Published in final edited form as:

Trends Biochem Sci. 2009 July ; 34(7): 324–331. doi:10.1016/j.tibs.2009.03.004.

The GAIT system: a gatekeeper of inflammatory gene expression

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Abstract

Functionally related genes are coregulated by specific RNA–protein interactions that direct transcript-selective translational control. In myeloid cells, interferon (IFN)- γ induces formation of the heterotetrameric, IFN- γ -activated inhibitor of translation (GAIT) complex comprising glutamyl-prolyl tRNA synthetase (EPRS), NS1-associated protein 1 (NSAP1), ribosomal protein L13a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This complex binds defined 3' untranslated region elements within a family of inflammatory mRNAs and suppresses their translation. IFN- γ -dependent phosphorylation, and consequent release of EPRS and L13a from the tRNA multisynthetase complex and 60S ribosomal subunit, respectively, regulates GAIT complex assembly. EPRS recognizes and binds target mRNAs, NSAP1 negatively regulates RNA binding, and L13a inhibits translation initiation by binding eukaryotic initiation factor 4G. Repression of a post-transcriptional regulon by the GAIT system might contribute to the resolution of chronic inflammation.

Transcript-selective translational control of gene expression

Gene expression is regulated at multiple synthetic and degradative steps, including transcription, splicing, mRNA transport, mRNA stability, translation, protein stability and post-translational modification. In many cases, translational control has evolved as an 'off-switch' mechanism to modulate protein expression because it exhibits key regulatory advantages. Foremost is the rapid responsiveness of the translational 'braking' mechanism due to its location as the final step in the process of genetic information transfer. Also, because translational control is non-destructive, that is, mRNA levels are unaltered, it can act as a 'rheostat', rapidly dialing up or down the level of a specific protein in a condition-dependent way. Finally, the decoupling of translational 'off-switches' from (mostly) transcriptional 'on-switches' permits negative regulation even in the continued presence of initiating factors. In the interferon (IFN)- γ -activated inhibitor of translation (GAIT) system, a single inflammatory stimulus (IFN- γ) activates both transcriptional 'on-switches' and translational 'off-switches' in a temporally staged manner.

Translational control can be subdivided into two major classes: global and transcript-selective. Global mechanisms regulate the expression of the majority of transcripts,

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generally by modifying an essential translation factor [1]. By contrast, transcript-selective mechanisms permit the translational regulation of a group of functionally-related mRNAs and are mediated proteins to cognate *cis*-elements in the 5' or 3'UTRs (untranslated regions) of target transcripts [2,3]. In eukaryotic cells, initiation is often the regulated step, and protein-mRNA interactions generally repress, rather than activate, translation [4,5]. Early studies focused on the 5'UTR as the site of translational control: for example, ferritin mRNA translation is inhibited by binding of iron regulatory protein to the 5'UTR iron-responsive element. More recently, investigators have recognized the special importance of the 3'UTR in regulating translation by *trans*-acting proteins and by microRNAs [2,3,6]. The mean length of human 3'UTRs is nearly four times greater than that of human 5'UTRs [7]. Moreover, mean 3'UTR length increases with evolutionary 'complexity', but mean 5'UTR lengths remain essentially constant [2]. These observations point towards the enormous potential of 3'UTR-based translational control, particularly in vertebrates. Here, we describe recent studies that have revealed a 3'UTR-mediated, cell-type-specific mechanism to suppress the translation of a family of inflammation-associated transcripts in response to IFN- γ .

Translational control by the GAIT pathway

Monocytes and macrophages provide an early line of defense against infection or injury. They are activated by cytokines to produce and secrete proteins and small molecules that can kill invading microorganisms. Overexpression of these toxic agents can be detrimental to host cells and tissues, contributing to chronic inflammatory disease. Therefore, host responses must be strictly controlled to limit the expression of injurious molecules. The 'resolution of inflammation' is an active program of regulated gene expression by which pro-inflammatory processes are restricted after neutralization or elimination of the initial insult [8]. Thus monocytes or macrophages, and their agonists, for example IFN- γ , can exhibit either pro- or anti-inflammatory responses depending on the context [9,10]. Early studies from our laboratory showed marked transcriptional induction of ceruloplasmin (Cp), an acute phase inflammatory protein, in human monocytes upon IFN- γ treatment [11]. However, Cp synthesis, as measured by pulse-labeling, was terminated almost completely after ~16 h, despite the presence of abundant Cp mRNA, a result indicative of translational inhibition. Total protein synthesis was unaltered, indicating transcript-selective inhibition. The inhibition was cell-type specific and observed only in myeloid cells (and related cell lines) [12]. The shift of Cp mRNA from the polyribosomal to non-polyribosomal pool indicated that inhibition occurred at the level of translation initiation.

Analysis of the Cp 3'UTR GAIT element

An RNA gel shift assay demonstrated that the Cp 3'UTR binds an IFN- γ -inducible protein/complex, termed the GAIT complex [13]. The functionality of the Cp 3'UTR was verified by *in vitro* translation; ligation of the 3'UTR downstream of a luciferase reporter conferred the translational silencing response to the heterologous transcript in rabbit reticulocyte lysates supplemented with lysate from IFN- γ -treated U937 cells. From these analyses, a minimal, 29-nt Cp 3'UTR GAIT RNA element was identified. A secondary structure consisting of a stem-loop with an asymmetric internal bulge was predicted by Mfold, an RNA-folding algorithm (<http://mfold.bioinfo.rpi.edu/>) [14], and was confirmed by mutation analysis (Figure 1). Most loop residues could be mutated without loss of binding or silencing activity; however, two residues in the bulge loop, A84 and U87, are essential, and mutation of either completely inactivated the element. Similar functional GAIT elements containing a stem-loop with an internal bulge were identified in the 3'UTR of multiple transcripts (Figure 1; see below).

Elucidation of the heterotetrameric GAIT complex

RNA affinity purification and the yeast three-hybrid approach [15] identified the four constituent proteins of the GAIT complex that binds the *Cp* GAIT RNA element but not an inactive mutant element. This complex is comprised of glutamyl-prolyl tRNA synthetase (EPRS), NS1-associated protein 1 (NSAP1, also known as SYNCRIP or heterogeneous nuclear ribonucleoprotein [hnRNP] Q1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13a [16,17]. The GAIT complex assembles in two distinct steps. In the first step, which occurs ~2 h after IFN- γ treatment, EPRS is released from its residence in the tRNA multisynthetase complex (MSC) and joins NSAP1 to form an inactive pre-GAIT complex that neither binds target mRNAs nor inhibits translation (Figure 2). About 12–14 h later, ribosomal protein L13a is released from the 60S ribosomal subunit and joins GAPDH and the pre-GAIT complex to form the functional GAIT complex. Elucidation of the mechanisms by which protein constituents are inducibly released from 'stable' macromolecular complexes remains a particularly exciting challenge [18] (Box 1).

Box 1

The depot hypothesis of large macromolecular complexes

Remarkably, two of the four proteins in the GAIT complex are inducibly released from parent macromolecular complexes previously thought to be stable, machine-like assemblies: EPRS from the MSC and L13a from the 60S ribosome subunit [16,17]. This discovery helped to formulate the generalized 'depot hypothesis', which holds that large macromolecular complexes can act as 'depots' or 'reservoirs' for regulatory proteins inducibly released by cellular signals to perform non-canonical functions distinct from those of the parent complexes [18]. The depot hypothesis expands the accepted concept that many (if not most) proteins are multifunctional, exhibiting secondary 'moonlighting' functions distinct from its 'principal' (or at least best characterized) function [62]. Moreover, certain proteins found in large macromolecular complexes, for example S19 from the ribosome and COP9 signalosome subunit 5 (CSN5, also called Jun activation domain-binding protein or JAB1), can exhibit distinct functions in isolation from the parent complex [63–65]. The novel concept in the depot hypothesis is that daughter proteins are released from the parent macromolecular complex and not derived from an existing pool or from *de novo* synthesis. Recent studies support the generality of the hypothesis as two other MSC components might undergo stimulus-dependent release: aminoacyl-tRNA synthetase-interacting, multifunctional protein 2 (AIMP2) and lysyl-tRNA synthetase (KRS) [66,67]. Although the molecular mechanisms underlying the release of EPRS and L13a from the MSC and 60S ribosome, respectively, remain unknown, recent insights from structural biology have provided clues. The X-ray crystallographic structure of archaeal 50S ribosomal subunit indicates that L13, the archaeal homolog of human L13a, is localized exclusively on the rRNA surface of the 60S subunit, with minimal penetration into the RNA and little contact with nearby proteins [68]. Similarly, cryo-electron microscopy has revealed EPRS at the MSC exterior, consistent with inducible release [69]. A phosphorylation-dependent alteration in L13a and EPRS conformation might be responsible for a protein–RNA or protein–protein 'uninteraction', which frees the protein from the parent macromolecular complexes for auxiliary functions.

Regulation of assembly and function of the GAIT nanomachine

IFN- γ -dependent assembly of the heterotetrameric GAIT complex is tightly regulated. Phosphorylation of both EPRS and L13a are essential events in the formation of a functional GAIT complex. Moreover, the complex is subject to both negative and positive regulation.

Phosphorylation-dependent release of EPRS from the tRNA MSC

EPRS has a crucial role in the GAIT pathway because it recognizes and directly binds GAIT element-bearing target mRNAs [17]. EPRS is a unique, bifunctional tRNA synthetase that consists of two catalytic cores, which catalyze the ligation of Glu and Pro to cognate tRNAs [19,20]. The catalytic domains are joined by a noncatalytic linker containing three tandem WHEP-TRS (or WHEP) domains (named after three tRNA synthetases that contain them, i.e. Trp(W)RS, His(H)RS and EPRS). The function of WHEP domains is unknown, but they bind RNA and protein with low affinity and specificity [19,20]. The presence of EPRS WHEP domains in nearly all metazoan species examined to date suggests an important function that has been retained for half a billion years or more [21,22]. The absence of WHEP domains in the separate ERS and PRS enzymes in bacteria and yeast indicates that they are not essential for synthetase activity but instead might be involved in a noncanonical function of the enzyme.

EPRS phosphorylation at one or more Ser residues is the earliest IFN- γ -inducible event in activating the GAIT translational silencing pathway. Phosphorylation induces EPRS release from the MSC and facilitates binding to cytoplasmic NSAP1; Ser kinase inhibitors prevent EPRS release and its interaction with NSAP1 [17]. Earlier studies showed that tRNA synthetase phosphorylation has little or no effect on synthetase activity [23], supporting a potential role for post-translational modifications in regulating synthetase noncanonical functions.

EPRS–target mRNA binding is subject to negative and positive regulation

EPRS binds the GAIT RNA element but, intriguingly, is unable to do so until ~14 h after its release from the MSC, and a domain analysis of EPRS binding activities provided an important clue as to why. Deletion analysis showed that the upstream pair of WHEP repeats (R1 and R2) in the EPRS linker directed phosphorylation-independent, high-affinity binding to *Cp* GAIT element RNA [24]. A similar analysis revealed that NSAP1 also bound EPRS in the linker domain; however, binding was phosphorylation-dependent and required the downstream pair of WHEP repeats (R2 and R3). Thus NSAP1 and GAIT element RNA share overlapping binding sites on EPRS, suggesting that NSAP1 might inhibit EPRS–RNA binding. Surface plasmon resonance (SPR) experiments with recombinant, purified GAIT constituent proteins revealed that high-affinity binding of phosphorylated EPRS to GAIT element RNA was completely blocked by pre-incubation with NSAP1. NSAP1 did not bind non-phosphorylated EPRS or inhibit its binding to RNA. Thus, EPRS phosphorylation is not required for binding to the GAIT RNA but is essential for NSAP1-mediated negative regulation.

The presence of NSAP1 inhibitory activity raised a question, namely, how is inhibition overcome 16 h after IFN- γ treatment? The restoration of RNA binding by the remaining components of the GAIT complex was investigated. Addition of either GAPDH or phosphorylated L13a (P-L13a) was ineffective; however, simultaneous addition of both proteins restored EPRS binding to GAIT element RNA even in the presence of NSAP1 [24]. Comparable reconstitution of translational inhibition activity *in vitro* established that the functional GAIT complex requires only these four proteins [24].

The mechanism underlying regulation of the GAIT complex was investigated by measuring Förster resonance energy transfer (FRET) between NSAP1 and phosphorylated EPRS linker, both fluorescently-labeled at their N-termini. Addition of phospho-L13a and GAPDH induced a conformational shift that increased the distance between NSAP1 and the N-terminus of EPRS linker by ~9 Å [24], a shift that might be sufficient to move NSAP1 away from the upstream WHEP domains and permit RNA binding. We expect that elucidation of

the specific EPRS phosphorylation sites and detailed structural analysis of GAIT constituents and their interaction sites will provide additional insight into the conformational switches that regulate GAIT complex activity.

Phosphorylation of ribosomal protein L13a and release from the 60S ribosomal subunit

Phosphorylation of L13a ~12–16 h post-IFN- γ treatment is obligatory for L13a release from the large, 60S ribosomal subunit and is a crucial rate-determining event in GAIT pathway activation [16,25]. Remarkably, nearly the entire cellular complement of L13a exits the ribosome without detectable inhibition of global protein synthesis [26]. Mass spectrometry and mutational studies revealed that IFN- γ -inducible L13a phosphorylation occurs only on Ser⁷⁷ [25] in an RXXS/T consensus kinase recognition motif [27–29]. *In vitro* and *in vivo* screening studies identified zipper-interacting protein kinase (ZIPK also known as death-associated protein kinase-3 or DAPK3), a member of the DAPK family, as the proximal kinase for L13a phosphorylation [25]. Another member of the same family, DAPK1 (referred to as DAPK) was identified as an upstream activator of ZIPK, indicating a mini DAPK–ZIPK signaling cascade, consistent with reports that DAPK can activate ZIPK [30].

Mechanism of L13a-mediated translational inhibition

Binding of proteins (or microRNAs) to 3'UTRs influences translation initiation at the distant 5'UTR. The 'closed-loop' model of mRNA translation provides a crucial clue. According to this model, an interaction between the 3'UTR and 5'UTR improves translation efficiency by ensuring that ribosomes released at the 3' end after the completion of protein synthesis are close to the 5' end to enable initiation of a new round of synthesis. In this model, the transcripts are 'circularized' by interaction of eukaryotic initiation factor 4G (eIF4G) at the 5' terminus with poly(A)-binding protein (PABP), which binds the poly(A) tail at the 3' terminus (Figure 2) [31]. Initial studies in the GAIT system sought to understand whether or not the GAIT complex interrupted transcript closure, thereby inhibiting protein synthesis. Despite multiple approaches, the GAIT complex was not found to disrupt any interactions at the termini [32]. Subsequent studies of the alternative hypothesis, that the GAIT complex does not impede circularization but rather depends on it, revealed that PABP depletion prevented translational silencing of a reporter transcript containing the GAIT element. Therefore, transcript circularization not only promotes efficient translation but also can be essential for transcript-selective translational control. The requirement for a poly(A) tail has been shown for translational inhibition by at least one microRNA, but the generality of this requirement for transcript closure has not been established [33,34].

Multiple mechanisms of transcript-selective translation inhibition have been shown, including inhibition of eIF4F (cap-binding complex) activity, recruitment of the 43S pre-initiation complex, scanning of the 43S complex to the initiation codon, and 60S ribosomal subunit joining [2,3,6]. L13a was considered a strong candidate for the translational repressor because its phosphorylation and release from the ribosome temporally coincided with translational silencing of target mRNAs. Indeed, P-L13a targets eIF4G, the component of the 5' cap-binding, translation-initiation complex that makes initial contact with the ribosome via interaction with eIF3 of the 43S translation preinitiation complex (which contains the 40S small ribosomal subunit) (Figure 2). Its large size, central positioning and manifold interactions render eIF4G a conspicuous target for translational control. Studies with the eIF4G-specific protease HRV-2A (human rhinovirus-2A) complemented by deletion mapping revealed that P-L13a binds eIF4G at its eIF3-binding site [35]. This interaction blocks recruitment of the eIF3-containing 43S ribosomal complex to GAIT element-bearing mRNAs, thereby blocking translation initiation. Inactivation or degradation of eIF4G are well-established RNA-independent mechanisms to achieve global translation

inhibition, for example during cellular stress [35]. However, the RNA-dependent eIF4G–P-L13a interaction provides a unique mechanism for transcript-selective translation control.

The GAIT system defines a post-transcriptional regulon of inflammatory genes

Studies from several laboratories have revealed the existence of ‘post-transcriptional regulons’, that is, functionally-related mRNAs containing structural elements in their UTRs that are specifically recognized and regulated by the same RNA-binding protein or complex [36–38]. The high cellular abundance of each of the GAIT proteins (and the robust release of L13a and EPRS from their parent complexes) suggests that sufficient GAIT complex is available for silencing multiple transcripts. Indeed, recent evidence suggests that the GAIT pathway regulates multiple pro-inflammatory mRNAs and thus forms a post-transcriptional regulon that has evolved to limit or resolve inflammation [25,39,40].

Pattern analysis identifies a GAIT element in the 3'UTR of VEGF-A and other mRNAs

PatSearch (<http://www.ba.itb.cnr.it/BIG/PatSearch/>), a folding energy-independent, pattern analysis algorithm, was used to search a 3'UTR database containing more than 200 000 sequences for a consensus GAIT element pattern, based on sequence and secondary structure of the human *Cp* GAIT element [41]. The algorithm found 55 human mRNAs with putative 3'UTR GAIT elements; only two mRNAs were predicted to contain 5'UTR GAIT elements, indicating marked positional specificity [39]. A Gene Ontology (GO)-based literature search [42] indicated that many putative GAIT-element-bearing transcripts were associated with the inflammatory response or induced by inflammatory agonists [39].

Vascular endothelial growth factor A (VEGF-A), a hallmark inflammatory protein that induces angiogenesis, was among the candidate mRNAs. Mfold, which calculates the optimal thermodynamic folding structure of RNA [43], revealed a bipartite stem-loop structure in the *VEGF-A* 3'UTR (nt 358–386) similar to the *Cp* GAIT element [13]. A series of experiments confirmed the authenticity of the *VEGF-A* GAIT element. The element conferred translational silencing to a heterologous reporter RNA in an *in vitro* translation system [39]. IFN- γ shifted *VEGF-A* mRNA from ribosome-rich, polysomal fractions to a ribosome-poor, ribonucleoprotein fraction. An RNA electrophoretic mobility shift assay (EMSA) ‘supershift’ experiment demonstrated an interaction with the four GAIT proteins. Moreover, suppression was not observed in L13a-deficient cells. In monocytic cells, IFN- γ induced VEGF-A protein expression after 8 h, followed by a return to the basal level after 24 h despite abundant *VEGF-A* mRNA, indicating delayed translational repression of endogenous VEGF-A. This translational inhibition decreased angiogenic activity of monocytic cells, as shown by reduced formation of capillary-like tubes by endothelial cells cultured in Matrigel™ [39].

Analysis of the kinase cascade (DAPK–ZIPK) responsible for L13a phosphorylation showed that expression and activity of both DAPK and ZIPK plummeted ~16 h after IFN- γ treatment, despite robust mRNA expression, suggesting the possibility of translational control [25]. The PatSearch algorithm revealed a candidate GAIT element in the human *DAPK* mRNA 3'UTR but not in *ZIPK*. Secondary structure analysis of the *ZIPK* 3'UTR by Mfold revealed a split stem-loop structure with some resemblance to the *Cp* and *VEGF-A* GAIT element structures (Figure 1). The crucial ‘A’ and ‘U’ nucleotides were present in the bulge of the *DAPK* GAIT element, as in *Cp* and *VEGF-A*, but were missing from the *ZIPK* GAIT element. Functionality of both the *DAPK* and *ZIPK* elements was shown by translational silencing of a heterologous reporter *in vitro*, by RNA EMSA and by supershift analysis [25]. Transfection of antisense morpholino oligomers complementary to the GAIT

elements of both kinases prevented their translational silencing, presumably by blocking GAIT complex binding. The morpholinos maintained long-term expression of the kinases as well as L13a in its active, phosphorylated state. The possibility of restricting macrophage inflammatory activity using morpholinos targeted against DAPK and ZIPK presents an exciting application with potential therapeutic benefit.

Translational silencing of DAPK and ZIPK can prevent the accumulation of these potentially injurious, pro-apoptotic kinases [30,44]. More importantly, DAPK, ZIPK and L13a form a negative-feedback module in which L13a phosphorylation activates the GAIT complex, represses kinase translation and returns L13a to the non-phosphorylated form, thereby restoring the cell to the basal state, and permitting re-stimulation by IFN- γ [25]. This network module could exemplify a principle of parsimonious pathway evolution in which the principal function of an existing system, in this case translational silencing of a regulon of inflammatory transcripts, is co-opted to downregulate the initial stimulus. Likewise, negative-feedback regulation of translation has been observed for the tyrosine kinase c-Src; binding of its phosphorylated substrate, hnRNP K, to the c-Src mRNA 3'UTR inhibits expression during erythroid maturation [45,46]. Thus, inhibition of kinase mRNA translation by binding of a phosphorylated substrate to the 3'UTR might represent a new 'network motif' in gene expression [47].

Multiple chemokine ligand and receptor mRNAs are GAIT pathway targets

Genome-wide microarray analysis of polysome-bound mRNAs isolated from IFN- γ -activated monocytic cells identified a family of mRNAs encoding multiple chemokine ligands and receptors as candidate GAIT pathway targets [40]. Structural prediction supported this finding. The presence of functional GAIT elements in transcripts encoding chemokine ligand and receptors CCL22, CCR3, CCR4 and CCR6, and also in APOL2 (apolipoprotein L 2), was validated by their ability to repress the translation of a heterologous reporter RNA in L13a-dependent fashion [40]. Importantly, overexpression of chemokines and their receptors is a major contributor to the pathogenesis of multiple inflammatory conditions, for example atherosclerosis and neuroinflammation [48,49]. Despite the expanding cohort of GAIT-targeted transcripts, consensus on the secondary structure of the element has not yet been achieved, except that all form stems split by their asymmetric bulge loops. Likewise, information is not yet available on the relative binding affinity of the GAIT elements in these transcripts for the GAIT complex, and it remains unknown whether a silencing hierarchy exists whereby certain transcripts are more effectively silenced than others.

Physiological functions of the GAIT system

Several unique features of the GAIT-mediated anti-inflammatory system distinguish it from established signaling pathways. Perhaps most obvious is the 12–16 h lag between stimulation by IFN- γ and translational silencing of targets, attributable to the delayed activation of the DAPK–ZIPK cascade that directs L13a phosphorylation. We can envision several advantages of this delayed translational silencing mechanism. A major function of the pathway could be to prevent excessive accumulation of inflammatory proteins that can potentially cause injury to host tissue. Thus the GAIT pathway joins the list of several inhibitory mechanisms that have evolved to restrict IFN- γ -inducible inflammatory gene expression. Previously recognized negative regulatory mechanisms act at the level of transcription inhibition; these mechanisms include phosphatase-mediated inactivation of crucial receptor-activated kinases, for example JAKs (Janus kinases) and MAPKs (mitogen-activated protein kinases) [50], proteasomal degradation of the STAT1 (signal transducer and activator of transcription 1) transcription factor [51], and inhibition of JAKs by interaction with SOCS (suppressors of cytokine signaling) [52]. The GAIT-mediated, post-

transcriptional mechanism provides the unique advantage of suppressing mRNAs that avoid early transcriptional blocks, for example transcripts that are highly stable or induced with delayed kinetics (Figure 3). Moreover, the GAIT pathway can silence mRNAs, which are induced by multiple IFN- γ -mediated transcriptional pathways, for example JAK-STAT or C/EBP- β (CCAAT-enhancer-binding protein- β). Early transcriptional induction by IFN- γ , followed by delayed translational suppression, is consistent with its paradoxical pro- and anti-inflammatory effects [9,10].

Little is known about physiological or pathological conditions that can influence GAIT pathway activity. Recent studies showed that hypoxic conditions increased the expression of the crucial angiogenic factor [53] and GAIT pathway target VEGF-A [39,54] through transcriptional upregulation, mRNA stabilization and internal ribosome entry site-mediated translation initiation [55]. Therefore, we addressed the consequences of concurrent exposure of monocytic cells to opposing regulatory signals from hypoxia and IFN- γ , stresses that are likely to exist in the cores of avascular, growing tumors or in the thickened intima of atherosclerotic lesions of blood vessels. Interestingly, hypoxia overcame GAIT-mediated *VEGF-A* mRNA translational silencing without disturbing formation of the active GAIT complex or silencing translation of another GAIT target, namely *Cp* mRNA [54]. Target-specific override of the GAIT pathway was directed by adjacent elements in the *VEGF-A* 3' UTR – the GAIT element and a binding site for hnRNP L functioning as a condition-dependent, binary conformational RNA switch. During normoxia, IFN- γ causes proteasomal degradation of hnRNP L and activation of the GAIT complex that stabilizes the conformer that represses *VEGF-A* translation. By preventing degradation and allowing binding of hnRNP L, hypoxia switches the UTR to the conformer that maintains high-level translation. The switching mechanism allows high-level *VEGF-A* expression to relieve tissue hypoxia while simultaneously permitting GAIT-mediated repression of harmful inflammatory proteins [54].

IFN- γ -mediated suppression of inflammatory gene expression implicates the DAPK-ZIPK-L13a axis in negative regulation of the inflammatory response of myeloid cells. The GAIT system can potentially fine-tune inflammatory gene expression in the presence of persistent inflammatory stimuli and also contribute to inflammation-resolution after the stimulus is eliminated. These findings provide an unexpected expansion of the function of the DAPK-ZIPK cascade, previously shown to have important roles in apoptosis and vesicle autophagy [30,44,56]. Because inflammation is an important contributor to tumorigenesis [57], the anti-inflammatory function of DAPK might contribute to its established tumor-suppressor activity [56,58]. Genetic defects in components of the GAIT pathway, or defects caused by environmental stress, might contribute to progression of chronic inflammatory disorders. Prolonged inflammatory gene expression contributes to malignant tumor progression [53,57]. Failure of tumor-associated macrophages to suppress VEGF-A expression could be particularly important because these cells regulate the angiogenic switch that drives the transition from small, dormant, avascular tumors to large, rapidly growing, vascularized tumors [59]. Thus, GAIT-mediated translational control of inflammatory transcripts might have a crucial role in protecting cells from inflammation and injury associated with tumorigenesis. In addition, recent studies suggest that genetic variation in two GAIT-pathway-related genes, *GAPDH* and *DAPK*, is associated with Alzheimer's disease [60,61]. Elucidation of the GAIT pathway could present alternative targets for novel anti-inflammatory therapeutic strategies that, owing to their target and cell specificity, might exhibit minimal adverse side effects.

Concluding remarks

In the decade following its discovery, notable progress has been made in understanding certain aspects of the GAIT system, for example its components and their interactions, the upstream activation pathways and several downstream RNA targets. However, there remain far more questions than answers. Among the immediate and future challenges are: (i) the determination of the specific domains involved in protein–protein and protein–RNA interactions and their consequent conformational changes; (ii) the determination of the structures of the pathway intermediates as well as the holo-GAIT complex; (iii) the elucidation of the signaling pathways responsible for EPRS phosphorylation and for the 16-h delay before L13a phosphorylation; (iv) the function of the pre-GAIT complex (if any); (v) the potential role of phosphatases in pathway inactivation; (vi) the function of GAPDH; and (vii) the role of nearby RNA elements, including microRNA-binding sites. Much more experimentation is needed to understand the physiological and pathophysiological importance of the GAIT pathway. Analysis of GAIT pathway evolution could provide insights into its function, as will a comprehensive analysis of the post-transcriptional regulon. Most importantly, we look forward to results from animal models that express genetically modified GAIT proteins and from association studies of variants in GAIT-related genes with human disease. We hope that answers to these and other fundamental questions will elucidate the mechanisms and functions of the GAIT system and, furthermore, that the insight gained from their elucidation will be applicable to depot systems yet to be discovered and to other complex transcript-selective translational control systems.

Acknowledgments

Our work is supported by awards from the National Institutes of Health (to P.L.F.) and by Postdoctoral Fellowships from the American Heart Association, Ohio Valley Affiliate (to A.A. and J.J.).

Glossary

Cp	ceruloplasmin. A copper protein produced by hepatocytes and cells of the myeloid lineage, including monocytes and macrophages
DAPK	death-associated protein kinase. A calmodulin-regulated, serine/threonine kinase implicated in apoptosis and tumor suppression
eIF4G	eukaryotic initiation factor 4G. A component of the cap-binding translation initiation complex that makes initial contact with the ribosome by interaction with eIF3 in the 40S-containing, 43S pre-initiation complex
EPRS	glutamyl-prolyl tRNA synthetase. A bifunctional tRNA synthetase that catalyzes the ligation of Glu and Pro to their cognate tRNAs. EPRS and 7 other synthetases reside in the cytoplasm within the tRNA multisynthetase complex
FRET	Förster resonance energy transfer. FRET provides a molecular ruler, accurately determining the distance between two fluorescently-labeled macromolecules
GAIT complex	interferon- γ activated inhibitor of translation complex. A heterotetrameric complex comprising EPRS, NSAP1, L13a and GAPDH that binds a structural 3'UTR GAIT element of target mRNAs and silences their translation

GAIT element	a defined structural RNA element present in the 3' UTR of several inflammatory mRNAs; the GAIT complex binds this element and suppresses translation
GAPDH	glyceraldehyde 3-phosphate dehydrogenase. A housekeeping enzyme that converts glyceraldehyde 3-phosphate to D-glycerate 1,3 bisphosphate during glycolysis
IFN-γ	interferon- γ . A type II pro- and anti-inflammatory cytokine known for anti-viral, immunoregulatory, and anti-tumor properties
L13a	ribosomal protein L13a. A 23 kDa, highly basic protein constituent of the 60S ribosomal subunit
MSC	tRNA multisynthetase complex. A cytosolic, 1.5 mDa complex containing nine tRNA synthetases (counting EPRS as two) and three non-synthetase proteins
NSAP1	NS1-associated protein (also referred to as SYNCRIP or hnRNP Q1). A splicing factor with several cytoplasmic activities including translational control
PABP	poly(A)-binding protein. A protein that interacts with both the 3' poly(A) tail of mRNA and eIF4G at the 5' terminus to maintain the closed-loop structure of translating mRNA
RRL	rabbit reticulocyte lysate. An RNA- and cell-free system containing all components required for efficient translation of exogenous mRNA
SPR	surface plasmon resonance. A sensitive, laser-based method that enables real-time quantitative analysis of protein–protein or protein–RNA binding kinetics
UTR	untranslated region. Upstream or downstream regions of an mRNA flanking the coding sequence which are not translated but offer post-transcriptional regulation of gene expression
VEGF	vascular endothelial growth factor. A critical factor involved in blood vessel growth and tumorigenesis
WHEP domain, a conserved helix-turn-helix domain of ~50 amino acids named after three vertebrate synthetases containing the domain	tryptophanyl (W), histidyl (H), and glutamyl-prolyl (EP) tRNA synthetases
ZIPK	zipper-interacting protein kinase, also known as DAPK3. A member of the death-associated protein kinase family involved in apoptosis and smooth muscle contractility

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Figure 1.

Secondary structures of GAIT elements. Secondary structures of functional 3' UTR GAIT elements as predicted by Mfold: ceruloplasmin (*Cp*, nt 78–106), vascular endothelial growth factor-A (*VEGF-A*, nt 358–386), death-associated protein kinase (*DAPK*, nt 1141–1169), zipper-interacting protein kinase (*ZIPK*, nt 174–206) and chemokine C-C motif ligand 22 (*CCL22*, nt 433–462) mRNAs. Nucleotides essential for Cp-GAIT activity are shown in red.

Figure 2.

GAIT-mediated translational control. IFN- γ treatment elicits an intricate translational regulatory pathway in which the GAIT complex modulates the expression of its target mRNAs. (i) IFN- γ induces phosphorylation and release of EPRS (green) from the tRNA multisynthetase complex (yellow). Phosphorylated EPRS (P-EPRS) interacts with NSAP1 (light red) and forms an inactive, pre-GAIT complex. (ii) Later, L13a (dark blue) is phosphorylated and released from the 60S ribosomal subunit. Phosphorylated L13a (P-L13a), with GAPDH (brown), joins the pre-GAIT complex to form the functional GAIT complex. (iii) The complex binds the GAIT element in the 3' UTR of target transcripts circularized by simultaneous interactions of poly(A)-binding protein (PABP, gray) with eIF4G (orange) and the poly(A) tail. (iv) P-L13a interacts with eIF4G in the translation initiation complex to block recruitment of the eIF3 (light blue)-containing 43S ribosomal complex subunit and repress translation. Cap-binding protein, eIF4E, is shown (dark orange).

Figure 3.

Early and late negative regulatory pathways of IFN- γ -driven gene expression programs. STAT1 and the GAIT complex regulate early transcription-induction (light red) and late translation-inhibition (light blue) regulons, respectively, with an intersection in both regulons (light violet). Dark red ellipses within the transcription-induction regulon denote early-IFN- γ -induced transcripts repressed by negative regulators of the JAK-STAT pathway (black); violet ellipses represent stable, IFN- γ -induced transcripts that evade transcriptional inhibition but are repressed by L13a (dark green) in the GAIT complex. Light blue ellipses denote transcripts induced by JAK-STAT-independent pathways, for example transcriptional induction by C/EBP- β . Abbreviations: IFN- γ -R, IFN- γ -receptor; PTP, protein tyrosine phosphatase.