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DISSERTATION

Immediate early response gene 2 (IER2), a novel regulator of the cell cycle inhibitor p21$^{waf1/cip1}$

Ausgeführt zum Zwecke der Erlangung
des akademischen Grades eines Doktors der Naturwissenschaften

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Theories crumble, but good observations never fade.

Through Rugged Ways to the Stars
Harlow Shapley (1885-1972)

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SUPPLEMENT

ABSTRACT

Immediate early genes (IEGs) are rapidly and transiently induced in response to several (extra)-cellular stimuli. Often, they encode transcription factors, and thus represent the first transcriptional program that precedes changes in multiple cellular processes including cell growth, differentiation, angiogenesis and inflammation. IEGs influence the activity of secondary mediators, that regulate the activation but also the termination of cellular processes. Their correctly timed regulation is therefore of utmost importance since its impairment carries the potential for deregulated cellular processes, including oncogenic transformation.

In a microarray-based gene expression study we previously identified immediate early response gene 2 (*IER2*) in the human endothelium. We consequently started to investigate the impact of this poorly described IEG with so far unknown protein function in our model system, primary human umbilical vein endothelial cells (HUVEC).

We found that endothelial cell proliferation- and angiogenesis-associated growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) rapidly and transiently induce *IER2* expression in HUVEC via the activation of the protein kinase C (PKC) signaling cascade. In addition, we discovered by using the phorbol ester PMA as a strong PKC activator and RNAi approaches that *IER2* is mainly regulated by the PKC delta (δ) isozyme. Moreover, we described for the first time that IER2 in the PKC signaling cascade suppresses PMA-induced cell cycle inhibitor $\textit{p21}^{\textit{waf1/cip1}}$ expression, acting at the level of transcription. Thereby, IER2 reverses the $p21^{waf1/cip1}$-mediated block of E2F-dependent transcription thus counteracting PMA-induced apoptosis in HUVEC. We subsequently provided evidence that IER2 is accumulative expressed in some cancer models suggesting a potential oncogenic function of IER2 with concomitant loss of tumor suppressor $p21^{waf1/cip1}$ expression in cancer.

Together, our findings propose that *IER2* expression is regulated upon PKC activation and posttranslational mechanisms. Moreover our data suggest that IER2 acts as a novel molecular regulator of the cell cycle inhibitor $p21^{waf1/cip1}$ by influencing the balance between $p21^{waf1/cip1}$-driven cell death towards E2F-mediated cell survival.

ZUSAMMENFASSUNG

Immediate early genes (IEGs) werden als Reaktion auf viele (extra)-zelluläre Reize schnell und transient induziert und sie sind oft Teil einer ersten Welle transkripioneller Regulation welche sodann Änderungen in einer Reihe von zellulären Prozessen wie Zellwachstum, Differenzierung, Angiogenese und Entzündung beeinflusst. IEGs haben Auswirkung auf die Biosynthese von Proteinen die u.a. als Transkriptionsfaktoren ihrerseits wiederum die Aktivierung aber auch die Beendigung zellulare Prozesse steuern. Falsche Regulation dieser Prozesse kann die Entstehung von Krankheiten wie Krebs fördern.

In einem Mikroarray-basierenden Genexpressions-Experiment wurde zuvor das immediate early response gene 2 (*IER2*) in menschlichen Endothelzellen identifiziert. In weiterer Folge untersuchten wir in unserem Modellsystem, primären humanen Nabelschnur Endothelzellen (HUVEC), dieses bis dato schlecht charakterisierte IEG auf dessen Funktion.

Wir konnten zeigen, dass Endothelzellproliferation und Angiogenese auslösende Wachstumsfaktoren wie der vascular endothelial growth factor (VEGF) oder basic fibroblast growth factor (bFGF) zu einer schnellen und transienten Induktion der *IER2* Expression in HUVEC über die Aktivierung der Protein Kinase C (PKC) Signalkaskade führen. In weiterer Folge fanden wir in Zellkulturversuchen mittels Phorbolester (PMA) Stimulierung und RNAi Experimenten, dass *IER2* hauptsächlich durch das Isoenzym PKC delta (δ) reguliert wird. Darüber hinaus konnten wir erstmals eine spezifische IER2 Protein Funktion zeigen: In der PKC Signalkaskade unterdrückt IER2 eine PMA-induzierte transkriptionell gesteuerte Expression des Zellzyklusinhibitors $p21^{waf1/cip1}$. Ferner bemerkten wir, dass die IER2 vermittelte Inhibierung von $\textit{p21}^{\textit{waf1/cip1}}$ zu einer veränderten E2F-abhängigen Transkription führt und dabei in HUVEC der PMA-induzierten Apoptose entgegenwirkt. IER2 war darüberhinaus in einigen Krebsmodellen überexpremiert, was auf eine mögliche onkogene Funktion von IER2 bei gleichzeitigem Verlust des Tumorsuppressors $p21^{waf1/cip1}$ hinweist.

Zusammenfassend können wir sagen, dass die *IER2* Expression durch PKC Aktivierung reguliert, und durch posttranslationale Mechanismen beeinflusst wird. IER2 als neuer molekularer Regulator des Zellzyklusinhibitors $p21^{waf1/cip1}$ könnte die Balance zwischen $p21^{waf1/cip1}$ vermittelten Zelltod in Richtung E2F-angetriebenem Zellwachstum verschieben.

LIST OF ABBREVIATIONS

AP-1	activator protein 1
aPKC	atypical PKC isozymes
ATP	adenosine triphosphate
BAX	BCL2-associated X protein
BCL-2	B-cell lymphoma 2 protein
cAMP	cyclic adenosine monophosphate
cdc2	cell division control 2
cdk(s)	cyclin dependent kinase(s)
Cip	cdk interacting protein
CKI	cyclin/cdk inhibitors
c-MYC	v-myc myelocytomatosis viral oncogene homolog
cPKC	conventional PKC
CRE	cAMP response element
CREB	cAMP response element-binding protein
DAG	diacylglycerol
ΔC	deleted C-terminus
DMSO	dimethyl sufoxide
ΔN	deleted N-terminus
DNA	deoxyribonucleic acid
E2F	E2F family of transcription factors
EGF	epidermal growth factor
EGR-1	early growth response protein 1
ELAM-1	endothelial-leukocyte adhesion molecule 1
ETR101	early TPA responsive 101
FCS	fetal calf serum
FGF	fibroblast growth factor
FOS	FBJ (Finkel, Biskis, and Jinkins) murine osteosarcoma viral oncogene homolog
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hr	human recombinant
HTLV-I	human T-cell leukemia virus type I
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1

IEG(s) immediate early gene(s)
IER2 immediate early response gene 2
IκB inhibitor of κB proteins
IKK IκB kinase
IL-1 interleukin-1
IP_3 inositol-(1,4,5)-trisphosphate
JNK c-Jun N-terminal kinase
JUN jun proto-oncogene
Kip kinase inhibitory protein
MAPK mitogen-activated protein kinase
MDM2 oncoprotein mouse double minute 2
MOI multiplicity of infection
NES nuclear export signal
NF-κB nuclear factor-κB
NLS nuclear localization signal
NORS non-regular secondary structure
nPKC novel PKC
Nurr77 nuclear receptor subfamily 4A
p21 cyclin-dependent kinase inhibitor 1
PARP poly ADP-ribose polymerase
PBS phosphate buffered saline
PCNA proliferating cell nuclear antigen
PEST peptide motif rich in proline (P), glutamate (E), serine (S), and threonine (T)
PIP_2 phosphatidylinositol-(4,5)-bisphosphate
pip92 proline-rich induced protein 92
PKA protein kinase A
PKC protein kinase C
PKD protein kinase D
PLC phospholipase C
PMA phorbol-12-myristate-13-acetate
pRb retinoblastoma tumour suppressor protein
qRT-PCR quantitative real-time reverse transcription-polymerase chain reaction
RNA ribonucleic acid
RNAi RNA interference

RT	room temperature
scr	scrambled/ non silencing control shRNA
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SP1	specific protein 1
SRE	serum-response element
SRF	serum response factor
Sumo	small ubiquitin-related modifer
TNFα	tumor necrosis factor α
tp53	tumor protein 53
TPA	12-O-tetradecanoylphorbol-13-acetate
VCAM-1	vascular cell adhesion protein 1
VEGF	vascular endothelial cell growth factor
Waf1	wild type tp53-activated fragment 1

1. INTRODUCTION

1.1. Inflammation

Throughout evolution, the immune system of humans as well as other vertebrates has developed two distinguished responses to protect the organism against diseases, mainly described as innate immunity and adaptive immunity. The innate immune system, also known as natural immunity or non-specific immune system recognizes pathogens based on conserved molecular structures and counteracts infections. The adaptive immune system, acquired or antigen-specific immunity, develops in time after pathogen contact. [1]

Inflammation represents the first response to extra- as well as intra-cellular harmful stimuli such as pathogen invasion or cellular damage and is thus a fundamental protective process in innate immunity. Classical signs of inflammation are redness, swelling, heat, and pain of the injured tissue and altered blood flow in its surrounded vascular system. This first step of protection and its subsequently following repair mechanisms is termed acute inflammation. Importantly, this beneficial system requires proper activation as well as termination signals otherwise leading to chronic inflammatory conditions. They are considered as a hallmark of several diseases, including inflammation-mediated and cardiovascular diseases (e.g., psoriasis, rheumatoid arthritis, and arteriosclerosis) as well as tumor development, tumor expansion, migration, and metastasis. [2-6]

1.1.1. Endothelial cells act as key players during inflammation and angiogenesis

Endothelial cells are central players in the inflammatory reaction, as they form a barrier between the circulating blood stream transporting immune cells and the surrounded tissue. It has become common knowledge that the endothelium is activated by e.g., an infection. At sites of inflammation leukocytes migrate from the blood into affected tissues across the endothelial cell layer. Given that endothelial cells are typically one of the most quiescent cell types, during inflammatory activation they dynamically react to the messengers released by infiltrating leukocytes. Thus, endothelial cells are able to amplify and re-direct the inflammatory response through three main mechanisms: i) adhesion molecule expression, leading to further recruitment of leukocytes to the site of inflammation, ii) cytokine production, acting in an autocrine and paracrine manner towards the surrounding tissue, and iii) reactivation of the cell cycle, leading to cell proliferation and to the formation of new blood vessels, also known as angiogenesis. [2, 3, 6-9]

1.2. Cellular signaling during inflammation and angiogenesis

Endothelial cells utilize diverse signaling pathways in a distinct and overlapping/interacting fashion to fulfill the above mentioned biological responses. In Figure 1, the main signaling pathways are schematically summarized: i) activation by first messengers such as cytokines and growth factors leads to ii) propagation of second messengers and its downstream targets. Specific signaling cascades then lead to iii) activation of a distinct transcriptional response resulting in iv) its respective biological outcome/functions.

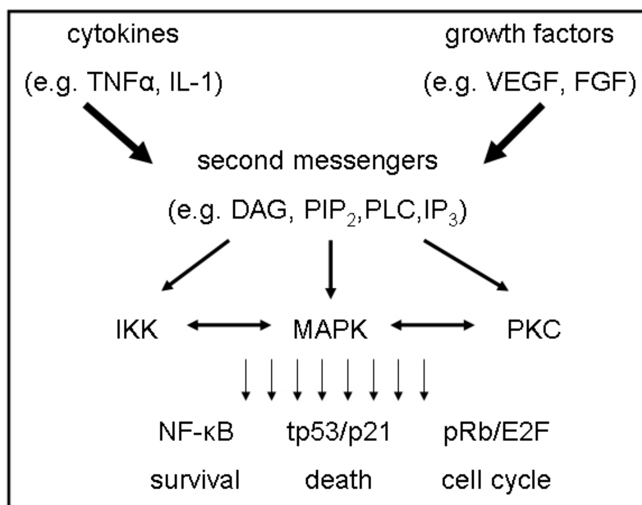


Fig.1: Schematic representation of endothelial cell signaling. TNFα: tumor necrosis factor α; IL-1: interleukin-1; VEGF: vascular endothelial cell growth factor; FGF: fibroblast growth factor; DAG: diacylglycerol; PIP_2: phosphatidylinositol-(4,5)-bisphosphate; PLC: phospholipase C; IP3: inositol-(1,4,5)-trisphosphate; IKK: IκB kinase; MAPK: mitogen-activated protein kinase; PKC: protein kinase C; NF-κB: nuclear factor-κB; tp53: tumor protein 53; p21: cell cycle inhibitor $p21^{waf1/cip1}$; pRb: retinoblastoma protein; E2F: E2F family of transcription factors.

1.2.1. First messengers

1.2.1.1. Inflammatory cytokines

One of the key regulators that are activated upon pro-inflammatory molecules which are released from immune cells, including cytokines such as interleukin-1 (IL-1) or tumor necrosis factor α (TNFα), is the dimeric transcription factor nuclear factor-κB (NF-κB). This transcription factor family comprises RelA (p65), RelB and c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively. [10] The exact regulation of the NF-κB response is of importance because sustained activation can lead to a switch from acute to chronic inflammation.

Basically, acute inflammation is a beneficial response. Nevertheless, a prolonged or not correctly terminated reaction results in disease. Some genes that are expressed at early stages of NF-κB activation can act as negative feedback regulators, thus terminating the inflammatory process. Drug-mediated inhibition, mimicking these feedback loops, is a novel approach leading to the resolution of an inflammatory process. Thereby, turning off prolonged

inflammatory gene expression can prevent chronic inflammation and further can lead to great benefit for patients suffering inflammation-related diseases. [11, 12]

In addition to regulation of innate and adaptive immune responses and inflammation NF-κB mediates divergent transcriptional programs including genes controlling programmed cell death (apoptosis), cell adhesion, proliferation, cellular-stress response and tissue remodeling. Furthermore, activated NF-κB is intimately linked via various cross-regulatory mechanisms that allow crosstalks throughout different signaling pathways. [13-15]

1.2.1.2. Growth factors

A large variety of molecules exhibits angiogenic activity. These include growth factors and chemokines, among others, such as vascular endothelial cell growth factor (VEGF) or fibroblast growth factors (FGF1 and FGF2). They bind to their specific receptors and downstream signaling results in gene activation and diverse biological mechanisms including cell differentiation, proliferation, and matrix dissolution. They all are critical for the growth of endothelial cells, fibroblasts, and smooth muscle cells. FGF1 stimulates the proliferation and differentiation of all cell types necessary for building an arterial vessel, VEGF primarily drives the formation of new capillaries. Besides FGF1, one of the most important functions of b(asic)FGF (FGF2) is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures, thus promoting angiogenesis. [16-20]

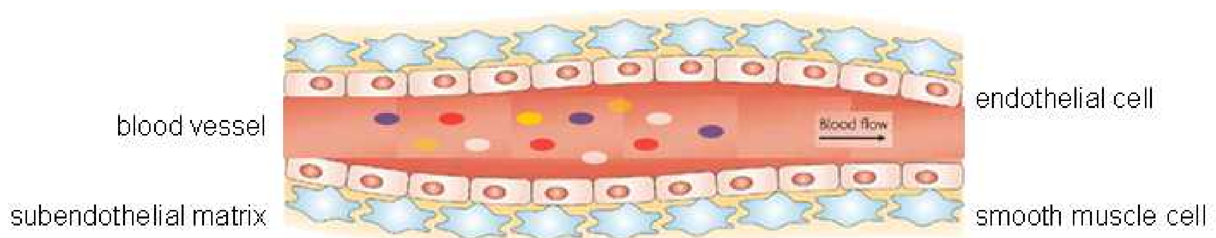


Fig.2: Schematic representation of a blood vessel supplying oxygen and nutrients, and transporting immune cells (colored dots) and encompassing endothelial cells, perivascular cells, and subendothelial matrix containing collagen and von Willebrand factor. Figure adapted from Verheul and Pinedo. [21]

Endothelial cell proliferation and angiogenesis-driven by both VEGF and bFGF are known to require activation of protein kinase C (PKC). This signaling cascade and its well characterized regulation of the cell cycle will be discussed in sections below.

1.2.2. Second messengers

1.2.2.1. Protein kinase C – isozymes and signaling

In several cell types stimuli such as serum, growth factors, phorbol esters, cytokines, calcium, and others regulate diverse cellular events as growth, cell death, differentiation, and angiogenesis through activation of protein kinase cascades. One of the most complex and important of these enzyme families is the protein kinase C (PKC), an ubiquitous enzyme with outstanding heterogeneity in its related signal transduction pathways and associated biological functions. [22]

The PKC family of signal transduction molecules and its isozymes differ in their structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. The serine/threonine kinases are classified as conventional (α, β1, β2, γ), novel (δ, ε, η, θ, μ), and atypical (ζ, λ) isozymes. Conventional PKC (cPKC) isoforms are calcium-, diacylglycerol (DAG)-, and phospholipid-dependent, while novel (nPKC) and atypical (aPKC) isozymes do not require calcium for their activation and the latter are not controlled by DAG either. PKC isoforms share certain basic structural features. These kinases contain a highly conserved catalytic domain consisting of motifs required for adenosine triphosphate (ATP) and substrate-binding and catalysis, and a regulatory domain that maintains the enzyme in an inactive conformation. [22-25]

Its involvement in signaling cascades was established by the demonstration that DAG, which is generated by agonist-induced hydrolysis of e.g., phosphoinositides, resulting in the activation of this enzyme family. Stimuli that bind to a variety of cell surface receptors (G protein-coupled receptors, receptor tyrosine kinases) can transiently produce DAG leading to PKC activation. Sustained enzyme activity, which is required for proliferation and differentiation, involves additional pathways such as the production of DAG through phosphatidylcholine metabolism. Additionally, altered PKC activity has been linked to various types of malignancies. These findings together suggest that DAG and/or related analogs can activate PKC *in vitro* and *in vivo*, indicating that PKC is a downstream target for a plethora of induced pathways. [26, 27]

1.2.2.2. Phorbol esters in PKC signaling

Since the discovery that PKC is the main receptor for tumor-promoting phorbol esters, natural products and pharmalogical analogs which can substitute for DAG in activating the enzyme,

PKC has been immediately linked to mitogenesis as well as to cancer development and progression. Unlike DAG, phorbol esters are more stable, thus, treatment of cells with these agents leads to a prolonged activation of the enzyme family. However, it should be noted that long-time phorbol ester stimulation (more than twenty-four hours) can potently decrease the enzyme activity following activation. Phorbol esters are tumor promoters, they are not carcinogenic by themselves, but increase the possibility of malignant cell transformation after the initiation event has been triggered by carcinogenic agents. The most common phorbol ester is TPA (12-O-tetradecanoylphorbol-13-acetate), also termed PMA (phorbol-12-myristate-13-acetate). [26, 28]

Concerning the complexities of PKC signaling and its multifaceted isozyme family covering distinct roles dependent on cellular background as well as in a stimulus-dependent fashion the focus is mainly set on the nPKC isozyme PKCδ, and their signaling in endothelial cells and the control of the cell cycle.

1.2.3. Third messengers

1.2.3.1. Immediate early genes

Immediate early genes (IEGs) are rapidly and transiently induced in response to a plethora of extra- as well as intra-cellular stimuli. IEGs act thereby as third messengers (Fig.3), which are directly responding on upstream activation, independent of *de novo* protein synthesis. In many cases, they encode transcription factors (e.g., *c-JUN*, *c-FOS*) or other regulatory factors. They act on different levels of signal transduction as i.e., transcriptional co-factors or DNA-binding proteins containing a basic helix-loop-helix leucine zipper (bHLH-Zip) region important for protein dimerization. Once activated, they represent the first cellular program that lead to secondary changes in a variety of cellular responses again including transcription factors and signaling molecules. Immediate early genes are therefore important early regulators of different pathophysiological aspects of a cell, e.g., growth, differentiation, and in the case of endothelial cells, angiogenesis and inflammation. [29-31]

IEGs are described to act cell type specific and more importantly, their specific function is mainly regulated by the duration of their expression. Prolonged activation of IEGs is linked to altered cell cycle regulation during proliferation and differentiation. Furthermore, IEGs often act as early feedback regulators that contribute to the termination of the (inflammatory) reaction (e.g., IκBα, the inhibitor of NF-κB). [12]

Most prominent and best characterized IEGs are transcription factors, containing e.g., FOS [FBJ (Finkel, Biskis, and Jinkins) murine osteosarcoma viral oncogene homolog] and JUN (jun proto-oncogene) protein family members forming the activator protein 1 (AP-1) transcription factor complex. *c-MYC* (v-myc myelocytomatosis viral oncogene homolog) as well as early growth response protein 1 (EGR-1) are classified as IEGs sharing the characteristic of influencing cell cycle regulation on the level of transcription which fits in line with the fact that most prominent IEGs, *c-FOS*, *c-JUN*, and *c-MYC*, are well characterized oncogenes. [30-32]

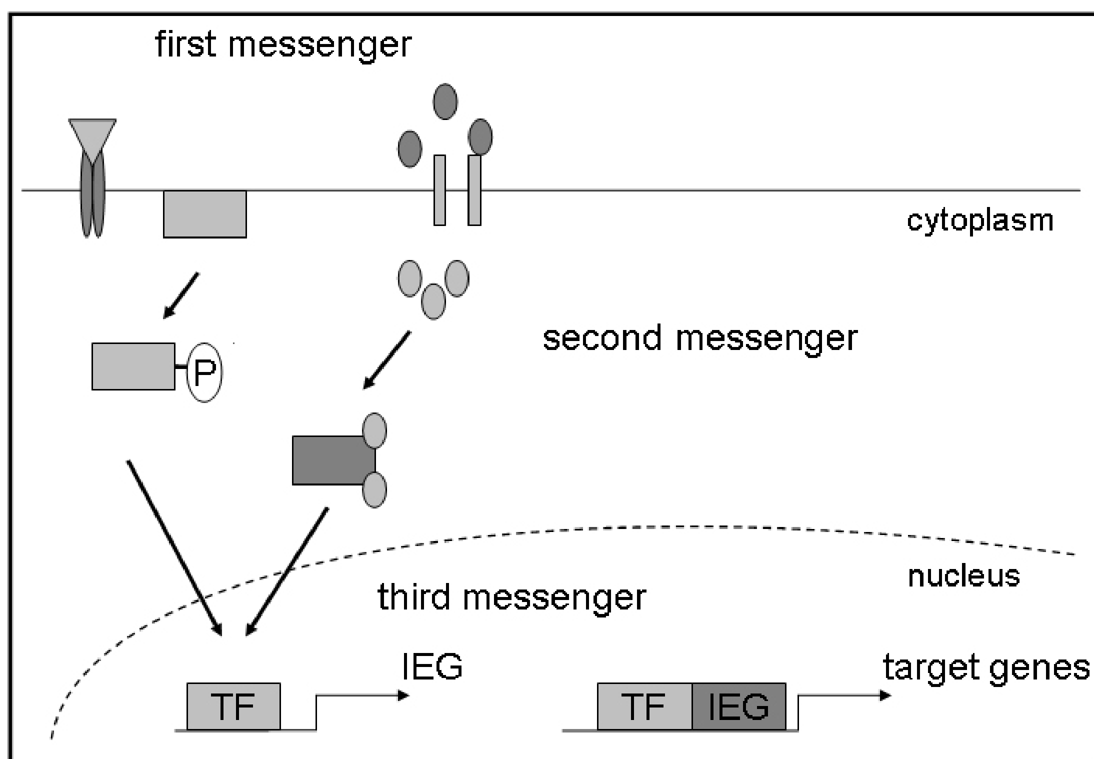


Fig.3: First, second, and third messengers act on different cellular levels. P: phosphorylation; TF: transcription factor; IEG: immediate early gene. Figure based on Yoneda *et al*. [33]

1.2.3.2. Immediate early response gene 2

In the late 1980ies and beginning 1990ies immediate early response gene 2 (IER2), also termed ETR101 (early TPA responsive) and its mouse homologue proline-rich induced protein 92 (pip92) was first reported as an immediate early gene rapidly induced by the phorbol ester TPA/PMA. Further experiments revealed that *IER2* is induced by a variety of different stimuli concluding serum, growth factors, and cytokines in various human, mouse and rat cell types. [34-37]

The *IER2* gene is located on chromosome 19p13.2 and is missing any intron. Furthermore, *IER2* comprises a unique GUUUG sequence in its 3' mRNA flanking region that is associated to be a mRNA degradation signal. The transcribed gene results in a 223 amino acid transiently expressed protein which is found in the nucleus as well as in the cytoplasm. [34, 37-39] IER2 protein sequence analysis (Fig.4) predicts one nuclear export signal (NES) and two nuclear localization signals (NLS), additionally a PEST domain (a peptide motif rich in proline (P), glutamate (E), serine (S), and threonine (T)) that is associated with short protein half-life. Hence, it is hypothesized that the PEST sequence acts as a signal peptide for protein degradation. [40] Using bioinformatics tools, a non-regular secondary structure (NORS) region that is involved in protein-protein interactions is predicted. In addition, the IER2 sequence contains posttranslational modification sites for sumoylation and ubiquitination as well as a number of serines predicted as phosphorylation sites mediated by protein kinase A (PKA) and protein kinase C (PKC). Controversially, IER2 sequence analysis reveals no potential DNA binding site ([34, 37], and bioinformatical analysis, see results).

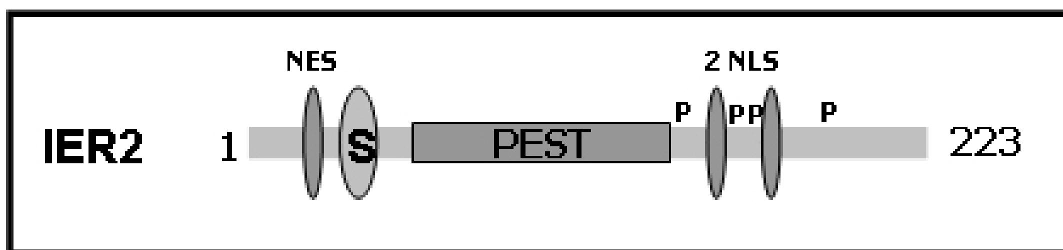


Fig.4: IER2 protein structure. NES: nuclear export signal; NLS: nuclear localization signal; PEST domain (a peptide motif rich in proline (P), glutamate (E), serine (S), and threonine (T)); S: sumoylation site;

IER2 was identified in a set of genes which are inducible by serum and growth factors e.g., fibroblast growth factor (FGF) in mouse fibroblasts. [36, 41] Its mRNA is expressed during the G0/G1 transition of quiescent to activated fibroblasts [34] as well as in fibroblasts treated with heavy metals. [42] Furthermore, *Ier2* is inducible in the rat pheochromocytoma cell line PC12 by addition of nerve growth factor (NGF) or epidermal growth factor (EGF) stimulation [34, 36] suggesting that neurogenic growth factor-induced expression of *pip92* is critical for the regulation of neuronal differentiation. *pip92* mRNA also accumulates in activated B- as well as T-cells. [35] Human *IER2* mRNA is induced in human promyelocytic leukemia cell line HL-60 undergoing macrophagic differentiation upon PMA stimulation activating protein kinase C (PKC) signaling. [37]

Sharing these characteristics with other IEGs, *pip92* promoter analysis further reveal that transcriptional activation by serum, growth factors, and PKC activator PMA is mediated through a serum-response element (SRE) consisting of both, Ets (E-twenty-six specific) and

CArG (CC,AT-rich,GG)-like elements similar to those found in other IEGs. Its activation appear to be mediated through a ternary complex involving members of the Ets family of transcription factors, especially ETS-like transcription factor 1 (Elk-1), and the serum response factor (SRF) binding to DNA. However, the *pip92* response element sequence is distinct from others (e.g., *c-fos*) but similar to nuclear receptor subfamily 4A (*nur77*) [43] forming not only ternary complexes but also binary complexes directly interacting with Elk-1, that alters the DNA binding properties of both, allowing them to bind a broader range of sequences. This spectra is extended hence the *IER2* promoter sequence additionally exposes a consensus cyclic adenosine monophosphate (cAMP) response element (CRE) and two κB binding sites. [44, 45] The first is supported by *pip92* mRNA accumulation by increased intra-cellular cAMP levels in fibroblasts. [34, 45] The latter are most likely used during *IER2* mRNA induction by pro-inflammatory stimuli (IL-1β, TNFα) shown in primary human endothelial cells [46] and a human cancer cell line (HeLa). [47] *IER2* is also expressed during blood coagulation in keratinocytes, a first step in wound repair, [48] and contrary in neutrophils upon co-stimulation with anti-inflammatory agents and inflammatory agonists. [49]

Rat hepatoma cells show *pip92* accumulation and a possible crosstalk between the extra-cellular signal-regulated kinase (ERK) cascade as well as the p38 mitogen-activated protein kinase pathway in insulin signaling is discussed. [50] Moreover, *pip92* levels are elevated during anisomycin-induced cell death by c-Jun N-terminal kinase (JNK) and p38-dependent activation of Elk-1 in fibroblasts and independent of ERK1/2 by activated rapidly growing fibrosarcoma protein (Raf1) in rat neuronal cells. Besides, rapid activation and followed deactivation of Ier2 synthesis involves a mitogen-activated protein kinase (MAPK)-dependent pathway. MAPK-independent Elk-1 phosphorylation demonstrates a steady state of Ier2 that is maintained for hours, which is linked to differentiation of these cells. [51, 52] Additionally, *pip92* is induced in a long-time kinetic showing pro-apoptotic activity which results in cell death in primary neurons after focal cerebral ischemia. [53]

Besides, the regulation of cell cycle progression is under the control of the transcription factor E2F. Interestingly, E2F1 and IER2, both, have the ability to bind to the promoter and act as transcriptional activators for the *ISYNA1*, a gene that encodes human inositol 1-phosphate synthase, playing important roles in several biological processes. [54] Furthermore, *IER2* is transcriptional activated in human T-cell leukemia virus type I (HTLV-I) infected cells and is regulated by Tax, a viral oncogene showing tumorigenic growth stimulating activity and

playing a role in adult T-cell leukemia (ATL). [44] In melanocytes exposed to ultraviolet (UV)-A radiation, an important carcinogen in melanoma development, *IER2* is one out of eleven overexpressed genes among 588 studied. Hence, *IER2* is suggested as a new candidate gene of melanoma development. [55] Moreover, *pip92* accumulates in highly proliferative cells [41] and *IER2* is increased in colon cancer, breast cancer, and metastatic melanomas. [56] Recently it was published that IER2 promotes tumor cell motility and metastasis and predicts poor survival of colorectal cancer patients. [39] Interestingly, IER2 shares weak homology to proto-oncogenes JUNB and JUND, [35] both located at the same region on chromosome 19. Translocations of this area are described in some cases of acute non-lymphoblastic leukemia and acute lymphoblastic leukemia. [44]

Summarizing, IER2 protein function is not completely understood so far, but is suggested to influence cell proliferation, apoptosis, and differentiation. If IER2 has oncogenic potential or represents a novel potent tumor marker has to be further investigated. However all these mechanisms are specific in a cell type and signal transduction pathway dependent manner.

1.3. The mammalian cell cycle

The cell cycle is regulated by the chronological activation and deactivation of protein kinases known as cyclin-dependent kinases (cdks). Cdk activation requires binding to cyclins, its regulatory subunits. Distinct cyclin/cdk complexes act at different cell cycle stages to control two key checkpoints, the G1/S and G2/M transitions. Early G1 progression is dependent on cdk4 and cdk6 assembly to one of the three D-type cyclins which are induced as a response to mitogens. Transition through G1/S phase requires activation of cdk2, first regulated by cyclin E in late G1 and followed by cyclin A after S phase entry. The G2/M checkpoint is regulated by cdc2 (cdk1) binding to cyclins A and B. [26, 27, 57]

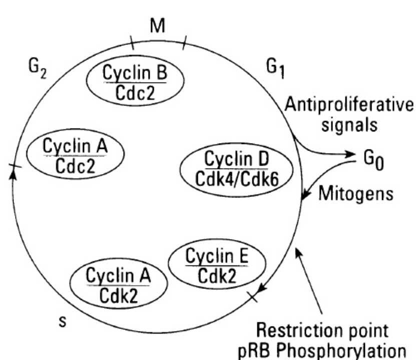


Fig.5: Schematic representation of cyclin/cdk protein complexes during the cell cycle. Figure from Shackelford *et al.* [57]

1.3.1. PKCδ bimodal regulates the cell cycle in endothelial cells

An increasing number of studies link PKC activation with the regulation of the cell cycle in the biodiversity of cell types. Most PKC-mediated effects on cell cycle progression have been described on its inhibitory function in the majority of assessed biological systems. However, there are also supporting data that associate PKC with positive control of both checkpoints, G1/S and G2/M. In vascular endothelial cells it has been described that time-dependent PKC activation essentially regulates G1/S phase progression. In this cellular context, PKC short-term activation early in G1 potentates G1/S transition, while activated PKC in mid-to-late G1, or long-time stimulation throughout G1, avoids S phase entry. [58, 59] This is in line with the expression of immediate early genes upon PKC agonist (phorbol esters) stimulation, suggesting a PKC-mediated mechanism that promotes cell proliferation in certain cell types. [60] Nevertheless, growth inhibitory effects of PKC signaling in endothelial cells can overcome the proliferative impulse.

PKC signaling is regulating the phosphorylation state of pocket proteins of the retinoblastoma family which comprises the retinoblastoma protein (pRb), p107, and p130. Thus, PKC activation in early G1 promotes phosphorylation of pocket proteins and growth factor-induced stimulation of DNA synthesis. Instead, activation of PKC in late G1 maintains pRb in an unphoshorylated (hypophosphorylated) state and inhibits G1/S phase transition. In addition PKC influences the expression and activity of the E2F transcription factor family. Phorbol ester-induced activation of PKC results in bimodal regulation of E2F1 levels in human umbilical vein endothelial cells depended on the timing of PKC activation in G1. [59] Furthermore, different PKC isozymes mediate distinct effects on E2F transcriptional activity. PKCα overexpression in fibroblasts inhibits, whereas PKCδ or PKCε overexpression enforces E2F activity. Noteworthy, overexpression of any one of these isoforms resulted in enhancement of phorbol ester-induced inhibition of E2F activity in late G1 phase. [26] Moreover, PKC induces inhibitory effects on cyclin E/cdk2 complex activity in vascular endothelial cells, and overexpression of PKCδ in rat microvascular endothelial cells significantly delays the induction of cyclin D1-associated kinase activity after serum stimulation. Indeed, although cyclin D activity was not directly measured, phorbol ester treatment inhibits cyclin D1 activity in human venous endothelial cells based on the presence of increased levels of cell cycle inhibitor $p21^{waf1/cip1}$ in cyclin D1/cdk4 complexes (see section below). [61, 62]

PKCδ generally executes pro-apoptotic signaling during DNA damage-induced apoptosis, but it also functions as an anti-apoptotic protein during receptor-initiated cell death. This bimodal function of PKCδ depends on e.g., its intra-cellular localization, its tyrosine phosphorylation, and on the presence of other pro- and anti-apoptotic target proteins and involved signaling pathways. In this regard, PKCδ in different cancer subtypes can specifically affect tumor survival or tumor suppression. [63]

1.3.2. p21$^{waf1/cip1}$ as a key target in of PKC-mediated cell cycle modulation

As a member of the Cip/Kip family of cyclin/cdk inhibitors (CKI), comprising p21$^{waf1/cip1}$, p27^{kip1}, and p57^{kip2}, respectively, p21$^{waf1/cip1}$ was first described as a powerful and universal inhibitor of cdks. [64] According to accumulating data, p21$^{waf1/cip1}$ functions as a regulator of the cell cycle by inhibiting cdks at, both, the G1/S and G2/M checkpoints. Moreover, p21$^{waf1/cip1}$ binds to cyclin/cdk complexes, thus preventing phosphorylation of the pRb and leading to a blockade of both, the E2F pathway and the cell cycle. [65] *p21$^{waf1/cip1}$* expression is generally controlled at the transcriptional level by either tumor protein tp53-dependent or -independent pathways, [[66, 67] but p21$^{waf1/cip1}$ expression can additionally be regulated at the post-transcriptional level. [68] However, p21$^{waf1/cip1}$ is more than a CKI. Besides the cell cycle regulatory role it is well documented that p21$^{waf1/cip1}$-dependent on its subcellular localization and involved regulatory pathways can directly regulate gene expression of pro- and anti-apoptotic genes. [69]

In summary it can be stated that p21$^{waf1/cip1}$ has quite controversial functions, namely it i) is implicated in promoting as well as protecting cells from apoptosis, ii) is associated to induce and inhibit cell survival, iii) is linked to inhibit and facilitate differentiation, and iv) shows tumor suppressor activities and paradoxical tumor-promoting activities in cell context- and stress-dependent manners. (Reviewed in [69, 70])

1.3.3. Influence of p21$^{waf1/cip1}$ on PKCδ-driven cell cycle regulation

PKCδ dual regulates the cell cycle in endothelial cells. Under non-stressed physiological conditions, p21$^{waf1/cip1}$ is expressed at low levels and promotes cell cycle progression. Under PKC-mediated stress conditions, short-time events early in G1 supports G1/S phase progression, while activated PKC in mid-to-late G1 and concomitant increased p21$^{waf1/cip1}$ expression prevent entry of cells into S phase. [26]

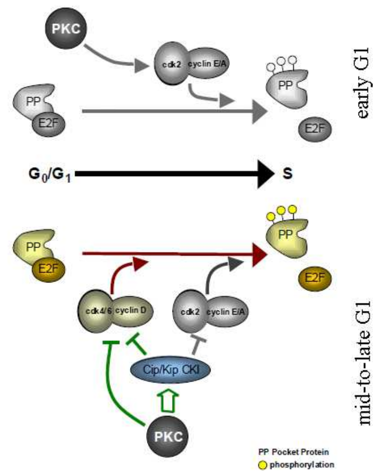


Fig.6: Bimodal PKC-mediated regulation of G1/S transition and its inhibition by p21$^{waf1/cip1}$. The upper panel (above the arrow indicating G0/G1→S progression) illustrates the consequences of PKC activation in early G1, while the lower part (below the arrow) represents PKC activation in mid-to-late G1 phase. PP: pocket proteins of retinoblastoma family. Modified from Black Review. [26]

In particular, PKCδ inhibits cell cycle progression in human vein endothelial cells by the inhibition of cyclin D1/cdk4/6 activity, and an increase in levels of cyclin-dependent kinase inhibitors (CKI), such as p21$^{waf1/cip1}$ and to lower extent p27^{kip1} (see Figure 6: highlighted in color is PKCδ p21$^{waf1/cip1}$ mechanism). [62, 71]

1.3.4. tp53-independent regulation of p21$^{waf1/cip1}$

Despite that p21$^{waf1/cip1}$ is one of the best studied tp53 downstream targets, various signals activate *p21*$^{waf1/cip1}$ transcription independent of the tp53 pathway. [72] These factors induce binding of different transcription factors to specific cis-acting elements located within the *p21*$^{waf1/cip1}$ promoter. Besides others, PMA and okadaic acid PKC-induced activation of its promoter is mediated by members of the specific protein 1 (Sp1) family of transcription factors, including Sp1 and Sp3. [73, 74] Noteworthy, control of p21$^{waf1/cip1}$ levels by PKC signaling appears to be overall complex, involving transcriptional and post-transcriptional mechanisms. [72, 75, 76] PKC can regulate p21$^{waf1/cip1}$ at the level of mRNA transcription, stabilization and translation, as well as by enhanced protein stability. Moreover, the mitogen-activated protein kinase (MAPK) cascade is involved in PKC agonist-mediated induction of p21$^{waf1/cip1}$. [26, 75] Chemical as well as genetically inhibition of the MAPK kinase 1 (MEK) inhibits DAG analog-induced *p21*$^{waf1/cip1}$ expression. [62, 75, 77]

1.3.5. p21$^{waf1/cip1}$ and PKCδ in cancer development and progression

As mentioned above, both p21$^{waf1/cip1}$ and PKCδ can exhibit pro-cancer or anti-cancer activity in different tumor cells due to heterogeneity in their regulation in a cell context- and stress-dependent manner. However, these opposing mechanisms can be explained based on the fact that malignant diseases such as cancer are heterogeneous cell formations and that diverse

mechanisms affect cancer development and progression/differentiation to metastatic cell clusters. PKC-mediated increases in $p21^{waf1/cip1}$ levels have been observed in melanoma cells, various leukemic cell lines, breast cancer cells, non-transformed intestinal epithelial cells, colon carcinoma cells as well as ovarian carcinoma cells. Furthermore, $p21^{waf1/cip1}$ is found to show decreased expression in some types of metastatic cancers, especially in late melanoma stages. [63, 78-80]

The complex signaling that regulates $p21^{waf1/cip1}$ expression and its biological functions warrants caution targeting $p21^{waf1/cip1}$ in cancer therapy. The goal is to selectively inhibit solely the oncogenic potential and not the tumor suppressor functions. Therefore, instead of direct targeting, affecting upstream or downstream factors of $p21^{waf1/cip1}$ that control its bimodal functions is the challenge. [78]

1.4. Aim of the Study

Acute inflammation, a beneficial system, requires proper activation as well as termination signals, since otherwise it may lead to chronic inflammatory conditions. These are considered as hallmarks of several diseases, including inflammation-mediated and cardiovascular diseases (e.g., psoriasis, rheumatoid arthritis, and arteriosclerosis) as well as tumor development, tumor progression, and metastasis. It is of outstanding importance to understand the complex mechanism behind the tight regulation of acute inflammation and malfunctioning signaling events that lead to a prolonged inflammatory stage. To find early regulators of this process might lead to new concepts of drug development and thereby to great benefit for patients suffering inflammation-related diseases.

Based on the observation that immediate early response gene 2 has been classified as an IEG and moreover found to be early induced in various cell types including human endothelial cells, [46] we define the overall aim of this project is to describe and clarify a functional role of IER2 in the human endothelium. We intend to evaluate if this protein with unknown function acts as a central player in the first transcriptional response upon pro-inflammatory stimuli. Furthermore, we address the question if IER2 is solely linked to inflammation or to other molecular mechanisms that are regulated in endothelial cells.

To provide a clear layout of our research program we define four specific aims:

i) Identification of promising first and second messengers that activate *IER2* expression
ii) Characterization of the IER2 protein and its regulation by posttranslational modifications that influence its expression
iii) Delineation of the impact of IER2 on downstream targets as well as influenced signaling cascades
iv) Elucidation of the biological role of IER2 and its impact on cellular mechanisms

Moreover, our findings will provide new information on the early gene regulation in the endothelium and we will gain more insight into the importance of proper regulation in the early steps of cellular signaling.

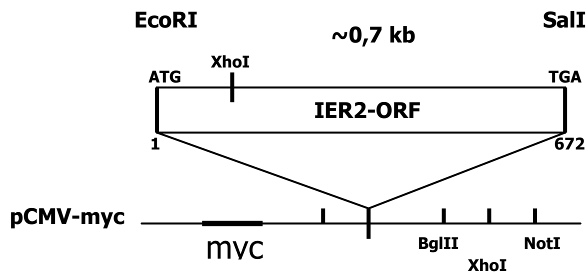
2. MATERIAL AND METHODS

2.1. Plasmids and cloning

2.1.1. IER2 plasmids

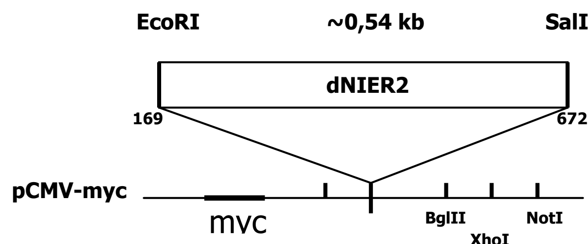
The coding sequence of human *IER2* was amplified by V. Kurtev from reverse transcribed cDNA of one hour IL-1β stimulated HUVEC mRNA by using the Expand High Fidelity^{PLUS} PCR System (Roche). The PCR product was digested with *EcoRI*/*SalI,* purified with GFX PCR columns (GE Healthcare illustra™) and ligated into *EcoRI*/*SalI* digested pCMV-myc vector (Clontech). Full length myc-IER2 (Map.1) and all further used plasmids were sequenced by Eurofins MWG Operon.

Map.1: pCMV-myc-IER2



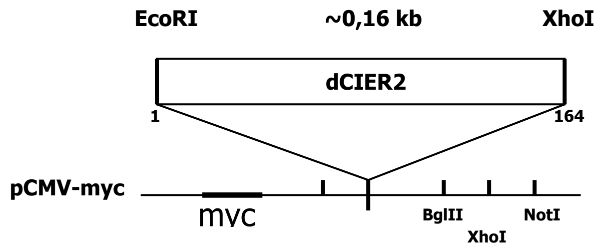
N- and C-terminal deletions (ΔN, ΔC) are described below in Map.2 and Map.3, respectively. Amino acids 57-223 of the human IER2 coding sequence were amplified by Expand High Fidelity^{PLUS} PCR from the vector pCMV-myc-IER2 (Map.1). The ~500bp PCR product was ligated into *EcoRI*/*SalI* digested pCMV-myc vector. Constructs were sequenced and tested for expression of the correct protein by Western Blotting after transient transfection into HEK293 cells.

Map.2: pCMV-myc-dNIER2



pCMV-myc-IER2 was cut with *XhoI*, purified with GFX PCR columns and re-ligated to achieve amino acids 1-56 of the human IER2 coding sequence. In frame clones with 12 amino acids exceeded stop codon were sequenced and analyzed by Western Blotting.

Map.3: pCMV-myc-dCIER2



IER2 plasmid cloning primers:

IER2 ORF fwd.: CGGAATTCCCATGGAAGTGCAGAAAGAG

IER2 ORF rev.: CGGTCGACTCAGAAGGCCACCAC

IER2dN aa57-223: CGGAATTCAGCCCGAGGTGTCGTTG

myc fwd.: GATCACCATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGGG

myc rev.: AATTCCCAGGTCCTCCTCTGAGATCAGCTTCTGCATTGATGCCATGGTG

2.1.2. Expression and reporter plasmids

The reporter plasmid p21$^{waf1/cip1}$-luciferase [81] was kindly provided by H. Rotheneder (Max F. Perutz Laboratories, Medical University of Vienna), the E2F1 [82] and 3xE2F-luciferase [83] plasmids were kind gifts from the former lab of E. Wintersberger (University of Vienna). pCMV-ßgal and tp53 expression plasmid were obtained from Clontech. p21$^{waf1/cip1}$-flag (pcDNA3.1-Flag, Invitrogen) was cloned by U. Resch (University of Cologne). Lentiviral overexpression plasmid (pWPT-GFP, Map.10) as well as knockdown plasmids (pLKOpuro.1, Map.4), packaging (psPAX2) and envelope plasmids (pMD2.G) were kindly provided by H. Stockinger (Medical University of Vienna). [84, 85]

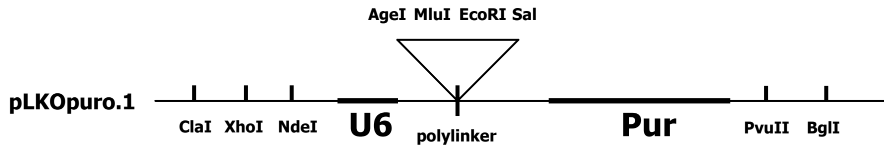
2.1.3. RNA interference

shRNA PKCδ and scrambled shRNA (scr), nontargeting any known human or mouse gene were obtained from Sigma (SHCLNG MISSION®shRNA 03251106MN).

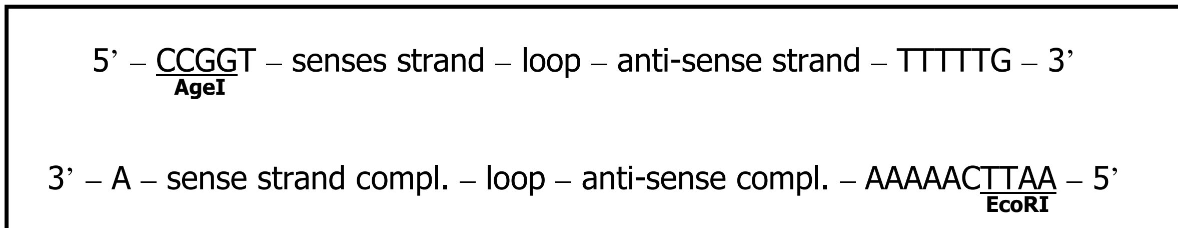
shRNA sequences silencing specific to different regions of IER2 (shIER2a and shIER2b) were selected by using the free available software RNAxs webserver http://rna.tbi.univie.ac.at /cgi-bin/RNAxs. Two target sequences, shIER2a at position 1713 (5´-GGACTGATCTAC TTTCACA-3´) and shIER2b at position 769 (5´-CCGTCTGGAAGAAAAGGAA-3´), were then used to design short hairpin constructs with *AgeI* restriction site on the sense strand and *EcoRI* site on the complementary strand (see scheme Map.5). Oligos were annealed by using 5µl sense (20µM) and 5µl antisense (20µM), 5µl 10xNEB buffer2 (New England Biolabs),

and 35µl ddH_2O. Annealing was performed for five minutes at 95°C followed by two hours at room temperature (RT). pLKOpuro.1 (Map.4) was digested by *AgeI/EcoRI* and ligated with annealed oligos. pLKOpuro.1-shIER2a and -b (Map.6) were sequenced by Eurofins MWG Operon.

Map.4: pLKOpuro.1



Map.5: scheme shRNA oligo design and sequence



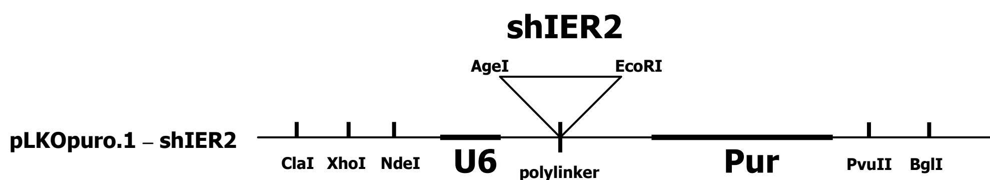
shIER2a

5´-CCGGTGGACTGATCTACTTTCACAttcaagagaTGTGAAAGTAGATCAGTCCTTTTTG-3´
5´-AATTCAAAAAGGACTGATCTACTTTCACATCTCTTGAATGTGAAAGTAGATCAGTCCA-3´

shIER2b

5´-CCGGTCCGTCTGGAAGAAAAGGAAttcaagagaTTCCTTTTCTTCCAGACGGTTTTTG-3´
5´-AATTCAAAAACCGTCTGGAAGAAAAGGAATCTCTTGAATTCCTTTTCTTCCAGACGGA-3´

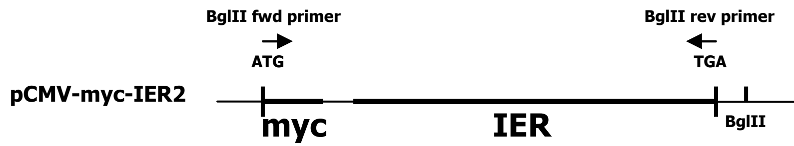
Map.6: pLKOpuro.1-shIER2a/b



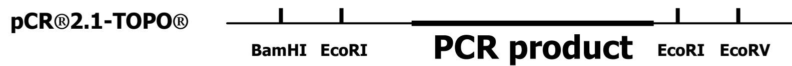
2.1.4. Overexpression (pwpt-GFP, pwpt-myc-IER2)

The human IER2 coding sequence, N-terminally fused to a myc-Tag, was amplified by PCR out of the vector pCMV-myc-IER2 (Map.7) using the primers *BglII*-IER2fwd. (5´-CGAGATCTACCATGGCATCAATGCAG-3´) and *BglII*-IER2rev. (5´-CGAGATCTTCAGAAGGCCACCACG-3´). The ~1kb PCR product was ligated into pCR®2.1-TOPO® (Invitrogen, Map.8) creating pCR®2.1-TOPO®-myc-IER2 (Map.9).

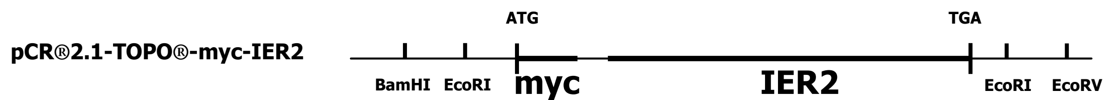
Map.7: pCMV-myc-IER2-*BglII*



Map.8: pCR®2.1-TOPO®

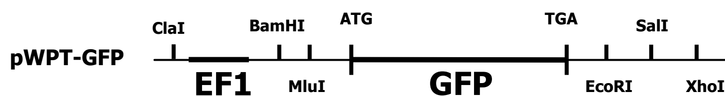


Map.9: pCR®2.1-TOPO®-myc-IER2

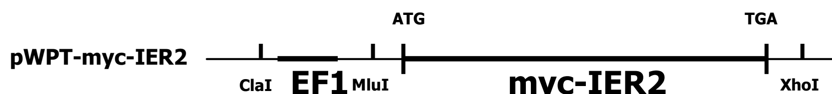


The lentiviral overexpression vector pWPT-GFP (Map.10) was digested with *BamHI* and *SalI* to cut out GFP. First, the vector was digested with *SalI*, followed by creating a blunt end with *Pfu* polymerase (Promega), and then digestion with *BamHI* was performed. pCR®2.1-TOPO®-myc-IER2 was cut with *BamHI/EcoRV* followed by ligation into digested pWPT vector to create pWPT-myc-IER2 (Map.11). Control digest was performed with *XhoI* and positive clones were sequenced.

Map.10: pWPT-GFP



Map.11: pWPT-myc-IER2



2.2. In vitro cell culture system

2.2.1. Cell culture

Human embryonic kidney cells (HEK293 and HEK293T) were obtained from ATCC and were cultured in DMEM (Bio-Whittaker) supplemented with 10% fetal calf serum (FCS, Sigma), 2mM L-glutamine (Sigma), penicillin (100U/ml), and streptomycin (100µg/ml).

Human umbilical vein endothelial cells were isolated from umbilical cords as described elsewhere [86] and maintained in M199 medium (Lonza) supplemented with 20% FCS, 2mM L-glutamine (Sigma), penicillin (100U/ml), streptomycin (100µg/ml), Heparin (5U/ml, and 25µg/ml endothelial cell growth supplement (ECGS, Promocell). Prostate cancer cell lines (LnCap, DU145, PC3) were kindly provided by J. Schmid (Medical University of Vienna) and maintained in RPMI 1640 medium (Sigma) supplemented with 10% FCS, penicillin (100U/ml), and streptomycin (100µg/ml). Colon cancer (Caco2, HCT-116), breast cancer (ZR-751, MCF7, MDA-MB-468, MDA-MB-231), and melanoma cells [87] were maintained with respective culture media. They, as well as protein extracts were kind gifts from C. Wiesner (University of Applied Sciences, Krems). All used cell lines and primary HUVEC cells were maintained in 5% CO_2 at 37°C.

2.2.2. Cytokines, growth factors, and inhibitors

Human recombinant (hr)-TNFα and hr-IL-1β were purchased from R&D systems. Both were stored as 10µg/ml stocks according to manufactures protocols and used at a final concentration of 10ng/ml. hr-bFGF (Roche) and hr-VEGF (Calbiochem) were used at final concentrations of 10ng/ml and 10µg/ml, respectively. Cytokine and growth factor stimulation was performed after serum starvation, maintaining cells 24 hours in media containing 2% FCS. Phorbol ester PMA (Sigma) was stored in dimethyl sufoxide (DMSO) and used at a final concentration of 50 to 250ng/ml as indicated. Ionomycin and the PKC inhibitor Rottlerin were obtained from Sigma and were used at final concentrations of 100ng/ml and 5µM, respectively. Rottlerin was added thirty minutes before harvesting cells for mRNA analysis and one hour for protein analysis. The proteasome inhibitor MG132 (Affinity) was used at a final concentration of 10µM for four hours. The transcriptional inhibitor Actinomycin D (Sigma) was stored as 1mg/ml stock in DMSO and used at a final concentration of 10µg/ml for one hour.

2.2.3. Transient transfection and reporter gene assays

HEK293 cells were transfected by the calcium phosphate method as described elsewhere. [88] For transient transfection cells were cultured in 6-well plates and transfected as described with the designated expression plasmids. Twenty-four hours post transfection cells were treated with indicated substances followed by mRNA or protein isolation. For analyzing protein knockdown efficiency cells were harvested forty-eight hours post transfection. For reporter gene assays, HEK293 cells were maintained in 24-well plates and transfected with

the indicated reporter and/or expression plasmids. Forty-eight hours post transfection cells were lysed in 1xPassive Lysis buffer (Promega) for one hour at room temperature (RT) followed by luciferase activity analysis using a Luminometer (Luminoskan *Ascent*, Labsystems). The reaction mix for luciferase activity measurements contained 25mM Glycin-Glycin (pH 7.8), 0.2mM D-Luciferin (Roche), 2mM ATP (pH 7.5) and 10mM $MgSO_4$. β-galactosidase was measured by adding chlorophenolred-β-D-galactopyranoside (CPRG, Roche) to cell lysates followed by absorbance analysis at 570nm using an ELISA reader. Experiments were performed in triplicates and luciferase values were normalized to β-gal expression and are depicted as mean fold induction. Error bars represent the standard deviation of the mean and are representatives of at least three independent experiments.

2.2.4. Lentivirus production and target cell infection

HEK293T cells were cultured in 6-well plates and transfected with 1µg lentiviral overexpression plasmid (pWPT-IER2) or knockdown plasmid (shPKCδ) in combination with 750ng packaging (psPAX2) and 250µg envelope plasmid (pMD2.G). [84, 85] Supernatants were harvested and pooled after twenty-four and forty-eight hours, followed by centrifugation at 1250 rpm for five minutes to remove any HEK293T cells. The lentiviral particles were stored at -20°C or at -80°C for long-time storage. For infecting target cells, HUVEC were seeded in 6-well plates and grown to approximately 70% confluence followed by changing to fresh media containing 5µg/ml polybrene. Then lentiviral particals were mixed with medium in a ratio of 1:1 or 1:2 approximately ending up at multiplicity of infection (MOI) of 100. mRNA was analyzed within three days, protein knockdown or overexpression and phenotypic assays within four days post infection.

2.3. Gene expression analysis

2.3.1. RNA isolation and cDNA synthesis

High Pure RNA Isolation Kit from Roche was used according to the manufactures protocol for extraction of total RNA. Cells were harvested and washed two times with phosphate buffered saline (PBS) and then mixed with Lysis buffer. High Pure filter tubes were loaded with cell lysates by a centrifugation step (5000rpm for five minutes) followed by DNAse digestion. After two washing steps total RNA was eluted with nuclease-free water and RNA concentration was assessed by photometry at wavelengths of 260 and 280nm. 1µg of total RNA was reverse transcribed in a total of 20µl using MuLV reverse transcriptase and random hexamers as primers (GeneAmp RNA PCR Kit, Applied Biosystems, Roche).

2.3.2. qRT-PCR analysis

For analyzing mRNA levels, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using 2.5µl of 1:10 diluted cDNA (12.5ng) as template. The Roche LightCycler was used for PCR utilizing the SYBR green detection method. All experiments were done in triplicates, the relative amount of mRNA was calculated by using the Pfaffl method and normalized to housekeeping genes (GAPDH or β-actin) as internal standard. Error bars represent the standard deviation of the mean. All oligonucleotide primers were designed using the freely available software Primer3 (http://frodo.wi.mit.edu/primer3) and sequences are listed in Table 1.

Table 1: qRT-PCR primer sequences (5´- 3´ direction)

Primer/gene	Forward	Reverse
IER2	CCAAAGTCAGCCGCAAACGA	TTTCTTCCAGACGGGCTTTCTTGC
ELAM-1	CCTGTGAAGCTCCCACTGA	GGCTTTTGGTAGCTTCCATCT
EGR-1	TGACCGCAGAGTCTTTTCCT	TGGGTTGGTCATGCTCACTA
PKCδ	GCCTCAACAAGCAAGGCTAC	AGGTGGGGCTCATGTAGTTG
IκBα	CACTTGCAGAGGGACAGGAT	CTGGCTGGGGATTTCTCTG
MEKK1	AGAGATGTCAAAGGTGCCAAT	ACCTCAGGTGCCATAAATGC
c-MYC	CGGGTAGTGGAAAACCAGCA	CAGCAGCTCGAATTTCTTCC
JUNB	AACAGCCCTTCTACCACGAC	GCTCGGTTTCAGGAGTTTGT
MDM2	CAATCAGCAGGAATCATCGGA	GATCAAAAGGACCTTGTACAAG
E2F1	AGATGGTTATGGTGATCAAAGCC	GGACTCTTCGGAGAACTTTCAGAT
Rb1	CTTGCATGGCTCTCAGATTCAC	CACCTTGAATCTGCTTGTCCTCT
tp53	GTG GAA GGA AAT TTG CGT GT	CCAGTGTGATGATGGTGAGG
p27	ACC CCT AGA GGG CAA GTA CG	GCG TGT CCT CAG AGT TAG CC
cyclin D1	GCCTCAACAAGCAAGGCTAC	AGGTGGGGCTCATGTAGTTG
cyclin E1	CTCCAGGAAGAGGAAGGCAA	TGAAGAAATGGCCAAAATCGA
p21	GAGCGATGGAACTTCGAC TT	CAGGTCCACATGGTCTTCCT
BCL-2	GAGGATTGTGGCCTTCTTTG	TTCAGAGACAGCCAGGAGAAA
GADD45b	TGATGAATGTGGACCCAGAC	GGTTCGTGACCAGGAGACAA
ICAM-1	CCATACAGGACACGAAGCTC	CCATACAGGACACGAAGCTC
VCAM-1	CCGGCTGGAGATTGAATTAC	TGTATCTCTGGGGGCAACAT
GAPDH	AGAAGGCTGGGGCTCATTT	CTAAGCAGTTGGTGGTGCAG
β-actin	TTTGAATGATGAGCCTTCGTCCCC	GGTCTCAAGTCAGTGTACAGGTAAGC

2.4. Protein extraction and Immunoblotting

2.4.1. Whole cell extract purification

For Western Blotting of transient transfected HEK293 cells and HUVEC, cell lysates were prepared using Laemmli sample buffer. Cells were rinsed twice with PBS, the pellet was resuspended in PBS and 2xLaemmli loading buffer. For HEK293 protein extracts from a 6-well 80µl 2xLaemmli were used and for HUVEC protein extracts from a 6-well 50µl 2xLaemmli were added to end up in 1µg/µl per sample. This was followed by three freeze-thaw cycles (liquid nitrogen, 95°C) before performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

For Western Blotting of reporter gene assay samples, cells were washed with PBS and resuspended in 1xPassive Lysis buffer (Promega) for one hour at RT. Cells from technical triplicate were pooled and 5xLaemmli loading buffer was added and followed by three freeze-thaw cycles before loading on SDS-polyacrylamide gels.

5xLaemmli loading buffer
50% (v/v) Glycerin
10% (w/v) SDS
0.4M Tris-HCl pH 6.8
5mg Bromphenol blue
10% (v/v) β-mercaptoenthanol

2xLaemmli loading buffer
20% (v/v) Glycerin
4% (w/v) SDS
0.125M Tris-HCl pH 6.8
5mg Bromphenol blue
10% (v/v) β-mercaptoenthanol

2.4.2. Nuclear and cytoplasmic fractionation

For endogenous IER2 and $p21^{waf1/cip1}$ localization HUVEC were seeded in $10cm^2$ dishes and maintained for forty-eight hours followed by PMA stimulation. For ectopic protein localization HEK293 cells were transfected in 6-well plates and forty-eight hours post transfection cells were stimulated with PMA for the indicated time points. Cells were harvested, washed twice with PBS and nuclear and cytoplasmic extracts were prepared. Pellets were lysed in hypotonic lysis buffer (Low Salt buffer) complemented with protease inhibitors (PI, Roche) for ten minutes on ice. Subsequently, 0.25% nonyl phenoxypoly-ethoxylethanol (NP40) was added for another fifteen minutes on ice to break the cytoplasmic membrane. After centrifugation at 3000rpm for five minutes supernatant (cytosolic protein fraction) was collected and mixed with 5xLaemmli loading buffer. Cell pellets were resuspended in hypotonic lysis buffer (complemented with PI and 0.25% NP40) for another fifteen minutes on ice to lyse remaining intact cells and to purify the nuclear protein fraction. After centrifugation supernatant was discarded and pellet (representing the nuclei fraction) was rinsed with ice cold PBS. Subsequently, cell pellets were lysed in High Salt buffer (>350mM NaCl) for fifteen minutes on ice. After centrifugation at 13200rpm for ten minutes supernatant (nuclear protein fraction) was collected.

Low Salt buffer
10mM Tris-HCl pH 7.5
10mM KCl
0.25% (v/v) NP40
protease inhibitors (PI)

High Salt buffer
20mM Tris-HCl pH 7.5
400 mM NaCl
0.5% (v/v) NP40
0.3% (v/v) TritonX 100

2.4.3. Western Blotting

Protein extracts were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Table 2) using the Bio-Rad Western Blotting System. Size separated proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham) by semi-dry blotting (Peqlab Semidry Blotter). Transfer efficiency was evaluated by PonceauS (Serva) staining and subsequently membranes were blocked in 5% non fat dry milk in PBS/Tween-20 (0.1%, Amersham) for 1h at RT. Incubation with the first antibody (Table 3) was performed over night at 4°C and horseradish peroxidase (HRP)-labelled secondary antibodies (see Table 4) were incubated in 2% non fat dry milk in PBS/Tween-20 for one hour at RT after three washing steps in PBS/Tween-20. Using chemiluminescence (WestPico, Pierce & ECL detection reagents, Amersham) and X-Ray films (SuperRX films, Fujifilm) respective proteins were visualized.

Table 2:

Separating gel	10%	12.5%	15%		Stacking gel	4%
H_2O	4.1ml	3.3ml	2.45ml		H_2O	3.1ml
Sol.A	2.5ml	2.5ml	2.5ml		Sol.B	1.25ml
Acrylamide	3.4ml	4.2ml	5.0ml		Acrylamide	0.65ml
APS (10%)	50µl	50µl	50µl		APS (10%)	50µl
TEMED	10µl	10µl	10µl		TEMED	10µl

Sol.A
1.5M Tris-HCl pH 8.8
0.4% SDS

Sol.B
0.5M Tris-HCl pH 6.8
0.4% SDS

Acrylamide
Acrylamide/Bis-Acrylamide
37.5:1; Serva

APS
10% (w/v) Ammoniumperoxydisulphate

TEMED
N,N,N´,N´-Tetramethylen-ethylendiamine

10xRunning buffer
0.25M Tris-Base
2M Glycine
1% SDS

10xTowbin buffer
0.25M Tris-Base
1.92M Glycine
1% SDS
pH 8.3

Densitometric analysis of cyclin D1 and p27 was done using the background-corrected integrated densities of the cyclin D1 and p27 bands normalized to the β-actin bands assessed by the ImageJ software freely available at http://rsb.info.nih.gov/ij/.

2.4.4. Antibody list

Table 3: first antibodies

Specifictiy	Provider	Cat. Number	Source	Dilution
IER2	Santa Cruz	sc-101980 (E-19)	rabbit	1:500-1:1000
p21	Santa Cruz	sc-397 (C-19)	mouse	1:1000
p27	Santa Cruz	sc-1641 (F-8)	mouse	1:1000
p27	Santa Cruz	sc-776 (M-197)	rabbit	1:1000
cyclin D1	Santa Cruz	sc-56302 (CD1.1)	mouse	1:1000
cyclin E	Santa Cruz	sc-56310 (13A3)	mouse	1:500
PCNA	Santa Cruz	sc-56 (PC10)	mouse	1:1000
PKCδ	Santa Cruz	sc-937 (C-20)	rabbit	1:1000
p-PKCδ Thr507	Santa Cruz	sc-11770	goat	1:500
PKCε	Santa Cruz	sc-214 (C-15)	rabbit	1:1000
p53	Santa Cruz	sc-6243 (FL-393)	rabbit	1:1000
c-MYC	Santa Cruz	sc-40 (9E10)	mouse	1:1000
SP1	Santa Cruz	sc-59 (PEP2)	rabbit	1:1000
NF-κB p65	Santa Cruz	sc-109 (A)	rabbit	1:1000
IκB-α	Santa Cruz	sc-371 (C-21)	rabbit	1:1000
EGR-1	Santa Cruz	sc-189X (C-19X)	rabbit	1:1000
PARP	Biomol	SA-250 (116/85kDa)	mouse	1:1000
PARP	cell signaling	9542P	rabbit	1:1000
cleaved PARP	cell signaling	9541P (D214)	rabbit	1:1000
anti-flag®	Sigma	F3165-1MG	mouse	1:2000
β-actin	Santa Cruz	sc-1616 (I-19)	goat	1:1000
GAPDH	chemicon	MAB374	mouse	1:100000

Table 4: secondary antibodies

Specifictiy	Provider	Cat. Number	Source	Dilution
α-rabbit IgG-HRP	GE Healthcare	NA934V	donkey	1:5000
α-mouse IgG-HRP	GE Healthcare	NA931V	sheep	1:5000
α-goat IgG-HRP	Santa Cruz	sc-2020	donkey	1:5000

2.5. Proliferation and apoptosis

HUVEC were left uninfected or were infected with control or IER2 expressing virus. Forty-eight hours post infection cells were stimulated with PMA (100ng/ml) for indicated time points and subsequently trypsinized for further analysis.

2.5.1. Cell count and alamarBlue® assay

To determine an effect on cell proliferation we analyzed the total cell number using a Neubauer counting camber. Experiments were performed in triplicates and repeated twice.

AlamarBlue® (Invitrogen) was used as a cell viability indicator. Viable, metabolically active cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of

viability and cytotoxicity. The amount of fluorescence produced is proportional to the number of living cells, using 560EX nm/ 590EM nm filter settings. Alternatively, the absorbance of alamarBlue® can be read on a UV-Vis spectrophotometer at 570 nm. Harvested cells (viable cells) were re-seeded with old media (to include dead cells) in 96-well plates in triplicates at a 10^4 cell density. After cells adhered/ sedimented to plates, 10µl alamarBlue® reagent per well was added. Within three to four hours resazurin was catalyzed to resorufin and the absorbance was recorded using an ELISA plate reader at 570nm. Cell viability was calculated, normalized to uninfected untreated control and depicted as viable cell in per cent compared to control.

2.5.2. Annexin V/7-AAD staining

Apoptosis is characterized by certain morphologic features including e.g., loss of plasma membrane asymmetry and attachment which in turn leads to the externalization of the membrane phospholipid phosphatidylserine (PS). [89] Annexin V is a 35-36 kDa phospholipids binding protein that has a high affinity for PS and binds to apoptotic cells that expose PS to the external cellular environment. Annexin V is conjugated to fluorochromes including Phycoerythrin (PE), used in combination with vital dyes such as 7-Amino-Actinomycin (7-AAD), and analyzed by flow cytometry. [90, 91] Viable cells with intact membranes exclude Annexin V and 7-AAD (double negative), early apoptotic cells are Annexin V positive and 7-AAD negative whereas the permeable membrane of late apoptotic or already dead cells are both, Annexin V and 7-AAD (double) positive.

For the assessment of apoptosis by Annexin V/7-AAD staining 10^4 cells were harvested into FACS tubes. Cells were washed two times with cold 1xAnnexin Binding buffer. Supernatant was discarded leaving approximately 100µl of residual fluid in the tube to avoid disturbing the pellet. Then 10µL Annexin V-PE (BD Biosciences Pharmingen, 1:10 diluted in 1xAnnexin Binding buffer) and 5µl 7-AAD viability staining solution (eBioscience) were added, shortly mixed and incubated for fifteen minutes in the dark at RT. Double stained cells were measured by flow cytometry (BD FACSCalibur™) and data were analyzed using the BD CellQuest™ Pro software.

<u>10xAnnexin Binding buffer:</u>
0.1 M Hepes
140mM NaCl
25mM $CaCl_2$
pH 7.4

2.5.3. PARP cleavage

As an established apoptosis indicator downstream of caspase activation we determined the cleavage of poly ADP-ribose polymerase (PARP) monitored by the detection of the 86 kDa cleaved product of PARP by Western blotting using anti-PARP specific antibodies recognizing both full length PARP and its cleaved fragment. [92, 93]

3. RESULTS

The basis of this study has been laid by a microarray analysis mimicking inflammatory activation in the endothelium, i.e. stimulation of primary human umbilical vein endothelial cells (HUVEC) with IL-1β. [46] Numerous genes that are linked to inflammation have been identified, some of them with immediate early kinetics. Besides, a number of functionally unexplored genes, which are assigned to various cellular processes, are also shown to be early induced in endothelial cells upon pro-inflammatory stimulation. One of the genes with unknown function was the immediate early response gene 2 (IER2). Herein, we aimed to investigate and characterize its function in the endothelium, as well as its role as a potential regulator influencing inflammatory signaling.

The rationale for choosing IER2 was that immediate early genes (IEGs) are rapidly and transiently induced in response to a plethora of extra- as well as intra-cellular stimuli. Thereby they encode in many cases transcription factors or other regulatory factors of high importance that act on different levels of signal transduction. Once activated, they represent the first cellular program that leads to secondary changes in a variety of cellular responses. IEGs are therefore important early regulators of different pathophysiological aspects of a cell, i.e., growth, differentiation, and in the case of endothelial cells, angiogenesis and inflammation. [31] It is of note that at the onset of inflammation some IEGs have been described to function, already at this early stage, as feedback mechanisms that contribute to the termination of the (inflammatory) reaction (e.g., NF-κB inhibitor IκBα, Tristetraprolin TTP). [12, 94, 95]

3.1. The IEG IER2 is regulated by a variety of inducers in HUVEC

3.1.1. Regulation of IER2 by pro-inflammatory cytokines

Based on the context above, HUVEC were treated with the classical pro-inflammatory cytokines TNFα and IL-1β, and analyzed for *IER2* expression using quantitative real-time PCR (qRT-PCR). Surprisingly, the experiments demonstrated that neither TNFα nor IL-1β led to a reproducible upregulation of *IER2* mRNA expression (Fig.7A/B, left panels, respectively). We used endothelial-leukocyte adhesion molecule 1 (ELAM-1) as positive control and we showed its typical expression pattern in this experimental setup (Fig.7A/B, right panels).

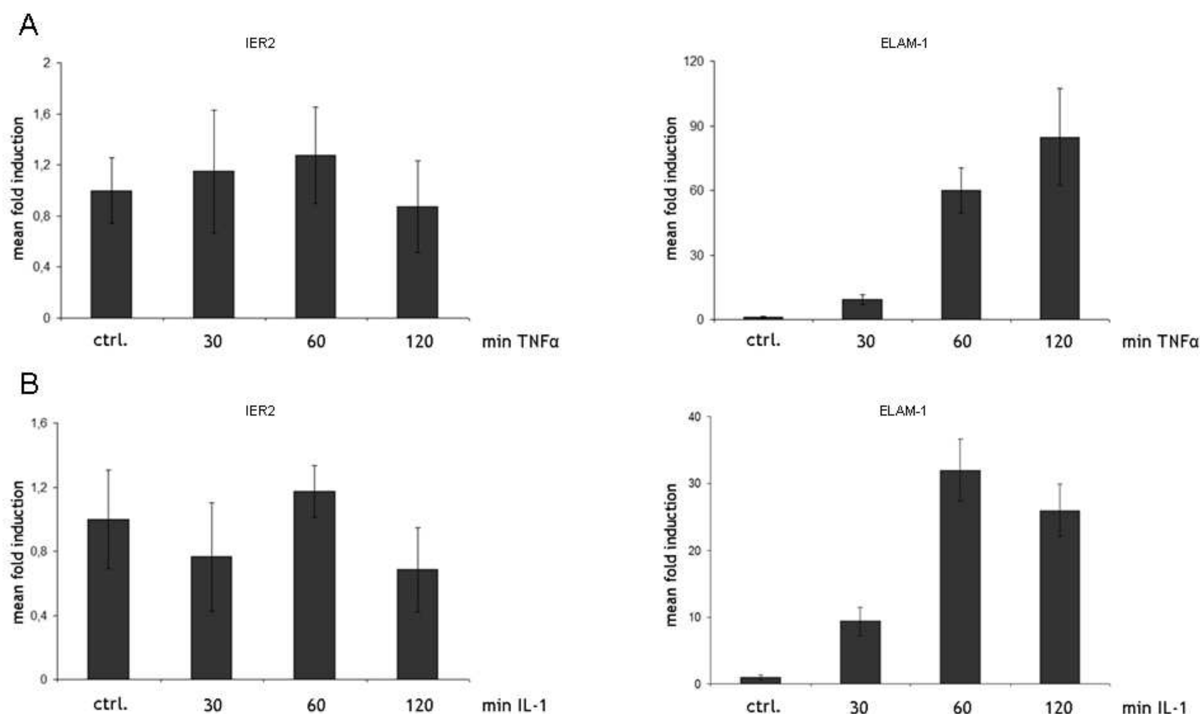


Fig.7: Pro-inflammatory cytokines induce ELAM-1 but not IER2 mRNA expression in HUVEC. Cells were stimulated with TNFα (A) or IL-1β (B) for indicated periods of time. qRT-PCR analysis of *IER2* (left panels) and *ELAM-1* (right panels) expression was performed and normalized to *GAPDH*. The results represent one of three independent experiments, done in triplicates. Error bars represent the standard deviation of the mean.

3.1.2. Potent activators of IER2 expression

Besides its role in inflammatory signaling, endothelial cells maintain the ability to re-enter the cell cycle and start to proliferate, an essential step for angiogenesis. As the expression of *IER2* has been shown to be activated by numerous growth factors in several cell types, we tested whether its expression was also regulated by these factors in HUVEC. Treatment with human recombinant (hr-)FGF or hr-VEGF indeed resulted in a fast and transient accumulation of *IER2* mRNA (Fig.8A). Expectedly, early growth response protein 1 (EGR-1), one of the best described IEGs upon growth factor stimulation, was monitored as internal experimental control and its expression peaked within thirty minutes of growth factor treatment in HUVEC (Fig.8B).

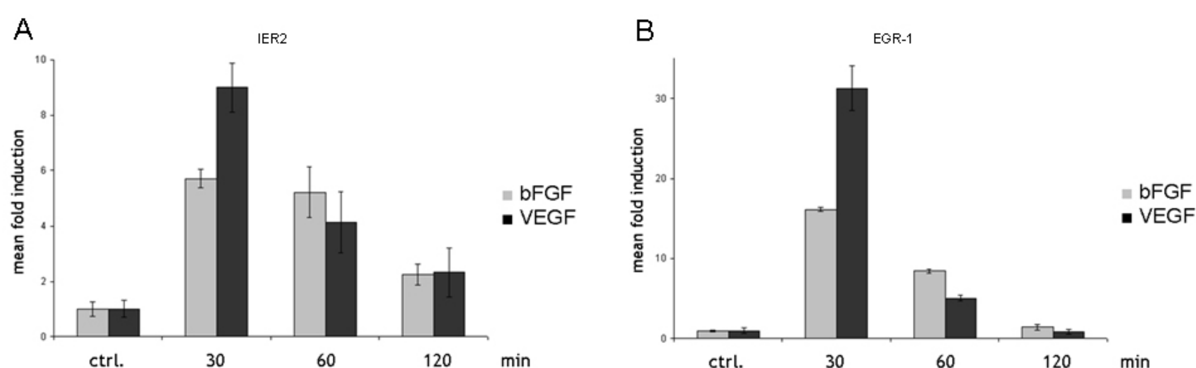


Fig.8: Growth factors potently induce IER2 and EGR-1 mRNA expression in HUVEC. Cells were stimulated with bFGF (gray bars) or VEGF (black bars) for indicated times and qRT-PCR analysis of *IER2* (A) and *EGR-1*

(B) expression was performed. Values were normalized to *GAPDH* and the results shown are representatives of three independent experiments, done in triplicates. Error bars represent the standard deviation of the mean.

It has been demonstrated that endothelial cell proliferation as well as angiogenesis driven by bFGF and VEGF, both require activation of the protein kinase C (PKC). [96, 97] A potent activator of PKC signaling is the phorbol ester PMA which activates the IER2 mouse homologue *pip92*. [34] Therefore, we analyzed *IER2* expression after PMA treatment and found that *IER2* was rapidly induced in HUVEC (Fig.9A). Thus, as well as through comparison with the immediate early expression pattern of *ELAM-1* (Fig.9B), IER2 could be clearly identified as a PMA-induced IEG in HUVEC.

Interestingly, when we evaluated the effect of increasing amounts of PMA on *IER2* expression (Fig.9C), we found that the latter was time but not concentration-dependent: *IER2* mRNA expression peaked within sixty minutes of treatment (Fig.9C), whereas the protein accumulated within up to four hours, before its levels started to decrease slowly (Fig.9D). Based on these initial results we state that IER2 induction occurs in a typical IEG-dependent manner upon PMA treatment in HUVEC. Therefore, we went on to further address the question of how the activation of the PKC signaling cascade might be involved in the regulation of IER2 expression in this context.

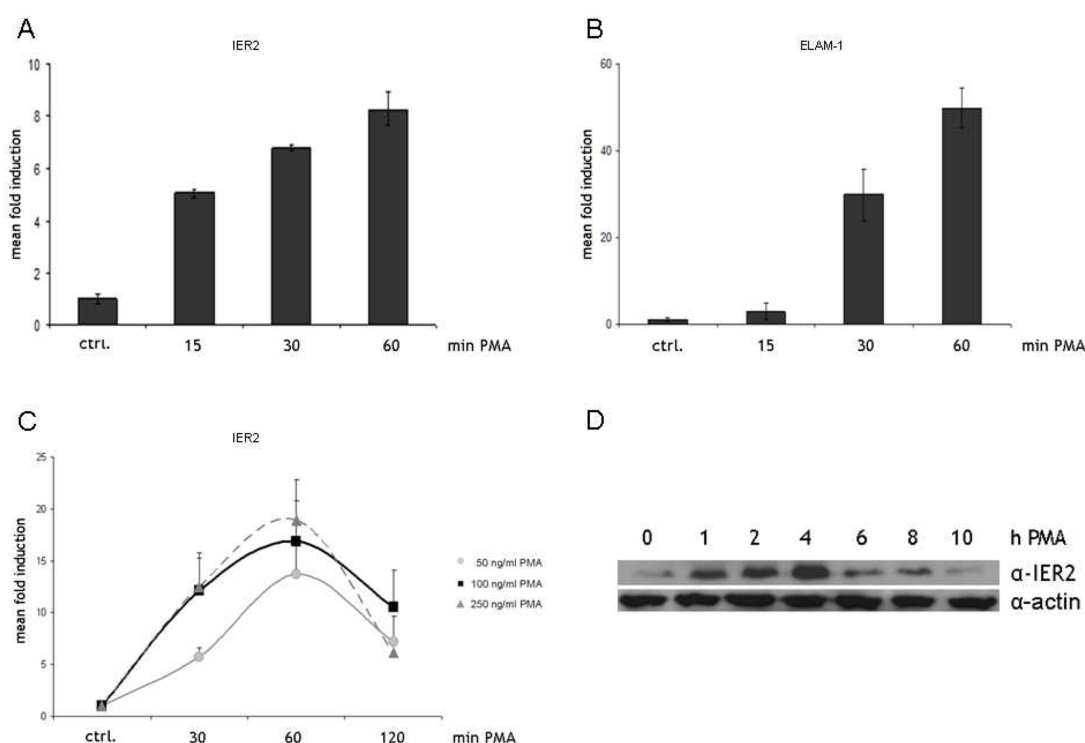


Fig.9: Phorbol ester PMA rapidly induces IER2 mRNA and protein expression in HUVEC. Cells were stimulated with increasing amounts of PMA for indicated time points. qRT-PCR analysis of *IER2* (A,C) and *ELAM-1* (B) expression was performed and normalized to *GAPDH*. The results represent one of three independent experiments, done in triplicates. Error bars represent the standard deviation of the mean. (D) Whole

cell extracts of PMA-induced HUVEC were analyzed by Western Blot analysis for the presence of endogenous IER2 protein. β-actin served as loading control.

3.2. PMA-induced IER2 expression depends on the PKC signaling pathway

Phorbol esters are tumor promoters which can substitute for DAG and activate cPKC as well as nPKC isozymes. We then raised the question whether inhibition of the PKC signaling cascade leads to suppressed IER2 expression.

3.2.1. PKCδ inhibition effectively blocks IER2 expression

Consequently we treated HUVEC with Rottlerin, a widely-used inhibitor of PKCδ, and found that PMA-induced *IER2* mRNA expression was strongly inhibited (Fig.10A, left panel). Since Rottlerin has also been shown to inhibit other PKC isozymes dependent on the concentration, we repressed PKCδ using a specific targeting short hairpin RNA (shPKCδ) and compared to non silencing control shRNA (scr). We observed that both, *IER2* and *PKCδ* mRNA expression were clearly reduced in shPKCδ expressing cells (Fig.10B). Additionally, HUVEC were stimulated with PMA in combination with Ionomycin to increase intra-cellular calcium levels thereby activating Ca^{2+}-dependent PKC isozymes on the one hand. On the other hand this co-treatment is used to stimulate cytokine production *in vitro*. We found that this combined stimulation resulted in weaker induction of *IER2* expression (Fig.10C) when compared to PMA only treatment. This accumulating data indicate for a specific nPKC-dependent regulation of *IER2* mRNA expression.

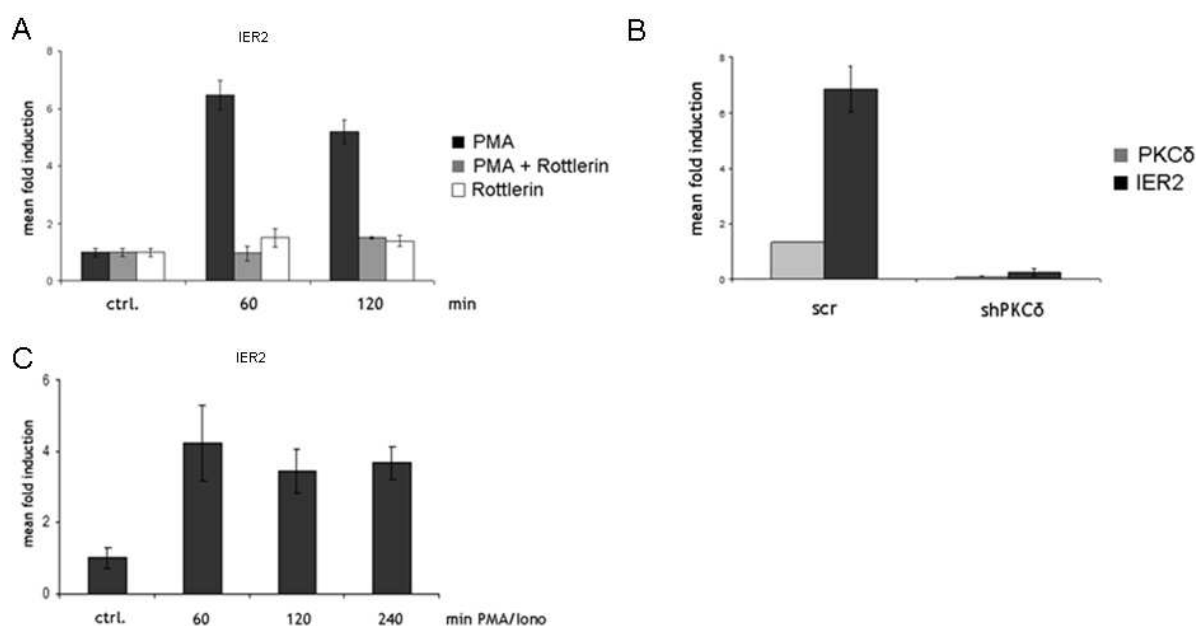


Fig.10: PMA-induced IER2 expression depends on PKCδ activity in HUVEC. A) Cells were treated with PMA (black bars), Rottlerin (white bars), or a combination of PMA and Rottlerin (gray bars). qRT-PCR analysis of *IER2* mRNA expression levels was performed. B) HUVEC were transduced with either a scrambled shRNA (scr) or a shRNA specific for silencing PKCδ (shPKCδ) lentivirus. 48 hours post infection cells were treated with PMA for 30 minutes and *PKCδ* (gray bars) and *IER2* (black bars) mRNA expression levels were assessed

by qRT-PCR. C) Cells were stimulated with PMA and Ionomycin for indicated time points and *IER2* mRNA expression levels were analyzed by qRT-PCR. Relative levels of expression were normalized to *GAPDH*. Error bars represent the standard deviation of the mean.

3.2.2. IER2 is a PKCδ-dependent cytosolic protein, degraded by the proteasome

IER2 was so far described as a protein which is located in the nucleus as well as in the cytosol in a stimulus-dependent and cell type specific manner. We therefore aimed to define the cellular compartment expressing the IER2 protein upon PKCδ activation. Using Western Blotting techniques after cell fractionation we found that endogenous IER2 was located in the cytosolic fraction of HUVEC upon PMA stimulation (Fig.11A).

Bioinformatical analysis indicated that a certain motif (PEST domain, see Introduction and text below) is putatively responsible for instability, suggesting a degradation mechanism for this protein. IEGs are often unstable key cell regulators with a fast turnover driven by poly-ubiquitination and subsequently followed degradation by the proteasome. Contrary it has been reported that e.g., c-Fos is degraded independently of any prior ubiquitination. [98] In this regard, we treated HUVEC with an inhibitor of proteasomal degradation (MG132). Interestingly, we found that IER2 protein was robustly accumulated in MG132 treated compared to untreated cells (Fig.11B) clearly indicating that the ubiquitin-proteasome system is involved in the regulation of IER2 protein degradation. In the next consequence, we wanted to investigate in which way this degradation process is influenced by activated PKC signaling. Therefore, we stimulated HUVEC with PMA or in a combination with MG132 (Fig.11C, left and right panel, respectively) and found a time-dependent stabilization of IER2 expression after four hours of treatment. In both cases, application of the proteasome inhibitor increased the amount of IER2 protein. Subsequently, we observed that IER2 protein expression was also reduced after four hours upon cotreatment with Rottlerin (Fig.11D), demonstrating IER2 protein degradation as a result of PKC signaling cascade inhibition.

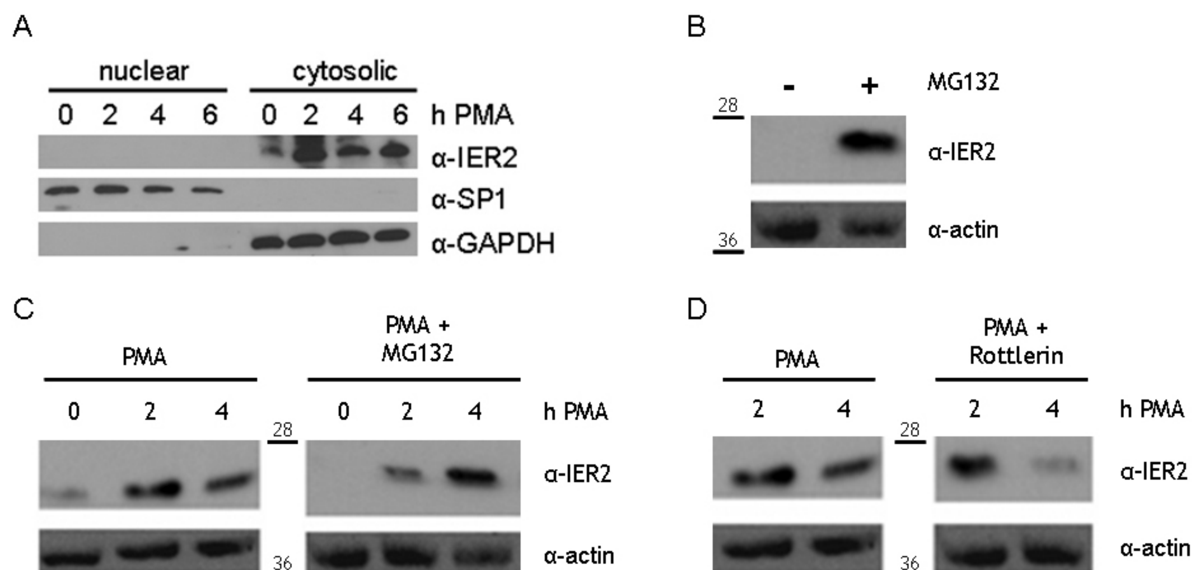


Fig.11: IER2 expression in the cytosol is PKCδ-dependent and degraded by the proteasome. A) HUVEC were stimulated with PMA for indicated time points followed by fractionation into nuclear and cytosolic cell extracts. They were analyzed by Western Blotting for the presence of endogenous IER2. SP1 and GAPDH served as nuclear or cytosolic loading controls, respectively. B) Cells were left untreated (-) or were treated (+) for 4 hours with the proteasome inhibitor MG132. C) Cells were stimulated for indicated periods of time with PMA (left panel) or in a combination with MG132 (right panel). D) Cells were stimulated for indicated times with PMA (left panel) or in a combination with Rottlerin (right panel). Whole cell extracts were analyzed for the presence of IER2, β-actin served as loading control.

These results indicate that IER2 is induced as well as showing prolonged/stabilized expression by activated PKC signaling. Furthermore, the proteasomal degradation pathway is involved in the regulation of IER2 protein levels. This poses the question of the multiplicity of stabilization and degradation pathways that apply to proteins depending on their intra-cellular localization.

3.2.3. Posttranslational modifications influence PMA-induced IER2 protein stability

As mentioned in the Introduction, we performed bioinformatical analysis of the IER2 protein sequence. As schematically depicted in Figure 12 the mature full length IER2 protein of 223 amino acids contains one nuclear export signal (NES) and two nuclear localization signal (NLS) domains. Furthermore, we predicted a PEST domain (a peptide motif rich in proline (P), glutamate (E), serine (S), and threonine (T)) that is associated with short intra-cellular protein half-life and a non-regular secondary structure (NORS) region (not depicted in scheme) that is involved in protein-protein interactions. Into special focus came the sequence domains for posttranslational modifications. We predicted a single sumoylation site in the IER2 sequence at position 51 using SumoSP 2.0 (Fig.12, depicted as S) as well as ubiquitination sites (not shown). NetPhosK 1.0 Server analyses revealed several serines

predicted as phosphorylation sites (Fig.12, depicted as P) mediated by protein kinase A (PKA) and protein kinase C (PKC), respectively.

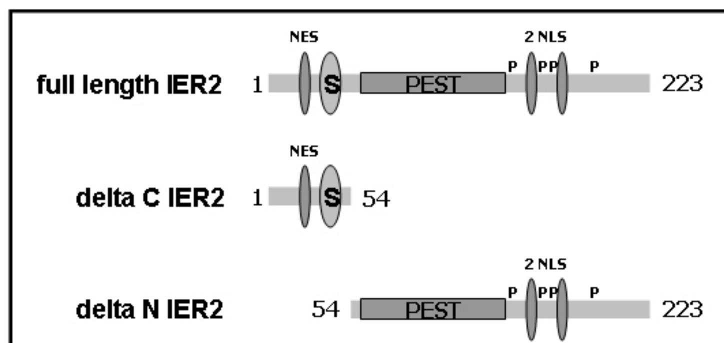


Fig.12: Schematic representation of IER2 protein with its bioinformatically predicted regulatory domains (abbreviations see text) as well as IER2 deletion constructs (delta N and delta C). All three expression plasmids are myc-tagged and IER2 levels can be detected with α-myc by Western Blotting.

Sumoylation is a posttranslational modification that covalently attaches small ubiquitin-related modifer (Sumo) proteins to respective residues of other proteins, so called sumoylation sites. This process leads to altered activity, stability or nuclear-cytosolic transport of proteins. [99] Since interaction of PKC signaling driven phosphorylation events and sumoylation influence protein stability [100, 101] we achieved further information on the regulation of IER2. We therefore performed protein stability assays using three different IER2 expression plasmids (Fig.12 and Material and Methods) and Western Blotting analyzing methods. HEK293 cells were transfected with respective IER2 plasmids, PMA stimulation activated IER2 by phosphorylation and IER2 degradation was achieved by addition of Rottlerin. We found that PMA application had no effect on ectopic full length myc-IER2 expression (Fig.13A, left panel). Additional PKC blockade led to reduced myc-IER2 levels (Fig.13A, right panel). Moreover, the delta C (ΔC) IER2 protein lacking its C-terminus after amino acid position 54 led to enhanced reduction of myc-IER2 expression (Fig.13B). In contrast, the expression of a truncated IER2 construct, missing its N-terminus (ΔN) including the sumoylation site, was not affected at all by PKC inhibition (Fig.13C). Furthermore, when cells were transfected with the respective plasmids followed by inhibition of the proteasomal degradation pathway we found that dCIER2 expression was not affected by MG132. In contrast, full length to low and dNIER2 to high extends showed the typical pattern of ubiquitination (Fig.13D), indicating that the N-terminus of IER2 appears to be the domain responsible for IER2 stability. In cooperation with U. Resch (University of Cologne), we additionally found by immunoprecipitaion that IER2 directly interacts with a member of the sumoylation complex, namely Sumo1. Upon PMA treatment Sumo1 levels were reduced after four to six hours (data not shown). We conclude that PKC activates and stabilizes IER2

expression. This is followed by sumoylation and subsequent ubiquitination of IER2 leading to its proteasomal degradation.

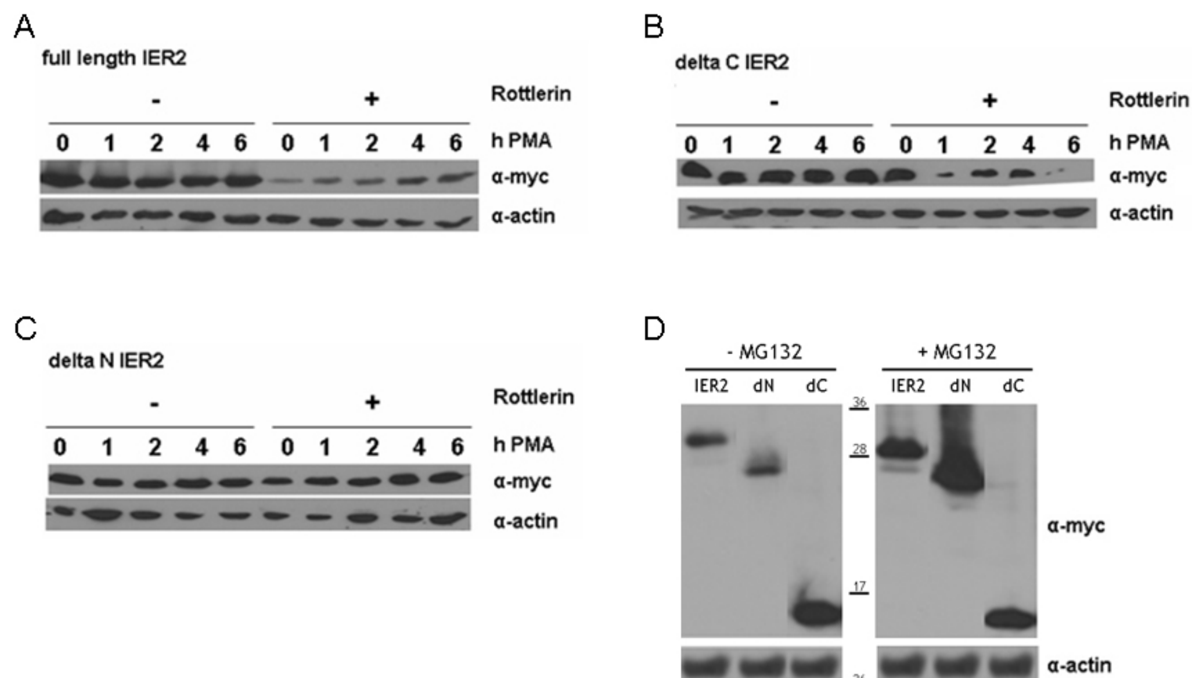


Fig.13: Phosphorylation and sumoylation events influence PMA-induced IER2 protein stability. HEK293 cells were transfected with 1µg of either full length IER2 (A) or deletion constructs ΔC (B) and ΔN (C), all myc-tagged. 24 hours post transfection cells were stimulated with PMA in the absence (-) or presence (+) of PKC inhibitor Rottlerin. D) Full length IER2, ΔN, and ΔC transfected cells were left untreated (-) or treated (+) with proteasome inhibitor MG132. Whole cell extracts were analyzed for the presence of myc-IER2 (α-myc) by Western Blotting, β-actin served as loading control.

Taken together, we provide evidence that PMA-induced IER2 expression in HUVEC is dependent on activated PKCδ signaling and that the cytosolic IER2 protein expression is affected by posttranslational modifications.

3.3. Role of IER2 in the PKC signaling cascade

To evaluate the impact of IER2 on PKCδ signaling we designed, created, and established lentiviral-mediated overexpression and knockdown tools (see Material and Methods).

3.3.1. Generation and testing of tools

As shown in Figure 14A infection of HUVEC with myc-tagged IER2 expressing virus led to strong expression of IER2 on the mRNA (upper panel) as well as on the protein level (lower panel). Knockdown of IER2 was not detectable in HUVEC because its expression under un-stimulated conditions was already at the borderline of detection (data not shown). Stimulating HUVEC with PMA after knocking down IER2 (shIER2), its mRNA expression was clearly reduced compared to control virus infected cells treated with PMA (Fig.14B). Using shIER2

plasmids for transient transfection in HEK293 cells, ectopic co-transfected myc-IER2 protein levels were strongly reduced as well (Fig.14C).

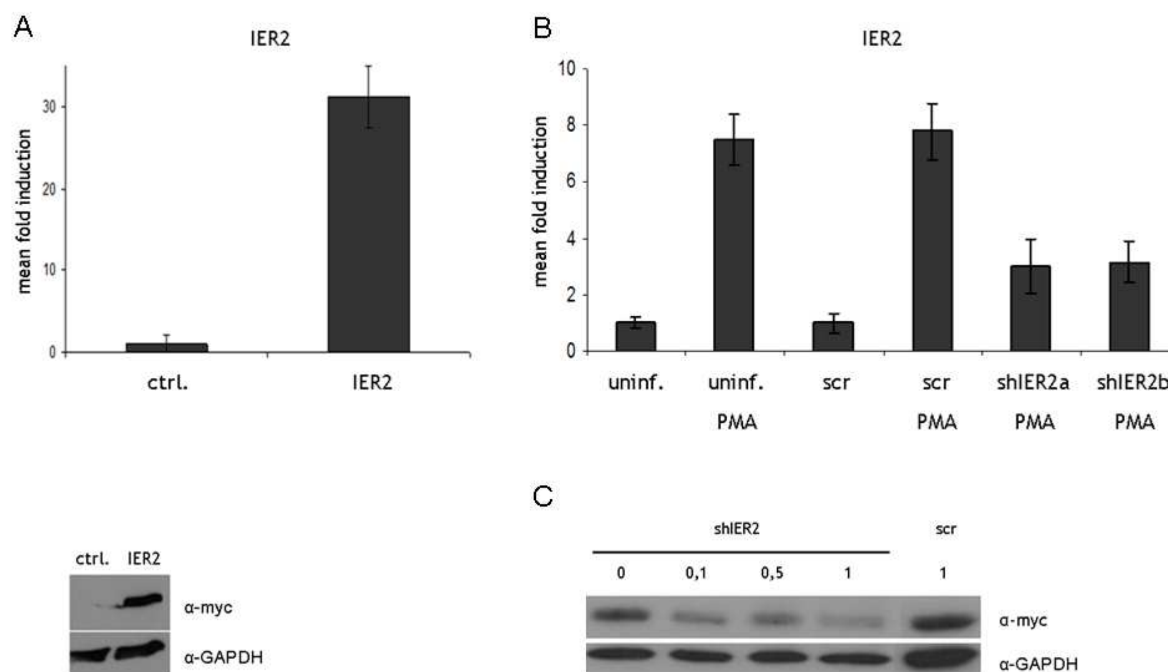


Fig.14: Lentiviral overexpression and knockdown tools. A) For lentiviral overexpression of myc-tagged IER2, HUVEC were infected with either control virus (ctrl.) or IER2 virus (IER2). 48 hours after infection cells were harvested and IER2 mRNA (upper panel) and protein (lower panel) expression levels were analyzed. B) HUVEC were left uninfected (uninf.), or were infected either with scrambled shRNA (scr) or shRNA targeting to different regions of IER2 (shIER2a and shIER2b). After 48 hours cells were treated with PMA for 30 minutes followed by qRT-PCR analysis of *IER2*. C) HEK293 cells were transfected with myc-tagged IER2 (1µg) and either scrambled shRNA (scr, 1µg) or specific shRNA (shIER2, 0 to 1 µg). IER2 (α-myc) protein levels were analyzed 48 hours post transfection.

Consequently, HUVEC were infected with control or ectopic expressing IER2 virus, followed by a PMA stimulation time course. In control cells IER2 showed its typical expression pattern upon PMA treatment peaking within sixty minutes. In contrast, overexpressing IER2 cells hardly reacted to the stimulus but showed at least a 30-fold increase of IER2 expression (Fig.15).

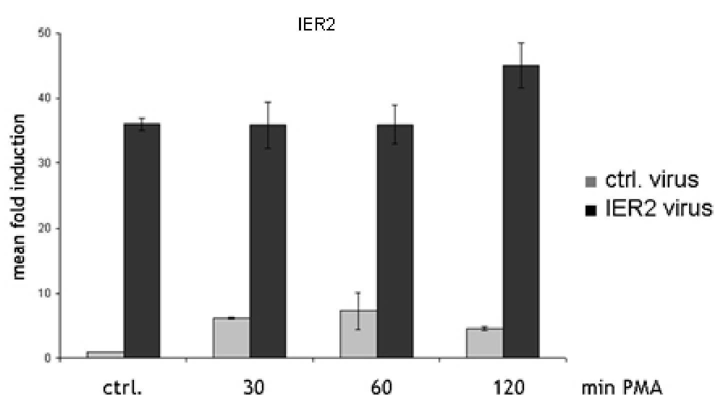


Fig.15: Lentiviral overexpression of IER2. For ectopic expression of IER2, HUVEC were infected with either control virus (ctrl., gray bars) or IER2 virus (black bars). 48 hours after infection cells were stimulated with PMA for indicated periods of time. *IER2* expression levels were analyzed by qRT-PCR and normalized to

GAPDH. Comparable results were obtained in at least three experiments performed in triplicates. Error bars represent the standard deviation of the mean.

3.3.2. IER2 suppresses PMA-induced p21$^{waf1/cip1}$ expression

To gain more insight into the biological function of IER2, we then screened for IER2 regulated genes using mRNAs from control or ectopic expressing IER2 virus infected HUVEC that were stimulated with PMA. In line with the fact that we found no IER2 activation upon cytokine stimulation, none of the tested pro-inflammatory genes showed alterations in their expression levels (e.g., ELAM-1, vascular cell adhesion protein 1 VCAM-1, intercellular adhesion molecule 1 ICAM-1, IκBα, data not shown). Surprisingly and most importantly, we found that in HUVEC ectopic IER2 expression in combination with PMA led to suppressed mRNA (Fig.16A) as well as protein (Fig.16B) induction levels of the cell cycle inhibitor p21$^{waf1/cip1}$.

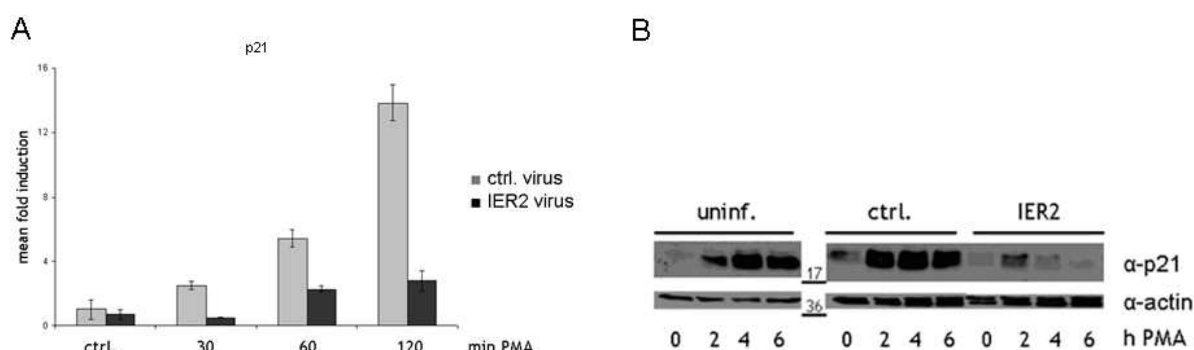


Fig.16: IER2 depletes PMA-induced p21$^{waf1/cip1}$ expression. A) HUVEC were either infected with control virus (ctrl., gray bars) or IER2 virus (black bars) and 48 hours after infection cells were stimulated with PMA for indicated periods of time. *p21* mRNA expression levels were analyzed by qRT-PCR and normalized to *GAPDH*. B) p21 protein expression was analyzed by Western Blotting from whole cell extracts, of either uninfected (uninf.), control- (ctrl.), or IER2-infected HUVEC after PMA treatment. β-actin served as loading control.

Since in our initial analysis IER2 was located in the cytoplasm we addressed the question in which cellular compartment p21$^{waf1/cip1}$ protein is affected by IER2 in our experimental setup. Traditionally, p21$^{waf1/cip1}$ has been shown as a nuclear protein leading to a cell cycle arrest [69, 102] but other studies also have demonstrated that p21$^{waf1/cip1}$ is localized in the cytosol influencing cell proliferation, apoptosis, and cell differentiation. [69, 102] We found that upon PMA stimulation of HUVEC endogenous p21$^{waf1/cip1}$ is located in the cytoplasm (Fig.17A). In addition, ectopic IER2 revealed its negative influence on cytosolic p21$^{waf1/cip1}$ expression in HEK293 cells (Fig.17B).

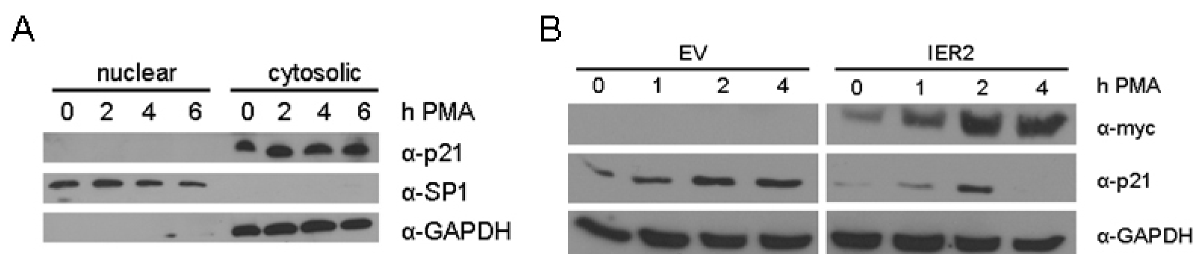


Fig.17: IER2 affects PMA-induced p21$^{waf1/cip1}$ in the cytosol. A) HUVEC were stimulated with PMA for indicated times, followed by fractionation into nuclear and cytosolic cell extracts. They were analyzed by Western Blotting for the presence of p21. SP1 and GAPDH served as nuclear or cytosolic loading controls, respectively. B) HEK293 cells were transfected with 1µg of myc-tagged IER2 and 24 hours post transfection cells were stimulated with PMA for indicated time points, followed by cell fractionation as in (A). Cytoplasmic extracts were analyzed for the presence of IER2 (α-myc) and p21. GAPDH served as loading control.

3.3.3. Full length IER2 is needed to attenuate p21$^{waf1/cip1}$ expression at the level of transcription

It has been reported that PMA-induced *p21*$^{waf1/cip1}$ via activation of the PKC signaling cascade, mainly the PKCδ isoform binds to the SP1 sites on the *p21*$^{waf1/cip1}$ promoter in a tp53-independent manner. Therefore, we addressed the question whether IER2 affects *p21*$^{waf1/cip1}$ expression at the level of transcription or if it supports posttranslational modifications that lead to p21$^{waf1/cip1}$ degradation. In fact, we found that in HEK293 cells transfected with a p21$^{waf1/cip1}$-luciferase expression plasmid and increasing amounts of IER2 inhibited PMA-induced p21$^{waf1/cip1}$-luciferase activity (Fig.18A, left panel). Western Blot analysis of the corresponding protein extracts confirmed that growing amounts of IER2 reduced p21$^{waf1/cip1}$ protein levels (Fig.18A, right panel). Next we transfected HEK293 cells with control or IER2 expression plasmid and increasing amounts of p21$^{waf1/cip1}$ followed by treatment with the proteasome inhibitor MG132 (Fig.18B). Control transfected cells treated with MG132 showed an enormous accumulation of p21$^{waf1/cip1}$ compared to untreated cells. In line with previous results (Fig.13D) even myc-IER2 was stabilized upon addition of the proteasome inhibitor. Interestingly, but corresponding to luciferase activity results, p21$^{waf1/cip1}$ expression in IER2 transfected cells was abolished independent of MG132 addition, concluding that IER2 influences *p21*$^{waf1/cip1}$ expression on the level of transcription.

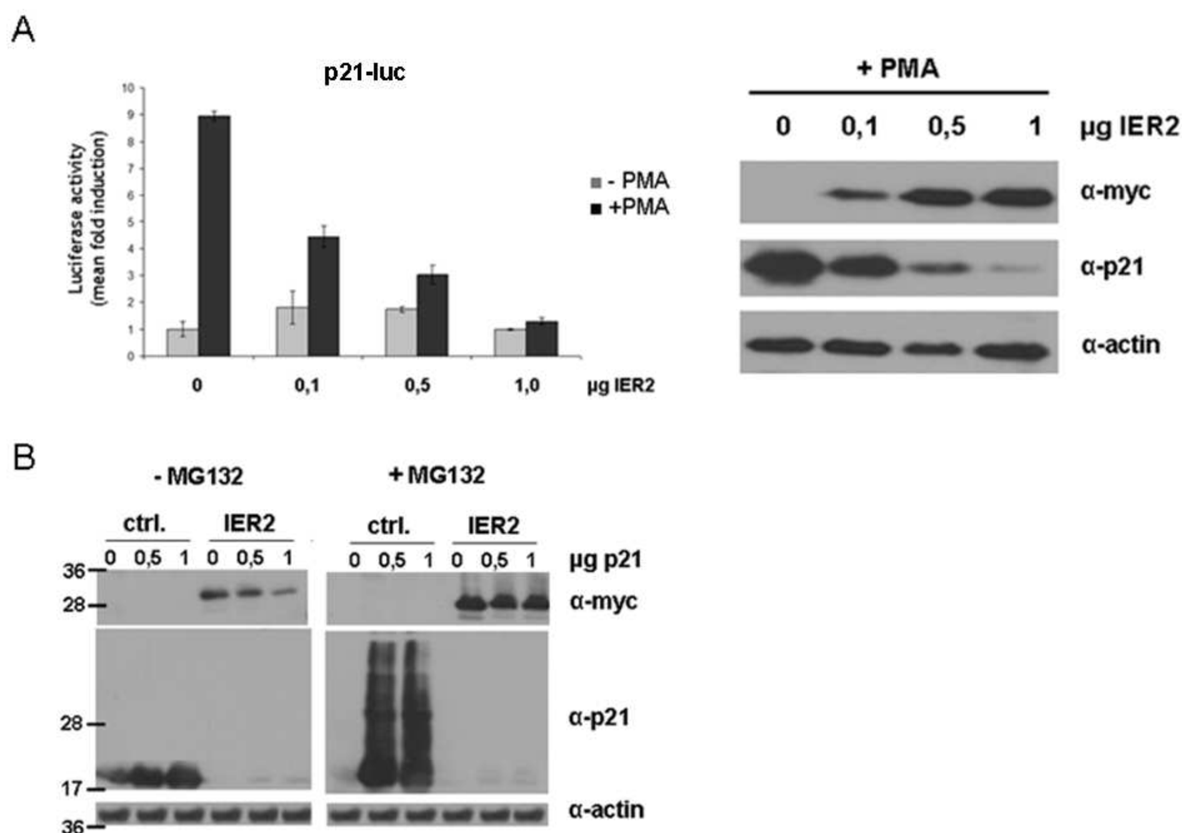


Fig.18: IER2 suppresses PMA-induced p21$^{waf1/cip1}$ expression at the transcriptional level. A, left) HEK293 cells were transfected with luciferase reporter gene driven by the *p21* promoter, in the presence of increasing amounts of (myc-) IER2 expression plasmid (0-1µg). Cells were left unstimulated (gray bars) or were induced with PMA (black bars) for 8 hours. Luciferase activities were analyzed, normalized to the activity of co-transfected β-Galactosidase (β-gal) and mean fold induction was calculated in relation to basal promoter activity (0µg, gray bar). Error bars represent the standard deviation of the mean. A, right) Western Blot of analyzed luciferase reporter assay samples for IER2 (α-myc) and p21 protein levels. B) HEK293 cells were co-transfected with 1µg of either control (ctrl.) or IER2 expression plasmid in combination with increasing amounts of p21 (0-1µg). 24 hours post transfection cells were left untreated (left panel, - MG132)) or treated with MG132 for 4 hours (right panel, + MG132). Whole cell extracts were analyzed by Western Blotting for the presence of myc-IER2 (α-myc) and p21. β-actin served as loading control.

Moreover, we found that p21$^{waf1/cip1}$-luciferase activity was also inducible by co-transfection of a p21$^{waf1/cip1}$ expression plasmid (Fig.19A, control (ctrl.)), confirming auto-regulation of p21$^{waf1/cip1}$. [78, 103, 104] As shown above, co-transfection of IER2 reduced p21$^{waf1/cip1}$-luciferase activity (Fig.19A, compare ctrl. to IER2). When testing truncated IER2 expression constructs (scheme Fig.12) missing its N- or C-terminus, we observed the phenomenon that the ΔN construct increased both, the basal as well as the p21$^{waf1/cip1}$ induced p21$^{waf1/cip1}$-luciferase activity (Fig.19A, dN) whereas the ΔC construct showed no effect on luciferase levels compared to control expression plasmid (Fig.19A, ΔC). Thereby, we conclude that only full length IER2 has the ability to inhibit p21$^{waf1/cip1}$-luciferase activity (Fig.19A, upper panel) as well as p21$^{waf1/cip1}$ protein expression (Fig.19A, lower panel) and that the deletion of its N-terminus results in a dominant negative protein.

*p21^waf1/cip1^* is regulated by a variety of different mechanisms and transcription factors. [69, 70, 103, 104] Consequently, we investigated whether IER2 influences p21$^{waf1/cip1}$ in a tp53-dependent or -independent manner, the latter is the preferred mechanism of PKCδ-induced *p21*$^{waf1/cip1}$ activation. Therefore, we co-transfected HEK293 cells with a p21$^{waf1/cip1}$-luciferase expression plasmid and increasing amounts of IER2 in the absence (EV) or presence (p53) of a tp53 expression plasmid. We observed that p21$^{waf1/cip1}$-luciferase activity was induced by tp53, the most prominent *p21*$^{waf1/cip1}$ regulator. Interestingly and in contrast to earlier results (Fig.18A and Fig19.A), increasing amounts of IER2 did not lead to changes in p21$^{waf1/cip1}$-luciferase activity (Fig.19B). Thereby, we showed that IER2-dependent loss of *p21*$^{waf1/cip1}$ expression is counteracted by tp53. We confirmed this finding by transfecting HEK293 cells with empty vector control (EV), IER2, p21$^{waf1/cip1}$, tp53 expression plasmid and its combinations, respectively. We found once more that IER2 reduced p21$^{waf1/cip1}$ levels (Fig.19C, compare lanes 2, 3, 5), however, this inhibitory mechanism was reversed by co-transfection of tp53 (Fig.19C, lanes 5, 7, 8) showing that *p21*$^{waf1/cip1}$ expression is rather controlled by tp53 signaling than affected by the PKC signaling cascade.

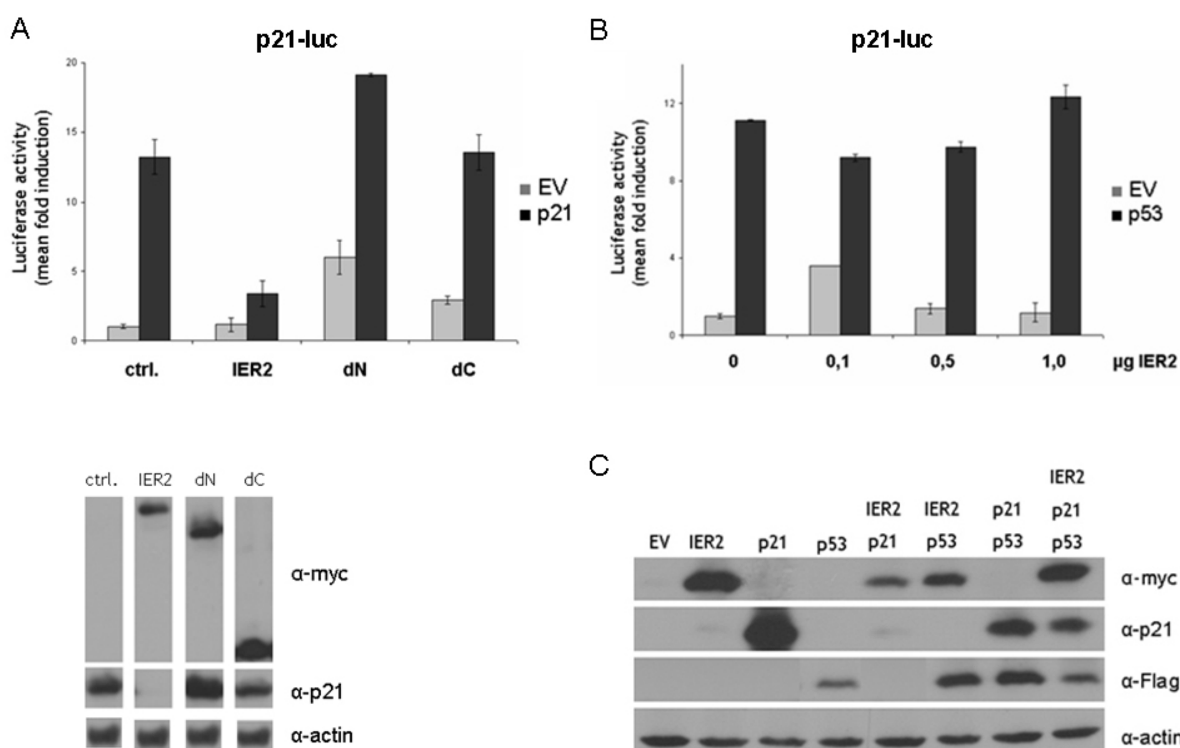


Fig.19: Full length IER2 affects p21$^{waf1/cip1}$ expression which is counteracted by tp53. HEK293 cells were transfected with a p21-luciferase reporter gene in the absence (EV, empty vector, gray bars) or presence of (A) p21 (1µg) or (B) tp53 (0,5µg) (black bars) expression plasmids. A) Cells were co-transfected with 1µg of either control expression plasmid (ctrl.), full length myc-IER2 or the deletion constructs ΔN and ΔC (see scheme Fig.12). Luciferase activities (upper panels) were analyzed, normalized to co-transfected β-gal and mean fold induction was calculated in relation to basal promoter activity (EV, ctrl.). Error bars represent the standard deviation of the mean. Protein levels (lower panel) of the luciferase reporter assay samples were analyzed for the expression of IER2 and deletion constructs (α-myc) and p21. β-actin served as loading control. B) Cells were co-transfected with either ctrl. (1µg) or with increasing amounts of myc-IER2 expression plasmid (0-1µg). Luciferase activities were analyzed as described above, mean fold induction was calculated in relation to basal

promoter activity (0µg IER2). C) HEK293 cells were transfected with EV, IER2, p21, tp53 expression plasmids, or their combinations. Myc-IER2 (α-myc), p21 as well as Flag-tp53 (α-Flag) were detected by Western Blotting. β-actin served as loading control.

In a complementary approach to substantiate our findings that the reduction of *p21$^{waf1/cip1}$* expression is based on IER2, we examined whether knockdown of IER2 could increase *p21$^{waf1/cip1}$* expression. Consequently, we co-transfected HEK293 cells with a p21$^{waf1/cip1}$-luciferase expression plasmid and added either IER2, p21$^{waf1/cip1}$, or IER2 and p21$^{waf1/cip1}$ combined with either scrambled shRNA (scr) or specific shRNA (shIER2) plasmids. Subsequently, we analyzed luciferase activity and protein levels observing that IER2 alone had no influence on p21$^{waf1/cip1}$-luciferase activity (Fig.20A, compare lane 1 and lane 4). Furthermore, co-expression of a p21$^{waf1/cip1}$ plasmid led to an increase of reporter activity independent of co-transfection of a non silencing control shRNA or a shRNA specific silencing IER2 (Fig.20A, compare lane 2 and lane 5). In line with earlier results, co-expression of IER2 and p21$^{waf1/cip1}$ plasmids led to a severe inhibition of p21$^{waf1/cip1}$-luciferase activity (Fig.20A, lane 3), whereas additional co-transfection of shIER2 partially reversed the inhibitory effect (Fig.20A, lane 6). Respective protein extracts confirmed the high efficiency of the knockdown, however, p21$^{waf1/cip1}$ protein expression was only slightly restored (Fig.20B).

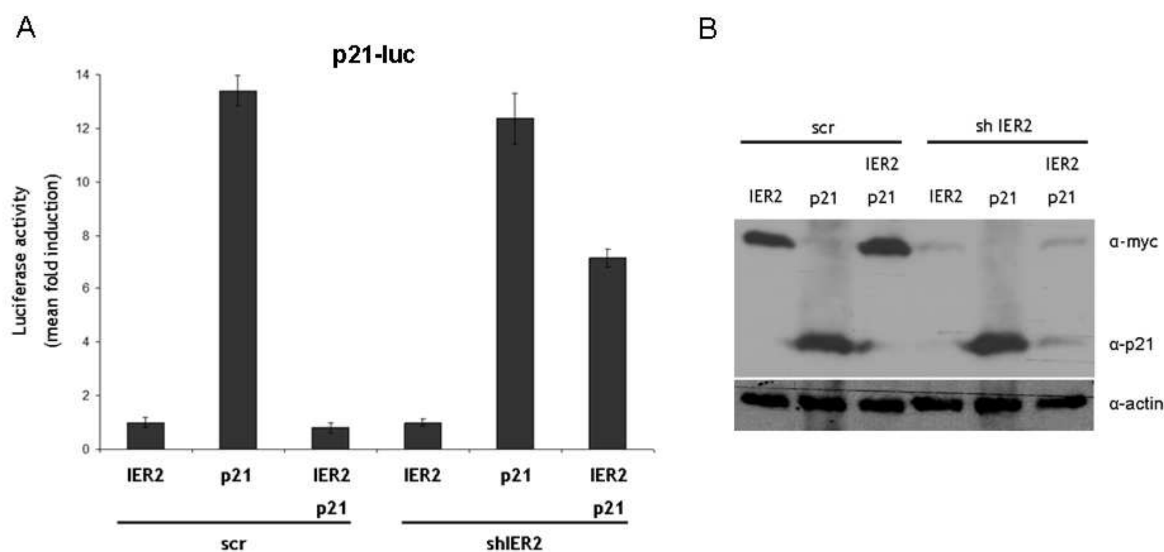


Fig.20: Blockade of IER2 expression restores p21$^{waf1/cip1}$ transcriptional activity. A) HEK293 cells were transfected with a luciferase reporter gene driven by the *p21* promoter. Luciferase activities were analyzed after coexpression of IER2, p21, or IER2 and p21 combined with either scrambled shRNA (scr) or specific shRNA (shIER2). Values were normalized to co-transfected β-gal and mean fold induction was calculated in relation to basal promoter activity. Error bars represent the standard deviation of the mean. B) Respective protein extracts of the luciferase assay were analyzed for myc-IER2 (α-myc) and p21 expression by Western blotting. β-actin served as loading control.

3.4. IER2 and its influence on cell survival and apoptosis

Altered *p21$^{waf1/cip1}$* expression has been shown to affect downstream events such as E2F-dependent transcription resulting in changes of cell cycle progression which in turn can lead to a switch between cell survival and death. Therefore we aimed to analyze the effect of IER2 on well-known p21$^{waf1/cip1}$ signaling complex key players, interacting partners, upstream regulators and downstream targets.

3.4.1. IER2 is not directly affecting p21$^{waf1/cip1}$-related gene expression but alters cell cycle-associated proteins

First, we performed screenings of p21$^{waf1/cip1}$ signaling-associated gene expression using mRNAs from control or ectopic expressing IER2 virus infected HUVEC that were stimulated with PMA. As estimated, we found that the expression of the most prominent p21$^{waf1/cip1}$ regulator *tp53* was not influenced by IER2 (Fig.21A) which is in line with earlier results (Fig.19C). Besides p21$^{waf1/cip1}$, the Cip/Kip family member cell cycle inhibitor p27^{kip1} is also PKC-dependently regulated, [105] however, we found no induction upon PMA stimulation within tested time points and as a consequence no influence of IER2 on *p27^{kip1}* (Fig.21B). Both cyclin-dependent kinase inhibitors (CKI) signal through the same pathway and are sufficient to promote assembly of the cyclin and cyclin-dependent kinase (cdk) complexes.

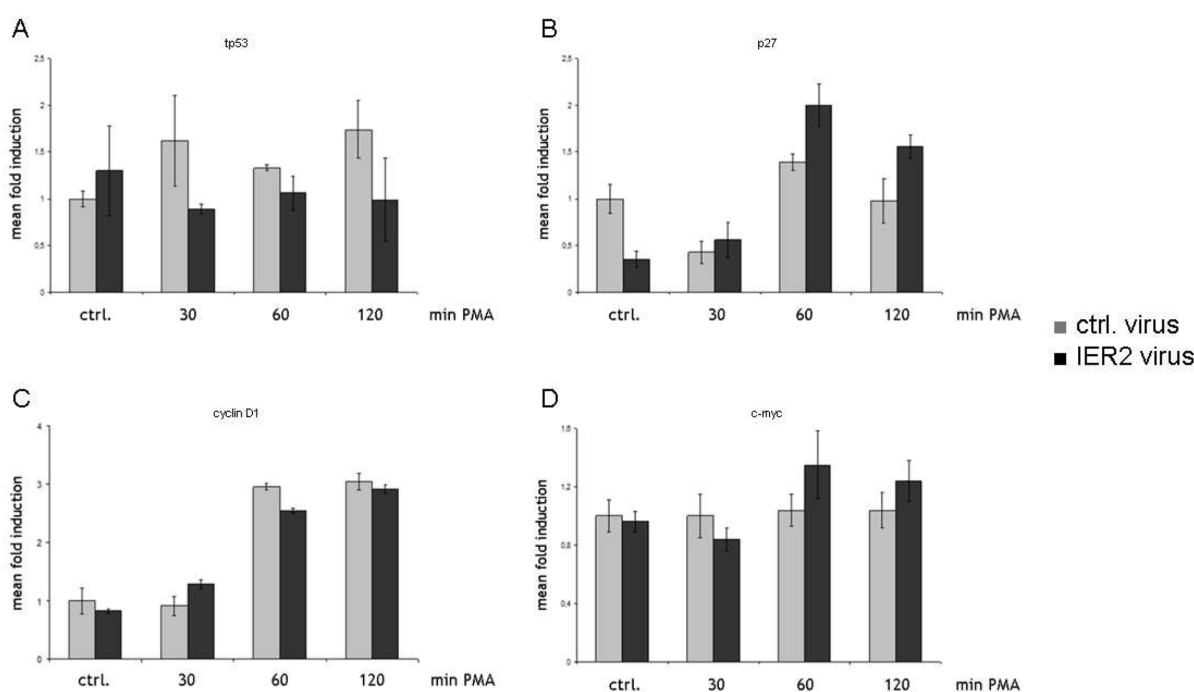


Fig.21: p21$^{waf1/cip1}$-related gene expression is unaffected by IER2. HUVEC were infected with either control (ctrl.) virus (gray bars) or IER2 virus (black bars) and 48 hours after infection cells were stimulated with PMA. *tp53* (A), *the cell cycle inhibitor p27* (B), *cyclin D1* (C), and *c-MYC* (D) expression levels were analyzed by qRT-PCR and normalized to GAPDH. Error bars represent the standard deviation of the mean.

As expected, we detected no alterations in the expression levels of tested cyclins such as *cyclin D1* or *cyclin E*, nor were *cdk 2/4/6* affected by IER2-dependent loss of $p21^{waf1/cip1}$ (Fig.21C, data not shown). In addition, the proto-oncogene c-MYC is described as a negative regulator of cell cycle inhibitor $p21^{waf1/cip1}$. [106] Importantly, *c-MYC* mRNA expression was not altered by IER2 (Fig.21D) demonstrating a specific IER2 influence on $p21^{waf1/cip1}$ expression and excluding a myc-tag side effect.

Finding no further transcriptional target of IER2 in the $p21^{waf1/cip1}$ signaling complex we assumed that IER2 contributes to altered cell cycle-related protein synthesis as a consequence of suppressed $p21^{waf1/cip1}$ expression. Therefore, we analyzed the expression of well-known $p21^{waf1/cip1}$ interaction partners. We transiently transfected HEK293 cells with myc-IER2 plasmid, $p21^{waf1/cip1}$ expression plasmid or with the combination of both and could show that ectopic IER2 abolished $p21^{waf1/cip1}$ protein expression (Fig.22A). Furthermore, we found that the protein levels of DNA synthesis and repair gene proliferating cell nuclear antigen (PCNA), interacting with $p21^{waf1/cip1}$ upon DNA damage, [107, 108] was not changed (Fig.22A). PKCδ expression transcriptionally regulates *IER2* as shown above (Fig.22B) and $p21^{waf1/cip1}$ through activation of its Sp1 binding site. [73] We showed that PKCδ protein levels are not changed in our experimental setup and thereby we excluded a feedback regulatory mechanism. Moreover, PKCδ inhibits cell cycle progression in HUVEC by inhibition of cyclin D1/cdk4/6 activity, and the Cip/Kip proteins interact with a variety of cyclin/cdk complexes through a conserved N-terminal domain. [105]

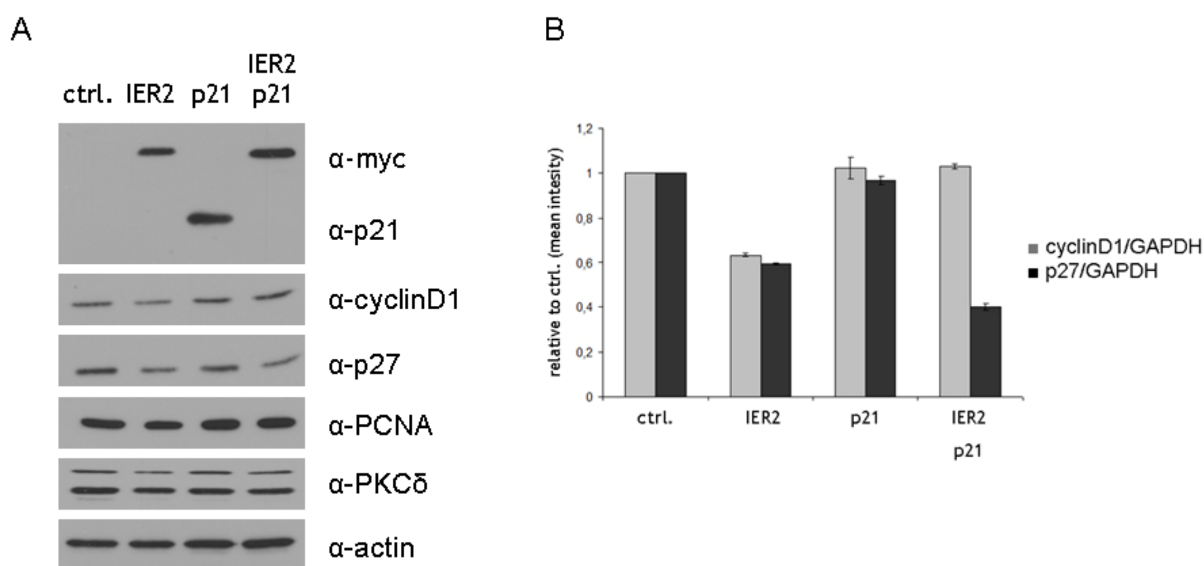


Fig.22: IER2-dependent loss of $p21^{waf1/cip1}$ leads to increased cyclin D1 and reduced p27 protein expression: A) HEK293 cells were transfected with either control expression plasmid (ctrl.), myc-IER2 or p21 expression plasmid or with the combination of IER2 and p21. 24 hours post transfection cells were harvested and whole cell extracts were analyzed by Western blotting for the presence of myc-IER2 (α-myc), p21, PCNA, cyclin D1, p27, and PKCδ. β-actin served as loading control. Results represent one of two experiments. B) Densiometric analysis

of of cyclin D1 and p27 protein levels normalized to loading control β-actin. Shown are mean intensities relatively expressed to control (ctrl.) levels of two experiments. Error bars represent the standard deviation of the mean.

We observed that ectopic expression of $p21^{waf1/cip1}$, similar to PMA-induced $p21^{waf1/cip1}$ levels (data not shown), led to a slight induction of both, cyclin D1 and cell cycle inhibitor $p27^{kip1}$ protein levels, when compared to IER2 expressing cells (Fig.22A/B). This is due to accumulation of $p21^{waf1/cip1}$ in cyclin D1/cdk4/6 complexes thereby inhibiting cyclin D1-associated kinase activity. [62] Of interest and contrary to obtained mRNA data in HUVEC (Fig.21B/C) was the observation that in HEK293 cells IER2 and concomitant loss of $p21^{waf1/cip1}$ expression resulted in a decrease of cell cycle inhibitor $p27^{kip1}$ protein levels. Furthermore, cyclin D1 protein levels remained elevated in these cells compared to $p21^{waf1/cip1}$ transfected cells even though $p21^{waf1/cip1}$ expression was abolished.

We concluded from our two approaches that IER2 is not directly affecting $p21^{waf1/cip1}$ signaling-associated genes and demonstrating a specific IER2 influence on *p21*$^{waf1/cip1}$ expression. Moreover, we found that IER2 alters $p21^{waf1/cip1}$ downstream protein expression that is linked to cell cycle regulation, namely accumulation of cyclin D1 and reduction of $p27^{kip1}$ protein levels.

3.4.2. IER2 reverses the $p21^{waf1/cip1}$-mediated block of E2F-dependent transcription

In addition, we supported the idea that downregulation of *p21*$^{waf1/cip1}$ expression driven by IER2 should result in aberrant cell cycle-associated transcription factor regulation, since $p21^{waf1/cip1}$ binds to cyclin/cdk complexes, thus preventing phosphorylation of the retinoblastoma protein and leading to a blockade of both, the E2F pathway and the cell cycle. [69, 70] Therefore, we analyzed the effect of IER2 expression on E2F activity. We used luciferase reporter genes driven by the *E2F* promoter and showed that IER2 (and thereby diminished *p21*$^{waf1/cip1}$ expression) activates E2F-luciferase activity after stimulation with PMA (Fig.23A) or by co-transfected $p21^{waf1/cip1}$ (Fig.23B), suggesting that IER2 reverses the $p21^{waf1/cip1}$-mediated block of E2F-dependent transcription. Moreover, we tested whether IER2 is a target of E2F and therefore transfected HEK293 cells with E2F1 plasmid, an activator of E2F-dependent transcription. We then performed qRT-PCR and found that *p21*$^{waf1/cip1}$ expression (Fig.23C, gray bars) is strongly induced upon E2F1 transfection compared to empty vector control as well as compared to transcription inhibitor Actinomycin

D treated cells (data not shown). In contrast, *IER2* expression was not at all induced by E2F1 (Fig.23C, black bars) concluding that *IER2* is not regulated by the transcription factor E2F1.

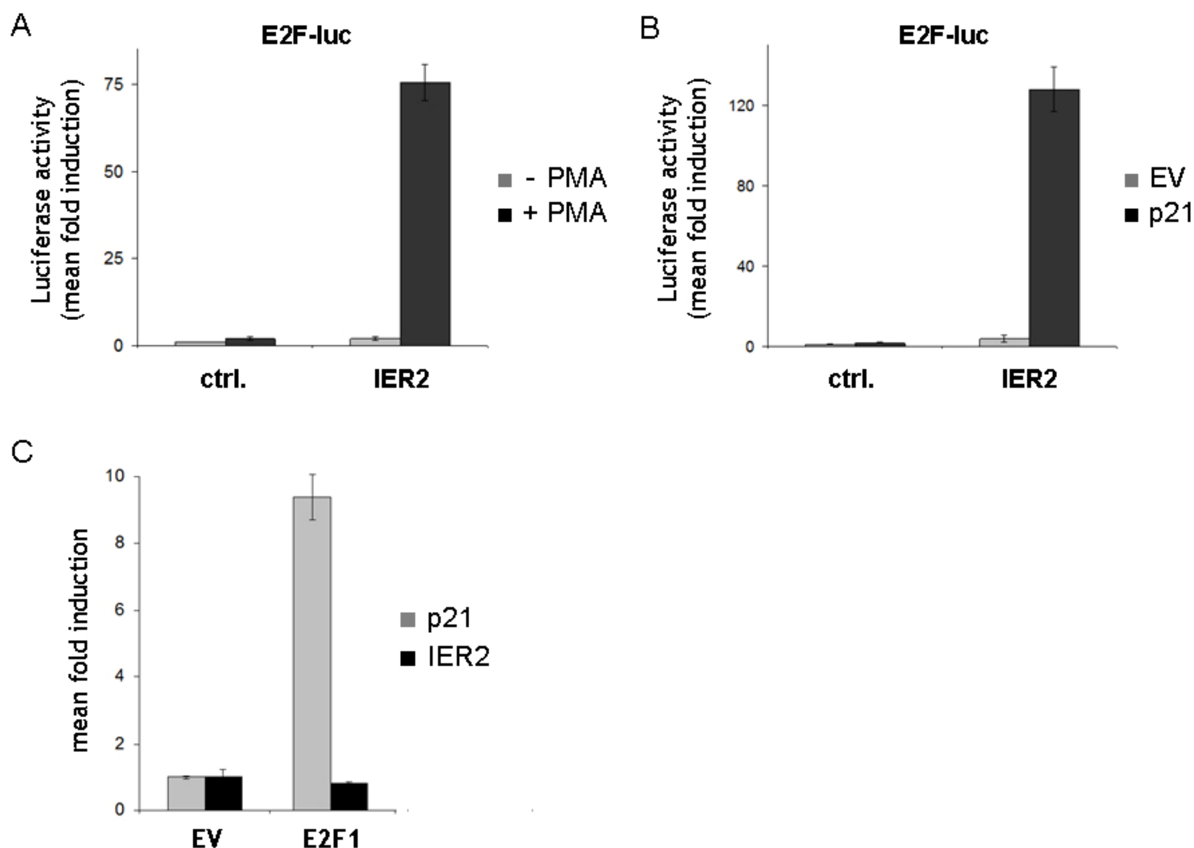


Fig.23: IER2 promotes PMA-induced E2F-activity and overrules the p21$^{waf1/cip1}$ block. HEK293 cells were transfected with luciferase reporter gene driven by the *E2F* promoter, in the absence or presence of IER2 expression plasmid (1µg). A) Cells were left unstimulated (gray bars) or were treated with PMA (black bars) for 8 hours. B) Cells were co-transfected with EV (gray bars) or p21 (1µg) (black bars) expression plasmids. Luciferase activities were analyzed, normalized to co-transfected β-gal and mean fold induction was calculated in relation to basal promoter activity (0µg, gray bar). Error bars represent the standard deviation of the mean. C) HEK293 cells were either transfected with empty vector control (EV) or E2F1 expression plasmid. 24 hours post transfection cells were harvested and *p21* (gray bars) and *IER2* (black bars) mRNA expression levels were analyzed by qRT-PCR and normalized to *GAPDH*. Error bars represent the standard deviation of the mean.

3.4.3. IER2 counteracts PMA-induced apoptosis in HUVEC

Long-time addition of PMA to culture medium inhibits human endothelial cell growth by increasing the levels of p21$^{waf1/cip1}$. [62] Therefore, we evaluated the impact of IER2 expression on HUVEC cell growth. In accordance with altered E2F transcriptional activity, we confirmed by using phase contrast light microscopy on the one hand that the cell density was reduced upon PMA treatment. On the other hand, we found that ectopic IER2 expression in addition to PMA stimulation resulted in an “endothelial cell-specific” proliferative morphology of these cells, showing less cobblestone but elongated cell shape, and they almost reached the same cell density when compared to HUVEC without treatment (Fig.24).

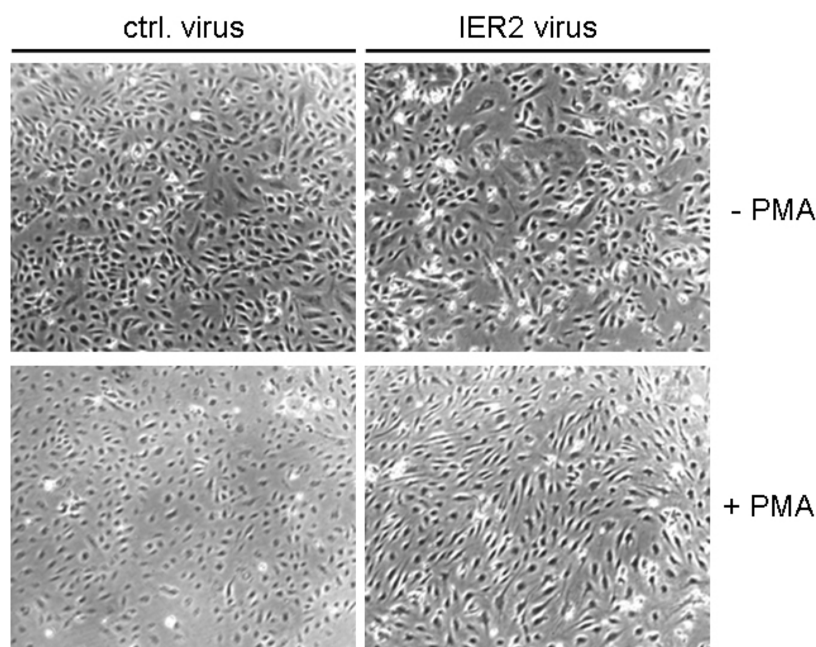


Fig.24: IER2 changes HUVEC morphology upon PMA treatment. HUVEC were infected with control (ctrl., left panel) or IER2 (right panel) virus. 24 hours post infection cells were treated with PMA for another 24 hours followed by phase contrast microscopy.

We found by measuring total cell number that uninfected (uninf.) and control (ctrl.) virus infected HUVEC were reduced by approximately 50% within twenty-four hours of PMA stimulation. In contrast to control cells, IER2 expressing cells almost doubled within the same time (Fig.25A). In addition, by measuring the cell viability using the alamarBlue® assay, we showed a drastic decrease of viable cells after PMA treatment within six to twelve hours of PMA application. In line with previous results, IER2 expressing cells remained viable upon PMA stimulation (Fig.25B). In order to measure apoptosis we used the Annexin V labeling assay in combination with 7-AAD staining followed by flow cytometry analysis. We observed that IER2 counteracts PMA-induced apoptosis in HUVEC (Fig.25C). As expected, approximately 50% of control virus infected HUVEC were either Annexin V positive or Annexin V/7-AAD double positive indicating early and late apoptotic stages (depicted as apoptotic cells in Fig.25C) within twenty-four hours of PMA treatment. We measured 30% less apoptotic cells in IER2 expressing cells compared to control cells. As a second approach to determine the influence of IER2 on apoptosis we determined the cleavage of poly ADP-ribose polymerase (PARP), a well-established apoptosis indicator downstream of caspase activation. We monitored the expression of the 86 kDa cleaved product of PARP by Western Blotting using an anti-PARP specific antibody that recognizes both, full length PARP and its cleaved fragment. Unexpectedly, PARP cleavage became evident already within four to six hours of PMA application. Interestingly, ectopic expression of IER2 completely inhibited

cleavage of PARP in this experiment (Fig.25D), once more demonstrating that IER2 counteracts PMA-induced apoptosis in HUVEC.

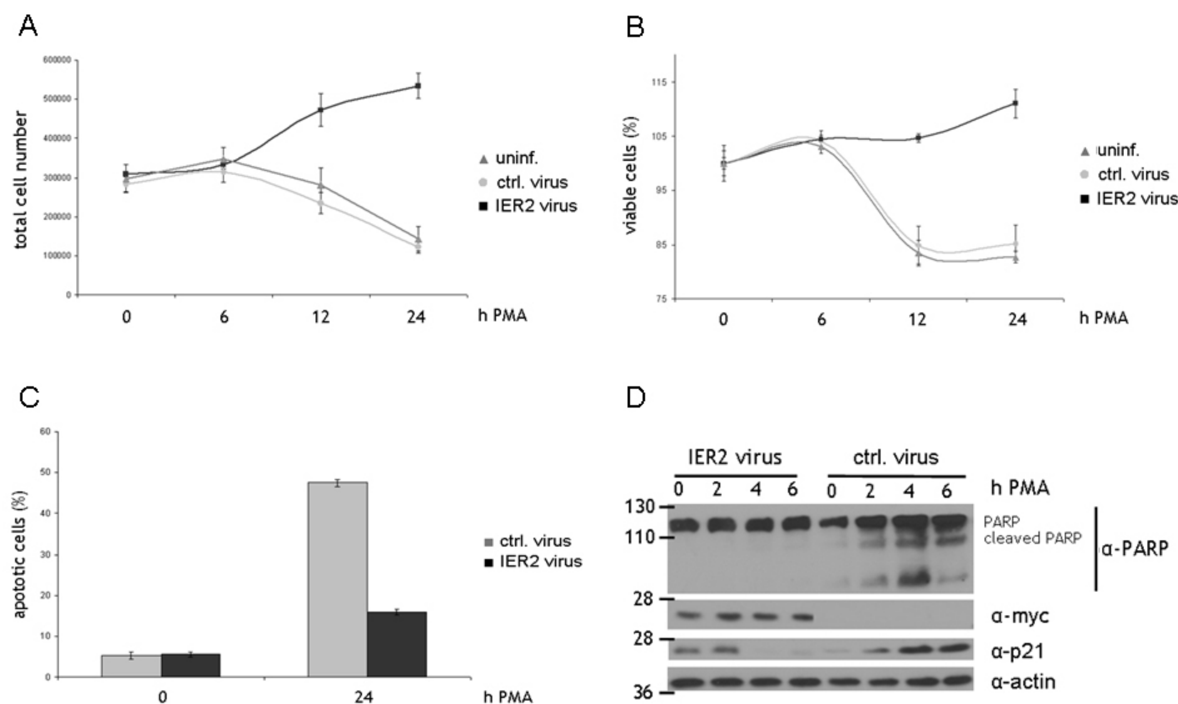


Fig.25: IER2 protects HUVEC from PMA-induced apoptosis. HUVEC were left uninfected (uninf., ▲), or were either infected with ctrl. (●) or IER2 (■) virus. 24 hours post infection cells were treated with PMA and cells were collected at indicated time points. Total cell numbers were counted (A) and cell viability was measured using the alamarBlue® assay (B). Apoptosis was assessed by FACS detecting Annexin V positive cells (C) and by Western Blotting showing PARP cleavage (D).

3.5. Relevance of IER2 in tumor development

Both, $p21^{waf1/cip1}$ and PKCδ can exhibit pro-cancer or anti-cancer activity in different tumor cells due to heterogeneity in their regulation in a cell context- and stress-dependent manner. PKC-mediated increases in $p21^{waf1/cip1}$ levels have been observed in melanoma cells, various leukemic cell lines, breast cancer cells, non-transformed intestinal epithelial cells, colon carcinoma cells and ovarian carcinoma cells. Furthermore, $p21^{waf1/cip1}$ shows decreased expression in some types of metastatic cancers, especially in late melanoma stages. [63, 78-80] We found that activated growth factor signaling via the PKC signaling cascade leads to early and transiently induced IER2 expression that is followed by a long lasting induction of *p21*$^{waf1/cip1}$ expression in a PKCδ-dependent manner. Elevated levels of growth factor receptors and/or their cognate ligands have been identified as a common component of unbalanced cellular signaling leading to aberrant proliferation and cell growth and hence tumor formation. [17] We showed that constitutive IER2 expression as a downstream consequence of abnormal growth factor signaling repressed *p21*$^{waf1/cip1}$ expression. This reversed the $p21^{waf1/cip1}$-mediated cell cycle block and resulted in changed cellular proliferation driven by E2F-dependent pro-survival mechanisms. In addition, recent publications have shown that IER2 levels are elevated in human breast

cancers, in colon cancers, and metastatic melanomas. [56] Therefore, we addressed the question if IER2 is expressed in various cancer entities and may serve as a prognostic marker in metastatic cancer subtypes.

3.5.1. IER2 in patient biopsies

We screened *IER2* mRNA levels in tumors and in the corresponding adjacent mucosa from patients suffering different stages of colon cancer (Table 5). Isolated RNA from patient samples were kindly provided by Prof. E. Kallay, Institute of Pathophysiology, Medical University of Vienna.

Table 5: Colon cancer patient samples. Muc.: adjacent mucosa, tumor free; Tu.: tumor; m: male; f: female; G: tumor differentiation grade; T: primary tumor size; N: lymph node infiltration grade; p: pathologic examination; ascd.: ascending colon; desc.: descending colon; rect.: rectum; sig.: sigmoid colon;

probe number	sample type	sex	age	diagnose	patient number
1. 2.	Muc. Tu.	M	81	G3 pT3/pN1 ascd.	1
3. 4.	Muc. Tu.	M	50	G3 pT3/pN2 desc.	2
5. 6.	Muc. Tu.	F	63	G3 pT2/pN0 rect.	3
7. 8.	Muc. Tu.	M	47	G3 pT3/pN2 rect.	4
9. 10.	Muc. Tu.	F	67	G2 pT3/pN0 sig.	5
11. 12.	Muc. Tu.	F	73	G3 pT3/PN0 rect.	6
13. 14.	Muc. Tu.	F	62	G3 pT3N2 ascd.	7
15. 16.	Muc. Tu.	F	63	G3 pT4N2 desc.	8
17. 18.	Muc. Tu.	M	69	G3 pT3N2 sig.	9
19. 20.	Muc. Tu.	M	73	G3 pT3N1 sig.	10

We performed quantitative real-time PCR analysis and surprisingly found that *IER2* mRNA expression was reduced in eight out of ten tumor samples compared to their related tumor free mucosa (Fig.26). These results were in contrast to our expectations, however, due to limited access to patient material we were hindered to test late stage tumors or metastatic colon cancer samples. Patient material collection and further analyses are ongoing and we expect in

these entities increased IER2 levels because it was recently shown that IER2 expression correlates with poor metastasis-free and overall survival in patients with colorectal adenocarcinomas. [39]

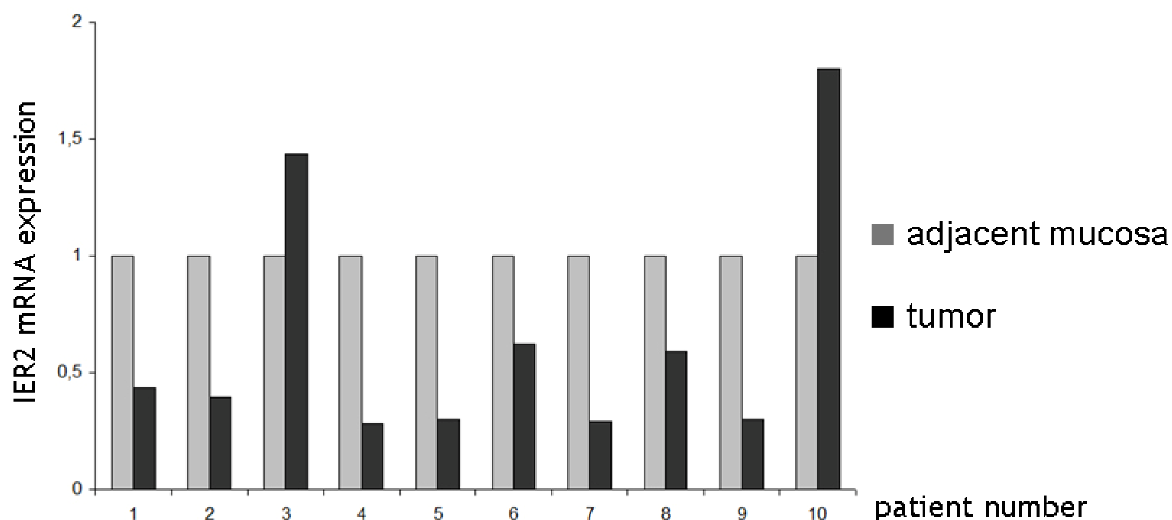


Fig.26: IER2 mRNA expression is suppressed in T2-T3 primary colon cancers. *IER2* mRNA expression was monitored in healthy adjacent mucosa and related tumor tissues of 10 patients with different sex, age, and diagnosis. Relative levels of expression were normalized to *GAPDH* and *β-actin* (data not shown). ct value of healthy tissue was set as one.

3.5.2. IER2 in human cancer cell lines

Furthermore, we tested several human cancer and metastatic cancer cell lines for IER2 protein expression levels (Table 6). Obtained protein lysates were analyzed by Western Blotting. We detected no IER2 protein expression in two tested colon cancer cell lines (Caco2, HCT-116) which is in line with the results of the obtained samples from colon cancer patients. Contrary, we identified that IER2 is strongly expressed in the androgen sensitive prostate LnCap cell line. Furthermore, we found weak IER2 expression in the androgen resistant DU145 compared to PC3 prostate cell line (Fig.27A). Although we tested only two cell lines, these findings reveal first evidence that IER2 could be involved in promoting metastasis as DU145 cells expose moderate metastatic potential in contrast to high metastatic PC3 cells. In addition, we tested three metastatic melanoma cell lines Mel-7, Mel-15, and Mel-17 [87] and found that IER2 is expressed in two (Mel-7, Mel-15) metastatic melanoma cell lines compared to healthy melanocytes. Controversy, IER2 was also expressed in one tested melanoma cell line (A-375). In addition, IER2 was not expressed in tested breast cancer (ZR-751, MCF7) or in metastatic breast cancer cell lines (MDA-MB-468, MDA-MB-231; Fig.27B). Screening $p21^{waf1/cip1}$ as well as tp53 protein levels we found at least one cell line, the spleen metastatic melanoma cell line Mel-7, which showed a severe reduction of $p21^{waf1/cip1}$ expression, normal/physiologic tp53 levels and intense IER2 expression. This

represents a IER2 specific and tp53-independent effect indicating a particular IER2 influence on oncogenic transformation, which has to be further investigated.

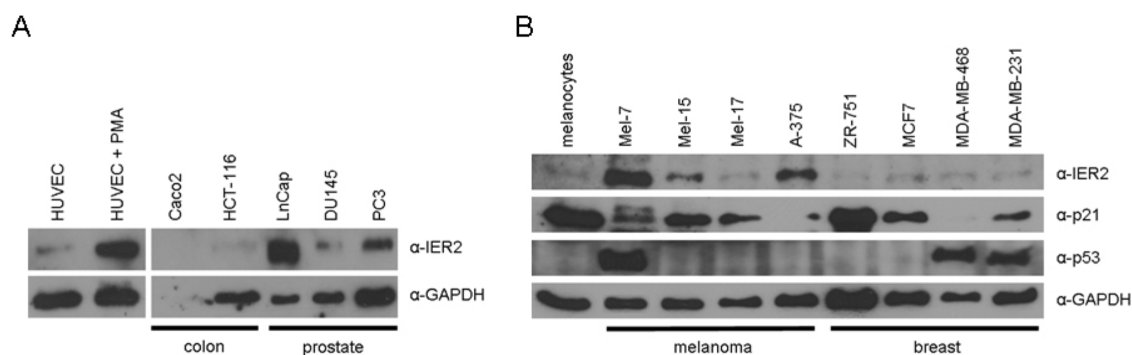


Fig.27: IER2 expression in cancer cell lines. Whole cell extracts of human colon cancer (Caco2, HCT-116), prostate cancer (LnCap, DU145, PC3), melanocytes, melanoma (Mel-7, Mel-15, Mel-17, A-375), and breast cancer (ZR-751, MCF7, MDA-MB-468, MDA-MB-231) cell lines were probed by Western Blotting with anti-IER2 (A,B) and p21, tp53 (B) antibodies. GAPDH served as loading control. Induction of IER2 in PMA stimulated HUVEC (A, left panel) is shown as control.

Information on tested human cancer cell lines and related IER2 protein expression levels is summarized in Table 6. A broader analysis of IER2 expression across human cancer biopsies and cell lines has to be performed to further contribute to the understanding of the IER2 function in tumor development or tumor progression.

Table 6: Tested cell lines. Prostate cancer cell lines were kindly provided by Johannes Schmid. Colon cancer, breast cancer, and melanoma samples were kind gifts from Christoph Wiesner. IER2 expression level legend: - stands for no detected IER2, ~ IER2 at the detection level, + for low IER2 and ++ for high IER2 expression.

cell line	origin	primary tumor / metastasis	additional info	IER2 level
Caco2	colon cancer			-
HCT-116	colon cancer			-
LnCap	prostate cancer	low metastatic potential	androgen sensitive	++
DU145	prostate cancer	moderate metastatic potential	androgen insensitive	~
PC3	prostate cancer	high metastatic potential	androgen insensitive	+
Mel	melanocytes			~
Mel-7	metastatic melanoma	spleen		++
Mel-15	metastatic melanoma	lymph node		+
Mel-17	metastatic melanoma	skin		~
A-375	malignant melanoma			++
ZR-751	breast cancer			~
MCF7	breast cancer			~
MDA-MB-468	metastatic breast cancer	high metastatic potential		~
MDA-MB-231	metastatic breast cancer	low metastatic potential		~

In summary, we confirm in this study that immediate early response gene 2 (IER2) expression is inducible by growth factors and by the tumor and angiogenesis promoting agent PMA leading to the activation of the PKCδ signaling cascade. Importantly, we present for the first time a biological function for this poorly characterized protein, namely that IER2 represses the expression of cell cycle inhibitor *p21*$^{waf1/cip1}$ on the level of transcription. As a consequence, IER2 can rescue human endothelial cells from PMA-induced apoptosis by reversing the p21$^{waf1/cip1}$-mediated blockage of E2F-dependent transcription. These findings of a novel regulator of p21$^{waf1/cip1}$ mighty increase the knowledge of malignancies that result from differentially regulated cell cycle progression resulting in enhanced proliferation or malfunctioning apoptotic processes. Thus, our data contribute to a better understanding of IER2, a putative novel player in the regulation of tumor progression and metastasis and therefore representing a potential target for prognostic and therapeutic strategies in cancer.

4. DISCUSSION

Chronic inflammatory conditions have been described as hallmarks of several diseases, including e.g., psoriasis, rheumatoid arthritis, and arteriosclerosis as well as tumor development, tumor progression, and metastasis. [2-6] They often develop as a consequence of inappropriately terminated acute inflammation. Therefore, it is of outstanding importance to understand the complex mechanism behind the tight regulation of acute inflammation and malfunctioning signaling events that propagate prolonged inflammatory conditions. [12] While all these processes have been studied intensively over the preceding decades, much less is known about early regulators of this process. However, evidence has accumulated that resolution is already determined during the activation phase of the initial response by so-called immediate early genes. The understanding of their function and mode of action might lead to new concepts of drug development and thereby to great benefit for patients suffering inflammation related diseases. [4]

Endothelial cells are central to the inflammatory reaction, as they form a barrier between the circulating blood stream transporting immune cells and the surrounded tissue. Moreover, this cell type is activated under inflammatory conditions and amplifies and re-directs the inflammatory response through three main mechanisms: i) adhesion molecule expression, leading to further recruitment of leukocytes to the site of inflammation, ii) cytokine production, acting in an autocrine and paracrine manner towards the surrounding tissue, and iii) reactivation of the cell cycle, leading to cell proliferation and to the formation of new blood vessels, also known as angiogenesis. [2, 3, 6-9]

In the course of our search of immediate early genes with potential negative regulatory function in HUVEC we found IER2. [46] The overall aim of this project was to elucidate the function of this hitherto poorly described protein in the human endothelium, especially due to its immediate early kinetics of expression, whether it may act as an early regulator in the first transcriptional response upon pro-inflammatory stimulation. Furthermore, we addressed the question if IER2 is solely linked to inflammation or to other processes that are important for the pathophysiology of endothelial cells.

Based on recently reported data [46, 47] we initially hypothesized that IER2 might represent a novel early negative regulator of NF-κB-driven inflammatory signaling in endothelial cells as it was shown for other genes. [12] However, for inexplicable reasons the reproducibility and

strength of the upregulation of *IER2* in response to TNFα or IL-1β was poor as compared to the array data. Increasing the concentrations of used cytokines and other modifications of the experimental setup (i.e., time of stimulation, serum-free conditions) resulted in no significant alterations of *IER2* expression. A possible explanation for these conflicting results is the fact that the *IER2* promoter contains a serum-response element (SRE) and that the *IER2* expression was monitored in non-serum starved conditions in the reported arrays. In addition, deletion analysis of the *IER2* mouse homologue *pip92* promoter indicated that *pip92* activation occurs primarily within the SRE region. [52] Moreover, M. St-Onge and colleagues found that *IER2* is induced within two hours upon stimulation with inflammatory agonists in neutrophils, also early players in the initial phase of inflammation. On the contrary, co-treatment with anti-inflammatory agents even enhances *IER2* expression in their hands. [49] These inconsistent findings together with our results indicate that *IER2* is an early regulated gene that is somehow associated with inflammatory conditions, but the underlying mechanism remains unclear.

The aim of further investigations was to find potent activators of *IER2* in HUVEC and to elucidate its biological function. *IER2* was rapidly and transiently induced by the growth factors VEGF and bFGF and its expression pattern exhibited classical features of an immediate early gene in HUVEC. Its mRNA levels accumulated within thirty minutes of growth factor stimulation and decreased to baseline levels after two hours. These results are in line with earlier findings that showed immediate early kinetics of *IER2* upon serum and growth factor stimulation (e.g., FGF, EGF, and NGF) in different mouse and human cell types. [34, 36, 41] Since *IER2* has been first described as a phorbol ester PMA-inducible gene [34] and concerning the role of PKC in VEGF- and bFGF-induced endothelial cell proliferation [18] we analyzed the effect of a potent activator of PKC signaling, the phorbol ester PMA on *IER2* expression. In line with the growth factor studies we found that *IER2* mRNA showed comparable kinetics peaking within thirty minutes to one hour of stimulation, and IER2 protein levels emerged shortly after accumulation of the *IER2* transcript. Important to note is that the protein did not undergo such a rapid turnover as compared to its mRNA. Conversely, the protein was stabilized and accumulated over approximately four hours and decreased below the level of detection after ten hours of PMA stimulation. This is in line with earlier studies in murine and rat cell types, where it was shown that *pip92* mRNA is induced within minutes and protein levels are decreased after six hours of serum stimulation but within one hour after bFGF stimulation. [34, 36]

It seems likely that the PEST domain within the IER2 sequences is important for the rapid degradation of IER2 protein. However, posttranslational modifications evoked by PMA stimulation seem to additionally support prolonged/ stabilized expression of IER2. Furthermore, after the period of stabilization, IER2 protein levels dropped until the initial undetectable state is reached. This decrease is due to ubiquitin-driven proteasomal degradation as its inhibition by MG132 effectively blocked IER2 degradation and led to high molecular mass ubiquitinated smear ladders (Fig.13D). Further experiments revealed that PMA-driven phosphorylation resulted in a time-dependent stabilization of IER2, and additional blocking of this phosphorylation demonstrated that the degradation of IER2 occurs as a result of PKC signaling inhibition. In addition, IER2 degradation is affected by sumoylation of the N-terminus of the protein which we demonstrated using truncated IER2 expression constructs. Taken together, the N-terminus of IER2 is important for protein stability and we describe for the first time a mechanism of IER2 degradation (Fig.28). Nevertheless, further analyses such as e.g., point mutations of the sumoylation and phosphorylation sites will be needed to completely understand the degradation process of IER2.

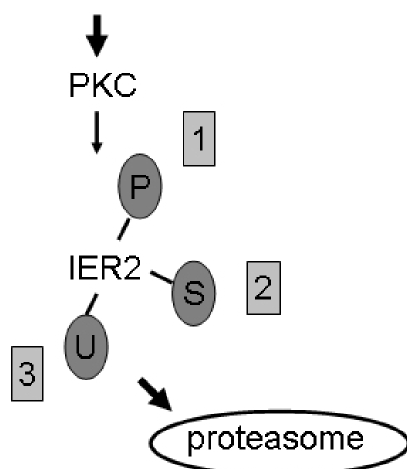


Fig.28: Proposed model of IER2 posttranslational modifications. PKC regulates IER2 on the level of transcription and by posttranslational phosphorylation. PMA activates PKC which in turn leads to phosphorylation of distinct PKC phosphorylation residues in IER2. IER2 is thereby activated and stabilized. Sumoylation of the IER2 N-terminus and subsequent ubiquitination lead to proteasomal degradation.

Moreover, we showed that PMA-induced IER2 protein accumulated in the cytosol which is in line with earlier findings described by Charles *et al.* but still remains elusive as IER2 contains two nuclear localization signals. [34] Moreover, *IER2* expression was time- but not concentration-dependent, at least in the range of 50 to 250ng/ml PMA, indicating that the used PMA concentrations were already in the saturation phase. Another explanation could be that IEGs are sensitively and time-dependently induced by many growth factors that activate

PKC signaling. [109] Therefore, analyses of a broader concentration range of growth factors will be necessary to understand the induction of *IER2* expression. Nevertheless, evidence has accumulated that growth factors and PKC signaling agonists such as PMA transcripionally activate *IER2*. To the best of our knowledge there were no data available that describe a specific function of IER2 in the PKC signaling cascade.

One important aspect of endothelial cells' functionality is the ability to form new blood vessels. Growth factors as VEGF and bFGF bind to their respective receptors and activate PKC signaling resulting in endothelial cell proliferation and angiogenesis. Furthermore, PMA is known to activate protein kinase C (PKC), [110, 111] nevertheless, a variety of PMA-driven effects on cultured endothelial cells have been described. These include the induction of migration, proliferation, and vessel formation [112, 113] whereas long-time application is rather described as a suppressor of proliferation and an activator of apoptotic endothelial cell death. [58, 62, 114] However, different PKC isoforms may have unique and even opposing functions that are important in endothelial cell proliferation and angiogenesis. Recent studies have focused on PKC isoforms α and δ and have indicated that both of these isozymes play a role in the regulation of angiogenesis. Taylor *et al.* and others have shown that PKCα and PKCδ are generally expressed in HUVEC. PMA stimulation results in a decrease in PKCα phosphorylation to undetectable levels after twenty-four hours, whereas PKCδ is phosphorylated within fifteen minutes but returns to basal levels after twelve hours. Decreases in total PKCα and PKCδ levels have been observed after forty-eight hours. Theses findings might explain the variable effects of PMA-activated PKC-driven mechanisms. [115, 116] In addition, overexpression of PKCδ in rat endothelial cells inhibits endothelial differentiation in a matrigel assay, [117] whereas downregulation of PKCα in HUVECs by antisense oligonucleotides also hinders vessel formation in a three-dimensional collagen angiogenesis assay. [118] Taken together, these data suggest that PKC isoforms have unique and possibly opposing functions during vessel assembly.

Important to note is that molecular targets for angiogenesis downstream of PKC remain elusive in endothelial cells. Therefore, we hypothesized that *IER2* is regulated by PKCα and/ or PKCδ thereby influencing endothelial cell proliferation and angiogenesis. Indeed, we found reduced *IER2* expression using the potent PKC inhibitor Staurosporine (data not shown), which is explained by the fact that PMA activates cPKC as well as nPKC isozymes. [119] Further examination revealed that PMA-induced IER2 expression and protein accumulation

was completely blocked by the addition of Rottlerin. This widely-used PKC inhibitor is selective for PKCδ in the used concentration of 5µM. [120] In contrast, we discovered that co-stimulation of PMA and Ionomycin resulted in a weaker induction of *IER2* compared to PMA only treatment which is due to increased intra-cellular calcium levels and thereby enhanced activation of cPKC. However, to clearly show a specific nPKC effect we additionally decreased PKCδ activity using RNA interference (RNAi). We showed that *IER2* mRNA levels accumulated within thirty minutes of PMA stimulation whereas its expression was at the limit of detection in PKCδ knockdown HUVEC. Together these findings indicate that PKCδ is the key driver of *IER2* expression in HUVEC as we showed that decreasing PKCδ activity (pharmacological or RNAi) suppressed *IER2* expression.

Furthermore, these results might explain the conflicting results we obtained from cytokine stimulation as it has been shown that PKC activation induces *ELAM-1* gene expression synergistically with IL-1β but not with TNFα, while such a synergistic effect is not observed upon the expression of *ICAM-1*, [121] demonstrating a link between PKC signaling and early regulated inflammation-associated gene expression. In addition, it has recently been shown that PKCδ selectively regulates/ phosphorylates protein kinase D (PKD), an activator of the transcription factor NF-κB in cells exposed to oxidative stress. [122, 123] Knockdown of PKCδ expression significantly inhibits PMA-stimulated NF-κB-luciferase activity suggesting that PMA-induced NF-κB-dependent transcription is driven by nPKC isoforms, particularly PKCδ. [124] Besides, PMA selectively causes the degradation of IkB kinases (IKKs) including IKK-γ and IKK-β, indicating that the IKK complex is predominantly degraded by the proteasome pathway in a PKC-dependent manner. [125] Moreover, the two κB binding sites in the *IER2* promoter led us to the assumption that *IER2* might be induced upon pro-inflammatory cytokine stimulation which involves NF-κB.

In reporter gene assays analyzing the transcriptional activity of NF-κB we observed that upon cytokine stimulation IER2 rescued NF-κB transduction (data not shown). Conversely, we found no *IER2* activation after cytokine-driven NF-κB activation nor an influence of IER2 on NF-κB target gene expression. This might be due to intra-cellular signaling crosstalks as *IER2* is activated upon PKC activation which in turns also activates *NF-κB*. Although we have evidence that IER2 inhibits NF-κB, we have so far no data on any influence on pro-inflammatory gene expression. It seems likely that nuclear translocation of p65 is not affected by IER2 but phosphorylation of nuclear NF-κB might be inactivated [126, 127] in the

presence of IER2 by competitive mechanisms of PKA and PKC. Nevertheless, we suppose that IER2 functions as a new regulator in pro-survival PKC–NF-κB signaling which, however, still remains elusive.

One of the paradigms that best exemplifies the functional versatility of PKC isozymes is the regulation of the cell cycle machinery. PKCδ regulates the cell cycle in endothelial cells in a bimodal way. Under non-stressed physiological conditions, the cell cycle inhibitor $p21^{waf1/cip1}$ is expressed at low levels and promotes cell cycle progression. Under PKC-mediated stress conditions, short-time events early in G1 support G1/S phase progression, while activated PKC in mid-to-late G1 and concomitant increased $p21^{waf1/cip1}$ expression prevent entry of cells into S phase. In particular, it has been shown in human vein endothelial cells that PKCδ inhibits cell cycle progression by inhibition of cyclin D1/cdk4/6 activity, and increases levels of cyclin-dependent kinase inhibitors (CKI), such as $p21^{waf1/cip1}$ and to lower extent $p27^{kip1}$. [62, 71] In line with that, we found that PMA-induced *p21*$^{waf1/cip1}$ expression within one to two hours, and protein levels also accumulated within two hours. This is due to a tp53-independent elevation of *p21*$^{waf1/cip1}$ expression by phorbol esters and a result of PKC-mediated mRNA stabilization. [76]

The most prominent finding of our study was that ectopic expression of IER2 suppressed PMA-induced *p21*$^{waf1/cip1}$ expression. More precisely, IER2 abolished PMA-induced $p21^{waf1/cip1}$ protein accumulation in the cytoplasm. Subcellular distribution influences the activity of $p21^{waf1/cip1}$, [128] and *in vitro* phosphorylation assays have further shown that PKCδ can directly phosphorylate $p21^{waf1/cip1}$ at Ser146, which triggers its cytosolic accumulation and influences the stabilization of $p21^{waf1/cip1}$. [129] Moreover, using reporter gene assays monitoring the activity of the *p21*$^{waf1/cip1}$ promoter we observed that increasing amounts of IER2 with concomitant activation of the PKC signaling cascade by PMA strongly inhibited *p21*$^{waf1/cip1}$ transcription. This finding is reminiscent of the function of other IEGs, most prominently the proto-oncogene c-MYC that has been shown to transcripitonally inhibit *p21*$^{waf1/cip1}$ expression. [106] In addition, knockdown of IER2 restored $p21^{waf1/cip1}$-luciferase activity. Western analysis of respective protein extracts confirmed the high efficiency of the IER2 knockdown although $p21^{waf1/cip1}$ protein levels were only slightly restored. This is possibly due to differences in expression kinetics or/ and protein stabilities of analyzed proteins.

Further experiments revealed that only full length IER2 was capable to attenuate $p21^{waf1/cip1}$ expression whereas truncated IER2 constructs disclosed opposing or even no effects on $p21^{waf1/cip1}$ activity (Fig.19A). Previous experiments indicated a regulatory role of the N-terminus of IER2 on protein stability. Interestingly, the ΔN construct even enhanced $p21^{waf1/cip1}$ activity, suggesting that the sumoylation-dependent degradation of IER2 may be important for IER2-dependent inhibition of $p21^{waf1/cip1}$. Moreover, the finding that the ΔC construct missing the C-terminus with the PKC-specific phosphorylation sites was without any effect demonstrated the importance of IER2 activation/ phosphorylation to execute its inhibitory function. Besides, we observed (auto-) regulation of $p21^{waf1/cip1}$-luciferase activity by co-transfection of a $p21^{waf1/cip1}$ expression plasmid. Little is known about the molecular mechanism and biological significance of the self-regulation of *p21*$^{waf1/cip1}$ but it might be explained by a positive feedback loop regulation of $p21^{waf1/cip1}$-associated transcription factors and co-activators, such as CCAAT enhancer binding protein α (C/EBPα), c-MYC, signal transducer and activator of transcription 3 (STAT3), E2F, SP1, and cAMP response element-binding protein (CREB)-binding protein (CBP)/p300. [78, 103, 104] As stated above, we found that IER2 regulated *p21*$^{waf1/cip1}$ on the level of transcription. Contrary, co-transfection of IER2 and $p21^{waf1/cip1}$ expression plasmids, both under a cytomegalovirus (CMV) promoter, in HEK293 cells completely reduced/ inhibited $p21^{waf1/cip1}$. This could be explained by the fact that both, IER2 and $p21^{waf1/cip1}$ were not expressed in HEK293 cells under non-stressed conditions; nevertheless, they were inducible with PMA (data not shown).

Moreover, we assumed that IER2 functions only in addition to PKCδ-triggered phosphorylation which we confirmed with truncated IER2 constructs (see above), and auto-regulation of *p21*$^{waf1/cip1}$ could promote the activation of IER2. On the other hand, post-transcriptional control of $p21^{waf1/cip1}$ has been linked to localization, phosphorylation and ubiquitination [102] and it has additionally been reported that $p21^{waf1/cip1}$ is degraded by the proteasome in ubiquitin-dependent and -independent manners. [130-132] We showed that inhibition of ubiquitin-driven proteasomal degradation with MG132 effectively blocked ectopic $p21^{waf1/cip1}$ degradation and led to high molecular mass ubiquitinated smear ladders, whereas co-transfection of IER2 completely suppressed $p21^{waf1/cip1}$ protein levels. These results indicated that IER2-dependent inhibition of $p21^{waf1/cip1}$, beside the transcriptional blockade, additionally affects ubiquitin-independent protein degradation. The posttranslational influence of IER2 on $p21^{waf1/cip1}$ stability will be further investigated.

As mentioned above, $p21^{waf1/cip1}$ is one of the best studied tp53 downstream targets. The induction of its expression by PMA is tp53-independent, [73, 74] whereas DNA damage leads to tp53-dependent induction of $\mathit{p21}^{\mathit{waf1/cip1}}$ transcription. [70] Indeed, $p21^{waf1/cip1}$-luciferase was activated by co-transfection of a tp53 expression plasmid. Conversely, IER2 was unable to inhibit tp53-mediated $p21^{waf1/cip1}$-luciferase activity and co-transfection of IER2, $p21^{waf1/cip1}$, and tp53 additionally prevented the IER2-driven reduction of $p21^{waf1/cip1}$ expression (Fig.19C). This could be due to the fact that IER2 influences $\mathit{p21}^{\mathit{waf1/cip1}}$ in a tp53-independent manner, but that the tp53–MDM2 (oncoprotein mouse double minute 2) pathway can override the PKCδ–IER2 pathway. The complexity of $p21^{waf1/cip1}$ regulation and signaling is complicated by the finding that MDM2 facilitates $p21^{waf1/cip1}$ degradation independent of both tp53 and ubiquitination. [133] These results rather argue for a specific role of IER2 in growth factor signaling than in response to DNA damage. In line with these findings, we observed no influence of IER2 on *tp53* transcription. Furthermore, the proto-oncogene c-MYC has been shown to transcripitonally inhibit $\mathit{p21}^{\mathit{waf1/cip1}}$ expression thereby overriding a $p21^{waf1/cip1}$-mediated cell cycle checkpoint. [106] Importantly, *c-MYC* expression was not affected by IER2 (Fig.21D) demonstrating a specific IER2 influence on $\mathit{p21}^{\mathit{waf1/cip1}}$ expression and also excluding a myc-tag side effect. Taken together, these observations reveal a to our knowledge novel activity for IER2 as an additional key regulator of the cell cycle inhibitor $p21^{waf1/cip1}$ that will further contribute to the understanding of the complex regulation of tp53-MDM2- $p21^{waf1/cip1}$ and PKC-IER2- $p21^{waf1/cip1}$ interfering signaling cascades.

Besides $p21^{waf1/cip1}$, regulation of the Cip/Kip family member cell cycle inhibitor $p27^{kip1}$ is also PKC-dependent. [105] However, we found no induction upon PMA stimulation within tested time points and as a consequence no influence of IER2 on $\mathit{p27}^{\mathit{kip1}}$ expression. This might be explained by the findings of Zezula *et al.* who showed that endothelial cells in G0 phase contain abundant amounts of $p27^{kip1}$ and low levels of $p21^{waf1/cip1}$, whereas after mitogenic stimulation (bFGF or phorbol ester phorbol-2,13-dibutyrate) levels of $p21^{waf1/cip1}$ are elevated and the levels of $p27^{kip1}$ remain unaltered. [62] Furthermore, it has been shown that $p27^{kip1}$ is unable to mediate PKC-induced cell cycle arrest in the absence of $p21^{waf1/cip1}$. Besides, $p27^{kip1}$ protein levels were repressed by IER2 whereas $p21^{waf1/cip1}$ elevated $p27^{kip1}$ to baseline levels, and the combination of IER2 and $p21^{waf1/cip1}$ resulted again in a drop of $p27^{kip1}$. The mechanism behind these effects remains elusive and warrants further investigation. Moreover, we detected no influence of IER2 on PMA-activated *cyclin D1* expression. In contrast, IER2 transfected HEK293 cells revealed decreased cyclin D1 protein

levels. This might be a result of PKCδ upregulation that has been shown to decrease cyclin D1 and cyclin E proteins co-immunoprecipitated with cdk6 and cdk2, respectively. [134] Interestingly, transfection of a p21$^{waf1/cip1}$ expression plasmid restored cyclin D1 levels to the initial level. Upon co-transfection of IER2 and p21$^{waf1/cip1}$ that resulted in undetectable levels of p21$^{waf1/cip1}$ we observed similar baseline cyclin D1 levels. On the one hand, this might be due to the fact that p21$^{waf1/cip1}$ induces growth arrest by inhibiting the activity of cyclin-dependent kinases (cdks) or of proliferating cell nuclear antigen (PCNA) [70] that is not affected in our experimental setup. On the other hand, p21$^{waf1/cip1}$ might act as an assembly factor for cdk/cyclin complexes. p21$^{waf1/cip1}$ has been found to promote the assembly of cdk4/6 and cyclin D *in vitro* and associates with cyclin D1/cdk4 complexes during cell cycle progression. [135] The role of p21$^{waf1/cip1}$ as an assembly activator or inhibitor depends on its expression level. At low and intermediate concentrations it is an assembly factor, while at high concentrations it is an inhibitor. [136, 137] This is an important issue for future experiments analysing the effect of increasing amounts of p21$^{waf1/cip1}$ and the influence of IER2 on phosphorylated cyclin D1 as well as total cyclin D1 levels and on the kinase activity. This will contribute to earlier findings which have shown that phorbol ester treatment inhibits cyclin D1 activity in human venous endothelial cells based on the presence of increased levels of cell cycle inhibitor p21$^{waf1/cip1}$ in cyclin D1/cdk4 complexes. [61, 62]

Consequences of altered *p21$^{waf1/cip1}$* expression have been shown to affect downstream events such as E2F-dependent transcription resulting in changes of cell cycle progression, which in turn can lead to a switch between cell survival and death. [70] It is commonly accepted that p21$^{waf1/cip1}$ influences cell cycle progression by controlling the activity of cdks that act on the retinoblastoma tumor suppressor protein (pRb). In a hypophosphorylated state, pRb associates with E2F transcription factors to prevent the activation of genes required for progression into S phase. Phosphorylation of pRb by G1 phase cdk complexes releases E2F and thereby enables progress through the cell cycle. [26] Indeed, using E2F-luciferase reporter assays we observed that IER2 counteracted the p21$^{waf1/cip1}$-mediated block of E2F-dependent transcriptional activity. Furthermore, we confirmed that *p21$^{waf1/cip1}$* expression was strongly induced by E2F1, a well-known activator of *p21$^{waf1/cip1}$* expression mediating a negative feedback loop. [128] Moreover, Delavaine *et al.* have suggested a p21$^{waf1/cip1}$-dependent and cdk/pRb activity-independent mechanism that facilitates the transcriptional regulation of E2F by association of p21$^{waf1/cip1}$ with E2F subunits. [138] This might also explain the weak effects of IER2 on cyclin D1, and additional experiments will address whether IER2 affects

the pRb status. In contrast to *p21$^{waf1/cip1}$*, *IER2* expression was not induced by E2F1, concluding that *IER2* is not regulated by the transcription factor E2F1. It has been recently shown that both E2F1 and IER2 have the ability to bind to the promoter and act as transcriptional activators for the gene *ISYNA1*, which encodes human inositol 1-phosphate synthase, playing important roles in several biological processes. [54] This finding suggests that IER2 may function as a transcriptional (co-) factor, similar to other IEGs that often act as transcription factors or transcriptional co-factors. [31] Nevertheless, further analysis of the IER2 regulation of *p21$^{waf1/cip1}$* and the divergent influence of all E2F transcription family members [139] will be needed to reveal deeper insights into the IER2-driven mechanism.

As mentioned above, long-time addition of PMA to culture medium inhibits human endothelial cell growth by increasing the levels of p21$^{waf1/cip1}$. [62] Indeed, upon microscopical examination we observed reduced cell density and changes in morphology in PMA treated HUVEC. They lost their cobblestone shape, adhesion, and turned into condensed, small, and round cells, resembling apoptotic endothelial cells. [140] It is important to note that ectopic IER2 expression in addition to PMA stimulation resulted in an "endothelial cell-specific" proliferative morphology of these cells, showing less cobblestone but elongated cell shape, and they almost reached the same cell density when compared to HUVEC without treatment (Fig.24). These findings further supported the idea that IER2 influences HUVEC survival as a consequence of IER2-mediated inhibition of *p21$^{waf1/cip1}$* and concomitant activation of E2F-dependent transcription. In fact, we found that the total cell number of PMA treated HUVEC was reduced by approximately 50% within twenty-four hours. Ectopic IER2 expression counteracted the PMA effect, and cells almost doubled within twenty-four hours. Moreover, we observed no changes in cell viability whereas PMA treated control cells demonstrated lower metabolic activity, depicted as per cent of viable cells (Fig.25B). In addition, IER2 rescued HUVEC from PMA-induced apoptosis as we measured 30% less apoptotic cells after twenty-four hours compared to control cells. It is important to note that IER2 expressing cells proliferated, although PMA was added to the culture medium. Nevertheless, a portion of these proliferating cells underwent apoptosis within twenty-four hours which seems to be a result of ineffective infection of all cells thus not reaching 100% efficiency. As a second approach to confirm whether IER counteracts PMA-induced apoptosis we assessed poly ADP-ribose polymerase (PARP) cleavage, which is an established apoptosis indicator downstream of caspase activation. During apoptosis, PARP, a 116 kDa protein that normally functions in DNA damage detection and repair, is cleaved by caspase-3 and caspase-

7. This cleavage effectively counteracts PARP-mediated DNA repair and triggers apoptosis. [92, 93] Interestingly, PMA-induced p21$^{waf1/cip1}$ as well as PARP cleavage within two to four hours, whereas in IER2 expressing cells PMA-induced p21$^{waf1/cip1}$ expression was inhibited and PARP cleavage suppressed. Thus, IER2 represents a novel so far not described regulator of cytosolic p21$^{waf1/cip1}$ and indicates a role as a regulator of cell proliferation and cell death. Important to note is that cytosolic p21$^{waf1/cip1}$ has been described to predominantly exert anti-apoptotic functions, [128] whereas we showed pro-apoptotic activity of cytosolic p21$^{waf1/cip1}$ in HUVEC. Similar results, however, in contrast to ours, have been shown by Levkau *et al*. They indicated that growth factor deprivation of HUVEC induces apoptosis via cdk2 upregulation, concomitant reduction of cytosolic p21$^{waf1/cip1}$ and emerging PARP cleavage within four to eight hours. [71] We highlight a more complex but specific IER2 function in HUVEC. In contrast to our results some studies have shown enhanced IER2 expression during the induction of apoptosis, and there is little evidence that IER2 expression might promote apoptosis. [53]

Nevertheless, others have reported that IER2 expression also correlates with cell differentiation, e.g., TPA/ PMA-induced differentiation of HL-60 cells [37] and during neuronal differentiation of rat hippocampal cell lines. [34, 51, 141] These findings, and also that PKCδ triggers p21$^{waf1/cip1}$ cytosolic accumulation [129] indicate that subcellular distribution of p21$^{waf1/cip1}$ is not the key mechanism of exerting its function and demonstrates further that cytosolic p21$^{waf1/cip1}$ in human endothelial cells might act as a pro- as well as anti-apoptotic protein. Nevertheless, we only reported that apoptosis concurred with p21$^{waf1/cip1}$ levels without determining whether p21$^{waf1/cip1}$ is required for the induction of apoptosis and indicating a clear pro-apoptotic p21$^{waf1/cip1}$ role. Further analyses will be needed to investigate the exact role of p21$^{waf1/cip1}$ under these conditions, e.g., p21$^{waf1/cip1}$-mediated inhibition of anti-apoptotic B-cell lymphoma 2 protein (BCL-2) or upregulation of pro-apoptotic BCL2-associated X protein (BAX).

Extensive research over the preceding decades identified growth factor receptors as crucial oncogenic mediators altering cellular signaling and their contribution to tumorigenesis. [142] In addition, PKC signaling was identified as the pathway involved in the carcinogenic effects of phorbol esters and further investigations revealed that PKCδ generally executes pro-apoptotic signaling during cellular stress-induced apoptosis, but also functions as an anti-apoptotic protein during receptor-initiated cell death. This dual function of PKCδ depends on

e.g., its intra-cellular localization, its tyrosine phosphorylation, and on the presence of other pro- and anti-apoptotic target proteins and involved signaling pathways. In this regard, PKCδ in different cancer subtypes can specifically affect tumor survival or tumor suppression. [63] Moreover, the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ was classified as a tumor suppressor in a tp53-dependent as well as -independent manner. However, extensive research indicated that $p21^{waf1/cip1}$ exhibits pro-cancer or anti-cancer activity in different tumor cells due to heterogeneity in their regulation in a cell context- and stress-dependent manner. [63, 78]

We showed that constitutive IER2 expression as a downstream consequence of abnormal growth factor signaling repressed $p21^{waf1/cip1}$ expression. This reversed the $p21^{waf1/cip1}$-mediated cell cycle block and resulted in changed cellular proliferation driven by E2F-dependent pro-survival mechanisms. Therefore, following up to the recognition of IER2 as a novel PKCδ-dependent regulator of $p21^{waf1/cip1}$ it was obvious to analyze IER2 expression and its contribution to cancer development. Recent publications have linked IER2 and cancer, however, without investigating the mechanism behind its altered expression. [39, 55, 56] In the experiments presented here, we analyzed *IER2* mRNA levels in tumors and in the corresponding adjacent mucosa from patients suffering different stages of colon cancer (Table 5). Alves *et al.* have shown enhanced *IER2* expression in primary colon cancer compared to normal colon mucosa using massively parallel signature sequencing (MPSS). [56] Contrary to our expectations, *IER2* mRNA expression was suppressed in eight out of ten tumor size T2-T3 primary colon cancers compared to their related tumor free mucosa, and additionally we detected no IER2 protein in two human colon cancer cell lines (Caco2, HCT-116). Nevertheless, it has recently been reported that IER2 expression correlates with poor metastasis-free and overall survival in patients with colorectal adenocarcinomas. [39] Therefore, analysis of a significant number of late stage tumors or metastatic colon cancer samples might reveal differences of IER2 expression and clarify whether IER2 contributes to tumorigenesis and metastasis. However, this has to be further carefully examined because we discovered varying IER2 levels within tested human cancer cell lines derived from four different origins. We detected low and high IER2 levels in DU145 and PC3 androgen resistant prostate cancer cell lines, respectively. It is important to note that they have revealed different metastatic potential in mouse xenograft experiments: DU145 cells cause moderate, whereas PC3 induce high rates of metastases. [143-145] In contrast, we also detected high intensity of IER2 in the androgen sensitive LnCap prostate cancer cell line exposing low

metastatic potential. [145, 146] This might be explained by the complexity of transcriptional and posttranslational regulation of androgen receptor signaling in prostate cancer. [147] Furthermore, we found no IER2 protein expression in tested breast cancer (ZR-751, MCF7) or in metastatic breast cancer cell lines (MDA-MB-468, MDA-MB-231; Fig.27B) although Alves *et al.* found a two-fold induction of *IER2* in breast cancer compared to normal breast tissue. [56] In addition, they have shown the greatest difference between normal melanocytes and metastatic melanoma tissues. We found IER2 in two metastatic melanoma cell lines Mel-7 (spleen) and Mel-15 (lymph node) compared to healthy melanocytes, but not in Mel-17 (skin). This might be explained by the fact that melanomas can metastasize either by the lymphatic or by the hematogenous route, beginning with either satellite or in-transit metastases next to the primary tumor in the skin, with regional lymph node metastasis, or with distant metastasis. [148] Conversely, IER2 also accumulated in the A-375 melanoma cell line, a low metastatic but amelanotic cell line. It is important to note that two cell lines, Mel-7 and A-375, exhibited strongly reduced levels of $p21^{waf1/cip1}$ but varying tp53 status, compared to melanocytes. This argues for differences in and participation of tp53-independent but IER2-$p21^{waf1/cip1}$-dependent mechanisms in cancer development.

Recently it has been shown that *IER2* is one out of eleven overexpressed genes in melanocytes exposed to UV-A radiation, an important carcinogen in melanoma development. In addition, the cell cycle inhibitor $p21^{waf1/cip1}$, among few other cell cycle regulators and stress proteins, is also induced. [55] Furthermore, $p21^{waf1/cip1}$ is a known checkpoint control in melanoma progression since it has been found to be often lost in late melanoma stages. [79, 80] Hence, it might be suggested that IER2 is already early regulated in melanoma development and contributes to $p21^{waf1/cip1}$ loss of function. Besides, it has been described that *IER2* is transcriptionally activated in human T-cell leukemia virus type I (HTLV-I) infected cells and is regulated by Tax, a viral oncogene showing tumorigenic growth stimulating activity and playing a role in adult T-cell leukemia (ATL). [44] Moreover, HTLV-1 Tax has been found to induce senescence, a growth arrest program, by upregulating the levels of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. [149] These reports additionally support our results and demonstrate a link between IER2 and $p21^{waf1/cip1}$ in cancer. Besides, IER2 has been found to share weak homology to JUNB and JUND, [35] both located at the same region on chromosome 19. These proto-oncogenes have both overlapping and independent functions controlling cellular proliferation, cell cycle arrest, senescence, and the response to stress [150] by the regulation of e.g., cyclin-dependent kinase inhibitor $p16^{INK4a}$ as a direct transcriptional

target of JunB. [151] These findings together could further indicate a specific function of IEGs influencing cell cycle regulators and act as proto-oncogenes.

Recapitulating, IER2 levels differed in analyzed cancer cell lines and patient biopsies. It is most likely that IER2 exerts its function in addition to the dual activity of both PKCδ and $p21^{waf1/cip1}$ in a cell type and cellular signaling specific manner. This is supported by our data as well as by Neeb *et al.* showing IER2 protein levels in one out of three high metastatic pancreatic, in six mammary (one high positive, other weak protein levels), and in one out of three prostate carcinoma cell lines with different metastatic potential. Nevertheless, IER2 expression significantly correlates with a short metastasis-free survival and with a poor overall survival in patients with colorectal adenocarcinomas. Moreover, they have found an IER2 specific effect only on migration; contrary to our data they have not observed any influence on cell proliferation. [39] This may be due to differences of the IER2 function in tumor cells and endothelial cell proliferation-mediated angiogenesis and blood vessel formation contributing to tumor progression and metastasis. Further ongoing experiments using IER2 knockdown tools should address IER2 in growth factor-driven cancer models. Analyzing high/ oncogenic IER2 levels and its contribution to cell proliferation, migration, cell death, chemo-resistance, and differentiation will lead to a better understanding of IER2 in cancer.

As summarized in our model (see below), we confirmed in this study that immediate early response gene 2 (IER2) expression is inducible by growth factors and by the tumor and angiogenesis promoting agent PMA leading to the activation of the PKCδ signaling cascade. Importantly, we present for the first time a biological and mechanistic function for this poorly characterized protein showing that IER2 represses the expression of cell cycle inhibitor *$p21^{waf1/cip1}$* on the level of transcription and by altered protein stability. As a consequence, IER2 rescues human endothelial cells from PMA-induced apoptosis by reversing the $p21^{waf1/cip1}$-mediated blockage of E2F-dependent transcription. We wish to point out that our experiments were carried out in cultures of primary human endothelial cells, thus we assume that the observations in this study are relevant to the normal endothelial cell cycle *in vivo*. Our findings of a novel regulator of $p21^{waf1/cip1}$ may be a further step to increase the knowledge of malignancies that result from differentially regulated cell cycle progression resulting in enhanced proliferation or malfunctioning apoptotic processes. IER2 represents a putative

novel player in the regulation of tumor progression and metastasis and therefore a potential target for prognostic and therapeutic strategies in cancer.

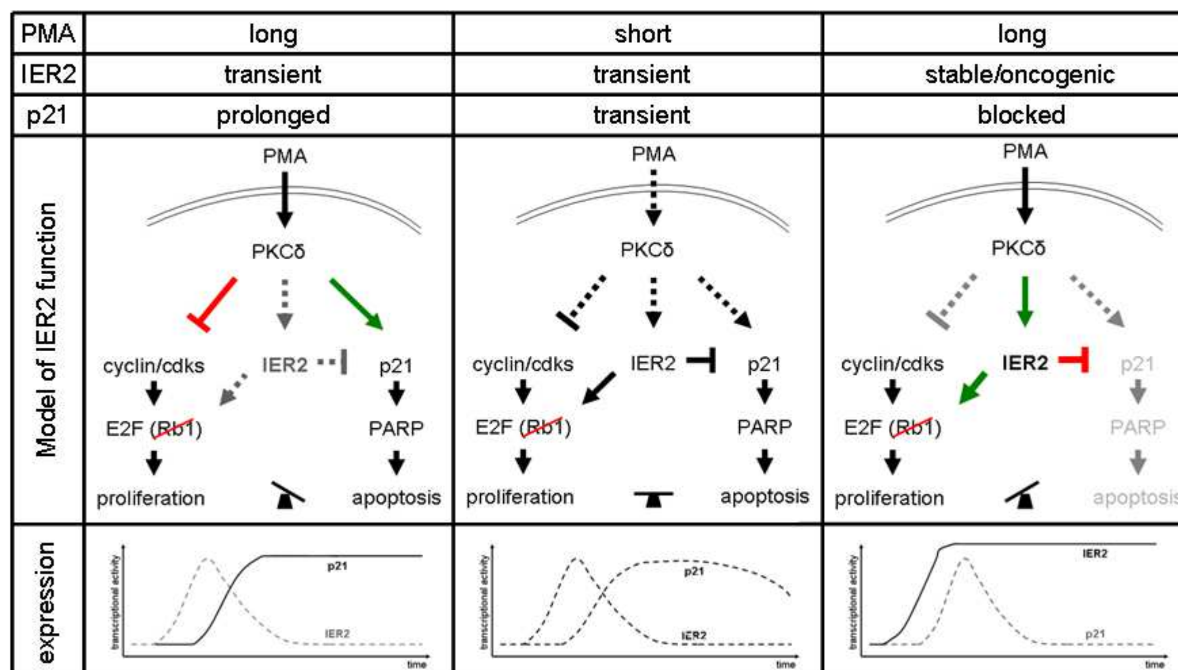


Fig.29: IER2 mediates a functional switch between cellular proliferation and cell death in HUVEC upon PMA treatment by suppressing $p21^{waf1/cip1}$ expression. The diacylglycerol analog PMA mimics growth factor stimulation and leads to the activation of the PKC signaling cascade. Short-time activation (dotted arrows) of the isoenzym PKCδ leads to induced and steady-state levels of cyclin/cdk complexes, the cell cycle inhibitor $p21^{waf1/cip1}$ as well as IER2 which promote G1/S phase progression of the cell cycle (middle panel). Appropriate and time-dependent regulation of pro-survival versus pro-apoptotic mechanisms result in a steady-state level of proliferation versus cell death. Long-time activation (black arrows) with PMA supports the functional switch in the dual effects of $p21^{waf1/cip1}$. Upon prolonged PKC activation, increased $p21^{waf1/cip1}$ expression prevents entry of cells into S phase and drives endothelial cell death (left panel). Alterations in this balanced signaling machinery, namely improper downregulation of IER2, promotes the (transcriptional) suppression of $p21^{waf1/cip1}$, thereby re-directing the pathway towards activated E2F activity and cellular proliferation (right panel). This IER2-controlled balance may constitute to a protective mechanism during the early state of growth factor stimulation and finally facilitate the unhindered, correct termination of the response.

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SUPPLEMENT

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Poster presentations and Meetings:

05/2011 Cincinnati Cancer Symposium Series: NF-κB, Cancer, Obesity, and Inflammation – Cincinnati
Christof E. Lemberger, Yvonne M. Schichl, and Rainer de Martin: Resolution of inflammation: Immediate early genes as negative feedback regulators in the endothelium (poster)
Yvonne M. Schichl, Ulrike Resch, **Christof E. Lemberger**, Dominik Stichlberger, and Rainer de Martin: Tristetraprolin – a molecular regulator of cell survival vs. death? (poster)

02/2011 Institute Retreat – Vienna
Christof E. Lemberger, and Rainer de Martin: Elucidation of immediate early response gene 2: a putative new $p21^{waf1/cip1}$ regulator? (oral presentation)

09/2010 Institute Retreat - Sopron
Christof E. Lemberger and Rainer de Martin
Elucidation of IER2 (immediate early gene 2) and its potential function in inflammation (poster)

09/2009 7th International Symposium on the Biology of Endothelial Cells – Vienna
Helped with organization, service for speakers and participants, instruction and service on technical equipment