Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Metabolism. Author manuscript; available in PMC 2020 April 01.

Published in final edited form as:

Metabolism. 2019 April ; 93: 75–85. doi:10.1016/j.metabol.2019.01.014.

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

Jørgen Sikkeland[1,5,§], **Torstein Lindstad**[1,§], **Hatice Zeynep Nenseth**[1], **Xavier Dezitter**[2], **Su Qu**[1], **Ridhwan M. Muhumed**[1], **Meric Erikci Ertunc**[1,3,4], **Margaret F. Gregor**[3], and **Fahri Saatcioglu**[1,5,*]

[1]Department of Biosciences, University of Oslo, Postboks 1066 Blindern, 0316 Oslo, Norway

[2]Plateforme de Binding et de Biologie Moléculaire, Institut de Chimie Pharmaceutique Albert Lespagnol, Faculté des Sciences Pharmaceutiques et Biologiques - Université de Lille, F-59006 Lille, France

[3]Department of Genetics and Complex Diseases and Sabri Ülker Center, Harvard TH Chan School of Public Health, Boston, MA 02115, USA

[4]Current address: Clayton Foundation Laboratories for Peptide Biology, Salk Institute for Biological Studies, La Jolla, CA 92037, USA

[5]Institute for Cancer Genetics and Informatics, Oslo University Hospital, 0310 Oslo, Norway

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 TNFα. 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 TNFα 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

*To whom correspondence should be addressed: Prof. Fahri Saatcioglu: Department of Biosciences, University of Oslo, Postboks 1066 Blindern, 0316 Oslo, Norway; Tel: 47-22854569; Fax: 47-22857207; fahris@ibv.uio.no.
§These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

immunoprecipitation, showed that induction of ER stress disrupts C/EBPα-mediated STAMP2 expression.

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

Keywords

STAMP2; ER stress; gene expression; iron reductase; protein translocation; adipocyte

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-κB) 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 IRE1α-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 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 (TNFα), consistent with earlier findings in mice [17].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

We have previously shown that CCAAT/enhancer binding protein alpha (C/EBPα) increases *Stamp2* promoter activity in HeLa cells [9]. As C/EBPα is down-regulated by TNFα [18], it is possible that two distinct regulatory pathways, adipogenic and inflammatory, control STAMP2 expression and/or action. In an independent study, both C/EBPα 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/EBPα 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% CO_2 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-2kb-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 μg/ml puromycin, surviving cells were pooled and expanded. All treatments were done by incubating cells with DMSO (Ctrl), 10 ng/ml TNFα (Sigma-Aldrich), 2 ng/ml Tu (Cambrex), or 300 nM Tg (Sigma-Aldrich) for 24 h unless otherwise indicated.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 μm 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 μm 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 μM 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-ΔR8.2) 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 μm filter (Millipore) and added to 3T3-L1 fibroblasts. 36 h post infection the 3T3-L1 cells were subjected to pool selection with 1 μg/ml puromycin for 7 days. To induce rat C/EBPα expression cells were treated with 500 ng/ml doxycycline (Sigma-Aldrich) for 48 h.

2.5. Reporter assays

The 2kb fragment upstream of the putative mouse *Stamp2* transcription initiation start site was amplified by PCR using Phusion proofreading 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 2kb insert was excised out with HindIII and XhoI 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 μl luciferin buffer containing 0.2 mM D-Luciferin (Anaspec) and 2 mM ATP, was injected to 10 μl 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 −2kb-ST2-LCU plasmid as template, a construct series with predicted NFκB and C/EBPα binding sites removed were created using Q5® Site-Directed Mutagenesis Kit (NEB). Primers forward/reverse (promoter length):

ACTTTGCTTCACAGCTCC/AAGGGCGAATTCTGCAGA (1229 bp);

GAGAACGTCCATCTGCGA/AAGGGCGAATTCTGCAGATA (531);

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

AAAGGAGTATCTCTCTGCC/AAGGGCGAATTCTGCAGA (387);

GCTCTGACTCTGTTAAAAATCAG/AAGGGCGAATTCTGCAGA (286);

ATGCCCTAACCATGTCAC/AAGGGCGAATTCTGCAGA (204).

2.7. Mutagenesis

Predicted C/EBP binding sites were mutated by the GeneTailor Site-Directed Mutagenesis system (Invitrogen) using the −2kb-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 nonspecific 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 $CaCl_2$, 0.8 mM $MgCl_2$, pH = 7.4) for one hour in the dark at 37°C. The amount of reduced Fe^{2+}-Ferrozine complex was detected by reading absorbance at 560 nm by a multiplate reader (Victor^2 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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2.12. Western blot analysis

Cells were washed in PBS and protein was extracted by incubating cells in lysis buffer (20 mM HEPES [pH7.7], 0.3 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 1% Triton X-100, 0.1% SDS with 1X 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) (1:10000) in 5% BSA in TBS-0.1% Tween. Western images were obtained with a Kodak 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 Lightycler480 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.

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. TNFα 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 (TNFα), or chemical ER stress inducers thapsigargin (Tg) or tunicamycin (Tu) for 3, 6 or 24 h. TNFα induced *Stamp2* mRNA expression at all time points reaching maximum levels at 24 h, consistent with previous reports [9, 17] (Figure 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 (Figure 1A). At all time points upon Tg and Tu treatment, but not that of TNFα, *Xbp1* splicing was increased confirming induction of ER stress and activation of UPR (Figure 1B).

Consistent with the above data, Western analysis showed that STAMP2 levels increased in response to TNFα, but significantly decreased upon Tg and Tu treatment (Figures 1C and 1D). 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/EBPα, consistent with previous findings (Figure 1E) [23, 24].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 Figure 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 (Figure 1G), a well-established adipokine that is downregulated by HFD and ER stress [25, 26].

STAMP2 predominantly localizes to the plasma membrane upon adipocyte differentiation as well as TNFα 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 TNFα treatment, there were no significant changes in the expression or localization of STAMP2 (Figure 2A). At 24 h, however, TNFα treatment increased both the expression and plasma membrane localization of STAMP2, consistent with previous findings [17] (Figures 2A-C). In contrast, STAMP2 expression not only decreased upon Tg or Tu treatment, consistent with the data presented above (Figure 1), but there was also a marked shift in its localization, resulting in loss from the plasma membrane (Figure 2D and 2E). 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 metalloreductase 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 metalloreductase 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 TNFα, Tg, or Tu. There was significant and comparable adipogenic conversion of both wild-type and *Stamp2*$^{-/-}$ cells (Figure 3A and 3B). In resting cells, iron reductase activity was decreased by 75% in *Stamp2*$^{-/-}$ cells compared with wild-type cells (Figure 3C). Upon TNFα treatment, iron reductase activity in wild-type adipocytes significantly increased, albeit less than would be expected by the TNFα-induced increase in STAMP2 expression, whereas there was no change in iron reductase activity in *Stamp2*$^{-/-}$ cells (Figure 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

adipocytes displayed similar response profiles as the wild-type primary adipocytes (Figure 3D).

3.3. The *Stamp2* promoter region contains several potential NF-κB and C/EBPα binding sites

TNFα was previously linked to murine STAMP2 expression in 3T3-L1 adipocytes [17]. In addition, we previously found that ectopic expression of C/EBPα 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 4kb region upstream of the predicted transcriptional start site (TSS) (Figure 4A). The full-length 4kb region (−4kb-ST2-LUC) and the 0–2kb region (−2kb-ST2-LUC) showed similar basal reporter activity; in contrast, the −4kb to −2kb region (−4–2kb-ST2-LUC) did not have activity (Figure 4B) suggesting that the functional *Stamp2* cis-acting sequences that mediate its expression lie within 2kb upstream of the predicted TSS. Using the bioinformatics tool PROMO [31, 32], we searched for and found potential Nuclear factor-kappaB (NF-κB) and C/EBPα binding sites within the 2kb 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 (Figure 4C). Reporter assays in HeLa cells showed that the C/EBPα binding site (−176–186) most proximal to the TSS by itself maintained approximately 50% of the promoter activity, while more distal C/EBPα binding site (−472–482) accounted for 20% activity compared to the complete −2kb region. The most distal C/EBPα binding site (−1267–1281) did not contribute to promoter activity. The two putative NF-κB binding sites were identified (−225–237 and −338–350) and at least the more distal site contributed to promoter activity.

3.4. TNFα increases *Stamp2* expression through activation of the NF-κB pathway

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

NF-κB is a transcriptional factor that mediates effects of TNFα in various tissues [34]. To test if NF-κB mediates TNFα-induced *Stamp2* expression, NF-κB-LUC or −2kb-ST2-LUC reporters were co-transfected into HeLa cells with either a control siRNA or one targeting NF-κB subunit *NFKB1 (p50)*. −2kb-ST2-LUC expression was decreased by 60% upon p50 knockdown (Figure 5C), correlating with the loss of *NFKB1* expression (Figure 5D). Consistently, ectopic expression of p50 increased −2kb-ST2-LUC and NF-κB-LUC activity 5-fold over that of an empty control (Figure 5E). These data indicate that NF-κB is required for TNFα-induced STAMP2 expression.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3.5. C/EBPα directly binds to the *Stamp2* promoter

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

We next tested if C/EBPα binds *in vivo* to the predicted sites (Figure 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/EBPα was absent at the proximal sites or the distal site in pre-adipocytes, but robustly bound to both sites upon differentiation (Figure 7B and 7C). This is consistent with our earlier findings indicating that STAMP2 levels are positively associated with normal C/EBPα expression during adipogenesis [21]. Furthermore, these data are consistent with similar findings where C/EBPα bound to the *aP2* promoter in a differentiation-dependent manner [35].

Since STAMP2 expression requires C/EBPα whose levels decrease in response to ER stress, this can potentially explain as to how ER stress strongly inhibits STAMP2 expression. In addition, previous work has shown that C/EBPα 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/EBPα recruitment in regulating *Stamp2* expression, 3T3-L1 adipocytes were treated with vehicle, TNFα, Tg, or Tu for 24h and subjected to ChIP analysis. Cells that were treated with Tg or Tu showed that C/EBPα recruitment to both the distal and the proximal sites was disrupted (Figure 7D and 7E). Interestingly, TNFα treatment also decreased C/EBPα association with both parts of the *Stamp2* promoter, albeit to a much lesser degree than by ER stress.

3.6. C/EBPα rescue reverses the effects of mild, but not severe ER stress on STAMP2 expression

To address if decreased C/EBPα binding to the *Stamp2* promoter in response to ER stress was due to disruption of C/EBPα recruitment, we established a 3T3-L1 cell line with doxycycline (dox) inducible C/EBPα expression. C/EBPα 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 (Figure 8A). 3T3-L1-C/EBPα 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/EBPα expression (Figure 8B). Dox treatment strongly induced C/EBPα expression which was maintained during Tu-induced ER stress (Figure 8C). However, Tg treatment reduced dox-induced C/EBPα expression which was approximately 30% of that in control cells (Figure 8C). The effect of C/EBPα 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 (Figure 8D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

This coincided with a marked induction of the ATF4/CHOP axis upon Tg induction, which was not activated by either TNFα or Tu (Figure 8B). ChIP analysis of C/EBPα expressing 3T3-L1 cells showed divergent responses to Tg and Tu: whereas C/EBPα recruitment to both the proximal and distal promoter was significantly reduced by Tg (Figure 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. TNFα.

We provide, using luciferase and ChIP assays, new insight to the regulation of the murine *Stamp2* promoter in adipocytes showing that both NF-κB and C/EBPα binding sites contribute to basal *Stamp2* expression via cis elements contained within the 2kb upstream region from the TSS. Furthermore, we show that TNFα induces *Stamp2* expression largely through NF-κB, and that ER stress inhibits C/EBPα 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 24h, 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 TNFα induces STAMP2 expression in adipocytes and adipose tissue [15–17, 38]. Consistently, we found that TNFα 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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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. TNFα-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 TNFα 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, TNFα, 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/EBPα 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/EBPα 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/EBPα. TNFα led to a small decrease in C/EBPα protein levels and recruitment to the *Stamp2* promoter. We also showed that both NF-κB and C/EBPα binding elements contribute to *Stamp2* promoter activity, but this is in contrast to previous findings 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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].

Acknowledgments:

This work was supported by grants from the Norwegian Research Council, Norwegian Cancer Society, Norway Health South East, Anders Jahre Fond, and University of Oslo to FS and from NIH to MEE (Roadmap Grant R90 DK071507) and MFG. TL received a travel grant from Lillemor Grobstok's Foundation. We thank Gökhan S. Hotamisligil for key suggestions for experimentation and for critically reading the manuscript.

Abbreviations:

STAMP2	six transmembrane protein of prostate 2
C/EBPα	CCAAT/enhancer binding protein alpha
TNFα	tumor necrosis factor alpha
Tg	thapsigargin
Tu	tunicamycin

References

[1]. Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev 2008;29:42–61. [PubMed: 18048764]

[2]. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annual review of immunology 2011;29:415–45.

[3]. Salvado L, Palomer X, Barroso E, Vazquez-Carrera M. Targeting endoplasmic reticulum stress in insulin resistance. Trends in endocrinology and metabolism: TEM 2015;26:438–48. [PubMed: 26078196]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[4]. Pagliassotti MJ, Kim PY, Estrada AL, Stewart CM, Gentile CL. Endoplasmic reticulum stress in obesity and obesity-related disorders: An expanded view. Metabolism 2016;65:1238–46. [PubMed: 27506731]

[5]. Gregor MG, Hotamisligil GS. Adipocyte stress: The endoplasmic reticulum and metabolic disease. Journal of lipid research 2007.

[6]. Gregor MF, Misch ES, Yang L, Hummasti S, Inouye KE, Lee AH, et al. The role of adipocyte XBP1 in metabolic regulation during lactation. Cell Rep 2013;3:1430–9. [PubMed: 23623498]

[7]. Usui M, Yamaguchi S, Tanji Y, Tominaga R, Ishigaki Y, Fukumoto M, et al. Atf6alpha-null mice are glucose intolerant due to pancreatic beta-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance. Metabolism 2012;61:1118–28. [PubMed: 22386934]

[8]. Sikkeland J, Sheng X, Jin Y, Saatcioglu F. STAMPing at the crossroads of normal physiology and disease states. Molecular and cellular endocrinology 2016;425:26–36. [PubMed: 26911931]

[9]. Wellen KE, Fucho R, Gregor MF, Furuhashi M, Morgan C, Lindstad T, et al. Coordinated regulation of nutrient and inflammatory responses by STAMP2 is essential for metabolic homeostasis. Cell 2007;129:537–48. [PubMed: 17482547]

[10]. Han L, Tang MX, Ti Y, Wang ZH, Wang J, Ding WY, et al. Overexpressing STAMP2 improves insulin resistance in diabetic ApoE(−)/(−)/LDLR(−)/(−) mice via macrophage polarization shift in adipose tissues. PLoS One 2013;8:e78903. [PubMed: 24236066]

[11]. Wang J, Han L, Wang ZH, Ding WY, Shang YY, Tang MX, et al. Overexpression of STAMP2 suppresses atherosclerosis and stabilizes plaques in diabetic mice. Journal of cellular and molecular medicine 2014;18:735–48. [PubMed: 24467451]

[12]. Kim HY, Park SY, Lee MH, Rho JH, Oh YJ, Jung HU, et al. Hepatic STAMP2 alleviates high fat diet-induced hepatic steatosis and insulin resistance. Journal of hepatology 2015.

[13]. Arner P, Stenson BM, Dungner E, Naslund E, Hoffstedt J, Ryden M, et al. Expression of six transmembrane protein of prostate 2 in human adipose tissue associates with adiposity and insulin resistance. J Clin Endocrinol Metab 2008;93:2249–54. [PubMed: 18381574]

[14]. Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Rotellar F, Valenti V, et al. Six-transmembrane epithelial antigen of prostate 4 and neutrophil gelatinase-associated lipocalin expression in visceral adipose tissue is related to iron status and inflammation in human obesity. European journal of nutrition 2013;52:1587–95. [PubMed: 23179203]

[15]. Moreno-Navarrete JM, Ortega F, Serrano M, Perez-Perez R, Sabater M, Ricart W, et al. Decreased STAMP2 expression in association with visceral adipose tissue dysfunction. J Clin Endocrinol Metab 2011;96:E1816–25. [PubMed: 21849520]

[16]. Zhang CM, Chi X, Wang B, Zhang M, Ni YH, Chen RH, et al. Downregulation of STEAP4, a highly-expressed TNF-alpha-inducible gene in adipose tissue, is associated with obesity in humans. Acta pharmacologica Sinica 2008;29:587–92. [PubMed: 18430367]

[17]. Moldes M, Lasnier F, Gauthereau X, Klein C, Pairault J, Feve B, et al. Tumor necrosis factor-alpha-induced adipose-related protein (TIARP), a cell-surface protein that is highly induced by tumor necrosis factor-alpha and adipose conversion. The Journal of biological chemistry 2001;276:33938–46. [PubMed: 11443137]

[18]. Cawthorn WP, Sethi JK. TNF-alpha and adipocyte biology. FEBS Lett 2008;582:117–31. [PubMed: 18037376]

[19]. Ramadoss P, Chiappini F, Bilban M, Hollenberg AN. Regulation of hepatic six transmembrane epithelial antigen of prostate 4 (STEAP4) expression by STAT3 and CCAAT/enhancer-binding protein alpha. The Journal of biological chemistry 2010;285:16453–66. [PubMed: 20304921]

[20]. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010;140:900–17. [PubMed: 20303879]

[21]. Sikkeland J, Saatcioglu F. Differential expression and function of stamp family proteins in adipocyte differentiation. PloS one 2013;8:e68249. [PubMed: 23874564]

[22]. Hausman DB, Park HJ, Hausman GJ. Isolation and culture of preadipocytes from rodent white adipose tissue. Methods Mol Biol 2008;456:201–19. [PubMed: 18516563]

[23]. Miller RS, Diaczok D, Cooke DW. Repression of GLUT4 expression by the endoplasmic reticulum stress response in 3T3-L1 adipocytes. Biochemical and biophysical research communications 2007;362:188–92. [PubMed: 17698029]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[24]. Lefterova MI, Mullican SE, Tomaru T, Qatanani M, Schupp M, Lazar MA. Endoplasmic reticulum stress regulates adipocyte resistin expression. Diabetes 2009;58:1879–86. [PubMed: 19491212]

[25]. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nature medicine 2001;7:941–6.

[26]. Mondal AK, Das SK, Varma V, Nolen GT, McGehee RE, Elbein SC, et al. Effect of endoplasmic reticulum stress on inflammation and adiponectin regulation in human adipocytes. Metabolic syndrome and related disorders 2012;10:297–306. [PubMed: 22545589]

[27]. Qin DN, Zhu JG, Ji CB, Chunmei S, Kou CZ, Zhu GZ, et al. Monoclonal antibody to six transmembrane epithelial antigen of prostate-4 influences insulin sensitivity by attenuating phosphorylation of P13K (P85) and Akt: possible mitochondrial mechanism. Journal of bioenergetics and biomembranes 2011;43:247–55. [PubMed: 21647634]

[28]. Ohgami RS, Campagna DR, McDonald A, Fleming MD. The Steap proteins are metalloreductases. Blood 2006;108:1388–94. [PubMed: 16609065]

[29]. Fernandez-Real JM, Manco M. Effects of iron overload on chronic metabolic diseases. The lancet Diabetes & endocrinology 2014;2:513–26. [PubMed: 24731656]

[30]. Le Lay S, Simard G, Martinez MC, Andriantsitohaina R. Oxidative stress and metabolic pathologies: from an adipocentric point of view. Oxidative medicine and cellular longevity 2014;2014:908539. [PubMed: 25143800]

[31]. Farre D, Roset R, Huerta M, Adsuara JE, Rosello L, Alba MM, et al. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res 2003;31:3651–3. [PubMed: 12824386]

[32]. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics 2002;18:333–4. [PubMed: 11847087]

[33]. Xu H, Hotamisligil GS. Signaling pathways utilized by tumor necrosis factor receptor 1 in adipocytes to suppress differentiation. FEBS Lett 2001;506:97–102. [PubMed: 11591379]

[34]. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell 2008;132:344–62. [PubMed: 18267068]

[35]. Tang QQ, Zhang JW, Daniel Lane M. Sequential gene promoter interactions of C/EBPbeta, C/EBPalpha, and PPARgamma during adipogenesis. Biochemical and biophysical research communications 2004;319:235–9. [PubMed: 15158467]

[36]. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, et al. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Molecular cell 1999;3:151–8. [PubMed: 10078198]

[37]. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol 2008;8:923–34. [PubMed: 19029988]

[38]. Chen XH, Zhao YP, Zhu C, Ji CB, Zhang CM, Zhu JG, et al. [Regulative role of TNFalpha on STEAP4 gene in matured human adipocytes]. Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics 2009;11:1008–11. [PubMed: 20113612]

[39]. Qin DN, Kou CZ, Ni YH, Zhang CM, Zhu JG, Zhu C, et al. Monoclonal antibody to the six-transmembrane epithelial antigen of prostate 4 promotes apoptosis and inhibits proliferation and glucose uptake in human adipocytes. International journal of molecular medicine 2010;26:803–11. [PubMed: 21042773]

[40]. Gauss GH, Kleven MD, Sendamarai AK, Fleming MD, Lawrence CM. The Crystal Structure of Six Transmembrane Antigen of the Prostate 4 (Steap4), a Ferri/Cuprireductase, Suggests a Novel Inter-Domain Flavin Binding Site. The Journal of biological chemistry 2013.

[41]. Jin Y, Wang L, Qu S, Sheng X, Kristian A, Maelandsmo GM, et al. STAMP2 increases oxidative stress and is critical for prostate cancer. EMBO molecular medicine 2015;7:315–31. [PubMed: 25680860]

[42]. Lambe T, Simpson RJ, Dawson S, Bouriez-Jones T, Crockford TL, Lepherd M, et al. Identification of a Steap3 endosomal targeting motif essential for normal iron metabolism. Blood 2009;113:1805–8. [PubMed: 18955558]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[43]. Batchvarova N, Wang XZ, Ron D. Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). EMBO J 1995;14:4654–61. [PubMed: 7588595]

[44]. Han J, Murthy R, Wood B, Song B, Wang S, Sun B, et al. ER stress signalling through eIF2alpha and CHOP, but not IRE1alpha, attenuates adipogenesis in mice. Diabetologia 2013;56:911–24. [PubMed: 23314846]

[45]. Fernandez-Real JM, Lopez-Bermejo A, Ricart W. Cross-talk between iron metabolism and diabetes. Diabetes 2002;51:2348–54. [PubMed: 12145144]

[46]. Tajima S, Ikeda Y, Sawada K, Yamano N, Horinouchi Y, Kihira Y, et al. Iron reduction by deferoxamine leads to amelioration of adiposity via the regulation of oxidative stress and inflammation in obese and type 2 diabetes KKAy mice. Am J Physiol Endocrinol Metab 2012;302:E77–86. [PubMed: 21917632]

[47]. Yan HF, Liu ZY, Guan ZA, Guo C. Deferoxamine ameliorates adipocyte dysfunction by modulating iron metabolism in ob/ob mice. Endocr Connect 2018;7:604–16. [PubMed: 29678877]

[48]. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods in enzymology 2011;490:71–92. [PubMed: 21266244]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Highlights

- STAMP2 is of importance in the understanding of metabolic disease
- ER stress affects STAMP2 expression, distribution, and function in adipocytes
- Loss of C/EBPα binding to *Stamp2* promoter under ER stress decrease STAMP2 levels
- This implicates STAMP2 as a link between ER dysfunction and metabolic disease

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

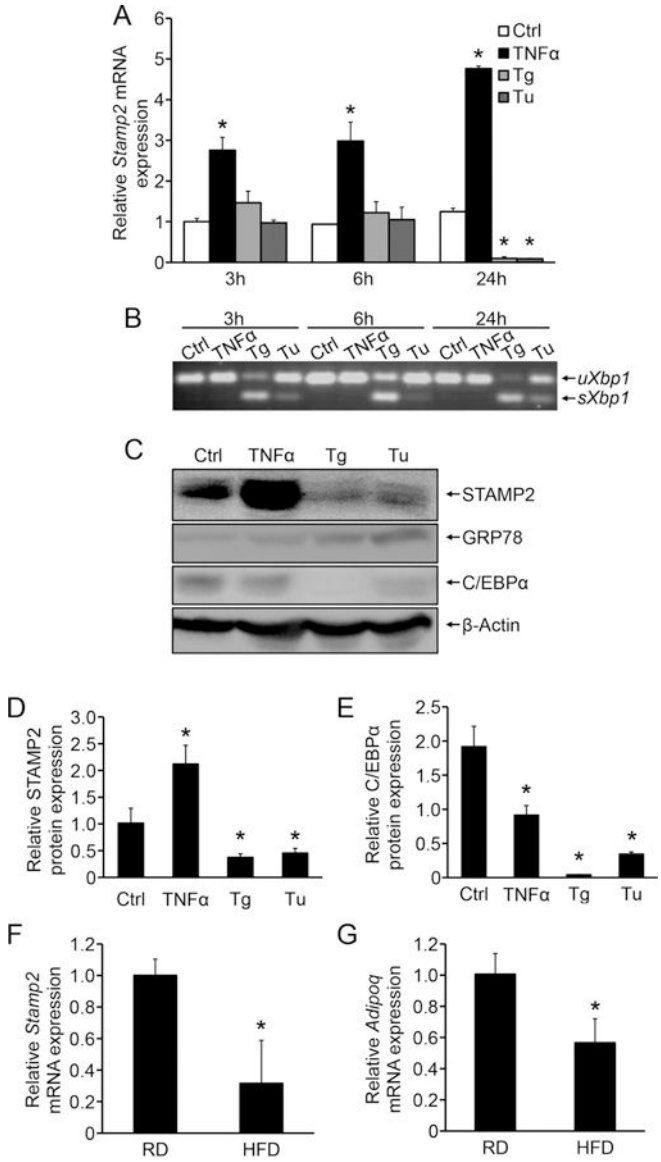

Figure 1.

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 *Stamp*2 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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

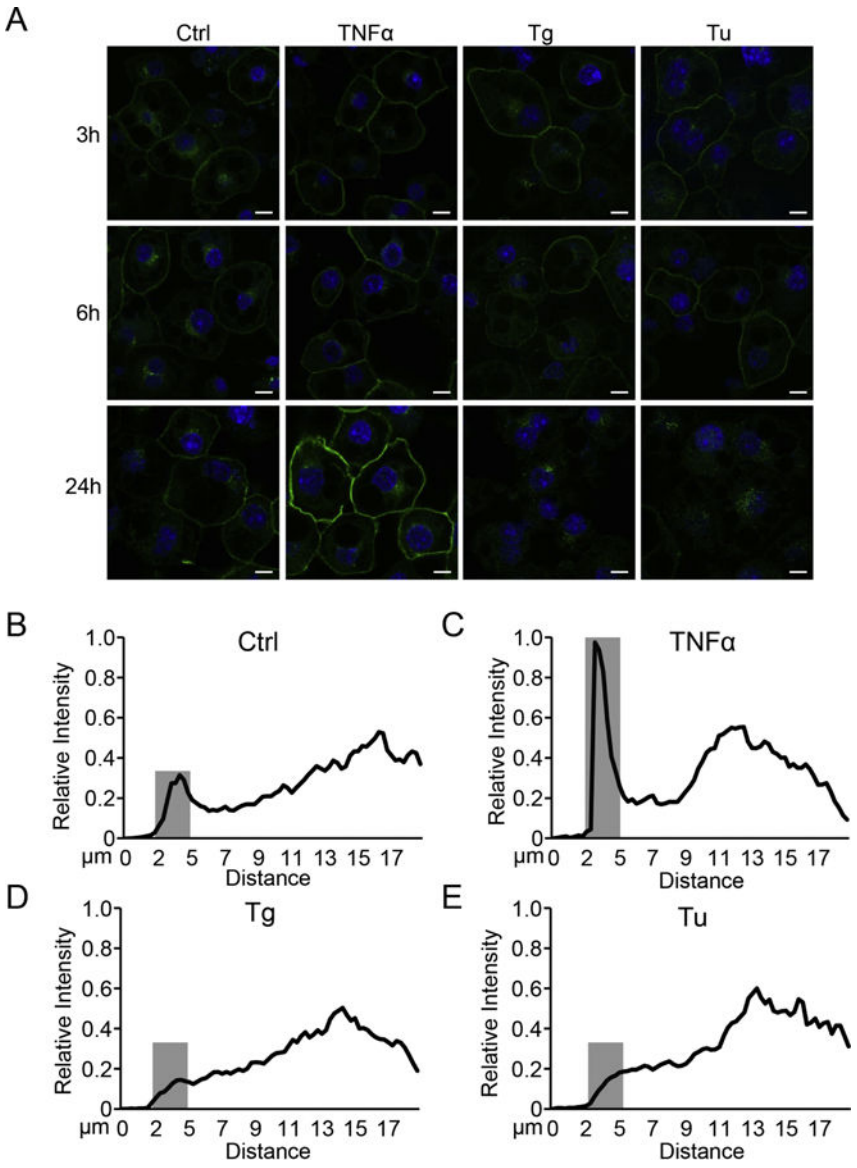

Figure 2.

Distinct regulation of STAMP2 subcellular distribution by TNFα and ER stress. (A) Immunofluorescence confocal microscopy analysis of differentiated 3T3-L1 adipocytes treated as in Figure 1 with TNFα, Tu and Tg to determine regulation of its subcellular localization. Antiserum against STAMP2 was used as described in Materials and Methods. Images are representative of three independent experiments, Scale bar = 10 μm. (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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

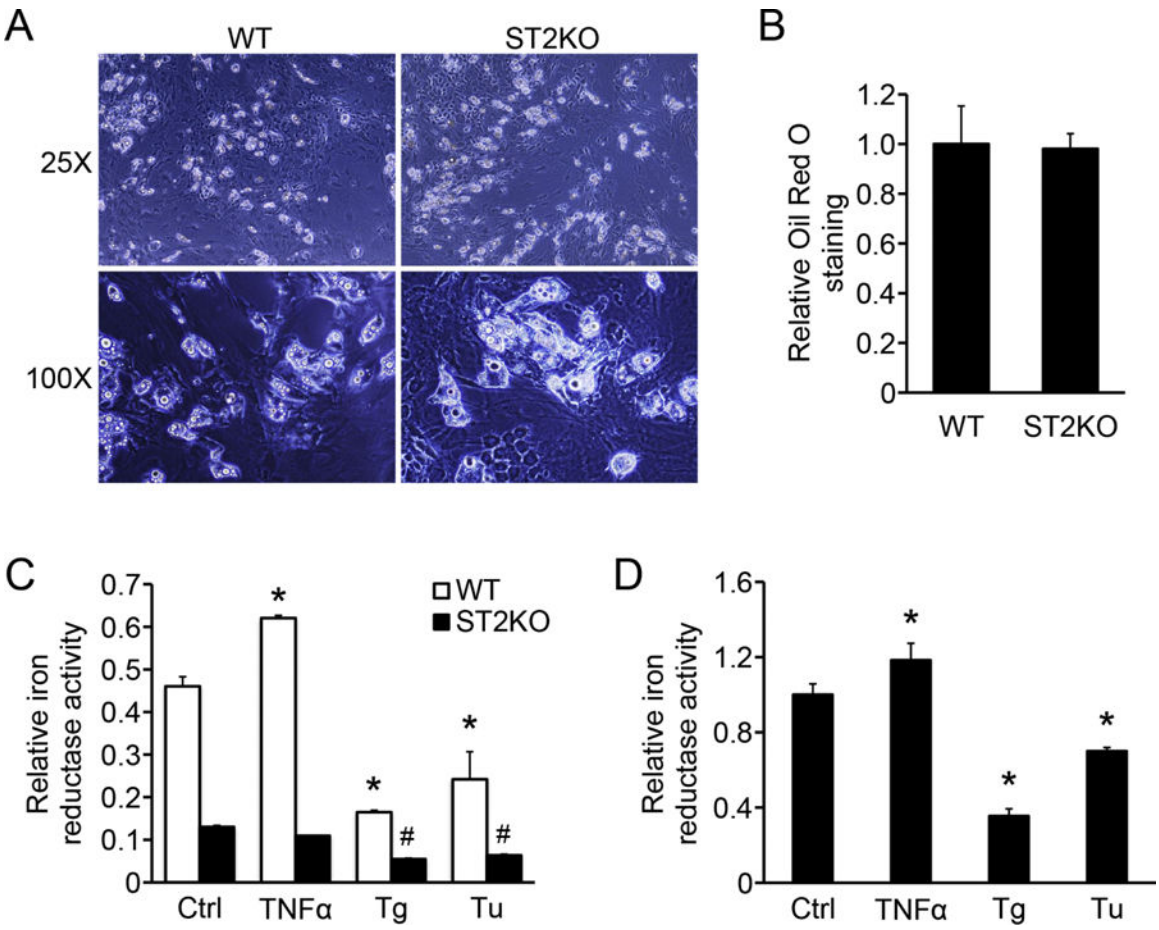

Figure 3.

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 (25x and 100x 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), TNFα (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 Materials and Methods. 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), TNFα (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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

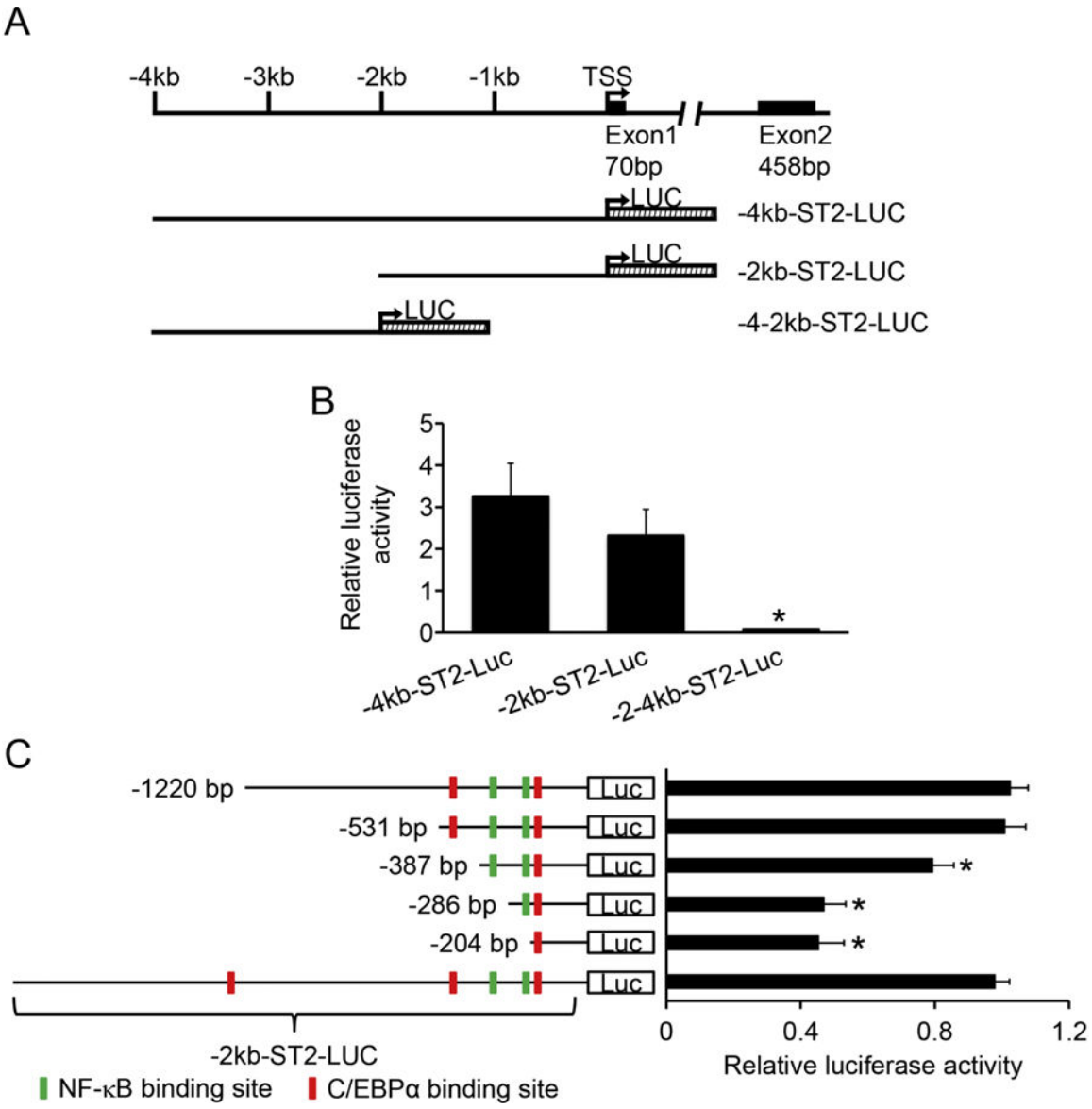

Figure 4.

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 −2kb 5' *Stamp2* promoter region with corresponding reporter activity in HeLa cells. Results shown are from 3 independent experiments, n=9.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

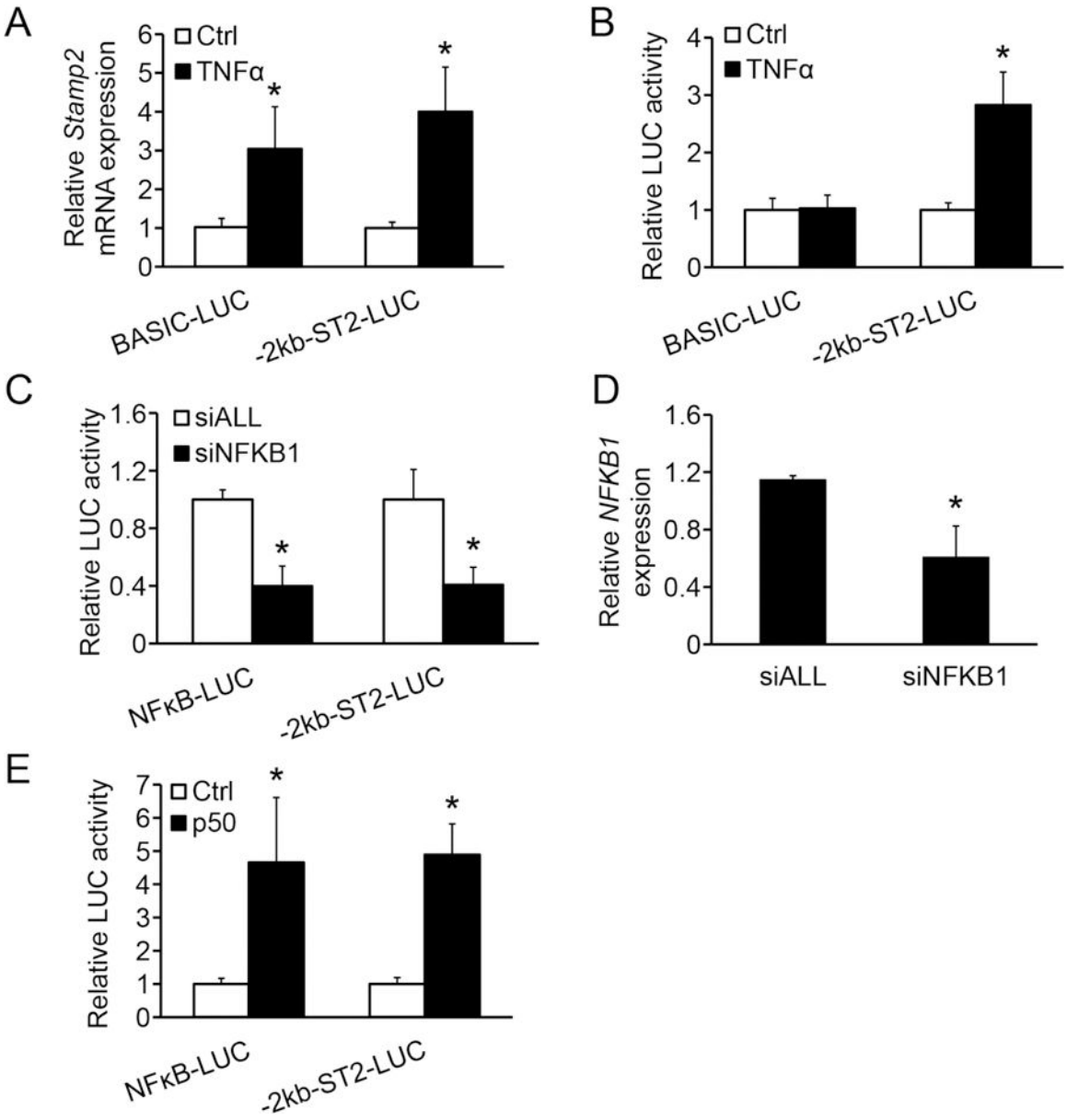

Figure 5.

Stamp2 promoter is activated by TNFα and p50. (A) 3T3-L1 cells with stable expression of either the −2kb-ST2-LUC or a BASIC-LUC reporters were differentiated into adipocytes, treated with TNFα (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 2xNFκB-LUC or −2kb-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)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

HeLa cells were co-transfected with either 2xNFκB-LUC or −2kb-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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

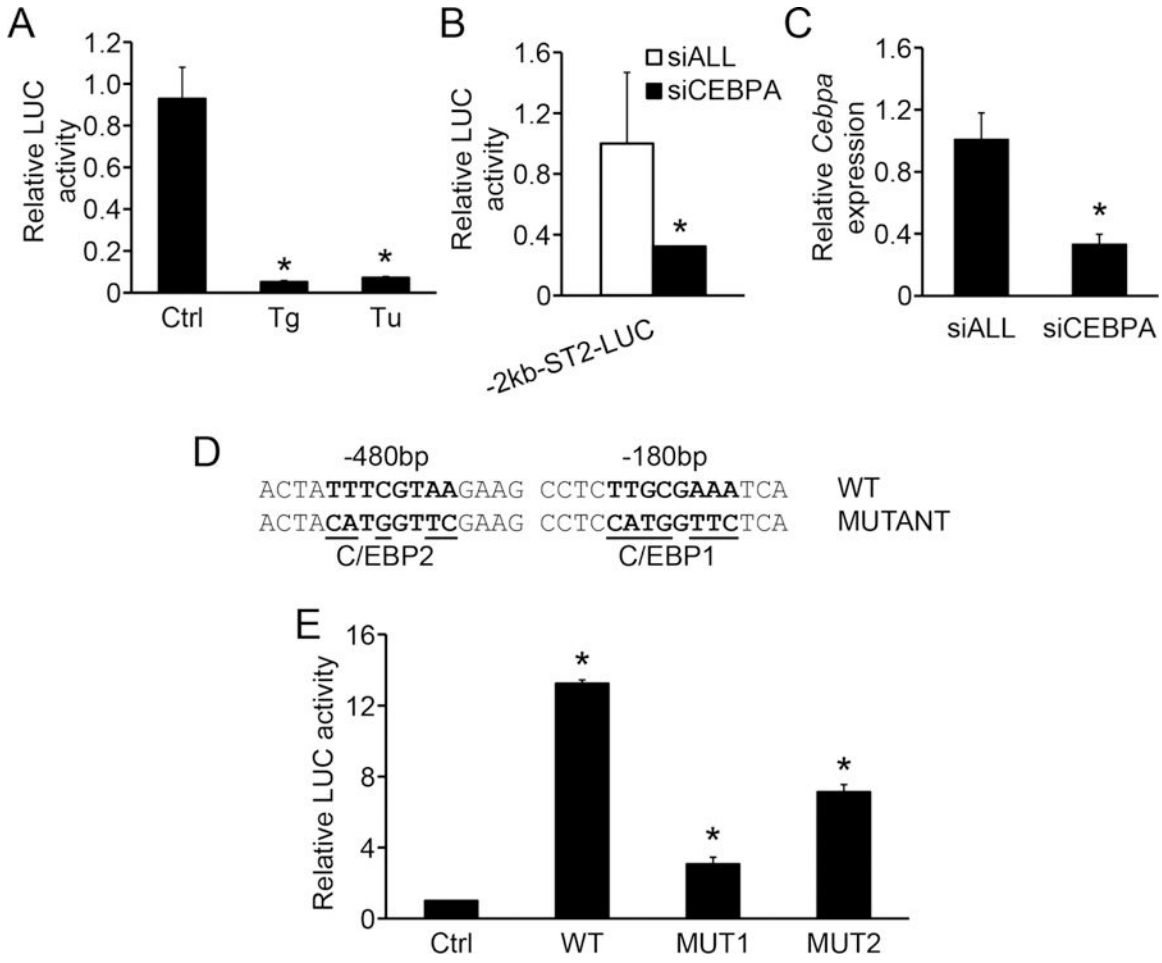

Figure 6.

Stamp2 promoter activity requires C/EBPα. (A) 3T3-L1 cells stably expressing the −2kb-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 −2kb-ST2-LUC reporter were transfected with either AllStar (siAll) or *Cepba* (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/EBPα 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/EBPα and either BASIC-LUC reporter plasmid (Ctrl), the wild-type −2kb-ST2-LUC, or a −2kb-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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

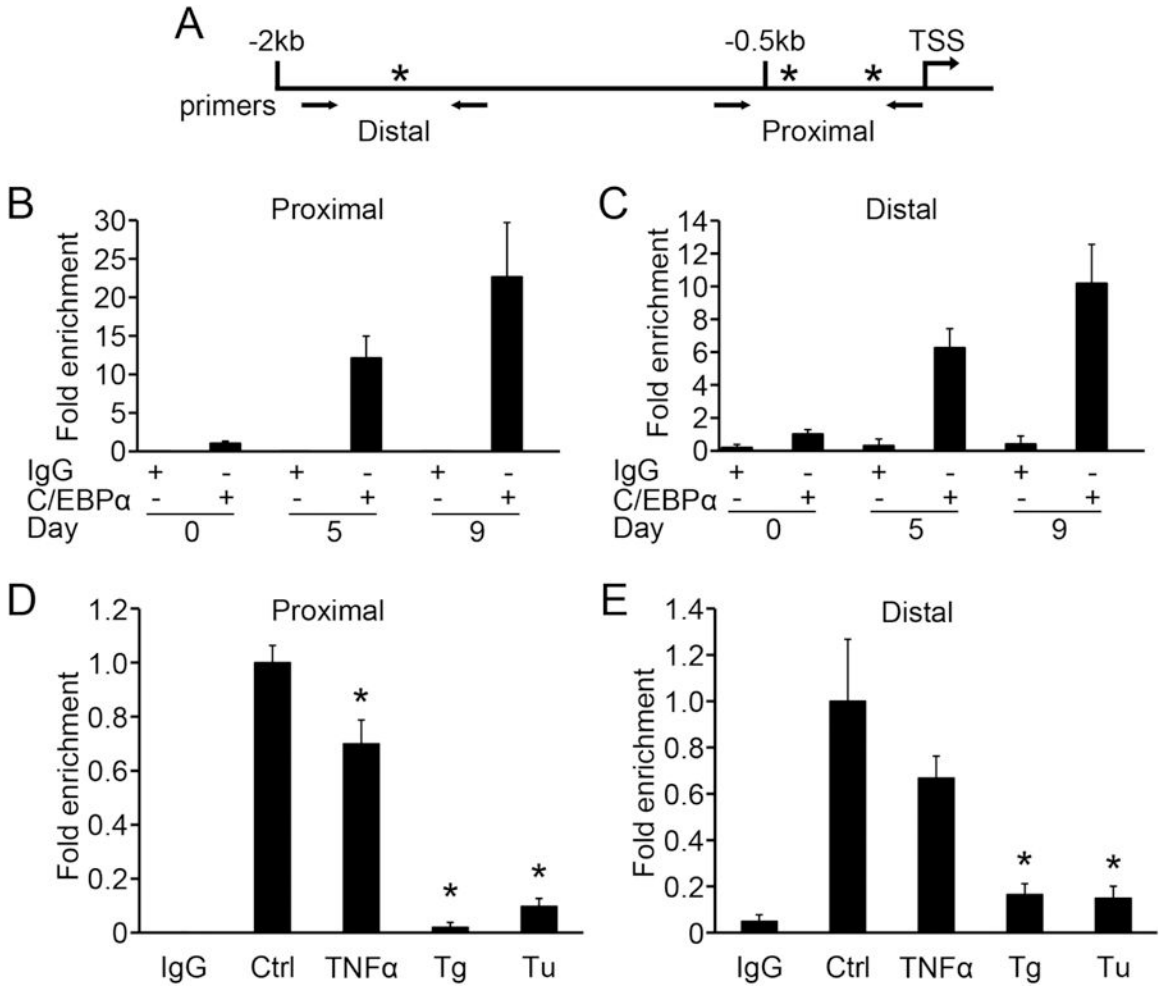

Figure 7.

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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

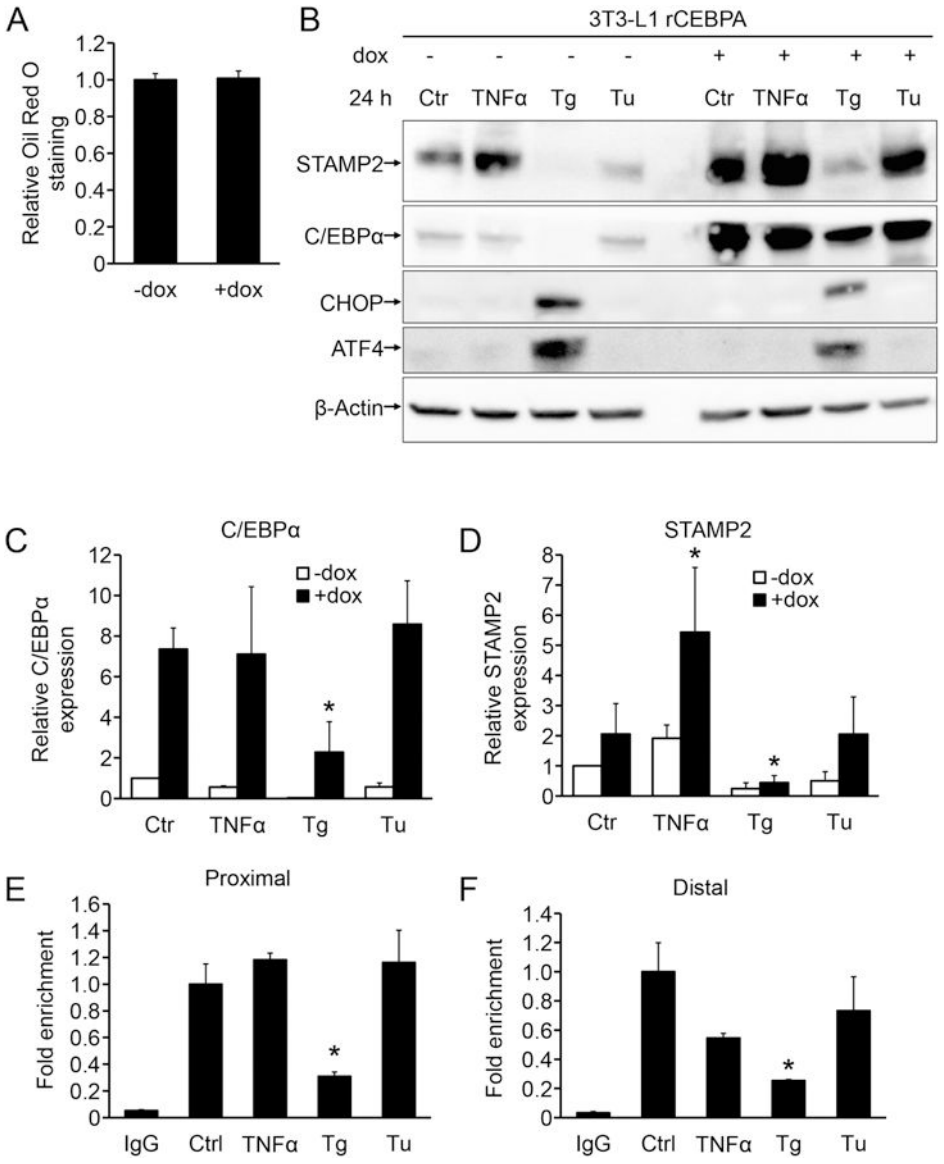

Figure 8.

C/EBPα 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/EBPα (−/+ Dox). (B) 3T3-L1 cells with inducible C/EBPα expression (+dox) were differentiated into adipocytes and were either left untreated, or incubated with Tg (300nM), or Tu (2ng/ml) for 24h 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/EBPα, 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/EBPα recruitment to the proximal and distal promoter regions of the *Stamp2* gene. Data shown are from two independent ChIP experiments done in triplicate.