



Inflammation and ER stress differentially regulate STAMP2 expression and localization in adipocytes

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ABSTRACT

Background: Chronic ER stress and dysfunction is a hallmark of obesity and a critical contributor to metaflammation, abnormal hormone action and altered substrate metabolism in metabolic tissues, such as liver and adipocytes. Lack of STAMP2 in lean mice induces inflammation and insulin resistance on a regular diet, and it is dysregulated in the adipose tissue of obese mice and humans. We hypothesized that the regulation of STAMP2 is disrupted by ER stress.

Methods: 3T3-L1 and MEF adipocytes were treated with ER stress inducers thapsigargin and tunicamycin, and inflammation inducer TNF α . The treatments effect on STAMP2 expression and enzymatic function was assessed. In addition, 3T3-L1 adipocytes and HEK cells were utilized for *Stamp2* promoter activity investigation performed with luciferase and ChIP assays.

Results: ER stress significantly reduced both STAMP2 mRNA and protein expression in cultured adipocytes whereas TNF α had the opposite effect. Concomitant with loss of STAMP2 expression during ER stress, intracellular localization of STAMP2 was altered and total iron reductase activity was reduced. *Stamp2* promoter analysis by reporter assays and chromatin immunoprecipitation, showed that induction of ER stress disrupts C/EBP α -mediated STAMP2 expression.

Conclusion: These data suggest a clear link between ER stress and quantitative and functional STAMP2-deficiency.

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1. Introduction

Proper endoplasmic reticulum (ER) activity is of paramount importance for cellular metabolism, function, and survival. In recent years, ER has also emerged as a critical regulator of systemic metabolic homeostasis. Chronic nutrient overload, inflammation and aberrant metabolic activity all lead to disruption of normal ER function, accumulation of unfolded proteins and activation of the three canonical branches of the unfolded protein response (UPR) [1]. Prolonged UPR activation, in turn, induces ER stress and impairs normal cellular

function through activation of the inflammatory and stress-signaling networks, such as nuclear factor-kappa B (NF- κ B) and c-Jun N-terminal kinase (JNK). In addition, reactive oxygen species (ROS) are produced, which are directly linked to the development of pathologies such as obesity, insulin resistance and type 2 diabetes [2–4]. The impact of ER function on metabolism and the underlying mechanisms have been studied in detail in hepatocytes and beta cells of the pancreas, but there is limited information on this in adipocytes. While ER function is linked to triglyceride uptake and release, cholesterol and nutrient sensing, as well as adipokine secretion, some of the canonical UPR pathways, such as IRE1 α -XBP1 and ATF6 branches and their targets, have limited impact on adipocyte metabolic function [5–7]. Hence, it is of interest to identify additional metabolically critical molecules that are linked to ER function and ER stress in adipocytes.

The six transmembrane protein of prostate (STAMP, also known as six transmembrane epithelial antigen of prostate – STEAP) family include transmembrane proteins with oxidoreductase-metalloreductase activity with links to inflammation, metabolic disorders, and cancer [8]. In mice, STAMP2 plays an important role in systemic metabolic

Abbreviations: STAMP2, six transmembrane protein of prostate 2; C/EBP α , CCAAT/enhancer binding protein alpha; TNF α , tumor necrosis factor alpha; Tg, thapsigargin; Tu, tunicamycin.

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homeostasis and its absence results in aberrant inflammatory responses to nutrients in adipose tissue [9]. *Stamp2*^{-/-} mice develop spontaneous metabolic disease on a regular diet, manifesting increased adiposity, inflammation, insulin resistance, glucose intolerance, mild hyperglycemia, dyslipidemia, and fatty liver disease. Recent studies have further found that STAMP2 has a protective role in both obese and genetic diabetic mouse models through improved insulin resistance and decreased atherosclerosis [10–12]. In humans, correlation between obesity and STAMP2 expression has been mixed: STAMP2 expression is up- [13,14] or down-regulated [15,16] in the adipose tissue of obese individuals compared to lean ones. Two of these studies [15,16] found that STAMP2 expression in human adipocytes is also induced by tumor necrosis factor alpha (TNF α), consistent with earlier findings in mice [17].

We have previously shown that CCAAT/enhancer binding protein alpha (C/EBP α) increases *Stamp2* promoter activity in HeLa cells [9]. As C/EBP α is down-regulated by TNF α [18], it is possible that two distinct regulatory pathways, adipogenic and inflammatory, control STAMP2 expression and/or action. In an independent study, both C/EBP α and signal transducer and activator of transcription 3 (STAT3) were found to bind to the *Stamp2* promoter in murine liver cells upon interleukin 6 (IL-6) treatment [19]. Interestingly, feeding and fasting cycles affected C/EBP α binding to the *Stamp2* promoter, but not that of STAT3 [19]. This suggests that transcriptional regulation of *Stamp2* expression may play a role in the response to nutritional and inflammatory stress and contributes to the protective effects of STAMP2 *in vivo*.

One potential point of intersection between nutritional and inflammatory pathways is the ER, both in healthy tissue and during metabolic disease. Obesity in mice and humans induces both ER stress and metabolic dysfunction, similar to defective STAMP2 expression or regulation [9,20]. Here we investigate potential regulation of STAMP2 expression/function by ER stress and delineate the factors that may be involved in this process.

2. Materials and Methods

2.1. Animal Experiments

Six-week-old male wild type littermates of the C57BL/6J (Jackson Labs) background bred in house, were randomly placed on a high-fat diet (Diet F3282; Bio-serve, French-town, NJ) or fed standard breeding chow (PicoLabs, Mouse Diet 20) for 8 weeks with *ad libitum* feeding. Minimal group sizes (4 animals in each) were chosen based on two-sided student *t*-test assuming 5% significance level, 90% power, and a high signal to noise ratio. All mice were maintained in separate cages on a 12-hour-light/12-hour-dark cycle in a pathogen-free barrier facility with free access to water. Body weight was measured every other week. The Institutional Animal Care and Use Committee (Harvard School of Public Health) approved all studies.

2.2. Cell Lines and Cell Culture

HeLa and 3T3-L1 cells were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 10% fetal bovine serum (FBS) (Saveen Werner), 50 U/ml penicillin-streptomycin (Lonza) and 2 mM L-Glutamine (Lonza) or DMEM:F12 Glutamax (Invitrogen) medium supplemented with 10% FBS and Penicillin-Streptomycin in a 5% CO₂ humidified atmosphere. 3T3-L1 cells were differentiated to adipocytes as previously reported [21]. To make stable reporter cells, linearized pGL2-BASIC-LUC or pGL2-BASIC-2 kb-ST2-LUC was co-transfected with the linearized selection vector pPur (Clontech) in a 5:1 ratio using Lipofectamine 2000 (Invitrogen) as transfection agent. After 10 days of selection with 2 μ g/ml puromycin, surviving cells were pooled and expanded. All treatments were done by incubating cells with DMSO (Ctrl), 10 ng/ml TNF α (Sigma-Aldrich), 2 ng/ml Tu

(Cambrex), or 300 nM Tg (Sigma-Aldrich) for 24 h unless otherwise indicated.

2.3. Isolation of Primary Preadipocytes

Visceral adipose tissue was dissected from euthanized 9–10 week-old male and female *Stamp2*-deficient (*St2*^{-/-}) or wild-type (WT) mice, previously reported [9]. The adipose tissue was digested with collagenase II and filtered through a 240 μ m nylon mesh. The adipocyte and stroma-vascular fraction (SVF) was separated by centrifugation at 50g for 5 min. The SVF fraction was further processed as previously reported [22]. Briefly, cells were centrifuged at 200g for 10 min to pellet the SVF cells and incubated with a red blood cell lysis buffer for 5 min to remove red blood cells. Cell clumps were then removed by a 20 μ m mesh nylon filter (Millipore), and the remaining cells were counted and plated in DMEM:F12 in 12-well plates. When cells reached confluency, they were differentiated into adipocytes by the standard adipogenesis protocol with 1 μ M pioglitazone added to the mix.

2.4. Lentivirus Production and Establishment of Stable 3T3-L1 Cell Line

pTriPz plasmid (Open Biosystems) containing rat *Cebpa* CDS was transfected together with a packaging plasmid (pCMV- Δ R8.2) and an envelope plasmid (pCMV-VGS-G) into HEK293T cells using Lipofectamine 3000 (ThermoFisher). 48 h post-transfection conditioned medium was harvested, filtered through a 0.45 μ m filter (Millipore) and added to 3T3-L1 fibroblasts. 36 h post-infection the 3T3-L1 cells were subjected to pool selection with 1 μ g/ml puromycin for 7 days. To induce rat C/EBP α expression cells were treated with 500 ng/ml doxycycline (Sigma-Aldrich) for 48 h.

2.5. Reporter Assays

The 2 kb fragment upstream of the putative mouse *Stamp2* transcription initiation start site was amplified by PCR using Phusion proof-reading polymerase (Finnzymes) and the BAC clone RP23-212F6 (Invitrogen) as a template. The PCR product was inserted into pCR-Blunt II-TOPO (Invitrogen) and sequenced. The 2 kb insert was excised out with *Hind*III and *Xho*I and ligated into the pGL2-BASIC reporter plasmid (Promega) using the same sites. Empty pcDNA3 and pcDNA3-p50 expression plasmids, or AllStar and *NFkB1* or *CEBPA* (all from Qiagen) siRNA were used in co-transfection experiments as indicated. Lipofectamine 3000 (Invitrogen), FuGene (Roche), or PEI (Sigma-Aldrich) were used as transfection agents. To detect luciferase activity, cells were lysed 48 h post-transfection in luciferase lysis buffer (25 mM Tris-HCl, pH 7.8, 2 mM DTT, 10% glycerol, 1% Triton-X). 100 μ l luciferin buffer containing 0.2 mM D-Luciferin (Anaspec) and 2 mM ATP, was injected to 10 μ l cell extract and luciferase activity was detected by the multiplate reader Victor 2 (PerkinElmer). Activity was normalized to protein concentration.

2.6. Serial Deletion

Using the -2 kb-ST2-LUC plasmid as template, a construct series with predicted NF κ B and C/EBP α binding sites removed were created using Q5[®] Site-Directed Mutagenesis Kit (NEB). Primers forward/reverse (promoter length):

ACTTTGCTTCACAGCTCC/AAGGGCGAATTCTGCAGA (1229 bp); GAG AACGTCATCTGCGA/AAGGGCGAATTCTGCAGATA (531 bp); AAAGGA GTATCTCTGCGC/AAGGGCGAATTCTGCAGA (387 bp); GCTCTGACTCT GTAAAAATCAG/AAGGGCGAATTCTGCAGA (286 bp); ATGCCCTAACC ATGTAC/AAGGGCGAATTCTGCAGA (204 bp).

2.7. Mutagenesis

Predicted C/EBP binding sites were mutated by the GeneTailor Site-Directed Mutagenesis system (Invitrogen) using the –2 kb-ST2-LCU promoter construct as template. C/EBP binding site 1 (–480 bp from TSS) was converted from TTGCGAAA to CATGGTTC. Binding site 2 (–180 bp from TSS) was converted from TTTCGTAA to CATGGTTC. All constructs were validated by sequencing.

2.8. ChIP Assay

ChIP experiments were carried out according to standard protocols (Upstate Biotechnology) with a crosslinking step (1% formaldehyde at 37 °C), followed by a quenching step with 125 mM glycine. Chromatin was sheared using the Bioruptor sonicator (Diagenode). Sonicated chromatin (350 µg) was immunoprecipitated with antibodies against anti-C/EBPα antibody (Santa Cruz, sc-61 or Abcam, ab40764) or a non-specific polyclonal antibody. Immune complexes were captured with protein A-agarose beads, and eluted in SDS buffer. Formaldehyde crosslinking was reversed, followed by DNA purification using phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) and quantified by qRT-PCR using the following primers: distal *Stamp2* promoter forward 5'-GTT CTT TTC TGG CCT ACA GAT AGT-3', reverse 5'-GGC ATC TCA CTC CTT AA GAG ACT-3'; Proximal *Stamp2* promoter forward 5'-GGC AGG AGA AAG ACA CCA CTA TT-3', reverse 5'-TTT AAG CCA AAG AGC GGA GGA G-3'.

2.9. Iron Reductase Assay

Cells were washed with PBS at 37 °C and incubated with iron uptake solution (50 µM Fe-3NTA, 1 mM Ferrozine [Sigma], 25 mM MES, 25 mM MOPS, 140 mM NaCl, 5 mM Glucose, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, pH = 7.4) for 1 h in the dark at 37 °C. The amount of reduced Fe²⁺-Ferrozine complex was detected by reading absorbance at 560 nm by a multiplate reader (Victor² PerkinElmer) normalized to total protein concentration (BioRad).

2.10. Oil Red O Staining

Cells were stained as previously reported [21].

2.11. Immunofluorescence

Cells grown on cover slips were washed with PBS and fixed in Methanol at –20 °C for 5 min. Cells were blocked with 1% BSA for 30 min before incubation with antisera against STAMP2 (Medprobe, 1:50) at 4 °C overnight and incubated with Alexa Fluor 488 goat anti-rabbit secondary antibodies (1:500) (Invitrogen) for 1 h at room temperature. Images were acquired with an Olympus FlowView FV1000 and analyzed with Image J.

2.12. Western Blot Analysis

Cells were washed in PBS and protein was extracted by incubating cells in lysis buffer (20 mM HEPES [pH 7.7], 0.3 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 1% Triton X-100, 0.1% SDS with 1× Protease inhibitor cocktail [Roche] and Phosphatase inhibitor cocktail [Roche]) for 1 h on ice. 50 µg of protein extract was resolved in an 8% polyacrylamide-SDS gel, blotted to a PVDF membrane and incubated with antisera against STAMP2 (Medprobe, 1:1000), GRP78 (Cell signaling, cs3183) (1:600) ATF4 (Cell signaling, cs1185) (1:1000), CHOP (Cell signaling, cs2895) (1:1000), C/EBPα (Santa Cruz, sc-61 or Abcam, ab40764) (1:500), aP2/FABP4 (Abcam, ab81605) (1:1000), or β-Actin (Cell signaling, cs47778) (1:10,000) in 5% BSA in TBS-0.1% Tween. Western images were obtained with a Kodak

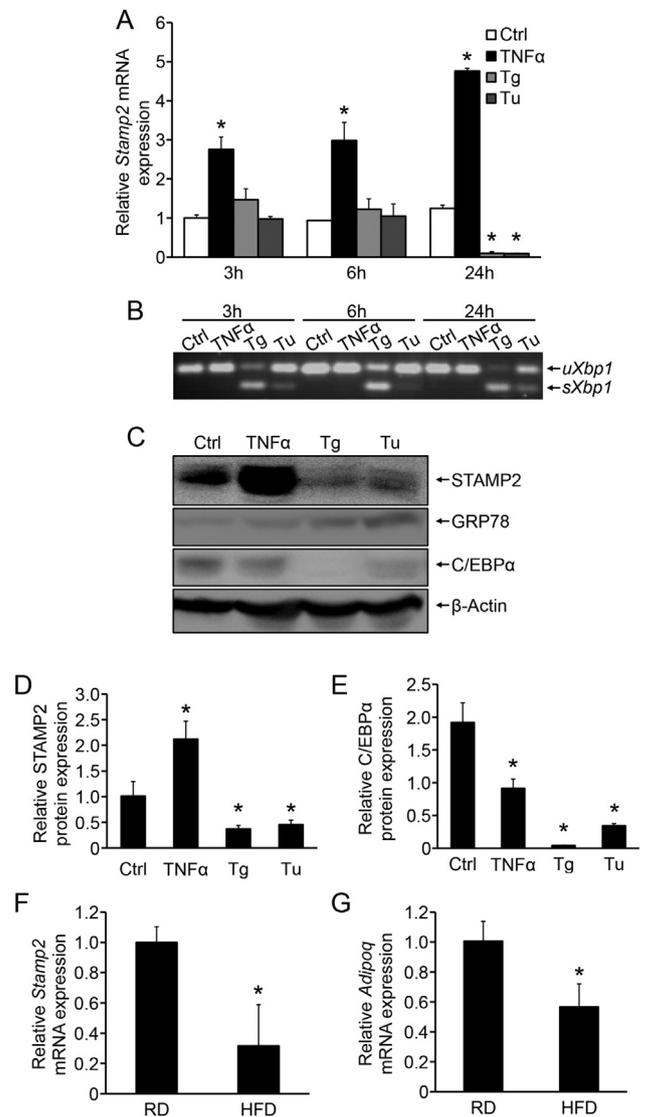


Fig. 1. ER stress and high-fat diet (HFD) suppress STAMP2 expression in adipocytes. (A) Analysis of *Stamp2* mRNA levels in 3T3-L1 adipocytes after treatment with DMSO (Ctrl), TNFα (10 ng/ml), Tg (300 nM), or Tu (2 ng/ml) for 3, 6 or 24 h by qRT-PCR. Results are from three independent experiments and data are presented as relative to 3 h DMSO treatment. The reference gene 36B4 was used to normalize expression. (B) Spliced and unspliced *Xbp1* (*sXbp1* and *uXbp1*, respectively) mRNA expression was assessed by PCR using the same cDNA as in A and after electrophoresis on a 3% agarose gel. The picture is representative of three independent experiments. (C) Western analysis showing STAMP2 protein levels in 3T3-L1 adipocytes after the same treatments as in panel A for 24 h. Representative STAMP2 blot, UPR marker GRP78, C/EBPα, and β-Actin as loading control. Quantification of STAMP2 (D) and C/EBPα (E) expression normalized to β-Actin, from two independent experiments, *n* = 5. (F and G) qRT-PCR analysis of *Stamp2* and *Adiponectin* mRNA from the adipocyte fraction of epididymal adipose tissue dissected from mice after 8 weeks on a regular diet (RD) or high-fat diet (HFD) (*n* = 4 for each group). Expression was normalized to 18S rRNA.

imaging station 4000R and the band intensities were quantified using Carestream Imaging Software.

2.13. Quantitative Reverse-Transcription PCR (qRT-PCR)

Total RNA was extracted from cells using the Trizol reagent (Invitrogen). Transcripts were converted to cDNA by the Superscript II (Invitrogen) reverse transcriptase using oligo(dT) primers. cDNA was quantified by the Lightcycler480 system (Roche) using the SYBR Green dye (Roche). The values were normalized to the expression of the ribosomal gene 36B4. All PCR products were analyzed by melting curve analysis. qRT-PCR primer sequences are available upon request.

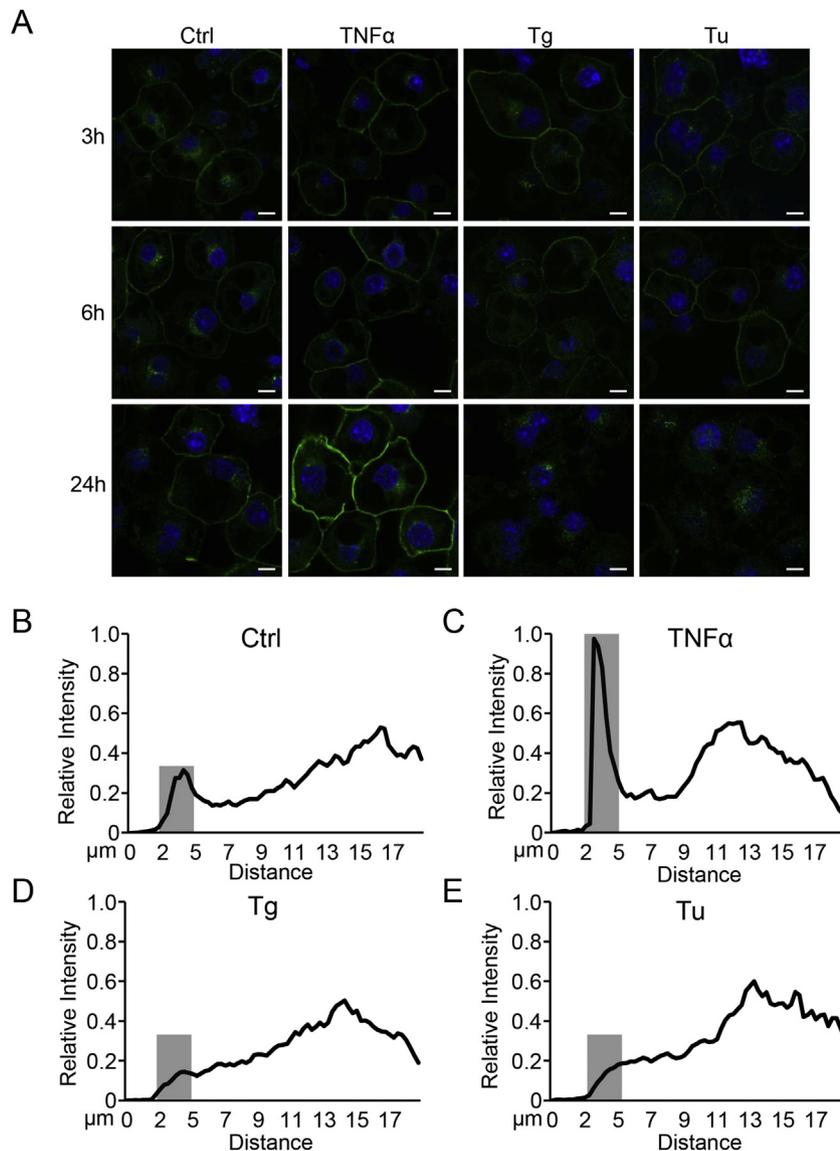


Fig. 2. Distinct regulation of STAMP2 subcellular distribution by TNF α and ER stress. (A) Immunofluorescence confocal microscopy analysis of differentiated 3T3-L1 adipocytes treated as in Fig. 1 with TNF α , Tu and Tg to determine regulation of its subcellular localization. Antiserum against STAMP2 was used as described in Section 2. Images are representative of three independent experiments, scale bar = 10 μ m. (B–E) Line scans (9 pixels width) from outside of the cell and in towards the nuclei with the indicated treatments. The intensity curve represents the average signal of the cells ($n = 20$). Shaded area indicates the plasma membrane.

2.14. Statistics

Data are presented as means and error bars represent standard deviation. Statistical analyses were performed using the Student's *t*-test, and *p*-values <0.05 were considered significant indicated with “*” or “#”.

3. Results

3.1. TNF α and ER Stress Divergently Affect STAMP2 Expression and Subcellular Distribution

To examine if STAMP2 expression is regulated by ER stress, 3T3-L1 adipocytes were incubated with DMSO (as control), an inflammatory cytokine (TNF α), or chemical ER stress inducers thapsigargin (Tg) or tunicamycin (Tu) for 3, 6 or 24 h. TNF α induced *Stamp2* mRNA expression at all time points reaching maximum levels at 24 h, consistent with previous reports [9,17] (Fig. 1A). In contrast, Tg and Tu had no significant effect on *Stamp2* mRNA expression at 3 and 6 h, but dramatically (>90%) reduced it by the 24 h time point (Fig. 1A). At all time points upon Tg and

Tu treatment, but not that of TNF α , *Xbp1* splicing was increased confirming induction of ER stress and activation of UPR (Fig. 1B).

Consistent with the above data, Western analysis showed that STAMP2 levels increased in response to TNF α , but significantly decreased upon Tg and Tu treatment (Fig. 1C and D). The decrease in STAMP2 expression in response to Tg and Tu was accompanied by an increase in the expression of ER-stress marker GRP78, but a decrease in C/EBP α , consistent with previous findings (Fig. 1E) [23,24].

Our previous work has shown that certain nutritional and dietary components can regulate *Stamp2* expression both *in vitro* and *in vivo* [9]. Since high-fat diet (HFD) is an established physiological ER stress inducer in adipose tissue [20], we investigated whether it may regulate *Stamp2* expression. Six-week-old male mice were fed *ad libitum* on either a control diet or a HFD for 8 weeks and *Stamp2* levels were measured in visceral adipose tissue by qRT-PCR analysis. As shown in Fig. 1F, *Stamp2* expression decreased by approximately 70% in the HFD group compared with controls, consistent with our previous findings [9]. It is of note that the magnitude of *Stamp2* downregulation is larger than that of adiponectin (Fig. 1G), a well-established adipokine that is down-regulated by HFD and ER stress [25,26].

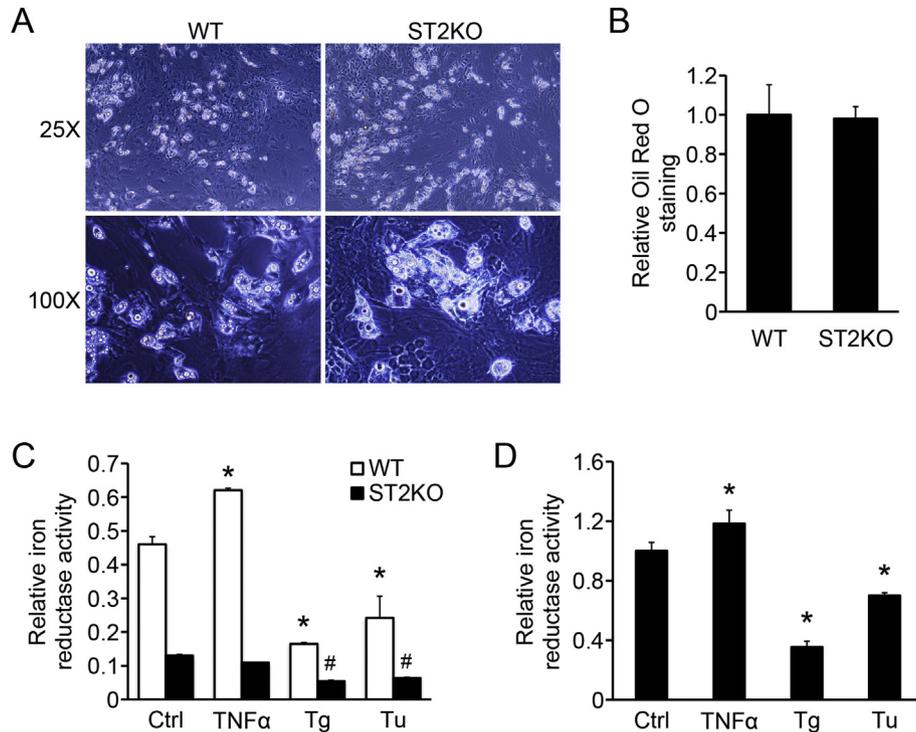


Fig. 3. STAMP2 contributes to endogenous iron reductase activity in adipocytes that is inhibited upon ER stress. (A) Isolated mouse embryonic fibroblasts (MEFs) from *wild-type* (WT) or *Stamp2*^{-/-} (ST2KO) mice were converted to adipocytes. Representative areas (25 \times and 100 \times magnification) are shown. Bright spots indicate lipid droplets. (B) Quantification of oil red O stained cells from A. (C) Cells from A were stimulated with either DMSO (Ctrl), TNF α (10 ng/ml), Tg (300 nM) or Tu (2 ng/ml) for 24 h after adipogenic conversion and used for the iron reductase assay as described in Section 2.11. Activity was normalized to total protein. Results shown are from one experiment done in duplicate. (D) Iron reductase activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were stimulated with either DMSO (Ctrl), TNF α (10 ng/ml), Tg (300 nM) or Tu (2 ng/ml) for 24 h and used in the iron reductase assay. Activity was normalized to total protein in each well. Results are from two independent experiments, $n = 6$.

STAMP2 predominantly localizes to the plasma membrane upon adipocyte differentiation as well as TNF α treatment [17]. Interestingly, STAMP2 localization to the plasma membrane has been linked to insulin sensitivity of mature adipocytes [27]. We therefore assessed whether STAMP2 intracellular localization is affected by ER stress. At earlier time points (3 and 6 h) of ER stress induction by Tg and Tu, or TNF α treatment, there were no significant changes in the expression or localization of STAMP2 (Fig. 2A). At 24 h, however, TNF α treatment increased both the expression and plasma membrane localization of STAMP2, consistent with previous findings [17] (Fig. 2A–C). In contrast, STAMP2 expression not only decreased upon Tg or Tu treatment, consistent with the data presented above (Fig. 1), but there was also a marked shift in its localization, resulting in loss from the plasma membrane (Fig. 2D and E). Some STAMP2 was still found in the perinuclear area suggesting a redistribution from the plasma membrane or retention in the ER and/or the trans-Golgi network before sorting.

3.2. STAMP2 Functions as an Iron Reductase in Adipocytes that Is Inhibited by ER Stress

STAMP2 is a metalloredoxase that can use both iron and copper as substrates [28]. It is also known that both iron levels and oxidative stress affect tissue inflammation and metabolic diseases [29,30]. In addition, STAMP2 levels are related to iron status in human visceral adipose tissue [14]. However, the iron reductase activity of STAMP2 in adipocytes remain uncharacterized. To determine if STAMP2 has metalloredoxase activity in adipocytes, we isolated preadipocytes from the adipose tissue of wild-type and *Stamp2*-deficient (*Stamp2*^{-/-}) mice, differentiated them into mature adipocytes *in vitro* and exposed them to TNF α , Tg, or Tu. There was significant and comparable adipogenic conversion of both wild-type and *Stamp2*^{-/-} cells (Fig. 3A and B). In resting cells, iron reductase activity was decreased by 75% in *Stamp2*^{-/-} cells compared with wild-type cells (Fig. 3C). Upon TNF α treatment, iron

reductase activity in wild-type adipocytes significantly increased, albeit less than would be expected by the TNF α -induced increase in STAMP2 expression, whereas there was no change in iron reductase activity in *Stamp2*^{-/-} cells (Fig. 3C). In contrast, treatment of wild-type cells with Tg or Tu strongly inhibited iron reductase activity almost down to the levels observed in DMSO-treated *Stamp2*^{-/-} cells (Ctrl). Tg and Tu treatment also significantly reduced the already low levels of iron reductase activity in *Stamp2*^{-/-} cells indicating that ER stress may regulate other iron reductases in adipocytes in addition to STAMP2. Upon the same treatments, the iron reductase activity of 3T3-L1 adipocytes displayed similar response profiles as the wild-type primary adipocytes (Fig. 3D).

3.3. The *Stamp2* Promoter Region Contains Several Potential NF- κ B and C/EBP α Binding Sites

TNF α was previously linked to murine STAMP2 expression in 3T3-L1 adipocytes [17]. In addition, we previously found that ectopic expression of C/EBP α strongly increased *Stamp2* promoter activation in a reporter assay in HeLa cells [9]. We analyzed the 5' flanking region of the murine *Stamp2* gene with a luciferase (LUC) reporter assay focusing on the 4 kb region upstream of the predicted transcriptional start site (TSS) (Fig. 4A). The full-length 4 kb region (-4 kb-ST2-LUC) and the 0-2 kb region (-2 kb-ST2-LUC) showed similar basal reporter activity; in contrast, the -4 kb to -2 kb region (-4 to 2 kb-ST2-LUC) did not have activity (Fig. 4B) suggesting that the functional *Stamp2* cis-acting sequences that mediate its expression lie within 2 kb upstream of the predicted TSS. Using the bioinformatics tool PROMO [31,32], we searched for and found potential Nuclear factor-kappaB (NF- κ B) and C/EBP α binding sites within the 2 kb upstream region. To assess the validity of these predictions, we constructed serial deletions in this area progressively removing putative binding sites from the 5' end (Fig. 4C). Reporter assays in HeLa cells showed that the C/EBP α binding site

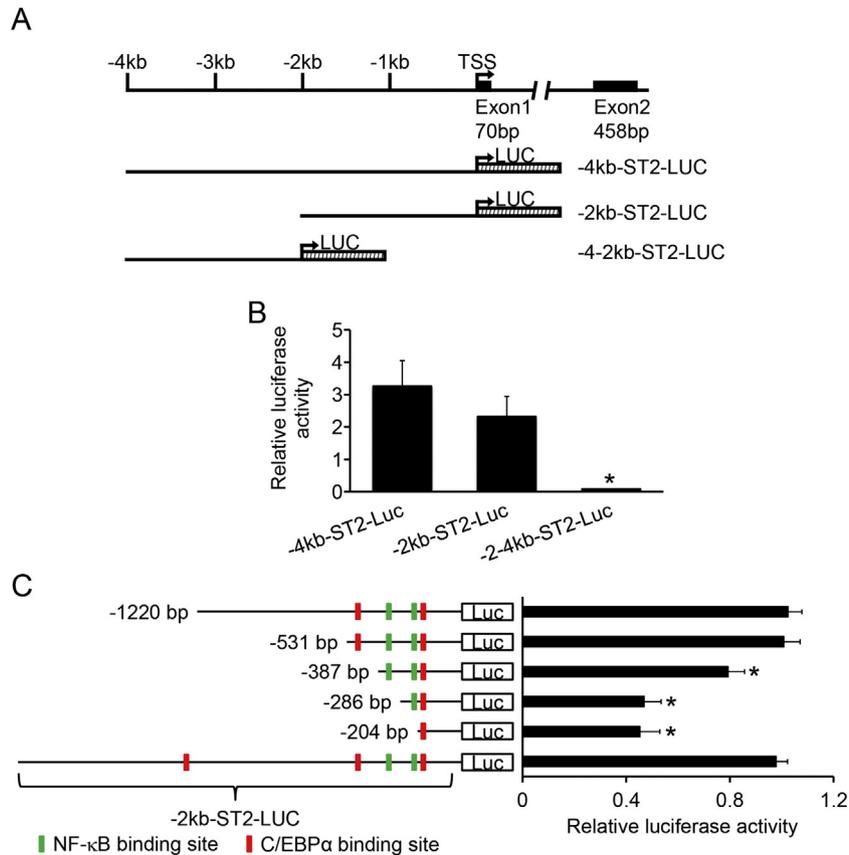


Fig. 4. Identification of functional elements in the *Stamp2* 5' flanking region. (A) Schematic representation of the 5' flanking region of the murine *Stamp2* gene and the different reporter constructs that were used. TSS: transcriptional start site. (B) *Stamp2* promoter activity analysis. HeLa cells were transfected with the indicated promoter constructs. After 48 h, cells were harvested and LUC activities were determined. Results shown are from two independent experiments, $n = 6$ for each sample. (C) Deletional analysis of *Stamp2* promoter activity. Schematic representation of serial deletion constructs of the -2 kb 5' *Stamp2* promoter region with corresponding reporter activity in HeLa cells. Results shown are from 3 independent experiments, $n = 9$.

(-176 to 186) most proximal to the TSS by itself maintained approximately 50% of the promoter activity, while more distal C/EBP α binding site (-472 to 482) accounted for 20% activity compared to the complete -2 kb region. The most distal C/EBP α binding site (-1267 to 1281) did not contribute to promoter activity. The two putative NF- κ B binding sites were identified (-225 to 237 and -338 to 350) and at least the more distal site contributed to promoter activity.

3.4. TNF α Increases *Stamp2* Expression through Activation of the NF- κ B Pathway

Induction of STAMP2 expression by TNF α in adipocytes is somewhat unexpected as TNF α inhibits expression of most other genes that are induced during adipogenesis [33]. In order to determine if regulation of STAMP2 expression by TNF α is mediated by cis-acting elements within the 2 kb upstream region, 3T3-L1 cells were stably transfected with -2 kb-ST2-LUC or the BASIC-LUC reporter as control. These cell lines were differentiated into adipocytes and then treated with TNF α which increased *Stamp2* mRNA expression in the two cell lines similarly without significant change in basal levels (Fig. 5A). In contrast, TNF α significantly increased LUC activity in -2 kb-ST2-LUC cells, but not that of BASIC-LUC.

NF- κ B is a transcriptional factor that mediates effects of TNF α in various tissues [34]. To test if NF- κ B mediates TNF α -induced *Stamp2* expression, NF- κ B-LUC or -2 kb-ST2-LUC reporters were co-transfected into HeLa cells with either a control siRNA or one targeting NF- κ B subunit *NFKB1* (*p50*). -2 kb-ST2-LUC expression was decreased by 60% upon *p50* knockdown (Fig. 5C), correlating with the loss of *NFKB1* expression (Fig. 5D). Consistently, ectopic expression of *p50* increased

-2 kb-ST2-LUC and NF- κ B-LUC activity 5-fold over that of an empty control (Fig. 5E). These data indicate that NF- κ B is required for TNF α -induced STAMP2 expression.

3.5. C/EBP α Directly Binds to the *Stamp2* Promoter

The data presented above showed that ER stress reduced both STAMP2 and C/EBP α expression (Fig. 1). Consistently, ER stress or siRNA-mediated C/EBP α knockdown significantly inhibited -2 kb-ST2-LUC expression in 3T3-L1 adipocytes (Fig. 6A–C) suggesting that C/EBP α is required for STAMP2 expression. To evaluate the potential contribution of the two predicted C/EBP α binding sites to basal *Stamp2* promoter activity (Fig. 4C) we subjected them to site-directed mutagenesis (Fig. 6D). Disruption of both sites significantly decreased C/EBP α -mediated activation of -2 kb-ST2-LUC where the disruption of the proximal site (C/EBP1) was most deleterious (Fig. 6E).

We next tested if C/EBP α binds *in vivo* to the predicted sites (Fig. 7A). To that end, we used chromatin immunoprecipitation (ChIP) assay in 3T3-L1 cells at day 0 (undifferentiated), day 5 (intermediate differentiation), and day 9 (mature adipocyte). C/EBP α was absent at the proximal sites or the distal site in pre-adipocytes, but robustly bound to both sites upon differentiation (Fig. 7B and C). This is consistent with our earlier findings indicating that STAMP2 levels are positively associated with normal C/EBP α expression during adipogenesis [21]. Furthermore, these data are consistent with similar findings where C/EBP α bound to the *ap2* promoter in a differentiation-dependent manner [35].

Since STAMP2 expression requires C/EBP α whose levels decrease in response to ER stress, this can potentially explain as to how ER stress

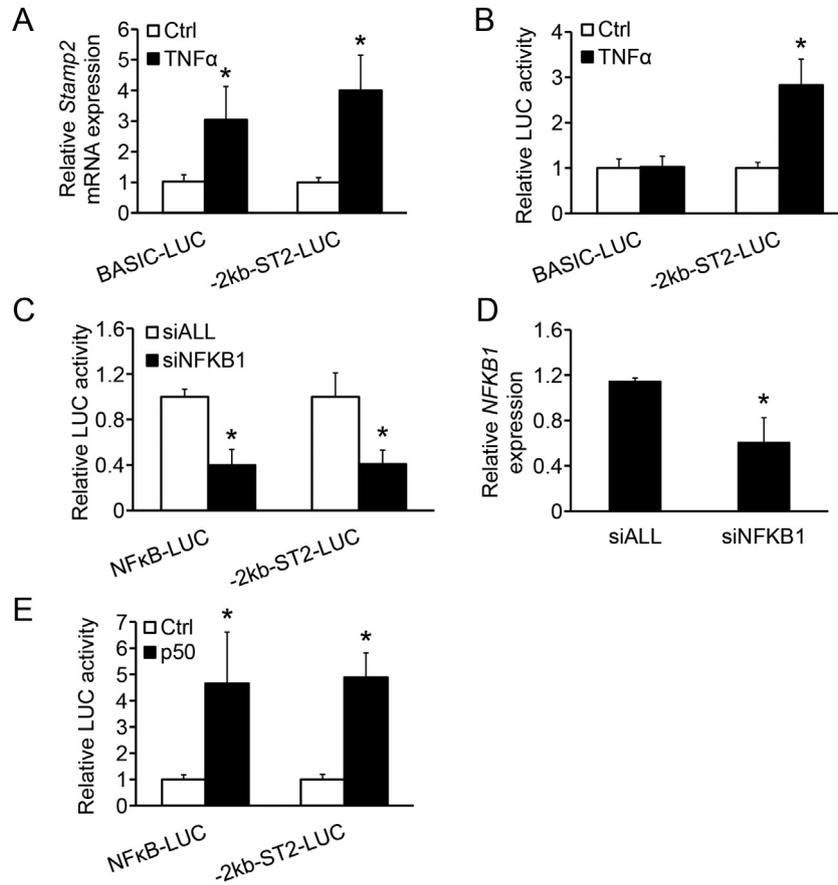


Fig. 5. *Stamp2* promoter is activated by TNF α and p50. (A) 3T3-L1 cells with stable expression of either the -2 kb-ST2-LUC or a BASIC-LUC reporters were differentiated into adipocytes, treated with TNF α (10 ng/ml) or vehicle (Ctrl) for 24 h and subjected to qRT-PCR analysis and (B) LUC assay. Values are presented as fold activity compared to vehicle. Results are from two independent experiments, $n = 9$ for each sample. (C) HeLa cells were co-transfected with either 2xNFKB-LUC or -2 kb-ST2-LUC (ST2-LUC), together with either AllStars (siALL) or NFKB1 (siNFKB1) siRNA. After 48 h, cells were harvested and LUC activity was determined. Values are presented as fold activity compared to control (siALL) from two independent experiments, $n = 6$ for each sample. (D) qRT-PCR analysis of NFKB1 (*p50*) mRNA from the same cells used in C. Values are presented as fold activity compared to control (siALL). Results are from two independent experiments, $n = 6$ for each sample. (E) HeLa cells were co-transfected with either 2xNFKB-LUC or -2 kb-ST2-LUC (ST2-LUC), together with either a p50 expression plasmid or an empty control plasmid. After 48 h, cells were harvested and LUC activity was determined. Values are presented as fold activity compared to control (Ctrl) from two independent experiments, $n = 6$ for each sample.

strongly inhibits STAMP2 expression. In addition, previous work has shown that C/EBP α recruitment to the *Stamp2* promoter increases in response to IL-6 in liver cells as well as in adipocytes [19]. To assess the potential role of C/EBP α recruitment in regulating *Stamp2* expression, 3T3-L1 adipocytes were treated with vehicle, TNF α , Tg, or Tu for 24 h and subjected to ChIP analysis. Cells that were treated with Tg or Tu showed that C/EBP α recruitment to both the distal and the proximal sites was disrupted (Fig. 7D and E). Interestingly, TNF α treatment also decreased C/EBP α association with both parts of the *Stamp2* promoter, albeit to a much lesser degree than by ER stress.

3.6. C/EBP α Rescue Reverses the Effects of Mild, but Not Severe ER Stress on STAMP2 Expression

To address if decreased C/EBP α binding to the *Stamp2* promoter in response to ER stress was due to disruption of C/EBP α recruitment, we established a 3T3-L1 cell line with doxycycline (dox) inducible C/EBP α expression. C/EBP α is a major adipogenic driver [36], but when its expression was induced after adipocyte maturation, it did not significantly affect differentiation measured with Oil Red O (Fig. 8A). 3T3-L1-C/EBP α cells were either left untreated, or treated with Tg (model for strong ER stress) or Tu (model for milder ER stress) in the presence or absence of C/EBP α expression (Fig. 8B). Dox treatment strongly induced C/EBP α expression which was maintained during Tu-induced ER stress (Fig. 8C). However, Tg treatment reduced dox-induced C/EBP α expression which was approximately 30% of that in control cells (Fig. 8C). The

effect of C/EBP α rescue was correlated with STAMP2 expression: STAMP2 levels of Tu-treated cells reached control levels, while those in Tg treated cells remained approximately 25% of control (Fig. 8D). This coincided with a marked induction of the ATF4/CHOP axis upon Tg induction, which was not activated by either TNF α or Tu (Fig. 8B). ChIP analysis of C/EBP α expressing 3T3-L1 cells showed divergent responses to Tg and Tu: whereas C/EBP α recruitment to both the proximal and distal promoter was significantly reduced by Tg (Fig. 8E–F), it remained at basal levels upon Tu treatment.

4. Discussion

Here, we have presented significant evidence, using cell models *in vitro*, showing that ER stress downregulates STAMP2 expression at both the mRNA and protein levels in adipocytes. In concert with loss of STAMP2 expression, we show that there are important changes to the spatio-temporal intracellular distribution of STAMP2 in response to ER stress. Moreover, we show for the first time that STAMP2 is responsible for the major part of the iron reductase activity in adipocytes, which is suppressed upon ER stress. The response of adipocytes to ER stress regarding STAMP2 expression, localization, and function is opposite to that observed by inflammatory pathway activation, *i.e.* TNF α .

We provide, using luciferase and ChIP assays, new insight to the regulation of the murine *Stamp2* promoter in adipocytes showing that both NF- κ B and C/EBP α binding sites contribute to basal *Stamp2* expression *via* cis elements contained within the 2 kb upstream region from the

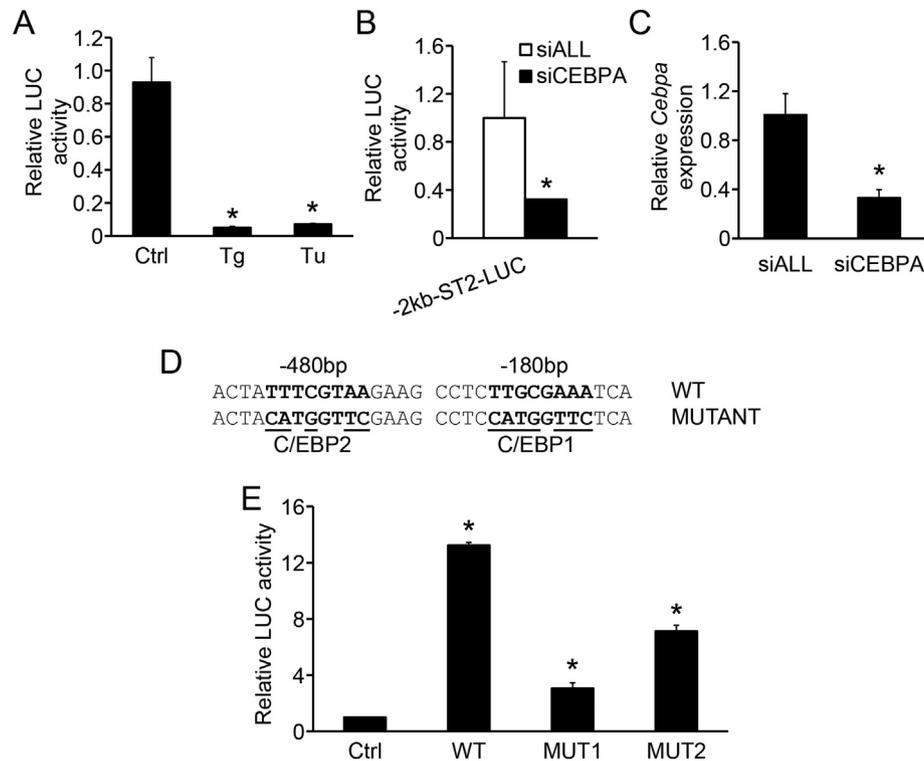


Fig. 6. *Stamp2* promoter activity requires C/EBP α . (A) 3T3-L1 cells stably expressing the -2 kb-ST2-LUC reporter were differentiated into adipocytes. They were then left untreated or incubated with Tg (300 nM), or Tu (2 ng/ml) for 24 h. Cells were then harvested and LUC activity was determined. Results are from two independent experiments, $n = 8$ for each sample. (B) 3T3-L1 cells with stable expression of the -2 kb-ST2-LUC reporter were transfected with either AllStar (siAll) or *Cebpa* (siCEBPA) targeted siRNA, and differentiated towards adipocytes for 4 days. Cells were then harvested and LUC activities were determined. Values are presented as fold activity compared to control (siAll) from two independent experiments, $n = 6$ for each sample. (C) qRT-PCR analysis of *CEBPA* mRNA from the same cells used in B. (D) Overview of two predicted C/EBP α binding motifs (C/EBP1 and 2) in the mouse *Stamp2* promoter. The nucleotides that are mutated are underlined. (E) HeLa cells were co-transfected with an expression vector for C/EBP α and either BASIC-LUC reporter plasmid (Ctrl), the wild-type -2 kb-ST2-LUC, or a -2 kb-ST2-LUC reporter construct mutated in C/EBP1 or C/EBP2 binding sites (MUT1 and MUT2, respectively). After 24 h, cells were harvested and LUC activities were determined. The data shown are from three independent experiments, $n = 9$ for each sample.

TSS. Furthermore, we show that TNF α induces *Stamp2* expression largely through NF- κ B, and that ER stress inhibits C/EBP α binding to the *Stamp2* promoter in an ER stress sensitive manner, which then affects STAMP2 expression.

Lack of STAMP2 induces inflammation in the liver and adipose tissue, and results in insulin resistance and dyslipidemia in lean mice on a regular diet [9]. Obesity, which has similar consequences, also induces ER stress which contributes to activation of stress and inflammatory networks, inhibition of insulin action, and impairment of systemic glucose homeostasis [3,37]. This integrated pathological network is critical for the development of chronic metabolic disease [20]. The pattern of decreased *Stamp2* mRNA and protein expression induced by experimental ER stress in adipocytes that we report, is similar to previous observations in VWAT of *ob/ob* mice [9,19], *ApoE*^{-/-}/*LDLR*^{-/-} diabetic model mice [10], and HFD-fed mice [9]. Interestingly, with up to 6 h of exposure to ER stress inducers, there was no significant change in *Stamp2* expression but robust inhibition at 24 h, highlighting the need for chronic exposure and suggesting that multiple factors are involved some of which themselves may be regulated by ER stress.

Previous work has shown that TNF α induces STAMP2 expression in adipocytes and adipose tissue [15–17,38]. Consistently, we found that TNF α treatment increased STAMP2 protein levels by about 2-fold in 3T3-L1 adipocytes, accompanied by a significant increase in the ratio of STAMP2 protein at the plasma membrane. In contrast, ER stress resulted in STAMP2 relocalization from the plasma membrane to the cytosol. Previous work suggested that STAMP2 plasma membrane localization and adipocyte insulin sensitivity are linked [27,39], indicating the potential significance of STAMP2 exclusion from the plasma membrane upon ER stress.

When ectopically expressed, STAMP2 has the highest iron reductase activity among the STAMP family members [28,40,41]. Here we provide the first evidence that endogenous STAMP2 has significant iron reductase activity in adipocytes, which is down-regulated by chronic ER stress. TNF α -induced STAMP2 protein expression (2-fold) was not proportionally reflected in the iron reductase activity that increased by about 20%. One reason for this could be that STAMP2 that is translocated to the plasma membrane upon TNF α treatment can no longer catalyze iron reduction. In support of this possibility, a previous report suggested that the iron reductase activity of STAMP2 may require the acidic environment of intracellular organelles [40]. Furthermore, mice with a point-mutant STAMP3 (STAMP3^{Y288H}) displayed impaired iron reductase activity in whole blood preparations compared to wild-type mice, which was linked to the inability of STAMP3^{Y288H} to internalize from the plasma membrane to endosomal vesicles [42]. Thus, TNF α , and possibly other cytokines, promote STAMP2 translocation to the plasma membrane where its iron reductase activity is inhibited which may not be required for its function therein.

In *ob/ob* mice, and to a large extent in HFD mouse models, STAMP2 expression is dysregulated in response to nutrients in the liver and adipose tissue [9,19]. C/EBP α levels are downregulated in VWAT in obese compared to lean mice [24] and similarly affected by experimental ER stress [23,24]. Consistently, we found that C/EBP α association with the *Stamp2* promoter was significantly reduced upon ER stress concomitant with diminished STAMP2 protein levels. These findings support a model where obesity-induced ER stress dysregulates STAMP2 expression in adipose tissue through C/EBP α . TNF α led to a small decrease in C/EBP α protein levels and recruitment to the *Stamp2* promoter. We also showed that both NF- κ B and C/EBP α binding elements contribute to *Stamp2* promoter activity, but this is in contrast to previous findings

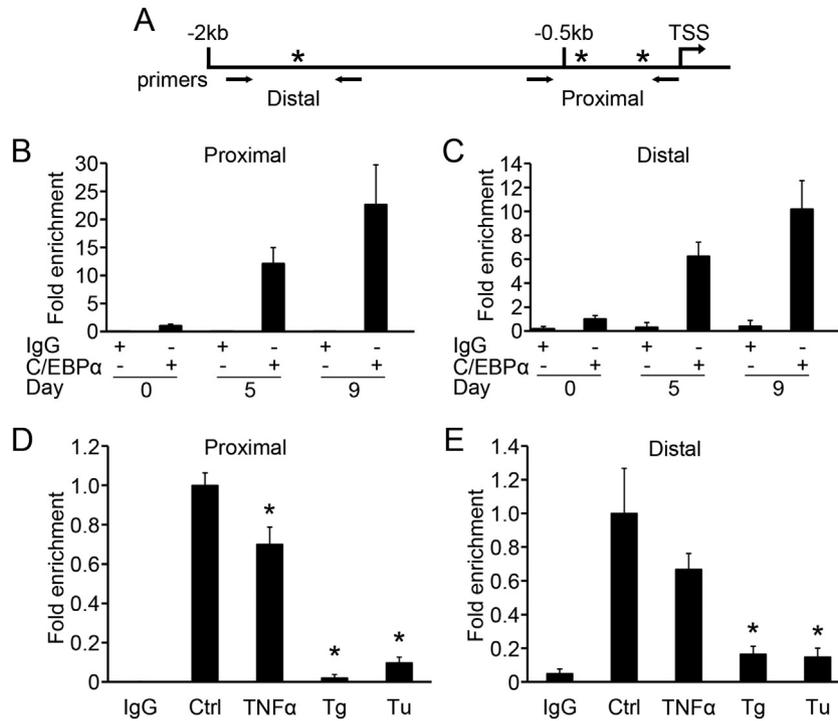


Fig. 7. C/EBPα binds to the *Stamp2* promoter that is disrupted by ER stress. (A) Location of the primers used in the ChIP assay targeting the proximal *Stamp2* promoter is shown, which contains the two predicted C/EBPα sites (labeled with *) and a more distal region containing one predicted site for C/EBPα. (B–C) 3T3-L1 pre-adipocytes or adipocytes at day 5 or day 9 after initiation of adipogenesis were fixed and ChIP assay was performed using a C/EBPα antibody or a control antiserum against IgG. Data shown are from one experiment done in duplicate representative for three independent C/EBPα promoter binding experiments. (D–E) 3T3-L1 cells were differentiated into adipocytes (day 9). They were then left untreated, or incubated (24 h) with TNFα (10 ng/ml), Tg (300 nM), or Tu (2 ng/ml) and analyzed for C/EBPα recruitment to the proximal and distal promoter regions of the *Stamp2* gene. Data shown are from two independent ChIP experiments.

that showed an increase in C/EBPα at the *Stamp2* promoter in response to interleukin-6 (IL-6), another cytokine, in liver cells [19]. However, induced expression of C/EBPα led to a more potent TNFα driven STAMP2 expression. A possible reason for this could be the neutralization of the negative feedback mechanism that TNFα enforces on C/EBPα levels [18]. It is currently unclear whether this reflects a differential regulation of C/EBPα expression/function with different cytokines in adipose vs. liver tissue. When C/EBPα was ectopically expressed, Tg treatment still significantly reduced C/EBPα expression and chromatin binding such that STAMP2 expression was decreased. In contrast, ectopic C/EBPα expression was not affected by Tu and its chromatin binding increased correlating with STAMP2 expression. This suggests that there is a critical level of C/EBPα that is required to induce STAMP2 expression. Another potential mechanism is the differential activation of the ATF4/CHOP axis in response to Tg and Tu considering that CHOP is an established inhibitor of C/EBPα [43,44]. These data suggest that distinct stress signals can affect STAMP2 expression through differential effects on C/EBPα levels.

Dysregulated STAMP2 expression in response to metabolic load is a feature of metabolic syndrome in obese mouse models [9,19]. In murine liver, C/EBPα regulates STAMP2 expression in response to nutrients [19], and we show here that ER stress disrupts this regulation. However, induced expression of C/EBPα may alleviate the disruption. In adipocytes, C/EBPα and PPARγ are master transcriptional regulators of a range of genes critical for adipose function, as well as each other [36]. PPARγ agonists are currently used clinically to treat type 2 diabetes, and could also restore STAMP2 regulation in diabetic patients. STAMP2 has significant iron reductase activity in adipocytes which is disrupted by ER stress. Free iron has been implicated in insulin resistance [45], and STAMP2 expression has been shown to negatively correlate with such iron in human visceral adipose tissue [14]. One way of possible intervention strategy in patients could therefore involve stimulation of iron metabolism in adipocytes, or removal of free iron from

circulation. The latter has been implemented using deferoxamine, an iron chelator agent [46,47].

Although well established in experimental settings, it is worth noting that the ER stress agents we used in these experiments significantly differ from the endogenous metabolic signals in terms of activity patterns, duration, and magnitude [48]. Our data are also largely limited to *in vitro* experiments. Thus, future *in vivo* confirmation of the data presented here, using additional ER stress agents and metabolic load is warranted. In addition, the interplay between the inflammation/ER stress signal axes still need further delineation. Although reduced by TNFα, it is not clear if a baseline of C/EBPα is needed for NF-κB driven STAMP2 expression. In future studies, it would be important to include additional inducers of inflammation. Furthermore, the effect of ER stress on inflammatory signaling is not addressed here. As a follow-up to the findings here, it would be of interest to investigate in adipocytes the potential feedback loop between STAMP2 and the ATF4–CHOP–C/EBPα axis, described in the context of prostate cancer [41].

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Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

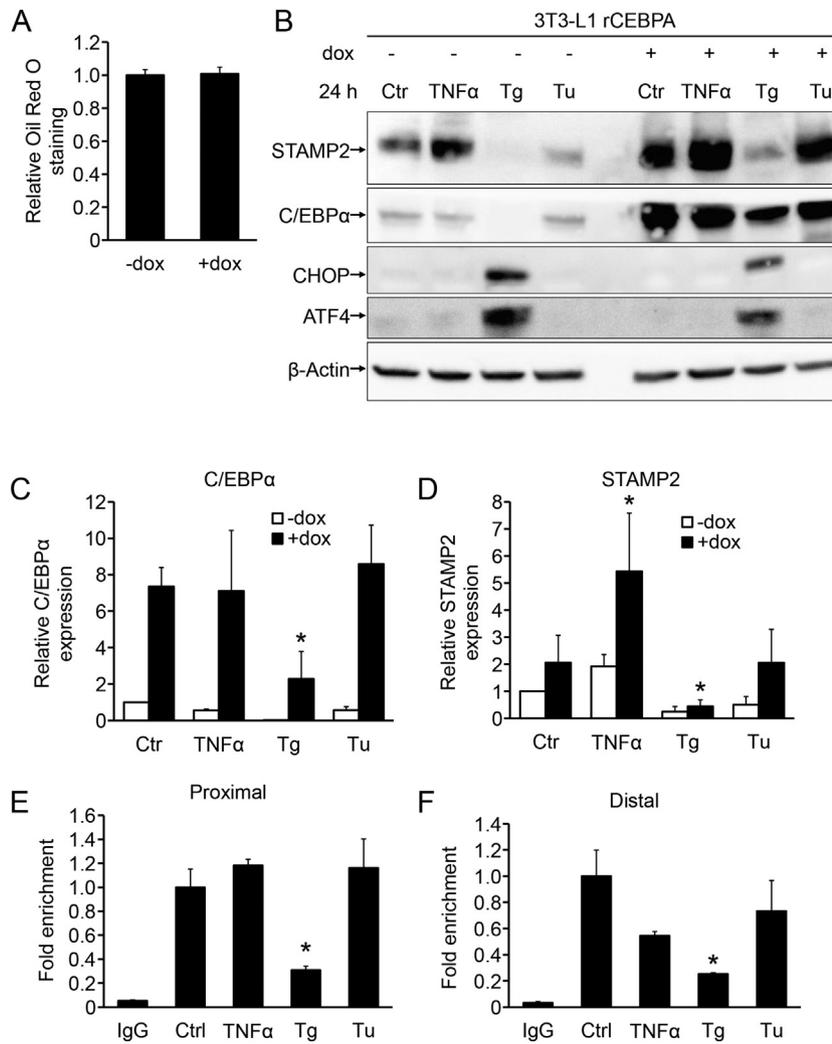


Fig. 8. C/EBP α expression rescues mild ER stress induced loss of STAMP2 expression. (A) Quantitative presentation of oil red O staining of 3T3-L1 adipocytes in the absence and presence of C/EBP α (-/+Dox). (B) 3T3-L1 cells with inducible C/EBP α expression (+dox) were differentiated into adipocytes and were either left untreated, or incubated with Tg (300 nM), or Tu (2 ng/ml) for 24 h in the presence or absence of dox. Cells were harvested and used for Western analysis. Shown are representative blots from three independent experiments for C/EBP α , STAMP2, CHOP, ATF4, and β -Actin as loading control. (C–D) Quantification of results from B. (E–F) ChIP analysis of cells treated as in (dox-induced cells only) for C/EBP α recruitment to the proximal and distal promoter regions of the *Stmp2* gene. Data shown are from two independent ChIP experiments done in triplicate.

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