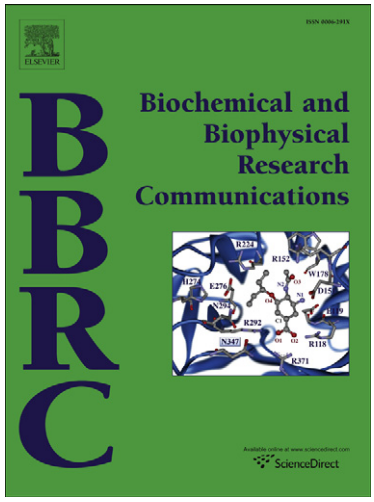

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Role of Activating Transcription Factor 3 in low glucose- and thapsigargin-induced apoptosis in cultured mouse islets

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Headings ATF3 knockout and beta-cell apoptosis

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ABSTRACT

In vitro, survival and function of rat pancreatic β-cells are optimally preserved in the presence of 10 mmol/l glucose (G10) and markedly altered by prolonged culture at either 2 mmol/l glucose (G2) or 30 mmol/l glucose (G30). The increase in islet cell apoptosis in G2 and G30 *vs.* G10 is preceded by parallel increases in the mRNA levels of the integrated stress response (ISR) gene Activating Transcription Factor 3 (*Atf3*) and its putative target and proapoptotic gene Growth arrest- and DNA damage-inducible gene 153 (*Gadd153* / *Chop*). In this study, we used islets from *Atf3* knockout (*Atf3*$^{-/-}$) mice to test the role of ATF3 in the stimulation of islet cell apoptosis under conditions associated with ISR activation. The glucose sensitivity of *Atf3*$^{-/-}$ and WT islets for the stimulation of insulin secretion and *Xbp1* mRNA splicing during 18h culture was similar, demonstrating that glucose metabolism was unaffected by *Atf3* deletion. However, the stimulation of islet cell apoptosis by the SERCA pump inhibitor thapsigargin was slightly but significantly reduced in *Atf3*$^{-/-}$ *vs.* WT islets despite similar level of expression of *Gadd153* and *Gadd34* mRNA. Also, the stimulation of islet cell apoptosis by 7 days of culture in G2 was slightly but significantly reduced in *Atf3*$^{-/-}$ *vs.* WT islets, and this effect was accompanied by a significant reduction in *Gadd153* mRNA expression. In conclusion, the increase in *Atf3* gene expression induced by thapsigargin and low glucose concentrations slightly contributes to the stimulation of islet cell apoptosis under these culture conditions.

Keywords : Apoptosis, pancreatic beta-cell, endoplasmic reticulum stress, integrated stress response, activating transcription factor 3

INTRODUCTION

Glucose is the main physiological stimulus of insulin secretion by pancreatic β-cells. In addition, glucose stimulates β-cell gene expression, protein synthesis, proliferation and growth, thereby contributing to the long-term maintenance of the functional β-cell mass [3]. *In vitro,* glucose-stimulated insulin secretion (GSIS) and survival of rat β-cells are optimally preserved during culture in the presence of 10 mmol/l glucose (G10) whereas they are markedly altered at either higher (30 mmol/l or G30) or lower (2 mmol/l or G2) glucose concentrations [2]. Interestingly, the mRNA levels of integrated stress response (ISR) genes follow an asymmetric V-shaped profile similar to that of apoptosis, suggesting that they could play a role in this deleterious effect of extreme glucose concentrations.

The ISR results from the phosphorylation of eukaryotic initiation factor 2α by eIF2α kinases under various types of stress, including endoplasmic reticulum (ER) stress, amino acid deprivation, virus infection and heme deficiency [9;10;20]. This ISR is characterized by increased expression of Activating Transcription Factor 4 (*Atf4*)-target genes, such as the proapoptotic transcription factor *Atf3*, its potential target Growth arrest- and DNA damage-inducible gene 153 (*Gadd153* or *Chop*) and *Gadd34* [6;13;19;21;22].

The *Atf3* gene encodes a member of the ATF/CREB family of transcription factors that share the basic region/leucine zipper DNA binding motif and bind to the ATF/CRE consensus sequence TGACGTCA [7]. Interestingly, *Atf3* expression is greatly increased in various cell types including rodent β-cells exposed to stress signals such as cytokines, nutrient deprivation, serum stimulation and calcium signaling, and this increase has been proposed to contribute to β-cell apoptosis in a number of studies [1;8;11;11;17;24]. We therefore tested whether *Atf3* plays a role in the stimulation of mouse islet cell apoptosis under conditions associated with ISR activation.

MATERIALS AND METHODS

Animals - *Atf3* knockout (*Atf3*$^{-/-}$) [11] and C57BL6/J wild type (WT) mice were bred under standard conditions. They were matched for sex and age (8 to 18 months) within each experiment.

Islet isolation and culture - Pancreatic islets from *Atf3*$^{-/-}$ and WT mice were isolated by collagenase digestion of the pancreas followed by density gradient centrifugation as described [15], except for the volume (2 ml) and concentration (1 mg/ml) of the collagenase solution used to inflate the pancreas. They were precultured for one week at 37°C in the presence of 5% CO_2 in serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing G10, 5 g/l bovine serum albumin (fraction V; Roche Diagnostics, Mannheim, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin. Islets were then cultured for 18h to 7 days in the same medium containing G2, G10 or G30 or G10 + 1 µmol/l TG (Sigma, St Louis, MO, USA) and processed for further analysis. All experiments were approved by the local ethics committee for animal experimentation (Projects 2004/UCL/MD/003 and UCL/MD/2009/009) and performed according to the Principles of Laboratory Animal Care.

Measurement of insulin secretion and islet insulin content - Insulin concentration was measured in the culture media and in total islet extracts by RIA using rat insulin as standard [12].

Morphological analysis - Islets were fixed for 4h in Bouin's fluid and embedded in paraffin. Four µm thick sections were then processed for haematoxylin and eosin staining. Images from

these sections were acquired with an Axioplan microscope coupled to an Axiocam HRc digital camera (using fixed camera settings) and analyzed with Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany).

Measurement of islet gene mRNA levels - Islet total RNA was extracted and reversed transcribed into cDNA as previously described [6]. Real-time PCR was performed with an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules,CA). Primers sequences and reactions conditions are shown in Suppl. Table 1.

Measurement of Xbp1 mRNA splicing - cDNAs corresponding to unspliced and spliced *Xbp1* mRNA (NM_001004210) were amplified simultaneously with TATA-box binding protein (*Tbp*) cDNA by duplex PCR using the Amplitaq Gold kit (Applied Biosystems, Foster City, CA, USA) (primers sequences in Suppl. Table 2). The amplicons were then separated by electrophoresis on an Agilent 2100 bioanalyzer using the Agilent DNA 1000 Assay kit (Agilent Technologies, Waldbronn, Germany). The spliced/total *Xbp1* mRNA ratio was calculated as an indicator of *Xbp1* mRNA splicing.

Measurement of islet cell apoptosis – Cytosolic histone-associated DNA fragments in islet cells were measured with the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) following manufacturer's instructions. Briefly, cytoplasmic DNA fragments (mono- and oligonucleosomes) were separated from nuclear DNA and measured using a mixture of anti-histone-biotin and anti-DNA-Peroxidase (POD) antibodies in streptavidin-coated wells. After addition of POD-substrate, absorbance of each sample was measured at 405 nm (reference wavelength: 490 nm). The data were normalized to the

absorbance of the positive control provided in the kit and corrected for differences in islet DNA content measured in the nuclear fraction by fluorimetry using SYBR Green I [18].

The percentage of apoptotic islet cells was also determined using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). DNA strand breaks (of mono- and oligonucleosomes as well as single strand breaks) were identified by labeling free 3'-OH termini with fluorescein-dUTP using Terminal deoxynucleotidyl transferase (TUNEL reaction). Reactions were performed on 4 µm-thick sections of paraffin-embedded islets. Islet cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The percentage of apoptotic cells was determined by counting fluorescein- and DAPI-positive nuclei on digital images obtained by fluorescence microscopy (FluoArc mounted on an Axioskop 40 coupled to a HBO 100 camera; Carl Zeiss, New York, NY) under standardized conditions (fluorescein: excitation/emission 475/540 nm; DAPI: excitation/emission 350/460 nm).

Statistical analysis - Results are means ± SEM for the indicated number of experiments. Statistical significance of differences between groups was assessed by two-way ANOVA followed by a test of Bonferroni. Differences were considered significant if $P < 0.05$.

RESULTS

Characteristics of WT and Atf3$^{-/-}$ mice - *Atf3$^{-/-}$* mice did not have any obvious phenotype compared to WT mice, consistent with the view that *Atf3* is not required under normal conditions but it is a stress-inducible gene. At the time of islet isolation (between 8 and 18 months of age), body weight and fed blood glucose levels were similar in both types of mice matched for age and sex (WT *vs.* *Atf3$^{-/-}$* mice: 23.9 ± 0.3 *vs.* 24.4 ± 0.6 g body weight and 7.0 ± 0.5 *vs.* 7.4 ± 0.5 mmol/l blood glucose; means ± SEM for 9 mice of each genotype). These results indicate that *Atf3* deletion does not induce major disturbances in glucose homeostasis.

Atf3 mRNA expression, insulin secretion and Xbp1 mRNA splicing in WT and Atf3$^{-/-}$ islets cultured in the presence of increasing glucose concentrations - We first tested the effects of 18h culture in the presence of different glucose concentrations on *Atf3* mRNA expression in WT mice. As shown in figure 1A, *Atf3* mRNA expression was minimal after culture in the presence of G10 and increased ~2 fold after 18h culture in G5 and G30, ~4 fold after culture in G2 and ~6 fold after culture in the presence of 1 µmol/l TG. As expected, *Atf3* mRNA was not detected in islets from *Atf3*$^{-/-}$ mice, even after treatment with TG.

To test if *Atf3* deletion alters β-cell function, we next measured insulin secretion during 18h culture in the presence of increasing glucose concentrations. As shown in figure 1B, glucose-stimulated insulin secretion followed a sigmoid curve in both types of islets, with a semi-maximal effect around G15 and a maximal effect at G30. The lack of difference between both types of islets suggests that β-cell glucose responsiveness is not altered by *Atf3* deletion.

We next tested the effect of *Atf3* deletion on the glucose stimulation of *Xbp1* mRNA splicing, an indicator of UPR activation, under similar culture conditions. In WT islets, glucose stimulated *Xbp1* mRNA splicing in a concentration-dependent manner between G2 and G30, but to a much smaller extent than did ER Ca^{2+} emptying with TG (Fig. 1C). These effects of glucose and TG were similar in *Atf3*$^{-/-}$ islets. Altogether, these results suggest that *Atf3* deletion impairs neither glucose sensitivity nor UPR activation in isolated mouse pancreatic islets.

Effects of glucose and TG on the morphology of WT and Atf3$^{-/-}$ *islets* – To test the effect of *Atf3* deletion on the stimulation of islet cell apoptosis by extreme glucose concentrations and TG, we studied the morphology of WT and *Atf3*$^{-/-}$ islets cultured for one week in the presence

of increasing glucose concentrations or G10 + TG (Fig. 2). In WT islets, one week culture in G30 *vs.* G10 did not affect the islet morphology. In contrast, one week culture in G2 *vs.*G10 markedly increased the number of apoptotic bodies (characterized by condensed chromatin) throughout the islets and induced islet cell atrophy, as shown by the increase in islet nuclei density. In comparison, two days of culture in the presence of TG also markedly increased the number of apoptotic bodies but the size of islet cells was almost normal and their cytoplasm contained large vacuoles. The morphological alterations induced by G2 and TG were similar in both types of islets, suggesting that *Atf3* deletion does not markedly reduce islet cell apoptosis under these conditions. However, this type of analysis does not allow precise quantification of islet cell apoptosis.

Effects of thapsigargin on islet cell apoptosis and ISR gene expression in WT and Atf3$^{-/-}$ islets - To better evaluate the effect of *Atf3* gene deletion on ER stress-induced apoptosis, we next quantified islet cell DNA fragmentation induced by culture in the presence of TG. As shown in figure 3 A and B, 48h culture in the presence of TG instead of G10 alone significantly increased the level of cytoplasmic histone-associated DNA fragments and the percentage of TUNEL-positive nuclei in WT islets. DNA fragmentation was of similar amplitude in *Atf3*$^{-/-}$ islets, but the percentage of TUNEL-positive nuclei was significantly reduced by 35% in *Atf3*$^{-/-}$ islets. This observation suggests that the stimulation of *Atf3* gene expression by TG slightly contributes to the stimulation of apoptosis in our experimental model.

We also measured the mRNA levels of other ISR-response genes, *Gadd153* and *Gadd34*, in WT and *Atf3*$^{-/-}$ islets. As shown in figure 3 C and D, *Gadd153* and *Gadd34* mRNA levels were minimal in G10 and similarly induced by TG in both types of islets, suggesting that *Atf3* deletion does not alter the induction by TG of other ER stress-response genes in isolated mouse pancreatic islets.

Effects of low and high glucose concentrations on cell apoptosis and ISR gene mRNA levels in WT and Atf3$^{-/-}$ islets - We next tested if *Atf3* gene deletion had an effect on islet cell apoptosis and ISR gene expression induced by extreme (low and high) glucose concentrations. As previously reported [14], DNA fragmentation and the percentage of TUNEL-positive islet cell nuclei significantly increased after one week culture in G2 *vs.* G10, but not after culture in G30 (Fig. 4). The increase in the level of cytoplasmic histone-associated DNA fragments and the percentage of TUNEL-positive nuclei were significantly lower in *Atf3*$^{-/-}$ *vs.* WT islets cultured in G2. These results suggest that ATF3 slightly contributes to the stimulation of mouse islet cell apoptosis by a low glucose concentration.

To evaluate whether the difference in apoptosis after culture in G2 resulted from differences in ISR activation, we next measured the mRNA levels of *Gadd153* and *Gadd34* after 18h and one week of culture under the same conditions. As shown in figure 4 (panels C and D), the stimulation of *Gadd153* and *Gadd34* mRNA levels by 18h culture in G2 or G10 + TG was similar in both types of islets. In contrast, the induction of *Gadd153* by one week culture in G2 was lower in *Atf3*$^{-/-}$ islets whereas no significant differences were observed for *Gadd34* mRNA levels.

DISCUSSION

This study demonstrates that the increase in *Atf3* gene expression induced by TG, a pharmacological inhibitor of SERCA pumps that potently induces ER stress, or by prolonged culture in a low glucose concentration, a condition associated with strong ISR activation, significantly contributed to the stimulation of mouse islet cell apoptosis under these culture conditions.

Mice lacking *Atf3* gene did not present major disturbances in glucose homeostasis and body weight gain, in agreement with the view that *Atf3* is only required under stress conditions [11]. In agreement, the glucose sensitivity of the islets for the stimulation of insulin secretion was not affected by *Atf3* deletion. Interestingly, the glucose stimulation of *Xbp1* mRNA splicing that we initially described in rat islets [6] and later confirmed in mouse islets [14] was also similar in *Atf3*$^{-/-}$ and WT islets, indicating that ATF3 does not influence that specific branch of the unfolded protein response (UPR). These results also demonstrate that the glucose sensitivity of *Atf3*$^{-/-}$ islets was not different from that of WT islets, excluding the possibility that differences between *Atf3*$^{-/-}$ and WT islet cell apoptosis during culture at various glucose concentrations result from difference in glucose metabolism.

Importantly, the glucose regulation of *Atf3* and *Gadd34* mRNA levels in mouse islets was similar to that in rat islets [6;14], with a minimum in G10, a large increase in G2 and a slight increase in G30. However, *Gadd153* mRNA levels and islet cell apoptosis only increased during prolonged culture in G2 but not G30 *vs.* G10, preventing us to study the role of ATF3 in glucotoxic islet cell apoptosis.

Earlier studies have lent support to the hypothesis that ATF3 plays a role in pancreatic β-cell apoptosis, but whether this was protective or deleterious depended on the experimental settings. In support of a proapoptotic effect of ATF3, it has been shown that transgenic mice overexpressing ATF3 in β-cells develop abnormal islets and defects secondary to β-cells deficiency [1], while *Atf3* knockout islets are partially protected from apoptosis induced by cytokines (IL-1β and IFN-γ) or NO [11]. ATF3 overexpression also enhanced β-cell apoptosis in streptozotocin (STZ)-treated cells while ATF3 knockdown significantly inhibited β-cell apoptosis induced by STZ or IFN-γ and TNF-α [17]. Recent studies also demonstrated that knockdown of *Atf3* attenuates ethanol-induced β-cell apoptosis [16] while *Atf3* exerts a deleterious effect on cell survival in the context of islet transplantation [24]. However, it has

been shown that *Atf3*$^{-/-}$ islets are not protected from apoptosis induced by palmitate and that *Atf3* knockdown in INS1E cells does not protect against apoptosis induced by palmitate or the SERCA pump inhibitor cyclopiazonic acid [5]. Moreover, it has recently been shown that ATF3 may play a role in prolactin-induced β cell growth during pregnancy [4]. In another recent study, the authors proposed a protective role of ATF3 during the development of T2D [23].

In the present study, *Atf3* deletion only slightly protected islet cells from TG-induced apoptosis, but this effect was unrelated to alterations in the amplitude of ISR gene expression. Regarding low glucose-induced apoptosis, the average data also show a small but significant beneficial effect of *Atf3* deletion. However, the results varied from one experiment to another, a strong protective effect of *Atf3* deletion being observed in half of our experiments while no effect was observed in the others. These differences were unrelated to mice age and gender (data not shown).

It had previously been shown that ATF3 exerts its action, at least in part, by regulating the expression of downstream target genes such as *Gadd34*, *Gadd153* [13;22]. Our results demonstrate that ATF3 is not involved in the rapid induction of *Gadd153* and *Gadd34* mRNA levels by low glucose and TG. However, although the induction of *Gadd153* by 18h culture in low glucose did not require ATF3, its increase after 7 days of culture in G2 was reduced to a similar extent as that of apoptosis. At this stage, it is impossible to say if the reduction in Gadd153 mRNA level is the cause or the consequence of the reduction in islet cell apoptosis by Atf3 deletion.

In conclusion, this study demonstrates that ATF3 is not required to maintain β-cell function. We also demonstrated that ATF3 is only slightly involved in TG-induced apoptosis and is not involved in ISR gene expression in isolated mouse islets. However, the increase in *Atf3*

induced by culture in a low glucose concentration slightly contributes to the stimulation of islet cell.

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Figure Legends

Figure 1. Effects of glucose and thapsigargin on *Atf3* mRNA expression (A), glucose-stimulated insulin secretion (B) and *Xbp1* mRNA splicing (spliced/total *Xbp1* mRNA levels)(C) in islets from WT and *Atf3*$^{-/-}$ mice - Islets isolated from Wild-Type (WT) mice (white symbols) and *Atf3*$^{-/-}$ mice (black symbols) were precultured for 1 week in serum-free RPMI medium containing G10 and 5 g/l BSA and then cultured for 18h in the presence of G2, G5, G10, G15, G20 and G30 (A, B) or in the presence of G2, G10 and G30 or G10 + 1 µmol/l thapsigargin (TG) (C). **A**, *Atf3* to *Tbp* mRNA ratio was measured by real-time PCR and expressed relative to the mRNA ratio in WT islets cultured in G10. *Atf3* mRNA was not detectable in islets from *Atf3*$^{-/-}$ mice (*Atf3*: threshold cycle (C_t) = 30.75 in WT islets cultured in G10, Ct >40 in *Atf3*$^{-/-}$ islets; *Tbp* : Ct = 28.6 and 28.1 in WT and *Atf3*$^{-/-}$ islets cultured in G10). **B**, insulin secretion was measured and expressed in percentage of the islet insulin content at the end of culture (mean ± SEM of insulin content in WT islets: 57.3 ± 3.1 ng/islet; mean ± SEM of insulin content in *Atf3*$^{-/-}$ islets: 62.4 ± 4.5 ng/islet). **C**, spliced/total *Xbp1* mRNA ratio were computed as an indicator of *Xbp1* mRNA splicing and expressed relative to the ratio in WT islets cultured in G10. Results are means ± SEM for 3 experiments. *, P<0.05 *vs.* G10 by two-way ANOVA and a post-test of Bonferroni.

Figure 2. Effects of glucose and thapsigargin on the morphology of WT and *Atf3*$^{-/-}$ islets - Islets from WT and *Atf3*$^{-/-}$ mice were cultured for one week in the presence of 2, 10 or 30 mmol/l glucose (G2, G10, G30) or for 2 days in the presence of G10 + 1 µmol/l TG as indicated. After culture, islets were fixed in Bouin's fluid and embedded in paraffin. Sections were then stained with haematoxylin/eosin (white stars: apoptotic bodies). The pink precipitate corresponds to the fixed BSA from the culture media. Islets shown are representative for 2 experiments.

Figure 3. Effects of thapsigargin on islet cell apoptosis (A, B) and ISR gene mRNA levels (C, D) in WT and *Atf3*$^{-/-}$ islets - After 1 week preculture, islets from WT (white bars) and *Atf3*$^{-/-}$ mice (black bars) were cultured for 18h to 48h in the presence of G10 or G10 + 1 µmol/l TG. **A**, Cytoplasmic histone-associated DNA fragments were measured as described in "methods" with a commercial ELISA. Absorbance values were divided by the absorbance of the positive control provided in the kit and corrected for variations in islet DNA content. They were then expressed relative to the value measured in WT islets cultured in G10. Results are means ± SEM for 4 experiments. **B**, the percentage of apoptotic islet cells was evaluated as the ratio TUNEL-positive/DAPI-positive nuclei on islet sections. Results are means ± SEM of all islets from 3 independent experiments (number of islets counted in each condition: from 42 to 88). **C-D**, *Gadd153* and *Gadd34* to *Tbp* mRNA ratios were determined by real time RT-PCR and expressed relative to the mRNA ratio in WT islets cultured in G10. Data are means ± SEM for 3 experiments. *, $P<0.05$ *vs.* G10 by two-way ANOVA followed by a test of Bonferroni.

Figure 4. Effects of *Atf3* gene inactivation on islet cell apoptosis (A, B) and ISR gene expression (C, D, E, F) after culture in the presence of increasing glucose concentrations - Islets from WT (white bars) and *Atf3*$^{-/-}$ mice (black bars) were cultured for 7 days (A, B, E, F) or 18h (C, D) in the presence of 2, 10 or 30 mmol/l glucose. **A-B**, The amount of DNA fragments and TUNEL-positive islet cells, measured as described in legend to figure 3, were used as indicators of cell apoptosis. For TUNEL, between 34 and 76 islets were counted for each condition **C-F**, *Gadd153* and *Gadd34* to *Tbp* mRNA ratios were normalized to the ratio in WT islets cultured in G10. Data are means ± SEM for 3 experiments. *, $P<0.05$ *vs.* G10 and $^{\#}$, $P<0.05$ *vs.* WT by two-way ANOVA followed by a test of Bonferroni.

Figure 1

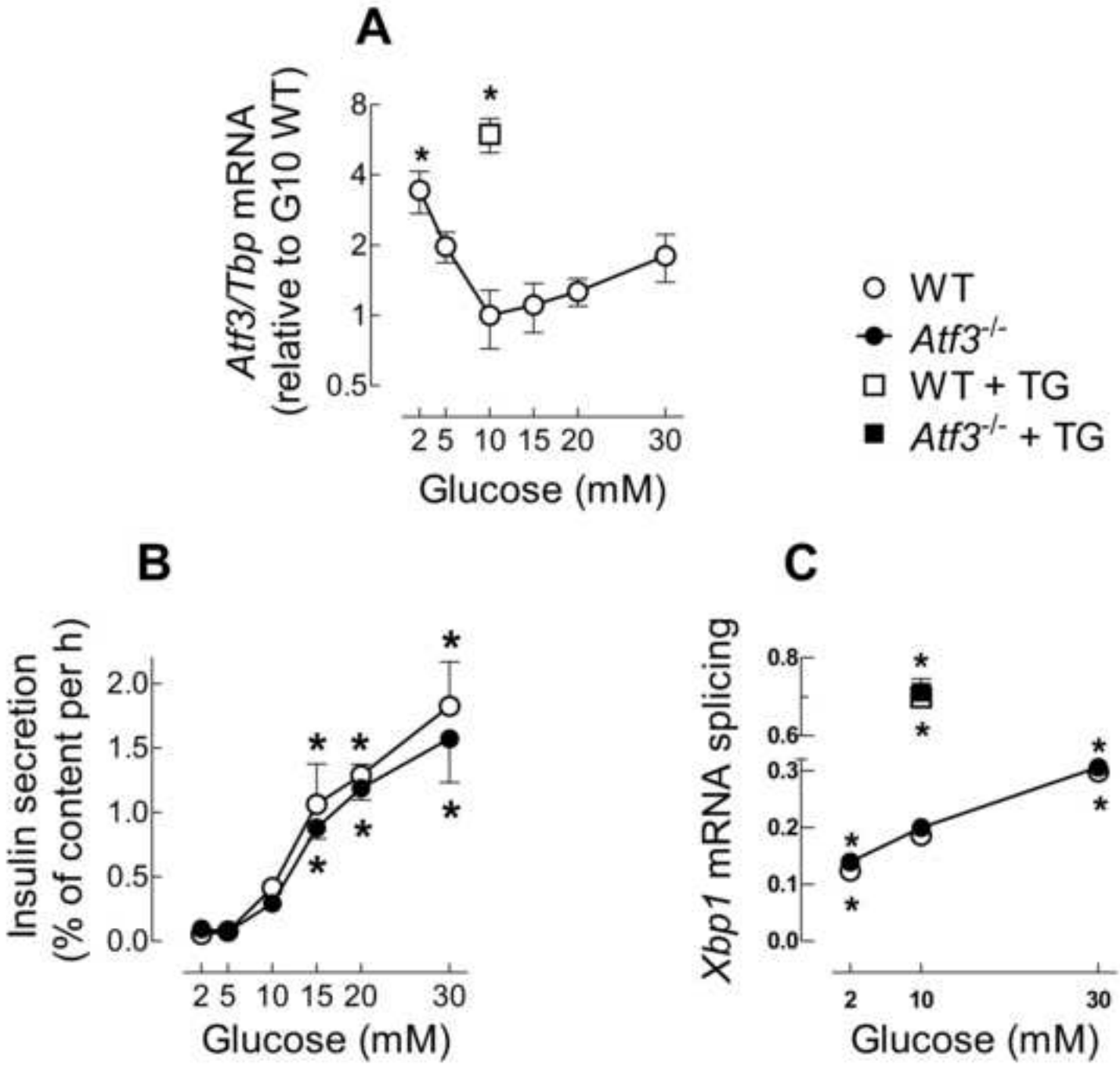


Figure 2

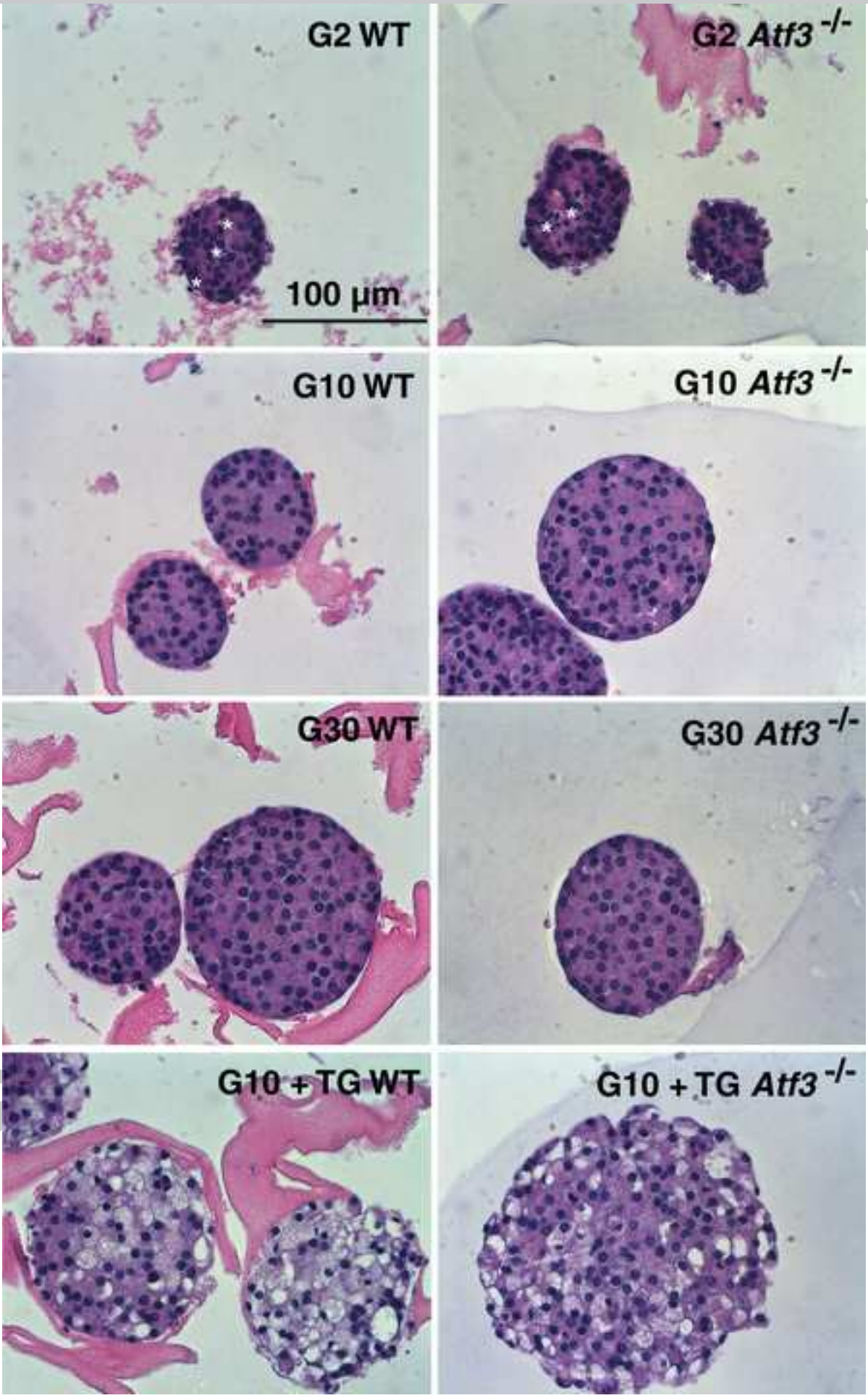


Figure 3

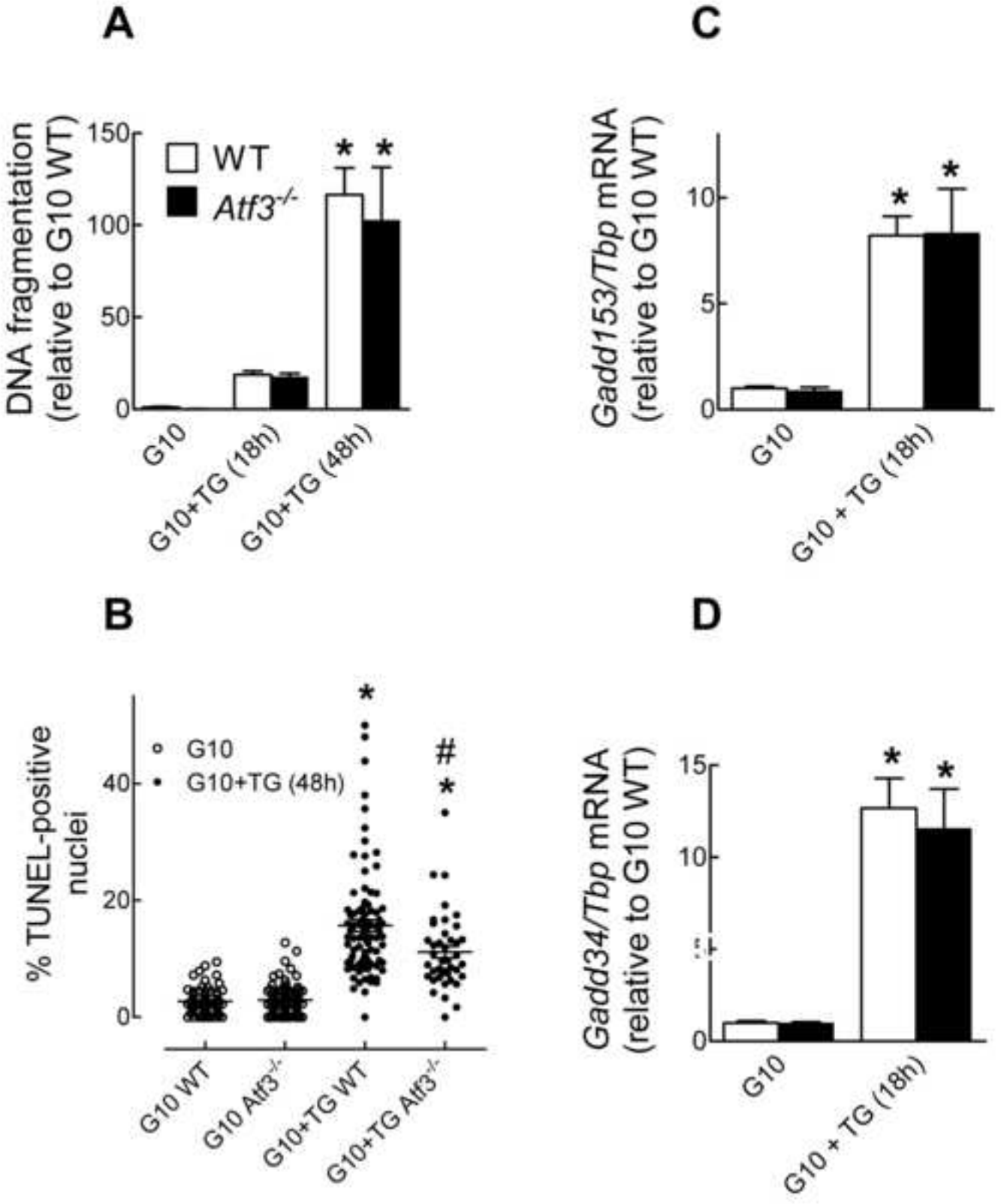
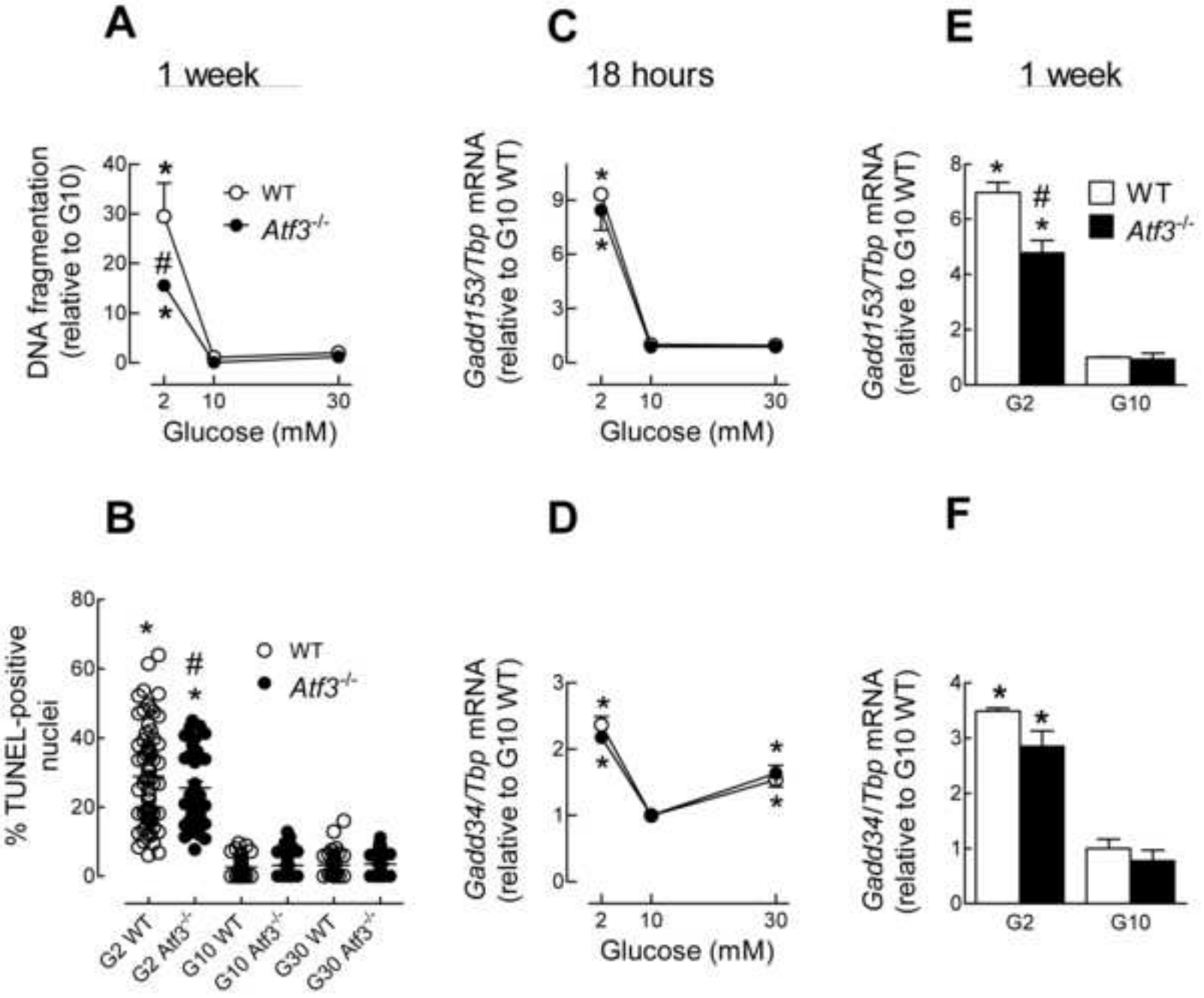


Figure 4



*Highlights

J Duprez & JC Jonas, ATF3 paper, Highlights

Atf3 partly contributes to ER stress-induced apoptosis in mouse islet cells

Atf3 partly contributes to low glucose-induced apoptosis in mouse islet cells

Atf3 deletion does not alter mouse islet glucose responsiveness