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## ATF6 $\alpha$ regulates airway hyperreactivity, smooth muscle proliferation, and contractility

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### Keywords

ATF6 $\alpha$ ; Airway smooth muscle; Asthma; ORMDL3

### To the Editor

ORMDL3, located on chromosome 17q21, is highly linked to asthma in genetic association studies<sup>1</sup>. The SNP linking ORMDL3 to asthma is associated with increased levels of ORMDL3 expression<sup>2</sup>. Mice overexpressing increased levels of human ORMDL3 have spontaneous increased levels of airway hyperreactivity (AHR) and increased airway smooth muscle (ASM) in the absence of airway inflammation<sup>3</sup>. ORMDL3 is localized to the endoplasmic reticulum (ER) and regulates several downstream pathways including the ER localized transcription factor ATF6 $\alpha$ <sup>4–7</sup>. Prior studies of hORMDL3<sup>zp3–Cre</sup> transgenic mice which overexpress human ORMDL3 have demonstrated that these mice have increased lung expression of ATF6 $\alpha$ <sup>3</sup> suggesting that ATF6 $\alpha$  may be one of the downstream pathways contributing to increased ASM and increased AHR in hORMDL3<sup>zp3–Cre</sup> transgenic mice.

In this study we have utilized ATF6 $\alpha$ <sup>–/–</sup> mice to determine whether ATF6 $\alpha$  contributes to AHR and could explain a downstream pathway from ORMDL3 to increased AHR. These studies (all methods described in the Supplementary On-Line Repository Methods) demonstrated that there was no difference in levels of ASM mass (Figure 1A–B) or AHR (Figure 1C) in naïve ATF6 $\alpha$ <sup>–/–</sup> mice compared to naïve WT mice. However house dust mite (HDM) challenged ATF6 $\alpha$ <sup>–/–</sup> mice had significantly less ASM mass compared to HDM challenged WT mice (Figure 1A–1B). This reduction in ASM mass in HDM challenged ATF6 $\alpha$ <sup>–/–</sup> mice was associated with reduced AHR to Mch compared to HDM challenged WT mice (p<0.05 Mch 24 mg/ml; p<0.01 Mch 48 mg/ml) (Figure 1C). There was no significant difference in levels of peribronchial fibrosis (Fig 1D), mucus (Fig 1E), BAL

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inflammation, peribronchial inflammation, or selected BAL cytokines (IL-5, IL-13, TGF- $\beta$ 1) comparing HDM challenged WT and HDM challenged ATF6 $\alpha^{-/-}$  mice (Supplementary On-Line Repository Figure E1A–K).

Levels of ORMDL3 were similar at baseline in the lungs of WT and ATF6 $\alpha^{-/-}$  mice (Fig 1F). ORMDL3 was significantly induced in the lungs of both WT and ATF6 $\alpha^{-/-}$  mice following HDM challenge. Interestingly, levels of lung ORMDL3 were induced to a higher degree in HDM challenged ATF6 $\alpha^{-/-}$  compared to HDM challenged WT mice. This suggests that there may be a feedback loop from ATF6 $\alpha$  to ORMDL3 such that ATF6 $\alpha$  inhibits allergen induced expression of ORMDL3 in the lung. When ATF6 $\alpha$  is inhibited (i.e. ATF6 $\alpha^{-/-}$  mice), the brake on inhibition of ORMDL3 is removed resulting in increased levels of ORMDL3 under conditions of HDM challenge. Thus, ATF6 $\alpha$  may be both downstream of ORMDL3<sup>4</sup>, as well as mediate a novel feedback loop to inhibit ORMDL3 under conditions of allergen challenge.

As our studies demonstrated that ATF6 $\alpha^{-/-}$  mice had reduced levels of HDM induced ASM and AHR without reduced levels of airway inflammation, we examined whether an intrinsic effect of ATF6 $\alpha$  in mouse ASM (mASM) cells could influence ASM contraction, proliferation, or apoptosis in vitro. These studies demonstrated that mASM cells from both WT and ATF6 $\alpha^{-/-}$  mice had similar low levels of spontaneous contraction as assessed in an ASM collagen gel contraction assay (Figure 2A). Incubation of WT ASM cells with either Mch (Figure 2A) or histamine (Figure 2B), resulted in a rapid increase in contraction by 15 minutes which persisted through 30 minutes. In contrast, incubation of ATF6 $\alpha^{-/-}$  deficient mASM cells resulted in a significantly reduced contractile response to agonists at 15 minutes which persisted through 30 minutes ( $p < 0.01$  histamine;  $p < 0.05$  Mch; ATF6 $\alpha^{-/-}$  mice vs WT mice) (Figure 2A, Figure 2B).

In addition, ATF6 $\alpha^{-/-}$  mASM cells had a minimal proliferative response to FBS 10% in vitro as assessed by BrdU incorporation, which was significantly less than that observed with WT mASM cells stimulated with FBS 10% ( $p < 0.001$ ; ATF6 $\alpha^{-/-}$  vs WT)(Figure 2C). Both WT and ATF6 $\alpha^{-/-}$  mASM cells had a similar minimal proliferative response to FBS 2% (Figure 2C). Thus, ATF6 $\alpha^{-/-}$  contributes to mASM cell proliferation under conditions of an increased mASM cell proliferative stimulus. Next, we examined whether the decrease in proliferation in ATF6 $\alpha^{-/-}$  mASM cells was due to apoptosis as assessed with a caspase 3/7 assay. Staurosporine, a known inducer of apoptosis<sup>8</sup>, induced a similar significant increase in mASM cell apoptosis in both WT mice ( $p < 0.001$ ) and ATF6 $\alpha^{-/-}$  mice ( $p < 0.001$ ) as detected by a significant increase in caspase3/7 activity (Figure 2D). Thus, ATF6 $\alpha$  does not modulate levels of apoptosis in proliferating mASM cells in vitro.

We also examined whether ATF6 $\alpha$  regulated human ASM (hASM) contraction, proliferation, and apoptosis in vitro. In these studies, we used siRNA to ATF6 $\alpha$  as compared to scrambled control siRNA to effectively knockdown ATF6 $\alpha$  mRNA ( $p < 0.001$ )(Figure 2E), and ATF6 $\alpha$  protein (Figure 2F) in primary hASM cells. Incubation of hASM cells transfected with ATF6 $\alpha$  siRNA with either Mch (Figure 2G) or histamine (Figure 2H), resulted in a significantly reduced contractile response to agonists at 15 minutes which persisted through 30 minutes ( $p < 0.01$  histamine;  $p < 0.001$  Mch; ATF6 $\alpha$  siRNA vs control

siRNA). The addition of histamine to ATF6 $\alpha$  siRNA treated cells further blocked contraction ( $p < 0.05$ ) (Figure 2H) through an at present unknown mechanism. There was no difference in contraction in non-transfected ASM cells compared to control siRNA treated ASM cells at baseline or in response to methacholine (Fig E2A), or histamine (Fig E2B). In addition, hASM cells knocked down with ATF6 $\alpha$  siRNA had a reduced proliferative response to FBS 2% or FBS 10% which was significantly less than that observed with control scrambled siRNA transfected hASM cells stimulated with either FBS 2% ( $p < 0.001$ ) (Figure 2I), and FBS 10% ( $p < 0.001$ ) (Figure 2I). Levels of apoptosis were not increased in either hASM cells transfected with ATF6 $\alpha$  siRNA or control siRNA and incubated with 0% FBS, 2% FBS or 10% FBS (Figure 2J). Levels of eotaxin-1 in hASM stimulated with IL-13 (Fig E1L) were no different in hASM cells in which ATF6 $\alpha$  is knocked down with siRNA or control siRNA. Thus, in vitro in hASM cells in which ATF6 $\alpha$  is knocked down with siRNA there is inhibition of contraction, and proliferation, but there is no effect on eotaxin-1 production.

In summary, in this study we have demonstrated that ATF6 $\alpha$  expression in mouse and human ASM cells in vitro plays an important role in ASM proliferation and contractility which are key features of asthma. Prior studies have also demonstrated that in epithelial cells ER resident protein 57 which is regulated by ATF6 $\alpha$ , plays a role in fibrosis and AHR<sup>E8,E9</sup>. In vivo studies with ATF6 $\alpha^{-/-}$  mice demonstrate an important role for ATF6 $\alpha$  in mediating increased levels of ASM and increased AHR in response to allergen challenge. In contrast, ATF6 $\alpha$  did not play a significant role in mediating allergen induced airway inflammation. As ORMDL3 regulates ATF6 $\alpha$ <sup>3,4</sup> these studies suggest that ATF6 $\alpha$  may be one of the important pathways downstream of ORMDL3 playing an important role in ASM and AHR changes characteristic of asthma. In this regard genes on chromosome 17q21 (ORMDL3, GSDMB)<sup>3,9</sup> and the downstream pathways of ORMDL3 (ATF6 $\alpha$ ) may play an important part in the structural changes in the airway, which contrasts with chromosome 5q which harbors genes (IL-4, IL-5, IL-9, IL-13) which play an important role in the immune and inflammatory response in asthma.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

<b>AHR</b>	Airway hyperreactivity
<b>ASM</b>	Airway smooth muscle
<b>ATF6<math>\alpha</math></b>	Activating transcription factor 6 $\alpha$
<b>ATF6<math>\alpha^{-/-}</math> mice</b>	Activating transcription factor 6 $\alpha$ deficient mice
<b>BAL</b>	Bronchoalveolar lavage
<b>ER</b>	Endoplasmic reticulum
<b>HDM</b>	House dust mite

<b>Mch</b>	Methacholine
<b>ORMDL3</b>	Orosomucoid like 3
<b>WT</b>	Wild type

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**Capsule Summary**

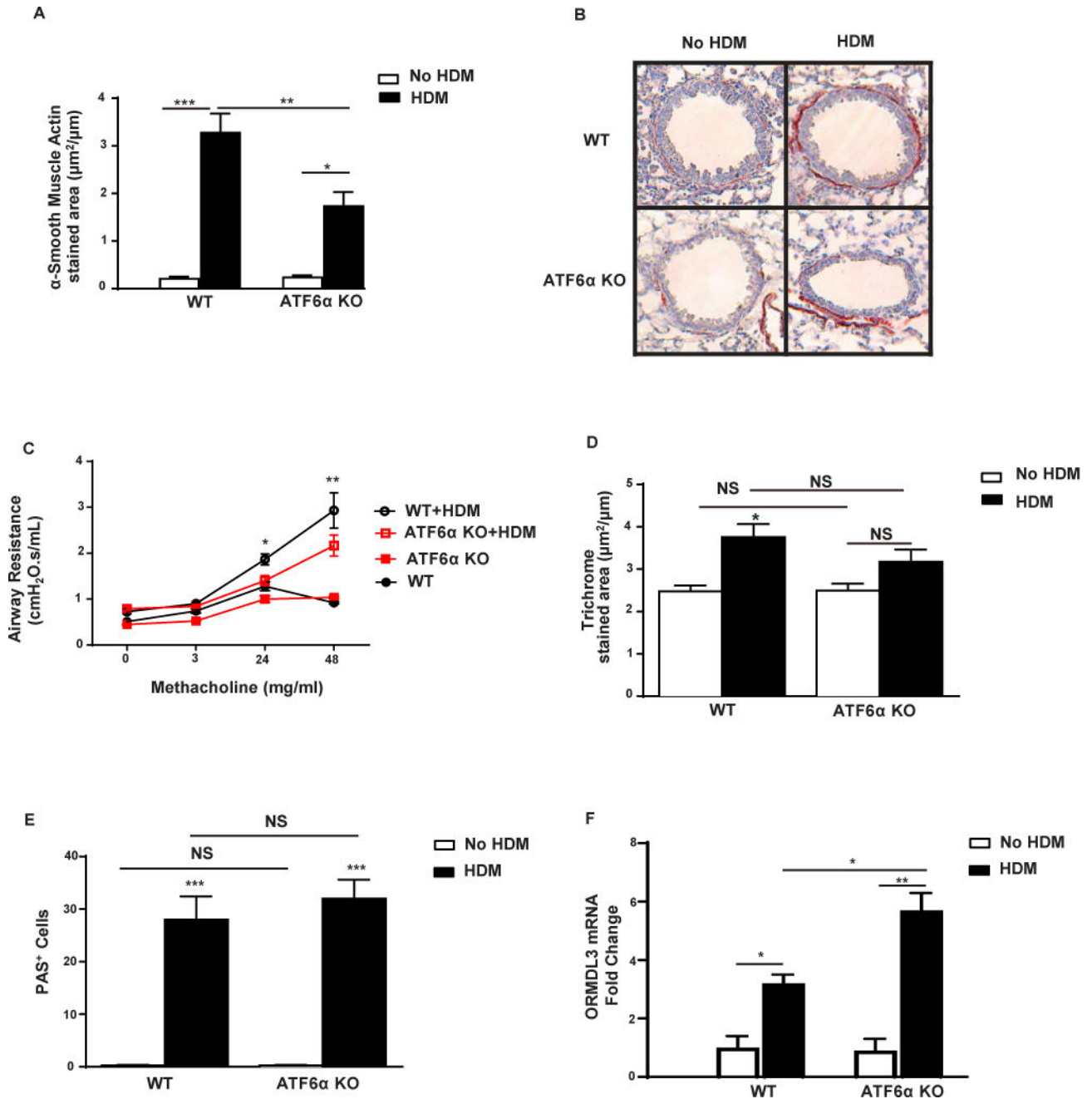
ORMDL3 on chromosome 17q21 is highly linked to asthma and regulates several downstream pathways including ATF6 $\alpha$ . We demonstrated that ATF6 $\alpha$  in airway smooth muscle (ASM) plays an important role in ASM contractility, proliferation, and AHR.

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**Figure 1. Reduced peribronchial smooth muscle and AHR in HDM challenged ATF6 $\alpha$ <sup>-/-</sup> mice** ATF6 $\alpha$ <sup>-/-</sup> or WT mice (n = 8 mice/group) were challenged with HDM intranasally on day 0, 7, 14, 21. On day 24, AHR to methacholine was assessed, and lung tissues were processed for immunohistochemistry and image analysis. **A–B**, Levels of peribronchial smooth muscle mass were quantitated by immunohistochemistry using an anti- $\alpha$ -smooth muscle actin Ab and image analysis (original magnification  $\times 200$ ). **C**, Airway resistance in response to methacholine was measured by flexiVent. WT + HDM vs WT,  $p < 0.001$  (Mch 48 mg/ml); ATF6 $\alpha$ <sup>-/-</sup> + HDM vs ATF6 $\alpha$ <sup>-/-</sup>,  $p < 0.01$  (Mch 48 mg/ml). Asterisk p values depicted in Figure are for WT+HDM vs ATF6 $\alpha$ <sup>-/-</sup> +HDM. **D**, Levels of peribronchial fibrosis were

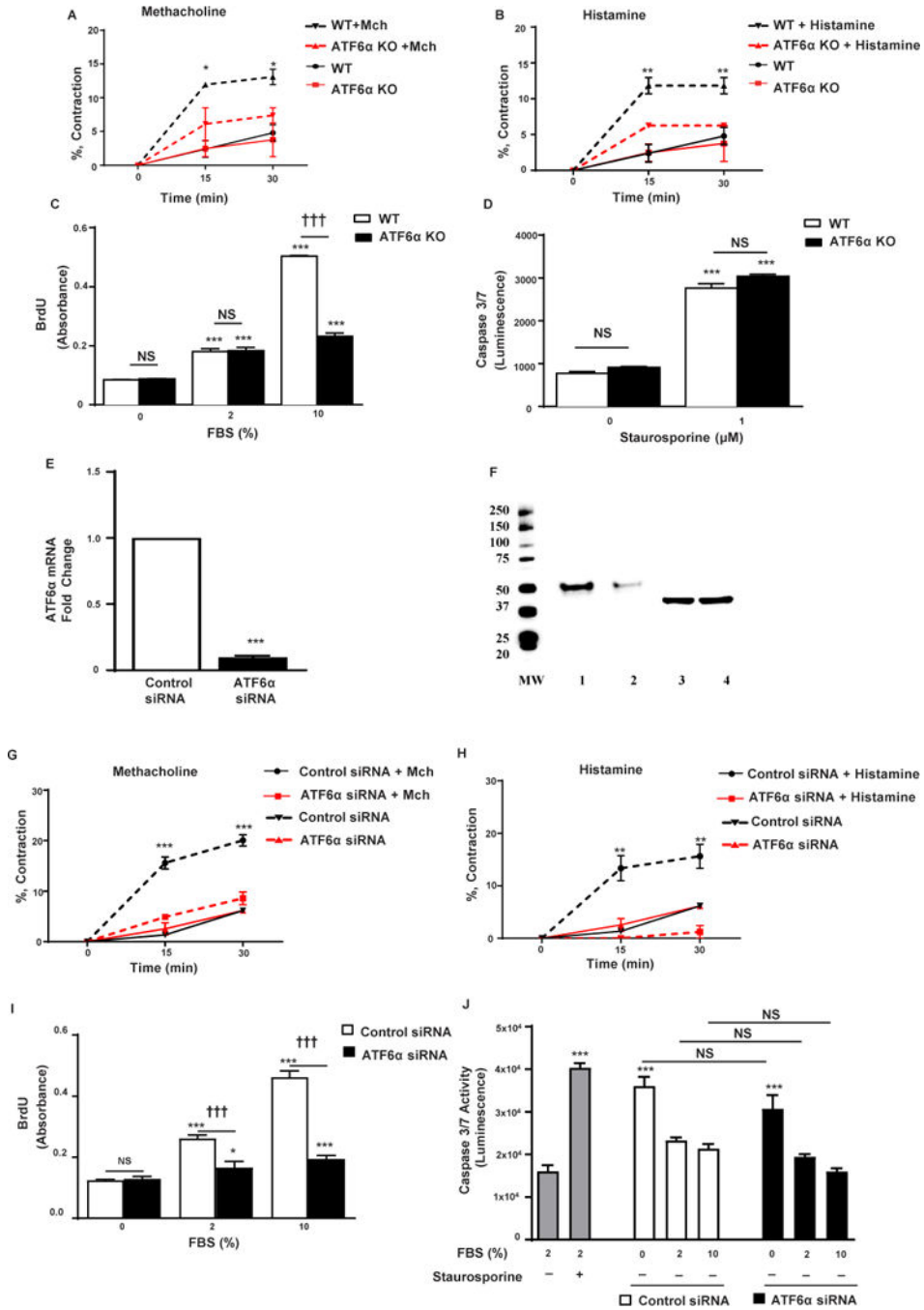
quantitated as the area of trichrome staining. **E**, Levels of lung mucus were quantitated by PAS staining. **F**, Levels of ORMDL3 mRNA were quantitated in the lungs of WT and ATF6 $\alpha^{-/-}$  mice before and after HDM challenge by RT-qPCR. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and NS (not significant).

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**Figure 2. Effect of ATF6α on mouse and human ASM contraction, proliferation, and apoptosis in vitro**

Levels of smooth muscle contraction in WT or ATF6α<sup>-/-</sup> mASM cells were assessed at baseline (time 0 min), as well as 15 and 30 min after incubation with either **A**, methacholine WT + Mch vs WT, p<0.05 (15 min), p<0.05 (30 min); ATF6α<sup>-/-</sup> + Mch vs ATF6α<sup>-/-</sup>, p=NS (15 min), p=NS (30 min); Asterisk p values depicted in Figure are for WT+ Mch vs ATF6α<sup>-/-</sup> + Mch, or **B**, histamine WT + histamine vs WT, p<0.01 (15 min), p<0.01 (30 min); ATF6α<sup>-/-</sup> + histamine vs ATF6α<sup>-/-</sup>, p<0.05 (15 min), p< NS (30 min); Asterisk p



values depicted in Figure are for WT+ histamine vs ATF6 $\alpha^{-/-}$  + histamine. With agonist-induced smooth muscle contraction the area of the gel decreases significantly. **C**, Cell proliferation was assessed by BrdU incorporation in WT or ATF6 $\alpha^{-/-}$  mASM cells incubated with either 0%, 2%, or 10% FBS. **D**, Caspase3/7 activity was measured by Caspase-Glo assay in WT or ATF6 $\alpha^{-/-}$  mASM cells cultured in the presence of staurosporine (1 $\mu$ M) or absence of staurosporine (0  $\mu$ M). **E**, The efficiency of siRNA knockdown of ATF6 $\alpha$  mRNA (RT-qPCR), and **F**, ATF6 $\alpha$  protein (western blot) in hASMS cells was assessed. Lane 1 and 2 are ATF6 $\alpha$ . Lane 3 and 4 are GAPDH. MW is molecular weight marker, lane 1 hASM control siRNA, lane 2 hASM ATF6 $\alpha$  siRNA, lane 3 hASM control siRNA, lane 4 hASM ATF6 $\alpha$  siRNA. **G–H**, Levels of smooth muscle contraction in ATF6 $\alpha$  siRNA or control siRNA transfected hASM cells were assessed at baseline (time 0 min), as well as 15 and 30 min after incubation with either **G**, methacholine, Control siRNA + Mch vs Control siRNA,  $p < 0.001$  (15 min),  $p < 0.001$  (30 min); ATF6 $\alpha$  siRNA+ Mch vs ATF6 $\alpha$  siRNA,  $p = \text{NS}$  (15 min),  $p = \text{NS}$  (30 min); Asterisk  $p$  values depicted in Figure are for Control siRNA+ Mch vs ATF6 $\alpha$  siRNA + Mch, or **H**, histamine, Control siRNA + histamine vs Control siRNA,  $p < 0.01$  (15 min),  $p < 0.01$  (30 min); ATF6 $\alpha$  siRNA+ histamine vs ATF6 $\alpha$  siRNA,  $p = \text{NS}$  (15 min),  $p < 0.05$  (30 min); Asterisk  $p$  values depicted in Figure are for Control siRNA+ histamine vs ATF6 $\alpha$  siRNA + histamine. **I**, Cell proliferation was assessed by BrdU incorporation in ATF6 $\alpha$  siRNA or control siRNA transfected hASM cells incubated with either 0%, 2%, or 10% FBS. **J**, Caspase3/7 activity was measured by Caspase-Glo assay in hASM cells cultured in 2% FBS in the presence or absence of staurosporine (grey bars). In addition ATF6 $\alpha$  siRNA or control siRNA transfected hASM cells were cultured in the presence or absence of FBS (0, 2, and 10%). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ††† $P < 0.001$ , and NS (not significant).