The Interferon Stimulated Gene 12 Inactivates Vasculoprotective Functions of NR4A Nuclear Receptors

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Rationale: Innate and adaptive immune responses alter numerous homeostatic processes that are controlled by nuclear hormone receptors. NR4A1 is a nuclear receptor that is induced in vascular pathologies, where it mediates protection.

Objective: The underlying mechanisms that regulate the activity of NR4A1 during vascular injury are not clear.

We therefore searched for modulators of NR4A1 function that are present during vascular inflammation.

Methods and Results: We report that the protein encoded by interferon stimulated gene 12 (ISG12), is a novel interaction partner of NR4A1 that inhibits the transcriptional activities of NR4A1 by mediating its Crm1-dependent nuclear export. Using 2 models of vascular injury, we show that ISG12-deficient mice are protected from neointima formation. This effect is dependent on the presence of NR4A1, as mice deficient for both ISG12 and NR4A1 exhibit neointima formation similar to wild-type mice.

Conclusions: These findings identify a previously unrecognized feedback loop activated by interferons that inhibits the vasculoprotective functions of NR4A nuclear receptors, providing a potential new therapeutic target for interferon-driven pathologies. (Circ Res. 2012;110:00-00.)

Key Words: vascular injury ▪ NR4A1 ▪ inflammation ▪ restenosis

Members of the nuclear receptor superfamily of transcription factors control diverse aspects of development, homeostasis and immunity.¹ This diversity of function is exemplified by the NR4A1 subfamily, consisting of NR4A1, NR4A2, and NR4A3. NR4A2 is required for the development of dopaminergic neurons,² and all 3 NR4A proteins regulate aspects of myeloid cell differentiation.³ Expression levels of NR4A proteins are regulated in muscle and liver by diet and NR4A proteins influence hepatic glucose metabolism.⁹ NR4A proteins are also highly inducible in macrophages and other cells types involved in inflammatory responses,⁵,⁶,¹⁰–¹³ suggesting roles in immunity. For example, a recent study identified a critical role for NR4A1 in the differentiation and survival of Ly6C⁻ monocytes.⁷

Endogenous ligands for members of the NR4A subfamily have not been identified, and the transcriptional activities of all 3 family members are thought to be mainly dependent on their expression levels. In addition, corepressors,¹⁴ coactivators,¹⁵–¹⁹ and posttranslational modifications²⁰–²² modulate the transcriptional activities of NR4A family members. Moreover, specific compounds have recently been identified that can function as transactivators or putative ligands for NR4A nuclear receptors, respectively.¹⁹,²²–²⁵

We and others have shown that NR4A1 (Nur77, Nak-1, TR3, NGFI-B) is upregulated in the inflamed vasculature, specifically in endothelial cells, smooth muscle cells, and monocytes.³,¹⁰–¹²,²⁶ In mice transgenic for a dominant negative form of NR4A1, a significantly higher degree of restenosis has been found in response to carotid artery ligation, indicating a protective role of NR4A1 in this specific model.²⁷ In human macrophages, NR4A1 has been shown to reduce the expression of proinflammatory genes.³ In addition, beneficial anti-inflammatory functions of another NR4A family member, NR4A2, have been demonstrated in micro-

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glia cells and astrocytes, in which NR4A2 was shown to recruit the CoREST corepressor complex, resulting in the clearance of NF-κB p65 and in transcriptional repression of NF-κB target genes.5

On the other hand, others have reported that NR4A1 is upregulated by inflammatory stimuli in a mouse macrophage cell line12 and that its overexpression induces the expression of inflammatory response genes.13 Therefore, while there is agreement that NR4A1 is upregulated in vascular pathologies,10–12,27 there are conflicting data and hypotheses on its functional role in inflammatory responses.3,13,27,28 One possible explanation for these observed discrepancies in the function of NR4A1 may be found in the modulatory activity of as yet unidentified factors. We therefore searched for possible modulators of NR4A1 activity that may influence its specific roles in vascular pathologies.

We report the identification of interferon stimulated gene 12 (ISG12, IFI27) as a novel modulator of the activity of NR4A nuclear receptors. Under normal conditions, ISG12 is expressed at low levels in the inner nuclear envelope and is strongly upregulated in response to inflammatory stimuli. We further found that ISG12−/− mice are completely protected from restenosis in vascular injury models, and that this was dependent on the presence of NR4A1. We therefore conclude that ISG12 is upregulated in the vasculature in injury and contributes to vascular pathologies by affecting transcriptional activities of “protective” nuclear receptors, such as NR4A1. Thus, ISG12 represents a hitherto unidentified layer of regulation for the activity of NR4A nuclear receptors that is highly relevant to vascular diseases and may serve as a potential target for novel therapeutic strategies.

Methods
An expanded Methods section is available in the Data Supplement.

Cell Culture and Transfections
Human umbilical endothelial cells (HUVECs)11 and human umbilical artery smooth muscle cells (HUASMCs)29 were cultured as previously described and used until the 5th (HUVECs) or 6th (HUASMCs) passage, respectively. Expression of the ISG12 and NR4A1 did not change during passaging. U937 cells were cultured as described previously.30 Mouse microvascular endothelial cells (MVECs) and smooth muscle cells (MVSMCs) were obtained and cultured as described previously.31 Mouse embryonic fibroblasts (MEFs) from wild-type and ISG12 deficient mice were obtained, cultured, and transfected as described previously.32 HUVECs and MEFs were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. HUASMCs were transfected using SuperFect (QIAGEN, Venlo, The Netherlands) according to the manufacturer’s protocol. Human embryonic kidney cells (293 cells) were cultured as recommended (ATCC, Manassas, VA). 293 cells were transiently transfected for luciferase reporter assays, cell fractionation experiments, western blot or microscopy using calcium–phosphate.33 Organelle lights were transfected according to the manufacturer’s instructions (Invitrogen). For siRNA transfection, 293 cells were transfected with polyethyleneimine34 using siRNAs from Ambion (Austin, TX). Efficiency of silencing was evaluated by q-PCR or by Western blot. Transfection mixtures always contained the same amount of DNA, and for cotransfections all vectors were used in equal ratios. In titration experiments, differences in the vector amounts were substituted with corresponding empty vectors.

Plasmids
Human full length ISG12 (AA1–122) was cloned into pCMV-myc (Clontech, Palo Alto, CA). ISG12 promoter luciferase constructs (−923 to +30, −730 to +30, −397 to +30) were cloned into pGL3. Additional ISG12 promoter constructs (deleted or mutated) used in this study were generated using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Constructs for full length EGFP-NR4A1 (AA1–598), dominant negative EGFP-NR4A1 (AA248–580), the luciferase reporter construct NBRE 4×, and pcDNA 3.1-NR4A1 (AA 1–598) were previously described.11 The constructs pXP1-NurRE-luciferase, DR5-luciferase, pCMX-NR4A2, and pCMX-NR4A3 were kindly provided by J. Drouin.35 Flag-CRM1 was a kind gift from W. Wang.

Yeast Library Construction and Yeast-2-Hybrid Screen
In brief, the yeast-2-hybrid screen for interaction partners of NR4A1 was performed using a yeast library generated with cDNA obtained from lipopolysaccharide (LPS)/tumor necrosis factor (TNF)α-activated human uterine microvascular endothelial cells. Yeast library construction was performed using the MATCHMAKER Library Construction and Screening Kit (Clontech) according to the manufacturer’s recommendations. For screening, a construct of NR4A1 coding for amino acids 248 to 557 lacking the transactivation domains 1 and 2 was cloned into pGBKT7.

Immunoprecipitation, Western Blotting, and Chromatin Immunoprecipitation
Protein interactions in transfected 293 cells were determined by immunoprecipitation of transfected or endogenous NR4A1, Lamin A, or ISG12, followed by immunoblotting of coprecipitated interaction partners. Chromatin immunoprecipitation (ChIP) was performed on 293 cells stimulated with or without IFNαx, respectively, using the EZ ChipTM kit (Upstate Inc). Subsequent PCR analyses were performed by LightCycler technology using the Fast Start SYBR Green I kit (Roche Diagnostics).

Immunocytochemistry and Microscopy
Immunocytochemistry was performed on transfected or not transfected: HUVECs, HUASMCs, 293 cells, or MEFs according to procedures previously described.36 In addition, the subcellular localization of NR4A1 in arterial cross-sections was also assessed by immunohistochemistry.
Fluorescence Resonance Energy Transfer
For fluorescence resonance energy transfer (FRET) experiments, cells were cotransfected with myc-ISG12 and an EGFP-NR4A1 construct or with the respective controls. After 24 hours, cells were fixed and processed for immunocytochemical staining of the myc-ISG12 using anti-myc antibody and Alexa 568-conjugated anti-mouse. A modified version of a method previously reported by König et al was used, in which FRET was assessed between EGFP-labeled NR4A1 and the immune complex of Alexa 568-labeled anti-myc antibody and myc-tagged ISG12. Images for FRET were recorded on a confocal microscope (Zeiss LSM510) or an epifluorescence microscope (Olympus AX-70) and analyzed using ImageJ software with the FRET and colocalization-analyzer plugin.

Isolation of Nuclear Fractions and Fractionation of Nuclear Envelopes
Isolation of nuclear extracts was performed as previously described. The presence of NR4A1 and histone H3 was determined in lysed nuclear fractions by immunoblotting and quantified using the Image J software. Additional isolation of nuclei for further lysis of nuclear fractions by immunoblotting and quantified using the ImageJ software with the FRET and colocalization-analyzer plugin.

Luciferase Assays
Cells (n=293) were transfected as outlined in the Figure and cotransfected with pRL-SV40 renilla vector internal transfection efficiency standard. Luciferase and renilla assays were performed 30 hours after transfection using a Dual Luciferase Assay Kit (Promega, Madison, WI) according to the manufacturer’s instructions. Luciferase activity was normalized to respective renilla values. All experiments were done in triplicates and repeated at least twice.

RNA Isolation and Quantitative RT-PCR
RNA isolated from pooled mouse arterial tissue (n=9) or from HUVECs, HUASMCs, U937 cells, MVSMCs, MVECs, and MEFs (n=3 each) was isolated using Trizol, reverse-transcribed, and analyzed by quantitative (Q)-PCR using LightCycler technology (Roche Diagnostics). Expression of target genes (ISG12, NR4A1, and MCP-1) was normalized to the expression of housekeeping gene hydroxymethylbilane synthase (PBGD) or cyclin B.

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from surrounding tissue and a nonconstrictive polyethylene cuff (PE50, 2.0 mm in length with a 0.965-mm outer and a 0.58-mm inner diameter; Becton Dickinson, San Jose, CA) was placed loosely around the femoral artery. After twenty-eight days after cuff placement, mice were anesthetized and subsequently perfused with PBS via the heart and both femoral arteries were obtained. The respective numbers of mice used are indicated in the respective figure legends.

Morphometric Analysis
Carotid and femoral arteries were fixed with 4% paraformaldehyde, snap-frozen with OCT media, and serial sections (6 μm) were obtained either from the site of ligation to the aortic arch or through the entire length of the cuffed femoral artery. Equivalent areas of control carotid or femoral arteries were analyzed in a parallel. Sections were stained with hematoxylin/eosin or stained for smooth muscle actin using a specific antibody (clone 1A4-FITC, Sigma, St Louis, MO), and counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). For ligated carotid arteries, a standardized reference point was set at which the vessel structure was not distorted by the ligation and where the elastic laminae were intact. Digital images of cross-sections at 0.7 mm, 1.7 mm, and 2.7 mm from the reference point were obtained with an F-View camera on an Olympus AX-70 microscope and analyzed morphometrically using the AnalySIS software package (Soft Imaging System; Muenster, Germany). In the femoral cuff model, 8 equal cross sections were used from each mouse for morphometric analysis. The circumferences of the lumen, internal elastic lamina, and external elastic lamina were measured, and media area, neointimal area, and neointima/media ratios were calculated as described previously for a rabbit restenosis model.

Statistical Analysis
Experimental values are expressed as mean±SEM if not otherwise indicated. Statistical significances were determined by unpaired Student t test or 1-way ANOVA followed by Bonferroni multiple comparison tests, as indicated. The significance of differences in morphometric analyses in Figures 4 and 5 were determined using the nonparametric Kruskal-Wallis test followed by Dunn multiple comparison tests.

Results
The Interferon Stimulated Gene 12 (ISG12) Interacts With NR4A1
We and others could show that NR4A1 is highly induced by proinflammatory stimuli. Moreover, we have previously demonstrated that TNFα is a potent inducer of NR4A1 expression in endothelial cells, which resulted in increased PAI-1 expression. To identify binding partners of NR4A1 in vascular cells we screened a cDNA library generated from TNFα/LPS activated microvascular endothelial cells employing a yeast two hybrid system. Using a truncated NR4A1 (amino acids (AA) 248–557) as bait, we identified the interferon stimulated gene 12 (ISG12, GI: 55925613) as novel interaction partner of NR4A1. Direct interaction of NR4A1 with ISG12 was confirmed in yeast by “back-transformation” (Online Figure I, A). The interaction of NR4A1 and ISG12 in mammalian cells was shown by coimmunoprecipitation of NR4A1 and ISG12. Using an antibody against NR4A1 that precipitated endogenous (Figure 1A, row 2, lane 1) and overexpressed full-length NR4A1 (Figure 1A, row 2, lane 2) in human embryonic kidney 293 cells, we could show that it coprecipitated overexpressed myc-tagged ISG12 (Figure 1A, row 1, lanes 1 and 2). In a reverse coimmunoprecipitation using an anti-ISG12 antibody, endogenous (Figure 1A, row 4, lane 1) and overexpressed...
Figure 1. ISG12 interacts with NR4A1 and decreases its transcriptional activity. A, Coimmunoprecipitation of ISG12 and NR4A1 in 293 cells: 293 cells were transfected with myc-ISG12 and NR4A1, as indicated on the bottom of the figure. Cell lysates were subjected to immunoprecipitation using anti-NR4A1 (rows 1–2), anti-ISG12 (rows 3–4), or control IgG (rows 5–6), and the presence of myc ISG12 and NR4A1 was detected by immunoblotting using anti-myc (WB1) or anti-NR4A1 antibodies (WB2), respectively. Shown is a representative example of 3 independent experiments. WCL indicates whole-cell lysate.

B, FRET analysis of the interaction between myc-ISG12 and EGFP-NR4A1 in 293 cells transfected with myc-ISG12, EGFP-NR4A1 or EGFP. ISG12 was detected using an anti-myc antibody followed by an Alexa fluor 568–conjugated 2nd antibody, and FRET between EGFP and Alexa fluor 568 was assessed as described in Experimental Procedures. The FRET index indicates the average FRET values obtained in each image (mean ± SEM, n = 75 cells). The corresponding representative images show FRET-signals at sites of colocalization.

C, ISG12 downregulates transcriptional activity of NR4A1; 293 cells were transfected with quadruplicated (4×) NBRE luciferase reporter constructs and cotransfected with NR4A1 and/or myc-ISG12, as indicated. Transcriptional activity was assessed in a dual luciferase reporter assay. Data represent mean ± SEM of fold induction above control (n = 4).

D, Inhibition of NR4A1 transcriptional activity on NBRE depends on the amount of ISG12; 293 cells were transfected with NBRE luciferase reporter constructs and cotransfected with NR4A1 and/or myc-ISG12, as indicated. Transcriptional activity was assessed in a dual luciferase reporter assay. Data represent mean ± SEM of percent inhibition of NR4A1 transcriptional activity (n = 3).
NR4A1 (Figure 1A, row 4, lane 2) was precipitated by ISG12. Moreover, FRET analysis of the interaction between myc-ISG12 and NR4A1 demonstrated the specific interaction between these 2 molecules (Figure 1B). FRET image analysis further suggested colocalization of NR4A1 and ISG12 in the area of the nuclear envelope (Figure 1B, lower panel, blue images).

Although NR4A1 has many cellular functions, it mainly acts as transcriptional factor. Therefore, we wanted to see if the interaction with ISG12 might modulate its transcriptional activity. For this purpose we employed a luciferase reporter system consisting of a quadruplicated NGFI-B response element (NBRE) with 1 binding site for monomeric NR4A1 and analyzed transcriptional activities of NR4A1 in the absence and presence of cotransfected myc-ISG12 (Figure 1C). Overexpression of NR4A1 increased the reporter gene activity more than 10-fold, and this was significantly reduced by \(\approx 50\%\) in the presence of coexpressed ISG12. This effect was directly dependent on the amount of ISG12 transfected as shown by the dose response of ISG12 inhibition (Figure 1D).

Furthermore, the fact that this inhibition reached saturation at a 1:20 ratio suggested that the ISG12-mediated effect is limited, possibly by components of the nuclear pore complex as previously hypothesized.

NR4A1 is a member of the NR4A family of nuclear receptors (NR4A1, NR4A2, and NR4A3) that are highly homologous in structure and function. Three different consensus binding sites exist for NR4A1 family members; NBRE represent binding sites for monomeric binding. Nurr responsive element (NurRE) represents binding sites for homodimeric and heterodimeric binding of all 3 members of the NR4A family, and DR5 is a binding site for NR4A1 or NR4A2 heterodimerized to the retinoic acid receptor RXR.35

To gain more insight into the functional consequences of the interaction between ISG12 and NR4A1, we analyzed transcriptional activities of all members of the NR4A family in the absence and presence of ISG12 using reporter assays for the 3 different consensus binding sites (Figure 1E through 1G). Overexpression of ISG12 significantly reduced transcriptional activities of NR4A1 and NR4A2 on all 3 different reporter constructs (NR4A1 and NR4A2 were downregulated by ISG12 on NBRE by 54.6±4.9% and 30.9±11.3%; on NurRE by 83.9±1.2% and 87.1±0.61%; and on DR5 by 63.5±1.8% and 54.5±3.3%, respectively). In contrast, ISG12 overexpression only downregulates NR4A3 activity on NurRE (by 39.1±4.8%) (Figure 1F), but not on NBRE (Figure 1E). As expected NR4A3, which does not form heterodimers with RXR, did not transactivate the DR5 reporter construct (Figure 1G). From these data, we conclude that ISG12 displays some selectivity within the NR4A family and predominantly downregulates the activities of NR4A1 and NR4A2.

To see whether the effect of ISG12 on transcriptional activity of NR4A1 is influenced by small coactivator molecules ("ligands") that have been shown to induce NR4A1 activity, we performed luciferase reporter assays in the absence or presence of 6-mercaptopurine or 9-cis-retinoic acid (9-cis-RA). The increased reporter activity induced by NR4A1 was significantly reduced in the presence of ISG12 independently of the presence of any of the added compounds (Figure 1H), suggesting this effect to independent of these "ligands."

Because the newly discovered activity of ISG12 was reminiscent of nuclear corepressors and most activity of corepressor complexes requires histone deacteylation, we tested whether ISG12 also mediates its effects via histone modification. We therefore studied if the inhibitory effect of ISG12 is still observed in the presence of the histone deacetylase (HDAC) inhibitor Trichostatine A. In luciferase reporter assays, Trichostatine A treatment did not influence the effect of ISG12 on NR4A1 transcriptional activity, whereas—as expected—it increased the activity of NR4A1 on NBRE when ISG12 was not cotransfected (Figure 1I). Thus, ISG12 mediates its inhibitory effects via a mechanism that is independent of HDAC activity.

The Inner Nuclear Envelope Protein ISG12 Decreases Transcriptional Activity of NR4A1 by Modulating Its Nucleo-Cytoplasmic Shuttling in a Crml-Dependent Manner

Because ISG12-mediated repression is independent of nuclear corepressor complexes that recruit HDAC and require HDAC activity, we wanted to determine the mechanisms by which ISG12 decreases NR4A1 transcriptional activity. Therefore, we first analyzed the intracellular distribution of ISG12. Previously, ISG12 was identified as a nuclear envelope protein, but recently 2 studies reported a mitochondrial localization of ISG12 in 3T3-L1 and fibrosarcoma cells. To identify the subcellular localization of ISG12 in our experimental settings, 293 cells were transfected with myc-ISG12 and its localization was assessed in association with fluorescent markers specific for different organelles (Organelle lights) by fluorescent microscopy (Online Figure I, B). ISG12 (red in rows 1 and 2, green in row 3) was found to be distributed in the nuclear membrane (green) and in the endoplasmic reticulum (green), and there was no colocalization with mitochondria (red). In addition, we could demonstrate the localization of ISG12 to the nuclear envelope and...
Figure 2. ISG12 mediates nuclear export of NR4A1 in Crm1-dependent fashion. A and B, Subcellular localization of endogenous NR4A1 in vascular cells. The localization of endogenous NR4A1 (green) was detected by confocal microscopy using anti-NR4A1 antibody and nuclear staining was done by 7-AAD (red) as described in Methods. Shown are representative images of 25 replicates. A, Localization of endogenous NR4A1 in HUASMCs. B, Localization of endogenous NR4A1 in HUVECs. C, Subcellular localization of NR4A1 in the presence or absence of cotransfected ISG12. HUVECs were cotransfected with the following constructs: myc-ISG12, EGFP-NR4A1, nuclear export deficient EGFP-dnNR4A1, as indicated and subcellular localization of NR4A1, and ISG12 was assessed by immunofluorescent microscopy. In parallel, experiment HUVECs were treated for 12 hours with the Crm1 inhibitor 4 ng/mL Leptomycine B (LMB). Panels on the right side indicate the percentage of total NR4A1 present in the nucleus under indicated conditions. Data show the mean ± SEM values of 500 cells from 3 independent experiments. D, ISG12 decreases the presence of NR4A1 in the nucleus; 293 cells were cotransfected with EGFP-NR4A1 and ISG12 as indicated. The amount of NR4A1 and Histone 3 (H3) was assessed in nuclear fractions by immunoblot analysis using anti-EGFP (WB1) and anti-H3 antibodies (WB2), respectively. The panel below shows the quantification of nuclear NR4A1 by densitometry. Data are mean ± SEM from 3 independent experiments.
endoplasmic reticulum in HUASMCs and HUVECs (Online Figure I, C through F). This data supported the data obtained in 293 cells. Further more we were able to confirm the nuclear localization of ISG12 by electron microscopy, which revealed a close association of ISG12 with nuclear pores (Online Figure I, G). Moreover, even after Triton X-100 preextraction of cultured cells, which has previously been shown to conserve only perinuclear proteins,48 ISG12 staining was preserved, further identifying it as a nuclear envelope protein (Online Figure I, H), as previously shown by Martensen.47 Cellular fractionation experiments of ISG12 transfected 293 cells further demonstrated the localization of ISG12 to the nuclear envelope (Online Figure II, A through E).

Because the majority of inner nuclear envelope proteins interacts with nuclear scaffold proteins such as lamins to tightly anchor the lamina to the inner nuclear membrane,49 we tested whether ISG12 had the ability to interact with lamins, such as Lamin A. Indeed, we could confirm the interaction between Lamin A and myc-ISG12 by coimmunoprecipitation using an anti–Lamin A antibody in lysates from 293 cells overexpressing ISG12 (Online Figure II, F, row 2, lane 2). Thus, we identified ISG12 as a novel interaction partner of NR4A1 and demonstrated its localization at the inner nuclear membrane, where it interacts with proteins of the lamin matrix, such as Lamin A. Therefore, we asked whether ISG12 mediates its effects by physically limiting the presence of NR4A1 in the nucleus. Under normal unstimulated conditions NR4A1 is predominantly localized to the nucleus of HUASMCs and HUVECs (Figure 2A and 2B). To answer whether the intracellular localization of NR4A1 is affected by ISG12, we studied the change in subcellular distribution of overexpressed EGFP-NR4A1 in the absence or presence of ISG12 by immunofluorescence cytochemistry. While a majority of transfected EGFP-tagged NR4A1 is found in the nucleus (Figure 2C, row 1, left panel), part of it is relocated to the cytoplasm when ISG12 is coexpressed (Figure 2C, row 1, middle and right panel). In fact, overexpression of ISG12 in HUVEC reduced the nuclear localization of transfected NR4A1 by ≈50% (Figure 2C, row 1, right graph). Importantly, when the same experiments were performed using an NR4A1 construct (AA 248–580) that cannot be exported from the nucleus,50 no influence of ISG12 on the subcellular distribution of that construct was seen (Figure 2C, row 2). This indicates that the ISG12 affects the nuclear/cytoplasmic distribution of NR4A1 by modulation of its nuclear export rather than having an effect on its nuclear import. Previously, it has been reported that NR4A1 export is Crm1 dependent. To analyze whether the ISG12-mediated export of NR4A1 is Crm1 dependent as well, we repeated the same experiments as shown in Figure 2C (row 2) in the presence of the Crm1 inhibitor Leptomycin B. Leptomycin B blocked nuclear export of NR4A1 in the absence as well as in the presence of ISG12, as in both instances nuclear localization of NR4A1 was >70% (Figure 2C, row 3, right graph). This indicates that the basal50,51 as well as ISG12 enhanced nuclear export of NR4A1 is Crm1 dependent. To quantify the effect of ISG12 on the cellular localization of NR4A1 using a different method, we determined the distribution of NR4A1 between the cytoplasm and the nucleus by analyzing nuclear fractions of cells in which EGFP-tagged NR4A1 was overexpressed in the absence or presence of myc-tagged ISG12 by Western blot (Figure 2D). In the presence of overexpressed ISG12, the amount of nuclear NR4A1 was decreased by 44.4±12.9% (Figure 2D, right graph), which compares well with the ≈50% reduction in nuclear localization as demonstrated by immunocytochemistry.

To further elucidate the mechanism by which ISG12 mediates the nuclear export of NR4A1 in Crm1-dependent manner, we performed coimmunoprecipitation experiments using cell lysates of 293 cells in which ISG12 was silenced and that were transfected with NR4A1 and/or flag-Crm1. As shown in Figure 2E, immunoprecipitation of endogenous and overexpressed NR4A1 results in the coprecipitation of Crm1. However, in cells in which ISG12 was silenced, the amount of Crm1 immunoprecipitated with NR4A1 was clearly reduced compared with cells transfected with scrambled siRNA. Thus, the formation of NR4A1/Crm1 complexes depends partially on the presence of ISG12.

Some investigators suggested that the nuclear export of NR4A1 is dependent on RXRα50–51 and its nuclear export sequence, whereas others have shown a reciprocal model in which RXRα export is dependent on NR4A1.52 To clarify this, we performed luciferase reporter assays for NR4A1 activity in the presence of overexpressed ISG12 in cells in which RXRα was silenced. The efficiency of the siRNA knockdown was confirmed by q-PCR and Western blot analysis (Online Figure II, G and H). These experiments demonstrated that RXRα had no effect on the capacity of ISG12 to downregulate NR4A1 transcriptional activity, indicating RXRα independence of this mechanism (Figure 2F).

Therefore, our findings identify ISG12 a novel interaction partner of NR4A1 that is localized at the inner nuclear envelope (Online Figures I and II, where it interacts with proteins of the nuclear lamina (Online Figure II, F) and decreases transcriptional activity by influencing the nuclear localization of the orphan nuclear receptor NR4A1 in a Crm1-dependent and RXR-independent manner (Figure 2E and 2F).

**ISG12 Is Induced by Interferons in Vascular Cells**

Because ISG12 was identified in a cDNA library of endothelial cells treated with TNFα and LPS, we first determined its expression in vascular cells in response to various inflamma-
Figure 3. Interferons induce ISG12 expression in vascular cells. A, Interferons induce ISG12 expression in HUVECs. HUVECs were treated for the indicated time with 100 U/mL TNFα, 10 μg/mL LPS, 1000 U/mL IFNα, or 100 ng/mL IFNγ, and the expression of ISG12 was analyzed by q-PCR. Data represent mean±SEM fold expression of ISG12 above control treated cells.

B, IFNα stimulates expression of ISG12 in vascular cells. Human aortic smooth muscle cells (HUASMCs) and the monocytic cell line U937 were stimulated with 1000 U/mL IFNα for 7.5 hours, and ISG12 expression was analyzed by q-PCR. Data represent mean±SEM fold expression of ISG12 above control treated cells.

C, Schematic map of the ISG12 promoter fragments as cloned into the pGL3 vector. Indicated are possible IREs that are binding sites for IRFs.

D, IFNα regulates ISG12 expression by the proximal promoter 150bp upstream of the transcription start site; 293 cells were transfected with ISG12 promoter fragments that differ in lengths and carry either mutations or deletions of the IRE core sequences, respectively. The 5′GAAA3′ core sequence was either mutated to 5′TAAT3′ indicated as m or entirely deleted indicated by d (see schematic representation). Subsequently, cells were treated with 1000 U/mL IFNα for 8 hours. Transcriptional activity was assessed with a dual luciferase reporter assay. Data represent mean±SEM of fold induction of IFNα stimulation normalized to respective unstimulated controls (n=3).

E, Interferon regulatory factor (IRF)-1, -3, and -4 bind to the ISG12 promoter on IFNα stimulation. 293 cells were treated with IFNα (1000 U/mL, 5 hours), and chromatin immunoprecipitation assays were performed using antibodies against IRF-1, IRF-3, and IRF-4 followed by q-PCR using primers specific for the ISG12 promoter.
tory stimuli. HUVECs were treated with cytokines and LPS, for various time points and analyzed by q-PCR. As shown in Figure 3A, the known potent ISG12 regulating cytokines interferon-alpha (IFNα) and interferon gamma (IFNγ) significantly upregulated ISG12 expression after 3.5 hours. We further analyzed the effect of IFNα on the expression of ISG12 in other vascular cells, and found that after 7.5 hours ISG12 was upregulated ~20-fold in vascular smooth muscle cells.

**Figure 4. ISG12 deficiency protects from neointima formation after vascular injury in mice.**

- **A.** Increased expression of ISG12, NR4A1, and MCP-1 in ligated carotid arteries of wild-type (wt) mice (n=9). Data represent mean±SEM fold increase of respective genes compared with control arteries. B, Ligation of the carotid artery does not result in neointima formation in ISG12 deficient mice. Representative images of carotid artery sections from wt and ISG12−/− mice, stained with hematoxylin/eosin or for smooth muscle actin, are shown. Quantification of the outer elastic lamina (EEL), the inner elastic lamina (IEL), the area of the neointima (NI), and the lumen area in ligated carotid arteries from wt (n=7) and ISG12−/− (n=7) mice. C, Cuffing of the femoral artery fails to induce neointima formation in ISG12−/− mice. Quantification of neointima (NI) area in cuffed femoral arteries of wt (n=4) and ISG12−/− (n=4) mice. D, IFNα induces nuclear export of NR4A1 in an ISG12-dependent manner. Mouse embryonic fibroblasts (MEFs) from wt and ISG12−/− were transfected with EGFP-NR4A1 and treated with IFNα (1000 U/mL for 5 hours) as indicated. The localization of EGFP-NR4A1 (green) was assessed by immunofluorescence microscopy. Nuclei are stained with PI (red). The graph (right panel) indicates mean±SEM percentage of nuclear localization of NR4A1 (n=500).

**Figure 3 (Continued).** Shown are representative examples of images from agarose gels of q-PCR products from 2 independent experiments. F, IFNα-mediated effects on NR4A1 transcriptional activity are dependent on ISG12. 293 cells were transfected with siISG12 or scrambled control, and cotransfected with a 4×NRE and/or NR4A1 and/or ISG12, as indicated. Subsequently, cells were stimulated with 1000 U/mL IFNα for 16 hours, and transcriptional activity was assessed in a dual luciferase reporter assay. Data represent mean±SEM of fold induction above control (n=3).
Figure 5. The vasculoprotective effect of ISG12 deficiency depends on NR4A1. A. The nuclear localization of NR4A1 is increased in the vessel walls of ligated carotid arteries of ISG12−/− mice. Sections of ligated carotid arteries of ISG12−/− and wt mice were stained with anti-NR4A1 antibodies (green) and nuclei were counterstained with propidium iodide (PI) (red). The images on the left show the bordered area that is enlarged in the images on the right. The yellow color indicates localization of NR4A1 in the nucleus. The quantification is shown in the graph. Data indicated are given as box plots of the percentage of NR4A1 that colocalize with the nuclear stain with anti-NR4A1 antibodies (green) and nuclei were counterstained with propidium iodide (PI) (red). The images on the left show the bordered area that is enlarged in the images on the right. The yellow color indicates localization of NR4A1 in the nucleus. The quantification is shown in the graph. B. Neointima formation was assessed by carotid artery ligation in wt, ISG12−/−, NR4A1−/−, and NR4A1−/−×ISG12−/− mice (7 mice per group). Representative images of control and ligated carotid arteries of ISG12−/−, NR4A1−/−, and NR4A1−/−×ISG12−/− mice stained with hematoxylin/eosin. Quantification of the intima/media ratio in ligated carotid arteries from wt, ISG12−/−, NR4A1−/−, and NR4A1−/−×ISG12−/− mice.

Because we have shown that IFNα is the major inducer of ISG12 (Figure 3A-B and Online Figure III, A through D), we tested if endogenous ISG12 induced by IFNα would also decrease NR4A1 transcriptional activity. Therefore, we performed NBRE luciferase reporter assays following stimulation with IFNα in cells, in which ISG12 expression was silenced by siRNA. The efficiency of the siRNA knockdown was confirmed by q-PCR (Online Figure III, E). As predicted, IFNα stimulation decreased the transcriptional activity of NR4A1 in cells transfected with scrambled siRNA, while this effect was lost when IFNα was induced by IFNα in cells, in which ISG12 expression was silenced by siRNA. The efficiency of the siRNA knockdown was confirmed by q-PCR (Online Figure III, E). As predicted, IFNα stimulation decreased the transcriptional activity of NR4A1 in cells transfected with scrambled siRNA, while this effect was lost when ISG12 was silenced (Figure 3F). In addition, IFNα decreased the transcriptional activity on the NR4A1 response elements, NurRE and DR5 (Online Figure III, G and H). Thus, IFNα decreases NR4A1 transcriptional activity and this effect is mediated through the presence of ISG12.

Furthermore, IFNγ, which activates ISG12 as potently as IFNα, was also found to downregulate the transcriptional activity of NR4A1 (Online Figure III, F).

Generation of ISG12-Deficient Mice
To obtain insight into the function of ISG12 in vivo, we generated ISG12−/− mice by targeted gene disruption as outlined in Online Figure IV, A. Successful gene deletion...
was confirmed by Southern blot analyses as well as by PCR for ISG12 in the spleens isolated from these mice (Online Figure IV, B and C). Male and female ISG12/H11002/H11002 mice exhibited no obvious gross phenotypic pathologies, having normal growth rates, survival, fertility, and litter sizes (data not shown). Routine histological analysis did not reveal any signs of organ pathology.

ISG12 Deficiency Protects From Vascular Injury In Vivo

To study the significance of ISG12 in vascular pathology, we tested its role in vascular injury using a carotid artery ligation model and a femoral artery cuff model. ISG12 is also potently induced by IFNα in both murine SMC and ECs (Online Figure III, C and D). We first determined the expression of ISG12 in response to carotid artery ligation in wild-type mice. Three weeks after ligation the expression of ISG12 was highly upregulated in the ligated arteries when compared with the contralateral unligated arteries (Figure 4A). Similarly, NR4A1, which has been shown to mediate protection in the same model, as well as the inflammatory marker gene monocyte chemoattractant protein-1 (MCP-1), which has been implicated in restenosis, were also found significantly upregulated in ligated carotid arteries of wild-type mice (Figure 4A). In ligated arteries of ISG12/H11002/H11002 mice, there was a similar upregulation of NR4A1 resulting in comparable levels as in wild-type mice, while upregulation of MCP-1 was absent, indicating a reduced inflammatory response (data not shown). We then used the carotid artery ligation model to determine the effect of ISG12 gene inactivation on neointima formation. As shown in Figure 4B, ligated carotid arteries of ISG12/H11002/H11002 mice retained a significantly larger lumen area than wild-type litter mates indicating a critical role for ISG12 in the vascular response to injury. In fact, ISG12/H11002/H11002 mice exhibited only negligible neointima formation as compared with wild-type mice while the vessel diameter (circumference of the external elastic lamina, EEL) was unaffected. Moreover, we observed no morphological differences between wild-type and ISG12/H11002/H11002 mice in unligated control carotid arteries, indicating that the absence of ISG12 does not influence vascular morphology under basal conditions where ISG12 is expressed only at low levels. In vascular injury (ligation), however, ISG12 is highly upregulated (Figure 4A) and is associated with increased neointima formation (Figure 4B). To demonstrate that the beneficial effect of ISG12 deficiency is not restricted to the specific model used, we employed the femoral artery cuff model (Figure 4C). Also in this model neointima formation in the ISG12/H11002/H11002 mice was less than 20% of that in wild-type mice. Based on this protective effect of ISG12 deficiency, our results suggest that ISG12 promotes neointima formation in response to vascular injury.

Based on our insights gained above, we hypothesized that the protective mechanism of ISG12 deficiency is mediated by
the transcriptional activity of NR4A1. Indeed, using MEFs transected with EGFP-NR4A1, we demonstrated by immunochemistry that IFNα stimulates the nuclear export of NR4A1 in wild-type but not ISG12−/− cells (Figure 4D). In fact, IFNα treatment reduced nuclear localization of NR4A1 significantly by ~40% only in wild-type but not in ISG12−/− MEFs. Moreover, we analyzed the presence of NR4A1 in the nuclei of cells in the vascular wall of ligated and nonligated carotid arteries of wild-type and ISG12 deficient mice 10 days after ligation (Figure 5A). In cells of the vascular wall of ligated arteries of wild-type mice only 5.6±0.5% of NR4A1 was located in the nuclei, whereas in cells of ISG12−/− mice, 10.0±0.7% of total NR4A1 was found in the nucleus (Figure 5A). In contrast, in control arteries no difference in the nuclear localization of NR4A1 was found between wild-type (5.9±0.7%) and ISG12−/− (6.5±0.5%) mice, indicating that only on injury when ISG12 is upregulated, the relative distribution of NR4A1 within the cell is affected. Thus, ISG12 mediates nuclear export of NR4A1, but only when induced by appropriate stimuli, such as vascular injury or IFNα.

To finally prove the protective effect of ISG12 deficiency is really dependent on the presence of NR4A1, we generated ISG12×NR4A1 double knockout mice by intercrossing ISG12−/− mice with NR4A1−/− mice. We then subjected the resulting double-deficient mice and indicated littermate controls to carotid artery ligation. As expected, ISG12 deficiency was protective against restenosis also in these mice, confirming our previous results. No additional increase of restenosis was observed in NR4A1−/− mice compared with wild-type mice, probably because maximum restenosis was already achieved in wild-type mice in this experimental set up. Importantly, ISG12×NR4A1 double-deficient littermates exhibited substantial restenosis similar to wild-type littermates (Figure 5B). Therefore, additional NR4A1 deficiency reversed the beneficial effect of ISG12 deficiency, indicating that the protective character of ISG12 deficiency is dependent on the presence of NR4A1.

Discussion

The vascular response to injury is a process that is involved in many vascular pathologies including atherosclerosis and restenosis. We here describe ISG12 as a novel modulator of the vascular response to injury. ISG12 is a member of a family of small interferon stimulated genes that are upregulated in cells on stimulation with interferon, but its function was so far unknown. In this report, we describe a molecular function for this gene and its role in vascular pathology.

We observed that ISG12 is upregulated at the site of vascular injury and influences activities of nuclear receptors including NR4A1 and NR4A2, thereby reducing their beneficial effects. This could explain why the naturally occurring upregulation of NR4A1 at sites of vascular injury cannot fully display its previously reported beneficial activities on restenosis. When ISG12−/− mice were analyzed for their susceptibility to develop vascular pathologies, we found that ISG12−/− mice were resistant to neointima formation after carotid artery ligation and in the femoral artery cuff model. However, when ISG12×NR4A1 double-deficient mice underwent carotid artery ligation, the degree of restenosis was not different from wild-type mice, indicating that the protective effect of ISG12 deficiency in carotid artery ligation is dependent on the presence of NR4A1. Although we cannot exclude a contribution of other members of the NR4A family, the major functional target for ISG12 in this model of restenosis is NR4A1, as the beneficial effect of ISG12 deficiency was completely reversed by additional deficiency of NR4A1.

In addition, we identified a molecular mechanism by which ISG12 influences NR4A1 transcriptional activity (Figure 6). We demonstrate that ISG12, localized in the inner nuclear membrane, promotes the nuclear export of NR4A1, thereby decreasing its nuclear localization and capability to transactivate. Thus ISG12 serves as a docking site which sequesters NR4A1 in the nuclear envelope. This interaction may result in a conformational change of NR4A1 that then exposes its nuclear export sequences (NES) on the surface of the molecule. Exposed NES recruit Crm1, which detaches itself and mediates the export of NR4A1 as a cargo to the cytoplasm through nuclear pores. Therefore inflammatory upregulation of ISG12 increases nuclear export of NR4A1, thereby decreasing its transcriptional activity. In this regard ISG12 represents a novel mechanism by which inflammation modulates the activities of nuclear receptors in vascular pathologies.

The recent findings that NR4A1 inhibits the development of atherosclerosis in mice points to another important vascular pathology that could be modulated by ISG12. Future experiments will aim at identifying the role of ISG12 in atherosclerosis and elucidate whether these effects are mediated through the ability of NR4A1 to control proinflammatory monocytes and/or macrophages, respectively.

In summary, our data indicate that ISG12 is a novel gene upregulated in the vasculature on injury, which contributes to vascular pathologies. This is mediated by increasing nuclear export of nuclear receptors, thereby downregulating their transcriptional activities. ISG12 therefore represents a target for novel therapeutic strategies for the treatment of vascular diseases.

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Disclosures

None.
References


What Is Known?

- Inflammatory processes modulate vascular response to injury, which is involved in vascular pathologies such as atherosclerosis and restenosis.

- The orphan nuclear receptor NR4A1 is upregulated in vascular injury and has been shown to protect from atherosclerosis and restenosis.

- ISG12 is a small transmembrane protein induced by IFNs with hitherto unidentified cellular function.

What New Information Does This Article Contribute?

- ISG12 is localized in nuclear envelopes of endothelial and smooth muscle cells in vasculature and is induced by vascular injury.

- ISG12 interacts with NR4A1 and downregulates its transcriptional activity by promoting its nuclear export, thereby decreasing the nuclear localization of NR4A1 and its ability to activate the expression of its target genes.

Novelty and Significance

- ISG12 antagonizes the protective functions of NR4A1 in the context of vascular injury in vivo.

The orphan nuclear receptor NR4A1, which is a member of the NR4A family of nuclear receptors, has been shown to protect from vascular pathologies such as atherosclerosis and restenosis. We identify ISG12 as a novel interaction partner of NR4A1 that is induced by vascular injury. We report that ISG12, which is localized in nuclear envelopes, facilitates the nuclear export of NR4A1 by a mechanism that depends on the nuclear export receptor Crm1 and thereby decreases the transcriptional activity of NR4A1. Newly generated ISG12-deficient mice were found to be protected from neointima formation in response to carotid ligation and femoral cuff placement. This effect was dependent on the presence of NR4A1, as mice double deficient in ISG12 and NR4A1 showed neointima formation similar to wild-type mice. Thus, our studies identify ISG12 as a novel negative regulator of NR4A1 activity and of its vasculoprotective effects.
The Interferon Stimulated Gene 12 Inactivates Vasculoprotective Functions of NR4A Nuclear Receptors

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Supplemental Information

Supplementary Methods
Yeast library and yeast-2-hybrid screen
For the construction of a yeast library, total RNA was isolated from activated human uterine microvascular endothelial cells stimulated with LPS for 4, 9 and 16 hours and TNFα for 2, 4, 9, and 16 hours, respectively, using Trizol (Invitrogen). mRNA was purified with oligo (dT)$_{16}$ labeled magnetic beads (Dynal, Oslo). Endothelial cell activation was confirmed by upregulation of IL-8 as revealed by Q-PCR. mRNAs isolated from the differently stimulated endothelial cells were pooled and first strand synthesis was performed by SMART technology based on template switching using modified oligo (dT)$_{16}$ primers in combination with MuLV reverse transcriptase. Following PCR, cDNAs and the linearized pGADT7-Rec vector containing the GAL4 activation domain (AD) were co-transformed into AH109 yeast strain (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS - GAL1TATA -HIS3, GAL2UAS - GAL2TATA - ADE2, URA3 : : MEL1UAS - MEL1TATA - lacZMEL1). For screening, a construct of NR4A1 coding for amino acids 248-557 and lacking the transactivation domains 1 and 2 was cloned into pGBK7 containing the GAL4 binding domain (Clontech), and the resulting construct was verified by sequencing with ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on a 310 Genetic Analyzer (Perkin Elmer, Wellesley, MA). Autoactivation of the bait was excluded by co-transformation with vector containing an empty AD plated on selective agar. The bait construct was transformed into Y187 yeast strain (MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met --, URA3 : : GAL1UAS - GAL1TATA - lacZMEL1). Mating was performed according to the manufacturer’s protocol with a AH109 yeast strain containing the endothelial cell library at 5x10$^8$cfu/ml (Clontech) with bacterial RNA as carrier nucleotides as described$^1$ resulting in about 1% mating efficiency. Positive colony selection was carried out on SD medium lacking leucine, tryptophan, adenine and histidine, and subsequently in the presence of 35 mM 3-amino-1,2,4-triazole (3-AT). Plasmid DNA from yeast was prepared by the method of Liang and Richardson$^2$, followed by electrotransformation into HB101 bacteria. Plasmids were isolated from bacteria by Fast plasmid™ mini kit (Eppendorf, Hamburg) and sequenced as described above. Positive interactions of bait and prey were confirmed by retransformation in yeast.

Immunoprecipitation and Western blotting
293 cells, transfected with the respective plasmids or scrambled control and siRNA for RXRa (Ambion, Austin, TX), were washed with PBS and lysed for 30 min. For Western blot analysis and confirmation of silencing by siRNA samples were lysed in Laemmlli buffer. Collected samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking in PBS/Tween20 containing 5% milk, membranes were incubated over night with 1:500 dilution of rabbit polyclonal anti RXR antibody (Santa Cruz Biotechnology, Santa Cruz, CA ). Afterwards membranes were incubated with the respective secondary HRP-labeled antibody (Amersham Biosciences, Fairfield, CT) for 45 min at room temperature, and developed using ECL Plus (Amersham Biosciences) according to the manufacturer’s protocol.

Cell lysis for immunoprecipitaton of NR4A1 and Lamin A was done in a buffer containing 2.7mM KCl, 1.5mM KH2PO4, 9.2mM Na2HPO4 2H2O, 150mM NaCl, 0.7% NP40, 0.3% Triton X-100 and a complete protease inhibitor cocktail (Roche Diagnostics, Basel). Cell lysis for immunoprecipitation of ISG12 was done in buffer containing 50mM sodium
HEPES of a pH 7.5, 150mM NaCl, 5 mM EDTA, 10mM NaF, 10mM Na3PO4, 1% v/v Triton X 100, 0.5% sodium deoxycholate, and 0.1% w/v SDS and a complete protease inhibitor cocktail (Roche Diagnostics). After lysis, the concentration of NaCl was adjusted to 350mM, and lysates were incubated for 4 hours at 4°C with 2μg of respective antibodies: polyclonal rabbit anti-NR4A1 antibody M210 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-ISG12 antibody ab14695 (Abcam, Cambridge, UK), polyclonal rabbit anti-Lamin A antibody ab2559 (Abcam), or control rabbit IgG (DAKO, Milan, Italy), together with protein A-sepharose beads (Amersham Biosciences, Buckinghamshire, UK). The resulting mixtures were washed five times with PBS, and beads were resuspended in Laemmli buffer to isolate precipitates from the beads by heating. Collected samples were subjected to 10% or 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking in PBS/Tween-20 containing 3% BSA, membranes were incubated for 1 hour at room temperature with following antibodies: monoclonal mouse anti-myc (Oncogene, San Diego, CA) or monoclonal mouse anti-NR4A1 (clone 2E4C6; a generous gift from Dr. Q. Weimin, Invitrogen/Zymed Laboratories Inc., San Francisco, CA), both diluted 1:250; polyclonal rabbit anti-actin (Sigma Aldrich, St. Louis, MO) diluted 1:5,000; monoclonal mouse anti-flag (F3165; Sigma Aldrich) diluted 1:1,000; polyclonal rabbit anti-Lamin A diluted 1:500; and polyclonal rabbit anti-ISG12 diluted 1:667 dilution. Subsequently, membranes were washed, incubated with the respective secondary HRP-labeled antibodies (Amersham Biosciences, Fairfield, CT) for 45 min at room temperature, and developed using ECL Plus (Amersham Biosciences) according to the manufacturer's protocol.

**Chromatin immunoprecipitation (ChIP)**

To obtain an initial scheme of factors binding to the ISG12 promoter, the MatInspector software (Genomatix, Munich) was used for analyses of potential binding sites for transcription factors up to 1,000 bp upstream of the transcriptional start. Subsequent ISG12 ChIP experiments were carried out in 293 cells treated with or without 1000U/ml human IFNα (R&D Systems, Minneapolis, MN) for 5 hours. Then, cells were lysed and ChIP assays were carried out using EZ Chip™ (Upstate Inc., Charlottesville, VA), as described by the manufacturer. For ChIP experiments, 2μg of rabbit anti-IRF-1 (H-205), anti-IRF-3 (FL-425), or anti-IRF-4 (H-140) (all Santa Cruz Biotechnology), or rabbit IgG, were used for immunoprecipitation, and PCR analysis was performed by LightCycler technology using the Fast Start SYBR Green I kit (Roche Diagnostics) using following (F) forward and (R) reverse primers: F 5' GCA CAC TCC CAT CCT TGA AT 3' and R 5' GTG TTC CCA AAG GGT GTG AT 3'. Analysis of the human GAPDH promoter was included in parallel experiments for control purposes.

**Isolation of nuclear fractions and fractionation of nuclear envelopes**

293 cells were grown in 6-well plates until sub-confluency and then transfected with EGFP-NR4A1 and/or myc-ISG12, respectively. Cell fractionation was performed 30 hours after transfection. Transfected cells were washed once with ice-cold PBS, scraped, and collected in falcon tubes. Afterwards, suspensions were centrifuged at 1500 rpm for 5 min and resuspended in buffer A (10mM HEPES, pH 7.9, with 1.5mM MgCl₂, 10mM KCl, 0.5mM Dithiothreitol (DTT) + protease inhibitor cocktail (PI)) and then pelleted at 1,500 rpm for 5 min. Pellets containing cells were resuspended in 4x buffer A of the packed cell volume and incubated on ice for 20 min. Suspensions were then homogenized using a glass homogenizer (50 strokes). Aliquots were separated for input control. The remaining lysates were centrifuged at 4,500 rpm for 6 min at 4°C, and the
resulting pellets were washed once with PBS, centrifuged again to sediment nuclei. Nuclei were lysed with a lysis buffer (50mM sodium HEPES, pH 7.5, with 150mM NaCl, 5 mM EDTA, 10mM NaF, 10mM Na3PO4, 1% v/v Triton X-100, 0.5% sodium deoxycholate, and 0.1% w/v SDS) on ice for 40 min. Total cell lysates and lysed nuclear fractions were then adjusted to equal concentrations in Laemmli buffer.

Isolation of a nuclei for separation of inner and outer nuclear envelope was done as previously described. In brief, cells were washed 2x with PBS, scraped, and pelleted at 1,000 rpm for 5 min at RT. The resulting pellets were resuspended in 5x volumes of a 0.25M STM buffer (50mM Tris-HCl, pH 7.4, with 0.25M sucrose, 1mM MgCl₂, 1mM DTT + PI), homogenized using a glass homogenizer (25 strokes), and centrifuged at 3,000rpm for 10 min. The resulting pellet was diluted with 0.25M STM and the molarity of this solution was adjusted to 1.5M sucrose by addition of 2.3M STM buffer (50mM Tris-HCl, pH 7.4, with 2.1M sucrose, 2mM MgCl₂, 1mM DTT + PI). This suspension was then placed on 57µl 2.3M STM buffer and centrifuged for 40 min at 16,000g. The resulting pellet of nuclei was then resuspended in 250µl STM and stored at -20°C. For further separation of nuclear envelopes, 50µl of nuclei was diluted in 250µl of 0.25M STM, and incubated with DNase (250µg/ml) and RNase (250µg/ml) for 1 h on ice. Digested nuclei were pelleted at 10,000 rpm for 10 min, and the pellet was resuspended in 125µl of 0.25M STM. Thereafter, the suspension was diluted in 125µl of 2M NaCl STM, incubated on ice for 60 min with gentle swirling, pelleted at 10,000 rpm for 10 min, and then resuspended in 120µl of 0.25M STM, yielding a nuclear envelope preparation. The subsequent separation of outer and inner nuclear membrane proteins was done in a 2-step procedure. First, 60µl of the nuclear envelope preparation was incubated with 60µl of a 2% citric acid 0.25M STM for 15 min on ice, and then pelleted at 5,000 rpm for 10 min. The resulting pellet containing inner nuclear envelope proteins was resuspended in 20µl of 0.25M STM and diluted with 2x Laemmli buffer. The supernatant containing the outer nuclear envelope proteins was used for trichloroacetic acid precipitation and diluted in 2x Laemmli buffer for further analyses.

Following nuclear fractionations, samples were separated on either 9% or 12% SDS PAGE, transferred onto PVDF membranes, and immunoblotting was performed as described above using following antibodies: monoclonal mouse anti-myc at a 1:250 dilution (Oncogene), polyclonal rabbit anti-EGFP (ab290; Abcam) at a 1:2,500 dilution, polyclonal rabbit anti-calreticulin (Abcam) at a 1:1,000 dilution, polyclonal rabbit anti-Lamin A (Abcam) at a 1:500 dilution, monoclonal mouse anti-flag (Sigma-Aldrich) 1:1000 dilution, or polyclonal rabbit anti-histone H3 at a 1:5,000 dilution (ab1791; Cell Signaling Technology, Danvers, MA).

Immunocytochemistry and microscopy
The subcellular localization of NR4A1 or ISG12 was evaluated by immunocytochemistry according to previously described protocol. In brief, cells were grown to 50-80% confluence in 2-well chamber slides (Nalge Nunc International, Naperville, IL) and transfected as indicated. In some experiments HUVECs were incubated with 4ng/ml of leptomycine B (Calbiochem, La Jolla, CA) for 12 hours experiments; MEFs were stimulated for 5 hours with 1,000 U murine IFNα (PBL Biomedical Laboratories, Piscataway, New Jersey). Thirty hours after transfection cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and then stained for NR4A1 with rabbit polyclonal anti-NR4A1 (Santa Cruz Biotechnology ) using dilution 1:300 or for myc-ISG12 with a monoclonal mouse anti-myc antibody (Oncogene) at a dilution of 1:100. Goat anti-mouse or anti-rabbit secondary antibodies conjugated to Alexa fluor 568 or Alexa fluor 488 were used at a 1:200 dilution (Molecular Probes, Eugene, Oregon). Nuclear stainings were performed using 7-AAD, DAPI or PI. Fluorescence
microscopy was performed either on a LSM-510 Meta confocal laser microscope (Zeiss, Gottingen, Germany) or on an Olympus AX-70 microscope. The relative distribution of NR4A1 between nucleus and cytoplasm was obtained by analyzing the integral signal intensity of all NR4A1 related pixels representing the nuclear region (defined by propidium iodide staining) and of those pixels representing the rest of the cellular cross section using the Analysis-Cell® software (Olympus Soft Imaging Solutions; Muenster, Germany). At least 500 cells per condition were analyzed. Results are expressed in percentage of detected cellular signal that is confined to the nuclear area.

For Triton pre-extraction, cells were rinsed once with PBS and then extracted with 1% Triton X-100 in 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2 and 2 mM CaCl2 for 30 seconds at room temperature. Afterwards cells were fixed, permeabilised, stained and analyzed by immunofluorescence microscopy as above.

To determine the nuclear localization of NR4A1 in carotid arteries of wild type and ISG12-/- mice, sections were stained with a rabbit anti-NR4A1 antibody (M210) diluted 1:100, followed by an Alexa 488-conjugated goat anti-rabbit secondary antibody diluted 1:300. Confocal images acquired on a LSM-510 Meta confocal laser microscope were analyzed as outlined above.

**Electron microscopy**

Immunoelectron microscopy was done on 293 cells expressing myc-ISG12. Cells were fixed for 1 h with 4% freshly depolymerized paraformaldehyde (pFA) in 0.1 M pPBS (pH 7.4), then washed 3 times with PBS, and blocked with PBS containing 50mM glycine to block excessive aldehyde. Embedding, trimming, and sectioning was essentially performed as previously described 5. For post-embedding immunogold staining, sections were blocked for 30 min in PBS (pH 7.4) containing 0.05% Tween-20 (PBST) and 1% BSA. Mouse anti-myc antibody (9E10, Calbiochem, San Diego) was diluted 1:100 in blocking buffer and sections were incubated for 3 h at room temperature. After washing, sections were incubated with a 5 (or 10) nm gold-conjugated goat anti-mouse antibody (Biocell), diluted 1:30 in PBST (pH 8.0). Finally, sections were washed with PBST (pH 8.0) and dH2O, then counterstained with 2% aqueous uranyl acetate for 45 min and finally analyzed with a Jeol 1200 EXII electron microscope. Control sections were blocked with anti-myc incubated in the presence of 10-fold excess of myc-protein for 2 h at 37°, and showed only minimal background.

**RNA isolation and quantitative RT-PCR (Q-PCR)**

Isolated arterial tissue from mice was immersed into ice-cold RNA later (Ambion, Austin, TX) and stored at -70°C until isolation. RNA from pooled mouse arterial tissue for Q-PCR was extracted using Trizol (Invitrogen) as described previously 6. 350ng of RNA was reverse transcribed with MuLV-reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems) and oligo (dT)16 primers. RNA from HUVECs, HUASMCs, U937 cells, MVSMCs, MVECs and MEFs was extracted using Trizol, and 900ng of total RNA was reverse transcribed as described above. The primers for mRNA expression of target genes were designed using the PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). Following forward (F) and reverse (R) primers were used: human ISG12: F 5’ TGT GAT TGG AGG AGT TGT GG 3’; R 5’ GAA CTT GGT CAA TCC GGA GA 3’; human RXRα: F5’AAT GAG GTG GAG TCG ACC AG3’, R5’ATG TTG TTG GTA ACA GGG TCG TT3’; mouse ISG12: F 5’ CTG CCA TAG GAG GAG CTC TG3’ and R 5’ATG GCA TTT GTT GAT GG G 3’; mouse NR4A1 F 5’CTC TCC GAA CCG TGA CAC TT3’ and R 5’ TTG TTT GCC AGG CAG ATG TA 3’; mouse MCP-1 as described 6; human PBGD F 5’ TCG AGT TCA GTG CCA TCA TC 3’ and R 5’ CAG GTA CAG TTG CCC ATC CT 3’, or mouse CycB F 5’ CAG
Q-PCR was performed by LightCycler technology using the Fast Start SYBR Green I kit (Roche Diagnostics). Relative quantification of target gene expression was performed using a mathematical model described by Pfaffl. The expression of the target molecule was normalized to the expression of PBGD and/or CycB.

**Generation of ISG12−/− and ISG12−/− X NR4A1−/− mice**

ISG12−/− mice were generated by targeted disruption of the murine ISG12 gene, which consists of 4 exons, separated by 3 introns (introns I to III), as outlined in Fig. II A. The cDNA sequence of mISG12 was used to design primers specific for different regions of the mISG12 gene. Using 129S/v genomic DNA as a template, the PCR reactions were optimized and used for screening of the genomic library generated by partial HindIII digestion of a 129S/v mouse genomic DNA and cloned into a RPCI.22 BAC vector (Invitrogen). Obtained genomic clones were used to prepare targeting vectors to inactivate the ISG12 gene in embryonic stem (ES) cells. In total, an 8.5kb homologous sequence of the ISG12 gene was introduced into a parental pPNT vector containing a neomycin phosphotransferase (neo) cassette and a herpes simplex virus pPNT.mlISG12. Briefly, a 4.7kb XhoI fragment containing 5’ UTR of mlISG12 gene was ligated into the XhoI site of pPNT generating plasmid pPNT1.mlISG12. Subsequently, a 3.8kb EcoRI fragment encompassing 3’ UTR of mlISG12 gene was cloned into the EcoRI site of the pBluescript®II KS(+) vector (Stratagene, La Jolla, CA). Subsequently, a 3.85 kb Sall-Smal fragment from this vector was Klenow filled and ligated into Asp718 site/Klenow filled pPNT1.mlISG12, yielding a targeting vector pPNT.mlISG12. Transfection of targeting vectors resulted in 4 out of 200 G418/Ganciclovir-double-selected clones that underwent the desired homologous recombination, as confirmed by comprehensive Southern blotting of the isolated genomic DNA from R1 embryonic stem (ES) cells derived from the 129S/v mouse strain (received from A. Nagy, Samuel Lunenfeld Institute, Toronto, Canada). Chimeric mice (F0), obtained by 8 cells-stage embryo aggregation of the targeted ES cell clones, were test-bred for germ line transmission with Swiss mice. The deleted ISG12 allele was transmitted to the offspring (50% 129S/v: 50% Swiss genetic background), yielding ISG12−/− mice. Intercrossing of these mice resulted in ISG12−/− mice as identified by Southern blot analysis of tail tip DNA using the 3’-external probe.

In order to generate ISG12−/− x NR4A1−/− double deficient mice and respective control mice, ISG12−/− mice were bred with NR4A1−/− mice in C57BL/6 background (kindly provided by J. Milbrandt, Washington University School of Medicine, St.Louis, USA).
Double heterozygous mice were obtained and subsequently bred to obtain littermates with the respective genotypes for carotid artery ligation experiments. Genotyping of mice with respect to the NR4A1 allele was done by Southern blotting using an exon 2 specific probe and BamHI genomic digests yielded a 4.9kb and a 6.6kb fragment for the wild type and recombined allele, respectively.

**GenBank accession numbers.**
Protein Homo sapiens ISG12, GI:55925614; protein Mus musculus ISG12 GI:44771124; protein Homo sapiens NR4A1 GI:21361342; protein Mus musculus NR4A1 GI:6754216; mRNA Homo sapiens ISG12 GI:55925613; mRNA Mus musculus ISG12 GI:44771123; mRNA Homo sapiens NR4A1 GI:21361342; mRNA Mus musculus NR4A1 GI:6754215; mRNA Homo sapiens Hydroxymethylbilane synthase HMBS (PBGD) GI:6693007; mRNA Mus musculus RXRa GI: 187954070; Mus musculus CycB GI:118130025
Supplemental References


Supplemental Figures

A. pGBK7NR4A1+ISG12  pGBK7+ISG12

B. Organelle lights™  ISG12  IGG12: Organelle lights™
   - Nuclear Envelope
   - Endoplasmic Reticulum
   - Mitochondria

C. ISG12  Calreticulin  Merge

D. ISG12  Lamin R1  Merge

E. IGG12  Calreticulin  Merge

F. ISG12  Lamin B1  Merge

G. control  TX100-preextraction
   - Nucleus
   - Cytoplasm

H. 20 µm

20 µm
Online Figure I. A. Interaction in yeast. The interaction of ISG12 and NR4A1 was confirmed by selective growth of yeast transformed with ISG12 fused to the activation domain of GAL4 and NR4A1 fused to the DNA binding domain of GAL4 or an empty DNA binding domain in pGBKT7, respectively. Positive colony selection was only observed when ISG12 and NR4A1 proteins interacted, enabling binding of the DNA binding domain to the activation domain of GAL4. B. Cellular localization of ISG12. 293 cells were transfected with myc-ISG12, the nuclear envelope, endoplasmic reticulum and the mitochondria were detected using organelle lights™ markers with respective specificities, and myc-ISG12 was detected using an anti-myc antibody as described in Experimental Procedures. Co-localization of ISG12 with organelle specific markers is indicated in yellow. Shown is a representative example of three experiments with 50 replicates each. C. – F. Cellular localization of ISG12 in vascular cells. HUASMCs and HUVECs were transfected with myc-ISG12, the endolplasmatic reticulum and the nuclear envelope were detected using anti-calreticulin and anti–lamin B1 antibody as described in Method section. Co-localization of ISG12 with cellular organelle markers is indicated in yellow. Shown are representative images from 50 replicates. (C.) Endoplasmic co-localization of ISG12 with calreticulin in HUASMCs. (D.) Co-localisation of ISG12 with the nuclear envelope marker Lamin B1 in HUASMCs. (E.) Endoplasmic co-localization of ISG12 with calreticulin in HUVECs. (F.) Co-localisation of ISG12 with the nuclear envelope marker Lamin B1 in HUVECs. G. Detection of ISG12 by electron microscopy. 293 cells were transfected with myc-ISG12 and analyzed by immunoelectron microscopy using a gold labeled secondary antibody. ISG12 (black dots) is found predominantly in the region between nucleus and cytoplasm and is associated with nuclear pores (highlighted in yellow). The left panel shows a selected electron microscopy image and the panels on the right represent a higher magnification of the area marked in red (n= 50 cells). H. Detection of perinuclear ISG12 in 293 cells. Cells were transfected with myc-ISG12, treated with Triton X-100 for 30 seconds, and then fixed with paraformaldehyde; or directly fixed. The localization of ISG12 (red) was assessed by immunofluorescence microscopy in control cells or in pre-extracted cells. Shown are representative images of 50 replicates.
Online Figure II. A-E. Subcellular localization of ISG12 in different cellular fractions. Western blot analysis of myc-ISG12 in whole cell lysates (A.), isolated nuclei (B.), isolated nuclear envelopes (C.) isolated inner nuclear envelopes (D.) and isolated outer nuclear envelopes (E.) of transfected 293 cells. 293 cells were transfected with myc-vector or myc-ISG12, respectively, and their subcellular fractions were subjected to immunoblotting using anti-calreticulin antibody (WB1) (ER and outer NE-marker), and anti-Lamin A (WB2) (inner NE-marker), and anti-myc antibodies (WB3). One representative example of 3 replicates is shown. (F.) Co-immunoprecipitation of ISG12 and Lamin A in 293 cells transfected with myc-ISG12 using an anti-Lamin A antibody. Membranes were immunoblotted with anti-Lamin A antibody (WB1) and anti-myc antibody (WB2), respectively. One representative example of 3 independent replicates is shown. G. – H. Efficiency of silencing of RXRα. (G.) 293 cells were transfected with siRXRα or a scrambled control. After 48 hrs, expression of RXRα was analyzed by Q-PCR. Data represent mean ± SEM fold expression of RXRα compared to scrambled control (n=2). (H.) 293 cells were transfected with siRXRα or a scrambled control. After 48 hrs, expression of RXRα was analyzed by Western blot using anti-RXRα antibody and loading was determined by reprobing the membrane using anti-actin antibody. One representative example of 2 independent replicates is shown.
Online Figure III. A. Time dependent upregulation of ISG12 by IFNα. 293 cells were stimulated with 1,000U/ml IFNα for indicated times and ISG12 mRNA expression was assessed by Q-PCR. Data shown are the mean ± SEM fold expression of ISG12 compared to 0 hr time point. B. IFNα stimulates expression of ISG12 mRNA in mouse embryonic fibroblasts (MEFs). MEFs were stimulated with 1,000U/ml IFNα for 7.5 hours, and ISG12 expression was analyzed by Q-PCR. Data represent mean ± SEM fold expression of ISG12 above of control treated cells. C. IFNα stimulates expression of ISG12, but not of NR4A1 in mouse microvascular smooth muscle cells. MVSMCs were stimulated with 1,000U/ml IFNα for 5 hours, and expression of ISG12 and NR4A1 was analyzed by Q-PCR. Data represent mean ± SEM fold expression of ISG12 above of control treated cells. D. IFNα stimulates expression of ISG12, but not of NR4A1 in mouse microvascular endothelial cells. MVECs were stimulated with 1,000U/ml IFNα for 5 hours, and expression of ISG12 and NR4A1 was analyzed by Q-PCR. Data represent mean ± SEM fold expression of ISG12 or NR4A1 above of control treated cells. E. Efficiency of silencing of ISG12. 293 cells were transfected with siISG12 or a scrambled control. After 48 hrs, expression of ISG12 was analyzed by Q-PCR. Data represent mean ± SEM fold expression of ISG12 compared to scrambled control (n=2). F. IFNγ decreases the transcriptional activity of NR4A1 on NBRE. 293 cells were transfected with NBRE and NR4A1, respectively. Cells were stimulated with 100 ng/ml IFNγ for 24 hours. Transcriptional activity was assessed in a dual luciferase reporter assay. Data represent mean ± SEM of fold upregulation above control (n=2). G.-H. IFNα decreases the transcriptional activity of NR4A1 on NurRE (G.) and DR5 (H.) binding sites. 293 cells were transfected with NurRE or DR5/RXR, and NR4A1 as indicated. Subsequently cells were stimulated with IFNα (1,000 U/ml) for 16 hrs. Transcriptional activity was assessed in a dual luciferase reporter assay. Data represent mean ± SEM of fold upregulation above control (n=4).
Online Figure IV. Generation of ISG12\textsuperscript{+/−} mice. A. Schematic presentation of the ISG12 gene deletion strategy. Black bars represent exons I to IV that were targeted by neo gene replacement. B. Southern blot analysis of the wt, ISG12\textsuperscript{+/−} and ISG12\textsuperscript{−/−} mice genomic DNA digested with HindIII and hybridized to a 3’-flanking external probe. C. RT-PCR of the ISG12 gene expressed in the spleens of wt and ISG12\textsuperscript{−/−} mice.