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REVIEW

Paradoxical role of Id proteins in regulating tumorigenic potential of lymphoid cells

Sumedha Roy (✉), Yuan Zhuang

Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA

Abstract **A family of transcription factors known as Id proteins, or inhibitor of DNA binding and differentiation, is capable of regulating cell proliferation, survival and differentiation, and is often upregulated in multiple types of tumors. Due to their ability to promote self-renewal, Id proteins have been considered as oncogenes, and potential therapeutic targets in cancer models. On the contrary, certain Id proteins are reported to act as tumor suppressors in the development of Burkitt's lymphoma in humans, and hepatosplenic and innate-like T cell lymphomas in mice. The contexts and mechanisms by which Id proteins can serve in such contradictory roles to determine tumor outcomes are still not well understood. In this review, we explore the roles of Id proteins in lymphocyte development and tumorigenesis, particularly with respect to inhibition of their canonical DNA binding partners known as E proteins. Transcriptional regulation by E proteins, and their antagonism by Id proteins, act as gatekeepers to ensure appropriate lymphocyte development at key checkpoints. We re-examine the derailment of these regulatory mechanisms in lymphocytes that facilitate tumor development. These mechanistic insights can allow better appreciation of the context-dependent roles of Id proteins in cancers and improve considerations for therapy.**

Keywords Id proteins; lymphoma; leukemia; T cells; B cells; tumor suppressor; oncogene

Introduction

Since their discovery almost three decades ago, Id proteins have been associated with the maintenance of a state of anaplasia or dedifferentiation in cells [1–5]. They are known to facilitate this through inhibition of functions of various classes of ubiquitous and tissue-specific helix–loop–helix (HLH) transcription factors, including E proteins, ETS, PAX, MYOD and RB proteins. The role of Id proteins in preventing cell differentiation and promoting stem cell behavior has begged the question regarding their potential in giving rise to cancer stem cells, and therefore as oncogenes [3–6]. In support of their oncogenic functions, Id proteins have been reported to have high expression in multiple tumor types, and to contribute to anaplasia, angiogenesis and metastasis of tumors. The upregulation of Id proteins can be mediated by upstream oncogenic events that promote their overexpression, increase their stability, or abolish their suppression by tumor suppressors. Id protein dysregulation has been implicated in causing a wide range of malignancies, including, but not limited to, tumors in the bladder, breast, prostate, liver and brain (summarized in [5]). However, gain-of-function alterations in Id genes in human tumors, that can conclusively verify the oncogenic nature of Id proteins, are a key piece to the puzzle that is still missing. Nonetheless, efforts spearheading the inhibition of Id proteins as potential therapeutic targets are underway [4,5,7]. The notion of Id proteins acting as oncogenes, however, has also now been challenged by recent data demonstrating their tumor suppressive roles in some cancers, most prominently in Burkitt's lymphoma. The overall role of Id proteins in normal development and tumorigenic pathways has been reviewed extensively [1–6].

Considering the different potential interaction partners and cell-specific functions of Id proteins, it is hard to uniformly and exhaustively predict the role of Id proteins in distinct tumor types. Blood cancers, including lymphomas and leukemias, are estimated to constitute up to 8% of new cancer cases diagnosed this year (Lymphoma and Leukemia Society statistics). In this review, we summarize the underlying pathways that drive either Id-mediated tumorigenesis or tumor suppression in lymphocytes. E proteins are the most typical interaction partners of Id

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Correspondence: Sumedha Roy, Sumedha.roy@dm.duke.edu, sumedharoy@gmail.com

proteins in lymphocytes, where these transcription factors are highly expressed, and play crucial roles in regulating cell differentiation and proliferation through direct inhibition, and mutual regulatory feedback loops. Additionally, phenotypic similarities between lymphocyte-derived tumors that develop in Id-overexpression and E protein null mutant mice suggest that Id proteins often mediate their functions in these tumors through inhibition of E protein activity [3].

Antagonism between E and Id proteins

Id proteins, including Id1, Id2, Id3 and Id4, are members of class V HLH proteins that can dimerize with other HLH protein classes, but unlike their binding partners, lack a DNA binding domain. They possess structural and conformational flexibility that is necessary for them to interact with different binding partners. All four Id protein family members share highly conserved HLH domains, but have differences in the N- and C-terminals, which can allow them to bind to diverse partner protein classes, albeit with varying binding preferences [4,7–9]. Among the different interaction partners, E proteins and the retinoblastoma protein, RB, are the only ones that are found to physically interact with Id proteins under physiological and disease conditions *in vivo*, as well as influence the expression of Id proteins. In this regard, the interaction of Id2 and RB is also suggested to be crucial for allowing uninhibited E protein activity [4].

E protein family members, E12, E47, HEB and E2-2, are classified as class I HLH proteins, and serve as key transcriptional regulators. These canonical interaction partners of Id proteins [4] are more commonly referred to as basic helix–loop–helix (bHLH) transcription factors because of the additional basic DNA binding region. These transcription factors form homo- or heterodimers with other factors through the HLH domains, and simultaneously identify and bind to E-box motifs in the regulatory regions of genes to facilitate their transcriptional activation or repression. The interaction between the HLH Id proteins and bHLH E proteins precludes the DNA binding ability of the latter, making Id proteins potent inhibitors of E protein function. Interestingly, the antagonistic interaction between E and Id proteins is utilized as gatekeepers or natural "brakes" in lymphocyte development, such that there is halt in progression into the next stage of development or maturation, until appropriate signals that regulate their expression tip the E/Id protein balance to allow developmental progression accompanied by the expression of appropriate target genes for the next stage. Overall these "brakes" ensure smooth regulation of cell proliferation and differentiation during development. The appropriate expression of, as well as the interaction between, E and Id proteins tightly control developmental outcomes, and therefore, their dysregulation causes the brakes to "fail," often resulting in cells being predisposed to malignant transformation. It is crucial to understand the various factors that can determine physiological versus tumorigenic outcomes driven by E and Id proteins (Fig. 1).

While we do not wish to enumerate all observations that have led to predominant beliefs about the oncogenic roles

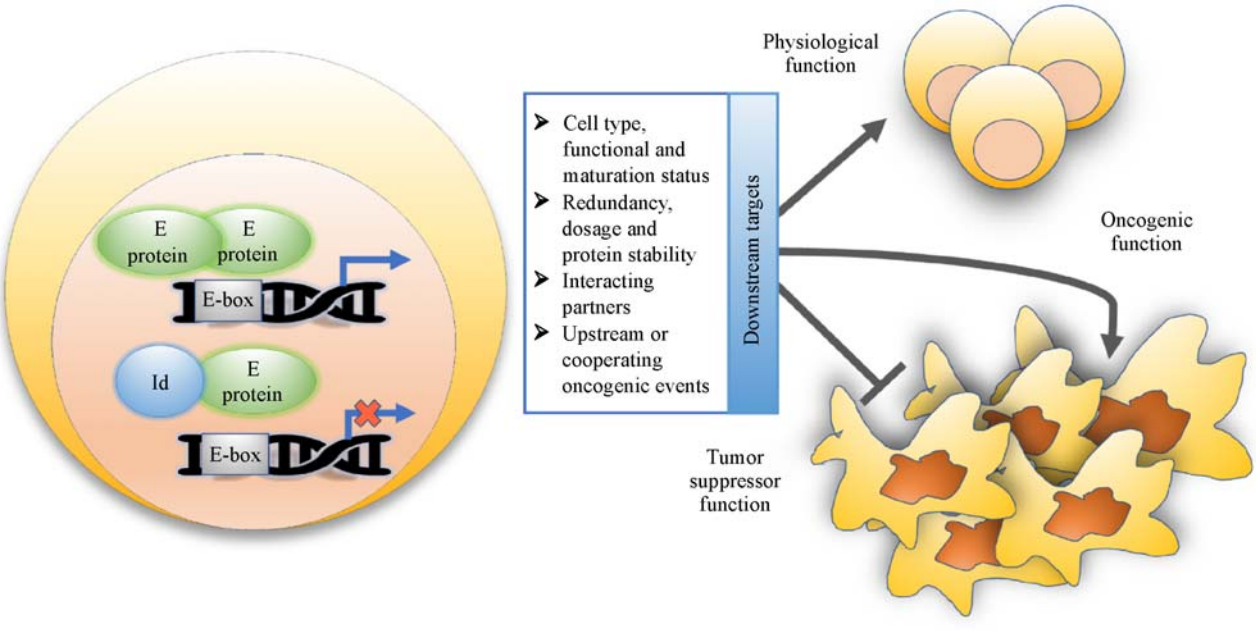

Fig. 1 Factors that influence context-dependent roles of E and Id proteins in development and cancer.

of Id proteins, and corresponding tumor suppressor roles of E proteins, it is worth mentioning a few of them to provide a historical perspective. The observation of induction of high Id expression in cells that are undifferentiated and proliferating supported the idea that Id proteins could contribute to anaplasia [3,10]. Even though just overexpression of Id proteins was not enough for immortalization of cell lines, it supported a pluripotent, stem cell-like fate in the presence of leukemia inhibitor factor (LIF) [4,11]. It was proposed that Id2 and Id3 may act together to control cyclin E (*Ccne1*) and cyclin A (*Ccna2*) [1]. Id proteins were also found to suppress the regulators of cell senescence, such as *Rb* and cyclin dependent kinase inhibitors *p16INK4a*, *p21* and *p27*, which can be activated by Ets and E protein family members [12,13]. In stark contrast, E2A was found to activate *p21*, and to play a role in apoptosis and growth suppression in NIH3T3 cells [14,15]. A forced MyoD-E47 dimer that can no longer bind to Id proteins can overcome the effects of Id1 overexpression to promote differentiation in target cells [1,16]. Id protein half-life is also extended by binding to E2A [17]. Therefore, the dynamics between E and Id proteins is crucial in maintaining a balance between cell proliferation, survival and differentiation either through their direct regulation of relevant downstream targets, or through indirect inhibition of binding to other HLH interaction partners, such as RB and ETS proteins.

In the sections below, we discuss the roles of E and Id proteins in regulating B and T lymphocyte development, as well as how their function is hijacked to cause lymphoma and leukemia in humans and mice. This derailment of E and Id proteins can change their roles from promoting hematopoietic differentiation to triggering oncogenic proliferation.

Physiological role of E and Id proteins in B cell development

E2A is essential for early stages of B cell development, as it is necessary for instructing a lineage-specific program [18–25]. E proteins induce the expression of FOXO1, which then works with E2A to induce EBF1, and all three activate PAX5 for B cell lineage determination [26]. As a result, a deficiency in E2A restricts B cell development at the pre-pro-B cell stage, which can however be rescued by forced Pax5 expression [27]. E proteins also support the survival and proliferation of pro-B cells [28]. A proliferative burst may be activated by a slight downregulation of E protein activity that follows pre-BCR signaling. E proteins can also regulate cell proliferation through cyclin D3 [25]. On the other hand, Id3 can induce apoptosis in progenitor B cells in a caspase-dependent fashion [29].

While E proteins play critical roles in early steps of B cell development, it has been shown that E proteins are dispensable for the generation and maintenance of mature, splenic B cells [27]. Nonetheless, E proteins regulate immunoglobulin κ locus rearrangements, somatic hypermutation (SHM) and class switch recombination (CSR). Their absence impairs germinal center (GC) B cell development through a mechanism that is independent of CSR, survival and proliferation, but is not fully characterized [27]. Id3 expression is low in GC B cells, but is essential to allow expression of genes for BCR signaling, cytokine signaling and CSR. Id3-deficient GC B cells are found to have a proliferation defect, which is important for CSR [30].

Tumor suppressor Id proteins counteract oncogenic E2A activity in B cell lymphomas

Burkitt's lymphoma (BL), typically characterized by a Myc translocation, is a type of non-Hodgkin lymphoma derived from malignant transformation of GC B cells [31,32]. It represents the first cancer type where the documentation of loss of function mutations in the *Id3* gene questioned the idea of Id proteins acting as only oncogenes [33–35]. Frequent mutations in *Id3* are found in BL patients, more frequently in adults, than in pediatric BL [36]. *Tcf3* (gene encoding E2A), which is otherwise highly expressed in GC B cells, as well as its target cell cycle regulator gene *Ccnd3* (cyclin D3), are also often mutated in BL [33–35]. While mutations in *Id3* are more common than mutations in *Tcf3*, some BL patients have mutations in both. The *Id3* mutations are usually bi-allelic inactivating mutations that block its binding to E2A [33]. *Tcf3* mutations on the other hand are mono-allelic, and most commonly affect the HLH dimerization domains of E47, but not E12. Some *Tcf3* mutants have broad binding profiles similar to WT E2A, but rare mutants can have slightly altered binding preference with respect to the E-box motif, thereby regulating different downstream targets [33]. BL samples that do not have mutations in Id3 have significantly higher expression of *Tcf3*, suggesting an important role for E protein-mediated tumorigenesis [34]. *Tcf3* expression has also been shown to be important for the survival of BL cells.

Interestingly, these lymphoma cells still retain some genes pertaining to their GC identity. Genes such as *Pou2af1*, *Cxcr4*, *Ltb*, which are normally expressed in GC B cells, are overexpressed in *Tcf3* mutant BL cells. In terms of cell cycle, Tcf3 mutants upregulate *Ccnd3* and *E2f2* and downregulate *Rb1* [35]. *Ccnd3* is important for cell cycle progression in GC B cells [37,38], and is mutated in a variety of BL forms, albeit at a lower frequency than other mutations [35]. These *Ccnd3* mutants have survival and proliferative advantage. Along similar lines, deleterious mutations are also found in the CDK6 inhibitor p16, and treating BL cell lines with CDK6 inhibitors induces cell death [35]. Overall, increased proliferation and survival of

the malignant GC B cells in BL is promoted through TCF3-mediated Ccnd3 upregulation and PI3K upregulation through SHP-1 repression downstream of tonic BCR signaling [33–35,39]. E2A can also promote survival in transformed pro-B cells independent of *Bcl2*, through the inhibition of caspase activity [40]. These expression profiles, and the dependency on E2A for BL survival, suggest that the normal GC B cell program is hijacked for tumorigenesis.

Even though Myc translocation next to one of three immunoglobulin loci is a hallmark in BL, and is capable of regulating cell cycle, growth and apoptosis, it is not enough to cause malignant transformation [41,42]. Myc translocation is therefore thought to be an early initiating event. *Id3* has also been mapped to be located in the minimal region of recurrent imbalance with respect to the Myc translocation, potentially explaining recurrent mutations in the gene [34,43]. Based on the evidence presented above, Id3 inactivation combined with Myc translocation may be considered a more representative hallmark of BL. Even then, a small percentage of patients with other non-BL lymphomas that have Myc translocations are reported to have *Id3* mutations as well [34]. The *Id3* mutations seem to be downstream of the Myc event, since a subset of B cell lymphomas that resemble BL features and gene expression patterns but don't have Myc translocation don't show mutations in *Id3* [44]. Myc also has dual roles in general in regulating cell cycle as well as apoptosis. Yet, the latter suppressive function is somehow overcome in BL, perhaps through the inactivating mutations in Id3 [45]. Inhibition of Pim3, a target of Myc in BL cell lines, leads to cell death in Myc-induced lymphomas independent of caspase activity [46].

Surprisingly, negative feedback regulation is still active in BL, as *Tcf3* mutants have increased expression of *Id1*, *Id2* and *Id3* [35]. This upregulation could be mediated by either E2A or Myc, since both can directly regulate Id proteins. *Id2* is found to be upregulated in Eμ-Myc BL, but its loss doesn't impact the survival or incidence of these lymphoma cells [47]. However, cells transfected to overexpress wild-type *Id3* seemed to have disadvantaged cell growth in cell culture, reflecting the tumor suppressor function of *Id3* in BL [34]. *Id4* expression is reduced through hypermethylation in Raji human BL cell line. Use of a demethylating agent reverses that to induce Id4 expression, which leads to cell cycle arrest and apoptosis [48]. TGF-β, which can induce Id3 to inhibit growth in progenitor B cells [29], can also inhibit growth of BL and B cell lymphoma cell lines by repression of *E2f1*, accompanied by activation of Smad signaling, p38 MAPK, and an increase in *Id1* and *Id2* expression [49,50].

In contrast, the role of Id3 in diffuse large B cell lymphomas (DLBCL), another type of mature B cell non-Hodgkin lymphoma, is yet to be fully ascertained. DLBCL is further classified based on gene expression profiling and different stages of differentiation as being derived from germinal center (GC) B cells, activated B cells (ABC) and primary mediastinal B cell lymphomas (PMBL). A recent study characterizing mutations in a large cohort of DLBCL patients did not find any mutations in *Id3* [51]. On the other hand, two other studies have reported a small percentage of patients from all three DLBCL subtypes to have mutations in *Id3* that affect its HLH region [39,52]. However, it remains to be determined if Id3 plays similar tumor suppressive roles in these DLBCL cases [53]. One of the studies also found *Tcf3* mutations that altered DNA binding specificity, whereas the other noted *Ccnd3* mutations among some DLBCL patients. When comparing the mutations in BL to DLBCL, it is crucial to note that the clinical diagnosis and classification of lymphomas as BL or DLBCL can strongly bias these results. Even though the World Health Organization (WHO) has laid out guidelines regarding the physiology and gene expression patterns that can be used to distinguish between these two, some mature B cell lymphomas, especially those derived from GC B cells, are hard to classify as either, and are placed in a third category known as B cell lymphoma unclassifiable (BCL-U). Given the contradictory reports on *Id3* mutations in DLBCL, *Id3* is not an ideal mutation to distinguish between BL and DLBCL. Recurrent *Id3* mutations are found in lymphomas classified as BCL-U, as compared to those classified as true DLBCL [54]. Combined mutations in *Id3-Ccnd3-Tcf3* have been suggested as a marker used to re-classify borderline BL/DLBCL cases as BL [39]. The age of patients in each study must also be considered since *Id3* mutation burden correlates with age [36,39]. Due to these reasons, the role of Id proteins in DLBCL cases remains ambiguous.

The discordance between *Id3* mutations in BL and DLBCL may partly be explained by the type of GC B cells they are derived from [45,55–57]. During the germinal centre reaction, activated B cells undergo repetitive cycles between somatic hyper mutation (SHM) and proliferation in the dark zone (DZ), and class switch recombination (CSR) and positive selection in the light zone (LZ). The SHM and CSR recombination events at the immunoglobulin loci mediated by activation-induced cytidine deaminase (AID) predispose GC B cells in both LZ and DZ to translocation events and tumorigenesis [58–60]. Based on gene expression patterns, BL cells have been found to resemble dark zone GC B cells, whereas DLBCL and most other GC-derived B cell lymphomas resemble light zone GC B cells [55,61]. DZ GC B cells, or centroblasts, are characterized by their high proliferation rate, and high expression of E2A and its target Ccnd3 [55,62]. Besides cell proliferation, E2A has also been shown to preferentially drive expression of typical genes important for centroblasts [62]. Id3, on the other hand, is upregulated by a negative feedback loop to keep E protein activity in check, and reduces with each cell cycle [63]. Interestingly

however, Myc, is typically expressed only when positively selected centrocytes are moving back to the DZ, where it is again suppressed [64,65]. Myc translocation patterns in sporadic BL suggest derivation from CSR events [66,67]. Therefore, one possibility is that low levels of AID activity [59,60,68] induce a Myc translocation in this window of transition from LZ to DZ, such that the cells undergo proliferation and further mutations upon reaching the DZ, ultimately leading to malignant transformation. Given the important physiological function of E2A in centroblasts, there might be a selection pressure among transformed cells for mutations that have enhanced E2A activity, allowing the cells to inherit their normal transcriptional identity and proliferative capacity. As a result, loss-of-function *Id3* mutations in centroblasts are likely to be selected for in the case of BL pathogenesis, in addition to the recurrent imbalance in the *Id3* locus upon Myc translocation [34]. However, it is possible to alternatively envision *Id3* or *Tcf3* mutation as the initiating oncogenic event for BL. In this case, the high E2A activity in mutated, highly proliferating DZ cells can potentially prime the *Myc* locus for a secondary translocation event, supported by AID expression and SHM. On the other hand, the selection pressure of oncogenic mutations in LZ centrocytes leading to DLBCL pathogenesis is likely to be different from BL, leading to frequent mutations in Bcl6 [69]. In support of preferential E2A function in centroblasts but not centrocytes, E2A is found to be important for BL survival, but does not affect DLBCL cell line survival [35]. Lymphomas that have intermediate phenotype between BL and DLBCL, or double-hit with Myc and Bcl2 and/or Bcl6, also tend to have frequent *Id3* mutations [53,54].

Oncogenic roles of Id proteins in B cell lymphomas

As highlighted in Fig. 1, the subtype of cells, and their functional and activation status, can determine the type of role played by E and Id proteins in lymphomas derived from those cells. While Id3 seems to play a tumor suppressor role in GC B cells that give rise to BL and perhaps GC B DLBCL, it may play oncogenic roles in other subtypes of B cell lymphomas. For instance, high expression of *Id1* and *Id3* genes in B cell adult lymphoblastic leukemia (B-ALL) leads to poor prognosis in adult patients [70]. *Id4* has also been found to be overexpressed as a result of a translocation in B-ALL [71]. Multiple inactivating mutations in *Prdm1* prevent Id3 suppression, such that *Id3* expression is always on in ABC DLBCL [72]. Follicular lymphomas upregulate *Id2* compared to healthy GC B cells [73].

While most B cell-derived lymphomas maintain expression of B cell factors, the loss of the B cell-specific program is a typical characteristic of mature B cells that give rise to Hodgkin-Reed/Sternberg (HRS) cells in classical Hodgkin's lymphoma (HL). *Id2* is highly overexpressed in HL, perhaps due to chromosomal gain, and contributes to the block in the B cell program [74]. This overexpression of Id2, along with ABF-1 (activated B cell factor 1), antagonizes E2A, resulting in reduced expression of B cell-specific genes like *Pou2f2*, *Cd19*, *Aicda*, *Cd79a*, *Ebf* and upregulation of T cell-specific genes such as *Gata3*, *Tcf7*, *Maf*, *Tbx21*, *Csf1r*. B cells are also found to hyperproliferate in mice lacking one copy of E2A [75,76]. High Id2 expression is considered a marker for HL [77], and an increase in Id1 expression is proposed to be a marker for cancer-initiating cells in HL [78]. Primary effusion lymphoma (PEL) is a type of non-Hodgkin lymphoma that is derived from post GC B cells that lose their B cell-specific genes to more closely resemble Hodgkin lymphomas. PEL samples are also found to lose E protein activity, partly due to the upregulation of Id2 and ABF-1. E2A overexpression leads to induction of apoptosis, suggesting an Id-mediated oncogenic function that suppresses E protein activity [79].

Oncogenic roles of E proteins in B cell lymphomas

The activating mutations in the E2A HLH region, that prevent Id-mediated suppression in BL patients are reflective of the oncogenic role of E proteins in BL. Nearly 50% of MALT (mucosal-associated lymphoid tissue) lymphoma cases, derived from marginal zone memory B cells aberrantly express *Tcf3*, even though it is not a memory cell marker, and may associate with poor treatment response [80]. There are translocations observed in B-ALL patients that give rise to oncogenic E2A fusion proteins. The fusion of E2A with HLF (hepatic leukemia factor) is found to contribute to B-ALL formation, particularly from pro-B cells [81]. E2A has been found to promote survival in transformed pro-B cells through the inhibition of caspase activity, and can therefore function as an oncogene in these cells [40]. In such patients, normal E2A targets are affected, but new oncogenic targets may also emerge. E2A is sufficient to drive the tumor, as long as it is overexpressed and/or dimerized with the fusion partner. The fusion protein requires both the E2A transactivating domain and the HLF basic region/leucine zipper dimerization domain [81,82]. Interestingly, transgenic mice expressing E2A-HLF have B and T cell maturation defects and give rise to T cell lymphomas [81]. Expressing the E2A-HLF fusion protein in 3T3 cells correlates with cell growth or density, supporting its oncogenic function. E2A-PBX1 is another fusion protein identified in humans with the pre-B cell ALL [82]. The fusion protein derived from the transactivating domain of E2A and DNA binding domain of homeobox gene PBX1 was originally identified

in human pediatric pre-B cell leukemias. The fusion protein is not sufficient to give rise to leukemia without secondary mutations since the premalignant cells are found to proliferate more, but also with more apoptosis [83,84]. E2A also lies in the chromosomal translocation breakpoint in pre-B cell leukemia, which leads to a chimeric fusion such that the dimerization and DNA binding domains of E2A are replaced by the DNA binding domain of the homeobox protein [85]. In cases such as those discussed above, where E2A targets are modified by the DNA binding domain of its fusion partner, the oncogenic role of E2A is no longer reflective of its physiological function.

Physiological role of E and Id proteins in T cell development

E proteins play crucial roles in early stages of T cell commitment, lineage specification and differentiation upon appropriate T cell receptor (TCR) expression [20,25,86–88]. In line with this, there is a significant developmental block at the early double negative (DN) stage in the absence of E2A [89,90]. E2A is found to activate Notch signaling to further induce T cell-specific genes like *Tcf1*, *Gata3* and *Bcl11b* [26]. E2A blocks developmental progression at pre-TCR and TCR selection checkpoints such that only a productive signal can activate downstream events that upregulate *Id3* expression to ultimately overcome the E2A-mediated block [88]. A proliferative burst after pre-TCR selection is associated with the induction of cyclin D3 (*Ccnd3*) by E protein family member E47 [91,92]. Several other target genes that help E47 in regulating cell survival, cell cycle and maturation have been elucidated, and involve the induction of JAK/STAT pathway and SOC signaling [93].

Tumor suppressive roles of E2A in T cell lymphomas

Malignant T cells can give rise to different kinds of lymphomas and leukemias in humans. A recurrent gene deletion is found in E2A in 70% of patients with Sezary syndrome, a type of T cell lymphoma, which is the first report demonstrating inactivating alterations in the E2A gene in human lymphomas [94]. This gene deletion results in upregulation of the cell cycle regulator *Cdk6*, which is a known E47 target [93] and can execute the anti-apoptotic and proliferative effects of NOTCH in these tumors. Interestingly, all chromosomal losses of E2A detected in these tumors were heterozygous. The authors propose that one copy of E2A is necessary for the survival of these cells, and therefore homozygous mutations are selected against. The remaining copy of WT E2A may also be post-translationally degraded by NOTCH1 [94].

Anaplastic large cell lymphoma (ALCL) is a type of peripheral T cell lymphoma (PTCL), where gene translocations are commonly observed. It was reported that the translocation that gives rise to an NPM-ALK fusion protein, also leads to abnormal upregulation of genes that are proximal to the breakpoint, including *Id2*. This *Id2* overexpression impairs E2A-mediated regulation of T cell specific genes like *Cd3*, *Lck*, *Fyn*, *Tcf7*, *Tbx21*, and *Gata3*, explaining the loss of T cell phenotype observed in ALCL cases. This supports tumor suppressor activity of E2A that is sensitive to Id protein inhibition [95].

T-ALL, or T cell acute lymphoblastic leukemia, is a classic example for the role played by E and Id proteins in the tumorigenic process. Activation of the *Tal1* (also known as SCL) or the *Tal2* genes is often a major causal factor for T-ALL, and is achieved by translocation and other mechanisms in over 60% of T-ALL patients [96–98]. TAL1 is a bHLH transcription factor that forms an obligate heterodimer with E2A and HEB. It is typically required for HSC generation, and is not expressed in the thymus. Human T-ALLs show no sign of changes in E2A expression, but it has long been hypothesized that TAL1 inactivates E2A or usurps its tumor suppressor activity to give rise to T-ALL. Similarly, *Lyl1* is another gene that is activated in T-ALL and can bind to E2A, but results in regulation of different target genes [89]. TAL1 and LYL1 are involved in the maintenance of short-term and long-term HSCs respectively, where they each function with E proteins in a complex. This may be reflective of gene targets that drive T-ALL [40]. The mechanisms of T-ALL tumorigenesis have been explored by using mouse models and human lymphoma cell lines. Aberrant TAL1 overexpression in human tumor as well as mouse models gives rise to a developmental block at the DP stage [98]. TAL1 is found to mediate lymphoma development through a regulatory complex that comprises of E2A, HEB, LMO1/2, GATA3, RUNX1 and TAL1, where the latter three transcription factors are also part of a positive autoregulatory loop. Knockdown of these components inhibits cell growth and induces apoptosis [98]. Haplo-insufficiency of either E2A or HEB leads to a block in development at the DN stage, and accelerated T-ALL development in mice with *Tal1* overexpression [96]. However, the dosage of E proteins is critical for lymphomagenesis as both E2A and HEB are required for the TAL1 complex. Co-repression of typical E2A targets was also observed, partly mediated by the mSin3A/HDAC1 complex.

A study done in the Jurkat T-ALL cell line, where *Tal1* is overexpressed and complexed with E2A, found low E2A transcriptional activity for an E-box reporter gene. Further, cells transduced with E-T/2 (E2A-Tal1) to restore E protein activity, underwent growth arrest and apoptosis, supporting an inhibition model where Tal1 inhibits the downstream tumor suppressive genes of E2A, such as p21, which could not be induced in Jurkat T cells [97]. While some normal E2A targets were still conserved in T-ALL

tumor cell lines, such as *Ptcra*, *Notch3*, *Rag1/2* and *Gfi1*, TAL1 was found to promote some genes that are otherwise suppressed by E2A and HEB, and vice versa. One of those genes, *TRIB2*, is normally repressed by E proteins, but upregulated in the context of TAL1 complex, perhaps through TAL1 and NOTCH1 activity. *TRIB2* has been reported as an oncogene in AML, and has now been shown to be important for T-ALL survival through downstream activation of XIAP, inhibitor of apoptosis [98,99]. TRIB2 can also control the expression of TAL1 partners GATA3 and RUNX1, and destabilizes E2A through proteasomes [99]. These observations support the tumor suppressor role of E2A in T-ALL.

Independent of the tumor suppressor roles of E2A observed in T-ALL, it was observed that E2A-deficient mice exhibit an early block in T cell development and develop lymphomas derived from immature DP or SP thymocytes [89,90,94]. Tumors, but not premalignant cells, overexpress c-Myc, which can possibly be explained by the gain of an extra copy of chromosome 15. But it is unclear if E2A and c-Myc work together, or if c-Myc is the sole culprit in this lymphoma model [89]. These thymocytes aren't hyperproliferating or resistant to apoptosis. Overexpression of E2A in cell lines derived from lymphomas in E2A-deficient mice led to programmed cell death in the cells rather than growth arrest [91]. On the other hand, homozygous knockout of *Id1* in these E2A null mice improved survival but they still failed to rescue the tumor phenotype. This can be explained by a dominant tumor suppressor role of E2A, rather than being a direct Id-mediated effect [90]. The function of Notch acting together with, or in opposition to E2A in T cell development is still open to debate [25]. Their role in E2A-deficient or T cell lymphomas is equally debatable [100–104]. Notch has been proposed to represent a second hit in T-ALL as Notch1 and Notch3 are found to be aberrantly activated by translocations and somatic mutations in a large fraction of T-ALL cases, where the tumor depends on Notch signaling for survival [103]. Others have reported that Notch activity is not essential for tumorigenesis in T cell derived tumors with impaired E protein activity [101].

Paradoxical role of Id proteins in T cell lymphomas

Since E proteins were suggested to play tumor suppressive roles in T cell-derived human and murine cancer models, it was natural to assume the oncogenic roles of Id proteins in these contexts, except in T-ALL where TAL1 overexpression modifies E2A targets through Id-independent mechanisms. The overexpression of Id2 in ALCL, PTCL and multiple other types of T cell lymphomas, and its induction by Myc, supported this hypothesis [105]. In mouse models expressing an *Id2* transgene, there is a developmental block at early steps of T cell development, accompanied by hyperproliferating T cell lymphomas in most mice [106]. These tumors have high level of *Myc* expression upon malignant transformation, but not in premalignant stage, therefore c-Myc may be important for the tumor, but is not driven by Id2 overexpression. Not all mice with the *Id2* transgene develop tumor, suggesting that it is an important but not sufficient event for causing tumorigenesis. It is likely that overexpression of *Id2* (or E protein deficiency) blocks further differentiation in proliferating cells, predisposing them to secondary mutations, that represent either early transformation events after which the T cells can independently rearrange their TCR, or divergent transformation events, that give rise to polyclonal tumors [106]. A similar phenotype is observed in *Id1* transgenic mice, such that there is a severe block at T cell progenitor step with massive apoptosis triggered in most T cells, ultimately leading to lymphoma development. c-Myc may play a role in this phenotype since it can function both as an oncogene, and inducer of apoptosis. Induction of p21 by E2A may partly be able to block cell cycle progression in these cells [107]. These studies support the oncogenic roles of Id proteins in T cell-derived lymphoma development.

In direct contrast, there is also strong evidence in favor of tumor suppressor roles played by Id proteins in a subset of lymphomas derived from innate-like T cells. Knocking out *Id3* in mice gives rise to γδT cell lymphomas, resembling hepatosplenic γδT cell lymphomas (HSTCL) in humans. There is no dysregulation of genes that are commonly found in αβ T cell lymphomas, such as those described in the previous sections, including *Lyl1*, *Tal1* and *p21*. However, an increase in Myc expression was observed among some samples [108]. We have recently found that Id2 and Id3 also play tumor suppressor roles in invariant natural killer T (iNKT-) and innate-like tumors in mice [109]. These lymphomas develop much more rapidly than the γδT cell lymphomas in Id3-deficient mice, and display signs of chromosomal instability. The dichotomy observed in the gene expression programs for premalignant innate-like T cells in these mice is interesting — there is both downregulation and upregulation of tumor suppressor, anti-proliferative and cell cycle arrest genes, demonstrating a somewhat "balanced" state. However, after a presumed second hit, lymphoma cells have enrichment for genes often dysregulated in cancers, cytokine-cytokine interaction genes and Nf-κB signaling, similar to pathways reported in human NK/T tumors. Similarly, deficiency of Id2 and/or Id3 in mice also causes an expansion of innate variant T_{FH}-like cells, and αβ T cell lymphomas in these Id2/Id3-deficient mice that display an increase in Myc expression and reduction in the tumor suppressor Cdkn2a [88,110]. There is evidence to suggest tumor suppressor functions of *Id4* in T cell tumors. Exogenous expression of Id4 in lymphoid tumor cell lines induces caspase-dependent apoptosis. Additionally, methylation is found

at the Id4 promoter locus in multiple tumor models, including chronic lymphocytic leukemia (CLL), and a mouse model of T/NK acute lymphoblastic leukemia upon malignant transformation, that correlates with reduced Id4 expression and lower patient survival [111–114]. Therefore, Id proteins can play both oncogenic and tumor suppressive roles in lymphomagenesis of T cells.

Concluding remarks

Id proteins have classically been considered as oncogenes based on their ability to promote stem cell-like properties, and their overexpression in different tumors. In this review, we investigate evidence in favor of or against the presumed oncogenic roles of Id proteins in lymphocytes, with respect to their inhibition of E protein activity. An oncogene is typically defined as a gene, which when mutated, confers cells with properties that facilitate adoption of a tumorigenic program [4]. Id proteins can upregulate anti-apoptotic and pro-survival factors that overcome programmed cell death, promoting tumorigenesis. In many cases, Id proteins are often dysregulated by upstream oncogenic events, such as Myc, Ras and Notch signaling, and also inhibited by tumor suppressor genes. The activation of Id3 by oncogenic Ras signaling leads to E protein inhibition, tipping the balance. This inhibition via Ras supports the role of E proteins as tumor suppressors [13,25]. However, overexpression of Id proteins is not enough to cause tumorigenesis in mice unless combined with anti-apoptotic genes [11,15,115,116]. It is also dangerous to draw conclusions about the oncogenic roles of E or Id proteins based on their expression levels or physiological activity, since these may be impaired or hijacked, irrespective of expression patterns. For instance, Id3 is overexpressed several fold in Burkitt's lymphoma samples [33–35], but its function of mediating E protein inhibition is blocked through mutations in *Id3* or *Tcf3*, revealing the tumor suppressive function, instead of the oncogenic role of Id3. Therefore, RNA expression data alone without validation of protein functions may not be sufficient to determine whether Id3 plays a tumor suppressing or promoting role.

A similar stringent criterion should also apply to the cases where Id proteins function as tumor suppressors. The observation of either loss-of-function mutations in Id3 or gain of function of E2A in Burkitt's lymphoma validates Id3's role as a tumor suppressor. Physiologically, E2A mediates germinal center B cell survival and function through critical genes, while the upregulation of Id proteins keeps their activity in check [63,117]. In the case of BL, the lack of E2A inhibition by mutated Id3 perpetrates a tonic BCR and PI3K signaling that drives GC B cell-derived lymphomagenesis. Id2 and/or Id3 also play tumor suppressor roles to prevent lymphomagenesis of γδ, iNKT and innate-like T cells. In these studies, tumorigenesis can be attributed to direct dysregulation of cell cycle, NF-κB, cytokine-cytokine receptor interaction and innate-like developmental genes by high E2A activity [108–110]. Interestingly, although most lymphomas found in Id3-deficient mice are $\gamma\delta TCR^+$, some morphologically identical tumors express typical B cell markers, B220 and CD19 in the absence T cell-related markers [108]. Even though it remains to be determined if these lymphomas are truly derived from B cells, this phenotype may somehow reflect the tumor suppressive role of Id3 in BL. Further, delineating the order and contribution of the predominant Id3 and Myc mutations in BL patients toward pathogenesis will greatly enhance our understanding of the downstream pathways that drive malignant transformation of GC DZ B cells.

E proteins promote survival and proliferation in B cell progenitors, while they play the opposite role in DN3 T cell progenitors [25]. It is suggested that despite recognizing the same E-box motif in immature thymocytes and pro-B cells, the downstream targets are starkly different, and may be determined by cooperating transcription factors. This can ultimately determine their role in tumor, and can explain their tumor suppressor role in T cells but oncogenic role in B cell lymphomas [88]. It is important to further consider that E2A functions as a heterodimer in concert with HEB in T cells, but as a homodimer in B cells. Strangely, the fusion proteins E2A-HLF and E2A-PBX1 that are responsible for B cell-driven lymphomagenesis in humans, give rise to T cell lymphomas and acute myeloid leukemia in transgenic mice [82].

To summarize, Id proteins and E proteins are powerful regulators of cell cycle and senescence, allowing them to interchangeably play tumor suppressive and oncogenic roles. In almost all cases, simple dysregulation of E and Id proteins is not sufficient to cause tumors, and a secondary mutation hit is often required. It is possible that the dysregulation of E and Id proteins causes a growth arrest at a stage where most cells are cycling, leading to accumulation of mutations that predispose these cells to lymphomageneses. This predisposition to accumulating mutations to undergo malignant transformation, referred to as cellular pliancy, however, differs between different cell types and developmental stages. Here we summarized the various factors that determine the downstream targets of E and Id proteins, and therefore their function in physiological development, tumor suppression or tumorigenesis (Fig. 1). This includes important considerations such as the target cell type, dosage of E proteins, or transient upregulation of Id proteins, and functional redundancy between family members. Given the complexity that determines the role of Id proteins in cancers, inhibition of Id proteins may be clinically beneficial in certain tumor settings, but not in others.

Compliance with ethics guidelines

Sumedha Roy and Yuan Zhuang declare no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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