Original Article

MOLECULAR METABOLISM

ASK1 (MAP3K5) is transcriptionally upregulated by E2F1 in adipose tissue in obesity, molecularly defining a human dys-metabolic obese phenotype

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ABSTRACT

Objective: Obesity variably disrupts human health, but molecular-based patients' health-risk stratification is limited. Adipose tissue (AT) stresses may link obesity with metabolic dysfunction, but how they signal in humans remains poorly-characterized. We hypothesized that a transcriptional AT stress-signaling cascade involving E2F1 and ASK1 (MAP3K5) molecularly defines high-risk obese subtype.

Methods: ASK1 expression in human AT biopsies was determined by real-time PCR analysis, and chromatin immunoprecipitation (ChIP) adopted to AT explants was used to evaluate the binding of E2F1 to the *ASK1* promoter. Dual luciferase assay was used to measure *ASK1* promoter activity in HEK293 cells. Effects of E2F1 knockout/knockdown in adipocytes was assessed utilizing mouse-embryonal-fibroblasts (MEF)-derived adipocyte-like cells from WT and E2F1$^{-/-}$ mice and by siRNA, respectively. ASK1 depletion in adipocytes was studied in MEF-derived adipocyte-like cells from WT and adipose tissue-specific ASK1 knockout mice (ASK1-ATKO).

Results: Human visceral-AT *ASK1* mRNA (N = 436) was associated with parameters of obesity-related cardio-metabolic morbidity. Adjustment for *E2F1* expression attenuated the association of *ASK1* with fasting glucose, insulin resistance, circulating IL-6, and lipids (triglycerides, HDL-cholesterol), even after adjusting for BMI. Chromatin-immunoprecipitation in human-AT explants revealed BMI-associated increased occupancy of the *ASK1* promoter by E2F1 ($r^2 = 0.847$, $p < 0.01$). In adipocytes, siRNA-mediated E2F1-knockdown, and MEF-derived adipocytes of *E2F1*-knockout mice, demonstrated decreased ASK1 expression and signaling to JNK. Mutation/truncation of an E2F1 binding site in h*ASK1* promoter decreased E2F1-induced *ASK1* promoter activity, whereas E2F1-mediated sensitization of *ASK1* promoter to further activation by TNFα was inhibited by JNK-inhibitor. Finally, MEF-derived adipocytes from adipocyte-specific *ASK1*-knockout mice exhibited lower leptin and higher adiponectin expression and secretion, and resistance to the effects of TNFα.

Conclusions: AT E2F1 −ASK1 molecularly defines a metabolically-detrimental obese sub-phenotype. Functionally, it may negatively affect AT endocrine function, linking AT stress to whole-body metabolic dysfunction.

Keywords Obesity; Transcriptional regulation; Sub-phenotypes; Adipose tissue; Adipocytes; Stress response

1. INTRODUCTION

Obese sub-phenotypes associated with increased cardio-metabolic risk are characterized by metabolically and endocrinologically dysfunctional AT that is thought to contribute to the development of obesity-associated morbidities [1—3]. As an underlying mechanism for dysfunctional AT, various AT stresses have been recognized in obesity, including inflammation, endoplasmic reticulum stress, hypoxia, and oxidative stress [4—8]. Much effort has been invested in understanding which types of besity-induced AT stress is most significant, and the emerging picture is of an inter-connected "network of stresses" [9]. Yet, the molecular pathways activated by these stresses, linking them with the functional alterations they are claimed to induce, are incompletely mapped. The interest in this extends beyond understanding the basic pathogenic mechanisms; such AT stress-response network(s), particularly when identified in human-AT, may constitute a molecular signature that could be used for improved patient stratification and identification of targets of future pharmacological therapies.

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Received February 15, 2017 • Accepted March 27, 2017 • Available online 6 May 2017

http://dx.doi.org/10.1016/j.molmet.2017.05.003

Several signaling pathways were reported to be activated in AT in obesity, mostly in mouse models [10–17]. In human-AT in obesity, one such stress-responsive pathway activated particularly in the intra-abdominal visceral fat is a MAP kinase signaling cascade comprised of MAP3K ASK1 (MAP3K5), MAP2Ks MKK4 and 3/6, MAPKs p38MAPK, and JNK [18,19]. We demonstrated that ASK1 expression (both mRNA and protein levels) was up-regulated in obesity and, more importantly, that *ASK1* mRNA levels in omental fat constituted, by multivariate analysis, a statistical predictor of whole-body insulin resistance independent of age, sex, BMI, and additional confounders [18]. In cellular systems, ASK1 has been recognized to respond to the same stresses that are implicated in AT in obesity, in particular to inflammation, oxidative and ER stresses [20–22]. Functionally related to obesity, genetic variants of *ASK1* that resulted in decreased ASK1 expression in muscle were associated with insulin resistance in Pima Indians [23]. In mice, whole-body knockout of *ASK1* predisposed to obesity, potentially by interfering with brown fat function [24]. These two studies assign a potential role for ASK1 in whole-body metabolic (dys)regulation. Yet, the impact of ASK1 over-expression in humans, particularly in white adipose tissue, and the molecular underpinnings for its elevated expression, remain unknown.

As a MAP kinase, ASK1 was mainly shown to be regulated by phosphorylation and/or oxidation of its inhibitory partner thioredoxin [22,25,26]. Yet, transcriptional regulation of ASK1 was also reported. The transcription factor E2F1, mainly known as a cell cycle regulator, binds directly to the *ASK1* promoter and up-regulates its expression in cancer cells [27–29]. Recently, we reported significant gene regulatory functions for E2F1 in adipocytes, cells that are post-mitotic [30]. E2F1 was up-regulated in omental AT in obesity in the adipocyte cell fraction, increasing not only the expression of several autophagy genes but also the autophagy process itself [30]. This finding is consistent with other studies implicating non-cell-cycle related, including metabolic, functions of E2F1 (and of other classical cell-cycle regulators) [31–35]. In the present study, we test the hypothesis that ASK1 expression and its downstream signals are regulated by E2F1 in human-AT in obesity. Both clinically and mechanistically, we systematically investigated the possibility that the E2F1-ASK1 network molecularly defines an obese sub-phenotype characterized by AT stress and metabolic dysfunction.

2. MATERIAL AND METHODS

2.1. Study population

Participants were from two cohorts, one in Beer-Sheva, Israel (N = 16) and one in Leipzig, Germany (N = 436), both of which have been described in a previous publication [30]. AT biopsies were analyzed exactly as described in previous publications [18,19,36–38]. All procedures were approved in advance by the ethics committee and were conducted in accordance with Declaration of Helsinki guidelines. In brief, participants were recruited before undergoing abdominal surgery (primarily bariatric surgery and elective cholecystectomy) after providing written informed consent. Paired subcutaneous (Sc) and Om AT biopsies were obtained during surgery and immediately delivered to the laboratory where they were processed for mRNA expression or chromatin immunoprecipitation (ChIP) studies.

2.2. Materials

Tissue culture media were from Biological Industries (Beit-Haemek, Israel). Indomethacin (I7378), dexamethasone (D4902), 3-isobutylmethylxanthine (IBMX) (I7018), and rosiglitazone (R2408) were from Sigma–Aldrich. Recombinant murine/human TNF (410-MT, 210-TA respectively) and murine IL-1β (401-ML) were purchased from R&D Systems Inc.

2.3. Cell culture

Human embryonic kidney (HEK) 293T cells (ATCC, Manassas, VA) were grown in DMEM containing 4.5 mM glucose, 10%FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Medium was changed every other day. For Western blot analysis experiments, HEK293 were treated with TNF (10 ng/ml) with or without SP600125 for 24 h. Mouse embryonal fibroblasts (MEF) from E2F1$^{-/-}$ and WT mice (kindly provided by Prof. Gustavo Leone, Department of molecular virology and genetics, College of Medicine and Public Health, Ohio State University, Ohio, USA) were cultured in DMEM containing 4.5 mM glucose, 10%FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Differentiation into adipocytes was performed as previously described [30,39]. For Western blot analyses and leptin/adiponectin concentration measurements, adipocyte-like MEFs were treated with TNFα (10 ng/ml) + IL-1β (10 ng/ml) for 24 h. Epididymal pre-adipocytes were grown and differentiated into mature adipocytes as previously described [40]. For quantitative Real-Time PCR experiments, epididymal adipocytes were treated for 4 or 24 h with one of the following treatments: TNFα (10 ng/ml)/Fas ligand (2 ng/ml)/Glucose oxidase (50 mU/ml) and Tunicamycin (5 μg/ml). For ASK1 depletion in adipocyte-like cells, we utilized MEF from adipocyte-specific ASK1-KO mice and control littermates. Targeted embryonic stem cell clones with exon 14 (whose deletion will lead to a frame shift) of ASK1 flanked by loxP sites and a FRT-flanked selection cassette were bought from the European Conditional Mouse Mutagenesis Program (EUCOMM) to generate floxed ASK1 mice. To obtain adipocyte-specific ASK1 depletion, homozygous ASK1 floxed mice were bred to mice that express the Cre enzyme driven by the adipocyte-specific adiponectin promoter (Adipoq-Cre mice) [41].

2.4. RNA extraction and quantitative real-time PCR

Total RNA from epididymal adipocytes or MEFs adipocyte-like cells was extracted using the RNeasy lipid tissue mini-kit (74804, Qiagen) and quantified using nano-drop. Then, 2 μg of RNA were reverse-transcribed with high capacity cDNA reverse transcriptase kit (4374966, Life Technologies Inc.). Taqman system (4369016, Life Technologies, Inc.) was used for real-time PCR amplification. Extraction of total RNA from paired human-AT biopsies (Leipzig cohort) was done according to a previously described protocol [18]. Relative gene expression was obtained after normalization to endogenous control genes, using formula $2^{-\Delta\Delta Ct}$ and specific primers (Supplementary Table 1).

2.5. E2f1 knockdown by small interfering RNA (siRNA) transfection

E2f1 and non-specific sequence control (*NS*) siRNAs ON-TARGET plus smart pools were obtained from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA). Each siRNA was transfected into differentiated epididymal adipocyte cell line at a concentration of 1 nmol/well by electroporation with GenePulser Xcell (BioRad Laboratories Inc., Hercules, CA). Briefly, epididymal adipocytes were trypsinized on the third day (of 6 days) of differentiation process from 150 mm culture plate dish. Cells were rinsed three times with PBS, centrifuged (5 min, 1200 rpm, room temperature), and after last wash supernatant was aspirated. Next, cells were re-suspended in 400 μl of Ingenio™ Electroporation Solution (Mirus Bio LLC, Madison, WI) with 4 nmol of *E2f1* or *NS* siRNA, and electroporation was performed in accordance with the manufacturer's instructions using the optimal program (170 V, 10 ms, two pulses). After electroporation, cells were cultured in

complete medium (DMEM 20% FCS). 24 h post-electroporation, cells were incubated with TNF(10 ng/ml) in DMEM 0.5%BSA for additional 24 h. Cells were rinsed 3 times with ice-cold PBS and subjected to either quantitative real-time PCR or Western blot analysis.

2.6. Transient transfection and promoter activity assay

HEK293 cells were seeded in 24 well plate in 1 ml/well of DMEM 10% FCS. 24 h later, medium was changed to a fresh 0.5 ml/well, and cells were transfected using jetPEI reagent (101-01N, Poly-plus transfection Inc.), according to manufacturer's instructions. Briefly, 200 ng/well of the expression plasmid (pCMV-empty/E2F1), 200 ng/well of the firefly luciferase reporter plasmids (*ASK1*^{WT}/*ASK1*^{m1}/*ASK1*^{m2}/*ASK1*^{m3}), and 2 ng/well of the *Renilla* luciferase reporter plasmid were used for each transfection. 4 h after transfection, medium was changed to fresh DMEM 10%FBS with or without TNFα (10 ng/ml) and with or without SP600125. Cells were lysed 24 h after incubation with or without TNFα 10 ng/ml and with or without SP600125 treatments by applying 100 μl of Passive Lysis Buffer of the Dual Luciferase Reporter Assay Kit (E-1910, Promega Corporation) into each well of the 24-well plate. 20 μl of cell lysate were used for the luciferase reporter assay with the same kit, according to the manufacturer's protocol. Luminescence intensity was quantified in a GloMax 20/20n Luminometer (Promega Corporation). The experiments were performed at least in triplicate. As a control for transfection efficiency, the firefly luciferase activity values were normalized to the *Renilla* luciferase activity values.

2.7. Promoter analysis and site-directed mutagenesis

Human *ASK1* promoter (1000 bp upstream to the transcription start site-TSS and 500 bp downstream) was analyzed for predicted E2F1 binding sites using MatInspector software (Genomatix Tools). Promoter analysis produced one predicted E2F1 binding site on *ASK1* promoter, which was located 384 bp upstream the TSS with a score probability of 0.84 (1-highest probability). Using site-directed mutagenesis kit (Stratagene, Santa Clara, CA), E2F1-predicted binding site on the ASK1-Luc plasmid was mutated at the core sequence (CGCG) generating 3 new mutated ASK1-Luc plasmids, as indicated in Figure 4B. Mutations/deletions were validated by sequencing.

2.8. Leptin and adiponectin concentrations

Leptin and adiponectin were measured in cultured media with or without TNFα (10 ng/ml) + IL-1β (10 ng/ml) treatment for 24 h by ELISA (MOB00, MRP300, respectively, R&D Systems Inc.).

2.9. Chromatin immunoprecipitation (ChIP)

ChIP from fresh human-AT explants was performed following a recently-described protocol [42]. Briefly, 1 g of Sc or Om AT was minced into small pieces using two pairs of sterile scissors. Next, samples were cross-linked with 1% of formaldehyde at 37 °C for 8 min. Cross-linking reaction was quenched using 0.125 M glycine. Samples were washed with ice cold PBS 3 times, and, following the last wash, adipocyte lysis buffer was added. After 15 min of incubation on ice, samples were homogenized using a Dounce homogenizer (loose pestle, 20 strokes, Wheaton). Nuclei were released after 20 additional strokes using a tight pestle. After sonication, the protein-DNA complexes were immunoprecipitated using anti-ICAM (sc-7891X, as negative control), anti-RNA poll 2 (sc-21750X, as positive control) and anti-E2F1 antibodies (sc-193X, all from Santa Cruz Biotechnology). After cross-linking reversal at 65 °C for 4 h, DNA was purified using the phenol-chloroform method. Primers (5′ to 3′) for end-point PCR or quantitative real-time PCR were as follows: *ASK1*- Fw − GTGCTGGACCGCTTTTACAATGC, Rv − CAGTTCAAGTCGATCGCATGGAC. Input (diluted 1/300) was used as a normalizing control.

2.10. Statistical analysis

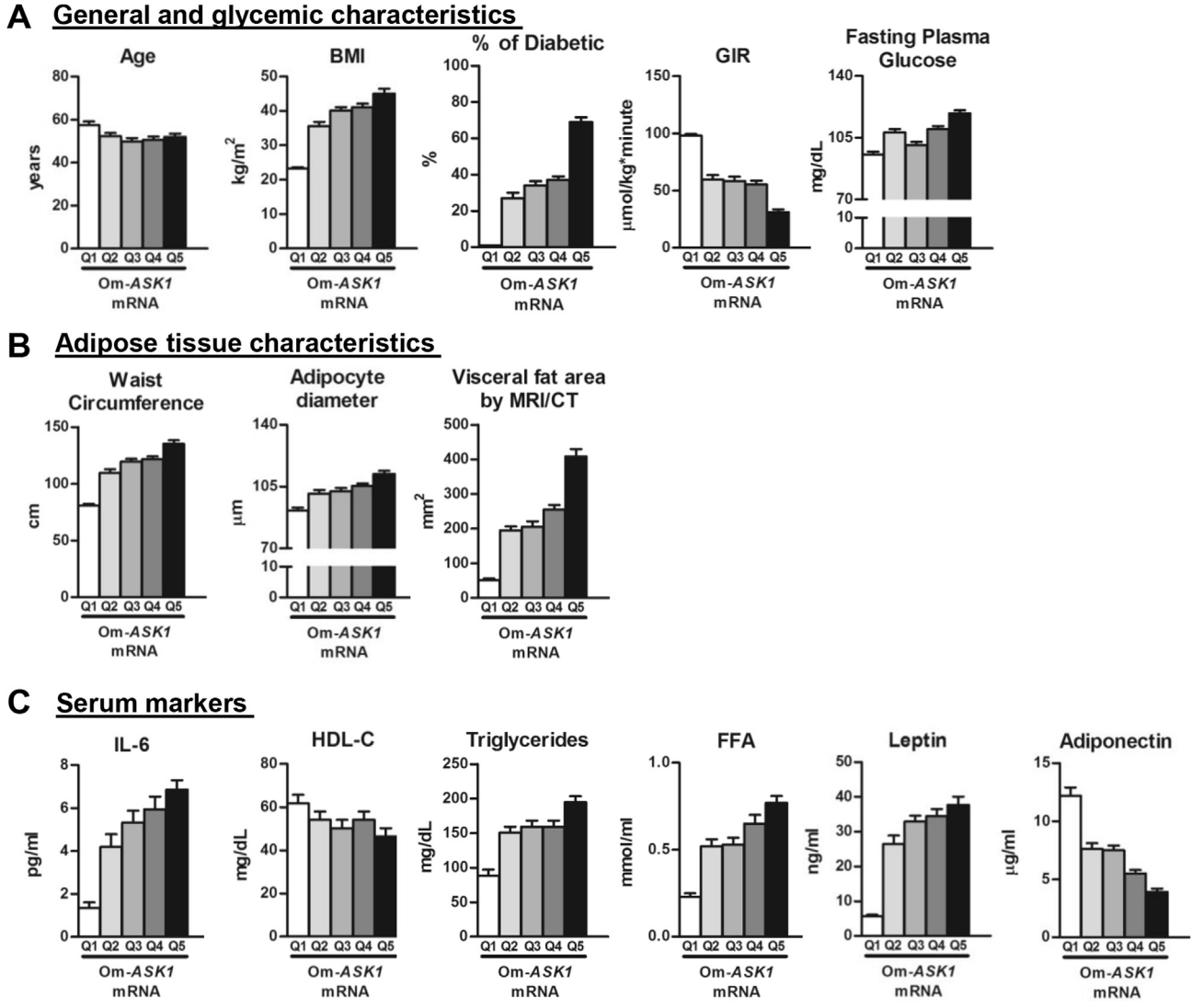
Data, expressed as the mean ± SD, and calculations were performed using GraphPad software. Statistically significant differences between two groups were evaluated using paired or unpaired Student's-t-test, as indicated in the figure legends. Correlation between BMI and E2F1 binding to *ASK1* promoter was assessed by linear regression. Clinical and biochemical characteristics of the study population were cross-classified across quintiles of visceral (Om) *ASK1* mRNA expression in order to control any un-normal variable distribution. *p* of trend was calculated across quintiles, and ANOVA with Tuckey post-hoc test was used to assess significance between each quintile pairs. We further assessed beta coefficients to Om-*ASK1* by performing step-wise multivariate linear regression models using SPSS statistical package.

3. RESULTS

3.1. A putative E2F1-ASK1 pathway in human adipose tissue in obesity

Previously, we reported that omental (Om) AT-*ASK1* mRNA levels were independent statistical predictors of whole-body insulin sensitivity by multivariate models that considered multiple potential confounders of this association (age, BMI, serum lipids and FFA, leptin, adiponectin, and IL-6) [18]. Moreover, we demonstrated that although AT-*ASK1* expression could be largely attributed in lean persons to the non-adipocyte stromal-vascular cells, the increased expression of this gene that accompanied visceral adiposity was largely contributed by the adipocytes. To assess the relation of Om-*ASK1* mRNA levels with additional clinical characteristics of the patients, with emphasis on obesity-related cardio-metabolic parameters, we utilized a larger, previously-described patient dataset [30], stratifying the data into quintiles of Om-*ASK1* mRNA (Table S1). Om-*ASK1* mRNA levels did not associate with age (Figure 1A). However, highly-significant associations (p of trend$<$0.05) were observed with BMI, fasting plasma glucose, and insulin resistance (as determined by hyper-insulinemic-euglycemic clamp), supporting the results of our previous work [18] (Figure 1A, Table S1). Furthermore, we found a significant association between Om-*ASK1* mRNA levels and adipocyte diameter and correlations with markers of obesity-associated cardio-metabolic risk, such as waist circumference and visceral fat area (Figure 1B). Moreover, high circulating levels of IL-6, triglycerides, free fatty acids (FFA), and leptin and low circulating levels of adiponectin were significantly associated with higher Om-*ASK1* mRNA levels ($p < 0.001$ for all, Figure 1C). Indeed, only 1% of the population in the lower Om-*ASK1* mRNA quintile was diagnosed with diabetes, whereas in the top quintile, more than 60% were diabetic patients (Figure 1A). Among the sub-group of obese patients (BMI $\geq$30 kg/m^2, N = 270), Om-*ASK1* mRNA associated with fasting glucose, HbA1c, insulin resistance, serum lipids, and with low circulating high-Mw-adiponectin, associations that remained significant even when further adjusting for BMI (in addition to age and sex) (Figure 1D). Furthermore, significant associations could be demonstrated in both obese subtypes categorized by abdominal fat distribution pattern (subcutaneously versus viscerally − obese) (Figure 1D).

MAP kinases are mainly thought to be regulated by phosphorylation. In cancer cells, transcriptional regulation of ASK1 was also demonstrated by the cell-cycle regulator E2F1 [27−29]. Recently, we reported that *E2F1* expression is increased in Om of intra-abdominally obese humans and may act to activate non-cell-cycle-related genes [30].



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Physiological parameter	Obese (all, N=270) Age, Sex, *ASK1* β	Sig	Obese (all, N=270) Age, Sex, *ASK1*, BMI β	Sig	Obese - SC (N=206) Age, Sex, *ASK1* β	Sig	Obese - Om (N=64) Age, Sex, *ASK1* β	Sig
Waist Circumference	**0.238**	**< 0.001**	0.010	0.751	**0.245**	**< 0.001**	0.227	0.062
Triglycerides	**0.163**	**0.025**	**0.200**	**0.009**	-0.011	0.9	0.102	0.525
HDL-C	**-0.280**	**< 0.001**	**-0.184**	**0.011**	-0.145	0.101	0.242	0.135
Fasting plasma glucose	**0.259**	**< 0.001**	**0.211**	**0.001**	**0.143**	**0.042**	0.174	0.199
HbA_{1c}	**0.391**	**< 0.001**	**0.336**	**< 0.001**	**0.205**	**0.004**	**0.274**	**0.043**
Fasting plasma insulin	**0.315**	**< 0.001**	**0.285**	**< 0.001**	**0.233**	**0.003**	**0.377**	**0.011**
HOMA-IR	**0.307**	**< 0.001**	**0.265**	**< 0.001**	**0.218**	**0.05**	**0.394**	**0.007**
GIR	**-0.377**	**< 0.001**	**-0.344**	**< 0.001**	-0.101	0.291	0.283	0.088
IL-6	**0.132**	**0.046**	0.098	0.146	**0.153**	**0.044**	0.204	0.149
Visceral fat area	**0.455**	**< 0.001**	**0.322**	**< 0.001**	**0.292**	**< 0.001**	**0.292**	**0.038**
Adipocyte diameter	**0.278**	**< 0.001**	0.104	0.090	**0.217**	**0.010**	**0.426**	**0.002**
Adiponectin	**-0.320**	**< 0.001**	**-0.254**	**< 0.001**	-0.102	0.183	0.248	0.07

Figure 1: Correlation of *ASK1* mRNA expression levels in human omental AT with different clinical parameters. (A–C) Clinical parameters of 436 persons were cross-classified across quintiles of Om-*ASK1* mRNA expression. *p* of trend (by linear regression) and ANOVA + post-hoc analysis of inter-quintile significance are presented in Table S1. **(D)** Multivariate analyses of the association between OM-*ASK1* mRNA and various clinical parameters among the entire obese subgroup (N = 270), and in obese patients with predominant subcutaneous obesity (Obese-SC, N = 206, defined as abdominal visceral/subcutaneous area<50% by L3-L4 CT or MRI imaging) or with predominant visceral adiposity (Obese-Om, N = 64). All models are adjusted for age and sex, and for the entire obese subgroup an additional model is presented also adjusted for BMI.

Therefore, we assessed whether increased BMI was associated in human-AT with greater occupancy of the *ASK1* promoter by E2F1. Using a chromatin immunoprecipitation (ChIP) protocol adopted to human-AT [42], a highly significant correlation between BMI and E2F1 binding to the *ASK1* promoter could be observed in both Om and Sc fat, though the effect of BMI was more robust for Om (Om: $r = 0.920$, $p < 0.001$; Sc: $r = 0.650$, $p = 0.009$, Figure 2A). Next, we adjusted the associations of *ASK1* with several obesity-related parameters for BMI and for E2F1 to assess if they may share a common mechanistic pathway. *ASK1* remained significantly associated with insulin resistance, high triglycerides, and, importantly, with low adiponectin, even after adjusting the model for BMI and E2F1 (Figure 2B). Yet, the associations of Om-*ASK1* with all parameters were attenuated by adjusting for the expression of *E2F1* mRNA in Om AT. Collectively, using a large human-AT biobank, we demonstrate that Om-*ASK1* expression may share a common path with *E2F1* in their association with major clinical parameters indicating AT dysfunction and increased cardio-metabolic risk. Moreover, molecularly, higher BMI associates with increased occupancy of the *ASK1* promoter by E2F1.

3.2. Molecular links between E2F1 and ASK1 regulation

To better define the molecular mechanisms and triggers for increased adipocyte *ASK1* expression and to evaluate the contribution of adipocytes, we utilized cellular models. A differentiated murine epididymal adipocyte cell line was utilized to determine potential individual triggers relevant to the AT milieu in obesity, which could increase *ASK1* mRNA levels. To this end, inflammatory stimuli such as the prototypical pro-inflammatory cytokine TNFα, and FasL, and an oxidative environment induced by adding the H_2O_2 generating enzyme glucose oxidase (GO) to the medium, increased *Ask1* mRNA by ~2-fold at 24 h (Figure 3A). At 4 h stimulation the responses differed, likely representing unique signaling components of the different stimuli that

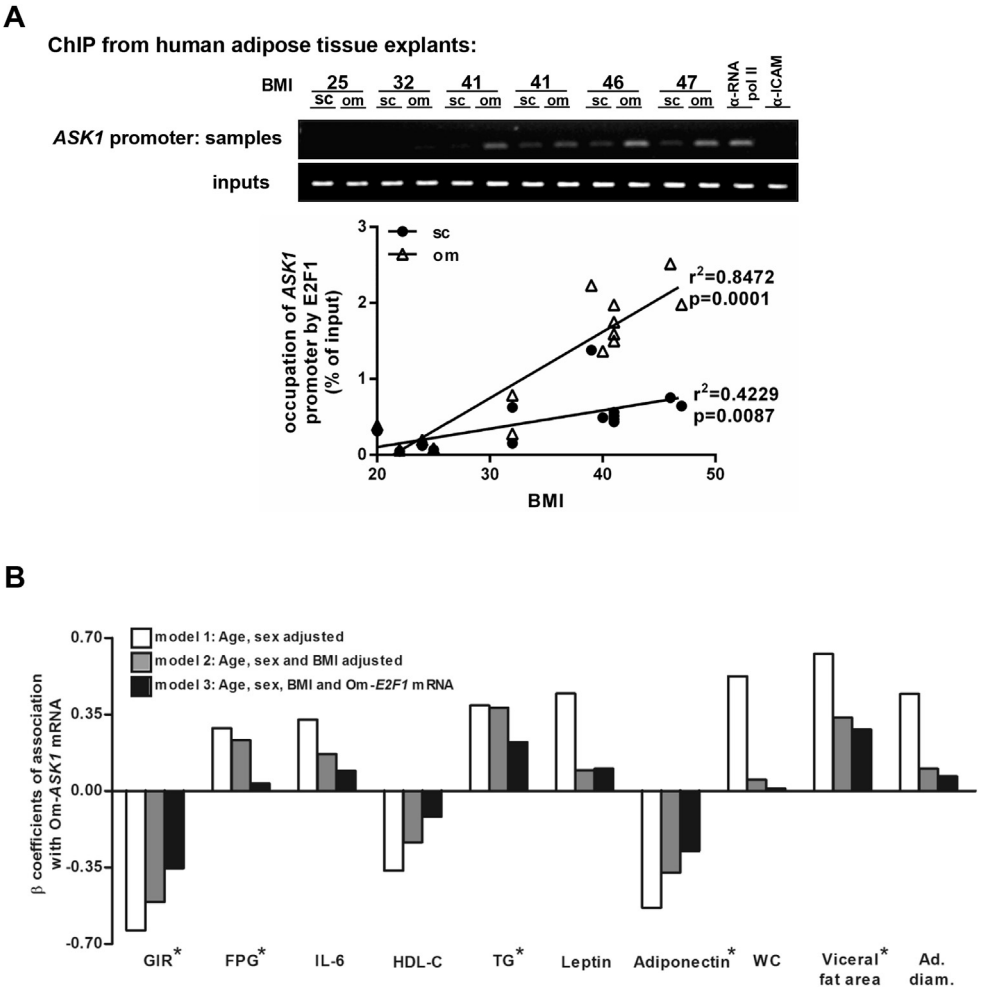

Figure 2: Effect of BMI on E2F1 binding to the *ASK1* promoter and associations between Om-*ASK1* expression levels and different clinical characteristics. (A) Formaldehyde cross-linked chromatin from 16 paired human (Om and Sc) ATs was subjected to ChIP experiments. Immunoprecipitation of E2F1 containing complexes was performed using anti-E2F1 antibody. Anti-POLR2 (polymerase RNA II), was used as positive control and anti-ICAM1 (intercellular adhesion molecule 1) was used as negative control. After isolation of bound DNA, end-point PCR and quantitative real time PCR were performed for a 300 bp region of the endogenous human *ASK1* promoter. Quantitative real time PCR results were analyzed using linear regression. Inputs indicate PCR performed on DNA (diluted 1:300) without any immunoprecipitation. **(B)** Multi variate models to assess associations between Om-*ASK1* mRNA levels and parameters shown in Figure 1 as continuous variables. Values are the β coefficient of association, with model 1 adjusted for age and sex, model 2 for age, sex, and BMI, and model 3 adjusted for age, sex, BMI, and Om-*E2F1* mRNA expression. * Associations with *p* values <0.05. GIR; glucose infusion rate during hyper-insulinemic euglycemic clamp, FPG; fasting plasma glucose, HDL-cholesterol; high-density lipoprotein cholesterol, TG; triglycerides, WC; waist circumference, ad. diam.; adipocyte diameter.

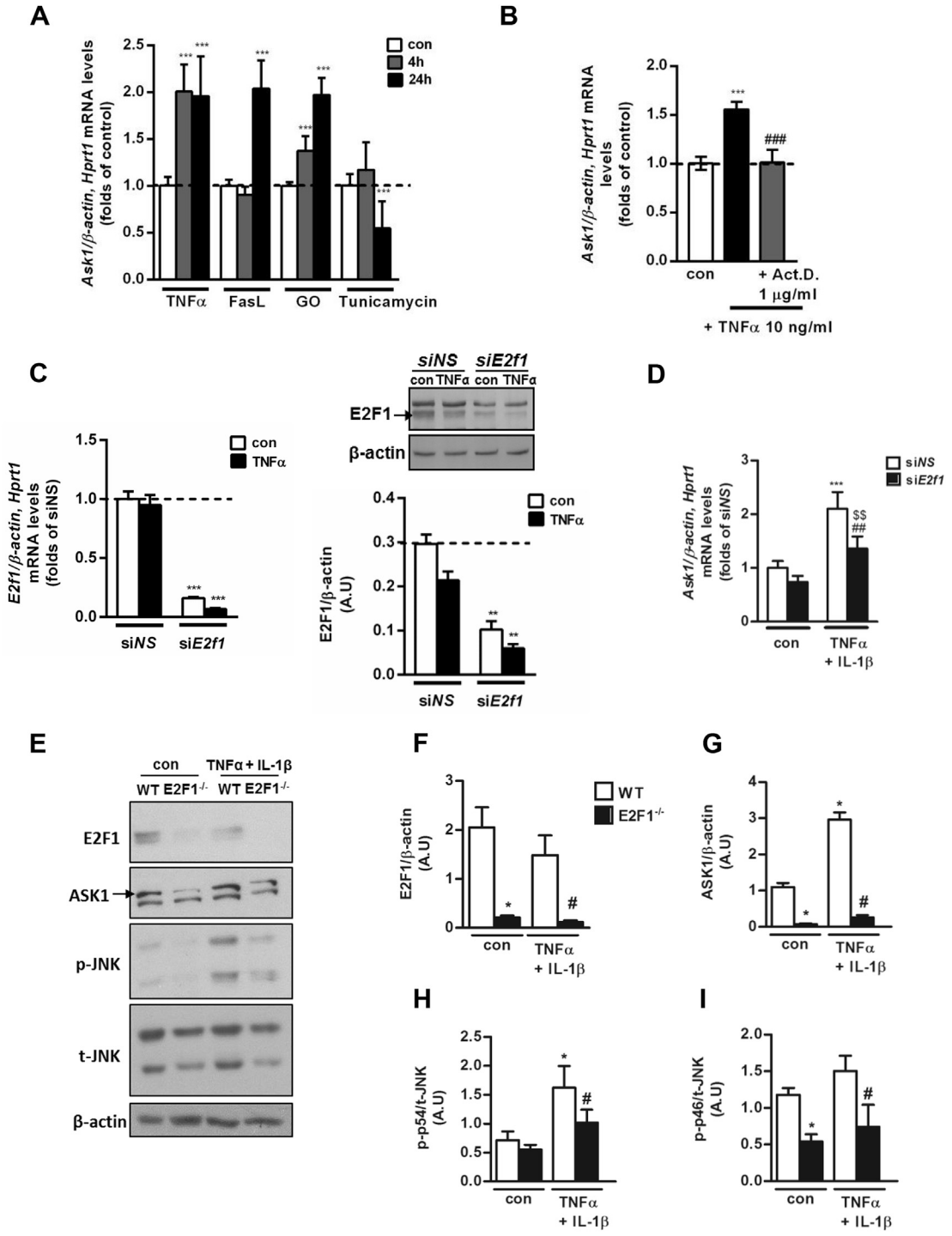

Figure 3: Increase in transcription of *ASK1* gene by mimickers of obesity-associated AT stresses is attenuated by absence of E2F1 in adipocytes. (A) Epididymal adipocytes were treated with several stress inducers (TNFα 10 ng/ml, FasL 1 ng/ml, GO 50 mU/ml, and Tunicamycin 5 μg/ml) for 4 or 24 h and subjected to quantitative real-time PCR analysis. The expression was normalized to β-actin and Hprt1. Results are mean ± SD of n = 5 independent experiments. $^{***}p < 0.001$ 4/24 h of each treatment vs. con. **(B)** Epididymal adipocytes were incubated with TNFα (10 ng/ml) or TNFα (10 ng/ml) + Act. D. (1 μg/ml) for 4 h and subjected to quantitative real-time PCR analysis. The expression was normalized to β-actin and Hprt1. Results are mean ± SD of n = 3 independent experiments. $^{***}p < 0.001$ TNFα only versus con. $^{\#\#\#}p < 0.001$ Act. D. **(C, D)** Epididymal adipocytes were transfected with. *E2f1* siRNA (si*E2f1*) or non-specific siRNA (si*NS*) using electroporation. 24 h after transfection, adipocytes were incubated with TNFα (10 ng/ml) for an additional 24 h and analyzed using either quantitative real-time PCR or Western blot. Each transcript expression was normalized to β-actin and Hprt1 mRNA levels. β-actin was used as a loading control on the gel. Results are mean ± SD of 3 independent experiments. $^{***}p < 0.001$ si*E2f1*con and TNFα vs. si*NS* con (middle graph), si*NS* TNFα vs. si*NS* con (right graph). $^{**}p < 0.01$ siE2f1 TNFα vs. siE2F1 con. $^{\#\#}p < 0.01$ si*E2f1* TNFα vs. si*NS* TNFα. MEF-derived adipocyte-like cells from WT and E2F1$^{-/-}$ were treated with TNFα 10 ng/ml + IL-1β 10 ng/ml for 24 h and subjected to Western blot analysis. Shown are representative blots **(E)** and densitometry graphs **(F–I)** of n = 3 independent experiments. β-actin was used as a loading control. $^{*}p < 0.05$ E2F1$^{-/-}$ con or WT TNFα 10 ng/ml + IL-1β 10 ng/ml vs. WT con. $^{\#}p < 0.05$ E2F1$^{-/-}$ TNFα 10 ng/ml + IL-1β 10 ng/ml vs. E2F1$^{-/-}$ con.

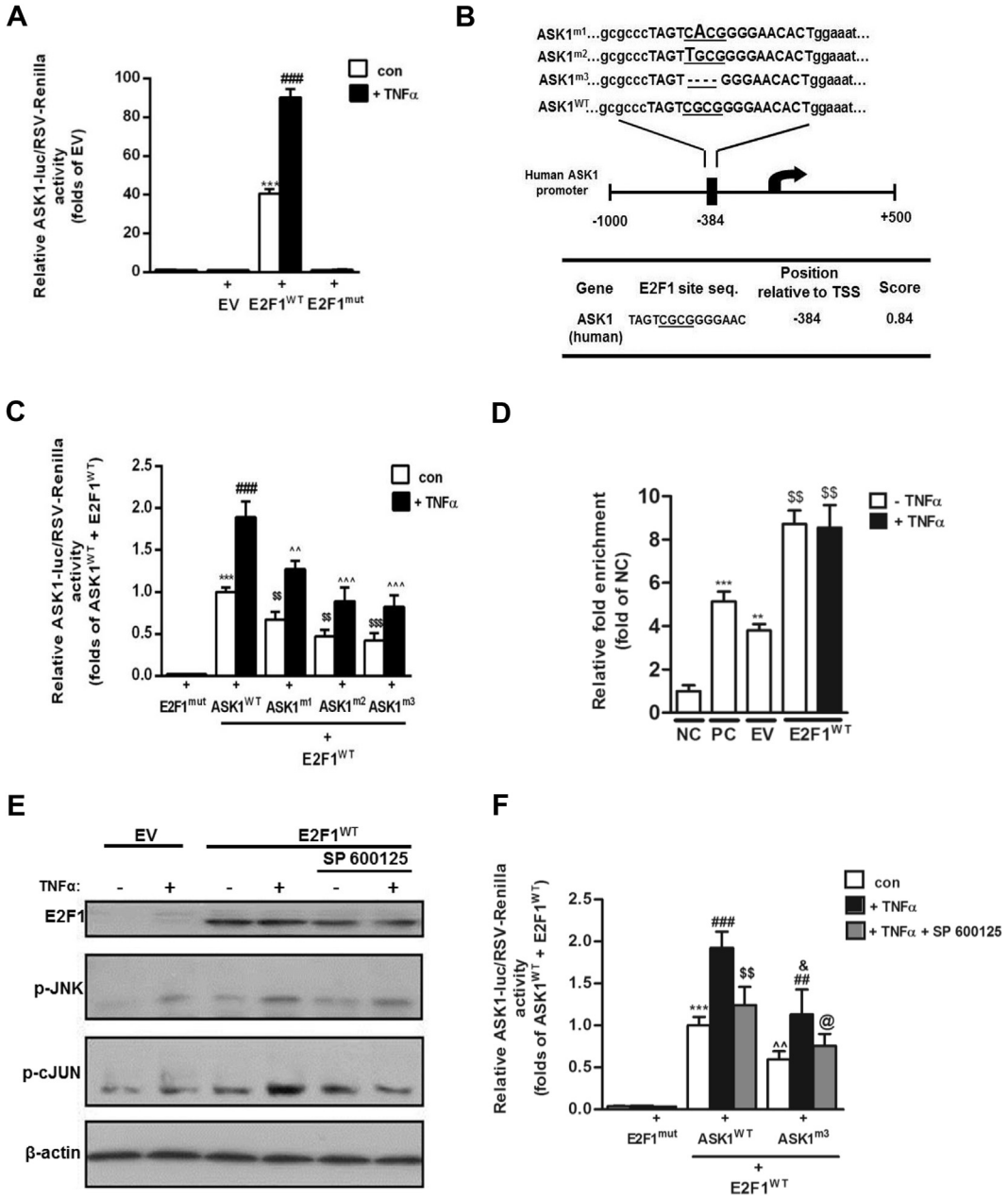

Figure 4: *ASK1* promoter activity is elevated by E2F1 over-expression and JNK phosphorylation input in HEK293 cells. (A) HEK293 cells were transfected with pA3-ASK1-Luc and an empty pcDNA3 (EV) or pcDNA3-E2F1 ($E2F1^{WT}$) or pcDNA3-E2F1-mut ($E2F1^{mut}$). After normalization to endogenous control (RSV-Renilla), an empty pcDNA3 (EV) was determined as basal luciferase activity. *ASK1*-promoter activity was measured after treatment with TNFα (10 ng/ml) for 24 h using Dual Luciferase Assay. Results are mean ± SD of n = 7 independent experiments. $^{***}p < 0.001$ con $E2F1^{WT}$ vs. con EV. $^{###}p < 0.001$ TNFα 10 ng/ml $E2F1^{WT}$ vs. con $E2F1^{WT}$. **(B)** Wild-type (WT) and mutated constructs of predicted E2F1-binding site on ASK1-promoter area by Met-Inspector software. E2F1 binding to the ASK1 promoter is mediated by physical interactions between E2F1 and a core sequence of the binding site (underlined sequence of four nucleotides). Two point mutations ($ASK1^{m1}$ and $ASK1^{m2}$) and one deletion mutation ($ASK1^{m3}$) of this core sequence were made by using mutagenesis kit as elaborated in the Methods, and their effect on ASK1 promoter activity is presented in **(C)**. After normalization to endogenous control (RSV-Renilla), mutated E2F1 ($E2F1^{mut}$) was determined as luciferase basal activity. ASK1-promoter activity was measured after treatment with TNFα (10 ng/ml) for 24 h using Dual Luciferase Assay. Results are mean ± SD of n = 5 independent experiments. $^{***}p < 0.001$ con $ASK1^{WT}$ vs. $E2F1^{mut}$. $^{###}p < 0.001$ TNFα $ASK1^{WT}$ vs. con $ASK1^{WT}$. $^{\$\$}p < 0.01$ con $ASK1^{mutants}$ vs. con $ASK1^{WT}$. $^{\wedge}p < 0.001/0.01$ respectively, TNFα $ASK1^{mutants}$ vs. TNFα $ASK1^{WT}$. **(D)** HEK-293 cells were transfected with an empty pcDNA3 (EV) or pcDNA3-E2F1 ($E2F1^{WT}$) and treated with TNFα for 24 h. Next, chromatin was cross-linked, fragmented and subjected to ChIP with α-RNA poll (as positive control − PC), α-ICAM (as negative control - NC), and α-E2F1 as the protein of interest, as indicated in the Methods. End-point PCR was done using purified DNA and specific primers for E2F1 binding site on the ASK1 promoter; inputs were diluted 1/300 and used as normalization control. Results are mean ± SD of n = 3 independent experiments. $^{**/***}p < 0.005/0.01$ respectively, vs. NC. $^{\$\$}p < 0.005$ vs. EV. HEK-293 cells were transfected with an empty pcDNA3 (EV) or pcDNA3-E2F1 ($E2F1^{WT}$) and treated with TNFα (10 ng/ml) and SP 600125 (50 μM). Shown are representative blots of Western blot analysis **(E)** of cell lysates prepared from HEK-293 cells. β-actin was used as a loading control. **(F)** HEK-293 cells were transfected with pA3-$ASK1^{WT}$ or $ASK1^{m3}$ and pcDNA3-E2F1-mut ($E2F1^{mut}$) or pcDNA3-E2F1 ($E2F1^{WT}$). After normalization to endogenous control (RSV-Renilla), pcDNA3-E2F1-mut ($E2F1^{mut}$) was determined as luciferase basal activity. ASK1-promoter activity was measured after treatment with TNFα (10 ng/ml) and SP 600125 (50 μM) for 24 h using Dual Luciferase Assay. Results are mean ± SD of n = 3 independent experiments. $^{***}p < 0.001$ con $ASK1^{WT}$ ($E2F1^{WT}$) vs. $E2F1^{mut}$. $^{###}p < 0.001$ TNFα $ASK1^{WT}$ vs. con $ASK1^{WT}$. $^{\$\$}p < 0.005$ TNFα + SP $ASK1^{WT}$ vs. TNFα $ASK1^{WT}$. $^{\wedge}p < 0.005$ con $ASK1^{m3}$ ($E2F1^{WT}$) vs. $E2F1^{mut}$. $^{\#\#}p < 0.01$ TNFα $ASK1^{m3}$ vs. con $ASK1^{m3}$. $^{\&}p < 0.05$ TNFα $ASK1^{m3}$ vs. TNFα $ASK1^{WT}$. $^{@}p < 0.05$ TNFα + SP $ASK1^{m3}$ vs. TNFα $ASK1^{m3}$.

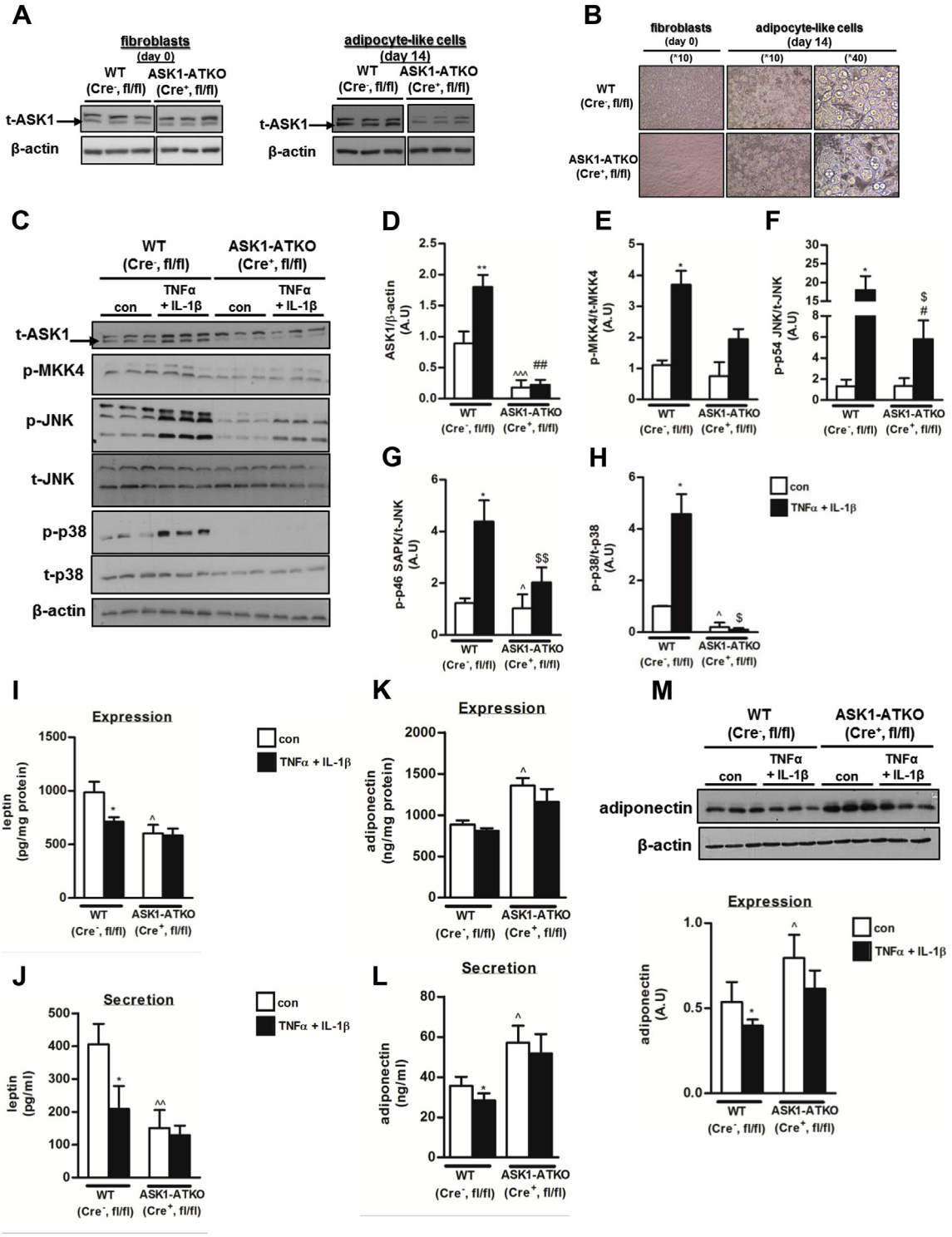

Figure 5: Effect of ASK1 depletion on phosphorylation of several members of MAP kinase pathway and on leptin/adiponectin expression and secretion in adipocytes. **(A)** ASK1 expression in MEF-derived fibroblasts (before differentiation into adipocytes) or MEF-derived adipocyte-like cells (after differentiation into adipocytes) from either WT or ASK1-ATKO mice was performed using Western blot analysis. Shown are representative blots from one of 5 independent experiments. Vertical white line indicates splicing of the same membrane for clarity of presentation. **(B)** Representative images of MEF-derived fibroblasts or MEF-derived adipocyte-like cells were obtained at 10X and 40X by light microscopy. MEF-derived adipocyte-like cells from WT and ASK1-ATKO were treated with TNFα 10 ng/ml + IL-1β 10 ng/ml for 24 h and subjected to Western blot analysis. Shown are representative blots **(C)** and densitometry graphs **(D–H)** of n = 6 independent experiments. β-actin was used as a loading control. $^{**/*}p < 0.01/0.05$ WT TNFα 10 ng/ml + IL-1β 10 ng/ml vs. WT con. $^{\wedge}p < 0.005/0.05$ ASK1-ATKO con vs. WT con. $^{\#\#/\#}p < 0.01/0.05$ ASK1-ATKO TNFα 10 ng/ml + IL-1β 10 ng/ml vs. WT TNFα 10 ng/ml + IL-1β 10 ng/ml $^{\$\$/\$}p < 0.01/0.05$ ASK1-ATKO TNFα 10 ng/ml + IL-1β 10 ng/ml vs. ASK1-ATKO con. To measure expression and secretion levels of leptin **(I, J)** or adiponectin **(K–M)**, medium (secretion and expression), and lysates (expression) were analyzed by ELISA (leptin and adiponectin) or by Western blot analysis (adiponectin). The results were normalized to cell protein concentrations. Results are mean ± SD of n = 6 independent experiments. $^{*}p < 0.05$ WT TNFα 10 ng/ml + IL-1β 10 ng/ml vs. WT con. $^{\wedge}p < 0.01/0.05$ ASK1-ATKO con vs. WT con.

resulted in different activation kinetics. Interestingly, tunicamycin, an inducer of ER stress, did not elicit a similar effect. Supporting the notion that the increased steady-state mRNA levels could be attributed to increased transcription (rather than to enhanced mRNA stability), we observed that actinomycin D, a global transcription inhibitor, fully prevented the increase in *Ask1* mRNA induced by TNFα (Figure 3B). Next, we set to dissect a putative role for E2f1 in adipocytes by siRNA-mediated knockdown and by utilizing MEFs from E2F1-KO ($E2F1^{-/-}$) mice that were differentiated into adipocyte-like cells. siRNA targeting *E2f1* decreased the mRNA and protein levels of this gene product in mature epididymal adipocytes by ~90% and 75%, respectively, compared to a non-targeting (*NS*) siRNA (Figure 3C). Under these conditions, TNFα-induced increase in *Ask1* mRNA was significantly inhibited by ~50% (Figure 3D). We previously demonstrated that despite the role of E2F1 in adipogenesis [35], under *in-vitro* conditions, MEFs from $E2F1^{-/-}$ mice can differentiate into adipocyte-like cells comparably to MEFs from wild-type (WT) mice, as assessed morphologically and by the similar induction of adipocyte-specific genes (Pparγ, *Cebp4α*, *Fabp4*, and *Glut4*) [30]. Utilizing these cells, we observed that in the absence of E2F1, "basal" ASK1 expression was markedly attenuated (Figure 3E–G). In addition, ASK1 expression was stimulated by 2.72 ± 0.20-fold by TNFα + IL-1β in the WT cells, but only by 1.93 ± 0.08-fold in the $E2F1^{-/-}$ adipocyte-like cells ($p = 0.029$). Consistently, both basal and TNFα + IL-1β-stimulated phosphorylation of JNK (a MAP kinase downstream of ASK1) was markedly attenuated (Figure 3E,H–I). Thus, in adipocyte cellular models, decreased E2F1 results in lower ASK1 expression and attenuated downstream signaling.

To elucidate how elevated E2F1 might increase ASK1 transcription, a luciferase reporter assay utilizing the human *ASK1* promoter region (−1000 to +500 bp) in HEK293 cells was performed. When cells were co-transfected with an empty vector plasmid (EV), promoter activity was low, and it was unresponsive to TNFα stimulation. Upon E2F1 over-expression, two phenomena were observed; basal *ASK1* promoter activity was greatly enhanced (by ~40-fold, Figure 4A), consistent with previous reports [28,29], and E2F1 over-expression permissively sensitized cells to respond to TNFα stimulus by "super-activating" *ASK1* promoter activity >2-fold (Figure 4A). Both the basal and TNFα-stimulated promoter activity required E2F1 binding to DNA, since expression of a mutant E2F1 that lacks DNA binding capacity failed to stimulate luciferase activity (Figure 4A). To further verify the role of direct binding of E2F1 to the ASK1 promoter, 3 mutants in the human *ASK1* promoter were generated targeting a single putative E2F1 binding site (see Methods for prediction approach) (Figure 4B). Both site mutants and a 4 bp deletion mutant significantly diminished *ASK1* promoter activity (Figure 4C). Yet, residual E2F1-related activity was still evident, and in particular, the 2-fold "super-induction" of *ASK1*-promoter −mediated luciferase activity by TNFα above basal activity was still evident. Intriguingly, ChIP analysis revealed that E2F1 over-expression indeed increased E2F1 binding to the *ASK1* promoter (Figure 4D). Yet, TNFα-induced "super-activation" of *ASK1* promoter activity under conditions of E2F1 over-expression (Figure 4A) was not associated with further binding of E2F1 to the *ASK1* promoter (Figure 4D). This suggests that although being permissive for *ASK1* promoter "super-activation," this effect of E2F1-overexpression is likely indirect, possibly being mediated by TNFα-induced JNK phosphorylation/activation. To address this possibility, we assessed whether a pharmacological JNK inhibitor, SP600125, could alleviate the TNFα-mediated activation of *ASK1* promoter activity under E2F1 overexpression. Indeed, TNFα induced JNK phosphorylation, and the phosphorylation of its substrate, c-JUN (Figure 4E). SP600125 inhibited JNK activity, since it did not affect TNFα-induced p-JNK while preventing the phosphorylation of its substrate c-Jun. Under these conditions, TNFα-induced super-activation of the *ASK1* promoter under conditions of E2F1 overexpression was nearly fully inhibited (Figure 4F). Collectively, we confirm that E2F1 over-expression activates *ASK1* promoter activity and identify a putative binding site for E2F1 in the human *ASK1* promoter. Importantly, we demonstrate that E2F1 plays a permissive, JNK-mediated, sensitization of the *ASK1* promoter to TNF-induced "super-activation."

3.3. Functional significance of adipocyte ASK1

Finally, to unravel the functional role of ASK1 in adipocytes, we utilized MEFs of wild-type (WT, Cre-, $ASK1^{fl/fl}$) and ASK1-AT-specific KO mice (Cre+, $ASK1^{fl/fl}$, ASK1-ATKO), which were differentiated into mature adipocyte-like cells. In non-differentiated MEF (before inducing adipogenesis), ASK1 expression was similar between the two genotypes (Figure 5A). After inducing adipogenesis, cells morphologically became adipocyte-like (Figure 5B), and ASK1 expression in the ASK1-ATKO adipocyte-like cells was lower than in the WT under basal conditions (Figure 5C,D). Moreover, induction of ASK1 expression by TNFα + IL-1β-was lower in the ASK1-ATKO adipocyte-like cells (Figure 5C,D). Next, we evaluated the effect of ASK1 absence on several members of the MAP kinase pathway downstream of ASK1. While only mild differences were observed in the expression levels of total JNK and total p-38 between the two types of cells, a significant reduction in basal and TNFα + IL-1β-stimulated phosphorylation of p-MKK4, p-JNK, and p-p38 was evident in the cells with lower ASK1 expression (Figure 5C,D). We next assessed the functional impact of reduced ASK1 expression. No significant differences between the WT and ASK1-ATKO − derived adipocyte like cells were detected in the phosphorylation of both basal and insulin-stimulated Akt (Ser-473, Thr- 308), or total levels of IRS-1 (Supplemental Fig. 1). In contrast, a significant difference in expression and secretion of the key adipokines leptin and adiponectin was observed using both ELISA and Western blot analysis. Whereas ASK1-ATKO adipocyte-like cells secreted lower basal levels of leptin than the WT cells (Figure 5E), the basal adiponectin secretion was significantly higher (Figure 5E). As expected, inflammatory conditions (TNFα + IL-1β) significantly lowered leptin and adiponectin secretion from WT adipocyte-like cells, but less so in the ASK1-ATKO adipocyte-like cells, indicating that ASK1 mediates the negative impact of inflammation on adipokine secretion (Figure 5E–G).

4. DISCUSSION

This study explores the hypothesis that ASK1, via transcriptional upregulation by E2F1, molecularly defines AT that supports a dys-metabolic obese phenotype in humans. We demonstrate associations between increased visceral-AT *ASK1* expression and multiple parameters of metabolically-unhealthy obesity, even beyond BMI and patients' sub-phenotyping by fat distribution pattern. Associations of *ASK1* with markers of dys-glycemia, dyslipidemia, and systemic inflammation were attenuated by adjusting for *E2F1* expression, suggesting that E2F1 and ASK1 share a common mechanistic pathway. At the molecular level, in human-AT, E2F1's occupancy of the *ASK1* promoter increases with BMI, more so in visceral than in subcutaneous fat, and two adipocyte cellular models of E2F1 knock-down/knockout demonstrate decreased ASK1 expression and attenuated downstream signaling. Furthermore, an E2F1 binding sequence in the human *ASK1* promoter was identified, and elevated E2F1 was shown to contribute to increased *ASK1* expression by both a direct transcriptional mechanism and by a JNK-mediated feed-forward loop.

These render E2F1 over-expressing cells hyper-sensitive to *ASK1* upregulation by inflammatory stimuli. Finally, the functional impact of ASK1 is demonstrated by adipocyte-specific ASK1-KO cells, which exhibit an improved adipokine secretory profile (secreting less leptin and more adiponectin, both basally and in response to an inflammatory stimulus).

Obese sub-phenotypes, particularly based on body-fat distribution patterns ('apples' vs 'pears'), were noticed decades ago [43]. Yet, with its growing prevalence, obesity requires a better characterization of obesity sub-phenotypes for a more stratified/personalized management of the disease. Differences in ATs' adaptation to chronic caloric surplus can serve as an effective means of patient stratification. For example, adipocyte size, indicating the reliance of AT expansion on hypertrophy, has been shown as a potentially clinically-useful tool to stratify obesity-related type 2 diabetes risk [44–46]. Other histopathological features of a dysfunctional AT include altered cellular composition of the tissue, in particular macrophage infiltration and lipid accumulation (foam cells) [36,38,47,48], and AT fibrosis [49,50]. Yet, a molecular definition of AT signatures that defines metabolically-unhealthy obese sub-phenotype(s) has not been systematically attempted. In oncology, molecular typing of tumors largely relies on the identification of different tumors' genetic perturbations, increasingly used to guide patients' management [51]. Genetic polymorphisms related to obesity sub-phenotypes and/or responses to therapy are just beginning to be unraveled [52]. Nevertheless, molecular pathways that mediate AT stresses could constitute promising leads for molecular-based obesity sub-phenotyping and personalized treatment.

Along this line, MAP kinases, and in particular the stress-activated MAP kinases [53], are promising candidates. Indeed, different members of this family of kinases, including p38MAP kinase, JNK, and upstream kinases such as the MAP3K's Tpl-2 and ASK1, and even MAP4K's, have been shown to regulate AT/adipocyte biology and to be activated in obesity [14–18,54]. MAP kinases are frequently shown to be regulated by rapid phosphorylation-dephosphorylation [55], and, indeed, phosphorylation-based activation of a MAP kinase pathway consisting of ASK1–MKK4,3/6–JNK/p38MAP kinase has been demonstrated in obese humans' visceral-AT [18,19]. Yet, we previously proposed that a significant level of regulation may be attributed to the expression level of these MAP kinases, reflecting a more chronic adaptation of AT in obesity [18]. Moreover, the association between *ASK1* mRNA levels in visceral-AT and insulin resistance, which remained significant even after adjusting for BMI, supported the notion that *ASK1* expression levels may define obese sub-phenotypes, i.e. that higher levels of visceral ASK1 may molecularly define obese persons with a more dys-metabolic "type" of obesity [18].

Findings of the present study provide a molecular mechanism for the elevated ASK1 expression in obesity and how it may be functionally linked to whole-body insulin resistance. The role our findings assign to increased E2F1 in upregulating ASK1 parallels the capacity of this transcription factor to regulate the expression of autophagy genes [56], reminiscent of previous observations in cancer cells [27–29]. The transcriptional activity of E2F1 is regulated at multiple levels, including expression, cellular (nuclear) localization, post-translational modifications, and multiple interacting proteins. Here, increased E2F1 expression parallels with increased E2F1 binding to the ASK1 promoter in human adipose tissue explants. Regardless of potential interrelations between the ASK1-MAP kinase pathway and autophagy activation, which have yet to be explored, both pathways downstream of E2F1 jointly form a signaling network that molecularly defines dysfunctional AT. The functional outcome of ASK1 activation in AT is linked to decreased adiponectin secretion. Intriguingly, up-regulated autophagy in obese adipose tissue was also shown to result in a dys-metabolic secretory function of adipocytes characterized by low adiponectin release, which induced insulin resistance in liver cells [57]. Our study assigns novel insights on the impact of dysregulated ASK1 expression beyond current literature. In either human muscle [23] or at the whole-body expression level in mice [24], it was decreased expression of ASK1 that was tied to insulin resistance and/or reduced energy expenditure, respectively. Here, we demonstrate upregulated ASK1 expression in visceral AT as a putative link between obesity-related AT stress and metabolic dysfunction.

In summary, we propose a stress signaling network composed of E2F1–ASK1-MAP kinase and E2F1-autophagy, which molecularly defines a metabolically-detrimental obese sub-phenotype in human adipose tissue. Functionally, by negatively affecting AT endocrine function, it may link obesity via adipose tissue stress to whole-body metabolic dysfunction.

ACKNOWLEDGMENTS

We would like to thank Prof. Gustavo Leone, Department of Molecular Virology and Genetics, College of Medicine and Public Health, Ohio State University, Ohio, USA, for the E2F1$^{-/-}$ and WT- MEFs. The contribution of Dr. Avi Shtevi for establishing the role of JNK in ASK1 super-activation by TNF is also acknowledged. This study was supported in part by grants from the Deutsche Forschungsge-meinschaft (DFG): SFB 1052/1: "Obesity mechanisms" (project B2 to A.R., project B1 to M.B., and project B4 to N.K.), and the Israel Science Foundation (to A.R., ISF 928-14).

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.05.003.

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