Promotion of food allergy by the unfolded protein response

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Abbreviations: 4-PBA, 4-phenylbutyric acid; α-gal, α-galactose; CC, collaborative cross; EA, egg allergy; e.c., epicutaneous; EW, egg white; EYP, egg yolk plasma; FA, food allergy; HC, heavy cream; HIO, human intestinal organoids; i.p., intraperitoneal; i.t., intratracheal; mAb, monoclonal antibody; MCT, medium chain triglycerides; NAC, N-

acetyl cysteine; o.g., oral gavage; PE, aqueous peanut extract; siRNA, small inhibitory ribonucleic acid; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response

Key Words: Cytokine, Egg, IgE, ER stress, Mast Cell, Mouse, Peanut, Saturated Fat

Abstract: Food allergy is common and increasing. We show that saturated fats, an important part of Western diet, promote food allergy by stimulating an epithelial unfolded protein response (UPR). This response activates a transcription factor, XBP1s, which is required for saturated fat induction of the allergy-promoting cytokines: IL-25, IL-33 and TSLP. XBP1s induction by saturated fat-rich egg, an important food allergen, is genetically controlled in vivo and strongly correlates in different mouse strains with egg allergy susceptibility. Consistent with this, the UPR-inhibiting drug metformin suppresses murine egg allergy and its use correlates with a protective effect in a human food allergy.

Introduction: Food allergy (FA) is a common problem in the US and other developed countries, with a prevalence of approximately 4% in adults and 8% in children[1-4] that has increased considerably in recent years[4, 5]. This disorder does not develop randomly to all foods; bovine milk, hen's eggs (henceforth referred to as milk and egg, respectively), peanuts, tree nuts, fish, crustaceans, sesame, wheat, and soy are responsible for most food allergy in North America and Europe[6-12]. Because ingestion of most potential antigens leads to tolerance rather than allergic immunity[13, 14], the observation that FA develops to a restricted set of foods suggests that these foods may have components with adjuvant properties that promote an allergic response, in addition to proteins and carbohydrates that have potent B and T cell epitopes.

Recently, we and others demonstrated a requirement for the pro-Th2 cytokines, IL-25, IL-33, and TSLP for the induction and maintenance of FA in a murine model[15-17]. To the extent that this is generalizable, it raises the possibility that putative adjuvants in allergenic foods promote allergy by stimulating gut pro-Th2 cytokine expression. In this regard, we also reported that saturated medium chain triglycerides (MCT), which promote the development of allergy to co-ingested ovalbumin and peanut extract (PE), stimulate increased IL-25, IL-33 and TSLP gene expression by intestinal epithelium[18]. More recent follow up experiments demonstrated that ingestion of saturated, but not unsaturated long chain triglycerides also promotes the development of FA and an IgE response (unpublished data).

These data suggested a possible mechanism for allergy pathogenesis, inasmuch as saturated, but not unsaturated long chain fatty acids cause cellular stress in hepatocytes, pancreatic islet cells, and some additional cell types that induces the unfolded protein

response (UPR)[19, 20]. The UPR relieves cellular stress through multiple mechanisms, including a general decrease in protein synthesis that is accompanied by increased synthesis of a select set of proteins that can counteract the stressors[21-23]. Classical UPR effects are initiated by transfer of the chaperonin BiP from 3 sensor molecules, PERK, IRE1α, and ATF6, to unfolded or misfolded proteins; this activates these sensor molecules[24, 25]. Activated PERK acts through eIF2α to induce the transcription factor ATF4. IRE1α autophosphorylates to acquire tyrosine kinase and RNA endonuclease activity that activate the NF-κB and JNK signaling pathways and modify XBP1 to generate its transcriptionally active metabolite, XBP1s. ATF6, when dissociated from BiP, is also processed to become an active transcription factor[25-28]. Because the pro-Th2 cytokines and the Th2 cytokines that they induce promote epithelial cell survival and recovery from stress[29, 30], we additionally hypothesized that one consequence of the intestinal epithelial UPR might be increased pro-Th2 cytokine expression. Because important food allergens, including milk and eggs, contain large amounts of saturated fat, we further hypothesized that these foods might, like purified saturated fats, induce an intestinal epithelial UPR, which could then promote food allergy by stimulating pro-Th2 cytokine expression. The data presented here support these hypotheses, show the importance of the IRE-1/XBP1 limb of the UPR in promoting a pro-Th2 cytokine response, demonstrate that peanuts and tree nuts also induce an epithelial UPR-dependent pro-Th2 cytokine response, reveal that agents that suppress the UPR can prevent murine FA and suppress established murine FA, and suggest that one of these agents also suppresses a form of human FA.

Methods:

Mice. BALB/c and C57BL/6 mice were bred in the Cincinnati Children's Hospital Medical Center (CCHMC) animal facility. ERN1$^{fl/fl}$ and XBP1$^{fl/fl}$ mice were a gift from Laurie Glimcher (Harvard University, Cambridge, MA) and were bred in our facility. They were both crossed to Vil-Cre mice, a gift from Simon Hogan (University of Michigan, Ann Arbor, MI). Collaborative cross (CC) mice (16 strains-CC004/TauUnc, CC005/TauUnc, CC006/TauUnc, CC011/Unc, CC012/GeniUnc, CC015/Unc, CC016/GeniUnc, CC019/TauUnc, CC024/GeniUnc, CC027/GeniUnc, CC037/TauUnc, CC038/GeniUnc, CC041/TauUnc, CC057/Unc, CC059/TauUnc, and CC060/Unc) were purchased from the University of North Carolina (UNC) Systems Genetics Core[31]. Mice of both sexes were used at age 8-12 wks. Cages of mice were randomly assigned to groups (without a specific randomization protocol), with the exception that groups were age- and sex-matched. Researchers were not blinded as to group. Animal work was approved by the CCHMC Institutional Animal Care and Use Committee.

Reagents. Sodium palmitate (CAS: 408-35-5), myristate (CAS: 544-63-8), palmitoleic acid (CAS: 373-49-9), oleate (CAS: 112-80-1), linoleate (CAS: 60-33-3), linolenate (CAS: 463-40-1), cholera toxin (C8052), papain (76216), N-acetyl-L-cysteine (NAC, CAS: 616-91-1), fatty-acid and low-endotoxin free bovine serum albumin (BSA, A8806) and *Salmonella enterica* lipopolysaccharide (LPS, L7770) were purchased from Millipore Sigma, USA. Etomoxir (11969), thioridazine (14400), orlistat (CAS: 96829-58-2), sodium 4-phenylbutyrate (4-PBA, 11323) and tauroursodeoxycholic acid (TUDCA, 20277) were purchased from Cayman chemicals, USA. KIRA6 (532281), STF083010 (412510), GSK2656157 (504651), salubrinal (324895), 4-(2-Aminoethyl)

benzenesulfonyl fluoride hydrochloride (AEBSF, 532586), BMS345541(401480), thapsigargin (InSolution™, 586006) and tunicamycin (InSolution™, 504570) were purchased from Calbiochem, USA. Medium chain triglycerides (MCT oil, Lot-32595117A, Nestle Health Science, Switzerland) was purchased from a local pharmacy. Metformin hydrochloride (Cat. no. 2864) was purchased from Tocris Bio-Techne Co, USA. Heavy cream (whole cream) and large hen's eggs were purchased at a local supermarket.

Fatty acid-BSA conjugate preparation. Saturated and unsaturated fatty acids are conjugated with fatty-acid and low-endotoxin free BSA as previously described[r] (Seahorse Bio Science, USA) with minor modifications. The molar ratio was maintained at 6:1 for all fatty acids tested. Stock solutions were stored at -80° C; freshly prepared solutions were used in experiments. Complexed fatty acids were added to serum-containing cell culture medium to achieve the required fatty acid concentration prior to the experiment. The pH of the medium did not differ significantly with the addition of a complexed fatty acid.

Allergen extracts. Supermarket-purchased unshelled roasted peanuts, soybeans, sesame seeds, shrimp, wheat flour and walnuts were individually shelled and ground, when necessary, with a commercial blender (Scovill Manufacturing Company, Avine, WI) while adding 2 parts 0.1 M ammonium bicarbonate (pH 9.0) to 1 part of the pulverized food. Suspensions were stirred for 4-12 hrs at room temperature and centrifuged twice at room temperature for 20 min at 9,000 - 10,000 RPM after briefly warming to 37° C. Supernatants were collected and dialyzed overnight against 0.9% NaCl. Dry ammonium sulfate was added while stirring to produce a 10% saturated solution. After centrifugation

at room temperature for 15 min at 10,000 RPM, supernatants were collected and ammonium sulfate added with stirring to produce an 80% saturated solution. The pellet produced by centrifugation at room temperature for 10 min at 10,000 RPM was collected and dissolved in 0.9% NaCl, then dialyzed overnight against 0.9% NaCl and centrifuged at room temperature for 30 min at 10,000 RPM. The food extract-containing supernatant was collected, sterile-filtered at 0.22 μm and stored at -20° C until use. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, Ill), according to the manufacturer's protocol

Cell cultures. Human colorectal adenocarcinoma cells (CACO-2, HTB-37) were purchased from ATCC (American Type Culture Collection, Manassas, USA) and maintained in Eagle's minimum essential medium (EMEM, Cat No-30-2003, ATCC) in the presence of 10% FBS and antibiotics. Human primary small intestinal epithelial cells (Cat: H-6051, Cell Biologics Inc, Chicago, USA) and human primary dermal epithelial cells (Cat: H-6066, Cell Biologics Inc, Chicago, USA) were maintained in complete human epithelial cell medium /w Kit (Cat: H-6621, Cell Biologics Inc, Chicago, USA). Cells (10^6) were plated 24-36 hrs before experiments and grew to an attached cell-monolayer with 80-90% confluence. Prior to the experiment, dead cells and debris were removed and fresh medium ± reagents was added, according to the experimental protocol. Cells were incubated with reagents for 6 or 24 hrs at 37° C and 5% CO_2, according to the experimental protocol and processed for RNA extraction, followed by quantitative-PCR.

Human intestinal organoids. Human intestinal organoids (HIOs) were generated by the CCHMC pluripotent stem cell facility (PSCF) using human embryonic stem cell line H1

(WA01; NIH Registration Number 0043) as previously described[32]. A cryopreserved batch of H1 cells, generated by the PSCF, was used as the source of cells for this study. The batch was authenticated by STR analysis (Genetica), karyotype analysis (CCHMC Cytogenetics core), and functional pluripotency (Scorecard assay; ThermoFisher). The passage number of H1 cells used for HIO formation was between p42 and p55. For HIO generation, H1 cells were plated in Matrigel-coated 24 well cell culture plates (Nunc) in mTeSR1 (StemCell Technologies). After 48 hrs, differentiation to definitive endoderm was initiated by changing to media containing 100 ng/mL activin A (Cell Guidance Systems) for 3 d with daily media changes. Definitive endoderm was then patterned to mid/hindgut endoderm using media containing 500 ng/ml of FGF4 (R&D Systems) and 3 μM CHIR99021 (StemCell Technologies) for 4 d with daily medium changes. At the mid/hindgut endoderm stage, floating spheroids were collected, embedded in Matrigel (Corning; 354234), and cultured in media containing 500 ng/ml of R-spondin1 (R&D Systems), 100 ng/ml of noggin (R&D Systems) and 100 ng/ml of EGF (R&D Systems), with medium changes every 3-4 d. Developing organoids were split to fresh Matrigel 14 days post-embedding and used for injection of palmitate, metformin, and TUDCA after 28 total days in Matrigel culture. Organoids were microinjected with a Nanoject II Auto Nanoliter Injector (Drammond Scientific Company, Broomall, PA, USA) using Drammond glass capillaries (Fisher, cat. 21-171-4). Capillaries were pulled on a horizontal bed puller (Sutter Instruments, Novato, CA, USA). Approximately 200 nl of fluid was injected per organoid; 12 organoids per group. Rhodamine-Dextran (Thermofisher, cat. D1824), 0.625mg/ml was added to the injected fluid to monitor leakage. Organoids were cultured for 24 hours after injection, after which they were

processed for RNA with TRIzol (Thermofisher, cat. 15596026) followed by RNeasy Plus Micro Kit Qiagen cat. 74034) according to manufacturer's instructions.

Gene silencing using siRNA. Small interfering RNA (siRNA) against human PERK (s18102), CHOP (s3996), ERN1 (s200432) and XBP1 (s14913) were purchased (Silencer® Select siRNA, ThermoFisher Scientific, USA) and transfected into cultured CACO-2 cells as per the manufacturer's protocol using Lipofectamine® RNAiMAX reagent (ThermoFisher Scientific, USA). Silencer® Select Negative Control No. 1 siRNA (4390843) was used as a negative control and human GAPDH (s5573) siRNA was used as a positive control. Efficiency of gene silencing relative to the HPRT gene was determined by RT-PCR.

RNA extraction and Quantitative (RT)-PCR. Cultured cells, organoids and mouse tissues were processed for total RNA extraction with a RNA Mini Kit (Cat: 12183020, Pure Link™, ThermoFisher, USA), using the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA per sample, using a High-Capacity cDNA Reverse Transcription Kit (Cat- 4368814, Applied BioSystems™, USA) and diluted 2x with PCR grade-H_2O. Bioinformatically validated QuantiTect PCR primer sets (Quagen, USA) were obtained for the human genes GAPDH (QT00273322), HPRT (QT00059066), PERK (EIF2AK3, QT00066003), BiP (HSPA5/HSP70, QT00096404), CHOP (DDIT3, QT00082278), ATF4 (QT00074466), ATF6 (QT00083370), IRE1 (ERN1, QT00025760), XBP1 (QT00068383), TSLP (QT00051464), IL-25(QT00211029), IL-33 (QT00041559), CXCL-1 (QT00199752), CXCL-8 (QT00000322), TNFAIP3 (QT00041853), NFkB1A (QT00063791), CCL2 (QT00212730), and for the mouse genes Gapdh (QT00199388), Perk (Eif2AK3,

QT00147329), Bip (Hspa5, QT00172361), Chop (Ddit3, QT01077769), Atf4 (QT00096033), Atf6 (QT00317961), Ire1(Ern1, QT00171787), Xbp1 (QT00109361), Tslp (QT00198261), Il-25 (QT00134645) and Il-33 (QT00135170). Quantitative (RT)-PCR was performed using 2 µl of synthesized cDNA, using PowerUp™ SYBR™ Green Master Mix (Cat: A25741, Applied BioSystems), following the manufacturer's protocol with a CFX96 (Bio-Rad, USA) qPCR system. For human XBP1s (forward 5'TGCTGAGTCCGCAGCAGGTG-3' and reverse 5'-GCTGGCAGGCTCTGGGGAAG-3') and mouse- Xbp1s (forward 5'-AAGAACACGCTTGGGAATGG-3', and reverse, 5'-CTGCACCTGCTGCGGAC-3'), primers were obtained from Genscript, USA.

Antibodies for in vivo experiments. Anti–IL-33 receptor (IL-33R, long form of ST2) mAb and anti–IL-25 mAb (clone 2C3, originally produced by Andrew McKenzie, Cambridge, United Kingdom) were obtained from Janssen Pharmaceuticals (Raritan, NJ)., anti-TSLP mAb (clone 28F12), was a gift of Dr Andrew Farr, University of Washington.

Western blot and Immunoprecipitation. CACO-2 cells (unstimulated or stimulated with sodium palmitate-BSA conjugate or with egg yolk plasma (EYP) for 24 hrs) were lysed with RIPA buffer. Lysates were normalized by protein concentration and validated by Western bloting against α/β tubulin protein. Lysates were incubated/ precipitated with anti-TSLP, anti-IL-25 or anti-IL-33 Abs for 1 hr at 4° C and incubated with protein G beads (Pierce Protein G Agarose (Thermofisher scientific, cat. 20399)) for another 2 hrs at 4° C, followed by centrifugation at 4° C. Beads were then boiled in complete Laemmle buffer and separated by electrophoresis on 4-20% PAAG gels, then transferred to PVDF membranes. Membranes were incubated with biotinylated anti-IL-25, anti-IL-33, or anti-

TSLP mAbs overnight at 4°C. All membranes were incubated 45 min with streptavidin-peroxidase at room temperature and visualized with ECL WB substrate. All antibodies (α/β tubulin, anti-IL33, anti-IL-33-biotin, anti-IL-25, anti-IL-25 biotin; anti-TSLP, anti-TSLP-biotin) were purchased from R&D Systems, USA Antibodies used for western blot: anti-IL-33 Abs (Nessy-1) (Enzo Life Sciences, Farmingdale, NY, cat. ALX-804-840; R&D Systems, BAF3625); anti-TSLP Abs (Thermofisher scientific, cat. 88-7497-22; R&D Systems cat. MAB1398); ani-IL-25 Abs (R&D Systems, AF1258; BAF1399), anti-alpha/beta tubulin Abs (Thermofisher scientific, cat MA1-19400). Immunopure streptavidin HRP conjugate (Thermofisher scientific, cat. 21126), Pierce ECL Western Blotting Substrate, (Thermofisher scientific, cat. 32106),

Preparation of egg antigen: Egg white (EW) and egg yolk from 12 fresh chicken eggs were separately pooled. The EW was dialyzed against deionized water and centrifuged to remove precipitate, then analyzed for protein concentration using a Pierce BCA Protein Assay Kit and pressure concentrated with a diaflo membrane with a 30 kDa cut-off to a concentration of 333 mg/ml. Egg yolk was diluted with an equal volume of 0.15M NaCl and centrifuged to remove egg yolk granules. The supernatant, egg yolk plasma (EYP) was added back to the dialyzed EW solution prior to pressure concentration to create a mixture that contained EYP from 3 eggs and EW from 12 eggs and had a final concentration of 333 mg of EW protein/ml.

Food allergen inoculation. For 24 hr experiments, mice were anesthetized with ketamine plus xylazine (Anased, Santa Cruz Animal Health, USA), then shaved on their backs with sterile electric clippers, 24 hr prior to sensitization. The next day, mice were fasted and epicutaneously (e.c.) sensitized with 100 µl of the food allergen adsorbed to sterile gauze,

placed on the shaved area, overlaid with Tegaderm™ (3M Science, USA), and secured with a cloth bandaid and waterproof tape. Mice were simultaneously sensitized intra-tracheally (i.t.) with 50 µg of the same food allergen in a volume of 40 µl. After 4 hrs of fasting, mice were inoculated by oral gavage (o.g.) with 10-100 mg of the food allergen and allowed 1hr later to eat normally. Tissues were collected from the sensitized mice 24 hrs later and processed for determination of gene expression. To induce food allergy (FA), mice were sensitized e.c. for 3 weeks using skin patching as described above. Sensitized mice were then inoculated o.g. 2x/week with 100 mg of EW/EYP after fasting for 4 hours until they reproducibly developed hypothermia and diarrhea by 1 hr after o.g. inoculation. Mice were then separated into groups and treated according to the individual experimental protocol. At the end of some experiments, mice were bled for serum and tissues were processed for RNA analysis. In a second model, EW prepared as above was adsorbed to Imject Alum (40 mg alum/ml) (Thermo Scientific, Rockford, IL) at a ratio of 0.5 mg EW to 10 mg alum by mixing at room temperature for 30 min. Mice were injected i.p. with 100 µl of this mixture and received a second i.p. injection 1 week later, with o.g. inoculations starting 1 week after that.

Administration of UPR antagonists. For in vitro experiments, different reagent stock solutions were prepared in DMSO or deionized H_2O, then diluted with culture medium and used immediately. Medium ± BSA or DMSO were used as negative controls. For in vivo experiments, metformin or TUDCA was provided in drinking water, with concentrations based on a daily estimated water intake of 5ml/day/mouse.

Measurement of MMCP-1 levels in mouse serum. MMCP-1 levels were measured with an ELISA kit (eBioscience, CA, USA) for mouse sera obtained 4 hours after antigen challenge, following the manufacturer's protocol.

Statistics. Student's-t test and the Wilcoxon rank-sum test were used, as appropriate, to determine statistical significance for comparisons of 2 groups. A 1-way ANOVA, 2-way ANOVA, Dunnet's test, Tukey multiple comparison test or Kruskal-Wallis test was used, as appropriate, to determine statistical significance for comparisons of >2 groups. For multivariate linear analysis, a least squares test was used, followed by multiple-ANOVA (MANOVA). The performance of multivariate linear analysis was cross-validated using K-fold cross-validation testing with a k value of 10 (10-fold), to measure average prediction error. A 2-tailed Fisher's exact test and Baptista-Pike test were used to assess the significance of differences between expected and observed results and to calculate odds ratios and 95% confidence intervals. Statistical analyses were performed using GraphPad Prism v 8.4 (GraphPad Software, USA) and/or for correlation and regression analysis, R Studio (Integrated Development for R., RStudio, Inc., USA). A p value of <0.05 was considered significant.

Results:

Maintenance of established murine egg allergy (EA) depends on pro-Th2 cytokines. Our previous report that established murine EA can be more completely and rapidly suppressed by a combination of mAbs to IL-25, TSLP and IL-33R than by any two of these mAbs was based on a model in which EA was induced and maintained by ingestion of a combination of egg white and MCT[15]. To determine whether more clinically relevant models of murine EA similarly depend on the three pro-Th2 cytokines, we induced EA by inoculating BALB/c mice i.t. or e.c. with egg white (EW) and egg yolk plasma (EYP, the liquid, fat-containing fraction of egg yolk), then inoculated them by oral gavage (o.g.) with EW + EYP until they developed hypothermia (a sign of shock[33, 34]) during the 30 minutes following o.g.. Mice were then treated for 21 days with anti-TSLP mAb, anti-TSLP plus anti-IL-33R (the most effective two mAb combination for suppressing the EW plus MCT model of EA[15]) or with anti-TSLP, anti-IL-33R and anti-IL-25 mAbs and evaluated for hypothermia induction. Hypothermia was partially suppressed in both models by anti-TSLP mAb or by the two-mAb combination and nearly completely suppressed by the combination of all three mAbs (Supplemental Fig. 1). Thus, disease maintenance is pro-Th2 cytokine-dependent in all three models of EA studied, all of which are characterized by sensitization in the absence of non-food adjuvants.

Saturated, but not unsaturated fats induce UPR-associated and pro-Th2 cytokine expression in transformed human intestinal epithelial cells. To initiate investigation of whether there is a connection between the UPR and the pro-Th2 cytokines that are essential for murine EA induction and maintenance, we cultured CACO-2 transformed

human large intestine epithelial cells with saturated or unsaturated long chain fatty acids and evaluated both UPR-associated and pro-Th2 cytokine gene expression 6 and 24 hours later. Results of this experiment confirmed the ability of saturated, but not unsaturated fatty acids to induce UPR-associated gene expression and also demonstrated that only the saturated fatty acids induced TSLP and IL-25 gene expression. In contrast, all of the saturated fatty acids and some of the unsaturated fatty acids induced IL-33 expression (Fig. 1A). Medium chain triglycerides and the lipid-containing fractions of two important causes of FA, milk (heavy cream) and eggs (EYP), also induced both UPR-associated and pro-Th2 cytokine gene expression by CACO-2 cells (Fig. 1B) and both EYP and palmitate increased pro-Th2 cytokine protein expression (Fig. 1C). The longer time required for EYP or heavy cream, as compared to palmitic acid, to induce pro-Th2 cytokine gene expression (compare Fig. 1A and 1B) suggested that the triglycerides in the allergenic foods might have to be hydrolyzed to glycerol and fatty acids to have a stimulatory effect; this would be consistent with the requirement that such hydrolysis is required for fat absorption by epithelial cells[35, 36]. Indeed, the lipase inhibitor, orlistat[37], blocked the abilities of heavy cream and EYP (but not palmitate) to induce UPR-associated and pro-Th2 cytokine gene expression (Fig. 1D and Supplemental Fig. 2). Thus, the triglyceride content of milk and EYP is required for their induction of the UPR and pro-Th2 cytokine response.

Because saturated and unsaturated fats and fatty acids differ in ability to induce the UPR and are catabolized differently in peroxisomes and mitochondria[38, 39], we evaluated whether inhibitors of peroxisomal and mitochondrial β-oxidation of saturated fatty acids (etomoxir and thioridazine, respectively), inhibit EYP, MCT, and palmitate

induction of UPR-associated and pro-Th2 cytokine genes in cultured CACO-2 cells. The ability of both of these agents to substantially inhibit the induction of all of the UPR-associated genes studied, as well as the three pro-Th2 cytokine genes (Fig. 1E and data not shown) is consistent with involvement of oxidative catabolism of EYP saturated fatty acids in UPR induction.

UPR inhibitors suppress human epithelial cell pro-Th2 cytokine expression. To more directly evaluate whether the UPR induces pro-Th2 gene expression in CACO-2 cells, we evaluated the abilities of three upstream UPR inhibitors to inhibit the ability of saturated fats and fatty acids to induce pro-Th2 cytokine expression by CACO-2 cells: metformin (thought to suppress the UPR primarily by increasing AMP kinase expression[40]); and 4-phenyl butyric acid (4-PBA) and tauroursodeoxycholic acid (TUDCA) (which suppress the UPR by acting as chaperonins for unfolded/misfolded proteins[41, 42]). Each of these UPR inhibitors strongly suppressed the pro-Th2 cytokine response by each of these lipid stimuli (Fig. 2A). Metformin also suppressed UPR and pro-Th2 cytokine gene expression responses in primary human intestinal and dermal epithelial cells that had been cultured with palmitate or EYP (Fig. 2B and Supplemental Fig. 3). To evaluate the ability of metformin and TUDCA to suppress pro-Th2 cytokine gene expression in non-transformed human cells were organized to resemble intestine, we generated hollow human intestinal organoids, which have an epithelial cell lining and a mesenchymal cell exterior[43], injected the lumens of these organoids with palmitate to induce increased UPR-associated and pro-Th2 cytokine gene expression, and further injected the organoid lumens with metformin or TUDCA. Palmitate strongly induced

both UPR-associated and pro-Th2 cytokine gene expression by the organoids and this expression was strongly suppressed by both metformin and TUDCA (Fig. 2C).

Because metformin has multiple effects, in addition to activating an enzyme, AMPK, which is know to suppress the UPR, we used CACO-2 cells to evaluate the XBP1s and pro-Th2 cytokine suppressive effects of AICAR, an agent that is a somewhat more specific AMPK antagonist than metformin[44]. Much like metformin, AICAR dose-dependently suppressed all of these responses (Fig. 2D).

To determine which of the three UPR pathways promote the pro-Th2 cytokine response, we evaluated the ability of five small molecule inhibitors to suppress palmitate- and EYP-induced pro-Th2 cytokine expression in cultured CACO-2 cells. Expression of these cytokines was strongly suppressed by KIRA6 (an IRE1 kinase inhibitor), by STF083010 (an IRE1 endonuclease inhibitor), and by AEFSF (an inhibitor of ATF6 activation), but not by GSK2656157 (an inhibitor of PERK activation) or by salubrinal (an inhibitor of eIF2a activation), even though all of these inhibitors had the expected effects on UPR-associated gene expression (Fig. 2E and Supplemental Fig. 4). These observations are most simply explained by an important role for XBP1s in the induction of pro-Th2 cytokine expression, because ATF6 strongly increases XBP1 transcription[45, 46] and IRE1 kinase activity greatly increases the IRE1 endonuclease activity needed to convert XBP1 to XBP1s[47]. To confirm an important role for the IRE1/XBP1 pathway in UPR induction of pro-Th2 cytokine responses, we evaluated the abilities of ERN1, XBP1 and PERK siRNA to suppress palmitate-induced CACO-2 cell XBP-1s, TSLP, and IL-25, and IL-33 responses. Results of this experiment were totally consistent with our results with the small molecule inhibitors: IL-25, IL-33, and TSLP responses were considerably

and significantly suppressed by ERN1, and XBP1s siRNA, while PERK siRNA failed to significantly inhibit these responses (Fig. 2F). These results were validated by the suppression of UPR-associated gene responses by these siRNAs, which completely matched expectations (Supplemental Figs. 5 and 6).

Because IRE-1α induces NF-κB[48] and NF-κB binds to TSLP and IL-33 gene promoters[49-51], we predicted that palmitate, EYP, and other UPR inducers would stimulate NF-κB gene expression and that the small molecule IKK antagonist BMS345541[52] would suppress palmitate- and EYP-induced pro-Th2 cytokine expression. These predictions were confirmed in an in vitro study with CACO-2 cells (Fig. 2G and Supplemental Fig. 7).

Additional allergens and a potent Th2 adjuvant induce a metformin-suppressible UPR and pro-Th2 cytokine response in epithelial cells. Because the UPR can be induced by multiple cell stress-associated mechanisms that are not associated with lipids[53], we investigated the ability of aqueous extracts of allergenic foods other than eggs and milk to induce UPR-associated and pro-Th2 cytokine gene expression and the ability of metformin to suppress these responses. A lipid-depleted, aqueous peanut extract (PE) strongly induced UPR-associated and pro-Th2 cytokine gene expression that, like EYP-induced expression, was metformin-suppressible (Figs. 3A). However, unlike induction of these responses by EYP, their induction by PE was lipase-independent (Fig. 3B). Aqueous walnut and codfish extracts also substantially induced expression of both UPR-associated and pro-Th2 cytokine genes, while an aqueous shrimp extract increased pro-Th2 cytokine genes considerably more than it induced UPR-associated genes. Aqueous wheat, sesame, and soybean extracts failed to substantially increase either UPR-

associated or pro-Th2 cytokine gene expression by CACO-2 cells (Fig. 3C and Supplemental Fig. 8). While 10 mM metformin strongly suppressed induction of UPR-associated gene expression by both walnut and fish extracts and suppressed walnut extract-induced pro-Th2 cytokine gene expression, it was less effective at suppressing induction of pro-Th2 cytokine gene expression by shrimp extract and no significant effect on pro-Th2 cytokine gene induction by fish extract (Fig. 3D and Supplemental Fig. 9).

To determine if our observations with potent food allergens might extend to an adjuvant that potently promotes a Th2/food allergy response and/or a potent allergen whose allergenicity is associated with its cysteine protease activity, we cultured CACO-2 cells with cholera toxin or papain, respectively, in the presence or absence of metformin. Results of this experiment demonstrate that cholera toxin and papain both induce strong UPR and pro-Th2 responses that are significantly inhibited by metformin (Fig. 3E).

Induction of the UPR and a pro-Th2 cytokine response by such diverse stimuli as saturated fatty acids, a toxin, a protease, and delipidated PE suggested that these stimuli induce the UPR through different mechanisms. To test this hypothesis for palmitate, EYP, and PE, we compared the ability of inhibitors of mitochondrial and peroxisomal β-oxidation to suppress the UPR-associated and pro-Th2 cytokine gene responses to these stimuli, with the thought that β-oxidation inhibitors would have a stronger suppressive effect on UPR induction by saturated fatty acids than on a mixture of proteins and complex carbohydrates. This hypothesis was confirmed in an in vitro study with CACO-2 cells (Fig. 1E). However, oxidative stress does appears to have a role in UPR and pro-Th2 cytokine induction by peanut as well as saturated fats, because the anti-oxidant N-acetylcysteine (NAC) suppresses CACO-2 cell UPR and pro-Th2 cytokine responses

induced by peanut extract as well as those induced by palmitate and EYP (Fig. 1F and 1G).

The UPR is not sufficient to stimulate a pro-Th2 cytokine response. These observations suggested that UPR induction by several foods contributes to their allergenicity by promoting an epithelial cell pro-Th2 cytokine response and that some UPR-associated molecules, such as the transcription factor XBP1s, are essential for pro-Th2 cytokine induction by these allergens. These observations, however, did not reveal whether the UPR is a sufficient stimulus for epithelial cell pro-Th2 cytokine induction. To approach this issue, we evaluated the ability of two additional strong UPR inducers to induce a pro-Th2 response by CACO-2 cells: thapsigargin (which induces the UPR by depleting endoplasmic reticulum Ca^{+2} through inhibition of the sarco/endoplasmic reticulum Ca^{+2} ATPase[54]) and tunicamycin (which induces the UPR by inhibiting the N-linked protein glycosylation required for normal protein folding and transit through the endoplasmic reticulum[55]). Although thapsigargin and tunicamycin were as effective as palmitate at inducing CACO-2 cell expression of classic UPR-associated molecules and their induction of these molecules was suppressed by metformin, they were much less effective than palmitate at inducing pro-Th2 cytokine gene expression (Supplemental Fig. 10A). Similar results were observed over a wide dose range (not shown). Failure of thapsigargin and tunicamycin to induce pro-Th2 cytokine genes did not result from their suppression of these genes, because thapsigargin and tunicamycin concentrations that were sufficient to induce the UPR did not suppress pro-Th2 cytokine gene induction by palmitate (Supplemental Fig. 10B). Similar to thapsigargin and tunicamycin, bacterial lipopolysaccharide induced CACO-2 cell expression of UPR-associated, but not pro-Th2

cytokine genes (Supplemental Fig. 11). Thus, although the UPR is required for induction of a pro-Th2 cytokine response by several important allergenic foods, additional, as yet unidentified stimuli, appear to also be required.

Egg induces UPR-dependent pro-Th2 cytokine gene expression in epithelial organs. To determine whether egg is as capable of inducing UPR-associated/pro-Th2 cytokine gene expression in vivo as it is in vitro, we patched shaved skin of BALB/c mice with EW + EYP and inoculated these mice i.t. and by o.g. with EW + EYP or saline (negative control) and 24 hours later evaluated skin, airway, and small intestine (proximal jejunum), respectively, for changes in UPR-associated and pro-Th2 cytokine gene expression. In the same experiments, we evaluated the ability of metformin to suppress the expression of the egg-induced genes. The results of these studies (Fig. 4A and Supplemental Figure 12) demonstrate metformin-suppressible egg induction of both UPR-associated and pro-Th2 cytokine genes in each of these organs. Because our in vitro small molecule inhibitor studies suggested an important role for the UPR-associated transcription factor XBP1s in EYP and palmitate induction of the pro-Th2 cytokines, we also evaluated whether selectively deleting the XBP1 or ERN1 gene from intestinal epithelium would block egg induction of an intestinal pro-Th2 cytokine response. XBP1 deletion caused the expected loss of XBP1 and XBP1s expression and also suppressed egg-induced intestinal IL-25, IL-33, and TSLP expression, without significantly suppressing induction of any other evaluated UPR-associated genes (Fig. 4B). Intestinal epithelial deletion of ERN1 had similar effects, except that it blocked ERN1, but not XBP1 expression (Fig. 4C). As expected, selective deletion of XBP1 or ERN1 from small

intestine epithelium had no effect on egg-induced skin or lung UPR or pro-Th2 cytokine gene expression (Supplemental Fig. 13).

Egg-induced 24 hour UPR-associated and pro-Th2 cytokine responses to EYP are genetically determined and predictive of food allergy development. Our observations that oral egg inoculation induces a UPR-dependent pro-Th2 cytokine response in BALB/c mice and that the pro-Th2 cytokines are essential to induce and maintain murine egg allergy suggested that EA should be induced by appropriate exposure to egg. This hypothesis, however, may seem contrary to the failure of the great majority of people who eat egg to develop this disorder. One possible explanation is that susceptibility is genetically determined. To investigate this possibility, we evaluated egg's ability to induce UPR and pro-Th2 gene expression as well as clinical allergy by inoculating one set of BALB/c, C57BL/6, and 16 inbred recombinant collaborative cross (CC) mouse strains[56] o.g., e.c., and i.t. with egg to induce a UPR-associated and pro-Th2 response in the inoculated organs 24 hours later, as well as the ability of e.c., followed by o.g. egg inoculation to induce hypothermia and diarrhea in a second set of the same mouse strains. The results of this study show great variation in the ability of egg inoculation to induce UPR and pro-Th2 gene responses as well as hypothermia and diarrheal responses in the different mouse strains (Fig. 5) Most importantly, we found a remarkable correlation between the ability of egg inoculation to induce a 24 hour IL-33/TSLP response in epithelial organs and the much later development of egg allergy (Fig. 5 and Supplemental Fig. 14, with the 24 hour jejunal TSLP response most predictive of later development of egg-induced shock (hypothermia). Analysis of the same day 1 data demonstrated strong correlations between development of the UPR-associated gene responses and the pro-Th2

cytokine responses, with (Supplemental Figs. 15-18). Although all pro-Th2 cytokines correlate positively with all UPR-associated molecules in all organs studied, TSLP generally correlates more strongly than IL-25 and IL-33, particularly in skin, and pro-Th2 cytokine correlations were less strong with CHOP, ATF4, and ATF6 and more strong (and always statistically significant) with ERN1, XBP1, and XBP1s, especially for TSLP (Supplemental Fig. 19).

Metformin suppresses egg allergy induction in mice. Our observations that: (1) pro-Th2 cytokines are required to induce EA; (2) 24 hour UPR and pro-Th2 cytokine responses correlate strongly with the later development of EA in skin-sensitized mice; and (3) egg induces metformin-suppressible pro-Th2 cytokine expression in epithelial interface organs, suggested that metformin might suppress the induction of EA in a skin sensitization model by blocking UPR-induced pro-Th2 cytokine production. Consistent with this hypothesis, adding metformin to drinking water while mice were being skin-sensitized to a mixture of EW + EYP, then challenged repeatedly by oral gavage with the same mixture, prevented hypothermia and diarrhea and decreased the mucosal mast cell response to oral EW + EYP challenge in egg-allergic mice (Fig. 6A).

The UPR inhibitors metformin and TUDCA can suppress established egg allergy. Because the pro-Th2 cytokines are required for the maintenance, in addition to the induction of food allergy[15], we hypothesized that UPR inhibition could have a therapeutic, in addition to a prophylactic effect in FA. To test this hypothesis, we sensitized mice e.c. with EW + EYP, then challenged them repeatedly o.g. with EW + EYP until they developed hypothermia and diarrhea. The mice were then treated with metformin (added to drinking water) while continuing bi-weekly o.g. with EW + EYP.

Mice that did not receive metformin continued to develop more severe disease while they received additional o.g. inoculations with EW + EYP; in contrast, EA was considerably ameliorated in mice that also received ~500 mg/kg/day or 1 gm/kg/day of metformin in their drinking water, with loss of diarrhea and substantially reduced hypothermia over a 4 to 5 week period (Fig. 6B). Even mice that had developed severe disease in response to 12 weeks of o.g. with EW + EYP improved considerably when treated with ~500 mg/kg of metformin, while it took 4-5 weeks for disease to worsen in metformin-treated mice when oral gavage with EW + EYP continued and metformin treatment was stopped (Fig. 6B). Treatment with TUDCA instead of metformin also suppressed established disease, although not as completely as metformin, even at a TUDCA dose of 2 gm/kg/day (Fig. 6C). An additional experiment with a similar protocol showed that metformin treatment of mice with established EA decreased mast cell (MMCP1) and intestinal UPR-associated and pro-Th2 cytokine gene expression, in addition to hypothermic and diarrheal responses to oral egg challenge (Fig. 6D).

Experiments were also performed to determine whether metformin treatment could suppress established EA when administered without egg, as would likely be the case if metformin were used to treat human EA. These experiments required switching to a model in which mice were initially sensitized i.p. with egg + alum, because mice that were initially skin-sensitized rapidly lost allergic responses when oral egg administration was stopped, even without metformin treatment. Using the egg plus alum sensitization model, egg allergic mice in which o.g. egg inoculation was stopped for 4 weeks after the 4^{th} o.g. and metformin treatment initiated when egg inoculation was stopped, developed less severe hypothermia when o.g. egg was resumed than mice that did not receive

metformin (Fig 6E). This was true even if metformin treatment were terminated when o.g. egg inoculation was resumed, although hypothermia, anaphylaxis-associated changes in mobility, and lethal anaphylaxis were further suppressed if metformin treatment were continued. Thus, metformin treatment can suppress established FA even if administered without the allergenic food.

Metformin appears to suppress α-gal syndrome (AGS). α-gal syndrome (AGS) is an IgE-mediated allergic response to mammalian meat and diary products[57]. It is induced by tick bites that sensitize to the oligosaccharide galactose-α-1,3-galactose (α-gal), which is shared by insects and most non-primate mammals[57]. Mammalian meat or diary ingestion exposes α-gal and can induce FA symptoms in sensitized individuals[57]. Evaluation of a cohort of 507 AGS adults shows that: (1) patients who initiated metformin after diagnosis with AGS had less risk of continuing to develop allergic reactions to dairy compared to AGS patients who did not use metformin; and (2) individuals who used metformin at the time of AGS diagnosis were underrepresented in the AGS cohort vs. metformin users in the general US population (Fig. 6F). This suggests that metformin may prevent human FA development and suppress established human FA, as we found for mice.

Discussion:

Our observations support the hypothesis that several clinically important food allergens promote an allergic response by inducing an epithelial cell UPR that, in turn, stimulates the pro-Th2 cytokine response that is essential to initiate and maintain food allergy. These studies demonstrate that: (1) the pro-Th2 cytokines are required to induce and maintain food allergy[15]; (2) egg, cream, PE, and walnut extract induce a human cell line, and, in the case of egg, primary human epithelial cells, human intestinal organoids, and mouse skin, lung, and jejunum to increase UPR and pro-Th2 cytokine gene expression; (3) increased pro-Th2 gene expression is accompanied by increased pro-Th2 cytokine secretion, at least for the human cell line; (4) drugs that globally suppress the UPR by inducing AMP kinase (metformin) or by acting as chaperonins (TUDCA and 4-PBA) also suppress the pro-Th2 cytokine response; (5) the pro-Th2 cytokine response is also suppressed by small molecule inhibitors of two UPR pathways that are both required to generate the transcription factor, XBP1s; (6) the pro-Th2 cytokine response by a human intestinal epithelial cell line is also suppressed by siRNAs that inhibit XBP1s expression; (7) induction of murine egg allergy is blocked by metformin and established egg allergy and the intestinal pro-Th2 response are suppressed by both metformin and TUDCA; (8) induction of an intestinal pro-Th2 response is suppressed by deletion of a functional IRE-1α or XBP1 gene in intestinal epithelium; (9) the ability of mice of different strains to develop a 24 hour intestinal UPR and pro-Th2 cytokine response correlates strongly with the likelihood that the same mouse strains will develop EA, a process that takes 2-3 months; and (10) there is a strong correlation between the induction

of the UPR and pro-Th2 cytokines in the jejunum of the same mouse strains 24 hours after egg ingestion.

Although most of these studies were performed using egg as a model allergen, our conclusions are likely to apply to milk, peanuts, and walnuts (as an example of tree nuts) as well. Each of these clinically important food allergens induced a UPR and pro-Th2 cytokine response, with both responses inhibited by metformin and TUDCA. Extracts of two additional clinically important food allergens, shrimp and fish (codfish), also induce a pro-Th2 cytokine response in the CACO-2 human intestinal cell line, but here the pro-Th2 cytokine response appears to be at least relatively UPR-independent. In the case of shrimp extract, a strong pro-Th2 response developed rapidly in the absence of a strong UPR response. Although codfish extract induced strong UPR-associated and pro-Th2 cytokine gene expression, only the UPR response was significantly suppressed by the doses of metformin and TUDCA used. In contrast to the ability of extracts of these six clinically important food allergens to induce a pro-Th2 cytokine response, extracts of three clinically important seed allergens (sesame, soy, and wheat) failed to induce a strong UPR or pro-Th2 cytokine response in CACO-2 cells. This suggests that the 9 most allergenic foods may fall into three groups that differ in their allergy pathogenesis: those that promote a UPR-dependent pro-Th2 cytokine response (eggs, milk, peanuts, tree nuts); those that induce a pro-Th2 cytokine response that is at least relatively UPR-independent (fish and crustaceans); and those that induce FA through a mechanism that does not require induction of an epithelial cell pro-Th2 cytokine response (wheat, soy, and sesame, although it is possible that our extraction process eliminated allergen components that induce UPR and pro-Th2 cytokine responses or that higher doses of

these allergens would induce UPR-associated and pro-Th2 cytokine gene expression). In vivo studies will be required to determine whether an in vitro UPR-dependent pro-Th2 response consistently predicts the ability of a UPR inhibitor to suppress intestinal allergy caused by each of those allergens, as is the case for egg.

Even within the first group of allergenic foods, the mechanism of UPR induction varies. For milk and egg, UPR induction was associated with their fat content, as shown by suppression with a lipase inhibitor that blocks fat absorption by preventing the hydrolysis of fats to fatty acids and glycerol. This is consistent with the ability of small molecule inhibitors of saturated fatty acid β-oxidation in peroxisomes and mitochondria to prevent UPR and pro-Th2 cytokine induction by EYP and purified palmitate. In contrast, PE was prepared in a way that removed fat. Consistent with this, UPR and pro-Th2 cytokine induction by PE was blocked little, if at all, by inhibitors of lipase or β-oxidation. The abilities of the allergenic cysteine protease, papain[58], and of cholera toxin, which increases 3',5'-cyclic AMP[59], to induce a metformin-suppressible pro-Th2 cytokine response in CACO-2 cells further illustrates that the UPR can stimulate a pro-Th2 cytokine response even when the UPR is induced by different mechanisms.

Although egg induces a strong local UPR and pro-Th2 cytokine gene response in BALB/c mice 24 hours after o.g., i.t., or e.c. inoculation, it only had a similar effect in two additional strains (CC024 and CC027, with CC027 > CC024) out of a total of 18 strains tested. Interestingly, one of these strains (CC027) also has an uncommon ability to develop food allergy in response to ingested peanut[60]. Importantly, the ability to induce a UPR response correlated strongly with induction of a pro-Th2 cytokine response in the tested strains and the ability to induce a pro-Th2 cytokine response in 24 hours correlated

well with the ability to induce FA following several e.c. and o.g. inoculations. This suggests that genetic polymorphisms primarily influence the ability of egg to promote an epithelial UPR, rather than the ability of the UPR to promote a pro-Th2 cytokine response or the ability of the pro-Th2 cytokine response to promote the development of EA in our model . These results support the view that genetic polymorphisms explain why only a minority of humans develop FA, despite the ubiquitous ingestion of allergenic foods. Focusing on 24 hour UPR induction and utilizing the genome sequencing that is already available for BALB/c, C57BL/6 and the 16 collaborative cross mouse strains that we tested should facilitate identification of the genes and their allelic variants that promote food allergy.

Although our hypothesis focuses on the connection between the UPR, the pro-Th2 cytokine response, and the development of FA, not all UPR inducers stimulate a strong epithelial pro-Th2 cytokine response. Thapsigargin and tunicamycin both induce expression of UPR-associated gene products, including XBP1s, to approximately the same extent as palmitate, but are much weaker inducers of pro-Th2 cytokine expression. This disparity does not result from inhibition of the pro-Th2 cytokine response by thapsigargin or tunicamycin, as shown by a mixing experiment. This suggests that UPR-associated signaling molecules and transcription factors, while necessary to induce an epithelial cell pro-Th2 response to several important food allergens, are not sufficient, although the putative additional required factor(s) has not yet been identified.

Nevertheless, our model makes biological and evolutionary sense. Helminthic and insect parasites have had an important influence on vertebrate evolution. These parasites stress epithelial cells in lung and gut (helminthes) and skin[29, 61, 62]. They

promote pro-Th2 cytokine responses in these organs[62-64], which, along with the type 2 cytokines they induce, protect the epithelial cells from parasite-induced stress and promote epithelial healing in addition to promoting parasite expulsion or sequestration[29, 65]. It seems appropriate, thus, to regard stress-induced pro-Th2 epithelial responses as an element of the UPR that evolved to protect against multicellular pathogenic parasites. In the relative absence of these parasites in the developed world in the 21st century, this initially protective mechanism may primarily protect against some viral disorders[66] , but also promote allergic responses[67, 68].

In addition to providing insight into allergenicity and FA pathogenesis, our observations suggest that drugs that suppress the UPR may be useful for the treatment of FA, or at least allergy to foods that induce a UPR-dependent pro-Th2 cytokine response, such as milk, eggs, peanuts, and tree nuts. This suggestion is validated in our studies in two mouse models of EA. Prophylactic treatment with metformin blocked the development of EA in mice inoculated with egg e.c and o.g. Metformin additionally had a dose-related ability to decrease both shock and diarrhea in mice in which disease had been established prior to initiation of treatment in this EA model, and to limit shock, including lethal anaphylaxis, in a more robust model in which EA was induced by i.p. sensitization with alum adjuvant, followed by o.g. inoculation. Studies in the latter model also provided the clinically relevant observation that metformin effectively suppresses EA even if administered to mice that are not then receiving egg, and that therapeutic effects of metformin treatment are still observed for weeks after metformin treatment is stopped. The strong therapeutic effects observed for metformin, at doses approximately equivalent on a gm/m^2 basis to the maximum doses used in humans may reflect, in part,

the critical role for intestinal epithelial cells in FA pathogenesis as the site of the UPR and pro-Th2 cytokine response and the high concentration of ingested metformin in the small intestine (30-300 times plasma levels[69]) after oral ingestion.

The therapeutic effects of metformin in mice raised the possibility that the same drug could be a useful therapy for food-allergic humans. Although therapeutic effects in mice do not guarantee similar effects in humans, particularly at tolerable drug doses, our observations with a human intestinal cell line, primary human epithelial cells, and human intestinal organoids indicate that egg and metformin have similar effects on human epithelial cells in vitro as they have in the mouse intestine in vivo. Furthermore, studies in a mouse model of another allergic disorder, asthma[70], and in asthmatic humans who have received metformin to treat diabetes mellitus suggest that metformin ameliorates this disease[71-73]; our serendipitous observations with α-gal allergy patients who have taken metformin for unrelated conditions suggests that this may be true for human FA as well, although all of these retrospective observations require confirmation in prospective studies. Although metformin can have gastrointestinal side effects that could complicate its use in food-allergic individuals, these untoward effects are usually mitigated by starting treatment with a low dose that is slowly increased, by using slow release metformin formulations, and by taking metformin with food[74-76]; indeed, approximately 75% of individuals who take metformin as a treatment for diabetes mellitus have no gastrointestinal side effects and 95% are able to continue taking the drug[74]. The ability of metformin in our in vivo murine studies to ameliorate EA without causing weight loss (a sensitive sign of gastrointestinal toxicity in humans) (data not shown), the ability of our α-gal allergy patients to tolerate this drug, metformin's use for >60 years with few

serious side effects in type 2 diabetics, and metformin's low cost and easy availability suggest that a prospective therapeutic trial of this drug in α-gal allergy patients and, if successful, in egg- and/or peanut-allergic individuals, is warranted.

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Figure legends:

Figure 1. Saturated fat induction of UPR-associated and pro-Th2 cytokine gene expression. A. CACO-2 cells were cultured with medium alone or with 100 μg of sodium salts of the fatty acids shown. After 6 hours, expression of the genes shown relative to GAPDH and culture with medium was determined by quantitative PCR. 8 cultures/group. Saturated fats induced stronger UPR-associated and pro-Th2 gene expression than unsaturated fats (2-way ANOVA, Tukey's multiple comparisons). B. CACO-2 cells were cultured with medium alone or with 10% MCT, EYP, EW, or heavy cream. After 6 and 24 hours, expression of the genes shown relative to GAPDH was determined by quantitative PCR. 8 cultures/group, MCT, EYP and HC significantly induced UPR genes at 6 hrs and pro-Th2 responses at 24 hrs, as compared to EW (2-way ANOVA, Tukey's multiple comparisons). C. CACO-2 cells were cultured with medium alone or with 10% EYP or 100 μM sodium palmitate (6 cultures/group) for 24 hrs. Cultured cells from each group were pooled and lysed. Lysates of the cultured cells were immunoprecipitated with mAbs to the pro-Th2 cytokines, resolved by gradient gel electrophoresis and stained with biotin-labeled mAbs to these cytokines, followed by streptavidin-peroxidase and ECL Western blot substrate. D. CACO-2 cells were cultured for 24 hours with medium alone or 100μM sodium palmitate, 10% v/v of MCT, 10% v/v of EYP or 10% v/v/ of HC ± the concentrations of orlistat indicated. Expression of the genes shown relative to GAPDH was determined by quantitative PCR. Orlistat significantly and dose-dependently inhibited pro-Th2 cytokine gene expression by MCT, EYP, and HC, but not palmitate (2-way ANOVA, Tukey's multiple comparisons). E. CACO-2 cells were cultured with

medium alone or with 100 μM sodium palmitate, EYP, or PE, ± 100 μM etomoxir or thioridazine. After 6 hour and 24 hours, expression of the UPR-associated genes and the pro-Th2 cytokine genes, respectively, were determined relative to GAPDH by quantitative PCR. 8 cultures/group. Etomoxir and thioridazine inhibited EYP- and palmitate-induced XBP1 and XBP1s expression at 6 hrs and pro-Th2 cytokine expression at 24 hours to a significantly greater extent than they inhibited PE-induced expression of these genes (2-way ANOVA, Tukey's multiple comparisons). F. CACO-2 cells were cultured with palmitate or EYP ± the concentrations of NAC shown for 6 or 24 hours. XBP1s and pro-Th2 cytokine expression relative to GAPDH were determined. NAC dose-dependently inhibited palmitate-induced XBP1s and pro-Th2 cytokine responses at 6 and 24 hours, and EYP-induced XBP1s at 6 hrs and pro-Th2 cytokines at 24 hrs (2-way ANOVA). G. CACO-2 cells were cultured for 24 hours with 10% PE ± 100 mM NAC. PE significantly increased XBP1s and pro-Th2 cytokine expression relative to GAPDH. Expression of the induced genes was significantly suppressed by NAC (2-way ANOVA, Tukey's multiple comparisons).

Figure 2. UPR inhibitors suppress pro-Th2 cytokine responses to saturated fats. A. CACO-2 cells were cultured for 24 hours with medium alone or the stimuli shown ± 10 mM metformin, TUDCA, or 4-PBA. Gene expression relative to GAPDH and culture with medium alone, is shown in the left panel. Percent suppression of gene expression by the 3 UPR inhibitors studied is shown in the other 3 panels (suppression of cultures with EW is not shown because of a lack of response to EW). 8 cultures/group. All UPR inhibitors significantly suppressed expression of all pro-Th2 cytokines, regardless of the

stimulus used (1-way ANOVA, Tukey's multiple comparisons). B. Primary human intestinal epithelial cells were cultured for 6 or 24 hours ± 100 μM palmitate or 10% v/v EYP and ± 10 mM metformin. 8 cultures/group. Expression of the genes shown relative to GAPDH was determined by quantitative PCR. Metformin significantly suppressed all responses to palmitate, the 6 hr XBP1s response to EYP, and the 24 hr pro-Th2 cytokine response to EYP (2-way ANOVA, Tukey's multiple comparisons). C. Human intestinal organoids were injected intraluminally with medium alone or with 100 μM sodium palmitate ± no inhibitor, or 10 mM metformin or TUDCA and cultured for 24 hrs. Expression of the genes shown relative to GAPDH and injection with medium alone is shown. Metformin and TUDCA significantly suppressed UPR and pro-Th2 cytokine responses (2-way ANOVA, Tukey's multiple comparisons). D. CACO-2 cells were cultured for 6 or 24 hours with 100 μM palmitate or 10% v/v EYP and the concentrations of AICAR shown. AICAR dose-dependently inhibited all responses to palmitate, 6 hr XBP1s responses to EYP, and 24 hr pro-Th2 cytokine responses to EYP (2-way ANOVA, Tukey's multiple comparisons). E. CACO-2 cells were cultured for the indicated times with 10 μM sodium palmitate or 10% v/v EYP ± the concentrations of the inhibitors shown. TSLP, IL-25, and IL-33 expression relative to GAPDH was determined by quantitative PCR. KIRA6, STF083010, and AEBSF dose-dependently suppressed the pro-Th2 cytokine responses to palmitate at 6 and 24 hours and to EYP at 24 hours (2-way ANOVA, Tukey's multiple comparisons). F. CACO-2 cells were cultured for 24 hrs ± 100 μM palmitate or 10% v/v EYP ± 5 pM of the siRNAs shown. Gene expression relative to GAPDH is shown. 8 cultures/group. XBP1s siRNA and ERN1 siRNA significantly suppressed the pro-Th2 responses to EYP and palmitate as compared to

scramble siRNA, GAPDH siRNA and PERK siRNA (2-way ANOVA). G. CACO-2 cells were cultured for 6 or 24 hours with medium, 100 μM palmitate, or 10% v/v EYP ± 100 μM BMS345541 (NF-κB inhibitor). XBP1s and pro-Th2 cytokine gene expression relative to GAPDH were determined by quantitative PCR. BMS345541 significantly inhibited all responses to palmitate, the 6 hr XBP1s response to EYP and PE and the 24 hr pro-Th2 cytokine responses to EYP and PE (2-way ANOVA, Tukey's multiple comparisons).

Figure 3. UPR-dependent pro-Th2 cytokine induction by non-lipid allergens. A. CACO-2 cells were cultured with medium alone or 10% v/v PE ± 10 mM metformin. Gene expression relative to GAPDH and culture with medium was determined after 6 hours for UPR-associated genes or after 24 hours for pro-Th2 cytokine genes. 8 cultures/group. Metformin significantly suppressed all of the responses shown (2-way ANOVA, Tukey's multiple comparisons). B. CACO-2 cells were cultured with medium alone, 10% v/v EYP, or 10% v/v PE ± 100 μM orlistat. Gene expression relative to GAPDH and culture with medium was determined after 6 hours for UPR-associated genes or after 24 hours for pro-Th2 genes for cells cultured with EYP or PE vs. medium. 8 cultures/group. Orlistat significantly suppressed EYP-induced UPR-associated responses at 6 hrs and all pro-Th2 cytokines at 24 hrs, but had no effect on PE-induced responses (2-way ANOVA, Tukey's multiple comparisons). C. CACO-2 cells were cultured with medium alone or 10% v/v EW, 100 μM palmitate, or 10% v/v of aqueous extracts of soy, sesame, shrimp, codfish, wheat, or walnut. Gene expression relative to GAPDH was determined after 6 and 24 hours. 8 cultures/group. Palmitate, walnut extract, and fish extract all significantly

stimulated the UPR-associated and pro-Th2 cytokine responses and shrimp extract significantly stimulated the pro-Th2 cytokine response (2-way ANOVA, Tukey's multiple comparisons). D. CACO-2 cells were cultured for 24 hours with medium alone, 100 μM sodium palmitate, or 10% v/v of fish extract, shrimp extract, or walnut extract ± 10 mM metformin. Gene expression relative to GAPDH was determined by quantitative PCR. 8 cultures/group. Metformin significantly inhibited expression of all genes tested except for the pro-Th2 cytokine genes for fish and IL-33 for shrimp (2-way ANOVA). E. CACO-2 cells were cultured for 6 or 24 hours with medium alone, 10 μg/ml cholera toxin, or 100 nM papain ± 10 mM metformin. Treatment with TUDCA had effects qualitatively similar, but less marked, as treatment with metformin (not shown). Gene expression relative to GAPDH was determined by quantitative PCR. 8 cultures/group. Cholera toxin significantly induced 6 hr XBP1 and XBP1s responses and a 24 hour pro-Th2 cytokine response. Papain significantly induced XBP1 and XBP1s and pro-Th2 cytokine responses at both 6 and 24 hours. All of these responses were significantly inhibited by metformin except for the 24 hr response to papain (2-way ANOVA, Tukey's multiple comparisons).

Figure 4. Egg induces XBP1- and ERN1-dependent intestinal epithelial pro-Th2 gene expression in vivo. A. BALB/c mice (6/group) were inoculated o.g. with EW + EYP or saline on day 0 and were provided with conventional drinking water or water supplemented with sufficient metformin to provide ~1 gm/kg/day starting 1 day prior to egg inoculation. One day later, proximal jejunum was obtained and RNA extracted. Expression of UPR-associated and pro-Th2 cytokine genes relative to GAPDH was

determined by quantitative PCR. Egg significantly increased and metformin significantly suppressed all of the egg-induced responses shown (2-way ANOVA, Tukey's multiple comparisons). B and C. C57BL/6 background mice homozygous for floxed XBP1 (B) or ERN1 (C) that expressed or lacked a villin-Cre transgene were inoculated o.g. with EW + EYP and evaluated 1 day later for proximal jejunal expression of UPR-associated and pro-Th2 genes relative to GAPDH, as in "A." Pro-Th2 cytokine responses to egg were significantly decreased in $XBP1^{fl/fl}$ Vil-Cre mice and ERN1, XBP1s, and pro-Th2 cytokine responses to egg were significantly decreased in $ERN1^{fl/fl}$ Vil-Cre mice (2-way ANOVA, Tukey's multiple comparisons).

Figure 5. Initial pro-Th2 cytokine responses to egg are genetically dependent and predict the development of egg allergy. In one experiment, BALB/c, C57BL/6, and 16 strains of collaborative cross mice (6-8/group) were inoculated e.c., i.t., and o.g. with saline or with EW + EYP and sacrificed one day later. Expression of IL-25, IL-33, and TSLP in jejunum, lungs, and inoculated skin relative to GAPDH were determined by quantitative PCR. In a second experiment, the same strains of mice were subjected to e.c. sensitization with EW + EYP followed by 18 o.g. challenges (2/week). Individual mice that developed diarrhea and/or hypothermia (temperature drop > 0.8°C) for at least 2 of the last 3 challenges were scored as having diarrhea and/or hypothermia, respectively. Dot plots show mean relative TSLP expression (X axis) and IL-33 expression (Y axis) in the first experiment for jejunum, lung, and skin. The percent of mice in the second experiment that developed hypothermia is indicated by the size of each circle, while the percent of mice that developed diarrhea is indicated by circle color. Strain names are indicated for

the mice that developed hypothermia and/or diarrhea in the second experiment. Day 1 IL-25 responses are not shown, but were similar to or less than day 1 IL-33 responses. r^2 and p values were determined by multiple linear regression analysis and are shown in each panel of the figure.

Figure 6. Inhibition of food allergy by UPR antagonists. A. BALB/c mice (6-8/group) were skin sensitized with EW + EYP for 3 weeks, then inoculated o.g. twice a week with 100 mg of EW + EYP for 9 weeks. Mice were provided with conventional drinking water or drinking water that provided ~0.5 or 1 gm/kg of metformin/day, starting 3 days before egg immunization. Hypothermia and diarrheal responses to the last oral gavage with EW + EYP are shown. MMCP1 levels were determined for sera obtained 4 hours after the last o.g. challenge. Metformin significantly suppressed hypothermia and diarrhea at 0.5g/kg/d and 1g/kg/d, and significantly suppressed MMCP1 at 1g/kg/d. (1-way ANOVA, Tukey's multiple comparisons). B. BALB/c mice (6/group) were skin sensitized with EW + EYP as in "A" until they all developed diarrhea and most developed hypothermia following o.g. with EW + EYP. Mice then continued to be challenged twice weekly o.g. with EW + EYP and provided with conventional drinking water (black squares), or drinking water that provided ~0.5 gm/kg (red circles) or ~1 gm/kg (green triangles) per day of metformin. Each symbol indicates values for a single mouse; some symbols are superimposed. Solid symbols indicate the presence of diarrhea. Starting with o.g. challenge 24, treatment with ~0.5 gm/kg of metformin/day was initiated for mice that did not initially receive metformin, the metformin dose was halved for mice that had earlier received 0.5 gm/kg/day and metformin was stopped for mice that had earlier received 1 gm/day. Metformin at both doses significantly suppressed diarrhea and hypothermia (2-

way ANOVA, Tukey's multiple comparisons). C. BALB/c mice (6/group) were skin sensitized with EW + EYP and challenged by o.g. as in Fig. 6A until they all developed diarrhea and hypothermia. Following the 21st o.g., mice continued to receive 2 o.g.s per week and were provided with conventional drinking water (black squares) or drinking water that contained ~0.5 gm/kg of TUDCA/day (red circles). TUDCA-treated mice developed significantly less severe hypothermia than mice that did not receive TUDCA (2-way ANOVA, Tukey's multiple comparisons). D. BALB/c mice (14-16/group) were skin sensitized with EW + EYP for (3 weeks), then challenged o.g. with EW + EYP for the number of times shown. One group of mice was administered 500 mg/kg/day of metformin via drinking water starting just prior to challenge #8. Hypothermia and diarrheal responses to each challenge are shown. Serum was drawn 4 hours after challenge #24 and MMPC1 levels were determined by ELISA. Segments of jejunum were obtained 4 hours after the last challenge for these mice and for untreated mice and levels of UPR-associated and pro-Th2 cytokine gene expression relative to GAPDH were determined by quantitative PCR. Metformin significantly suppressed MMCP1 levels (unpaired t test) as well as diarrhea and hypothermia (2-way ANOVA, Tukey's multiple comparisons) and UPR and pro-Th2 cytokine gene expression, except for BiP (2-way ANOVA, Tukey's multiple comparisons). E. BALB/c mice (8/group) were sensitized to egg by i.p. injection with EW adsorbed to alum on days 0 and 7, then challenged o.g. with EW twice a week for 2 weeks, by which time all developed hypothermia in response to challenge. Mice were then rested for 4 weeks. Sufficient metformin in drinking water to provide ~1 g/kg/day was initiated for 2 groups of mice after challenge 4 and terminated for one of these groups at the time of challenge 5 (green line), but continued

for the other group for the duration of the experiment (blue line). Deaths from anaphylaxis, changes in rectal temperature, and spontaneous movement during the hour after each challenge are shown. The experiment was terminated when all of the mice that never received metformin had died. Continued treatment with metformin was significantly more effective than terminated treatment with metformin, which was significantly better than no metformin at suppressing hypothermia, decreased mobility, and diarrhea (2-way ANOVA, Tukey's multiple comparisons). Any metformin treatment decreased mortality (Mantel-Cox test). F. Upper panel: A cohort of 507 adults with α-gal syndrome (AGS) was analyzed for whether they were now able to tolerate dairy products and for whether they had initiated metformin treatment for any reason after being diagnosed with AGS. Patients who initiated metformin after being diagnosed with AGS had less risk of continuing to develop allergic reactions to dairy compared to AGS patients who did not use metformin (odds ratio <0.1, 95% confidence interval 0.041 – 0.255, p<.0001). Lower panel: The same patient cohort was analyzed for the number of individuals who were taking metformin at the time of AGS diagnosis, vs. the number in the general US population who would be expected to be taking metformin, based on the age-adjusted prevalence of type 2 diabetes, the age distribution of the AGS cohort, and the use of metformin by ~80% of type 2 diabetics in the US. Individuals who used metformin at time of AGS diagnosis were underrepresented in the AGS cohort vs. age-matched metformin users in the general US population (odds ratio <0.085, 95% confidence interval 0.034 – 0.186, p<.0001). 2-tailed Fisher's exact test and Baptista-Pike test.

Supplemental Figure Legends:

Supplemental Figure 1. Pro-Th2 cytokine-dependence of egg allergy maintenance. BALB/c mice (6/group) were sensitized to egg by patching shaved skin for three weeks with 0.1 mg of EYP + EW (left panel), or by i.t. inoculation with 50 μg of EW + EYP three times a week for three weeks (right panel), then inoculated o.g. with 100 mg of EW + EYP every other day until they consistently developed hypothermia after egg inoculation. All mice then continued to be inoculated o.g. with EW + EYP but were also treated i.p. with isotype control mAbs or the combinations of mAbs to IL-25 (0.5 mg), IL-33R (0.1 mg), and/or TSLP (0.5 mg) shown twice a week for 4 weeks. Development of hypothermia following the final o.g. with EW + EYP was determined for each group. For both experiments, hypothermia was less severe in groups treated with anti-TSLP mAb than isotype control mAb and in groups treated with all 3 anti-cytokine mAbs than in groups treated with 1 or 2 of these mAbs (2-way ANOVA, Tukey's multiple comparisons and Dunnett's test).

Supplemental Figure 2. UPR induction by EYP, cream, and medium chain triglycerides requires fats to be hydrolyzed: CACO-2 cells were cultured with the stimuli ± the concentrations of orlistat shown. Expression of the UPR-related genes relative to GAPDH was determined by quantitative PCR after 6 hours of culture. 8 cultures per point. Orlistat significantly decreased the UPR-associated gene responses to MCT, EYP, and HC but not to palmitate (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 3. Palmitate and EYP induce UPR-associated and pro-Th2 cytokine gene responses that are suppressed by metformin in human primary intestinal and dermal epithelial cells. Primary human dermal and intestinal epithelial cells were cultured for 6 or 24 hours ± 100 μM palmitate or 10% v/v EYP ± 10 mM metformin. 6 cultures/group. Expression of the genes shown relative to GAPDH was determined by quantitative PCR. Responses of cells cultured with EW closely resembled responses of cells cultured with medium (not shown). Metformin significantly suppressed all responses to palmitate, the 6 hour UPR-associated responses to EYP, and the 24 hour pro-Th2 cytokine responses to EYP (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 4. Effects of selective UPR inhibitors on expression of UPR-associated genes. Expression of the indicated UPR genes relative to GAPDH was determined in the same experiment shown in Fig. 2E. KIRA6, STF083010 and AEBSF significantly decreased XBP1s, GSK2656157 and salubrinal significantly decreased ATF4, AEBSF significantly decreased BiP and XBP1, and salubrinal significantly decreased CHOP (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figures 5 and 6. Effects of siRNAs specific for UPR-associated molecules on gene expression of these molecules. Expression of the indicated UPR genes relative to GAPDH was determined in the same experiment shown in Fig. 2F. GAPDH siRNA significantly decreased GAPDH expression; PERK siRNA significantly decreased PERK and ATF4 expression; ERN1 siRNA significantly decreased ERN1 and XBP1s expression; and XBP1 siRNA significantly decreased XBP1 and XBP1s expression (2-

way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 7. Induction of NF-κB-related genes by food allergens and UPR stimuli. CACO-2 cells were cultured for 6 or 24 hours ± 100 μM palmitate or thapsigargin, 1 μg/ml tunicamycin, 10 μg/ml LPS, or 10% v/v of EYP or PE ± 100 μM BMS345541. Expression of the genes shown relative to GAPDH was determined by quantitative PCR. All of the stimuli tested significantly increased expression of all of the NF-κB-associated genes at both 6 and 24 hours. BMS345541 significantly decreased expression of all of the NF-κB associated genes at either 6 or 24 hours (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 8. Palmitate, walnut, and codfish are more potent than shrimp at inducing UPR-associated genes. CACO-2 cell expression of UPR-associated and pro-Th2 genes relative to GAPDH and culture with vehicle in response to the stimuli shown was determined after 6 and 24 hours of culture in the same experiment shown in Fig. 3C. Palmitate and walnut, but not shrimp, significantly increased UPR-associated gene expression at 6 and 24 hrs. Palmitate, walnut and shrimp significantly increased pro-Th2 cytokine expression at 24 hrs (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 9. Metformin suppresses fish- and walnut-induced CACO-2 cell expression of UPR-associated genes. CACO-2 cell expression of UPR-associated genes relative to GAPDH in response to the stimuli shown was determined after 6 hours of culture in the same experiment shown in Fig. 3D. Palmitate, fish and walnut significantly

increased UPR-associated gene expression at 6 hrs; this was significantly suppressed by metformin. UPR-associated genes induced by shrimp were not consistently significantly suppressed by metformin (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 10. Thapsigargin and tunicamycin only weakly induce pro-Th2 cytokine expression. A. CACO-2 cells were cultured for 6 or 24 hrs with 100 μM sodium palmitate or thapsigargin, or 1 μg/ml tunicamycin ± 10 mM metformin. Expression of the UPR-related genes relative to GAPDH was determined by quantitative PCR after 6 hours of culture. 6 cultures per point. Palmitate, tunicamycin, and thapsigargin all similarly and significantly increased expression of all of the UPR-associated cytokine genes. Palmitate increased expression of all of the pro-Th2 cytokine genes to a significantly greater extent than did tunicamycin or thapsigargin (2-way ANOVA, Tukey's multiple comparisons) B. CACO-2 cells were cultured for 6 or 24 hours with 50 μM sodium palmitate, 100 μM thapsigargin, 1 μg/ml tunicamycin, 50 μM palmitate + 100 μM thapsigargin, or 50 μM palmitate + 1 μg/ml tunicamycin. Expression of the UPR-related genes relative to GAPDH was determined by quantitative PCR after 6 hours of culture. 6 cultures per point. Neither tunicamycin nor thapsigargin significantly suppressed palmitate induction of the pro-Th2 cytokine genes (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 11. LPS induces CACO-2 cell expression of UPR-associated, but not pro-Th2 cytokine genes. CACO-2 cells were cultured for 6 hrs ± 10 μg of *Salmonella enterica* LPS. Expression of the UPR-related, pro-Th2, and NF-κB-related genes relative to GAPDH was determined by quantitative PCR. Expression of UPR- and NF-κB-related,

but not pro-Th2 cytokine genes was significantly increased by culture with LPS. Similar results were seen after 24 hours of culture (not shown) (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 12. Egg induces UPR-associated and pro-Th2 gene expression in skin and airways. BALB/c mice (6/group) were shaved and patched with EW + EYP or saline and inoculated i.t. with EW + EYP or saline. Mice were provided with conventional drinking water or water supplemented with sufficient metformin to provide ~1 gm/kg/day starting 1 day prior to egg inoculation. One day after egg inoculation, skin and lung and airways were obtained and RNA extracted. Expression of UPR-associated and pro-Th2 cytokine genes relative to GAPDH was determined by quantitative PCR. Egg significantly induced metformin-inhibitable expression of all of the UPR-associated and pro-Th2 cytokine genes in airways and skin (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 13. Selective deletion of XBP1 or ERN1 in small intestine does not affect the UPR-associated and pro-Th2 cytokine response to egg in skin and airways. C57BL/6 background mice homozygous for floxed XBP1 or ERN1 that expressed or lacked a villin-Cre transgene were shaved and patched with EW + EYP or saline and inoculated i.t. with EW + EYP or saline. One day later, mice were evaluated for expression of UPR-associated and pro-Th2 genes by patched skin and by lungs and airways as in Supplemental Fig. 11. Villin-Cre did not significantly suppress UPR-associated gene or pro-Th2 cytokine gene expression in skin or airways in response to

egg in either ERN1$^{flox/flox}$ or XBP1$^{flox/flox}$ mice (2-way ANOVA, Tukey's multiple comparisons).

Supplemental Figure 14. Correlations between pro-Th2 cytokine gene expression and signs and symptoms of egg allergy. IL-25, IL-33, and TSLP expression in lung and airways, jejunum, and skin 24 hours after local egg inoculation was correlated with numbers of mice of each strain that developed hypothermia or diarrhea or with the magnitude of their MMCP1 responses to o.g. challenge with egg after developing EA. r^2 and p values for the individual correlations for each strain were determined by multiple linear regression analysis (least squares test), followed by multiple-ANOVA.

Supplemental Figures 15-19. Correlations between pro-Th2 cytokine and UPR gene expression among different mouse strains. UPR-associated and pro-Th2 gene expression were determined for the 24 hour experiment described in Fig. 5. Dot plots show mean relative expression of each of the pro-Th2 cytokine genes vs. each of the UPR-associated genes for lung, jejunum, and skin for each strain of mice tested. r^2 and p values are shown in each figure panel for each pro-Th2 cytokine and were determined by multiple linear regression analysis (least squares test), followed by multiple-ANOVA. Data are summarized in supplemental Fig. 19.

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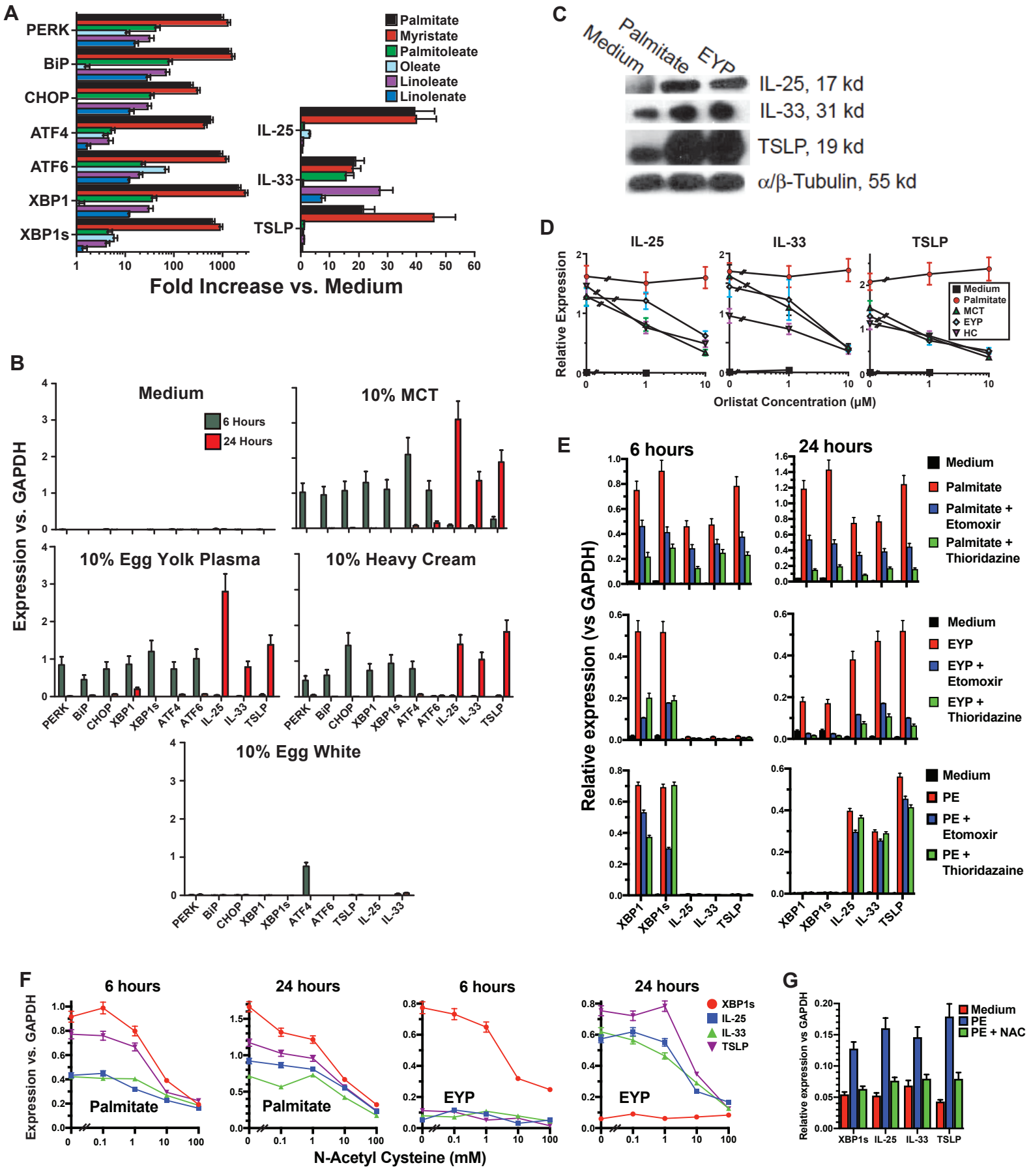
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IL-33
TSLP
Relative Expression
Orlistat Concentration (μM)
Medium
Palmitate
MCT
EYP
HC
B
Medium
10% MCT
10% Egg Yolk Plasma
10% Heavy Cream
10% Egg White
6 Hours
24 Hours
Expression vs. GAPDH
E
6 hours
24 hours
Relative expression (vs GAPDH)
Medium
Palmitate
Palmitate + Etomoxir
Palmitate + Thioridazine
EYP
EYP + Etomoxir
EYP + Thioridazine
PE
PE + Etomoxir
PE + Thioridazaine
F
6 hours
24 hours
Expression vs. GAPDH
Palmitate
EYP
N-Acetyl Cysteine (mM)
XBP1s
IL-25
IL-33
TSLP
G
Relative expression vs GAPDH
Medium
PE
PE + NAC

Figure 1

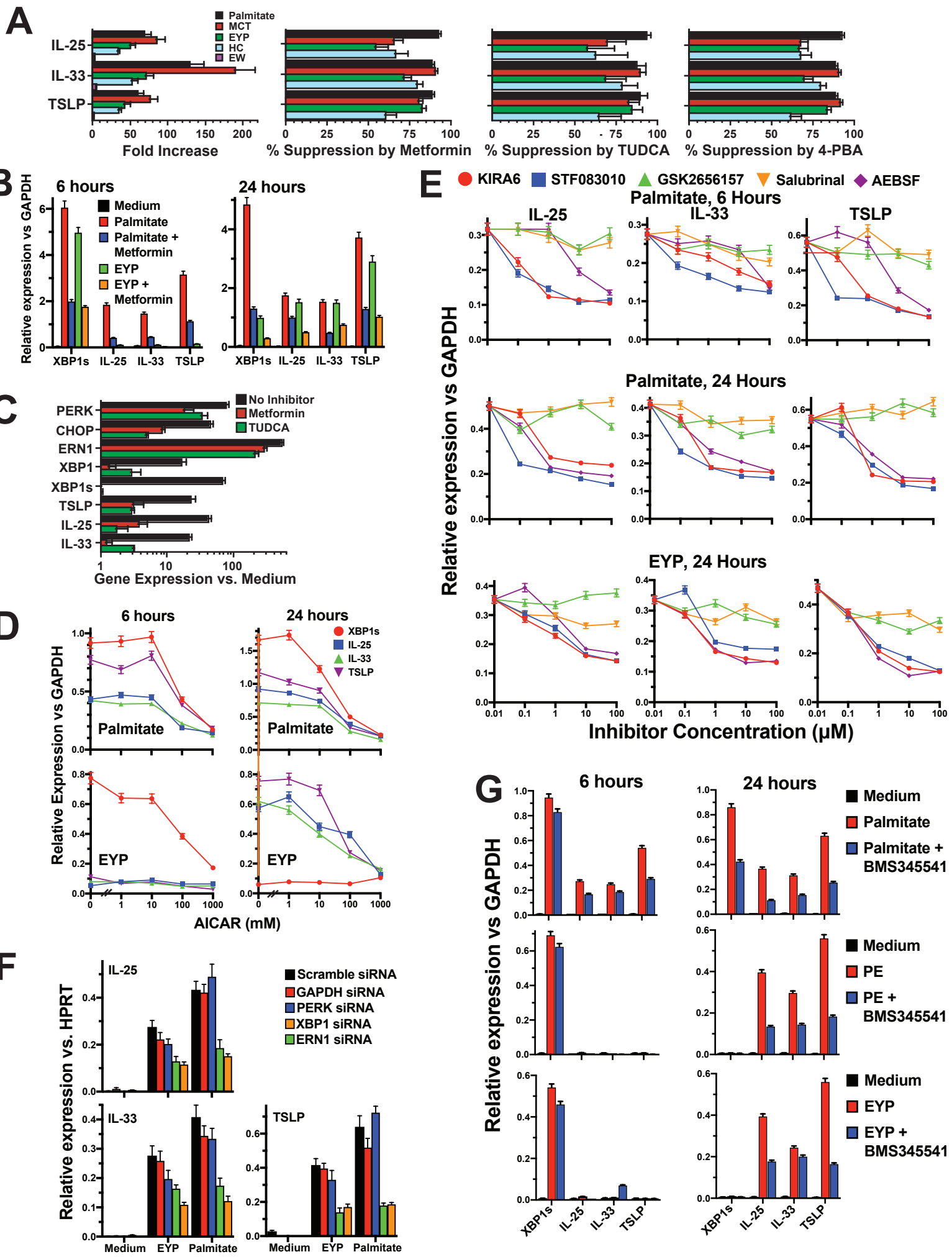
A
Palmitate
MCT
EYP
HC
EW
IL-25
IL-33
TSLP
Fold Increase
% Suppression by Metformin
% Suppression by TUDCA
% Suppression by 4-PBA
B
6 hours
24 hours
Medium
Palmitate
Palmitate + Metformin
EYP
EYP + Metformin
Relative expression vs GAPDH
XBP1s
IL-25
IL-33
TSLP
C
No Inhibitor
Metformin
TUDCA
PERK
CHOP
ERN1
XBP1
XBP1s
TSLP
IL-25
IL-33
Gene Expression vs. Medium
D
6 hours
24 hours
XBP1s
IL-25
IL-33
TSLP
Palmitate
EYP
Relative Expression vs GAPDH
AICAR (mM)
E
KIRA6
STF083010
GSK2656157
Salubrinal
AEBSF
Palmitate, 6 Hours
IL-25
IL-33
TSLP
Palmitate, 24 Hours
EYP, 24 Hours
Relative expression vs GAPDH
Inhibitor Concentration (μM)
F
Scramble siRNA
GAPDH siRNA
PERK siRNA
XBP1 siRNA
ERN1 siRNA
IL-25
IL-33
TSLP
Relative expression vs. HPRT
Medium
EYP
Palmitate
G
6 hours
24 hours
Medium
Palmitate
Palmitate + BMS345541
PE
PE + BMS345541
EYP
EYP + BMS345541
Relative expression vs GAPDH
XBP1s
IL-25
IL-33
TSLP

Figure 2

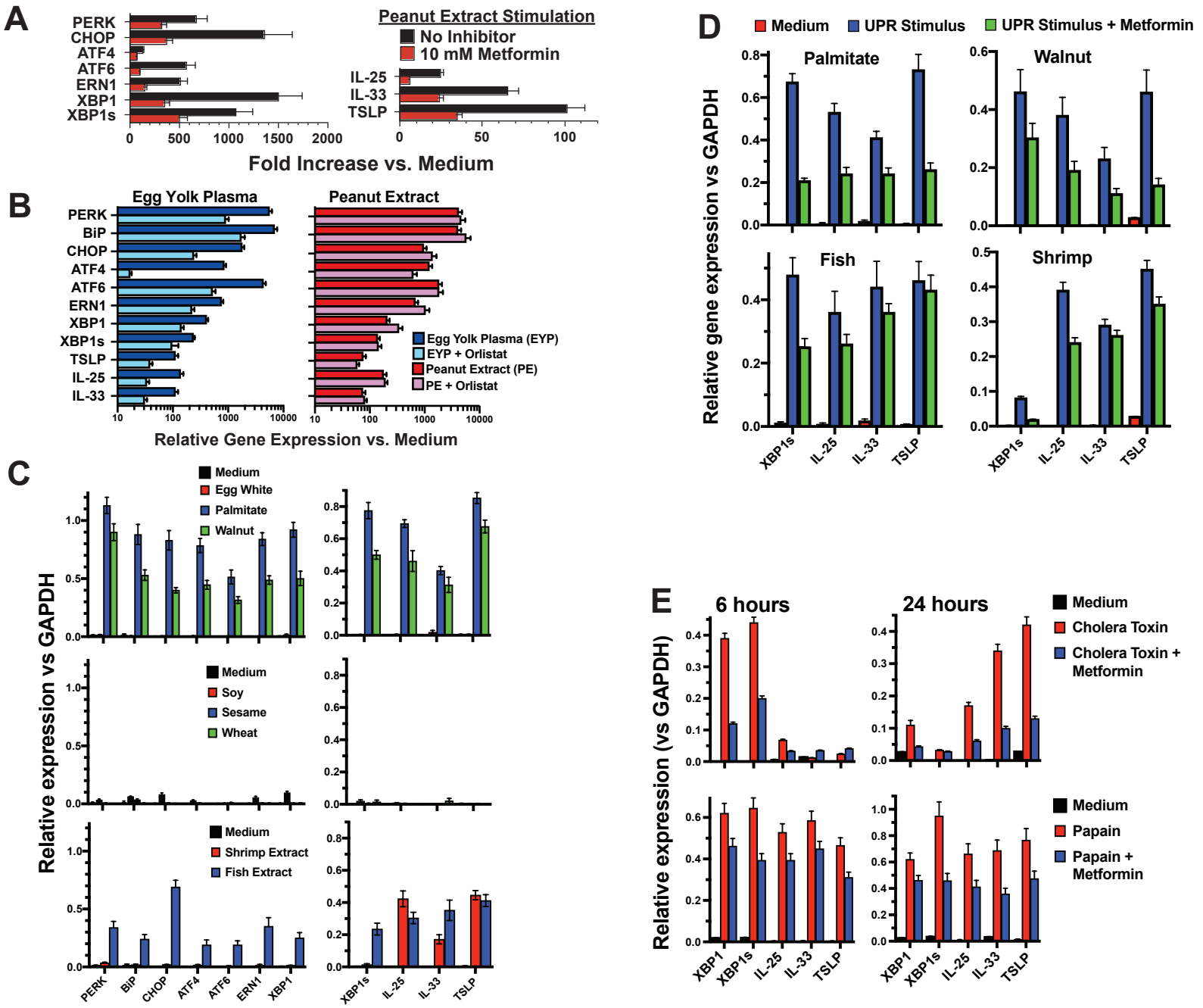
A
PERK
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
0
500
1000
1500
2000
Peanut Extract Stimulation
No Inhibitor
10 mM Metformin
IL-25
IL-33
TSLP
0
50
100
Fold Increase vs. Medium
B
Egg Yolk Plasma
Peanut Extract
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
TSLP
IL-25
IL-33
10
100
1000
10000
Egg Yolk Plasma (EYP)
EYP + Orlistat
Peanut Extract (PE)
PE + Orlistat
Relative Gene Expression vs. Medium
C
Medium
Egg White
Palmitate
Walnut
Medium
Soy
Sesame
Wheat
Medium
Shrimp Extract
Fish Extract
Relative expression vs GAPDH
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP
D
Medium
UPR Stimulus
UPR Stimulus + Metformin
Palmitate
Walnut
Fish
Shrimp
Relative gene expression vs GAPDH
XBP1s
IL-25
IL-33
TSLP
E
6 hours
24 hours
Medium
Cholera Toxin
Cholera Toxin + Metformin
Medium
Papain
Papain + Metformin
Relative expression (vs GAPDH)
XBP1
XBP1s
IL-25
IL-33
TSLP

Figure 3

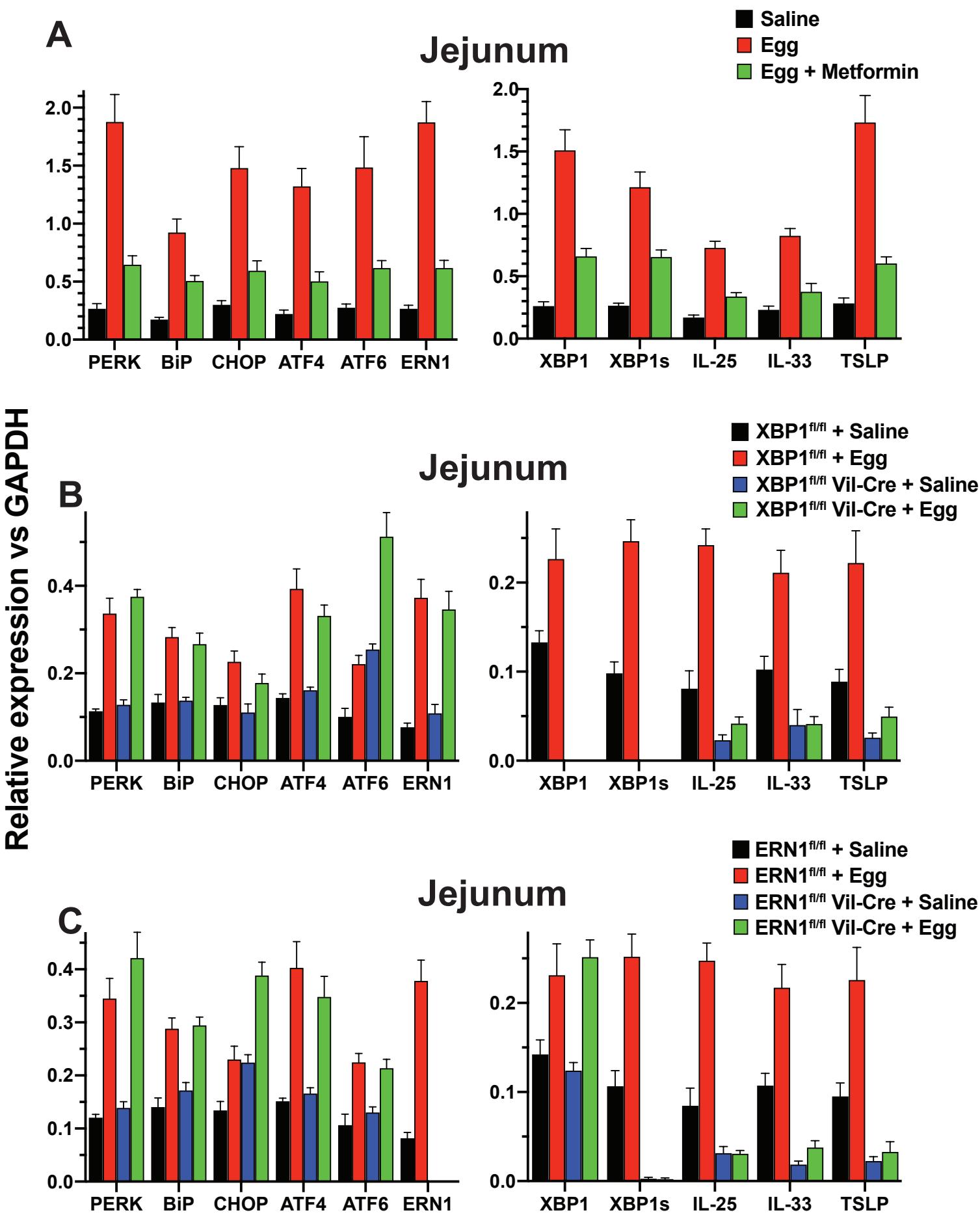
A
Jejunum
Saline
Egg
Egg + Metformin
2.0
1.5
1.0
0.5
0.0
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP
B
Jejunum
XBP1fl/fl + Saline
XBP1fl/fl + Egg
XBP1fl/fl Vil-Cre + Saline
XBP1fl/fl Vil-Cre + Egg
0.4
0.2
0.1
0.0
C
Jejunum
ERN1fl/fl + Saline
ERN1fl/fl + Egg
ERN1fl/fl Vil-Cre + Saline
ERN1fl/fl Vil-Cre + Egg
0.3
Relative expression vs GAPDH

Figure 4

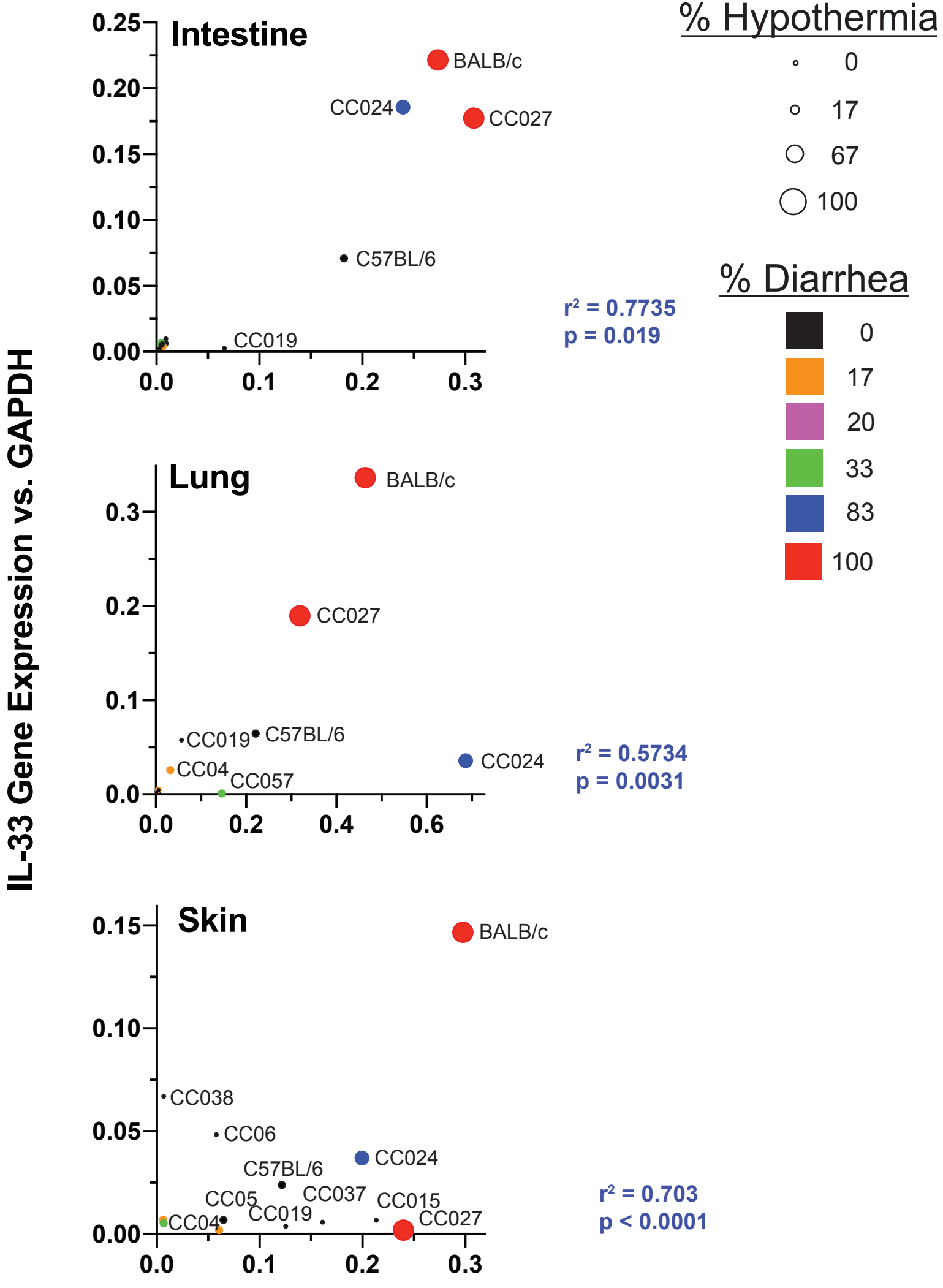
% Hypothermia
0
17
67
100
% Diarrhea
0
17
20
33
83
100
Intestine
BALB/c
CC024
CC027
C57BL/6
CC019
r² = 0.7735
p = 0.019
Lung
BALB/c
CC027
CC019
C57BL/6
CC024
CC04
CC057
r² = 0.5734
p = 0.0031
Skin
BALB/c
CC038
CC06
CC024
C57BL/6
CC05
CC037
CC015
CC019
CC04
CC027
r² = 0.703
p < 0.0001
IL-33 Gene Expression vs. GAPDH
TSLP Gene Expression vs. GAPDH

Figure 5

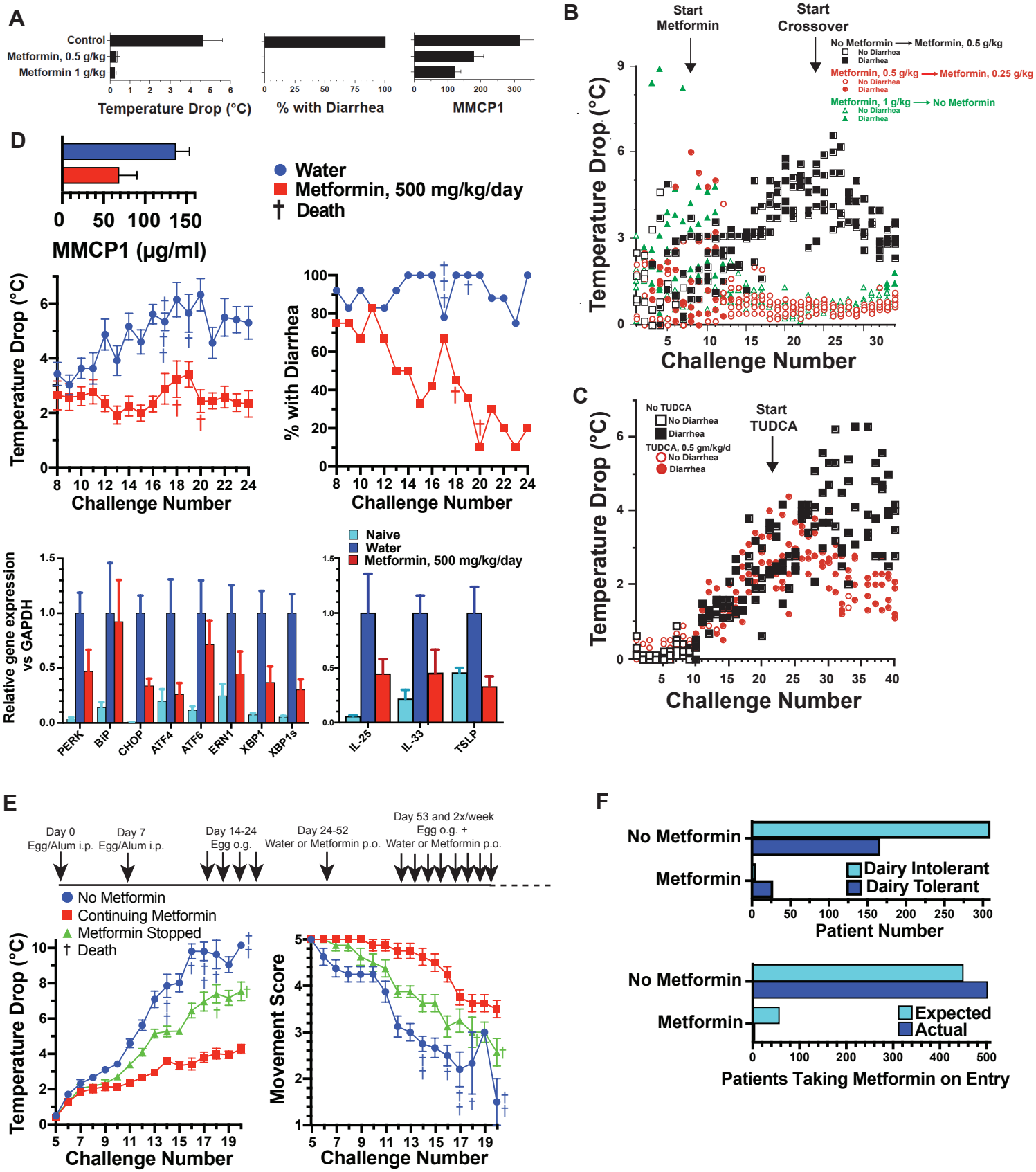
A
Control
Metformin, 0.5 g/kg
Metformin 1 g/kg
Temperature Drop (°C)
% with Diarrhea
MMCP1
B
Start Metformin
Start Crossover
No Metformin → Metformin, 0.5 g/kg
No Diarrhea
Diarrhea
Metformin, 0.5 g/kg → Metformin, 0.25 g/kg
No Diarrhea
Diarrhea
Metformin, 1 g/kg → No Metformin
No Diarrhea
Diarrhea
Temperature Drop (°C)
Challenge Number
C
No TUDCA
No Diarrhea
Diarrhea
TUDCA, 0.5 gm/kg/d
No Diarrhea
Diarrhea
Start TUDCA
Temperature Drop (°C)
Challenge Number
D
MMCP1 (µg/ml)
Water
Metformin, 500 mg/kg/day
† Death
Temperature Drop (°C)
Challenge Number
% with Diarrhea
Challenge Number
Relative gene expression vs GAPDH
Naive
Water
Metformin, 500 mg/kg/day
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP
E
Day 0 Egg/Alum i.p.
Day 7 Egg/Alum i.p.
Day 14-24 Egg o.g.
Day 24-52 Water or Metformin p.o.
Day 53 and 2x/week Egg o.g. + Water or Metformin p.o.
No Metformin
Continuing Metformin
Metformin Stopped
† Death
Temperature Drop (°C)
Challenge Number
Movement Score
Challenge Number
F
No Metformin
Metformin
Dairy Intolerant
Dairy Tolerant
Patient Number
No Metformin
Metformin
Expected
Actual
Patients Taking Metformin on Entry

Figure 6

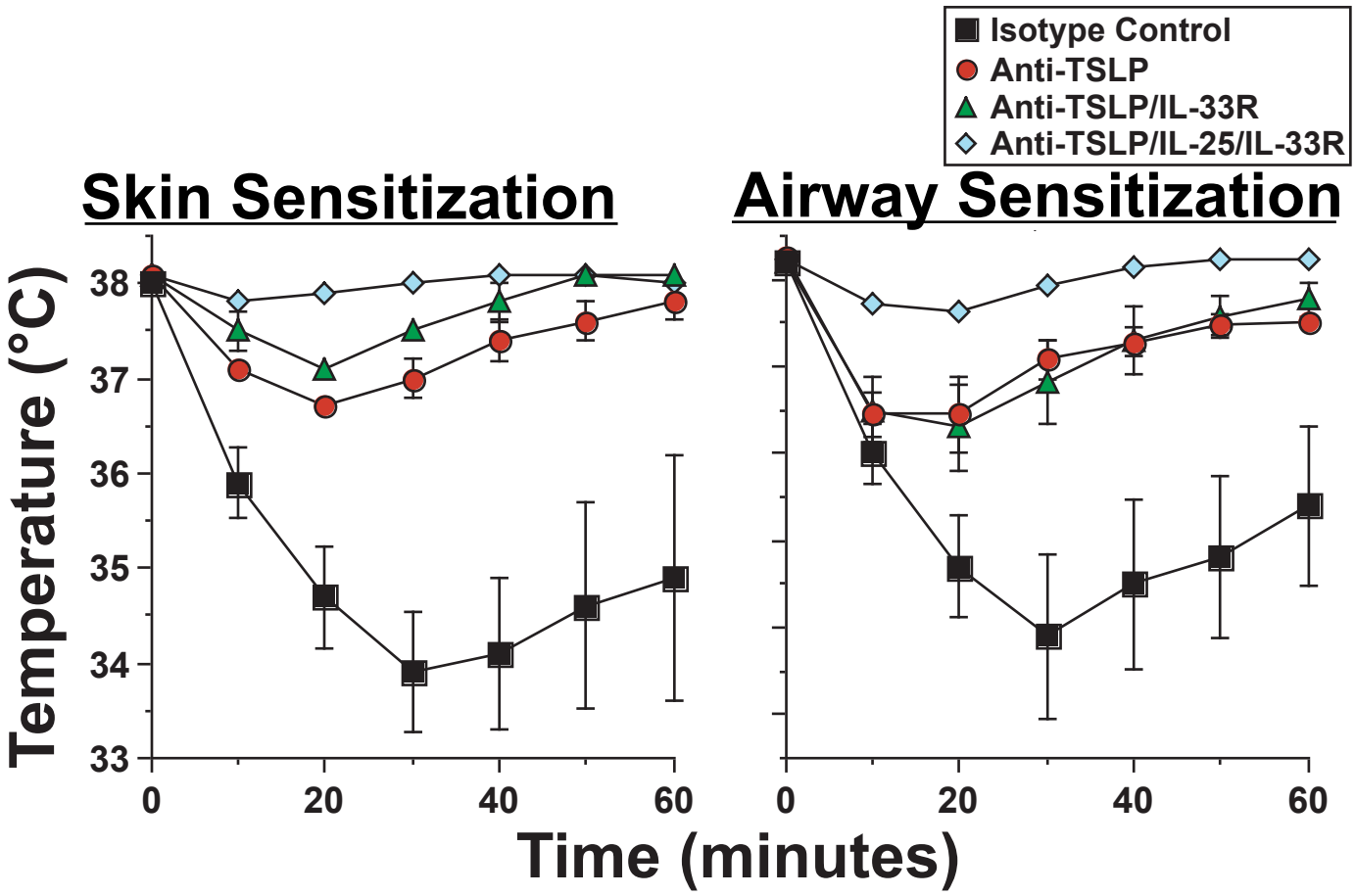
Isotype Control
Anti-TSLP
Anti-TSLP/IL-33R
Anti-TSLP/IL-25/IL-33R
Skin Sensitization
Airway Sensitization
Temperature (°C)
38
37
36
35
34
33
0
20
40
60
Time (minutes)

Figure S1

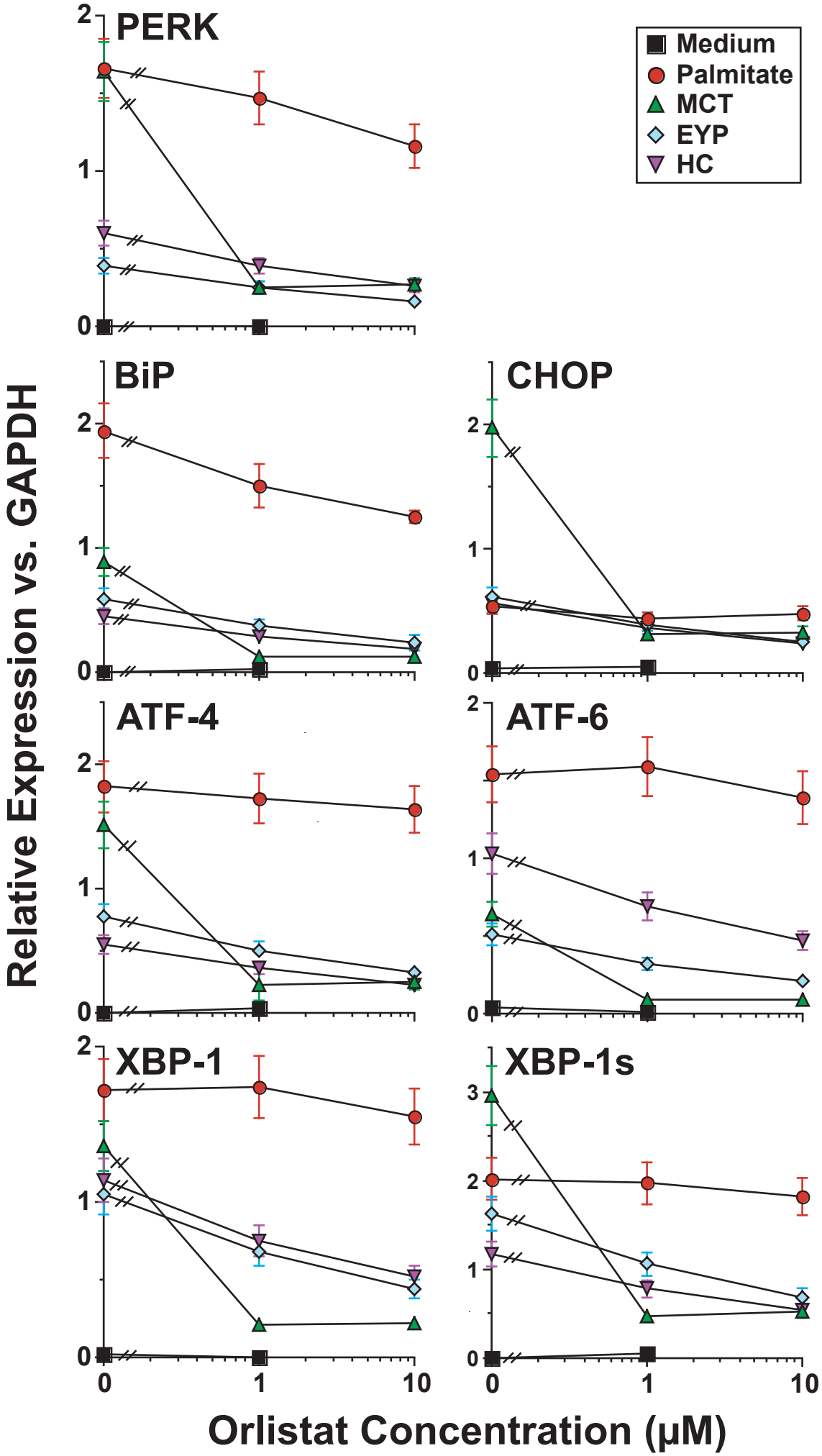
PERK
BiP
CHOP
ATF-4
ATF-6
XBP-1
XBP-1s
Medium
Palmitate
MCT
EYP
HC
Relative Expression vs. GAPDH
Orlistat Concentration (μM)
0
1
10
2
3

Figure S2

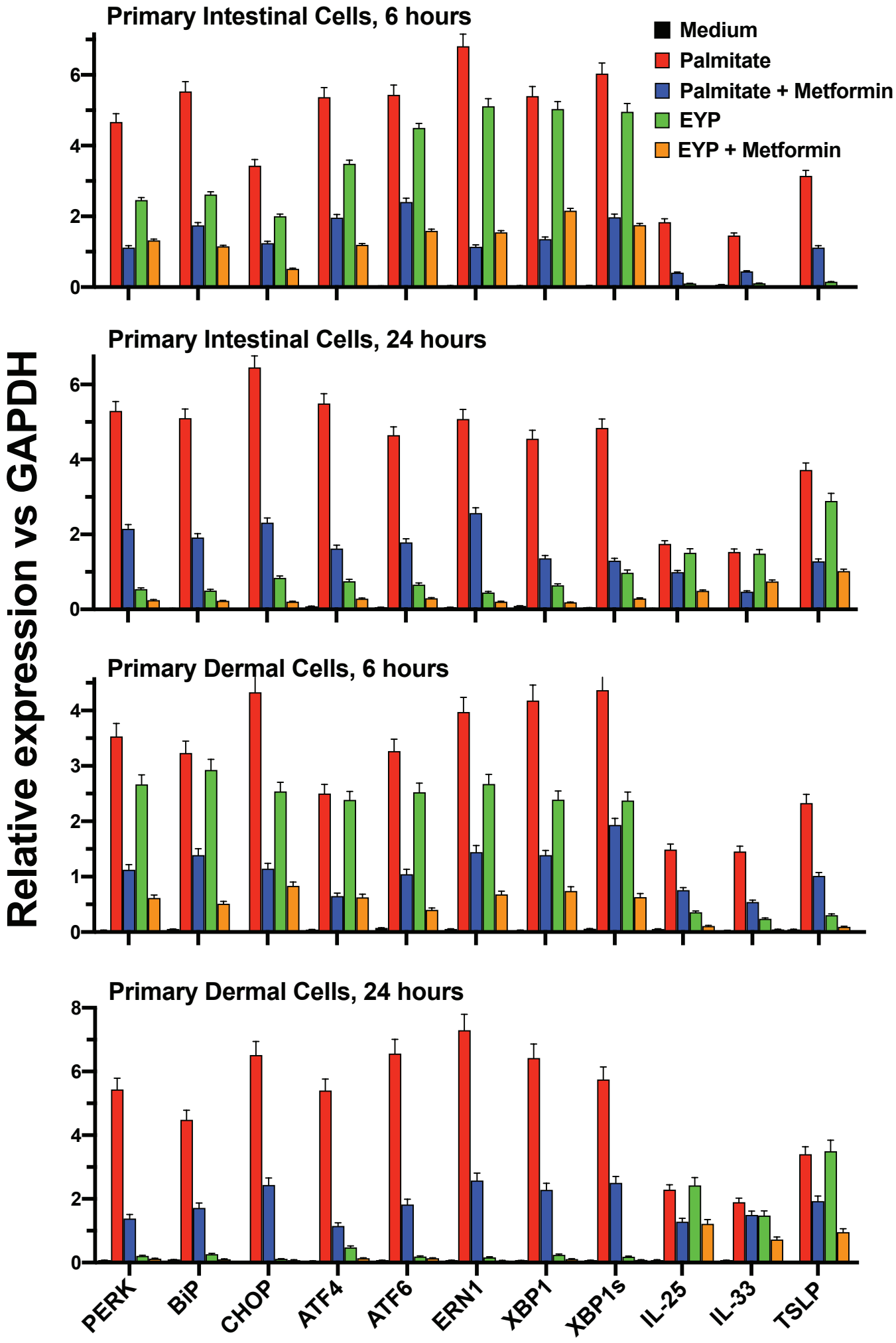
Primary Intestinal Cells, 6 hours
Medium
Palmitate
Palmitate + Metformin
EYP
EYP + Metformin
Primary Intestinal Cells, 24 hours
Primary Dermal Cells, 6 hours
Primary Dermal Cells, 24 hours
Relative expression vs GAPDH
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP

Figure S3

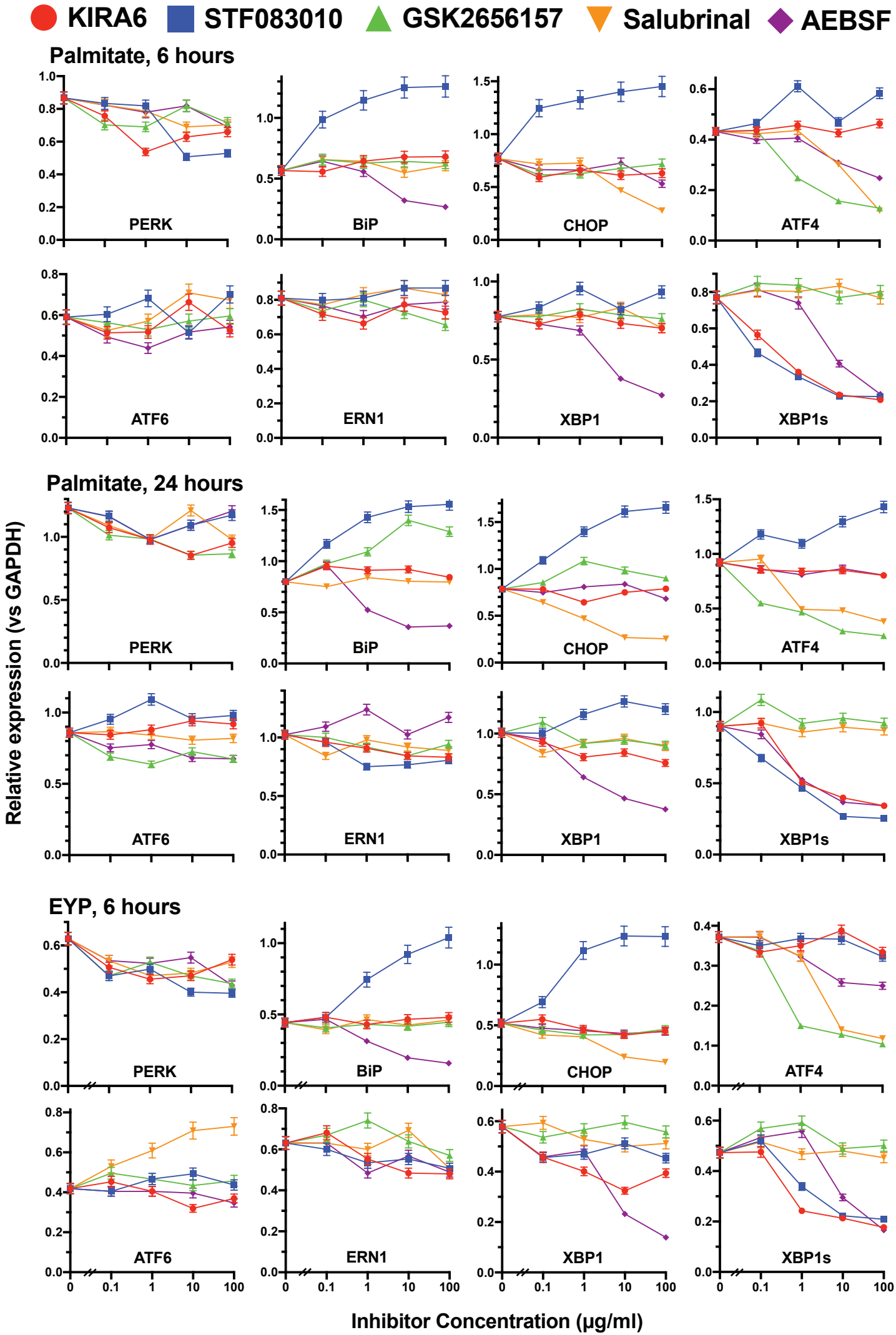
KIRA6
STF083010
GSK2656157
Salubrinal
AEBSF
Palmitate, 6 hours
Palmitate, 24 hours
EYP, 6 hours
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
Relative expression (vs GAPDH)
Inhibitor Concentration (µg/ml)

Figure S4

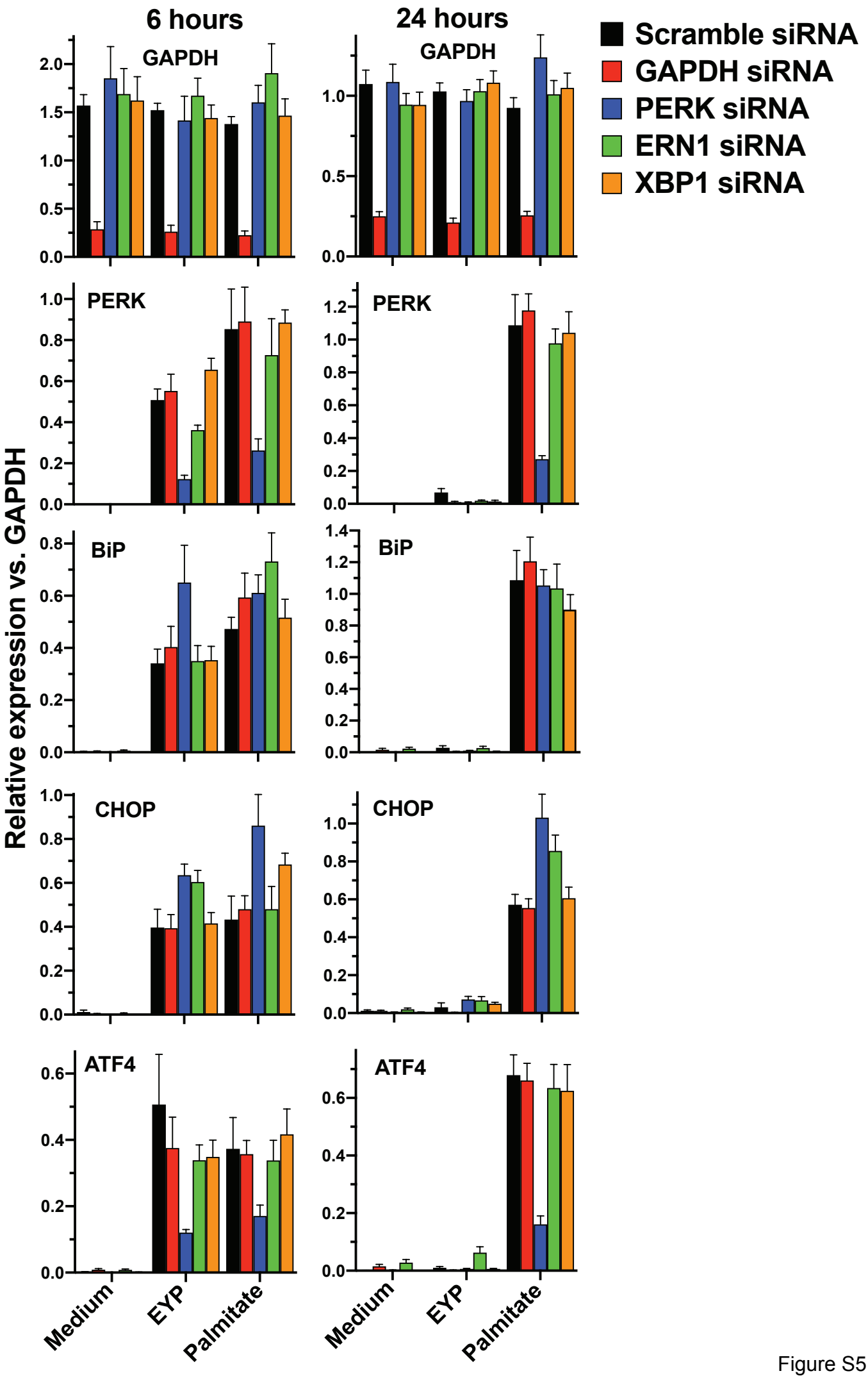
6 hours
24 hours
Scramble siRNA
GAPDH siRNA
PERK siRNA
ERN1 siRNA
XBP1 siRNA
Relative expression vs. GAPDH
GAPDH
PERK
BiP
CHOP
ATF4
Medium
EYP
Palmitate

Figure S5

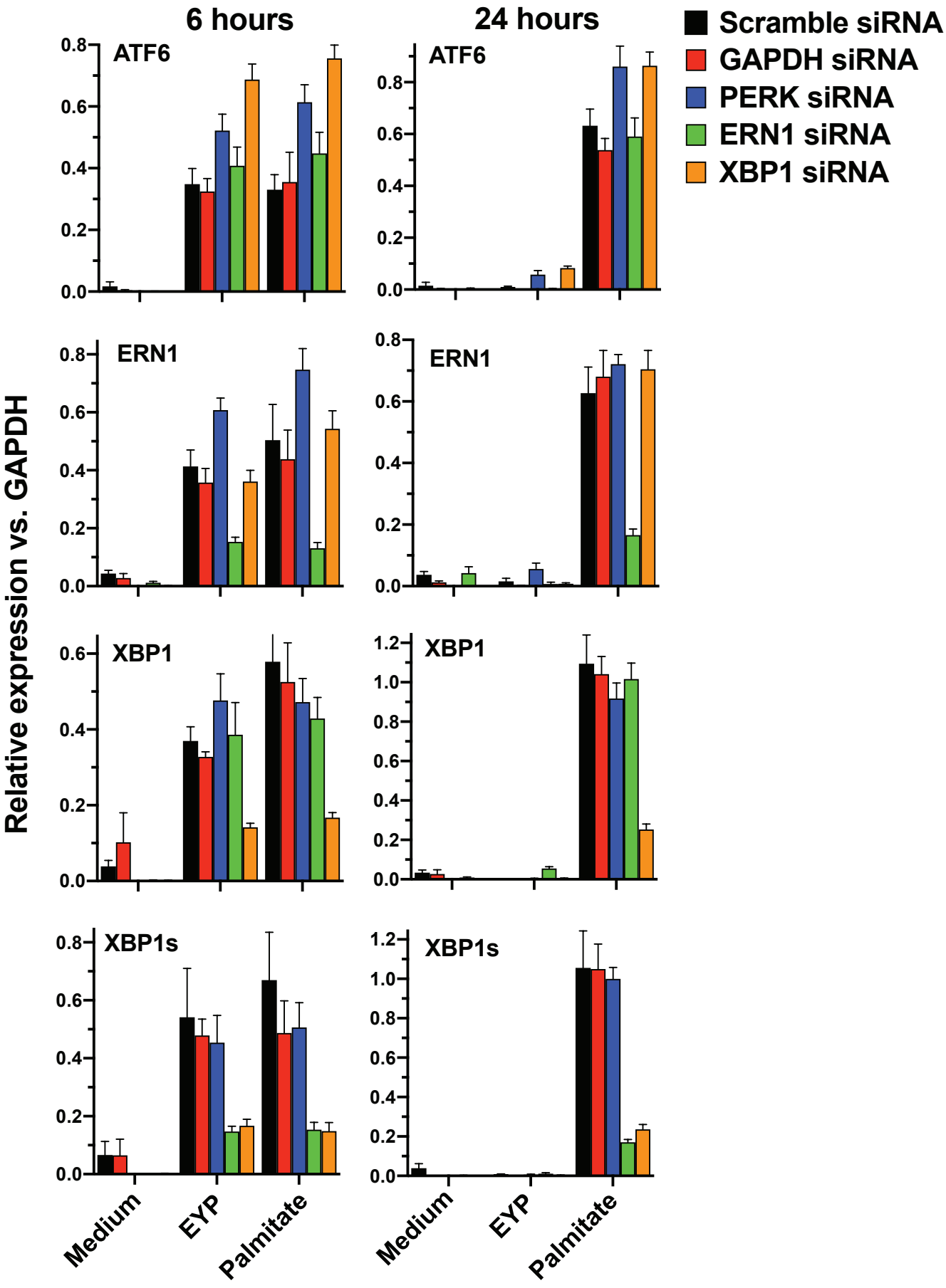
6 hours
24 hours
Scramble siRNA
GAPDH siRNA
PERK siRNA
ERN1 siRNA
XBP1 siRNA
Relative expression vs. GAPDH
ATF6
ERN1
XBP1
XBP1s
Medium
EYP
Palmitate

Figure S6

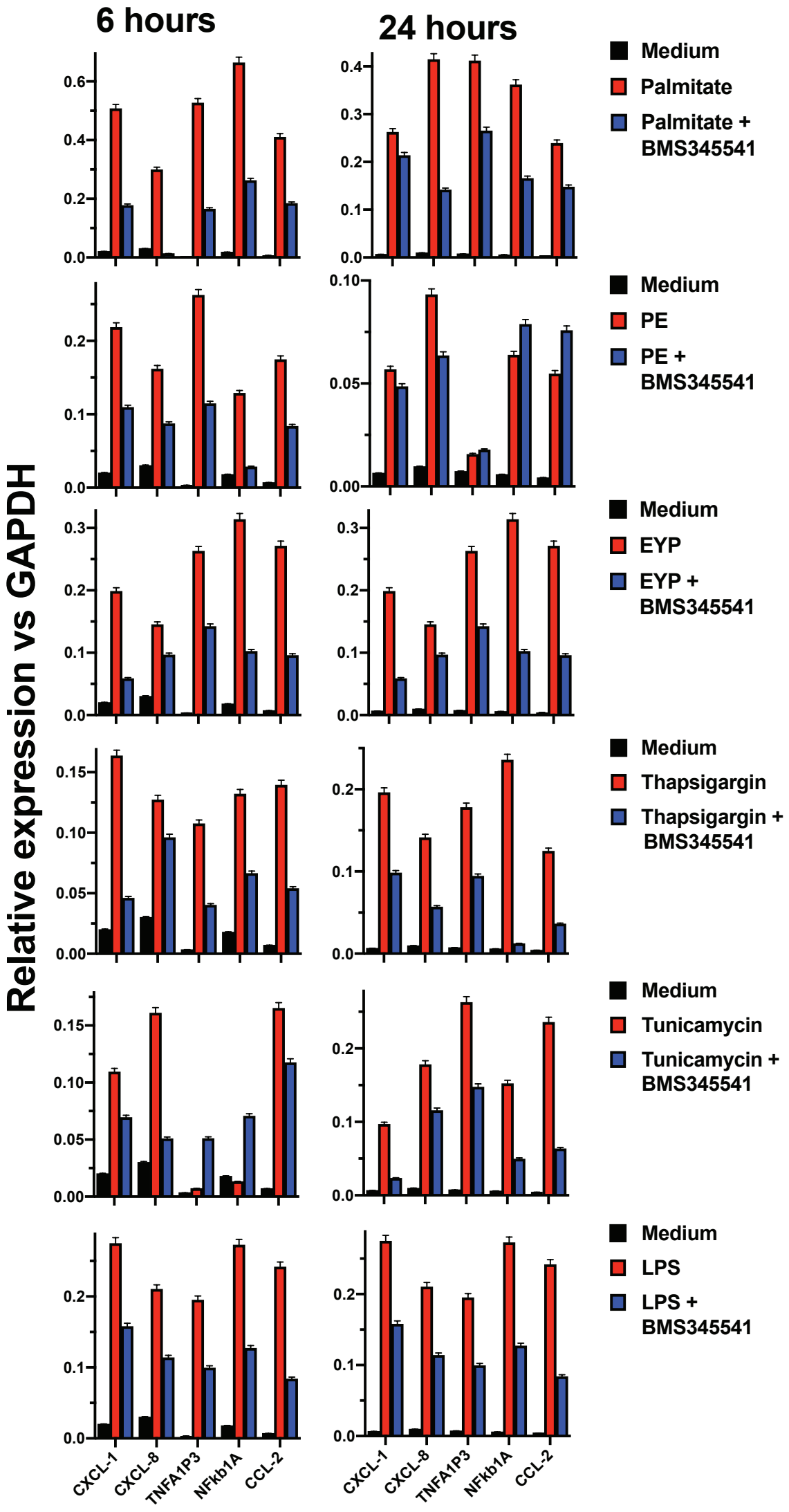
6 hours
24 hours
Relative expression vs GAPDH
Medium
Palmitate
Palmitate + BMS345541
Medium
PE
PE + BMS345541
Medium
EYP
EYP + BMS345541
Medium
Thapsigargin
Thapsigargin + BMS345541
Medium
Tunicamycin
Tunicamycin + BMS345541
Medium
LPS
LPS + BMS345541
CXCL-1
CXCL-8
TNFA1P3
NFkb1A
CCL-2

Figure S7

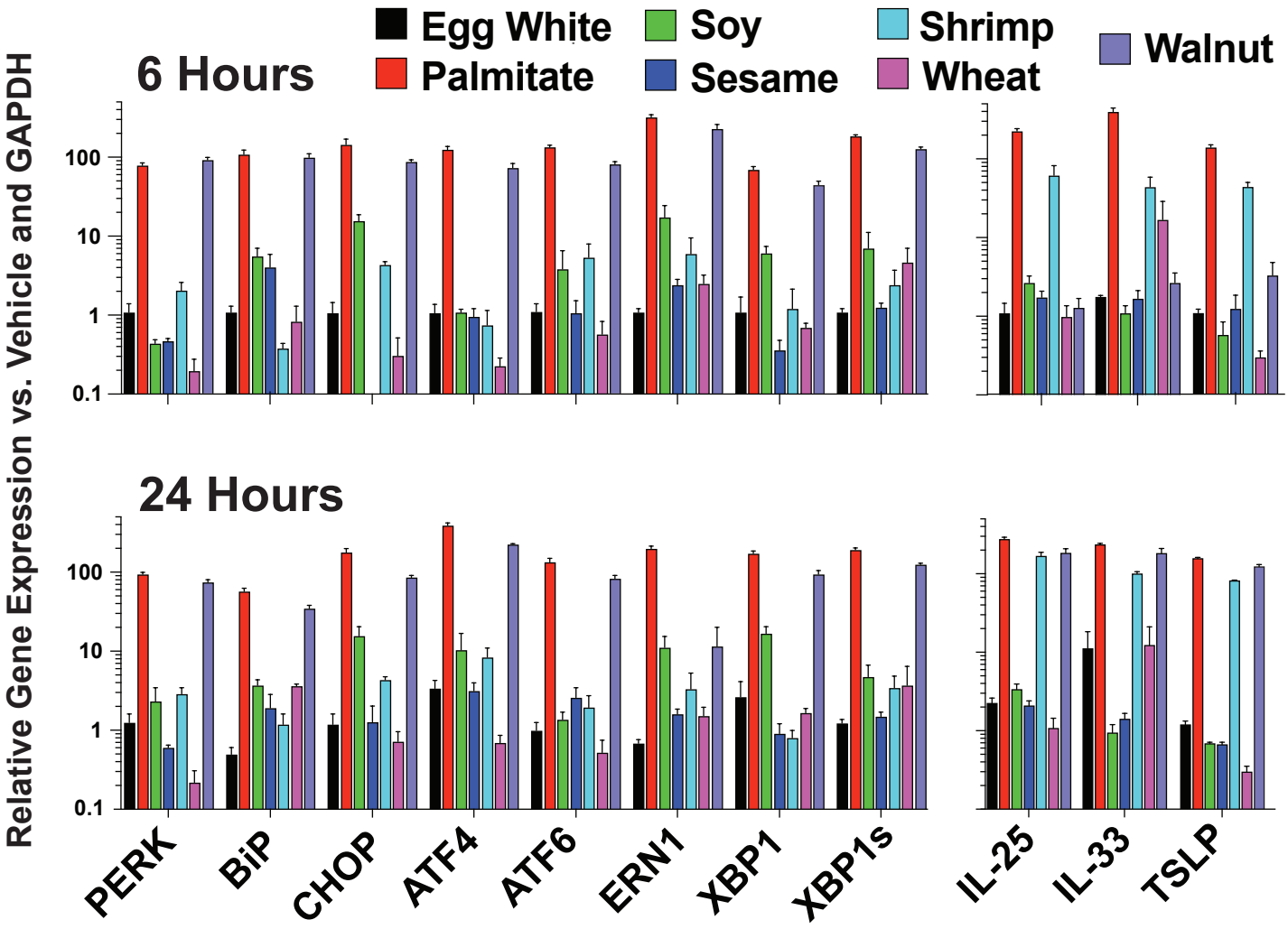
Egg White
Palmitate
Soy
Sesame
Shrimp
Wheat
Walnut
6 Hours
24 Hours
Relative Gene Expression vs. Vehicle and GAPDH
100
10
1
0.1
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP

Figure S8

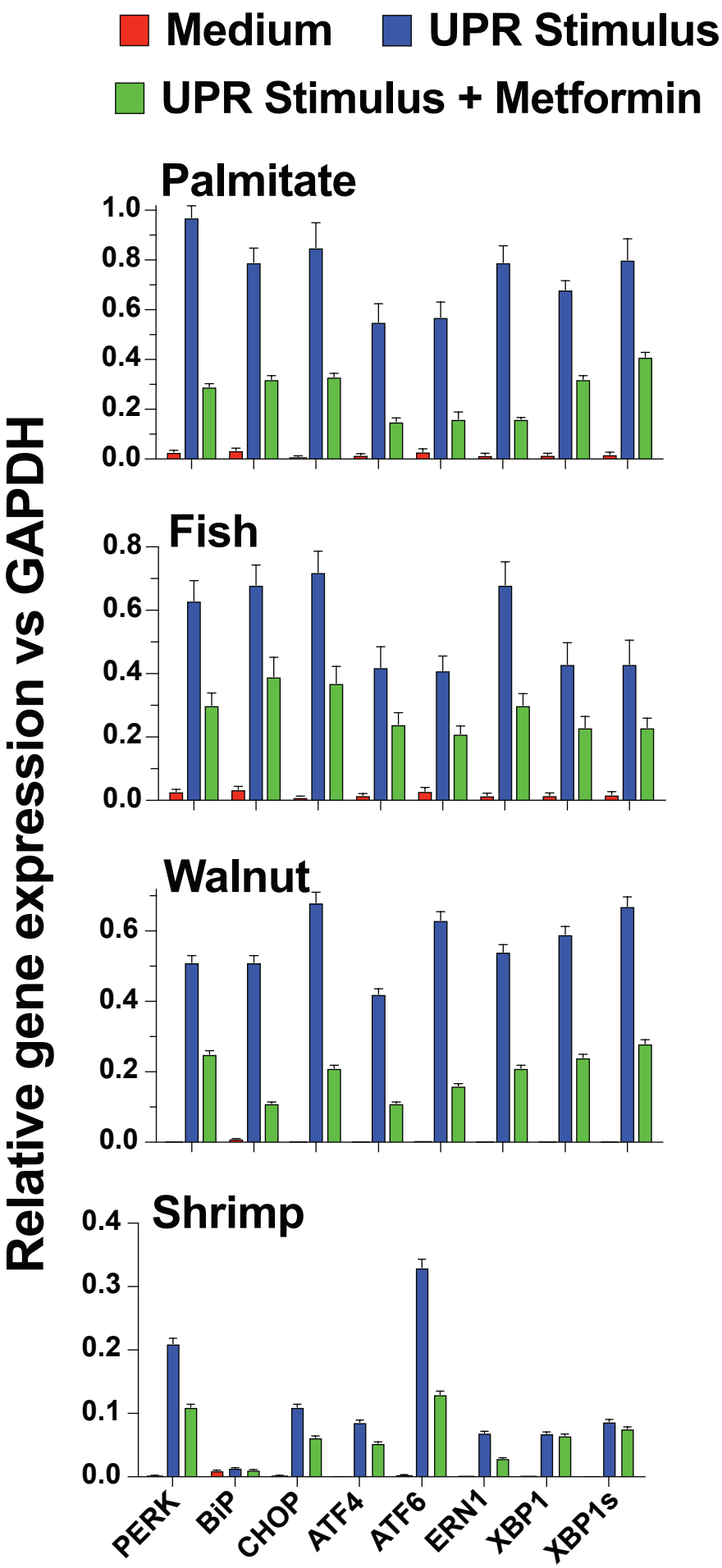
Medium
UPR Stimulus
UPR Stimulus + Metformin
Palmitate
Fish
Walnut
Shrimp
Relative gene expression vs GAPDH
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s

Figure S9

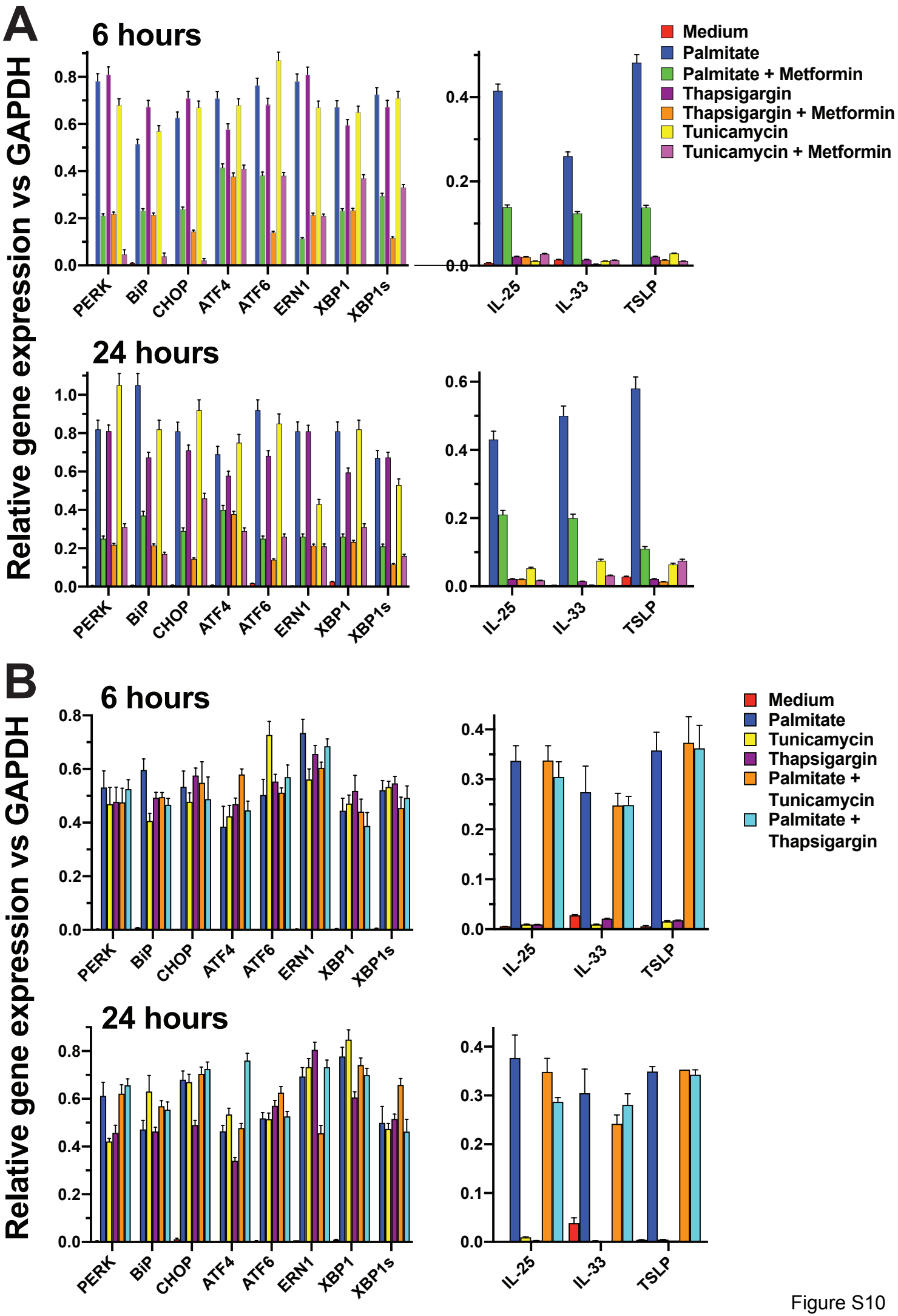
A
6 hours
24 hours
Relative gene expression vs GAPDH
Medium
Palmitate
Palmitate + Metformin
Thapsigargin
Thapsigargin + Metformin
Tunicamycin
Tunicamycin + Metformin
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP
B
6 hours
24 hours
Relative gene expression vs GAPDH
Medium
Palmitate
Tunicamycin
Thapsigargin
Palmitate + Tunicamycin
Palmitate + Thapsigargin

Figure S10

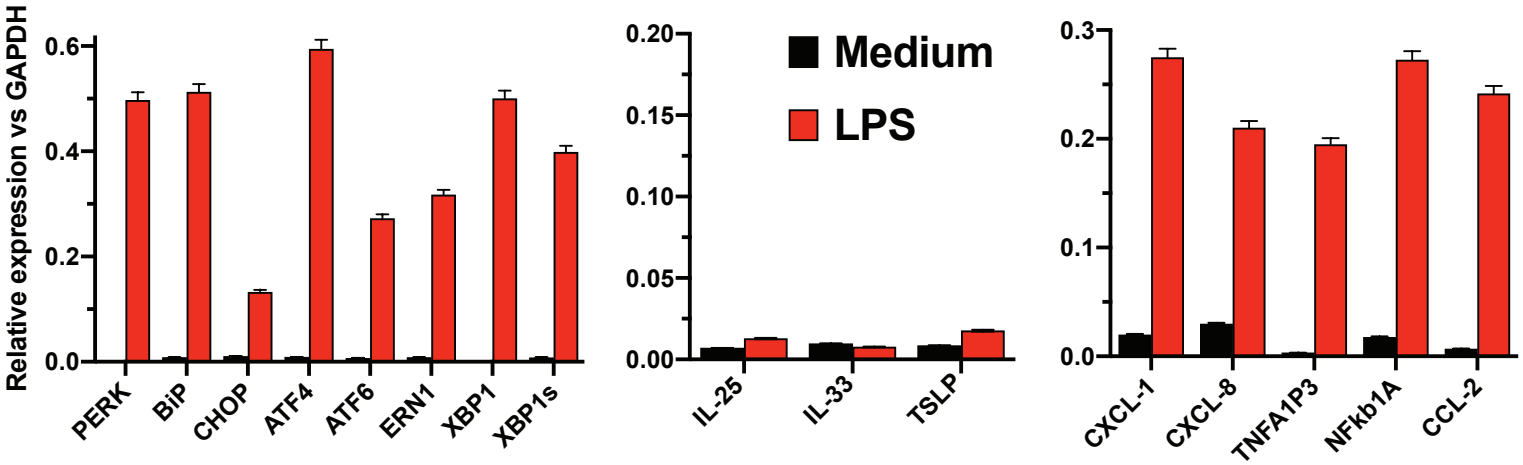
Relative expression vs GAPDH
0.6
0.4
0.2
0.0
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
0.20
0.15
0.10
0.05
0.00
IL-25
IL-33
TSLP
Medium
LPS
0.3
0.2
0.1
0.0
CXCL-1
CXCL-8
TNFA1P3
NFkb1A
CCL-2

Figure S11

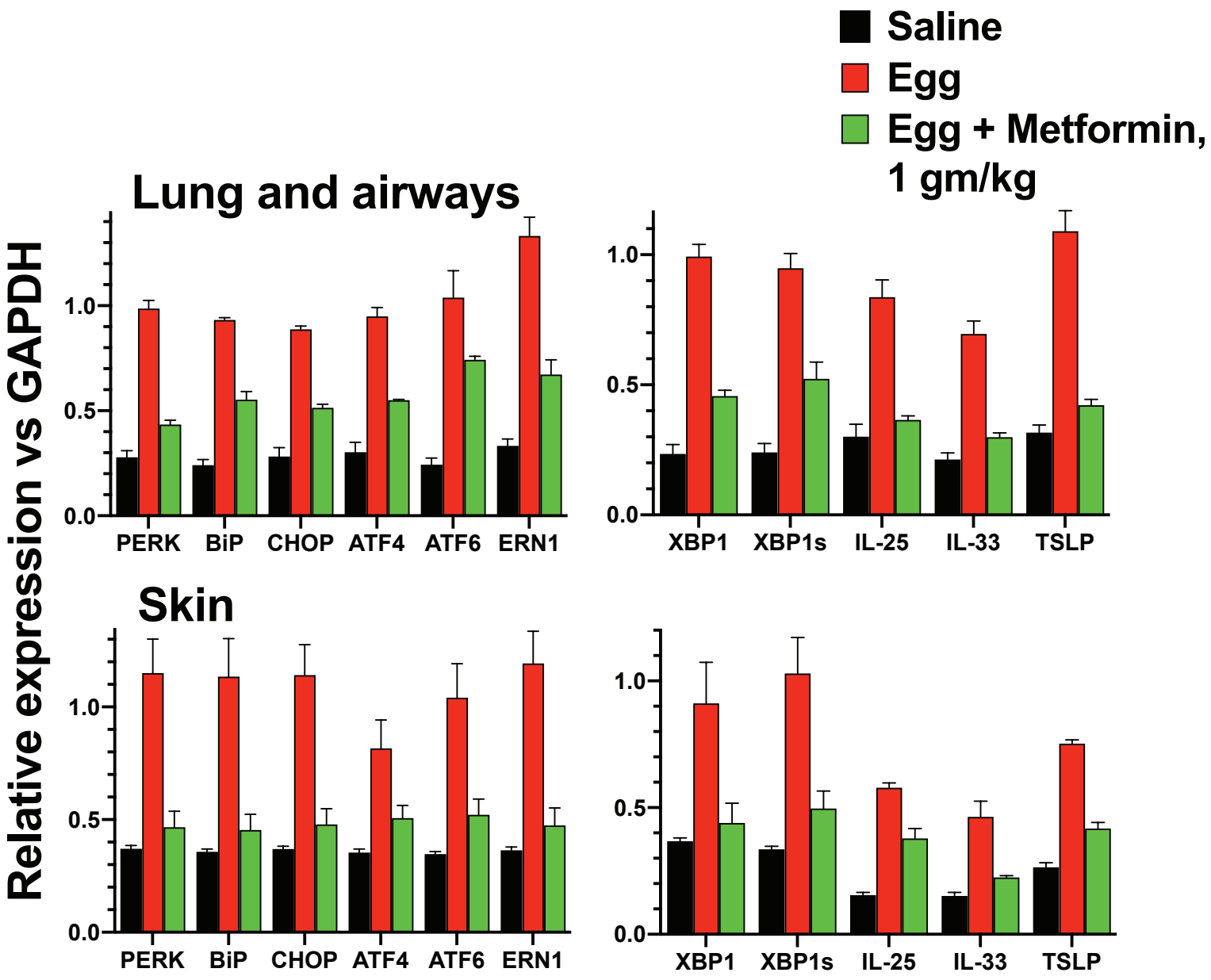
Saline
Egg
Egg + Metformin, 1 gm/kg
Lung and airways
Skin
Relative expression vs GAPDH
1.0
0.5
0.0
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP

Figure S12

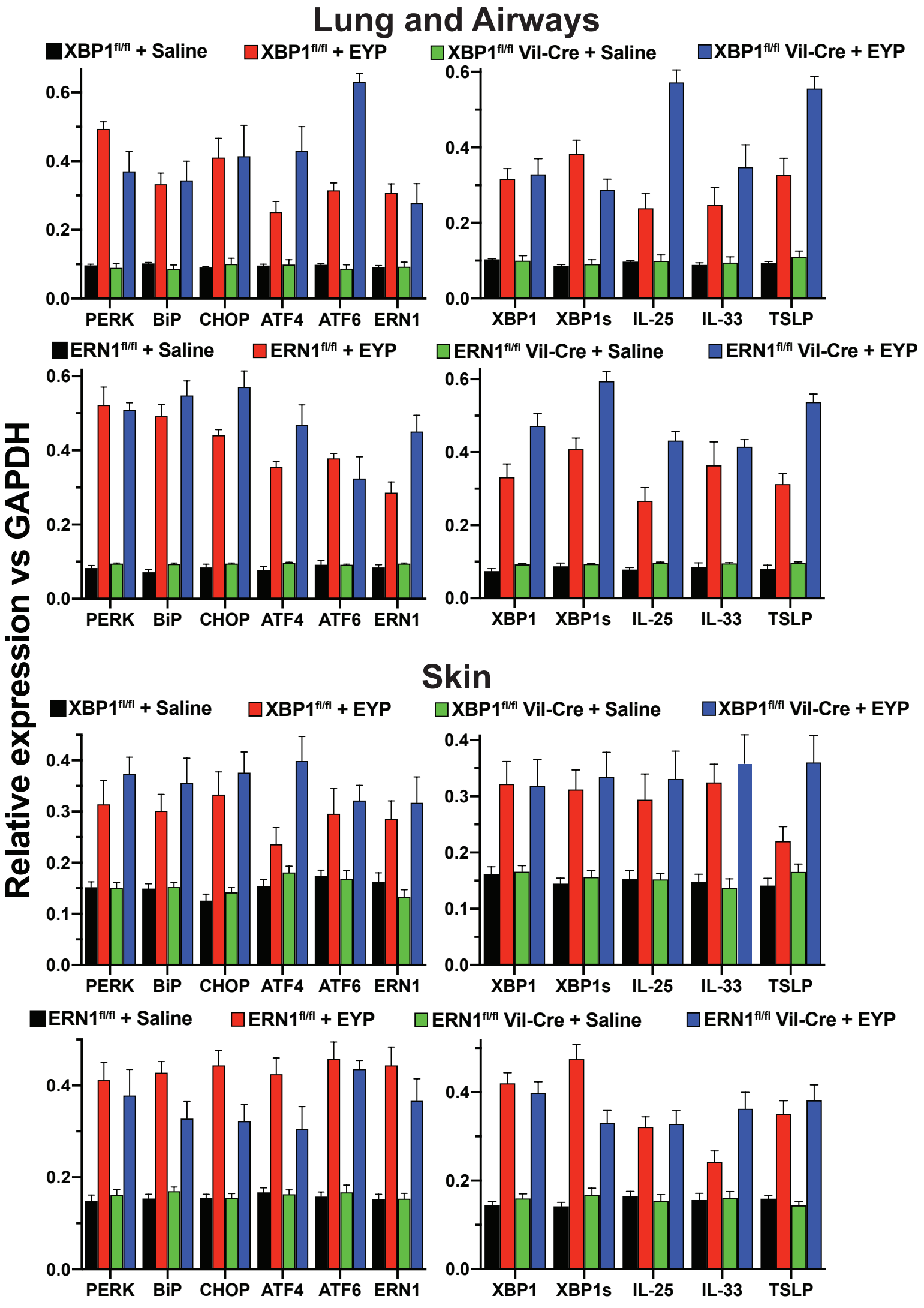
Lung and Airways
XBP1fl/fl + Saline
XBP1fl/fl + EYP
XBP1fl/fl Vil-Cre + Saline
XBP1fl/fl Vil-Cre + EYP
ERN1fl/fl + Saline
ERN1fl/fl + EYP
ERN1fl/fl Vil-Cre + Saline
ERN1fl/fl Vil-Cre + EYP
Relative expression vs GAPDH
0.0
0.2
0.4
0.6
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s
IL-25
IL-33
TSLP
Skin
0.0
0.1
0.2
0.3
0.4

Figure S13

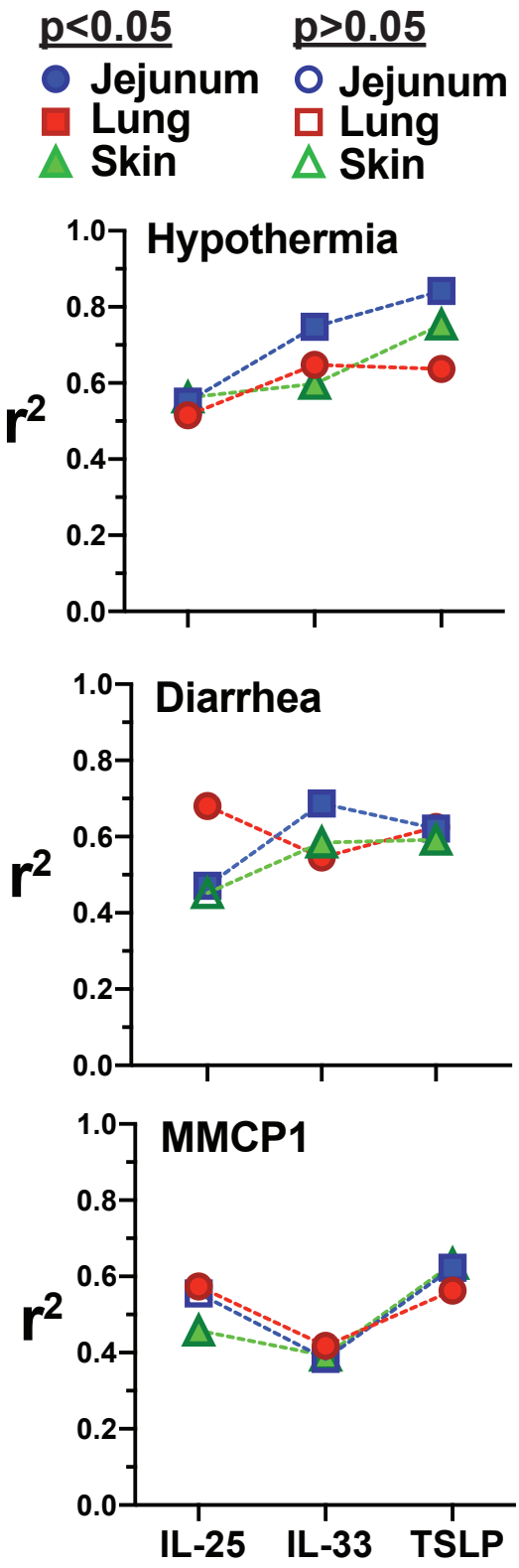

Figure S14

Correlations between pro-Th2 cytokines and diarrhea, hypothermia, MMCP1

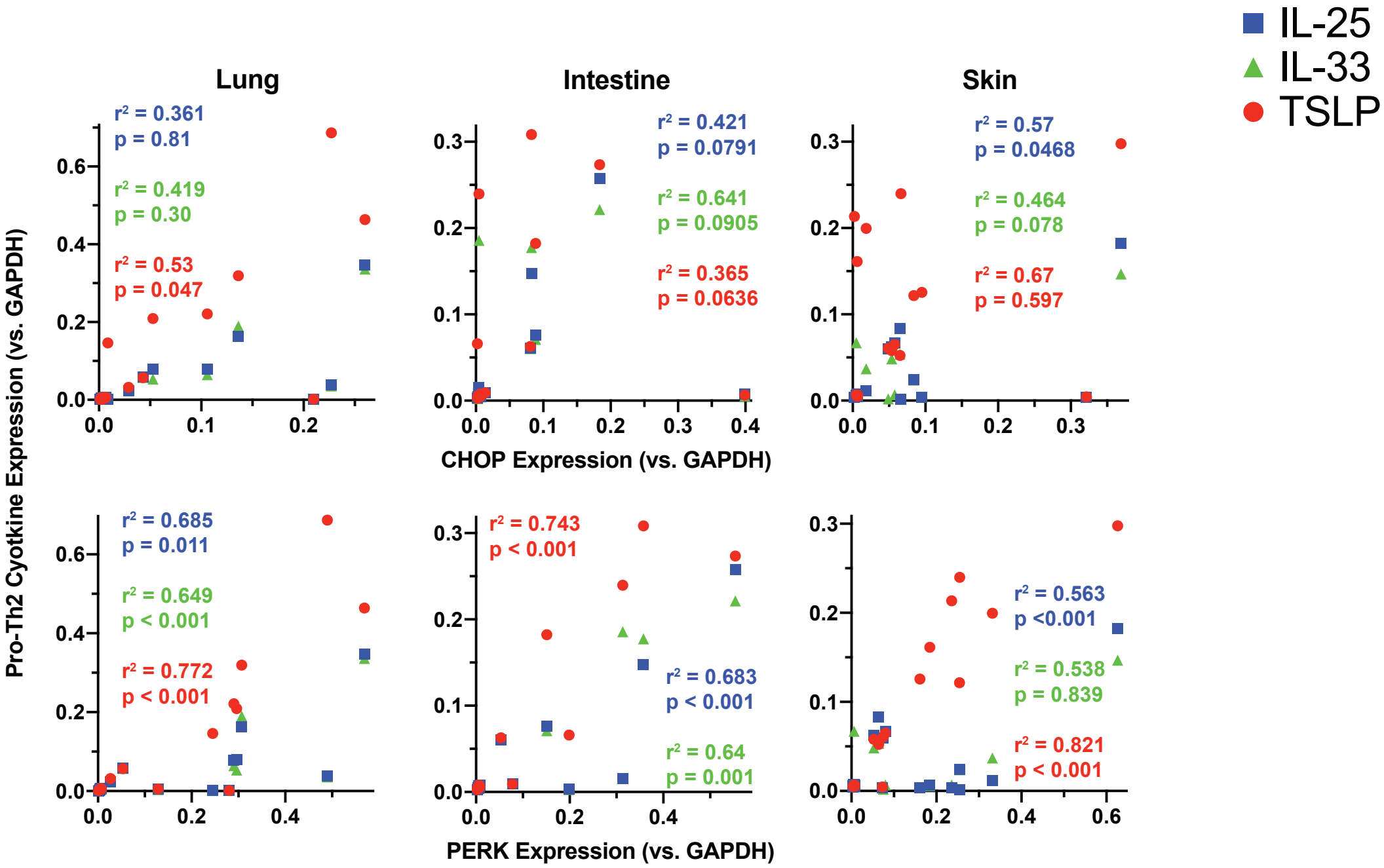
IL-25
IL-33
TSLP
Lung
Intestine
Skin
r² = 0.361
p = 0.81
r² = 0.419
p = 0.30
r² = 0.53
p = 0.047
r² = 0.421
p = 0.0791
r² = 0.641
p = 0.0905
r² = 0.365
p = 0.0636
r² = 0.57
p = 0.0468
r² = 0.464
p = 0.078
r² = 0.67
p = 0.597
CHOP Expression (vs. GAPDH)
Pro-Th2 Cyotkine Expression (vs. GAPDH)
r² = 0.685
p = 0.011
r² = 0.649
p < 0.001
r² = 0.772
p < 0.001
r² = 0.743
p < 0.001
r² = 0.683
p < 0.001
r² = 0.64
p = 0.001
r² = 0.563
p <0.001
r² = 0.538
p = 0.839
r² = 0.821
p < 0.001
PERK Expression (vs. GAPDH)

Figure S15

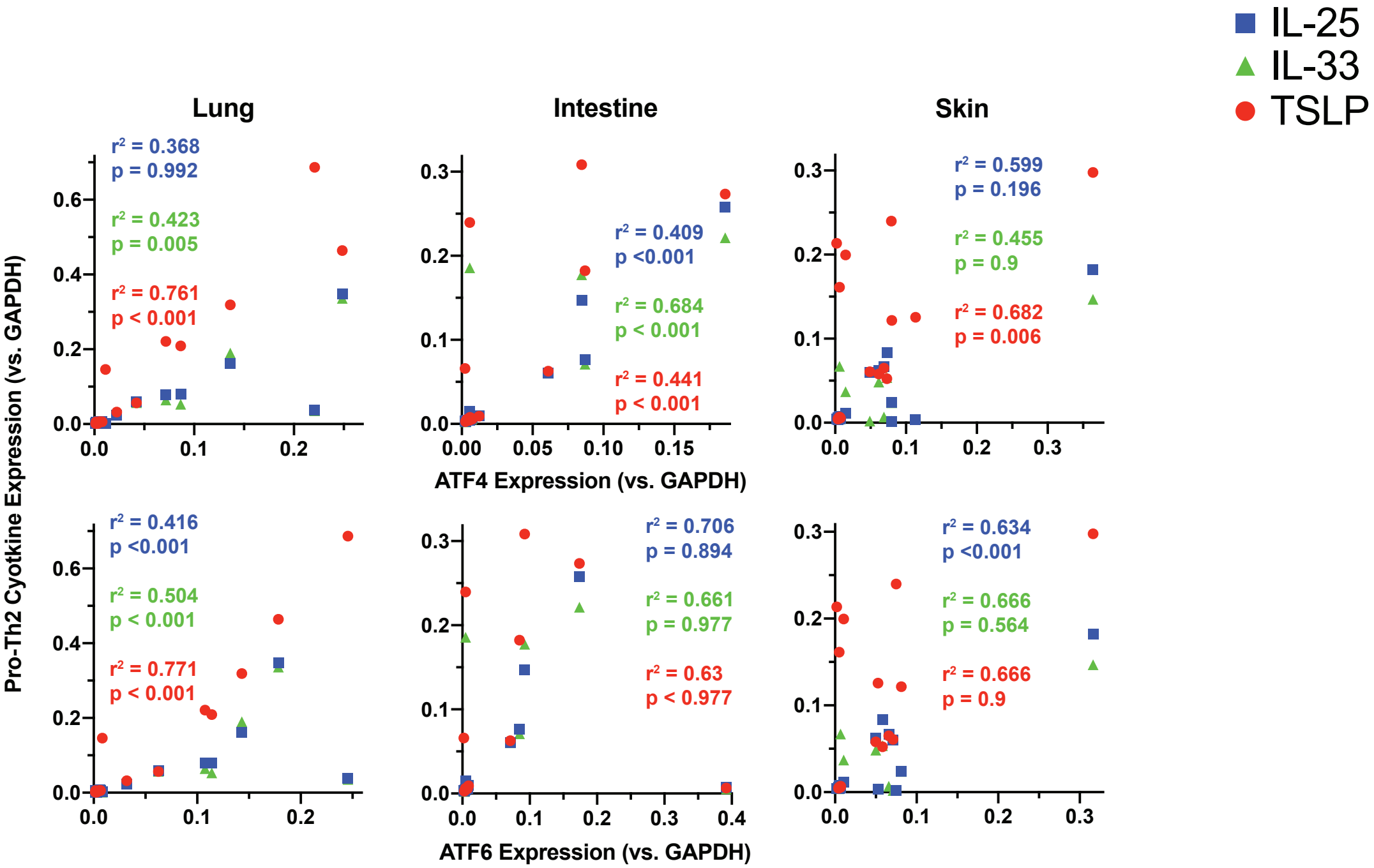
IL-25
IL-33
TSLP
Lung
Intestine
Skin
r² = 0.368
p = 0.992
r² = 0.423
p = 0.005
r² = 0.761
p < 0.001
r² = 0.409
p <0.001
r² = 0.684
p < 0.001
r² = 0.441
p < 0.001
r² = 0.599
p = 0.196
r² = 0.455
p = 0.9
r² = 0.682
p = 0.006
ATF4 Expression (vs. GAPDH)
r² = 0.416
p <0.001
r² = 0.504
p < 0.001
r² = 0.771
p < 0.001
r² = 0.706
p = 0.894
r² = 0.661
p = 0.977
r² = 0.63
p < 0.977
r² = 0.634
p <0.001
r² = 0.666
p = 0.564
r² = 0.666
p = 0.9
ATF6 Expression (vs. GAPDH)
Pro-Th2 Cyotkine Expression (vs. GAPDH)

Figure S16

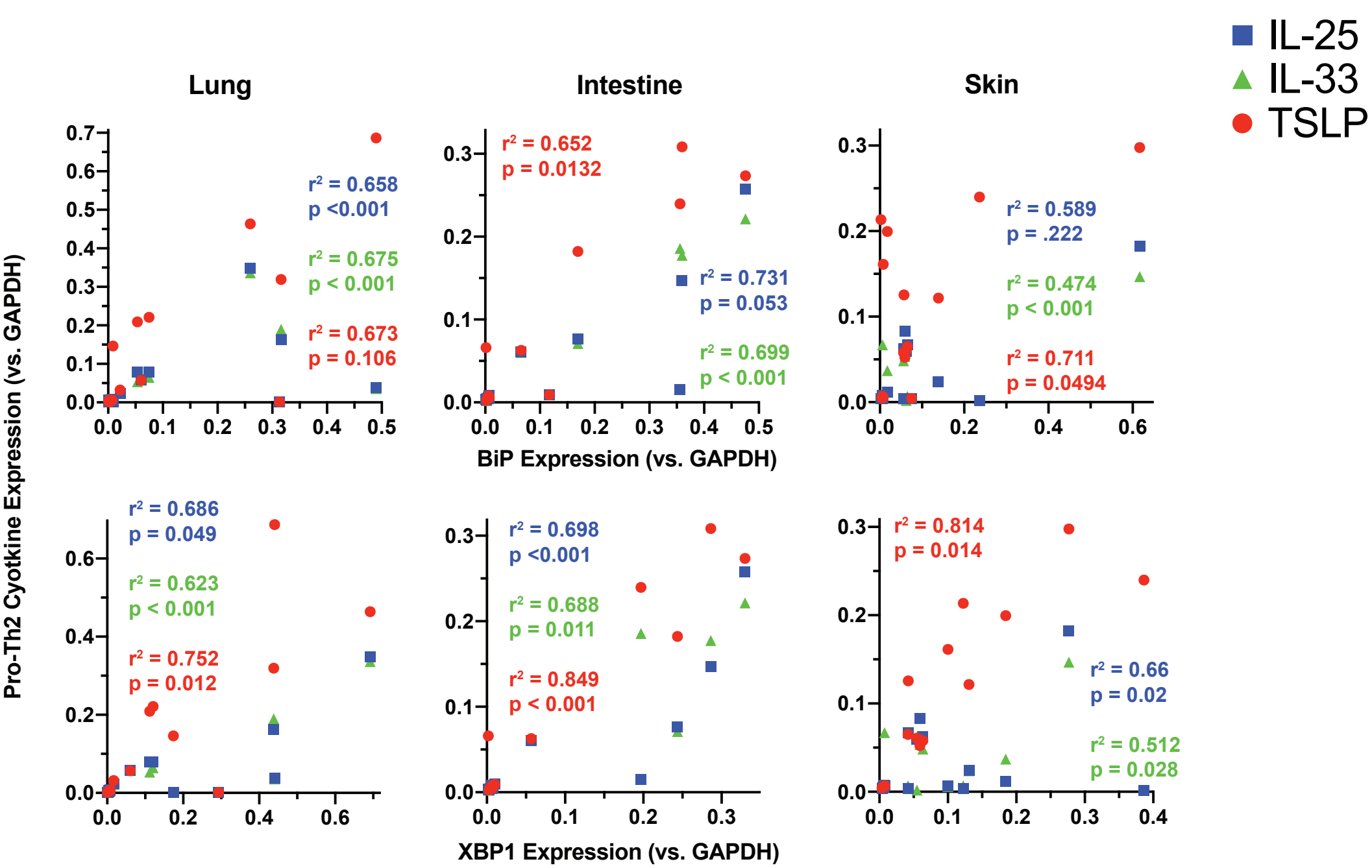
IL-25
IL-33
TSLP
Lung
Intestine
Skin
Pro-Th2 Cyotkine Expression (vs. GAPDH)
BiP Expression (vs. GAPDH)
XBP1 Expression (vs. GAPDH)
r² = 0.658
p <0.001
r² = 0.675
p < 0.001
r² = 0.673
p = 0.106
r² = 0.652
p = 0.0132
r² = 0.731
p = 0.053
r² = 0.699
p < 0.001
r² = 0.589
p = .222
r² = 0.474
p < 0.001
r² = 0.711
p = 0.0494
r² = 0.686
p = 0.049
r² = 0.623
p < 0.001
r² = 0.752
p = 0.012
r² = 0.698
p <0.001
r² = 0.688
p = 0.011
r² = 0.849
p < 0.001
r² = 0.814
p = 0.014
r² = 0.66
p = 0.02
r² = 0.512
p = 0.028

Figure S17

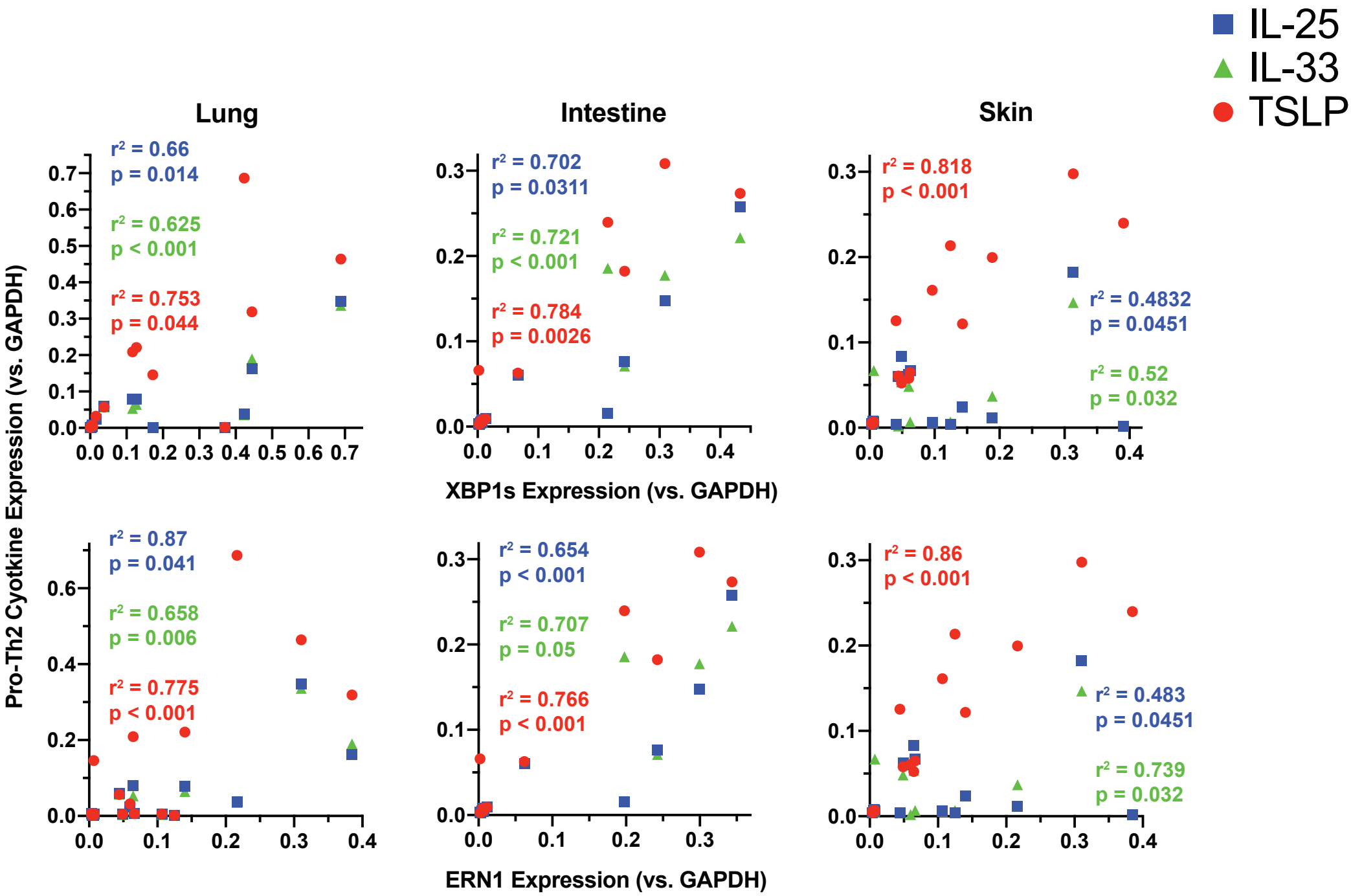
IL-25
IL-33
TSLP
Lung
Intestine
Skin
r² = 0.66
p = 0.014
r² = 0.625
p < 0.001
r² = 0.753
p = 0.044
r² = 0.702
p = 0.0311
r² = 0.721
p < 0.001
r² = 0.784
p = 0.0026
r² = 0.818
p < 0.001
r² = 0.4832
p = 0.0451
r² = 0.52
p = 0.032
XBP1s Expression (vs. GAPDH)
Pro-Th2 Cyotkine Expression (vs. GAPDH)
r² = 0.87
p = 0.041
r² = 0.658
p = 0.006
r² = 0.775
p < 0.001
r² = 0.654
p < 0.001
r² = 0.707
p = 0.05
r² = 0.766
p < 0.001
r² = 0.86
p < 0.001
r² = 0.483
p = 0.0451
r² = 0.739
p = 0.032
ERN1 Expression (vs. GAPDH)

Figure S18

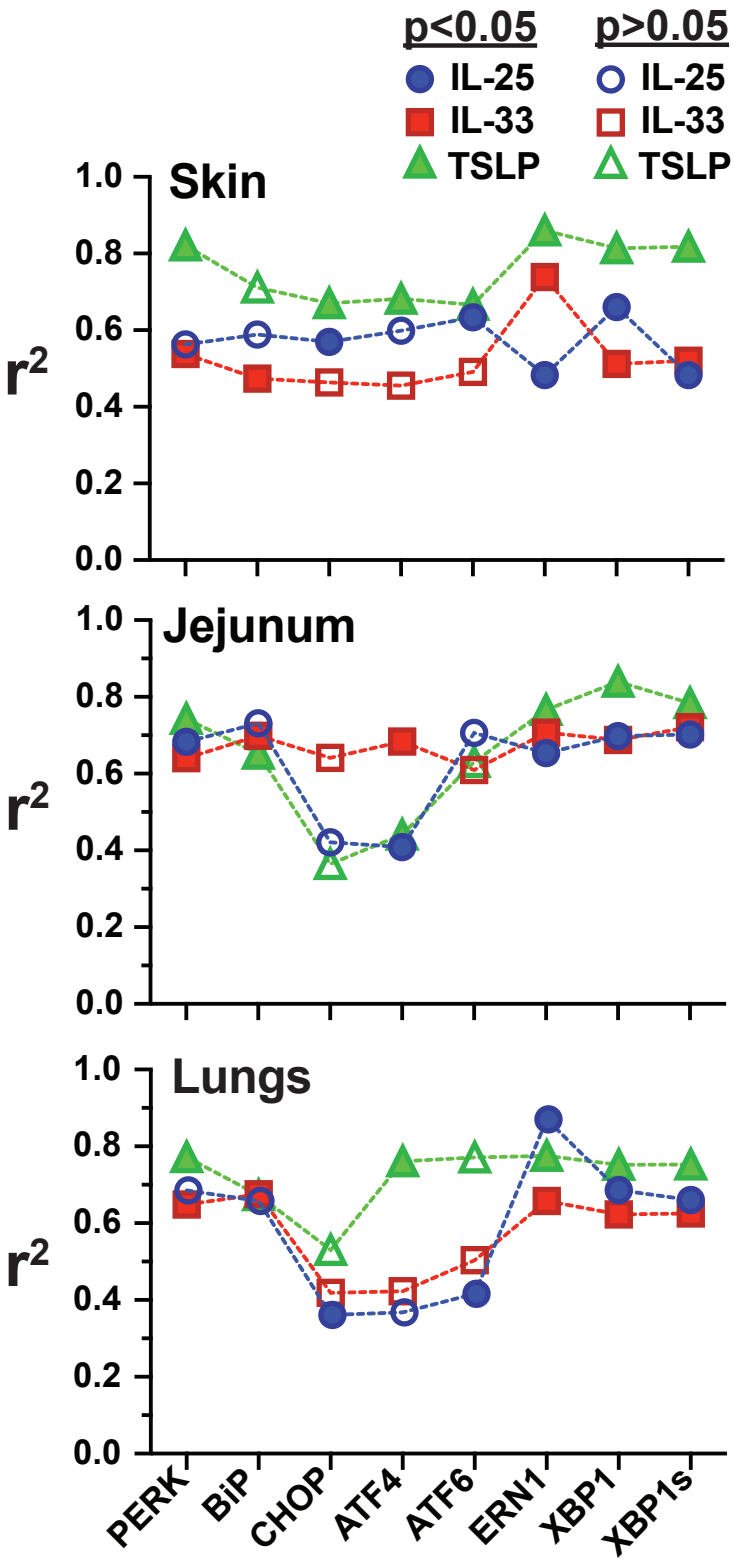
p<0.05
p>0.05
IL-25
IL-33
TSLP
IL-25
IL-33
TSLP
Skin
Jejunum
Lungs
r2
1.0
0.8
0.6
0.4
0.2
0.0
PERK
BiP
CHOP
ATF4
ATF6
ERN1
XBP1
XBP1s

Figure S19