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Review of Ets1 structure, function, and roles in immunity

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Abstract

The Ets1 transcription factor is a member of the Ets gene family and is highly conserved throughout evolution. Ets1 is known to regulate a number of important biological processes in normal cells and in tumors. In particular, Ets1 has been associated with regulation of immune cell function and with an aggressive behavior in tumors that express it at high levels. Here we review and summarize the general features of Ets1 and describe its roles in immunity and autoimmunity, with a focus on its roles in B lymphocytes. We also review evidence that suggests that Ets1 may play a role in malignant transformation of hematopoietic malignancies including B cell malignancies.

Keywords

Ets1; B lymphocyte; Transcription factor; Gene regulation

Introduction to Ets1

Ets1 (also known as *ets*, *c-ets*, *c-ets-1*, *ets-1*, or *Tpl-1*) is the founding member of the family of Ets transcription factors. The Ets gene family is comprised of 28 genes in humans and 27 in mice. Ets genes are evolutionarily conserved across the metazoan (multicellular animal) lineages, including very primitive animals such as sponges. However, Ets genes are not found in choanoflagellates [1], simple Eukaryotes that live as single cells or small colonies and are considered to be the closest living relatives of metazoan animals. This suggests that the first Ets genes arose within very primitive multicellular animals, probably at least 600–700 million years ago. As described below, the amino acid sequences in Ets1 that bind to DNA adopt a structure known as a winged helix-turn-helix motif. This motif itself is of even more ancient origin and is found in DNA binding proteins in both Eukaryotes and Prokaryotes [2].

Mouse and human Ets1 proteins are highly similar throughout their lengths and differ from each other in only 12 amino acids (out of a total of 440 in mouse and 441 in human) and some of those changes are conservative in nature (97 % amino acid identity, 99 % similarity) (Table 1). Even Ets1 proteins from distantly related species such as the marsupial *Monodelphis domestica* (opossum) and the bird *Gallus gallus* (chicken) exhibit ~95 % amino acid sequence identity with mouse Ets1. Ets1 homologs from amphibians and fish exhibit ~80–90 % amino acid identity to mouse Ets1. The extensive conservation of the Ets1 proteins throughout their entire length is unusual. As shown in Table 1, several other well-studied and conserved transcription factors, including the closely related Ets2 protein, show

significantly less conservation overall as compared to Ets1. However, it is interesting to note that Pax5, a crucial regulator of B cell differentiation, represents another transcription factor that is very highly conserved across the 300 million years that separates mouse and chicken. Even protein domains that are not normally well conserved in transcription factors, such as the transactivation domain, have extensive conservation in Ets1 proteins across a variety of species. Therefore, essentially the entire amino acid sequence of Ets1 is under selective pressure, indicating that it plays an essential non-redundant role in metazoan development and/or differentiation.

Within the Ets gene family of mammals, Ets1 is most closely related to Ets2 (55 % overall amino acid identity, 70 % similarity) (Fig. 1a). These two proteins share similar domain structure as described below in more detail. In its chromosomal locus, *Ets1* is closely linked to another Ets family gene *Fli1*, with the two genes being oriented in a head-to-head fashion (Fig. 1b). Similarly, *Ets2* is linked to the *Fli1*-related Ets family member *Erg* in a head-to-head orientation. These Ets family genes probably arose from a series of duplications during evolution [3]. Some organisms, including all vertebrates that have been analyzed, have both *Ets1* and *Ets2* homologous genes, while other organisms have only a single *Ets1/Ets2*-like gene. For example, the fruit fly *Drosophila melanogaster* has a single gene homologous to *Ets1/Ets2*, which is referred to as *Pointed* [4]. Based on currently reported Ets1/Ets2 sequences, it appears that the duplication event giving rise to the *Ets1/Fli1* and *Ets2/Erg* pairs of genes occurred during the split of vertebrates from invertebrates.

The fact that Ets1 and Ets2 show similar domain structure and are nearly identical in their DNA binding domains (Fig. 1a) suggests that they may regulate similar or identical target genes and respond to similar signaling pathways. However, the sequence differences between the two proteins could be important in interactions with additional transcription factors and co-factors thus allowing Ets1 and Ets2 to differentially modulate cellular processes. In addition, Ets1 and Ets2 display differing expression patterns, which likely also contributes to their differential functions in vivo. The phenotypes of mice lacking Ets1 and Ets2 are not the same, indicating their independent roles. However, mice lacking Ets1 and carrying a hypomorphic allele of Ets2 have additional phenotypes not seen in either of the single deficient mice, indicating that these genes also have overlapping roles in development [5].

Organization of Ets1 genomic locus

The major isoforms of mouse and human *Ets1* genes are encoded by 8 exons, which are designated as exon A (first exon) followed by exons III–IX (last seven exons) (blue boxes in Fig. 2). In chickens, a minor isoform of the protein (referred to as p68) has been described that lacks exon A and instead initiates transcription at an alternate promoter sequence located upstream [6]. This p68 isoform was reported to contain two additional exons I and II encoding 85 novel N-terminal amino acids. Analysis of currently available nucleotide databases indicate that there is an additional non-coding exon (which we designate exon Ia) present in the chicken *Ets1* locus that splices to exons I and II. Similarly, humans appear to produce a p68 isoform of Ets1 as cDNA sequences encoding human Ets1 isoforms homologous to p68 of chickens are found in nucleotide databases and exons Ia, I, and II are present in genomic DNA (green boxes in Figs. 2, 3). Sequences homologous to exons Ia, I, and II are also present in the mouse genome, indicating that there may also be a mouse homolog of p68. However, it is unclear if these give rise to any functional proteins, since no mouse cDNA sequences encoding the putative exons spliced to downstream exons III–IX are presently found in nucleotide databases. The amino acid sequences encoded by exons Ia, I, and II are not as highly conserved as the remaining sequences of Ets1, suggesting that they are not under as strong a selective pressure. However, interestingly the amino acid sequences encoded by exons I and II are more homologous to the N-terminal sequences of

Ets2 than they are to the amino acid sequences of exon A of Ets1 [7]. Thus, exons Ia, I, and II likely represent the ancestral exons of this gene, with exon A being acquired later and becoming the major transcriptional start site.

The regulatory sequences that control Ets1 expression in lymphoid lineages, the major sites of expression in adult organisms, are unknown. Transgenic mice carrying either 2.4 or 5.3 kb of mouse sequences containing the promoter of the p54 isoform of Ets1 showed expression of the transgene during embryonic development in the neural tube, a site of endogenous Ets1 expression, but not in other tissues [8]. Including an additional 9 kb of sequences from the beginning of the intron downstream of exon A was not sufficient to drive transgene expression in the lymphoid system, although expression was detected in blood vessels, another site of endogenous Ets1 expression. Therefore, enhancers that drive lymphoid-specific expression of Ets1 must be located outside of the 14 kb (−5 to +9 kb) surrounding the Ets1 transcriptional start site.

Structure of the Ets1 protein

The major isoform of Ets1 in mice is 440 amino acids long and in humans is 441 amino acids long. This isoform has been referred to as p51 or p54 in various publications. In both species, a second isoform of Ets1 derived from alternative splicing of exon VII of the gene gives rise to a protein of 353 amino acids in mice and 354 amino acids in humans (Fig. 3). This isoform, which accounts for around 10 % of the total Ets1 protein in lymphocytes, is referred to as p42. Since the alternatively spliced exon in this case encodes sequences of the autoinhibitory domain described in more detail below, the p42 isoform of Ets1 is more active in binding to DNA than the full-length p51/p54 isoform [9]. A third isoform of the protein arises from splicing out of exons III through VI of Ets1 and gives rise to a protein of 225 amino acids in humans (Fig. 3) [10]. A similar isoform of Ets1 in mice has not yet been described. This isoform is designated p27 and lacks the ERK2 phosphorylation site, the Pointed domain and the acidic transactivation domain described below, but retains the DNA binding and autoinhibitory domains. Thus, it binds to DNA and can affect gene expression, apparently as a dominant-negative inhibitor of full-length Ets1 [10]. Finally, as described above, a fourth isoform of Ets1 that lacks sequences encoded by exon A is found in some organisms. Instead, this particular isoform of the protein, designated p68, has a novel amino terminus derived from exons Ia, I, and II (Fig. 3) [6].

Ets1 and other Ets transcription factors bind to DNA via an ~85 amino acid winged-helix-turn-helix DNA binding motif known as the Ets domain [11]. Ets1 binds to DNA as a monomer [12–14]. The DNA binding function of this domain is modulated by an autoinhibitory module, which is composed of two alpha helices, HI-1 and HI-2, and a serine-rich region that lie N-terminal to the Ets domain as well as two alpha helices H4 and H5 that lie C-terminal to the Ets domain [15, 16]. Both the regions N-terminal to the Ets domain and the regions C-terminal to it are required for autoinhibition [17]. The helices in this autoinhibitory module pack with each other to form a helical bundle that associates with the Ets DNA binding domain on the opposite face from the region that contacts DNA. Serines within the serine-rich region can be phosphorylated by serine/threonine kinases [18–20]. Studies in primary B cells and B cell lines have shown that this phosphorylation is crucially dependent on mobilization of intracellular calcium and is likely mediated by calcium/calmodulin-dependent (CAM) kinases [20, 21]. Similarly, Ets1 phosphorylation is also calcium-dependent in thymocytes [18]. Phosphorylation of these serines is known to reinforce the autoinhibitory function and prevent Ets1 binding to DNA, while removal of the phosphates increases Ets1 binding to DNA [19, 22–25]. A model has been proposed that suggests that the mechanism employed by the autoinhibitory domain to inhibit association of Ets1 with DNA involves changes to the flexibility of the Ets domain [26]. In this model,

binding of Ets1 to DNA requires a certain degree of flexibility in the Ets domain to allow it to adopt a high-affinity DNA binding conformation. The autoinhibitory module, particularly when the serines in the serine-rich region are phosphorylated, functions to reduce this flexibility and make the Ets domain more rigid.

In vivo, the activity of the autoinhibitory region may be abrogated by interaction of Ets1 with other transcription factors to form multiprotein complexes. For instance, the autoinhibitory module of Ets1 has been shown to interact with Runx1, Pax5, TFE3, and USF in a fashion to relieve autoinhibition [27–30]. Furthermore, the autoinhibitory module of Ets1 can also interact with an adjacent molecule of Ets1 to relieve autoinhibitory function when two Ets DNA binding sites are present in the correct orientation and spacing [31]. Thus, the exact layout of binding sites within an enhancer or promoter segment to either relieve or allow autoinhibition of Ets1 to occur may strongly influence whether or not Ets1 actually binds to particular site.

Ets1 harbors an acidic transactivation domain in the middle portion of the protein [32, 33]. Ets1 also contains an ~80-amino-acid conserved region in the N-terminus of the protein termed the “Pointed” or “Sterile alpha motif (SAM)” domain, which is involved in protein–protein interactions [34–37]. In Ets1, the Pointed domain serves as a docking site for ERK2 [36], which subsequently phosphorylates Ets1 at a conserved threonine residue (T38) [38–40]. ERK2 also phosphorylates a conserved serine residue (S41), which is not located in a consensus ERK phosphorylation sequence [41]. The phosphorylation of T38 is correlated with enhanced transcriptional activation by Ets1 via its stimulation of CBP recruitment [39, 41–43].

Regulation of Ets1 by ubiquitination and sumoylation

SUMO and ubiquitin are small (8–12 kD) conserved proteins that can be attached via lysine residues to other proteins, where they function to modulate their activities. Ubiquitination of target proteins depends on the activities of three separate enzymes. The first enzyme is a ubiquitin-activating enzyme called E1; the second enzyme is a ubiquitin-conjugating enzyme (E2) and the third enzyme is the ubiquitin ligase E3. Similarly, sumoylation also depends on an E1, E2, and E3 enzyme, although the enzymes are not the same as those that attach ubiquitin to proteins.

Ets1 can be modified both by ubiquitination and by sumoylation. In 1997, Ets1 was shown to interact with human UBC9, an enzyme originally reported as a ubiquitin ligase, but later discovered to be a E2 SUMO conjugating enzyme [44]. This therefore was the first indication that Ets1 might be sumoylated. Indeed, it was later discovered that Ets1 is subject to sumoylation and that the relevant E3 ligase was PIASy [45, 46]. Since both UBC9 and PIASy are involved in sumoylating Ets1, it would be expected that they would affect Ets1 function in a similar fashion. However, UBC9 was reported to stimulate Ets1 transcriptional activity [44], whereas PIASy was reported to inhibit Ets1 transcriptional activity [45, 46]. Mutation of the sumoylation sites in Ets1 (K15 and K227) lead to increased transcriptional activity by Ets1 [45, 46], supporting a negative role for sumoylation on Ets1 transcriptional function. Furthermore, it should be noted that sumoylation of Ets1 is important for Ets1 to interact with DAXX, a component of PML nuclear bodies that is associated with the nuclear matrix and involved in repressing Ets1-dependent gene activation [47]. Altogether, the majority of the evidence indicates that sumoylation of Ets1 probably inhibits its activity in transactivating genes.

The major sumoylation sites of Ets1 have been defined as lysines 15 and 227 of the protein [45, 46, 48]. Lysine 15 is at the N-terminus of the protein and associates with UBC9, which mediates the sumoylation [48]. Addition of SUMO to the N-terminus of Ets1 does not

modify the structure of Ets1 or SUMO and instead results in the addition of a globular SUMO protein onto a flexible, relatively unstructured N-terminal Ets1 domain (the so-called beads-on-a-string model) [48]. Ets1 can be de-sumoylated by the enzyme SENP1 [45, 46]. Sumoylation of Ets1 does not appear to regulate the stability of the protein to any great extent as the K15R, K227R double mutant of Ets1 that lacks sumoylation sites had similar stability as wild-type Ets1 [46, 49]. However, over-expression of PIASy increases Ets1 protein stability by preventing proteasomal degradation, an effect which is not mediated by altering ubiquitination of Ets1 [49]. Although the mechanism is unclear, it would appear that PIASy binds to Ets1 and affects its stability in a manner that does not involve SUMOylation or ubiquitination of Ets1.

Ets1 can also be ubiquitinated with K48-linked polyubiquitin chains that lead to Ets1 degradation via the proteasome [46, 49]. The lysine residue(s) of Ets1 involved in ubiquitination remain unknown, although the major sumoylation sites do not appear to be involved [46]. Deletion of amino acids 139–300 prevents ubiquitination, suggesting that this region may be involved in either recruiting ubiquitin ligases or in serving as a ubiquitin acceptor site [49]. Altogether, it is evident that both ubiquitination and sumoylation as well as interaction with PIASy in the absence of sumoylation can modulate Ets1 activities.

Expression patterns of Ets1

In mice, Ets1 seems to be highly expressed in many tissues during embryonic and early post-natal development [50, 51]. However, in adult mice, under normal conditions expression of Ets1 is much more restricted and is found at the high levels mainly in immune tissues such as thymus, spleen, and lymph node [50, 52–54]. Adult humans and chickens show a similar pattern where high expression of Ets1 is found mainly in lymphoid tissues [55–57]. Ets1 is expressed in B cells, T cells, NK cells, and NK T cells [21, 50, 56–62] and is inducible in other, non-lymphoid cell types in response to certain stimuli (for example, [63–65]).

Since the major p54 isoform of Ets1 and the minor p68 isoform initiate at different promoters, there is a possibility that their expression patterns are different. In most studies done thus far, the overall expression of Ets1 was measured using probes or antibodies that recognize all isoforms of Ets1. One exception to this is for chicken p68 protein, which was shown to be absent from lymphoid cells, but found in stromal cells of the spleen [66]. cDNA sequences encoding human p68 have been isolated from undifferentiated embryonic stem cells and primitive neuroectoderm as well as the hormone-responsive tissues such as endometrium and breast. Further studies are required to better understand the expression pattern of p68 proteins as compared to p54 proteins.

Multiple Ets gene family members are expressed in all cells tested, including B cells (Hollenhorst, 2004). In the human Raji B cell line, 17 of 27 human Ets genes tested were expressed at detectable levels. However, the most highly expressed of these genes was *ETSI*, followed by *GABPA*, *ELK4* (SAP1), *SPII* (PU.1), *FLII*, and *SPIB*. *ETSI* is also the most highly expressed Ets family member in the spleen, a tissue rich in B cells (Hollenhorst, 2004). Ets1 mRNA is detected in pro-B, pre-B, and immature/mature B cells sorted from the bone marrow as well as in mature splenic B cells [21, 58, 59, 62]. Gene expression microarrays have shown Ets1 to be high in naive B cells, downregulated in germinal center B cells and plasma cells and re-expressed in memory B cells [67]. We have also shown that Ets1 is downregulated in B cells differentiating into plasma cells in response to CpG oligonucleotide stimulation [62]. The downregulation of Ets1 in plasma cells is essential to their formation as enforced expression of Ets1 blocks plasma cell differentiation in response to the Toll-like receptor 9 (TLR9) ligand CpG oligodeoxynucleotide [62]. In fact, Ets1

upregulates the expression of the key B cell-restricted transcription factor Pax5 and inhibits the activity of the plasma cell transcription factor Blimp1 to inhibit B cell differentiation to plasma cells [62]. In summary, Ets1 is high in resting B cells (naïve B cells and memory B cells), but low in activated B cells and its downregulation is essential to terminal differentiation of the B cell lineage (summarized in Table 2). Similarly, Ets1 is also downregulated during T cell activation [68].

The expression of high levels of Ets1 in lymphocytes suggests that it plays an important role in their development and/or functional differentiation. Indeed, knockout mouse studies support a role for Ets1 in these processes, as described below in detail. This review will focus on the role of Ets1 in regulating B cell development and functional responses.

Targeted alleles of Ets1

Ets1 function in mice and particularly in lymphoid cells has been studied by the generation of gene-targeted knockout alleles of *Ets1*. Several knockout alleles of *Ets1* have been reported and are listed in the Mouse Genome Informatics (MGI) website. The *Ets1^{tm1Fwa}* knockout allele was developed in the laboratory of Fred Alt and targets the last two exons of the gene (exon VIII and IX), resulting in a null allele [69]. The *Ets1^{tm1Jml}* allele was developed in the laboratory of Jeffrey Leiden and targets exons IV and V [70]. We have also referred to this allele as the *Ets1^P* allele, a designation chosen to indicate that the sequences encoding the Pointed domain are deleted. Although originally reported as a null allele [70], this allele actually produces a small amount of protein lacking the sequences encoded by the deleted exons [71]. Because the amount of protein produced by the *Ets1^{tm1Jml}* allele is very low, it functions essentially as a null allele and has a phenotype very similar to the *Ets1^{tm1Fwa}* allele.

In 2002, an additional *Ets1*-targeted allele was reported by the Hertzog laboratory [72]. In this allele, exons III–VI of mouse *Ets1* are flanked by loxP sites, creating a floxed allele that could be used for tissue-specific deletion of the *Ets1* gene. Using this floxed allele, the *Ets1* gene was deleted in embryonic stem (ES) cells to yield heterozygous cells, which were subsequently selected in high concentrations of G418 to derive homozygous Ets1-deficient ES cells [72]. The ES cells lacking Ets1 were demonstrated to have defects in p53 responses to UV light [72]. Unfortunately, this floxed allele has not given rise to gene-targeted *Ets1* knockout mice so far.

A fourth knockout allele *Ets1^{tm1.1Dds}* developed in the laboratory of Demetri Spyropoulos targets exon VII of the gene. This exon is normally subject to alternative splicing and when it is spliced out or deleted by gene targeting the remaining *Ets1* exons encode the p42 isoform of the protein lacking autoinhibitory sequences and thus able to bind DNA constitutively [9, 73, 74]. Because of this, the *Ets1^{tm1.1Dds}* allele is a gain-of-function allele, unlike the other two knockout alleles of *Ets1*. The phenotype of mice carrying this fourth targeted allele of *Ets1* is in most respects opposite to that found in mice carrying the first two alleles [75]. It should be noted though that this mutant allele of *Ets1* produces increased amounts of mRNA and protein, as compared to the wild-type allele and hence some of the phenotypes obtained may be due to increased expression of Ets1, rather than expression of a hyper-active form of Ets1.

Ets1 in T cells

Loss of Ets1 has profound effects on B cells, T cells, and NK cells, each of which has high levels of expression of Ets1 under normal physiological conditions. We have recently reviewed some of the immune defects found in *Ets1* knockout mice, with a focus on the involvement of Ets1 in production of cytokines by T-helper cells [76]. Thus, in this review,

we will briefly summarize the roles of Ets1 in CD4⁺ T helper cells and report new studies in this area. We will also discuss in more detail the roles of Ets1 in CD8⁺ T cells.

Ets1 knockout mice have a variety of defects in the T cell lineage including aberrant thymic differentiation, reduced peripheral T cell numbers, reduced IL-2 production, a skewing towards a memory/effector phenotype and impairments in the production of Th1 and Th2 cytokines [69, 70, 76–83]. Recently, it was shown that Ets1 is important for maintaining the expression of CD127 (IL7R α) in peripheral T cells [84]. Lack of CD127 leads to survival defects and a failure to expand when Ets1-deficient CD4⁺ T cells are transferred to lymphopenic hosts [84]. Ets1-deficient T cells also fail to give rise to normal numbers of T-regulatory (Treg) cells and those that develop are functionally impaired [85], likely due to a deficiency in expressing FoxP3 [85, 86]. However, in contrast to the decreases in Th1, Th2, and Treg development found in *Ets1* knockout mice, Th17 cells are increased in these animals [80]. Potentially, reduced production of IL-2 by Ets1-deficient CD4⁺ T cells could influence T-helper cell differentiation as IL-2 inhibits Th17 generation, while promoting development of Tregs [87].

Ets1-deficient Th1 T cells seem to be confused in their differentiation status. Unlike wild-type Th1 cells, Ets1-deficient Th1 cells express several genes that are normally expressed in Th2 cells including IL-10, IL-24, and the chemokine receptor Ccr8 [82, 88]. Ets1 appears to control normal Th1 specification in a number of ways. First, Ets1 has been shown to cooperate with the transcription factor T-bet to promote expression of the Th1-specific cytokine interferon- γ [82]. Furthermore, Ets1 is proposed to recruit HDAC enzymes to the regulatory regions of Th2-specific genes to repress their expression [88].

Ets1 is expressed in CD8⁺ T cells [53, 58] and mice lacking Ets1 have defects in CD8 T cell development and function. *Ets1*^{-/-} CD8 single-positive (SP) thymocytes express less surface CD8 than wild-type CD8 SP thymocytes [70]. In fact, Ets1 is proposed to regulate the activity of an enhancer element located in the last exon of the CD8 α gene [89]. We showed that there are relatively fewer CD8 SP thymocytes with a mature phenotype in the thymus of *Ets1*^{-/-} mice and that the defect in their development is cell-intrinsic [81]. Similar observations have also been made by others [78]. This is likely due to a failure of CD4⁺CD8⁺ double-positive (DP) thymocytes that have committed to become CD8 SP cells to downregulate CD4 and/or to maintain CD8 expression. The failure to downregulate CD4 in CD8-committed T cells is also evidenced by the presence of some CD4⁺CD8⁺ mature peripheral T cells in Ets1-deficient mice [78, 81]. Ets1 has been shown to regulate expression of the Runx3 gene in thymocytes that are committed to become CD8 cells [78]. Runx3 is essential for silencing CD4 expression and maintaining CD8 expression in CD8-committed cells and hence that it impaired expression in *Ets1* knockout thymocytes is likely contributed to the failure to properly regulate the CD4 and CD8 co-receptors in these cells. The number of CD8 SP thymocytes are increased in mice carrying a gain-of-function allele of *Ets1* (Ets1 Δ exonVII mice or *Ets1*^{tm1.1Dds} mice) [75].

Ets1 is thought to regulate the expression of IL12r β 2, a key component of the IL-12 receptor, in antigen-stimulated CD8⁺ T cells [77]. The upregulation of IL-12 receptor allows the T cells to respond to IL-12 and differentiate into effectors. CD8⁺ T cells isolated from *Ets1* knockout mice and stimulated with anti-CD3 plus anti-CD28 to activate them expressed less IL12r β 2 and lower levels of effector-related genes (IFN γ , granzyme B, and perforin) than wild-type CD8 T cells cultured under the same conditions. The survival and recovery of *Ets1*^{-/-} CD8⁺ T cells was also impaired under these conditions [77]. As mentioned above, mature CD8 SP thymocytes and naive peripheral CD8⁺ T cells from *Ets1* knockout mice express lower levels of CD127 (IL7R α) than do controls [84]. Furthermore, the levels of CD127 on different subsets of human CD8 T cells are correlated with the levels of Ets1

expressed by these cells [84]. Altogether, these data indicate that Ets1 controls the differentiation CD8⁺ T cells as well as CD4⁺ T cells.

Ets1 in B cells

Several aberrations in B cell differentiation are also noted in Ets1-deficient mice, although it is not yet clear how many of the alterations are B cell-intrinsic and how many are driven by changes in the T cell compartment. The most striking B cell aberration is enhanced differentiation into IgM- and IgG-secreting plasma cells that accumulate to high numbers in the peripheral lymphoid organs and bone marrow ([60, 69] and unpublished data). The serum titers of IgM, IgG1, and IgE are increased [60, 69, 85], while titers of IgG2a are decreased [90]. A portion of the antibody secreted is autoantibody, recognizing a variety of different self-antigens including DNA, histone, and myelin basic protein [71, 85]. These autoantibodies deposit in the kidney as immune complexes, but fail to induce proteinuria [71]. Both B cell-extrinsic and B cell-intrinsic mechanisms have been described to explain these alterations in B cell differentiation. Mouly et al. [85] demonstrated that autoantibodies and increased serum IgG1 and IgE were associated with decreased numbers and function of Tregs in Ets1-deficient mice, supporting a B cell-extrinsic function of Ets1. However, we showed that B cells purified from the spleens of Ets1-deficient mice and cultured in vitro are hyper-responsive to stimulation with the TLR9 ligand CpG ODN and undergo enhanced differentiation into IgM-secreting plasma cells [71]. As T cells and other cell types are largely depleted from these cultures, this would suggest a B cell-intrinsic role for Ets1 in blocking plasma cell differentiation. Supporting a B cell-intrinsic function for Ets1, we have also shown that enforced expression of Ets1 during TLR-induced B cell differentiation maintains Pax5 expression and suppresses Blimp1 activity to prevent TLR-induced plasma cell differentiation [62]. The B cell defects described in Ets1 deficient mice are summarized in Table 3.

In addition to the increase in plasma cells noted in *Ets1* knockout mice, these mice also have a defect in generation or maintenance of marginal zone type B cells and reduced numbers of transitional type 2 B cells [71, 91]. There is conflicting data on the presence of B-1 type B cells in the peritoneal cavity [71, 91]. Follicular B cells in the peripheral lymphoid organs express higher than normal surface levels of CD23, CD80, and CD86 [71, 91], suggesting an activated phenotype. Ets1-deficient B cells have a defect in isotype-switching to IgG2a in the presence of interferon- γ caused by failure to activate the T-bet locus properly [90]. There are also partial defects in bone marrow B cell development with reduced cellularity and inefficient transition from pro-B to pre-B cell stages [91].

Ets1 in NK cells

Ets1 is also important for the development and function of natural killer (NK) cells [60, 92] and NK T cells [93]. There are reduced numbers of NK cells and reduced numbers of NK progenitors in the bone marrow and reduced numbers of NK T cells in the thymus, spleen, and liver of *Ets1*^{-/-} mice [60, 92, 93]. Interestingly, the few mature NK cells that develop in *Ets1* knockout mice have a phenotype resembling that of cells chronically stimulated by IL-15 [92]. The decrease in NK cell numbers is due to a cell-intrinsic requirement for Ets1 in NK cells as assessed by mixed bone marrow chimeras [92]. Furthermore, there is reduced cytolytic activity of both splenocytes and purified *Ets1*^{-/-} NK cells towards NK target cells [60], potentially as a result of decreased expression of NK activating receptors and/or deficiencies in degranulation of the cells [92]. Gene expression profiling identified a cohort of genes under the control of Ets1 in NK cells, including T-bet, which is also regulated by Ets1 in B cells. Ets1 also regulates the expression of the *Idb2* gene, which is important in NK cell development [92].

Autoimmunity

Given the high expression of Ets1 in lymphocytes, this gene has attracted attention as a potential contributor to autoimmune disease pathogenesis. An early study examined Ets1 expression in peripheral blood mononuclear cells (PBMCs) from a small number of systemic lupus erythematosus (SLE) patients as compared to a single normal control [94]. In this analysis, it appeared that Ets1 expression might be ~twofold higher in the SLE PBMCs than in the control. In the same study, the authors also examined Ets1 expression in splenic T cells from NZB/W F1 autoimmune mice as compared to non-autoimmune DBA/2 and NZW mice [94]. No significant difference in Ets1 expression was noted among these mouse strains. The data in this study are not quantitative and derived from only a small number of samples. They also conflict with later studies indicating reduced expression of Ets1 in PBMCs from SLE patients (see below).

A locus on mouse chromosome 9, referred to as the *Idd2* region or the *Tlf* region, has been linked to autoimmune diabetes and increased T cell counts in the periphery of non-obese diabetic (NOD) mice [95, 96]. The *Ets1* gene is on chromosome 9 near the *Thy1* locus, which was originally linked to *Idd2* [96, 97]. However, the *Ets1* gene itself has been eliminated as a candidate for this autoimmune locus [98].

In 1990, Aparicio et al. [99] published a study in which they examined restriction fragment length polymorphisms (RFLPs) in the *ETS1* gene of a small cohort of Japanese diabetes patients. A polymorphism in an *Ava* II fragment was detected in the *ETS1* locus that was associated with diabetes, but the association was not statistically significant after correction for multiple comparisons.

In 2000, the first significant evidence supporting a role of Ets1 in autoimmunity was published. A study of CA repeat polymorphisms downstream of the human *ETS1* gene showed that particular alleles of *ETS1* were associated with disease symptoms in SLE patients [100]. More recently genome-wide association studies (GWAS) in Chinese and Thai lupus patients have identified disease susceptibility single-nucleotide polymorphisms (SNPs) in the *ETS1* locus [101, 102]. One such SNP designated rs1128334 lies in the 3' UTR of the *ETS1* gene (odds ratio of 1.29 with a *p* value of 2.33×10^{-11}). Direct sequencing of cDNA derived from individuals that are heterozygous for the disease-associated SNP indicates that less Ets1 mRNA is present from the disease-associated allele as compared to the normal allele [102]. Therefore, the rs1128334 SNP regulates steady-state levels of Ets1 mRNA, most likely by interfering with message stability, potentially via altering binding sites for microRNAs. Another study measured Ets1 mRNA levels in PBMCs derived from lupus patients and controls and demonstrated that lupus patients have lower Ets1 mRNA than do normal controls [103]. The associations of SNPs in *ETS1* with lupus susceptibility have been replicated in independent Asian populations [101, 104] and further shown to associate with particular clinical features including age of disease onset, malar and discoid type rashes, photosensitivity, arthritis, serositis, renal disorder, hematologic disorder, immunologic disorder, and anti-nuclear antibody levels [104, 105]. A recent study has shown that at least one of the SNPs associated with lupus susceptibility in Asian populations is also associated with lupus susceptibility in European and American populations [106].

Meta-analyses of multiple genome-wide association studies has suggested an association of SNPs in the *ETS1* locus with rheumatoid arthritis in Japanese populations and with psoriasis in European populations [107, 108]. Recently, examination of a single *ETS1* SNP rs11221332 located in the intron separating exons A and of III the gene has confirmed an

association with rheumatoid arthritis susceptibility (odds ratio of 1.5 with a *p* value of 0.04) [109].

GWAS studies in European populations have also implicated the *ETS1* locus in celiac disease, another disorder of misregulated immunity [110, 111]. The SNPs identified in the celiac disease study are at the 5' end of the *ETS1* gene, rather than the 3' UTR and downstream region that was identified in SLE patients. Indeed, the celiac disease-associated SNPs overlap with the *ETS1* p54 promoter and exon A. These SNPs could potentially affect transcription of the *ETS1* gene, although this remains to be tested.

There is also evidence that microRNAs and Ets1 are coordinately involved in autoimmune diseases. For instance, the levels of miR326, a micro RNA that is highly expressed in Th17 cells, is correlated with disease severity in multiple sclerosis (MS) patients and mice with the MS-analogous condition experimental autoimmune encephalomyelitis (EAE) [112]. miR326 has been shown to target Ets1 and leads to impaired translation of the Ets1 mRNA [112]. Since Ets1 is a negative regulator of Th17 cells, the downregulation of Ets1 leads to increased numbers of disease-causing Th17 cells and increased severity of MS. This study also showed reduced levels of Ets1 protein in CD4⁺ T cells derived from MS patients with active disease [112].

Ets1 has also been shown to regulate the promoter of miR146a, a negative regulator of the interferon pathway whose expression is decreased in PBMCs of SLE patients [113]. A SNP near the Ets1 binding site of the promoter of miR146a was shown to inhibit Ets1 binding and transactivation of this microRNA [113]. In contrast, SNPs in miR146a or Ets1 failed to show any disease association in a relatively small cohort of patients with autoimmune uveitis [114].

Ets1 in hematopoietic tumors

Ets1 is an oncogene whose expression is frequently upregulated in a variety of human tumors of different tissue origins (for example, [115–120]). Using transgenic mouse models, where we are able to inducibly over-express Ets1 in stratified squamous epithelial cells, we have demonstrated that Ets1 induces a number of pro-oncogenic changes when it is expressed in epithelial cells including a block to terminal differentiation accompanied by high secretion of matrix metalloproteases (Mmps), epidermal growth factor ligands, and inflammatory mediators [121, 122]. It is likely that one or more of these activities of Ets1 contributes to its oncogenic activities in naturally arising tumors.

Ets1 was originally cloned as part of an oncogenic fusion protein gag-mybets encoded by the chicken leukemia retro-virus E26 [123, 124]. The E26 virus induces mixed myeloid/erythroid leukemias in chickens, an activity that is dependent on both the myb and Ets1 portions of the fusion protein [125–128]. Another retrovirus, avian myeloblastosis virus (AMV), which contains the oncogene myb (but not Ets1) induces purely myeloid tumors. Interestingly, myeloblasts harboring E26 do not spontaneously differentiate in culture [129], unlike those harboring AMV, suggesting a role for Ets1 in blocking differentiation of early hematopoietic progenitors. As described above, Ets1 blocks the terminal differentiation of squamous epithelial cells [121, 122] and of B lymphocytes [62]. Thus, together these data would suggest that Ets1 has a common activity of inhibiting cellular differentiation in a number of different contexts. This activity might contribute to its pro-oncogenic effects by keeping cells in an immature and proliferative state.

In rats, the retrovirus Moloney murine leukemia virus (MoMuLV) shows recurrent integration in a locus within the *Ets1* p54 promoter region (i.e., designated the Tpl-1 locus) during T lymphoma tumor progression [130, 131]. Integration into this locus is rapidly

selected for in cultured lymphoma cells. However, the steady-state levels of Ets1 mRNA and protein are only slightly elevated (~twofold or less) in tumors carrying a provirus in the Tpl-1 locus [131]. The relatively minor induction of Ets1 mRNA and protein is somewhat surprising given the strong selective advantage of provirus integration into the Tpl-1 locus. It is possible that this viral integration event, instead of promoting over-expression of Ets1, might instead function to prevent Ets1 down-regulation or silencing in lymphoma cell lines. At any rate, these observations indicate that Ets1 plays an important, but as yet unidentified, role in T lymphoma tumor progression induced by MoMuLV.

Several Ets family genes are involved in chromosomal translocations that result in over-expression of the relevant Ets factor and/or production of oncogenic fusion proteins. The potential involvement of *Ets1* in such chromosomal translocations has also been investigated. A number of studies in the late 1980s and early 1990s showed chromosomal translocations in the 11q23-24 region of the human genome, near where the *ETS1* gene is located [132–145]. In these studies, the *ETS1* gene was frequently translocated onto an alternate chromosome. However, when probes in the *ETS1* locus were used to test for rearrangement of this gene by Southern blotting, rearrangements were not detected in most cases [132, 134, 138, 139, 146–150]. Indeed, many 11q23 translocations were later shown to involve the *MLL* gene, which is located approximately 10 Mb centromeric to *ETS1* [151]. A few studies did show rearrangement of the *ETS1* gene in Southern blotting [135, 137, 140]. However, it has been suggested that bands attributed to a rearrangement of *ETS1* might instead arise from a fragile site that is artifactually cleaved during sample processing [148] or to polymorphisms in restriction enzyme cleavage sites [146]. In general, it appears that translocations of the *ETS1* gene are uncommon in hematopoietic malignancies and that if misregulation of Ets1 is noted in these tumors, it might occur through alternative mechanisms.

Other studies have supported amplification of the *ETS1* oncogene in hematopoietic malignancies [135, 140, 152, 152]. More recently, copy number analysis on marginal zone B cell lymphomas of the gastrointestinal tract revealed amplification of the *ETS1* gene along with some flanking genes in the more aggressive large cell variants of these tumors [154]. Furthermore, analysis of the genomes of diffuse large B cell lymphomas by direct sequencing demonstrated amplification or missense mutations of *ETS1* in some DLBCL tumors [155, 156] and in pediatric acute myeloid leukemia [157]. Ets1 expression levels are a poor prognostic marker for diffuse large B cell lymphoma [158]. Therefore, it is possible that Ets1 may function as an oncogene in certain B cell malignancies, where its expression is upregulated via gene amplification or where it is mutated. However, further studies will be required to confirm this.

Despite the studies supporting the functional significance of Ets1 in B cell lymphomas, other studies have indicated that one or both copies the *ETS1* gene are deleted in some tumors [159–162], potentially arguing for a tumor suppressive activity of Ets1. Thus, it would seem that the role of *Ets1* in tumors may be more complex than anticipated. Depending on the tumor type and the exact constellation of other genetic changes and mutations, Ets1 might serve as either a tumor suppressor or as an oncogene in B cell malignancies. It will be important to clarify the exact role of *Ets1* in different types of human B cell tumors and to identify which genetic mutations it may cooperate with to drive oncogenesis and which mutations it might antagonize to block tumor progression.

Conclusions

In this review, we have summarized published studies implicating the Ets1 transcription factor in regulating immune function and preventing autoimmunity as well as information

that potentially implicates Ets1 as an oncogene in hematopoietic malignancies including B cell tumors. Although these studies have provided us with a clearer picture of Ets1 function, many questions about Ets1 structure, regulation, and biological functions remain. Some examples of interesting questions are: Why is the entire amino acid sequence of Ets1 so highly conserved? What are the unique roles of Ets1 as contrasted to its overlapping roles with other Ets family proteins? What are the specific roles of different isoforms of Ets1? What are the genomic sequences that control lymphocyte-specific expression of Ets1 in adult vertebrates? What are relevant target genes for Ets1 in B cells and other immune cells? How much of the B cell phenotype found in Ets-deficient mice is due to cell-intrinsic roles for Ets1 versus cell-extrinsic roles? Can we further understand how Ets1 regulates the immune system to prevent autoimmune disease? What if any role does Ets1 play in B cell-derived tumors? Additional studies focused on Ets1 will help us to garner a better understanding of the importance and relevance of Ets1 to important medical problems such as cancer and autoimmunity.

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Fig. 1.

Conservation of Ets1 structure and chromosomal organization. **a** Diagram comparing the protein structure of mouse Ets1 to mouse Ets2. The major protein domains, including the Pointed domain, the acidic transactivation domain, the autoinhibitory domains, and the Ets DNA binding domain, are indicated. Also shown is the conserved MAP kinase phosphorylation site (T38 in Ets1 and T72 in Ets2) found at the N-terminus of each protein. **b** Chromosomal organization of the mouse *Ets1/Fli1* and *Ets2/Erg* loci. Note that in both cases the two related genes are oriented in a head-to-head fashion. The size of the *arrows* indicates the approximate length of each gene including introns. Note that the coding sequences of Ets1 and Ets2 are nearly the same length and similarly the coding sequences of Fli1 and Erg are nearly the same length. Thus, the changes in the size of the *arrows* simply reflects differences in intron sizes in these genes

Fig. 2.

Exon/intron structure of human *ETS1*. Shown are the exons of the human *ETS1* gene. Exons included in the major transcript are shown in *blue* (*dark blue* indicates the open reading frame, *light blue* is 3' and 5' UTRs). Exons Ia, I and II encoding the N-terminus of the p68 isoform of Ets1 (shown in *green* with the *darker green* indicating the open reading frame) derive from an alternate promoter located upstream and splice to exon III (as shown in the *dotted lines*). The first exon of the major transcript of *FLII* is also shown as a *red box*. Also indicated by *dashed lines* are the splicing events that delete exon VII (to generate the p42 isoform) and that delete exons III–VI (to generate the p27 isoform)

Fig. 3.

Isoforms of Ets1 protein. Shown are the four isoforms of Ets1 protein currently reported in the literature. The p54 isoform is the most abundant, followed by the p42 isoform, while the p68 and p27 isoforms are produced in very low amounts in tissues that have been tested so far. Also indicated are the sumoylation sites (K15 and K227), the ERK phosphorylation sites (T38, designated by the *red circle* atop the *black line*) and four CAM kinase phosphorylation sites (S251, S257, S282, and S285, designated by *blue circles* atop the *black lines*)

Table 1

Amino acid identity in a sampling of different transcription factors

Transcription factor ^a	Mouse/human identity (%)	Mouse/opossum identity (%)	Mouse/chick identity (%)
Ets1 (p54)	97	95	95
Pax5	99	97	94
Irf4	92	88	84
Fli1	97	90	85
Ets2	91	80	76
HoxA1	94	83	71
Blimp1	89	80	79
Bcl6	95	52	79
c-myc	91	68	63
p53	77	72	62

^a Rows highlighted in *bold* represent transcription factors that are more highly conserved across evolutionary time

Table 2

Expression pattern of Ets1 in B cells

Stage of B cell differentiation	Expression of Ets1	References
Pro-B cells	Yes	[58, 59]
Pre-B cells	Yes	[58]
Bone marrow IgM+ B cells	Yes	[58]
Naïve mature B cells	High	[67]
Germinal center B cells	Low	[67]
Memory B cells	High	[67]
Plasma cells from in vitro LPS stimulation	Low/absent	[62]
Plasma cells from T-dependent antigen immunization	Low/absent	[67]

Table 3

Summary of B cell defects in Ets1-deficient mice

Cell type	Aberrations found in Ets1-deficient B cells
Bone marrow development	Partial defect in transitioning from pro-B to pre-B cell stage [83, 91]
Splenic differentiation	Failure to develop or maintain marginal zone B cell type; conflicting data concerning development of B-1 B cells; reduced numbers of T2 transitional B cells [71, 83, 91]
Activation	Enhanced expression of activation markers such as CD80 and CD86; increased secretion of autoantibodies; enhanced differentiation into IgM- and IgG-secreting plasma cells [69, 71, 85]
Isotype switching	Enhanced switching to IgG1 and IgE; Reduced switching to IgG2a [85, 90]
In vitro responses	Increased plasma cell responses when cultured with TLR9 ligand CpG DNA; modest decrease in proliferation to LPS or to anti- CD40+ anti-IgM [69, 71]