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## S100A6 regulates endothelial cell cycle progression by attenuating antiproliferative STAT1 signaling

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### Abstract

**Objective**—S100A6, a member of the S100-protein family, has been described as relevant for cell cycle entry and progression in endothelial cells (ECs). The molecular mechanism conferring S100A6's proliferative actions, however, remained elusive.

**Approach and Results**—Originating from the clinically relevant observation of enhanced S100A6 protein expression in proliferating ECs in remodeling coronary and carotid arteries, our study unveiled S100A6 as a suppressor of antiproliferative signal transducers and activators of transcription 1 (STAT1) signaling. Discovery of the molecular liaison was enabled by combining gene expression time series analysis with bioinformatic pathway modeling in S100A6 silenced human ECs stimulated with vascular endothelial growth factor A (VEGF-A). This unbiased approach led to successful identification and experimental validation of interferon-inducible

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transmembrane protein 1 (IFITM1) and protein inhibitors of activated STAT (PIAS) as key components of the link between S100A6 and STAT1.

**Conclusions**—Given the important role of coordinated EC cell cycle activity for integrity and reconstitution of the inner lining of arterial blood vessels in health and disease, STAT1 suppression by S100A6 may represent a promising therapeutic target to facilitate reendothelialization in damaged vessels.

### Keywords

Cell Cycle; Endothelium; PIAS; S100A6; STAT1

## Introduction

Coordinated endothelial cell (EC) cell cycle activity is vital for the integrity and reconstitution of the innermost lining of arterial blood vessels in health and disease. The endothelium keeps the underlying smooth muscle cell (SMC) layer in a quiescent state via paracrine signaling, cell-cell interactions and by shielding it from circulating growth factors. Efficient reendothelialization after intimal injury becomes clinically important, e.g. after percutaneous arterial angioplasty, bearing the risk of obstructive neointima formation due to SMC proliferation and migration.<sup>1, 2</sup> Although the use of growth-inhibitory drug-eluting stents has significantly reduced the restenosis rate, deciphering the molecular mechanisms that might facilitate or hinder vascular reendothelialization remains clinically relevant in efforts to improve therapeutic approaches.

In this regard, S100A6, also known as calcyclin, attracted attention due to its putative role as a cell cycle regulator in ECs.<sup>3</sup> It is a member of the S100 family of low molecular weight calcium ( $\text{Ca}^{2+}$ )-binding proteins. This group comprises more than 20 isoforms to date, each of which are characterized by a cell- and tissue type-specific expression pattern.<sup>4</sup> The S100 family constitutes the largest subgroup within the EF-hand  $\text{Ca}^{2+}$ -binding protein superfamily including familiar  $\text{Ca}^{2+}$ -buffer and  $\text{Ca}^{2+}$ -sensor proteins such as parvalbumin and calmodulin, respectively. Mostly acting as molecular decoders and transducers of intracellular  $\text{Ca}^{2+}$  signals via interaction with intracellular target proteins, S100 proteins are key components of the cell's versatile molecular toolkit to translate  $\text{Ca}^{2+}$  signals into specific actions. These extend from  $\text{Ca}^{2+}$ -dependent regulation of muscle contraction, membrane excitability, secretion, motility, signaling and differentiation, to general features such as cell cycle activity.<sup>5</sup> Although previous reports highlighted a potential role for S100A6 in modulating p44/42 (ERK1/2) and beta-catenin signaling,<sup>6</sup> the molecular mechanism by which S100A6 potentially impacts cell cycle entry and progression in ECs remains elusive.

Inhibition of cell cycle entry in a number of cell types depends on a family of latent cytoplasmic transcription factors known as signal transducers and activators of transcription (STAT). Biochemical and molecular analyses have indicated that STAT activity is regulated predominantly by phosphorylation on specific tyrosine residues (Tyr-701 in case of STAT1), which causes STAT dimerization.<sup>7, 8</sup> STAT1 homodimers then translocate into the nucleus and bind to consensus DNA binding sites that represent enhancer sequences, e.g. for a

variety of antiproliferative genes<sup>7, 8</sup>. One group of antiproliferative genes that are expressed in response to activated STAT1 encompasses interferon-inducible (IFI) proteins. Specifically, interferon-induced transmembrane protein 1 (IFITM1) is induced by STAT1 signaling, thereby leading to downstream activation of p53/p21 and cell cycle arrest.<sup>9, 10</sup>

Great progress has been made in better understanding the regulation of STAT activity.<sup>11</sup> Protein inhibitors of activated STAT 1-4 (PIAS1-4) have been identified to mitigate STAT signaling at different levels.<sup>12</sup> PIAS1, for example, negatively interferes with the DNA binding activity of STAT1, thereby acting as an inhibitory transcriptional co-regulator.<sup>13</sup> Furthermore, PIAS attenuates phosphorylation of STAT1-Tyr-701 and makes tyrosine phosphorylated STAT1 more susceptible to inactivation by nuclear phosphatases (e.g. TC45).<sup>14, 15</sup> In addition, suppressors of cytokine signaling (SOCS) exert a negative feedback evoked by nuclear STAT activity.<sup>16</sup> SOCS bind to phosphorylated non-receptor tyrosine janus kinases 1 and 2 (JAK 1/2) and their receptors to interrupt the transmission between JAK and STAT molecules.<sup>17</sup> A link between the cell cycle regulator S100A6 and the antiproliferative STAT1 cascade and PIAS has not been reported yet.

Originating from the clinically relevant observation of enhanced intimal S100A6 protein expression in remodeling coronary and carotid arteries, we combined time-resolved transcriptome data with bioinformatic analysis to elucidate the molecular pathways through which S100A6 impacts EC cell cycle activation. This unbiased approach led to successful identification and validation of key components of the *in silico* modeled pathway, and, for the first time, linked S100A6 to antiproliferative STAT1/IFITM1 signaling through the modulation of PIAS activity. Hence, augmented S100A6 expression during vascular remodeling may facilitate reendothelialization by controlling antiproliferative STAT1 signaling. In addition, these novel findings advance our understanding about the role of S100A6 beyond molecular vascular medicine. S100A6/STAT1 signaling may also represent a valuable therapeutic target in oncology given the established role of S100A6 as a biomarker in various cancer types and molecular driver of tumorigenesis.<sup>6, 18–20</sup>

## Materials and methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### S100A6 is upregulated during vascular remodeling after arterial endothelial cell injury

We found S100A6 protein to be expressed in all layers, and particularly abundant in ECs (the intimal layer), of both porcine coronary and rat carotid arteries (Figure 1A and Supplemental Figure IA, B and C). When vascular remodeling was induced by intimal injury due to experimental stent implantation or balloon angioplasty, we observed an increase in the immunofluorescence (IF) and immunohistochemical (IHC) S100A6 signal in proliferating vascular cells of both arteries (Figure 1A and Supplemental Figure IA, B and C). When arterial reendothelialization had occurred, the S100A6 signal returned to baseline and was largely confined to ECs again (Figure 1A and Supplemental Figure IA). These observations in clinically relevant *in vivo* endothelial injury models prompted investigation

of the regulation of S100A6 expression *in vitro*. Proliferation was induced in quiescent and cell cycle synchronized cultured human umbilical vein endothelial cells (HUVECs) as well as vascular SMCs by VEGF-A stimulation. VEGF-A exposure led to a 4.7-fold increase in endothelial S100A6 protein expression within 72 hours (Figure 1B), which was confirmed by S100A6 IF staining (Figure 1C). Similar results were observed in human SMCs (Supplemental Figure ID). In this study, we focused on the biological significance of S100A6 in endothelial cell proliferation, given the importance of efficient arterial reendothelialization to mitigate vascular remodeling.

### **S100A6 is required for human endothelial cell cycle activation and proliferation**

S100A6 siRNA knockdown was carried out in HUVECs, which led to a 60% reduction of S100A6 protein levels that persisted upon VEGF-A stimulation (Figure 2A). Control experiments showing unchanged S100A4 and S100B expression in response to S100A6 siRNA treatment confirmed the specificity of our experimental approach (Supplemental Figure IIA). As a result, we observed a profound decrease in the VEGF-A stimulated proliferation rate in S100A6 silenced HUVECs compared to the control siRNA treated group using three independent methods: 1) Reduced cell numbers were assessed by nuclear DAPI staining (Supplemental Figure IIB) while 2) diminished DNA synthesis was measured by nuclear incorporation of fluorescent EdU as well as 3) decreased anti-Ki67 IF signal (Figure 2B). To further investigate migration as a crucial EC function in the setting of vascular injury repair, we employed a wound-healing chemokinesis assay that showed a trend towards reduced migration of S100A6 depleted cells (Supplemental Figure IIC). Additionally, an *in vitro* angiogenesis (Matrigel tube-formation) assay, assessing both migration and proliferation capacity of ECs, was carried out and revealed an almost 50% reduction in tube formation capabilities of S100A6 knockdown ECs (Supplemental Figure IIC). Taken together, these results indicated that S100A6 is indispensable for EC function, especially proliferation. In a next step, we combined time-resolved transcriptome data and bioinformatic analyses to decipher the molecular pathways transmitting the effect of S100A6 depletion in EC cell cycle regulation.

### **Dynamic functional transcriptome analysis reveals antiproliferative signaling in S100A6 depleted human endothelial cells**

We used Illumina HT-12v4 human bead-arrays to quantify the HUVEC transcriptome response in siRNA mediated S100A6 knockdown and control samples over 24 hours after VEGF-A stimulation. A principal component analysis (PCA) on the normalized and Entrez ID filtered expression time series revealed a quantitative, rather than qualitative separation of S100A6 knockdown and control siRNA treated HUVEC transcriptomes over time (Figure 3A). This is evident from the green and black trajectories marking the knockdown and control samples in PCA space. Albeit similar in shape, there is a shift along the second principal component PC2, which indicates the change in related subsets of genes, yet at different regulatory strength. Next, we assessed the functional relevance of gene expression over time and performed a Gene Set Enrichment Analysis (GSEA) using the human Gene Ontology (GO) gene sets.<sup>21</sup> The analysis showed a decelerated activation of cell cycle/cell division gene pathways after VEGF-A stimulation as a result of S100A6 knockdown. Heatmaps in Figure 3B indicate an early up-regulation of such pathways at 12h (marked by

red box) in control samples, while delayed activation in S100A6 knockdown HUVECs is present only after 24h. In line with the quantitatively different cellular responses, regulation of several cyclins and cyclin-dependent kinases to VEGF-A stimulation was mitigated after S100A6 knockdown, reflecting delayed cell cycle entry (Supplemental Figures IIIA and IIIB). To systematically evaluate the S100A6 dependent effect of VEGF-A stimulation on HUVECs, we performed a paired GSEA on the shared duplicate time points in knockdown versus control cells using the Consensus Pathway Database gene sets.<sup>22</sup> The analysis unveiled a strong and persistent up-regulation of a group of STAT1 responsive interferon-inducible genes with tetratricopeptide repeats (IFI and IFIT) and transmembrane proteins (IFITM) upon S100A6 depletion (Figures 3C and 3D and Supplemental Figure IIIC). The finding is supported by the previously reported role of IFITM1 as an inhibitor of cell cycle entry and progression in malignant cells.<sup>9</sup> Therefore, we continued by focusing on IFITM1 as a putative key driver of the cellular phenotype observed.

### **IFITM1 silencing restores proliferation capacity in S100A6 knockdown endothelial cells**

S100A6 depletion resulted in a constitutive upregulation of both the IFITM1 gene transcript and its respective protein (Figures 4A and 4B), although the response dynamics to VEGF-A stimulation differed over time. The excessive (up to 25fold) IFITM1 mRNA expression translates into a 1.8–2.5fold IFITM1 protein expression in S100A6 knockdown ECs suggesting that IFITM1 might be subject to post-translational modifications in line with previous studies concluding that especially regulators of cell-cycle specific functions tend to have unstable protein.<sup>23</sup> IFITM proteins have been reported to be regulated by palmitoylation and ubiquitination, for example.<sup>24, 25</sup> This might explain our finding that the increase in IFITM1 protein both in control and S100A6 knockdown cells is partially overcome by short-term VEGF-A treatment. To test the impact of increased IFITM1 protein levels, we conducted a dual S100A6/IFITM1 siRNA-mediated knockdown in HUVECs. IFITM1 silencing partially rescued the diminished proliferation rate in S100A6 knockdown cells in response to VEGF-A as measured by nuclear DAPI staining and nuclear EdU incorporation (Figure 4C). IFITM1 knockout alone did not result in a significantly different EC proliferation rate from control cells at baseline (data not shown) and in presence of VEGF-A stimulation (Supplemental Figure IVA). Interestingly, in addition to the plasma membrane, IFITM1 was previously shown to be localized on the endoplasmic reticulum (ER), the golgi apparatus and cytoplasmic vesicles, indicating the possibility to influence expression and activity of proteins, including cell cycle regulators.<sup>9, 26</sup> Analysis of the IFITM1 protein localization pattern in HUVECs (Supplemental Figure IVC) and carotid arteries at baseline or after injury (Supplemental Figure IVD) show a granulated pattern within the cell, likewise pointing to an organelle- and vesicular localization, additionally, we cannot exclude nuclear localization.

These results not only supported the significance of the predicted role of most differentially expressed genes for the cellular phenotype as mentioned above, but also identified IFITM1 as the first relevant molecular component in the antiproliferative pathway downstream of negative S100A6 expression changes. The next step was to ascertain regulators within this signaling pathway.

### **S100A6 depletion causes increased STAT1 activation in endothelial cells**

ChIPseq data from the ENCODE project indicated STAT1 and STAT2 as well as nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB) and DNA Polymerase Epsilon Catalytic Subunit A (POL2/POLE) as regulators of the IFITM1 promoter (Supplemental Figure VA).<sup>27</sup> Those results were in line with previously published experimental data.<sup>10</sup> Guided by IFI/IFITM1 transcriptome changes, subsequent analysis of upstream STAT1 activity unveiled enhanced STAT1 mRNA and protein levels together with increased tyrosine (Tyr-701) phosphorylation (Figures 5A, 5B and Supplemental Figure VB) in S100A6 depleted HUVECs over time. In contrast, STAT2 and POL2 were neither significantly regulated at the transcriptional (Supplemental Figure VC) nor at the translational level (Supplemental Figure VD). Likewise, NFKB-p65 mRNA (encoded by the RELA gene) and respective protein levels did not change significantly over time (Supplemental Figure VC and VD). Of note, S100A6 knockdown was sufficient to persistently activate STAT1 throughout VEGF-A stimulation (Figure 5B). Interestingly, VEGF-A stimulation in control siRNA treated cells did not significantly increase STAT1 protein expression or phosphorylation over time. For a more comprehensive *in silico* assessment of putatively regulated STAT1 target genes, we matched our transcriptome data with previously identified STAT1 target genes.<sup>28</sup> We found 301 out of 1440 target genes to be significantly regulated (q-value<0.05) upon S100A6 knockdown in HUVECs (Supplemental Table I), including PARP9, IFI35, CXCL10 and IRF7 (Supplemental Figure VE). Taken together, these data suggest a potentially pivotal role for STAT1 activation in the antiproliferative effects of S100A6 depletion in ECs.

### **STAT1 knockdown prevents IFITM1 upregulation and restores proliferation in S100A6 depleted endothelial cells**

To assess whether increased STAT1 activity actually accounts for the antiproliferative cellular phenotype, we subjected HUVECs to dual S100A6/STAT1 siRNA-mediated gene silencing. In line with our *in silico* pathway analysis, STAT1 depletion suppressed downstream IFITM1 upregulation in S100A6 knockdown ECs at baseline and after VEGF-A stimulation (Figure 5C) and in turn restored the proliferation capacity. Figure 5C depicts representative cell culture and IF EdU incorporation images. In line with previous findings,<sup>29, 30</sup> Ki67 protein upregulation in S100A6/STAT1 gene silenced HUVECs surpassed the VEGF-A mediated Ki67 increase in corresponding control groups (Figure 5C). STAT1 knockdown alone also lead to a relative decrease in IFITM1 expression. However, the increase in cell proliferation was relatively mild (Supplemental Figure VIA). Interestingly, while STAT1 knockdown decreased IFITM1, it did not affect S100A6 protein expression (Supplemental Figure VIB). IFITM1 on the other hand, did not affect STAT1 expression (Supplemental Figure IVB). These results confirmed STAT1 as an upstream activator of IFITM1 in S100A6 depleted ECs and validated the *in silico* pathway prediction.

### **S100A6 regulates STAT1 activity in endothelial cells through PIAS modulation**

To determine the mechanism by which S100A6 might impact STAT1 activity, we first analyzed the phosphorylation status of the non-receptor tyrosine kinases JAK1 and JAK2 (Supplemental Figure VIIB). Unchanged JAK1/2 activation largely ruled out direct actions



on the kinases or paracrine effects that might have resulted in STAT1 activation in S100A6 depleted HUVECs. Hypothesizing that S100A6 might operate independent of JAK1/2, we drew our attention to negative regulators of STAT1. We observed persistent downregulation of PIAS genes in S100A6 depleted ECs (Figure 6A), which was significant for the concerted downregulation of all four PIAS isoforms (PIAS1-4, shown as PIAS gene set, Figure 6B) over time. In line with gene expression, protein level of the STAT1 inhibitor PIAS1 was diminished in S100A6 depleted ECs, both at baseline and after VEGF-A stimulation (Figure 6C). A trend towards increased total and phosphorylated STAT1 protein (Supplemental Figure VIIIA), as well as reduced Ki67 positive nuclear counts (Figure 6D) in PIAS1 depleted ECs confirmed a relevant role of PIAS1 in EC proliferation. Combined PIAS1/STAT1 knockdown in HUVECs restored the mildly reduced Ki67 expression observed in PIAS1 depleted ECs (Supplemental Figure VIIIA). Both individual STAT1 knockdown (Supplemental Figure VIB) and IFITM1 knockdown in HUVECs (Supplemental Figure IVB) did not change the expression of PIAS1. These results further corroborated the hierarchy of the signaling pathway. A possible additive effect of PIAS2-4 in this setting seems likely.

More reports emerged suggesting that the STAT1 signaling pathway, including negative regulators such as PIAS, might be targeted by miRNAs via post-transcriptional regulation.<sup>31, 32</sup> Concordantly, applying miRNA-mRNA interaction prediction, our serial transcriptome analysis unveiled that certain miRNAs, e.g. miR548c, negatively correlate with the expression of PIAS genes (Figure 6E and Supplemental Figure VIIIB).

We also considered additional mechanisms of putative S100A6 and STAT1 interactions. Tyrosine phosphatases constitute a second class of negative regulators of STAT. Previous studies indicated that cytoplasmic tyrosine phosphatases SH2-containing phosphatase 1 and 2 (SHP-1, SHP-2, genes PTPN6 and PTPN11) and protein tyrosine phosphatase 1B (PTP1B, gene PTPN1) are involved in dephosphorylating activated STAT1.<sup>33</sup> However, none of the genes encoding for above proteins were differentially expressed in S100A6 depleted HUVECs (Supplemental Figure VIIC). A third type of repressor, suppressors of cytokine signaling (SOCS), represents a negative feedback loop evoked by nuclear STAT.<sup>16</sup> SOCS proteins bind to phosphorylated non-receptor tyrosine kinases interrupting the transmission between JAK and STAT molecules. Interestingly, the SOCS1-3 gene set was transiently downregulated in S100A6 knockdown HUVECs (Supplemental Figure VIID). However, SOCS proteins function in part through ubiquitin-dependent degradation and inhibition of the autocatalytic domain of JAK1/2.<sup>11, 16</sup> Unchanged protein levels and phosphorylation of the latter therefore argued against a meaningful role of diminished SOCS expression in S100A6 depleted ECs (Supplemental Figure VIIIB). In summary, these data suggest that S100A6 is necessary to prevent induction and activation of STAT1 in endothelial cells most likely via PIAS.

### **S100A6 is not sufficient to alter endothelial STAT1 signaling**

We further sought to understand whether S100A6 was sufficient to alter STAT1 signaling in endothelial cells. We applied an adenoviral gene transfer approach to overexpress S100A6 in HUVECs (Figure 7A). S100A6 overexpression neither changed STAT1 nor IFITM1 or

PIAS1 expression, indicating that S100A6 is necessary for endothelial proliferation by controlling STAT1 signaling, but not sufficient to down-modulate the pathway.

### **VEGF-A mediated pro-proliferative signaling in endothelial cells is not disrupted by S100A6 knockdown**

We finally determined, whether reduced HUVEC S100A6 would interfere with VEGF-A induced cell cycle entry and progression. Interestingly, PI3K-AKT-MAPK signaling pathways remained unchanged at the transcriptional level according to GSEA (Supplemental Figure IXA). In line with this result, neither phosphorylation nor protein abundance of AKT and p44/p42 were altered in S100A6 depleted HUVECs (Supplemental Figure IXB and IXC). Of note, a trend towards a premature ERK1/2 deactivation was observed after 24h of VEGF-A stimulation in S100A6 depleted cells and is therefore rather a possible late downstream effector of the pathway than a cause for the reduced proliferation observed in S100A6 depleted HUVECs. These data suggest that the antiproliferative effect of STAT1 activation in S100A6 knockdown cells is independent of AKT or MAPK signaling.

### **Discussion**

Previous studies demonstrated a role for S100A6 in the regulation of cell cycle entry and progression of HUVECs.<sup>3</sup> S100A6 gene silencing caused significant accumulation of HUVECs in G1/S and G2/M phase transition and cell cycle arrest. Similar phenotypes were observed both in fibroblasts and cancerous cells.<sup>19, 34, 35</sup> The underlying molecular pathways, however, by which S100A6 might affect EC proliferation remained elusive.

Based on experimental data supporting a role for S100A6 in efficient reendothelialization of injured arteries, this is the first report that causatively links S100A6 to the attenuation of antiproliferative STAT1 signaling in ECs. Discovery of this unexpected molecular liaison was guided by the time-resolved transcriptional STAT1 dependent “fingerprint” in S100A6 gene silenced HUVECs. The unbiased origin subsequently fueled bioinformatic analysis of the putative molecular pathway, which in turn guided *in vitro* experimental validation of predicted key factors. Given S100A6’s widespread expression in mammalian cells, it is tempting to speculate that the biological impact of the S100A6/STAT1 signaling axis might not be limited to EC and vascular molecular medicine. S100A6 might act as a general attenuator of STAT1 signaling with potential relevance to various fields in molecular medicine, but that requires further investigation.

Focusing on the most differentially regulated genes that are expected to shape the antiproliferative phenotype, our time-resolved transcriptome analysis first identified IFITM1 as a downstream molecular effector, also owing to its previously described role as a cell cycle inhibitor amidst other candidates.<sup>9</sup> Of note, S100A6 gene silencing was sufficient to upregulate IFITM1 as well as other IFI proteins prior to and throughout VEGF-A stimulation.

The key role of IFITM1 was validated by partially restoring the proliferative capacity in VEGF-A stimulated S100A6 depleted HUVECs when IFITM1 protein was diminished. This result is in line with a previous report showing that IFITM1 stabilizes p53 in cancer cells by



directly inhibiting Thr55 phosphorylation. Enhanced p53 translational activity consecutively induced the cyclin-dependent kinase inhibitor 1 (p21).<sup>9</sup> Subsequent GSEA analysis in S100A6 depleted HUVECs confirmed concordant transcriptional changes in various cell cycle pathways over time such as *cell division* or *G1/S*. IFITM1's increased abundance might therefore present a molecular barrier for endothelial cell cycle entry rather than being an irrevocable blockade.

In support of this argument, we observed an expression delay across numerous relevant cyclins such as E1, L2, Y and D2, as well as various cyclin-dependent kinase genes in S100A6 silenced HUVECs. This pattern, and the fact that IFITM1 knockdown was not sufficient to fully restore the proliferative capacity in S100A6 depleted HUVECs or independently drive EC proliferation argues against the notion that the antiproliferative effect can solely be pinpointed to an IFITM1/p53-dependent mechanism. Other inhibitory mechanisms might synergize and impede cell cycle entry and progression at multiple levels.

Leveraging previously published CHIPseq data on STAT1 responsive elements within the IFITM1 promoter, bioinformatic analysis predicted upstream STAT1 activation as the most likely transcriptional effector for the antiproliferative transcriptional signature in S100A6 depleted HUVECs. Our experimental data strongly supported this argument since STAT1 signaling was significantly enhanced in S100A6 knockdown HUVECs and STAT1 depletion rescued expression of the pan-cell cycle marker Ki67 protein and the cell proliferation rate in response to VEGF-A stimulation. Of note, STAT1 depletion in S100A6 knockdown cells that prevented IFITM1 upregulation resulted in greater restoration of proliferative capacity than IFITM1 siRNA-mediated inhibition alone. This suggests again that STAT1 signaling governs a multifactorial set of downstream cell cycle inhibitors in this setting, e.g. other interferon inducible proteins highly regulated upon S100A6 depletion.

The JAK-STAT signaling pathway usually transmits information from a variety of ligands including cytokines, hormones and growth factors, and their receptors to gene promoters on DNA in the cell nucleus and thereby modulates cellular activities.<sup>36</sup> Stimulation of the receptor results in JAK activation, which in turn leads to STAT phosphorylation, dimerization and subsequent translocation into the nucleus.<sup>11, 36</sup> It is a major signaling alternative to classical second messenger systems. Our data however indicate that STAT1 signaling due to diminished S100A6 protein levels in ECs depends neither on receptor stimulation nor on JAK1/2 activation.

Since our results ruled out JAK1/2 as upstream STAT1 activators in response to S100A6 knockdown, we focused on molecular mechanisms that might result in STAT1 disinhibition. Among those factors known to restrict STAT1 activity, PIAS genes emanated from our transcriptome time series data and consecutive *in silico* analysis as most likely candidates. PIAS1 protein was actually found to be decreased in S100A6 depleted HUVECs prior to VEGF-A stimulation and this suppression endured over time. Previous studies showed that PIAS1 apparently exerts a high degree of specificity towards STAT1 inhibition.<sup>13</sup> PIAS1 depleted endothelial cells exhibited a tendency for attenuated proliferation and STAT1 upregulation with increased STAT1 activity. One explanation for the rather mild phenotype of PIAS1 depleted HUVECs is that S100A6 might regulate a more complex signaling

module. For example, our data indicate that PIAS2-4 will most likely add to the effect in this setting. Another reason is that STAT1 expression and activity are not significantly increased in VEGF-A stimulated HUVECs. In agreement with this observation, we also see a mild effect of STAT1 silencing for VEGF-A stimulated HUVEC proliferation, as opposed to the significant effect that STAT1 silencing has in S100A6 depleted cells, after interferon  $\gamma$  treatment or *in vivo*.<sup>30</sup> These results are in line with previous studies showing that PIAS1 overexpression inhibits STAT1 mediated gene expression and VEGF-induced proliferation after interferon  $\gamma$  treatment.<sup>13, 30</sup> However, it has also been reported that PIAS1 induction resulted in reduced expression of p21 and thereby enhanced proliferation and PIAS1 silencing lead to growth arrest in human osteosarcoma cells.<sup>37</sup> Further experimentation is needed to decipher the role of other PIAS proteins in S100A6 depended STAT1 regulation and the functional consequence of PIAS1 overexpression in S100A6 depleted HUVECs.

Several inhibitory mechanisms of PIAS have been described. For example, PIAS1 has been shown to negatively interfere with the DNA binding activity of STAT1, thereby acting as an inhibitory transcriptional co-regulator.<sup>13</sup> Furthermore, SUMOylation by PIAS makes tyrosine phosphorylated STAT1 more susceptible to inactivation by nuclear phosphatases (e.g. TC45).<sup>15</sup> Both mechanisms could be an explanation for the prolonged STAT1 expression and activity as well as the upregulation of STAT1 target genes in S100A6 knockdown HUVECs. However, since S100A6 knockdown as the single stimulus increased STAT1 activity (Supplemental Figure VIIA), it seems likely that an additional PIAS-STAT1 inhibitory mechanism is directly influenced by S100A6 in the cytoplasm. Droscher et al. have shown that SUMOylation of STAT1 precludes phosphorylation of the Tyr-701 residue.<sup>14</sup> Their data also suggests that SUMO interferes in the very moment that STAT1 is phosphorylated at the receptor-level (i.e. by the receptor tyrosine kinase itself or the non-receptor tyrosine-kinases). This mechanism seems to suggest that SUMOylation, for example by PIAS1, is a way the cell maintains active STAT1 homeostasis required to balance pro- and antiproliferative signaling. However, we cannot exclude that additional (yet unknown) mechanisms have an effect on STAT1 upon S100A6 depletion in ECs.

Nevertheless, PIAS downregulation in S100A6 depleted HUVECs appears as an evident mechanism to facilitate STAT1 disinhibition. It explains why S100A6 knockdown does not interfere with the pro-proliferative signaling pathway and does not hamper receptor- or JAK activity. Importantly, PIAS proteins can also negatively interfere with NF $\kappa$ B signaling.<sup>38</sup> Unchanged NF $\kappa$ B expression and activation levels nevertheless support the notion that altered PIAS activity in S100A6 knockdown HUVECs might indeed be limited to STAT1 activation. It has previously been described that PIAS might be regulated by microRNAs (miRNAs).<sup>31, 32, 39</sup> Our serial transcriptome analysis unveiled that certain miRNAs, e.g. miR548c, negatively correlate with the expression of PIAS1 and PIAS3. However, a mechanistic interaction of miRNAs and PIAS genes would require further validation.

Although SOCS genes, which constitute an inducible feedback mechanism for the JAK1/2-STAT1 pathway, were also moderately expressed over time, unchanged JAK1/2 protein levels and phosphorylation largely ruled out a relevant role for enhanced STAT1 activity. S100A6 knockdown nonetheless seems to interrupt the feedback mechanism, since elevated STAT1 activity would have been expected to result in increased SOCS protein levels in our

experimental setting.<sup>16, 17</sup> Unchanged STAT1 signaling upon S100A6 overexpression and intact VEGF-A mediated proliferative signaling through ERK1/2 and AKT eventually corroborated the notion that S100A6 protein may act as an independent molecular brake that facilitates cell cycle entry by preventing simultaneous antiproliferative STAT1 signaling. Upregulation of S100A6 by VEGF-A in HUVECs is in line with this notion and previous findings by Bao et al. showing that forced S100A6 expression has no to at best minimal effects on endothelial cell proliferation further substantiate our results.<sup>3</sup> Our findings of a “premature” ERK1/2 deactivation after 24h of VEGF-A stimulation in S100A6 depleted cells could underline this possible mechanism of how IFITM1 regulates its downstream effectors.

Overall, our study sheds new light onto S100A6’s role as a cell cycle regulator in ECs through discovery of a hitherto unknown link to STAT1 signaling (Figure 7B). Our *in vivo* results indicate a relevant role for S100A6 and STAT1 signaling in efficient reendothelialization after intimal injury. Interestingly, Fludarabine, a pharmacological STAT1 inhibitor, was therapeutically tested in a rat carotid artery balloon injury model before and resulted in reduced neointimal hyperplasia through inhibition of smooth muscle cell proliferation.<sup>40</sup> Multiple studies have confirmed that STAT1 is directly stimulated by Angiotensin II in SMCs and responsible for Angiotensin induced proliferation of this cell type. We could confirm that STAT1 (Supplemental Figure VIC) and its downstream effector IFITM1 (Supplemental Figure IVD) were expressed in neointima-forming SMCs during the vascular remodeling process after balloon injury of carotid arteries. Because of the disrupted vascular architecture upon injury that complicates endothelial cell discrimination, further research is warranted to clarify lineage specific roles of S100A6. Nevertheless, since the data presented in this study, and previous reports investigating angiogenesis in STAT1<sup>-/-</sup> mice,<sup>30</sup> suggest that, contrary to SMCs, STAT1 inhibits proliferation in ECs, it appears that STAT1 resumes opposing functions, alike Angiotensin II itself, depending on the stimulus and cell lineage. Therefore, Fludarabine’s inhibition of STAT1 could exert double positive effects as a therapeutic agent to prevent excessive neointima formation after vascular injury due to inhibited SMC proliferation and unhindered EC proliferation. However, given Fludarabine’s ability to inhibit DNA synthesis and cytotoxic effects in endothelial cells (data not shown),<sup>41</sup> the positive effects of STAT1 inhibition unlikely unfold in endothelial cells. Therefore, characterization of upstream and downstream effectors such as S100A6 and IFITM1 might stimulate novel therapeutic strategies.

Discovery of the unexpected molecular link between S100A6 and STAT1 became possible by combining transcriptome time series analysis with bioinformatic pathway modeling and subsequent experimental validation. Further *in vivo* studies are warranted to test the therapeutic impact of the candidates that emerged from this study such as IFITM1 or S100A6 to facilitate healing of the arterial intimal layer. Likewise, additional studies are necessary to decipher the role of S100A6 for SMC proliferation. Manipulating vascular S100A6 expression might bear the risk of being a double-edged sword and cell-type specific formulations might be necessary to safely exploit its inherent therapeutic potential. Originating from an unbiased approach, our strategy nevertheless has the potential for a comprehensive characterization of novel signaling pathways with relevance for molecular medicine.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>EC</b>	Endothelial Cell
<b>GSEA</b>	Gene Set Enrichment Analysis
<b>GO</b>	Gene Ontology
<b>IFITM1</b>	Interferon-inducible Transmembrane Protein 1
<b>PCA</b>	Principal Component Analysis
<b>PIAS</b>	Protein Inhibitor of Activated STAT
<b>SMC</b>	Smooth Muscle Cell
<b>STAT1</b>	Signal Transducers and Activators of Transcription 1
<b>VEGF-A</b>	Vascular Endothelial Growth Factor A

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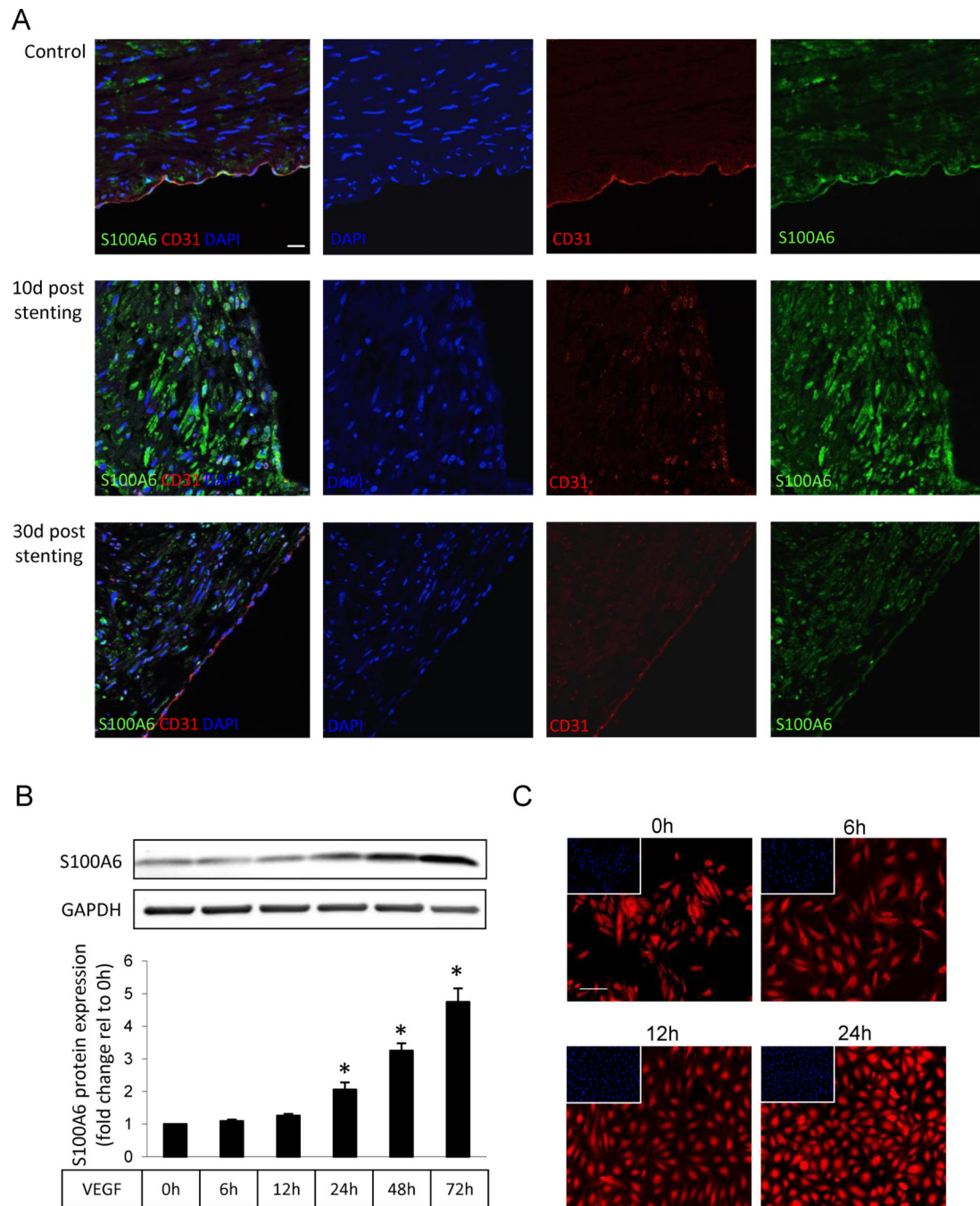
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**Highlights**

- S100A6 orchestrates novel signaling module in endothelial cell cycle regulation
- Bioinformatic and molecular biology techniques enabled to uncover unknown link between S100A6 and the suppression of anti-proliferative signal transducers and activators of transcription 1 (STAT1) signaling in endothelial cells
- Interferon-inducible transmembrane protein 1 (IFITM1) and protein inhibitors of activated STAT (PIAS) revealed as key components of the link between S100A6 and STAT1



**Figure 1.**

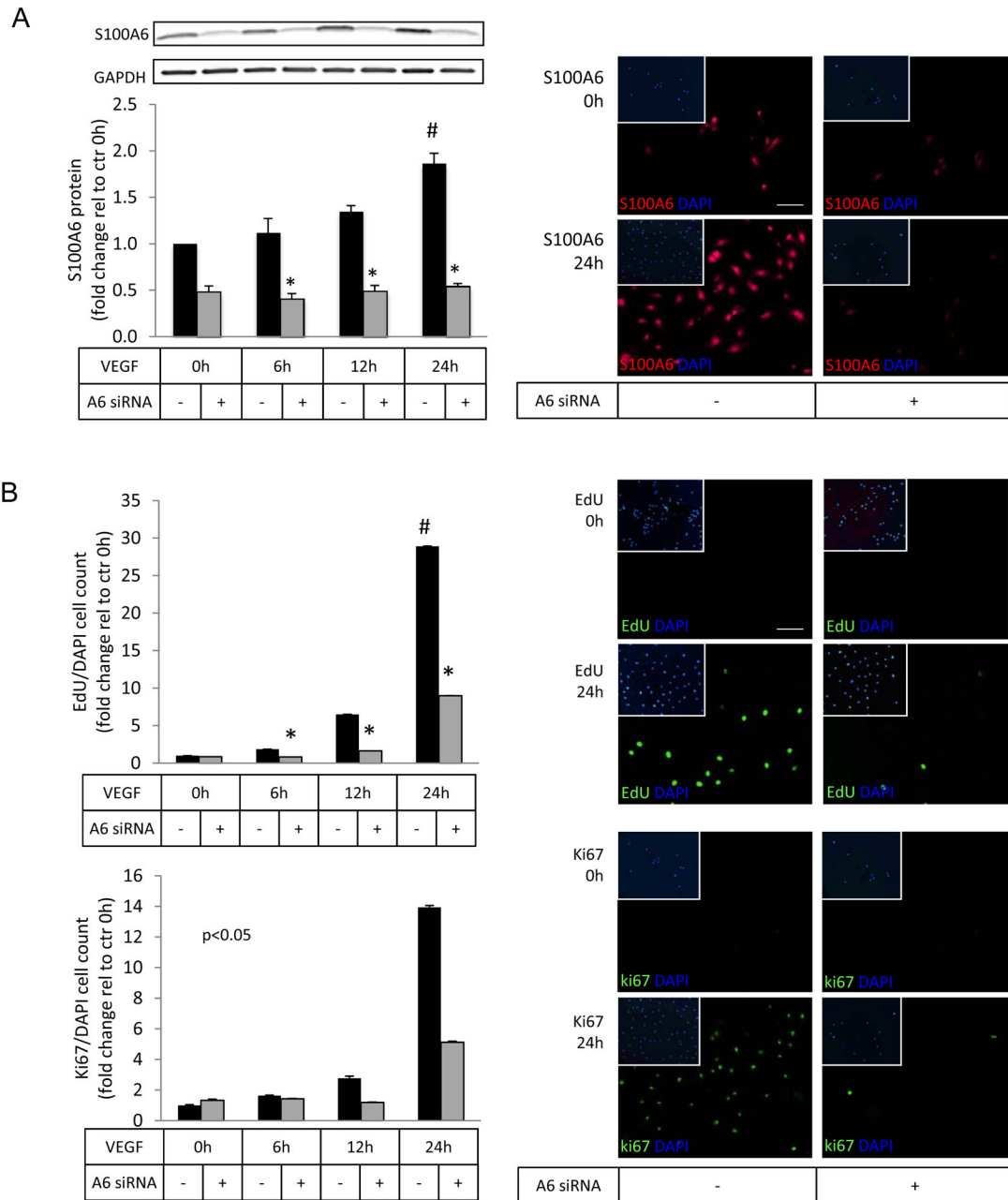
S100A6 is upregulated during the vascular remodeling process after endothelial cell injury and upon growth factor stimulation in endothelial cells

A Representative immunofluorescence (IF) stainings of S100A6 localization in porcine coronary arteries after percutaneous coronary intervention. Upper panel: no stent implantation control, right coronary artery (RCA); center panel: 15 days post stent implantation, left anterior descending coronary artery (LAD); lower panel: 30 days post

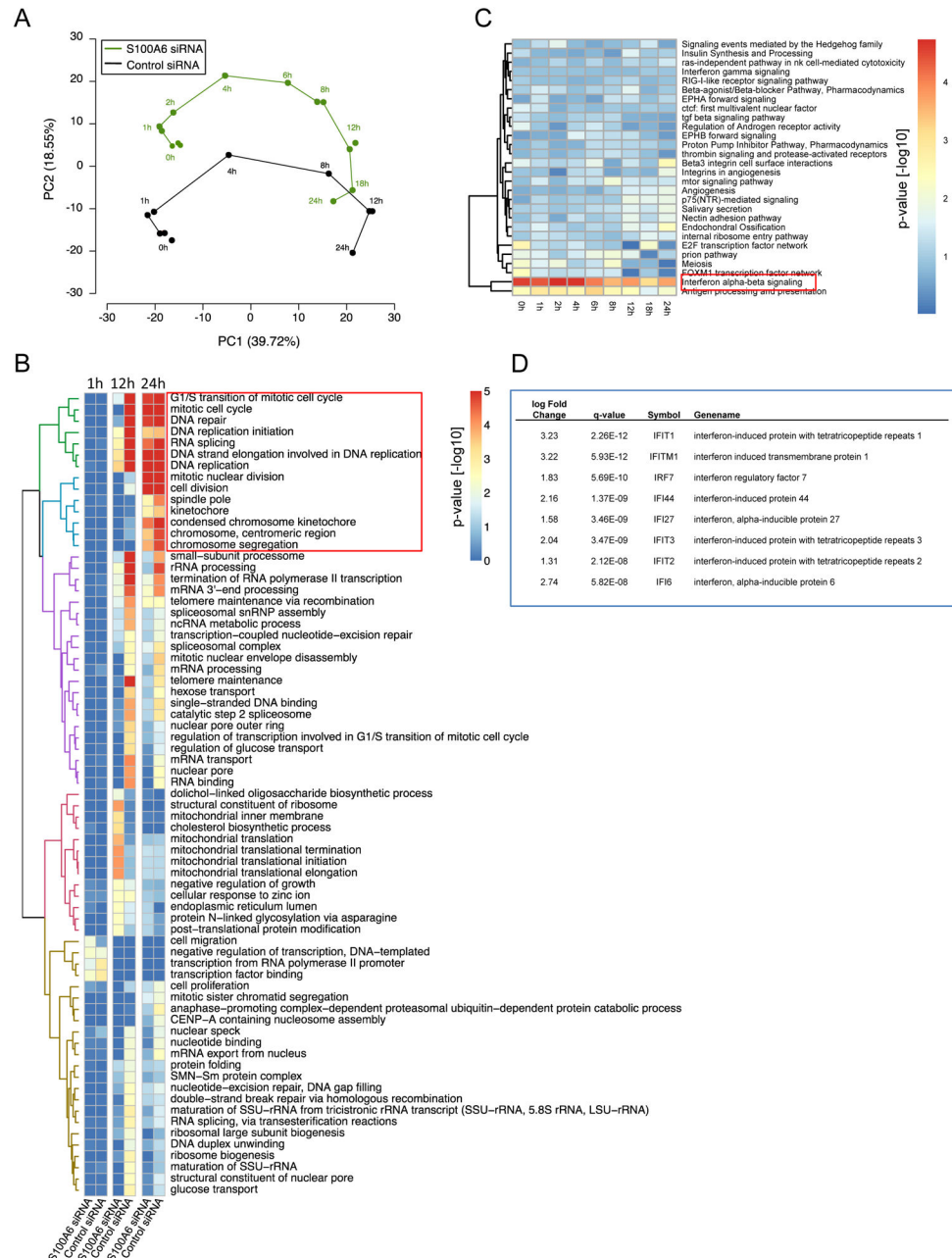
stent implantation, LAD. (63x magnification, scale bar 10µm, green = S100A6, red = CD31, blue = DAPI).

B Immunoblot showing elevated S100A6 expression in HUVEC after VEGF-A stimulation for indicated time points (n=6 individual experiments, each performed in duplicate; \*p <0.05 vs 0h time point/no stimulation, Friedman test with subsequent Dunn's correction for multiple comparisons).

C Representative IF stainings of upregulated S100A6 expression in HUVECs after 6h, 12h and 24h of VEGF-A stimulation (20x magnification, scale bar 40µm, red = S100A6, blue = nuclear DAPI).



B Left: S100A6 knockdown and control HUVECs were stimulated with VEGF-A for 24h and cell proliferation was measured as 1) (upper panel) EdU positive cells versus the total number of cells, i.e. nuclear DAPI stained cells (n=5 individual experiments; \*p<0.05 vs corresponding control siRNA treated cells, #p<0.05 vs control siRNA treated cells at 0h, Friedman test for repeated measures and non-parametric exact test with subsequent correction for individual time point comparisons) and 2) (lower panel) by counting Ki67 positive cells versus the total number of cells (n=4 individual experiments; \*p<0.013 S100A6 vs control siRNA treated cells, Friedman test for repeated measures). Right: Representative IF images of VEGF-A stimulated S100A6 knockdown and control HUVECs after EdU detection (upper panels) and Ki67 staining (lower panels) (20x magnification, scale bar 40µm, green = EdU/Ki67, blue = nuclear DAPI).



**Figure 3.**

Transcriptome time series analysis reveals antiproliferative signaling in S100A6 depleted human endothelial cells

A Principal component analysis of the transcriptome time series (from control and S100A6 siRNA treated HUVECs after a time course of VEGF-A stimulation for indicated time points). Axes depict the first two principal components, accounting for 39.72% and 18.55% of the total variance.

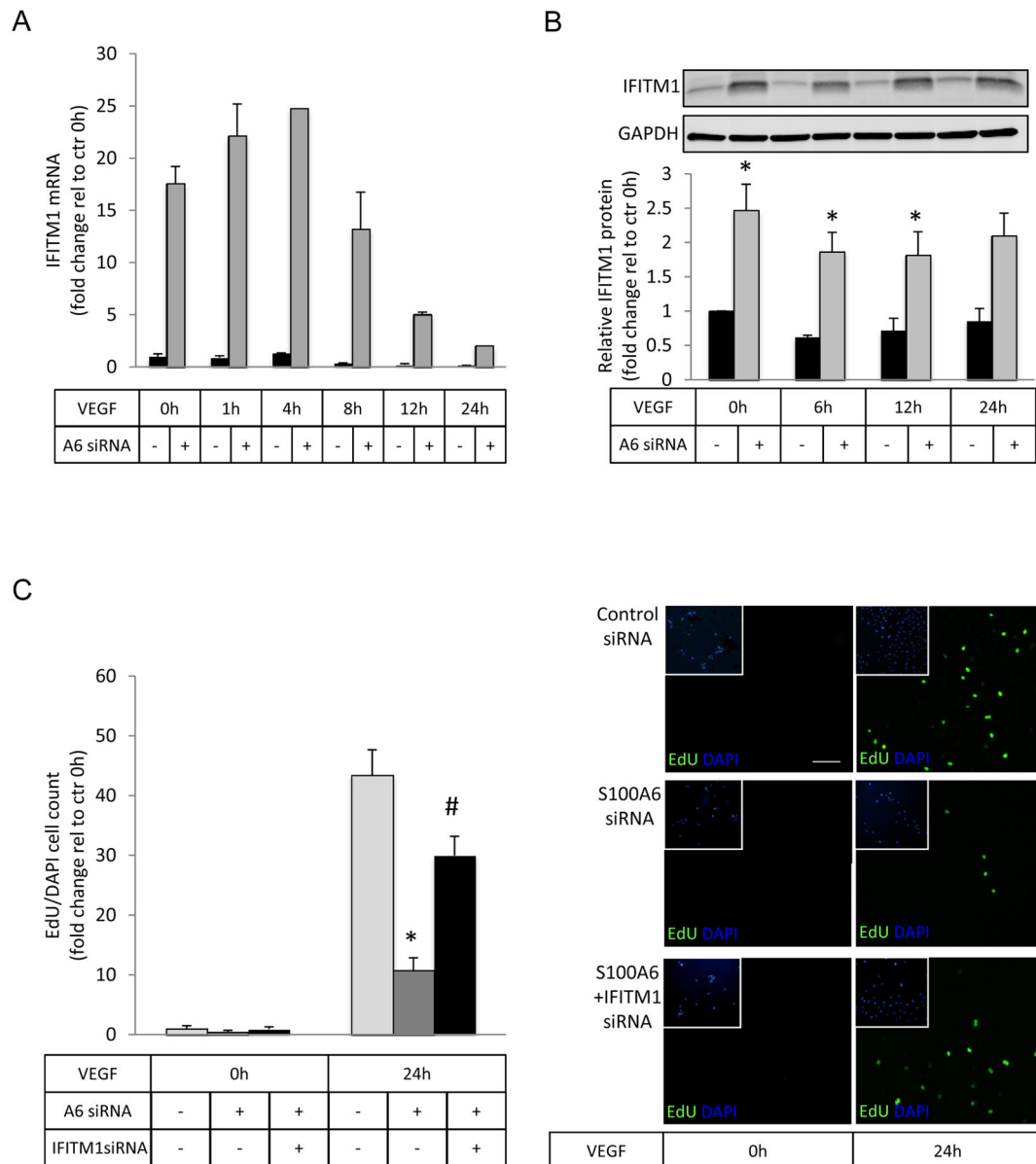
B Gene set enrichment analysis (GSEA) of knockdown and control samples at 1, 12 and 24h relative to their respective pre-stimulus controls at 0h. The heatmap represents gene sets



having a ( $-\log_{10}$ -transformed) p-value $>2$  in at least one time point and condition. Rows were hierarchically clustered using complete linkage for agglomeration based on the Euclidean distance of the fold change values. Colors mark the five largest clusters. Red box indicates gene sets related to cell cell cycle, division and DNA repair.

C Paired GSEA of S100A6 knockdown versus control siRNA HUVECs over time using the Consensus Pathway Database. Heatmap depicts the ( $-\log_{10}$  transformed) p-value significance of gene set upregulation relative to control samples for each time point. Rows were hierarchically clustered as in B.

D Table showing the most regulated interferon-inducible (IFI) genes and their significance level upon S100A6 knockdown when compared to siRNA control treated cells at time point 0h of the transcriptome time series analysis (q-value was calculated from a moderated t-test with false discovery rate (FDR) correction using the R limma package).

**Figure 4.**

Interferon-inducible transmembrane protein 1 (IFITM1) is among the most regulated genes upon S100A6 knockdown and a key regulator of S100A6 cell signaling

A RT-qPCR analysis for IFITM1 from RNA samples used in the illumina beadarrays was carried out to confirm the results of the transcriptome time series analysis (error bars were drawn when replicates were available).

B Cell lysates from control and S100A6 siRNA treated HUVECs after a time course of VEGF-A stimulation were collected and immunoblotted for IFITM1 (n=6 individual; \*p<0.05 vs corresponding control siRNA treated cells, Friedman test with subsequent Dunn's correction for multiple comparisons).

C Left: S100A6, S100A6/IFITM1 knockdown and control HUVECs were stimulated with VEGF-A for 24h and EdU incorporation was measured as EdU positive cells vs the total

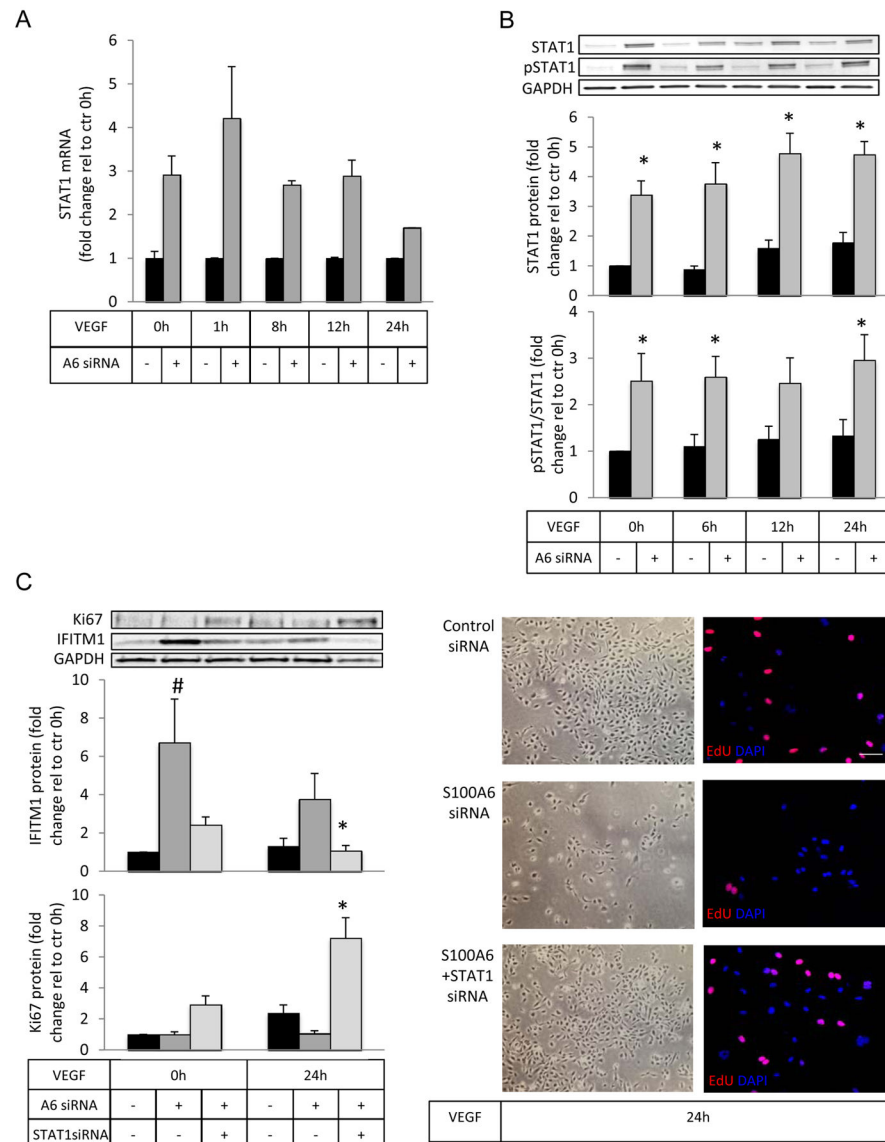
number of cells (i.e. nuclear DAPI stained cells) (n=6 individual experiments; \*p<0.05 vs corresponding control siRNA treated cells, #p=0.062 vs S100A6 siRNA treated cells with 24h of VEGF-A stimulation, Friedman test for repeated measures, Wilcoxon signed rank test with subsequent correction for individual time point comparisons). Right: Representative IF images of serum/growth factor starved and VEGF-A stimulated control, S100A6 and S100A6/IFITM1 knockdown HUVECs after EdU detection (20x magnification, scale bar 40µm, green = EdU, blue = nuclear DAPI).

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**Figure 5.**

Elevated IFITM1 expression and antiproliferative phenotype in S100A6 depleted endothelial cells is caused by STAT1 activation

A RT-qPCR analysis for STAT1 from RNA samples used in the gene array was carried out to confirm the results of the transcriptome time series analysis (error bars were drawn when replicates were available).

B Cell lysates from control and S100A6 siRNA treated HUVECs after a time course of VEGF-A stimulation were collected and immunoblotted for STAT1 and Tyr-701 phosphorylated STAT1 (n=6 individual experiments; \*p<0.05 vs corresponding control siRNA treated cells, Friedman test and subsequent Dunn's correction for multiple comparisons).

C Left: Cell lysates from S100A6, S100A6/STAT1 knockdown and control HUVECs were collected after over night starvation and following VEGF-A stimulation and were

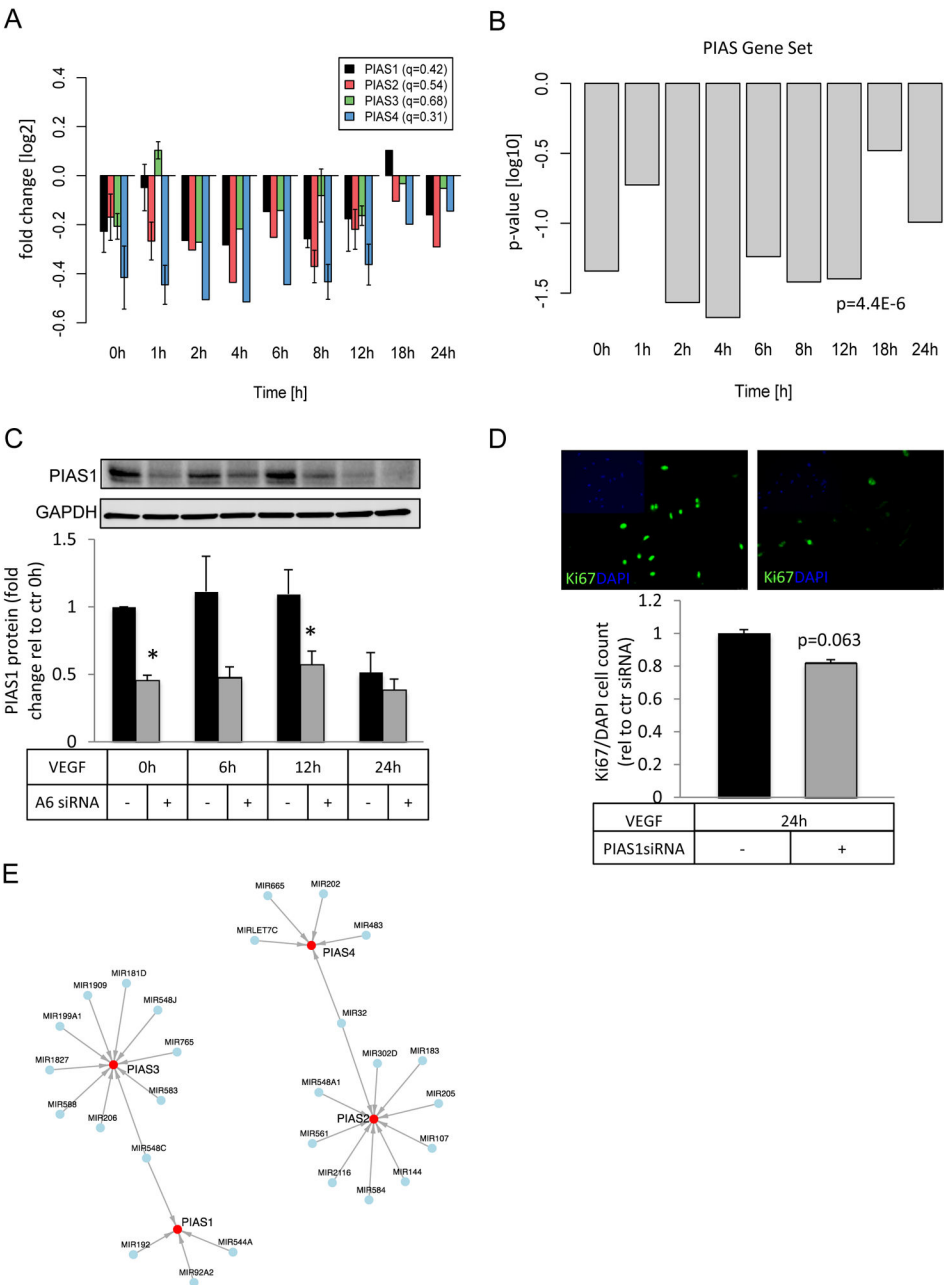
immunoblotted for Ki67 (n=6 individual experiments) and IFITM1 (n=4 individual experiments for 0h time point, n=6 individual experiments for 24h time point; #p<0.05 vs corresponding control siRNA treated cells, \*p<0.05 vs S100A6 siRNA treated cells at 24h, Friedman-Dunn). Right: Representative cell culture images from S100A6, S100A6/STAT1 knockdown and control HUVECs 24h after VEGF-A stimulation (4x magnification), and representative IF images from the corresponding experiment's EdU incorporation (20x magnification, scale bar 50µm, red = EdU, blue = nuclear DAPI).

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**Figure 6.**  
S100A6 regulates STAT1 activity in endothelial cells through PIAS modulation  
A Transcriptome time series was analyzed for PIAS1-4 expression (PIAS mRNA expression represented as log<sub>2</sub> fold difference over time in S100A6 knockdown cells relative to the respective controls (error bars were drawn when replicates were available and represent  $\pm$  standard deviation; q-values (in figure legend) denote the FDR corrected significance of differential regulation over time).  
B Barplot of the significance of combined PIAS1-4 regulation in S100A6 depleted cells pairwise compared to the respective control over time using GSEA (y-axis represents log<sub>10</sub>

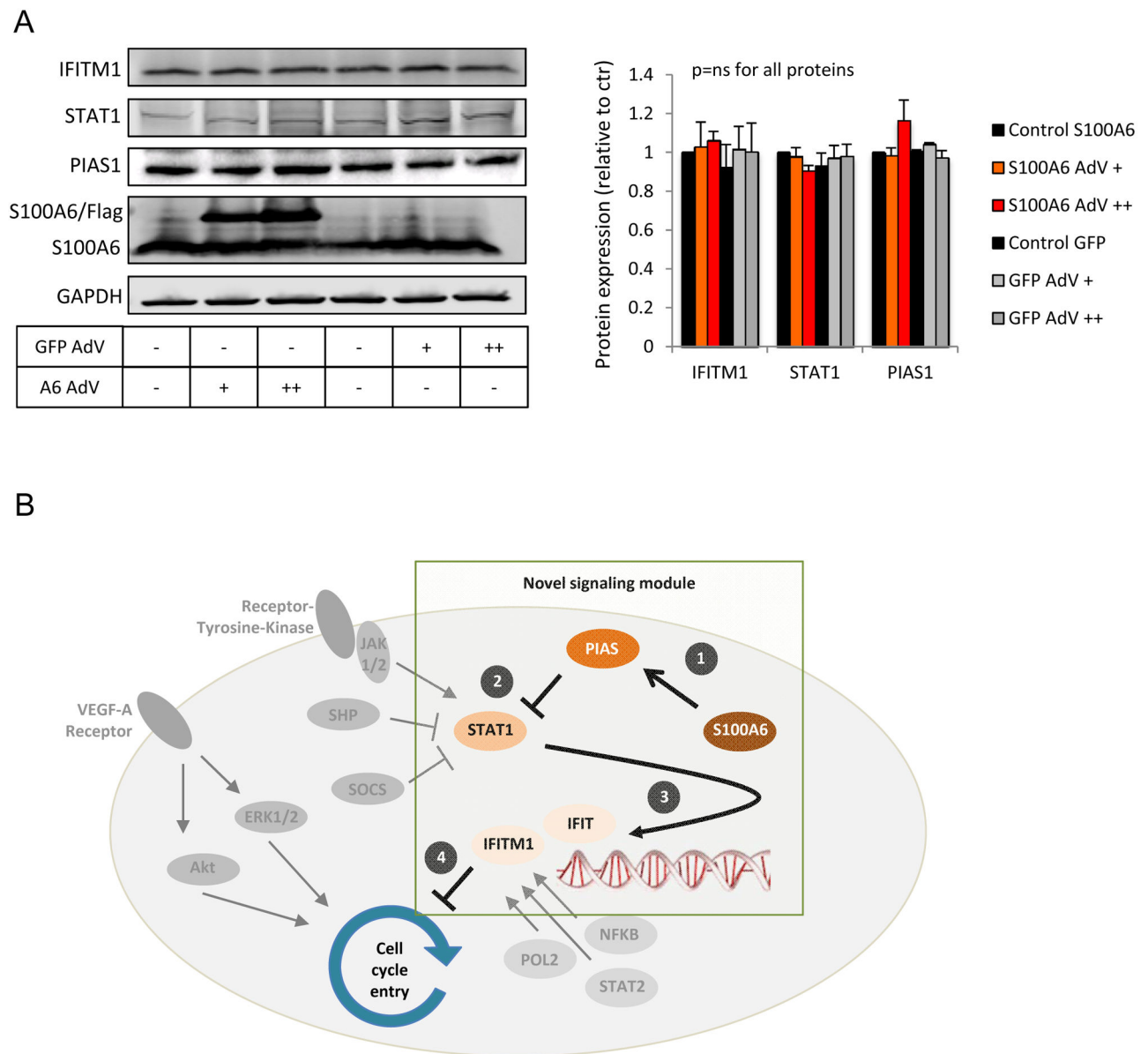


transformed p-value significance; overall significance of the PIAS gene set down-regulation for the time course is  $p=4.4E-6$ ).

C Cell lysates from control and S100A6 siRNA treated HUVECs after VEGF-A stimulation for indicated time points were collected and immunoblotted for PIAS1 protein (n=6 individual experiments;  $*p<0.05$  vs corresponding control siRNA treated cells, Friedman test with subsequent Dunn's correction for multiple comparisons).

D Synchronized PIAS1 knockdown and control HUVECs were stimulated with VEGF-A for 24h and cell proliferation was measured by counting Ki67 positive cells over the total number of cells (n=4 individual experiments;  $p=0.063$  vs control siRNA treated cells, Wilcoxon signed rank test).

E miRNA target network for PIAS1-4 genes. Predicted miRNA-mRNA interactions have been extracted from different miRNA databases (edges were drawn, if at least 2 out of 8 databases predicted an interaction and if the miRNA-mRNA expression values had a significant anti-correlation ( $p<0.2$ )).

**Figure 7.**

Novel signaling module linking S100A6 to control of STAT1 signaling

A Cell lysates from S100A6 Adenovirus (FLAG tagged) and control GFP Adenovirus infected HUVECs (48h) were collected and immunoblotted for IFITM1, PIAS1, STAT1, S100A6 and FLAG (n=3 individual experiments).

B The schematic highlights the molecular control of anti-proliferative STAT1 signaling in ECs via S100A6. Our findings indicate that S100A6 might constitutively facilitate activity of the STAT1 suppressor PIAS (1). Loss of S100A6 expression subsequently entails STAT1 activation via loss of PIAS activity independent of JAK1/2, SHP and SOCS signaling (2). STAT1 in turn activates a set of anti-proliferative genes including IFITM1 besides others (3) that oppose cell cycle entry in part most likely via previously described p53/p21 activation. Of note, VEGF-A mediated proliferative signaling pathways such as ERK1/2 and AKT are

not affected by anti-proliferative STAT1 signaling indicating downstream competitive integration of pro- and anti-proliferative signals for cell cycle decision entry. Colored elements highlight *in silico* predicted and experimentally validated components of the novel S100A6/STAT1/IFITM1 signaling module. Grey elements are not affected by S100A6 signaling as predicted by *in silico* modeling and experimental validation. In summary, S100A6 might be indispensable for timely cell cycle entry by attenuating anti-proliferative STAT1 signaling.

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