidence, the blockade of BMP signaling by the small molecular antagonist DMH2 decreased the expression of Id1 and Id3, and reduced the growth of lung cancer cells expressing Oct4 or nestin [146].

Id proteins and chemoresistance: Id proteins have been implicated in the resistance to chemotherapy in a variety of human cancers. For example, overexpression of Id1 reduced titanium dioxide-induced cytotoxicity in H1299 non-small cell lung cancer cells [147]. Conversely, knockdown of Id1 in prostate cancer cells led to increased sensitivity to taxol-induced apoptosis [148]. In another setting, the overexpression of Id1 in CNE1 nasopharyngeal carcinoma cells suppressed drug-induced apoptosis in response to nine anti-cancer drugs, whereas knockdown of Id1 exhibited the opposite effect [149]. Similar observations have also been

made in human bladder cancer cells [150]. Likewise, the induction of Id1 by nicotine was shown to confer resistance to gemcitabine-induced apoptosis in pancreatic cancer cells *in vitro* [37]. Together, all these studies strongly suggest that Id1 is able to protect cancer cells against anticancer drug-induced apoptosis. Therefore, alternative therapies encompassing suppressors of Id1 might be a potential approach to improve the efficiency of traditional chemotherapeutic drugs.

Id protein regulated chemosensitivity in human cancers is mediated through multiple signaling pathways. For example, it has been shown that Id1 expression protects esophageal squamous carcinoma cells from tumor necrosis factor (TNF)-alpha-induced apoptosis by upregulating and activating Bcl-2 [62]. Furthermore, Id1 reduces Bax expression and increases Bcl-2 expression by upregulating P53 and NF-kB, thereby protecting breast cancer cells from taxol-induced apoptosis [151]. In prostate cancer cells, the increased sensitivity to the taxol-induced apoptosis observed after knocking down Id1 resulted in the activation of c-Jun N-terminal kinase (JNK) signaling [148]. The attenuating effect of Id1 on titanium dioxide-induced cytoxicity described above was mediated in part through JNK and/or ERK signaling [147]. Moreover, in prostate cancer cells, the activation of Akt signaling by the physical interaction between Id1 and caveolin-1 was demonstrated to be essential for the resistance to taxol-induced apoptosis [111]. In esophageal cancer cells, competitive binding between Id1 and E2F1 to cell division cycle protein 20 (Cdc20) protected the E2F1 protein from Cdc20-mediated degradation, and subsequently increased chemoresistance to 5-fluorouracil through E2F1-dependent induction of IGF2 and thymidylate synthase expression [152]. Stat3-induced Id1 expression was shown to mediate the inhibitory effect of leukemia inhibitory factor (LIF) on P53 and P53-mediated apoptosis by upregulating MDM2 expression and promoting resistance to chemotherapeutic agents in human colorectal cancer cells [153]. Vascular endothelial cells have also been reported to rescue chronic lymphatic leukemia cells from spontaneous and drug-induced cell death via an Id protein-coupled redox-dependent mechanism [68]. TGF-β signaling is activated by 5-fluorouracil treatment in drug resistant colorectal cancer cells, and inhibition of TGF-β RI by its specific inhibitor was able to restore the chemosensitivity to 5-fluorouracil by reducing the expression of BCL2-like 1 (BCL2L1) and Id1 [154].

Chemotherapeutic drugs modulating Id1 expression also play important roles in chemoresistance. For example, both Id1 gene and protein expression were found to be downregualted following exposure to nine anticancer agents in five different cancer cell lines including nasopharyngeal (CNE1), cervical (HeLa), breast (MCF7), hepatocarcinoma (Huh7) and prostate cancers (PC3). This was accompanied by increased drug-induced apoptosis [149]. Treatment of human breast cancer cells with gamma-tocotrienol inhibits Id1 and NF-kB expression by modulating their upstream regulators such as Src, Smad1/5/8, Fak and LOX, all of which contribute to the induction of apoptosis and promotion of chemosensitivity [155]. Accordingly, partially restoration of Id1 protected cells from gamma-tocotrienol-induced apoptosis [155]. The resistance of human esophageal squamous cell carcinoma cells to etoposide-induced cell apoptosis was found to be enhanced by Id1 by inhibiting caspase 3 activity and PARP cleavage in a c-Jun/c-Fos-dependent manner [53]. Id1 upregulation by etoposide was mediated through the transcription factor activator protein 1 (AP1) binding sites within the Id1 promoter [53]. Cancer stem cells have an inherent resistance to chemotherapy and radiation, and as stemness inducing factors, the knockdown of Id1 and Id3 was shown to increase the sensitivity of human colon cancer-initiating cells to the chemotherapeutic agent oxaliplatin [143]. Conversely, enhanced cell self-renewal capacity was observed in mouse glioblastoma cells following the induction of Id1 by PGE2 resulting in resistance to radiation-induced DNA damage [44].

Importantly, it is necessary to note that some studies have contradicted the chemoresistance inducing effect of Id proteins and have found that Id expression correlates with the induction of apoptosis. For instance, Id3 has been reported to sensitize A430 human squamous carcinoma cells to cisplatin and 5-fluorouracil treatment [69]. These studies are however much fewer than those that show the enhancement of chemoresistance.

A promising molecular target for cancer therapy

Overexpression of Id proteins has been associated with tumor progression and metastasis in many types of human cancers, suggesting that the modulation of Id protein expression at the transcriptional or translational level could be an attractive strategy for anticancer therapy. Knockdown studies have shown that the functions of Id proteins can be reduced by Id-specific antisense oligonucleotides or small interfering RNAs, resulting in growth arrest and induction of apoptosis in various types of tumor cells. However, the use of antisense technology to inhibit Id protein expression is still not possible in human cancer patients.

Cannabidiol (CBD), a non-toxic cannabinoid, was demonstrated to downregulate Id1 expression and upregulate Id2 expression in human breast cancer cells, thereby inhibiting cell proliferation and invasion *in vitro* [156]. *In vivo* studies further showed that CBD treatment could downregulate Id1 expression and reduce primary tumor growth and secondary lung metastases in immune competent mouse models of breast cancer [157]. In human glioblastoma cells, CBD not only downregulated Id1 gene expression and inhibited cell invasiveness, but also decreased neurosphere formation [96]. Moreover, these results were mirrored *in vivo* as treatment of mice with CBD inhibited Id1 expression and reduced tumor growth in an orthotopic model [96]. Along similar lines, the inhibition of Id1 by CBD in murine AE9a expressing leukemia cells was shown to impair cell growth through induction of cell cycle arrest and apoptosis *in vitro* and prolong the median survival of the AE9a leukemia mice [158]. Recent studies in human aggressive salivary cancer have also confirmed the CBD mediated downregulation of Id1 gene expression and subsequent anticancer effects both *in vitro* and *in vivo* [93]. Taken together, these results suggest that CBD might have the potential to become a non-toxic exogenous agent for the treatment of cancer patients, especially those with documented overexpression of Id proteins.

2-Methoxyestradiol, a metabolite of 17-B-estradiol, was shown to downregulate the gene and protein expression of Id1 in mouse and human mammary tumors and endothelial cell lines, indicating an anti-angiogenic potential for the substance [159]. MK615, an extract of the Japanese traditional medicine apricot, was found to also reduce the gene and protein expression of Id1 by inhibiting the phosphorylation of ERK1/2, and subsequently inhibit cell growth in A375 melanoma cells [160]. Ma et al [161] reported that epigallocatechin-3-gallate inhibited proliferation and promoted apoptosis in poorly differentiated human gastric cancer cells by downregulating Id1 mRNA and protein expression. Berberine, a pure compound originally derived from Chinese medical herb has also been shown to inhibit cell growth, migration, invasion and pro-angiogenic activities in hepatocellular carcinoma *in vitro*, and to suppress primary and metastatic tumor growth *in vivo* [162]. The downregulation of Id1 was identified to be a novel mechanism of the anti-growth and anti-invasive effects of berberine in hepatocellular carcinoma cells [162]. Still, an arabinogalactan from flowers of Panax notoginseng has been demonstrated to reduce the migration and tube formation of endothelial cells and inhibit angiogenesis by inhibiting BMP2/Smad2/Id1 signaling [163].

Inhibition of Id function at the protein level by generating libraries of small peptides or aptamers has also been evaluated as a promising anticancer approach. Since structural similarities exist between the Id proteins and bHLH binding partners, drugs targeting Id proteins must be selective for the Id-bHLH interaction and not affect bHLH-bHLH dimerization [164]. Furthermore, since Id1 and Id3 have overlapping biological functions and can compensate for each other, inhibition of either Id protein might not be sufficient for a satisfactory antitumor effect [73]. A peptide-conjugated antisense oligonucleotide was able to effectively downregulate Id1 in endothelial cells, inhibiting primary tumor growth and metastasis in MDA-MB-435S aggressive breast cancer xenografts as well as Lewis lung carcinoma (LLC) allografts [164]. Similarly, a peptide aptamer Id1/3-PA7 delivered into cells selectively inhibited the intracellular functions of Id1 and Id3 proteins, and did not affect bHLH dimerization [165]. In breast cancer cells, Id1/3-PA7 inhibited the heterodimerzation of E47 with Id1 or Id3, enhanced E-box promoter activity, increased the expression of cyclin-dependent kinase

inhibitors 1A and 1B tumor suppressors and induced the cleavage of poly ADP ribose polymerase, which resulted in cell cycle arrest and apoptosis [165]. Similar effects of Id1/3-PA7 have also been shown in ovarian cancer ES2 and PA-1 cells [165].

In a different approach, Ciarapica and colleagues used a dominant interfering HLH dimerization mutant, 13I. This was able to strongly bind Id2, preventing it from interacting with bHLH proteins [166]. Treatment of human neuroblastoma cells with 13I induced cell differentiation, inhibited proliferation and VEGF expression, enhanced cell responsiveness to all-trans retinoic acid and attenuate tumorigenic properties *in vitro* [166].

All these findings suggest that inhibition of Id proteins functions is a viable strategy that could have significant implications in the treatment of cancer.

Conclusions and perspectives

The expression of Id proteins is tightly regulated; however, deregulation is common in a wide variety of human cancers. A large body of research has already tried to decipher and understand how these molecules contribute to human cancer progression and metastasis. Nonetheless, further investigation is warranted to fill the gaps in our understanding of these molecules, particularly as it relates to effects that are contextual and cancer cell type specific.

Id proteins have been implicated in virtually all aspects of tumor progression including cell proliferation, migration, invasion, EMT, angiogenesis, tumorigenesis and metastasis. With a footprint in all of these processess, it becomes increasingly important to not only identify and catalogue the functional mediators responsible for the individual oncogenic events, but also understand how the interactions leading up to the events are orchestrated. Overexpression of Id proteins has been correlated with advanced tumor stages and poor prognosis and this means they could potentially be used as biomarkers.

Importantly, increasing evidence demonstrates that Id proteins are involved in the protection against anticancer drugs-induced cell apoptosis, and unraveling the molecular mechanism contributing to the conferment of this survival benefit would be of significance. The critical role of Id proteins playing in maintaining the cancer stem cell population may play an important role in drug resistance. More so, this stemness maintaining trait means that Id proteins and their downstream targets are potential candidates for differentiation therapy in cancer. Differentiation therapy reactivates the endogenous differentiation programs in cancer cells with subsequent tumor cellular maturation and concurrent loss of the tumor phenotype [167].

Moreover, Id proteins are potentially attractive molecular targets for anticancer therapy. The development of nontoxic compounds against Id proteins for translation into clinical care has witnessed some progress, and is also of paramount importance. Already promising results have been documented with the non-toxic cannabinoid compound CBD, but further studies are needed to determine whether it can be used in other types of human cancer besides breast cancer and glioblastoma. Efforts to develop new CBD analogs targeting Id proteins may be warranted. Since the biological functions of Id proteins in human cancers are diverse, it is important to identify particular cancer types where the targeting of these proteins could be of benefit. For example, Id4 has been reported to suppress MMP2-mediated invasion of glioblastoma-derived cells [4], pharmacological inhibition of Id1, Id2 and Id3 proteins should be highly specific to avoid inhibition of Id4.

As clinical oncology advances towards personalized cancer medicine, efforts are needed to find tools for identifying and stratifying patients that may benefit from Id targeted therapies and also for finding biomarkers that will predict response to therapy. These efforts also need to consider which other conventional chemotherapeutic agents can be co-administered with anti-Id therapy. Overall, an enhanced understanding of Id protein-regulated signaling pathways and molecules is critical for developing novel and more effective therapeutic strategies for the treatment of cancer patients.

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