Protection of Extraribosomal RPL13a by GAPDH and Dysregulation by *S*-Nitrosylation

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SUMMARY

Multiple eukaryotic ribosomal proteins (RP) are co-opted for extraribosomal "moonlighting" activities, but paradoxically, RPs exhibit rapid turnover when not ribosome-bound. In one illustrative case of a functional extraribosomal RP, interferon (IFN)-γ induces ribosome release of L13a and assembly into the IFN-Gamma-Activated Inhibitor of Translation (GAIT) complex for translational control of a subset of inflammation-related proteins. Here we show GAPDH functions as a chaperone, shielding newly released L13a from proteasomal degradation. However, GAPDH protective activity is lost following cell treatment with oxidatively-modified low density lipoprotein and IFN-γ. These agonists stimulate *S*-nitrosylation at Cys^{247} of GAPDH which fails to interact with L13a, causing proteasomal degradation of essentially the entire cell complement of L13a, and defective translational control. Evolution of extraribosomal RP activities might require co-evolution of protective chaperones, and pathological disruption of either protein, or their interaction, presents an alternative mechanism of diseases due to RP defects, and targets for therapeutic intervention.

INTRODUCTION

Eukaryotic cells from yeast to mammals contain precise, stoichiometric amounts of ribosomal proteins (RP) maintained by transcriptional and translational mechanisms. However, as an additional safeguard, excess unassembled RPs are degraded rapidly, with a half-time ranging from seconds to minutes (Warner, 1999). The discovery of important extraribosomal, "moonlighting" functions by multiple eukaryotic RPs (Warner and McIntosh, 2009) presents an apparent paradox, namely, how are free RPs stabilized to permit these persistent activities? As a corollary, regulation (or dysregulation) of the stabilization process can influence RP extraribosomal activity, and possibly contribute to pathology. RP L13a, a component of the large ribosomal subunit conserved in bacteria, archaea, and eukarya, exhibits an extraribosomal function as a critical component of the IFN-Gamma-Activated Inhibitor of Translation (GAIT) complex in human myeloid cells

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(Mazumder et al., 2003; Mukhopadhyay et al., 2008). Elucidation of physiological and pathophysiological mechanisms regulating L13a survival outside the ribosome can shed light on these fundamental questions.

The GAIT complex inhibits translation of a select group of inflammation-related transcripts in human myeloid cells (Mazumder et al., 2003; Yao et al., 2012). Translational silencing requires binding of the heterotetrameric GAIT complex to the $3'$-UTR of target mRNAs including ceruloplasmin (Cp), vascular endothelial growth factor (VEGF)-A, and several chemokines and their receptors (Mukhopadhyay et al., 2009; Ray and Fox, 2007; Sampath et al., 2003; Vyas et al., 2009). Other GAIT complex constituents are glutamyl-prolyl-tRNA synthetase (EPRS), NS1-associated protein-1 (NSAP1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sampath et al., 2004). Remarkably, two GAIT complex proteins are inducibly released from parent macromolecular complexes following IFN-γ-stimulated phosphorylation: EPRS from the aminoacyl-tRNA multisynthetase complex (Sampath et al., 2004) and L13a from the 60S, large ribosomal subunit (Mazumder et al., 2003). L13a release from the ribosome requires phosphor-ylation of Ser[77] by zipper-interacting protein kinase (ZIPK) (Mukhopadhyay et al., 2008). GAIT complex assembly occurs in two stages. Initially, phospho-EPRS binds NSAP1 to form a non-functional, pre-GAIT complex. About 16 hr later, phospho-L13a and GAPDH join the pre-GAIT complex to form the functional GAIT complex.

Inflammation is an orchestrated response to trauma, e.g., injury or microbial invasion, characterized by induced expression and activation of signaling cascades and defensive molecules, and gradually diminished upon injury repair or elimination of the trauma stimulus (Anderson, 2010; Lindemann et al., 2005; Medzhitov and Horng, 2009; Nathan, 2002). We have proposed that delayed activation of the GAIT complex permits initial high-level expression of certain inflammation-related proteins, but subsequently silences their expression to prevent unchecked injury to host tissues (Mukhopadhyay et al., 2009). Thus, the GAIT system, via transcript-selective, delayed translational repression activity, has the potential to restrict the duration and magnitude of the inflammatory response, and contribute to the resolution of inflammation. Conversely, condition-dependent or genetic defects in the GAIT system or its upstream activation pathways, might permit persistent over-expression of injurious GAIT system-regulated products and thereby contribute to chronic inflammatory disorders. However, conditions or effectors that inactivate L13a or other GAIT complex constituents, and prevent translational silencing of GAIT system target mRNAs have not been identified.

A principal component of the inflammatory response is cellular overproduction of reactive oxygen and nitrogen species that promote pathological modification of lipids and proteins, implicated in cardiovascular and neurodegenerative disease progression (Libby et al., 2002). Oxidatively-modified LDL (LDL$_{ox}$) is one such factor detected in plasma, cerebrospinal fluid, and inflammatory tissues, and is associated with risk for Alzheimer's disease (Kankaanpaa et al., 2009), end-stage renal disease (Shoji et al., 2002), and atherosclerosis (Podrez et al., 2002; Stocker and Keaney, 2004). Here we show that LDL$_{ox}$ prevents formation of an active GAIT complex in myeloid cells thereby prolonging production of VEGF-A and Cp. Our results provide evidence for physiological regulation and pathological dysregulation of the stability of an extraribosomal RP.

RESULTS AND DISCUSSION

To explore the influence of pathological conditions on GAIT-mediated translational control we investigated the potential effect of LDL$_{ox}$ on GAIT activity in myeloid cells. Human peripheral blood monocytes (PBM) were incubated with IFN-γ in the presence of human

LDL_{ox} generated by *in vitro* exposure of freshly isolated LDL to the physiological, leukocyte oxidation system consisting of myeloperoxidase (MPO), NO_2^-, and H_2O_2 (Podrez et al., 2000). The effect of LDL_{ox} on endogenous expression of GAIT targets was investigated. Immunoblot analysis of cell lysates revealed induction of VEGF-A and ceruloplasmin (Cp) protein after 8 hr of IFN-γ treatment, followed by marked suppression after 24 hr (Fig. 1A, top 3 panels). Delayed suppression of both proteins is due to GAIT-mediated translational silencing (Mazumder and Fox, 1999; Ray and Fox, 2007). In contrast, treatment of cells with IFN-γ in the presence of LDL_{ox} caused high-level expression of VEGF-A and Cp even after 24 hr. The enhancement of GAIT target expression at 24 h was specific as shown by the absence of any effect on β-actin expression, or total protein synthesis as determined by [^{35}S]Met/Cys labeling of PBM protein (Fig. 1A, bottom). Persistent high-level expression of VEGF-A and Cp in lysates from LDL_{ox}-treated cells was not due to increased mRNA, consistent with the GAIT-mediated post-transcriptional control mechanism (Fig. 1B). The effect of LDL_{ox} on transcript-selective translation was determined by *in vitro* translation of a GAIT element-bearing Luc reporter in rabbit reticulocyte lysate (RRL). Cytosolic lysates from U937 cells treated with IFN-γ for 24 hr inhibited Luc RNA translation, as expected (Fig. 1C). Co-treatment of cells with IFN-γ and LDL_{ox} prevented the translation inhibition completely. Translation of T7 gene 10 RNA, a control transcript that lacks the GAIT element, was unaffected indicating target specificity.

To explore the mechanism underlying GAIT inhibition by LDL_{ox}, we examined GAIT complex assembly. After stimulation with IFN-γ and LDL_{ox} for 24 hr, NSAP1 was immunoprecipitated and the interaction with the other three GAIT complex components determined by immunoblot analysis. The pre-GAIT complex, formed by interaction of EPRS with NSAP1 about 2 hr after IFN-γ treatment, assembled normally. However, L13a and GAPDH, components unique to the mature GAIT complex, did not bind NSAP1 in cells treated with IFN-γ and LDL_{ox} (Fig. 1D). Remarkably, LDL_{ox} caused a near-complete disappearance of L13a after 24 hr, but levels of other GAIT complex proteins were unaltered; native LDL did not alter expression of GAIT proteins (Fig. 1E, left). LDL_{ox} did not reduce L13a mRNA expression, indicating a post-transcriptional regulatory mechanism (Fig. 1E, right). Neither IFN-γ nor LDL_{ox} by itself reduced L13a expression, indicating an absolute requirement for both agonists (Fig. 1F). To establish degradation of L13a, IFN-γ and LDL_{ox} were added to cells in the presence of MG132, a proteasome inhibitor. MG132 prevented L13a depletion at 24 hr, substantiating proteasomal degradation (Fig. 1G). Proteins are generally targeted for proteasome degradation by polyubiquitinylation at one or more Lys residues by the coordinated action of ubiquitin activating enzyme E1, conjugating enzyme E2, and E3 ligase. Cells were transfected with cDNA encoding HA-tagged ubiquitin in the presence of MG132 to prevent proteasomal degradation of ubiquitinylated protein. Ubiquitinylation was detected with anti-HA tag antibody following immunoprecipitation with anti-L13a antibody. Cell treatment with LDL_{ox} for 24 hr induced robust polyubiquitinylation of L13a that was blocked by UBEI-41, an E1 inhibitor (Fig. 1H). Treatment of cells with LDL_{ox} or IFN-γ alone did not induce L13a ubiquitinylation confirming the requirement for both.

IFN-γ induces ZIPK-mediated L13a phosphorylation at Ser^{77}, and consequent release from the large ribosome subunit (Mukhopadhyay et al., 2008). We investigated whether L13a phosphorylation is required for ubiquitinylation and degradation. Cells were transfected with plasmid encoding Flag-tagged L13a bearing a non-phosphorylatable Ser-to-Ala mutation at the Ser^{77} phospho-site. The wild-type transfectant was almost completely degraded whereas mutated L13a was not susceptible to LDL_{ox}-mediated degradation (Fig. 2A, top 3 panels). Endogenous RPs L6 and L28 were not degraded revealing exceptional target selectivity. Similar results were observed when ZIPK, the proximal L13a kinase, was specifically inhibited by siRNA (Fig. 2A, bottom 3 panels). To investigate the specific role of

phosphorylation in L13a ubiquitinylation, U937 cells were co-transfected with HA-tagged ubiquitin and Flag-tagged wild-type or S77A mutant L13a. L13a phosphorylation was determined by immunoprecipitation with anti-Flag antibody and immunoblot analysis with anti-phosphoserine. Wild-type L13a was phosphorylated and robustly polyubiquitinylated by IFN-γ and LDL_{ox} (in the presence of MG132 to prevent degradation), but the S77A mutant was not susceptible to either covalent modification (Fig. 2B). Similarly, L13a phosphorylation and ubiquitinylation were inhibited by siRNA-mediated ZIPK suppression (Fig. 2C). Phosphorylation is required for L13a release from the ribosome, but might independently be required for L13a recognition by the E1–E2–E3 system. The latter possibility was investigated in a cell-free system. Flag-tagged L13a was incubated with biotin-ubiquitin and a reconstituted ubiquitinylation system containing recombinant E1 and E2, and cell lysate to provide E3 ligase (and L13a kinase activity in the 24-hr lysate). Wild-type L13a was robustly polyubiquitinylated by the E1–E2–E3 cascade; however, modification was prevented by substitution of the S77A mutant, by pre-treatment with alkaline phosphatase, or after addition of a 0-hr lysate lacking L13a kinase activity (Fig. 2D). Thus, L13a phosphorylation is required not only for ribosome release, but also for recognition by the ubiquitinylation system.

The second phase of GAIT complex assembly requires both GAPDH and L13a. We considered the possible role of GAPDH in determining L13a stability. To investigate their interaction in the absence of other GAIT components, L13a and phospho-L13a were incubated with immobilized glutathione-S-transferase (GST)–GAPDH fusion protein, and the interaction detected by immunoblot. GAPDH bound phospho-L13a, but not unmodified L13a (Fig. 3A). Importantly, treatment of cells with LDL_{ox} for 24 hr (in the presence of MG132 to prevent L13a degradation) completely blocked the IFN-γ-driven interaction of GAPDH and L13a, suggesting that the interaction might be critical for maintenance of free cellular L13a (Fig. 3B). We considered the possibility that GAPDH functions as a shield that binds phospho-L13a and prevents its degradation. GAPDH-deficient cells were generated using siRNA targeted against GAPDH in the presence of pyruvate, the 3-carbon end-product of glycolysis, to minimize bioenergetic imbalance (Hara et al., 2005). GAPDH depletion, even in the absence of LDL_{ox}, caused specific and near-complete degradation of L13a after 24-h treatment with IFN-γ (Fig. 3C). Similarly, depletion of GAPDH induced phosphorylation-dependent polyubiquinylation of L13a in the absence of LDL_{ox} (Fig. 3D).

We examined the possibility that LDL_{ox} induced a post-translational modification of GAPDH that blocked its ability to shield L13a. Cell treatment with LDL_{ox} induced formation of *S*-nitrosylated GAPDH (SNO-GAPDH) as determined by immunoblot analysis with anti-SNO-Cys antibody (Fig. 3E). Modification did not require IFN-γ. Lys-acetylation was also detected, but this modification was not stimulus-dependent. Lys- and Arg-methylation, and Ser-, Tyr-, and Thr-phosphorylation were not detected (not shown). *S*-nitrosylation was confirmed by the "biotin-switch" procedure that replaces Cys nitrosyl groups with biotin for subsequent detection by avidin (Jaffrey et al., 2001). IFN-γ plus LDL_{ox} induced robust *S*-nitrosylation of GAPDH in human PBM (Fig. 3F). To determine the Cys residue *S*-nitrosylated by LDL_{ox}, U937 cells were transfected with human HA-GAPDH cDNAs containing Cys-to-Ser mutations at each of the three positions 152, 156, and 247. LDL_{ox} plus IFN-γ induced *S*-nitrosylation of GAPDH bearing mutations at Cys[152] and Cys[156] but not at Cys[247], indicating Cys[247] is the primary LDL_{ox}-inducible target (Fig. 4A, Supplemental Fig. S1A). *S*-nitrosylation of Cys[247] was confirmed by mass spectrometric analysis of GAPDH isolated from cells treated with LDL_{ox} plus IFN-γ (Supplemental Fig. S1B). Transfection of the C247S GAPDH mutant protected L13a from degradation and restored IFN-γ-mediated translational silencing of VEGF-A in the presence of LDL_{ox}; this result also shows that other constituents of the GAIT complex are not impaired (Fig. 4B). Importantly, LDL_{ox} *S*-nitrosylates an amino acid distinct from the

Cys^{150} residue (the rat equivalent of human Cys^{152}) *S*-nitrosylated by lipopolysaccharide (LPS) and other apoptotic stimuli (Hara et al., 2005). Indeed, treatment of human PBM with IFN-γ plus LPS induced specific *S*-nitrosylation of Cys^{152} and failed to induce L13a degradation (Fig. 4C). Together, these results indicate that GAPDH protects L13a from degradation, and that LDL$_{ox}$-induced *S*-nitrosylation of GAPDH inactivates the protective function.

Protein *S*-nitrosylation is a ubiquitous, post-translational effector mechanism. Dysregulation of protein *S*-nitrosylation is associated with diverse pathological conditions (Foster et al., 2009). A concept is emerging in which altered modification of a specific disease-related protein, rather than a global nitrosative effect, contributes to disease phenotype. In a well-studied example, apoptotic stimulation of murine macrophages with lipopoly-saccharide (LPS) triggers *S*-nitrosylation of GAPDH to facilitate binding and nuclear translocation of Siah1, contributing to cytotoxicity. (Hara et al., 2005). In our experiments LDL$_{ox}$ induces *S*-nitrosylation at human GAPDH Cys^{247} (equivalent to rat Cys^{245}), a residue distant from the active-site Cys^{152} (equivalent to rat Cys^{150}) residue modified following apoptotic stimulation by LPS (Hara et al., 2005). Differential cysteine S-nitrosylation suggests that LDL$_{ox}$ and LPS, in presence of IFN-γ, trigger distinct proinflammatory signaling pathways. Sequence motifs associated with *S*-nitrosylation are not well-defined; however, a modified acid-base motif has been proposed in which charged residues are concentrated at the solvent-exposed surface of the protein near the NO-Cys site, which may not be surface-exposed (Marino and Gladyshev, 2010). The X-ray crystal structure of human GAPDH indicates the Cys^{247} site is not exposed to the surface, but charged residues nearby are exposed (Fig. 4D). Moreover, because mutation of human Cys^{247}, in contrast to Cys^{152}, is far from the active-site and does not alter GAPDH enzymatic activity (Nakajima et al., 2009), even extensive modification is unlikely to negatively influence cell energy balance.

GAPDH is an archetype multi-functional protein; in addition to its principal role in glycolysis, GAPDH acts as reversible metabolic sensor that responds to oxidative stress (Zheng et al., 2003). Our new results suggest an unexpected protective function of GAPDH. In its native form, GAPDH binds phosphorylated L13a upon release from the 60S ribosomal subunit, and protects it from degradation by the ubiquitin-proteasome system (Fig. 4E). The stoichiometry of ribosomal proteins is generally enforced by rapid degradation of newly synthesized ribosomal proteins not bound to ribosome sub-complexes during assembly (Nomura, 1999). Several ribosomal proteins are protected and controlled by heat shock proteins during ribosome biogenesis (Kim et al., 2006). However, one can envisage that a specialized mechanism is required for protection of a free ribosomal protein released from a mature ribosome as in the case of L13a. Possibly, the mechanism is generalizable and GAPDH binds and protects other ribosomal proteins with extra-ribosomal functions (Warner and McIntosh, 2009). *S*-nitrosylation of GAPDH results in complete loss of the protective function, and consequent ubiquitinylation and degradation of phospho-L13a and loss of GAIT complex activity.

Ribosomal proteins are implicated in multiple pathologies including developmental impairment (Warner and McIntosh, 2009), malignant peripheral nerve tumors (Amsterdam et al., 2004), glucose tolerance and diabetes (Ruvinsky et al., 2005), Diamond-Blackfan anemia (Flygare and Karlsson, 2007; Warner and McIntosh, 2009), and cleft palate and abnormal thumbs (Draptchinskaia et al., 1999; Gazda et al., 2008). In all of these cases, the pathology can be traced to genetic modification of a ribosomal protein. In contrast, selective, stimulus-mediated degradation of L13a is remarkable in that the phenotype is not due to genetic alteration, but rather is a consequence of environment-dependent modification. Moreover, the pathophysiological significance of L13a is unique in that it is the extraribosomal function that is subject to dysregulation. The possible role of extraribosomal

functions of other RPs in pathogenesis, for example in Diamond-Blackfan anemia, has been considered, but remains uncertain (Flygare and Karlsson, 2007; Warner and McIntosh, 2009). Our findings suggest that in seeking the etiological underpinnings of RP-related disease, attention should be given not just to the ribosome and their associated RPs, but also to extraribosomal RPs and their binding partners.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, Site-directed Mutagenesis, and Recombinant Proteins

See Extended Experimental Procedures.

Cell Culture and Transfection

Human U937 monocytic cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS). BMDM were harvested and cultured from femurs of mice in C57BL/6J background under a protocol approved by the Cleveland Clinic Institutional Animal Care and Use Committee. PBM from healthy clinical donors were isolated by leukapheresis and countercurrent centrifugal elutriation under a Cleveland Clinic Institutional Review Board-approved protocol that adhered to American Association of Blood Bank guidelines. Cells were pre-incubated for 1 hr in medium containing 1% FBS before treatment with IFN-γ (500 units/ml), lipoproteins (50 μg/ml), and other reagents for up to 24 hr. In some experiments, U937 cells were transfected with plasmids (5 μg DNA) expressing HA-ubiquitin, Flag-L13a (wild-type or S77A), or HA-GAPDH (wild-type or Cys mutants) using Human Monocyte Nucleofector Kit (Lonza, Walkersville, MD). Cell transfection with siRNA against ZIPK or GAPDH was done according to manufacturer's protocol and allowed to recover 24 hr before use.

Quantitative RT-PCR, *in Vitro* Transcription, and *in Vitro* Translation

Total RNA (10 ng) from U937 cells was reverse-transcribed, and analyzed by quantitative RT-PCR using primers specific for VEGF-A, Cp, β-actin, or L13a. Capped, poly(A)-tailed Luc reporter and T7 gene 10 RNAs were prepared using Message Machine SP6 and T7 Kit (Ambion). DNA template preparation and mRNA purification were as described (Sampath et al., 2004). For determination of GAIT activity, cell lysates (1 μg) were pre-incubated with reporter and control mRNAs, and *in vitro* translation determined by incorporation of [^{35}S]methionine in RRL, followed by autoradiography (Jia et al., 2008).

Determination of L13a Ubiquitinylation

To determine L13a polyubiquitinylation, U937 cells (1×10^7 cells) were transfected with plasmid encoding HA-ubiquitin (5 μg, or 2.5 μg when co-transfected with 2.5 μg Flag-L13a), and then incubated with IFN-γ and LDL for up to 24 hr in the presence of MG132 (5 μM) to prevent proteasomal degradation. Cell lysates (1 mg) were immunoprecipi-tated with anti-L13a or -Flag antibody, and immunoblotted with antibodies against HA-tag, L13a, and P-Ser. For *in vitro* reconstitution of L13a ubiquitinylation, purified Flag-tagged L13a (1 μg of wild-type or S77A mutant) was pre-incubated with U937 cell lys-ate, and then incubated with a mixture E1 and E2 enzymes, biotin-ubiquitin, and cell lysate as a source of L13a kinase and E3 ligase. Recombinant L13a was immunopre-cipitated with anti-Flag tag antibody, and biotin-ubiquitin detected with streptavidin-HRP.

Determination of GAPDH-L13a Interaction

Purified GST-GAPDH was immobilized on glutathione-agarose beads, and incubated with L13a (unmodified or phosphorylated) expressed and phosphorylated as described

(Mukhopadhyay et al., 2008). After washing, bound proteins were heat-denatured in SDS gel-loading solution and analyzed by immunoblotting.

Biotin-Switch Determination of *S*-Nitrosylation of GAPDH

U937 cell (1×10^7 cells) lysates were labeled by the biotin-switch method (Jaffrey et al., 2001) with the *S*-Nitrosylation Protein Detection Assay Kit, immunoprecipitated with anti-GAPDH antibody, and detected with avidin-HRP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- GAPDH acts as a shield, protecting free ribosomal protein L13a from degradation

- Condition-dependent degradation of an extraribosomal ribosomal protein

- S-Nitrosylation of GAPDH at Cys[247] suppresses its protective activity

- Degradation of unprotected L13a inactivates GAIT system and induces VEGF-A expression
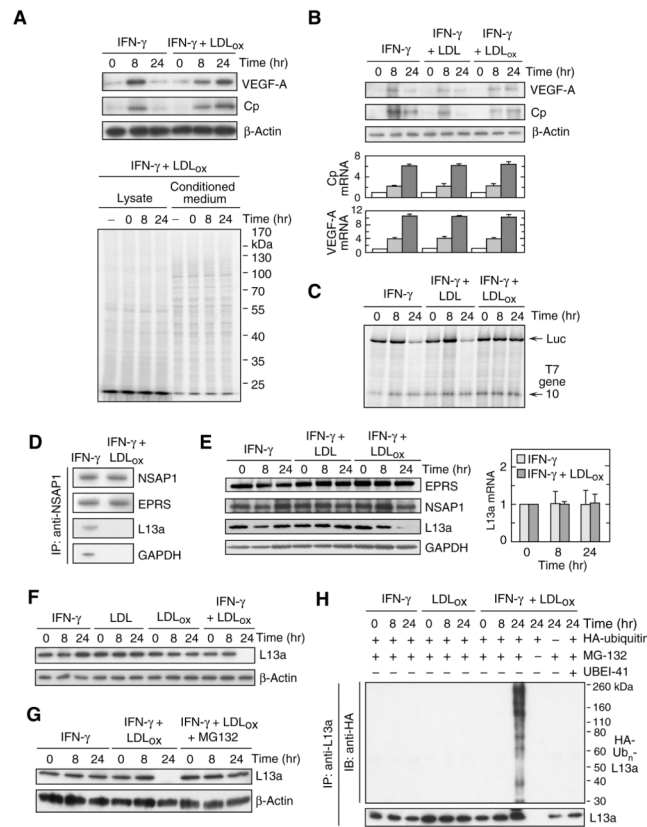
Figure 1. LDL$_{ox}$ suppresses GAIT activity by selective degradation of ribosomal protein L13a

(A) Effect of IFN-γ and LDL$_{ox}$ on GAIT target gene expression. Human PBM were incubated with IFN-γ (500 unit/ml) in the presence of LDL$_{ox}$ (50 μg/ml). Cell lysates (40 μg protein) were immunoblotted with anti-Cp, anti-VEGF-A and anti-β-actin antibodies (upper 3 panels). PBM were subjected to metabolic labeling for up to 24 h in the presence of [^{35}S]Met/Cys and IFN-γ plus LDLox. Conditioned media and lysates were analyzed by SDS-PAGE and autoradiography (lower panel).

(B) Lack of effect of IFN-γ and LDL$_{ox}$ on GAIT target mRNA expression. Human U937 monocytic cells were treated with IFN-γ in presence of unmodified LDL or LDL$_{ox}$, and lysates immunoblotted as above (top 3 panels). Cp, VEGF-A, and β-actin mRNA were determined by quantitative RT-PCR with appropriate primers; results were normalized to β-actin mRNA and expressed as mean ± SEM (n = 3 experiments).

(C) LDL$_{ox}$ suppresses translational silencing activity of the GAIT pathway. U937 cells were incubated with IFN-γ (500 unit/ml) in the presence of LDL or LDL$_{ox}$ (50 μg/ml) for 8 or 24 hr. Cp GAIT element-bearing Luc reporter and T7 gene 10 RNA (200 ng of each) were subjected to *in vitro* translation in rabbit reticulocyte lysate (RRL) in the presence of cell lysate (1 μg) and ^{35}S-methionine.

(D) LDL$_{ox}$ represses GAIT complex assembly. After cell treatment for 24 hr with IFN-γ or IFN-γ plus LDL$_{ox}$, lysates (1 mg) were immunoprecipitated with anti-NSAP1 antibody and immunoblotted with anti-NSAP1, -EPRS, -L13a and -GAPDH antibodies.

(E) IFN-γ plus LDL$_{ox}$ represses L13a expression. U937 cells were incubated with IFN-γ and LDL or LDL$_{ox}$ for up to 24 hr. Expression of GAIT complex proteins was determined by immunoblot analysis (left). L13a mRNA (right) was determined by quantitative RT-PCR, normalized to expression in untreated cells (mean ± SEM, n = 3 experiments).

(F) LDL$_{ox}$ by itself does not degrade L13a. Cells were treated as in (A) and L13a and β-actin determined by immunoblot.

(G) Proteasome degradation of L13a. Cells were incubated with IFN-γ plus LDL$_{ox}$ in presence or absence of MG132 (5 μM); MG132 was added after 4 and 12 hr for the 8- and 24-hr incubations, respectively. Cell lysates were immunoblotted with anti-L13a or anti-β-actin antibodies.

(H) Poly-ubiquitinylation of L13a. U937 cells were transfected with plasmid encoding HA-ubiquitin and then treated with IFN-γ, LDL$_{ox}$, MG132, and UBEI-41 (25 μM) as indicated. Lysates were subjected to immunoprecipitation with anti-L13a antibody and immunoblot analysis with anti-HA tag and -L13a antibodies.
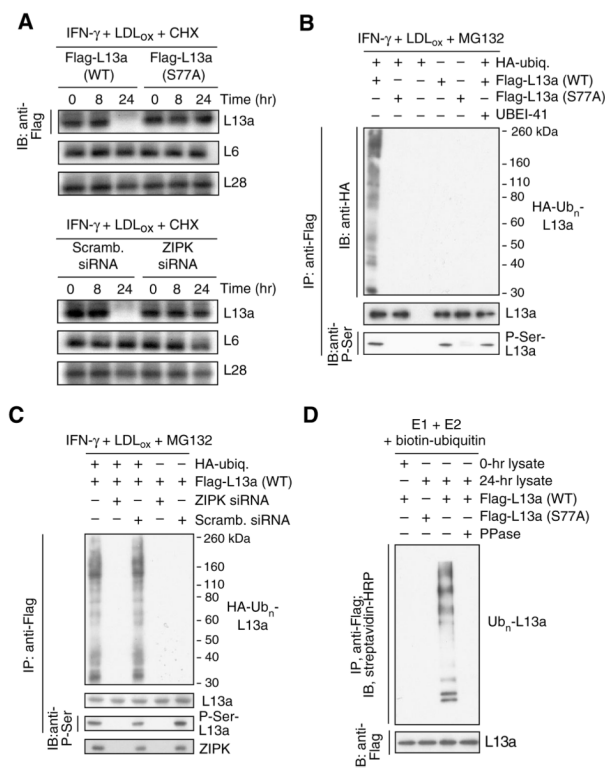
Figure 2. Phosphorylation-dependence of L13a ubiquitinylation and degradation

(A) L13a degradation is phosphorylation-dependent. U937 cells were transfected with plasmids encoding Flag-tagged wild-type (WT) or phospho-null (S77A) L13a. After recovery, cells were treated with IFN-γ plus LDL$_{ox}$ in the presence of cycloheximide (CHX) to inhibit de novo synthesis. Lysates were immunoblotted with anti-Flag, -L6, and -L28 antibodies (top 3 panels). Cells were transfected with ZIP kinase (or scrambled) siRNA and then incubated and immunoblotted as above (bottom 3 panels).

(B) L13a poly-ubiquitinylation is phosphorylation-dependent. U937 cells were co-transfected with plasmids encoding HA-ubiquitin and Flag-tagged wild-type or S77A mutant L13a. After recovery, cells were treated with IFN-γ plus LDL$_{ox}$ and with MG132 and UBEI-41 as in Figure 1H. L13a in cell lysates was immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA, -L13a, and -phospho-Ser antibodies.

(C) Poly-ubiquitinylation requires ZIP kinase (ZIPK). U937 cells were co-transfected with plasmids encoding HA-ubiquitin and Flag-L13a, and with ZIPK (or scrambled) siRNA. Cells were treated as above, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA (top), anti-L13a (second panel), or anti-phospho-Ser (third panel) antibodies. ZIPK was detected in 16 hr lysates by immunoblot with anti-ZIPK antibody (bottom).

(D) L13a phosphorylation is required for E3 ligase recognition. Flag-tagged L13a was incubated with biotin-ubiquitin and a reconstituted ubiquitinylation system containing recombinant E1 and E2 and lysate from cells treated with IFN-γ for 24 hr (or 0 hr as negative control) to provide E3 ligase and L13a kinase activity.
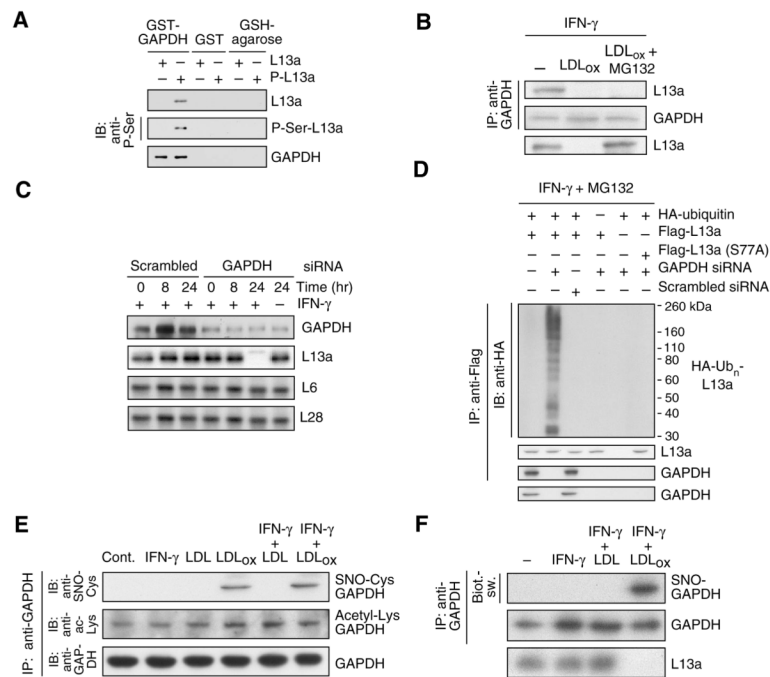
Figure 3. GAPDH binds phospho-L13a and protects it from degradation
(A) GAPDH binds phospho-L13a in absence of other GAIT components. Unmodified and phosphorylated L13a (P-L13a, prepared by treatment with ZIPK) were incubated with GST-GAPDH or GST immobilized to glutathione (GSH)-agarose beads. After washing, binding was detected by immunoblot analysis with anti-L13a, -phospho-Ser and -GAPDH antibodies.
(B) LDL$_{ox}$ blocks GAPDH binding to phospho-L13a. Cells were treated with IFN-γ, LDL$_{ox,}$ and MG132 as shown. Cell lysates were immunoprecipitated with anti-GAPDH antibody, and immunoblotted with anti-L13a and -GAPDH antibodies. Total L13a was detected by immunoblot with anti-L13a antibody.
(C) GAPDH protects L13a from degradation. U937 cells were transfected with GAPDH (or scrambled) siRNA. After recovery, cells were treated with IFN-γ, and lysates immunoblotted with anti-GAPDH, -L13a, -L6, and -L28 antibodies.
(D) GAPDH prevents ubiquitinylation of phospho-L13a in cells. U937 cells were co-transfected with GAPDH (or scrambled) siRNA and with plasmids encoding HA-ubiquitin and Flag-tagged L13a (wild-type or S77A mutant). After recovery, cells were incubated with IFN-γ and MG132 for 24 hr. Lysates were immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA, -L13a, and -GAPDH antibodies. Total GAPDH was determined by immunoblot with anti-GAPDH antibody.
(E) LDL$_{ox}$ induces S-nitrosylation of GAPDH. Cells were treated with IFN-γ, LDL, and LDL$_{ox}$. Lysates were immunoprecipitated with anti-GAPDH antibody and immunoblotted with anti-GAPDH, -S-nitrosocysteine (SNO-Cys), and -acetyl-Lys antibodies.
(F) Determination of GAPDH S-nitrosylation by biotin-switch assay. S-nitrosylation of endogenous GAPDH and L13a degradation were determined in human PBM treated with IFN-γ in presence of LDL or LDL$_{ox}$ for 24 hr. GAPDH was immunoprecipitated and S-nitrosylation detected with avidin-HRP in the biotin-switch assay. Efficiency of immunoprecipitation was shown by immunoblot analysis with anti-GAPDH antibody.
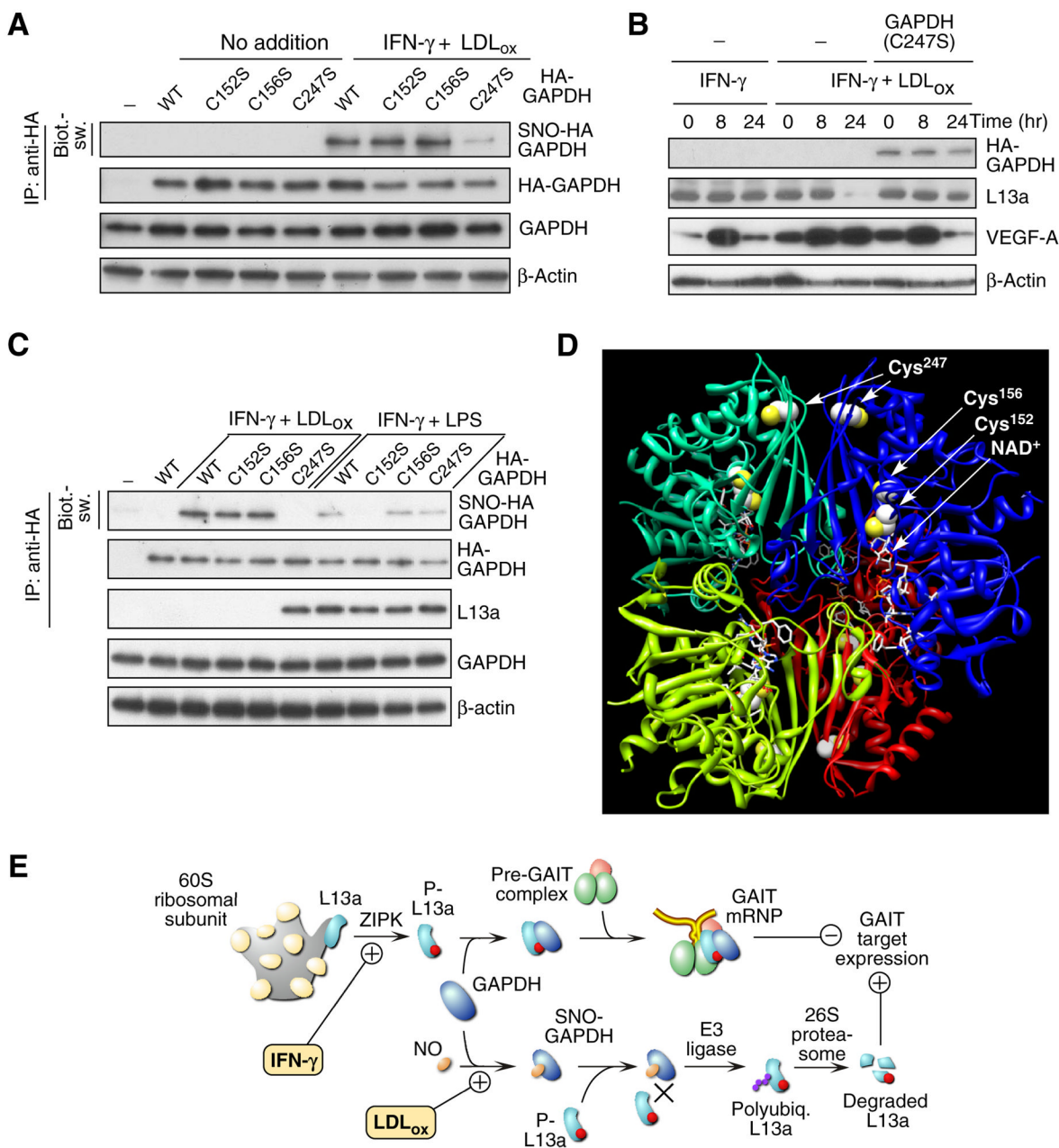
Figure 4. Effect of LDL$_{ox}$ on GAIT target gene expression and schematic

(A) LDL$_{ox}$ induces S-nitrosylation of GAPDH at Cys^{247}. Mouse BMDM were transfected with plasmids encoding wild-type HA-GAPDH, or C152S, C156S, or C247S mutants. Cells were treated with IFN-γ and LDL$_{ox}$ for 24 hr. S-nitrosylation of transfected GAPDH was detected in lysates by immunoprecipitation with anti-HA tag antibody followed by biotin-switch analysis. Total transfected HA-GAPDH was determined by immunoprecipitation with anti-HA tag antibody followed by immunoblot analysis with anti-GAPDH antibody. Total GAPDH and β-actin were determined by immunoblot analysis.

(B) Expression of GAPDH C247S mutant restores translational silencing of VEGF-A. Cells were transfected with plasmid encoding HA-tagged C247S mutant GADPH and treated with IFN-γ or IFN-γ plus LDL$_{ox}$ for up to 24 hr. Expression of HA-GAPDH, L13a, VEGF-A, and β-actin was detected by immunoblot analysis.

(C) LDL$_{ox}$ and LPS Induce GAPDH S-nitrosylation at different sites. Human PBM were transfected with plasmids encoding wild-type HA-GAPDH, or C152S, C156S, or C247S mutants. *S*-nitrosylation of transfected GAPDH in presence of IFN-γ plus LDL$_{ox}$ or IFN-γ plus LPS (10 μg/ml) was detected by biotin-switch. Transfected HA-GAPDH and GAPDH-bound L13a were determined by immunoprecipitation with anti-HA tag antibody followed by immunoblot analysis with anti-GAPDH and anti-L13a antibodies. Total GAPDH and β-actin were determined by immunoblot analysis.

(D) Three-dimensional structure of GAPDH. Ribbon diagram of tetrameric GAPDH structure with individual subunits shown in different colors. Cys residues are shown in spacefill with sulfur atoms highlighted (yellow). NAD$^+$ in active site is shown (stick model).

(E) Schematic of GAIT pathway activation and its disruption by LDL$_{ox}$. IFN-γ induces GAIT complex assembly that causes translational repression of inflammation-related, GAIT target genes (upper pathway). LDL$_{ox}$ induces GAPDH S-nitrosylation and consequent L13a degradation, causing GAIT pathway dysregulation and prolonging GAIT target gene expression (lower pathway).

See also Figure S1.