The agonist-independent intracellular localization of platelet-activating factor receptor regulates retinal vasculature.

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Abstract

The canonical G-protein coupled receptor (GPCR) signaling is thought to involve activation of the receptor exclusively at the cell-surface, followed by its binding to heterotrimeric G-proteins, arrestins to trigger intracellular signaling cascades, and finally, termination of signaling by internalization of the receptor. It is now known that several GPCRs continue to signal after internalization in the early endosomes. Since the initial discoveries of nuclear binding sites for GPCR ligands in the 1980s, more than 30 GPCRs have been detected at the cell nuclei. In this regard, our group first reported presence of platelet-activating factor receptor (Ptafr) at intracellular sites, including at the nuclei of piglet brain microvascular endothelial cells. But the mechanism of localization and physiologic functions of most nuclear GPCRs, including Ptafr, remain poorly understood.

We hereby provide evidence for nuclear localization of Ptafr in primary human retinal microvascular endothelial cells. We demonstrate that Rab11a (member of Ras superfamily of small GTPases) and importin-5 act as molecular chaperones during the nuclear translocation of Ptafr. Even though Ptafr does not contain a functional nuclear localization signal, a putative internalization motif at the C-terminus with previously unknown function is essential for the translocation. This process is independent of stimulation of the cells with circulating (extracellular) platelet-activating factor (PAF) as well as its intracellular biosynthesis. Moreover, using a cell-surface impermeable biotin tag, we show that nuclear Ptafr likely comes from trans-golgi network. To our knowledge, this is a first report unraveling molecular mechanism involved in agonist-independent nuclear localization of any GPCR.

In isolated nuclei, Ptafr activates endothelial nitric oxide synthase (eNOS, also known as Nos3). We further demonstrate that cell-surface Ptafr is responsible for upregulation of pro-inflammatory cytokines including interleukin-1β; while intracellular Ptafr primarily mediates PAF-induced expression of *Nos3* and vascular endothelial growth factor-a (*Vegfa*) in cultured cells (*in vitro*), and in isolated microvessels from the rat blood-brain barrier (*ex vivo*).

Several pro-inflammatory phospholipid mediators, including PAF, are elevated in human ischemic retinopathies such as retinopathy of prematurity (ROP). The underdeveloped retinal neuro-vasculature of preterm infants is particularly susceptible to the damage by these agents; ensuing in vaso-obliteration during first phase of ROP. The resulting compensatory increase in vaso-proliferative factors is responsible for pathological neovascularization; a hallmark of the second phase of ROP. Using oxygen-induced retinopathy model in rodents to mimic changes seen in human ROP, we show that cell surface Ptafr controls vaso-obliteration (in line with our previously report); while intracellular Ptafr is responsible for *in vivo* retinal angiogenesis.

Together with recent studies reporting functional nuclear GPCR signaling in developmental vascularization and osteogenesis, our results highlight the importance of subcellular localization of a GPCR in determining its signaling outcomes. Developing new pharmacologic approaches to target intracellular receptors may thus help to achieve more specificity and less side effects.

Key words: nuclear GPCR, platelet-activating factor receptor, Rab GTPase, importin, angiogenesis, Ptafr, VEGF

Résumé

La signalisation classique d'un récepteur couplé aux protéines G (GPCR) implique l'activation exclusive du récepteur à la surface des cellules. Une fois activé, ce récepteur se lie à des protéines G hétérotrimériques déclenchant une cascade de signalisation intracellulaire. Les protéines arrestines bloquent quant à elles cette liaison du récepteur aux protéines G hétérotrimériques mettant ainsi fin à la signalisation par l'internalisation du récepteur. On sait maintenant que beaucoup de GPCR poursuivent leur signalisation après leur internalisation dans les endosomes précoces. Depuis les premières découvertes de sites de liaison nucléaires pour des ligands des GPCRs vers la fin des années 1980, plus de trente GPCRs ont été détectés dans les noyaux de cellules et tissus. À cet égard, notre laboratoire a été le premier à démontrer la présence du récepteur du facteur d'activation plaquettaire (rPAF) au niveau de sites intracellulaires, notamment au niveau du noyau cellulaire des cellules endothéliales microvasculaires du cerveau de porcelet. Cependant, le mécanisme de la localisation et les fonctions physiologiques de la plupart des GPCRs nucléaires, y compris rPAF, sont peu connus.

Cette thèse présente une preuve du mécanisme de localisation nucléaire du rPAF dans les cellules rétiniennes humaines primaires microvasculaires endothéliales. Nous démontrons que Rab11a (membre de la superfamille des petites GTPases appelée Ras) et importin-5 agissent comme chaperons moléculaires au cours de la translocation nucléaire du rPAF. Même si rPAF ne possède pas un signal fonctionnel de localisation nucléaire (SLN), nous montrons qu'un motif conservé d'internalisation à l'extrémité C terminal, dont la fonction était inconnue jusqu'à maintenant, demeure essentiel pour la translocation. Ce processus est indépendant de la stimulation des cellules par le facteur d'activation plaquettaire (PAF) circulant (extracellulaire), de même que de sa biosynthèse intracellulaire. La biotinylation de la membrane plasmique a permis de déterminer que le rPAF nucléaire vient directement du réseau golgien trans. À notre connaissance, c'est la première fois qu'on élucide, parmi les GPCRs, un mécanisme moléculaire impliqué dans la localisation nucléaire qui est agoniste indépendant.

Dans des noyaux isolés, le rPAF active l'oxyde nitrique synthase endothélial (eNOS, également connue sous le nom de NOS3). Nous démontrons que le rPAF à la surface cellulaire est responsable de la régulation positive des cytokines pro-inflammatoires, notamment

l'interleukine-1β (IL-1β) ainsi que le facteur de nécrose tumorale α (TNFα); tandis que le rPAF intracellulaire permet principalement l'induction par le PAF de l'expression du *Nos3* et du facteur de croissance de l'endothélium vasculaire-A (*Vegfa*) dans des cellules en culture (*in vitro*) et dans les microvaisseaux fraîchement isolés de la barrière hématoencéphalique chez le rat (*ex vivo*).

Plusieurs médiateurs de phospholipides pro-inflammatoires, y compris PAF, sont élevés dans les rétinopathies ischémiques humaines telles que la rétinopathie du prématuré (ROP). La rétine sous-développée des prématurés est particulièrement sensible aux dommages causés par ces agents comme la vaso-oblitération qui caractérise la première phase de la ROP. Il y a alors une augmentation compensatoire des facteurs de croissance vasculaire donnant lieu à une néovascularisation pathologique et à la deuxième phase de la ROP. Grâce au modèle de la rétinopathie induite par l'oxygène chez les rongeurs nous pouvons imiter les changements observés dans la ROP humaine. Nous montrons que le rPAF à la surface cellulaire contrôle la vaso-oblitération (comme nous l'avions rapporté précédemment); alors que le rPAF intracellulaire est responsable de l'angiogenèse in vivo dans la rétine. En accord avec de récentes études démontrant une signalisation fonctionnelle des GPCR nucléaires au niveau du développement de la vascularisation et de l'ostéogenèse, nos résultats mettent en évidence l'importance de la localisation subcellulaire des GPCR ainsi que de leur voie de signalisation. Ces résultats permettront de développer de nouvelles approches pharmacologiques pour cibler des récepteurs intracellulaires afin d'atteindre une plus grande spécificité tout en ayant moins d'effets secondaires.

Mots clés: GPCR nucléaire, facteur d'activation plaquettaire (PAF), Rab GTPase, importin, angiogenèse, récepteur du facteur d'activation plaquettaire (rPAF), facteur de croissance de l'endothélium vasculaire (VEGF).

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Preface

The following are original findings reported in this study:

1) We unravel a novel trafficking pathway for agonist-independent nuclear translocation of a G-protein coupled receptor (GPCR).

2) This is a first report of a functional interaction between rab GTPase and an importin to govern nuclear translocation of a GPCR.

3) We, for the first time, identify unique and distinct set of genes induced by cell-surface and nuclear sub-populations of the same receptor (platelet-activating factor receptor) in human retinal microvascular endothelial cells.

4) We confirm the intracellular function of platelet-activating factor receptor, using *in vitro* (cell culture), *ex vivo* (intact rodent brain microvessels), and *in vivo* approaches.

5) We demonstrate that Ptafr can execute two different actions in an animal model of retinopathy of prematurity, based on its subcellular localization.

Contribution of Authors

The thesis is manuscript-based in accordance with the guidelines from the Faculty of Graduate Studies and Research of McGill University. Here is the description of the authors' contributions to both manuscripts included in the thesis, highlighting the contribution of the candidate.

Chapter-3 (paper-1)

High-resolution imaging and function of Nuclear G-protein coupled receptors (GPCRs) Bhosle V. K., Gobeil F., Jr., Rivera J. C., Ribeiro-da-Silva A., Chemtob S. (2015) Methods Mol Biol 1234: 81-97.

The candidate modified first three out of the total four protocols to their current form, performed all the experiments, and wrote the manuscript. F. Gobeil Jr. performed experiments and wrote the section on GPCR functions. J. C. Rivera drew the first figure. A. Ribeiro-da-Silva provided advice for and supervised electron microscopy. S. Chemtob provided scientific advice on the experiments, supervised the work, and edited the manuscript.

Chapter-4 (paper-2)

Nuclear localization of platelet-activating factor receptor controls retinal neovascularization

Bhosle V. K., Rivera J. C., Zhou T. E., Omri S., Sanchez M., Hamel D., Zhu T., Rouget R., Al Rabea A., Hou X., Lahaie I., Ribeiro-da-Silva A., Chemtob S. (In revision, manuscript submitted to *Cell Discovery*)

VKB and SC conceptualized and designed the experiments. VKB, T(E)Z, JCR, SO, TZ, AAR performed the experiments and analyzed the data. JCR, XH and IL helped in writing and conducting animal protocols. MS and DH participated in scientific discussion and helped in troubleshooting the experiments. ARdS provided advice on imaging techniques. SC and ARdS co-supervised the study. VKB wrote the first draft and all authors contributed in revision of the manuscript.

Appendix-B (paper-3)

Subcellular GPCR signaling hints at greater therapeutic selectivity

Joyal J. S.*, Bhosle V. K.*, Chemtob S. (2015) Expert Opin Ther Targets 19: 717-21.

Note- *- These authors contributed equally to the work.

V.K. Bhosle and J.-S. Joyal performed literature search and wrote the manuscript. S. Chemtob edited the manuscript and provided advice.

List of abbreviations

ACH	acetylcholine
Ang	angiopoietin
BBB	blood-brain barrier
BS^3	bis(sulfosuccinimidyl) suberate
CHO	Chinese hamster ovary
CNS	central nervous system
COX	cyclooxygenase
cPLA2	cytosolic phospholipase A2
CPP	cell-penetrating peptide
EC	endothelial cells
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GEF	Guanine exchange factor
GPCR	G-protein coupled receptor
HEK	human embryonic kidney
hRMEC	human retinal microvascular endothelial cells

IGF	insulin-like growth factor
IHC	immunohistochemistry
IL-1β	interleukin-1β
INM	inner nuclear membrane
iNOS	inducible nitric oxide synthase
IR	ischemic retinopathies
JAK	Janus-activated kinase
LPA	lysophosphatidic acid
MAP	mitogen-activated protein
MTS	mitochondrial targeting sequence
NE	nuclear envelope
NLS	nuclear localization signal
NO	nitric oxide
NOS	nitric oxide synthase
NPC	nuclear pore complex
NV	neovascularization
OIR	oxygen-induced retinopathy
OLGS	oligodendrocytes
ONM	outer nuclear membrane
PAF	platelet-activating factor
PAF-AH	platelet-activating factor acetylhydrolase
PAR2	proteinase-activated receptor-2

PG	prostaglandin
PLA2	phospholipase A2
PM	plasma membrane
Ptafr	platelet-activating factor receptor
RGC	retinal ganglion cells
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SNX	sortin-nexin
TGN	trans-golgi network
TM	transmembrane
TNF	tumor necrosis factor
TXA2	thromboxane A2
VEGF	vascular endothelial growth factor
VO	vaso-obliteration

List of Official Gene Names

The databases used for official gene names and symbols:

1. human genes- approved by HUGO Gene Nomenclature Committee (www.genenames.org)
2. rat and mouse genes- www.informatics.jax.org/mgihome/nomen/gene.shtml

Per convention, gene names are italicized and protein names (symbols) are not italicized.

Following is a list of commonly used gene names, symbols (non-human gene symbol is given in brackets):

F2RL1 (*F2rl1*)	coagulation factor II receptor-like 1 (unofficial symbol- PAR-2)
IL1B (Il1b)	interleukin 1 beta (unofficial symbol- IL-1β)
IPO5 (*Ipo5*)	importin 5
KDR (*Kdr*)	kinase insert domain receptor (previous name- vascular endothelial growth factor receptor 2)
LBR (*Lbr*)	lamin B receptor
NOS3 (*Nos3*)	nitric oxide synthase 3 (previous name- endothelial nitric oxide synthase)
PTAFR (*Ptafr*)	platelet-activating factor receptor
RAB11A (*Rab11a*)	RAB11A GTPase
TBXAS1 (*Tbxas1*)	thromboxane A synthase 1

CHAPTER 1

INTRODUCTION

1. Introduction:

The phrase “intracrine GPCR signaling” was first introduced in the late 1980s to denote mechanism of action of a peptide hormone, angiotensin-II, mediated by binding to its nuclear receptors in cardiovascular tissues (1). The initial findings of nuclear binding sites for GPCR ligands using radioligand binding assays (2, 3) were corroborated in 1990s by use of various immunological methods of detection (4, 5). With regard to topic of this thesis, presence of functional binding sites for platelet-activating factor (PAF) was first reported in rat liver nuclei (6) and our group confirmed existence of functional nuclear platelet-activating factor receptors (Ptafr) in piglet brain microvascular endothelial cells (7). In addition to more than 30 GPCRs which have been detected at the cell nuclei so far, various tyrosine kinase receptors are also found at the nucleus (8, 9). More recently, independent research groups have confirmed presence of functional, intracellular GPCRs at the endosomes (10), at the mitochondria (11) and, for our interest, at the nucleus (12-14). Therefore, these transmembrane receptors can no longer be considered to be functional exclusively at the plasma membrane.

The intracellular trafficking of GPCRs is tightly regulated by small GTPases of the Ras superfamily; especially members of Rab and Arf families. These small GTPases also exhibit specific subcellular compartmentalization (15, 16). Moreover, another member of the Ras superfamily, Ran GTPase, has been implicated in the trafficking of inner nuclear membrane resident proteins (17). For the latter purpose, Ran GTPase interacts with members of karyopherin family, specifically importins, which recognise the proteins for nuclear trafficking (18). Recently, two karyopherins, importin-β1 and transportin were shown to regulate nuclear translocation of different GPCRs (13, 14).

Hence, we hypothesized that nuclear translocation of Ptafr in microvascular endothelial cells requires specific small GTPase and importin as molecular chaperones.

The PAF and PAF-like lipids are known to exert diverse effects on the affected vasculature by binding to Ptafr expressed on vascular endothelial cells. The biosynthesis of PAF can occur via one of two pathways, viz. remodeling of membrane phospholipids or via *de novo* synthesis. The former is believed to be the dominant source of PAF and its derivatives during inflammation (19) and an enzyme required for PAF synthesis, cytosolic phospholipase A2, is also localized at nuclear membrane (20) and some of the newly synthesized PAF is retained in vascular endothelial cells (21); which hints towards the intracellular action of PAF. We previously reported that Ptafr activates expression of cyclooxygenase-2, a major enzyme involved in the prostanoid biosynthesis, in isolated nuclei by binding to pertussis-toxin sensitive heterotrimeric G-proteins (7). In the present study, we further explore intracrine PAF signaling, with focus on various vascular endothelial cell growth factors, in intact primary cells using two Ptafr antagonists with different membrane permeability properties. We also confirm the *in vitro* results in an *ex vivo* system of isolated rat brain microvessels. These findings are particularly relevant in light of recent reports of PAF-induced angiogenesis in experimental cancer models (22, 23).

In last part of this thesis, we study *in vivo* action of PAF on the retinal vasculature. The retina is a rich source of membrane lipids and fatty acids (20% of dry weight) which are used to produce pro-inflammatory phospholipid mediators, including PAF and PAF-like lipids, during ischemic retinopathies such as retinopathy of prematurity (ROP) (24). The neurovascular retina of preterm infants is underdeveloped and is particularly vulnerable to injury by pro-inflammatory mediators in a setting of variable tissue oxygen levels; resulting in retinal vaso-obliteration (first phase) followed by pathologic

neovascularization (second phase) during ROP (25). The characteristic retinal changes seen in human ROP subjects can be experimentally produced in rodents using oxygen-induced retinopathy (OIR) models (26). As reported previously, PAF plays a role in hyperoxia-induced vasoobliteration during the first phase of OIR (27). However, molecular basis of PAF-induced vaso-obliteration and delayed effects of PAF during neovascularization phase remain poorly understood. We hypothesized that PAF has distinct actions in two phases of OIR, which are governed by subcellular localization of Ptafr in retinal microvascular endothelial cells. These findings might have implications in understanding of signaling pathways involved in PAF-induced vascular changes in other disorders such as age-related macular degeneration (28) and hypoxic-ischemic brain injury (29, 30).

CHAPTER 2

LITERATURE REVIEW

Section- 2.1 Platelet-activating factor (PAF) signaling

2.1.1 Historical perspectives

Platelet-activating factor (PAF) was discovered as a soluble factor responsible for antigen-induced histamine release from rabbit platelets in 1960s (31) and was later identified in leukocytes (32, 33). French immunologist, Jacques Benveniste, named it "platelet-activating factor (PAF)" (34) and first reported its role in anaphylaxis (35, 36). His team was also involved in chemical characterization of PAF as a phospholipid (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine) (37, 38). The biological activity of PAF is dependent on alkyl group at sn-1 position, acetyl group at sn-2 position (38) and on nature of the polar head group (39). The structural analogs of PAF can vary in the length of 1-O-alkyl side-chain (most common side-chains contain 18 or 16 carbons) (40).

2.1.2 Biosynthesis and catabolism of PAF

The earliest evidence for enzymatic synthesis of PAF was provided by Wykle et al., who showed that acetyltransferase activity is essential to synthesize PAF from its non-acetylated precursor lyso-PAF and acetyl-CoA (41). The enzyme has been identified as lyso-PAF acetyltransferase (official name- 1-alkylglycerophosphocholine O-acetyltransferase; EC 2.3.1.67) and is present in the microsomal compartment in cells (41, 42). The production of PAF from cellular membrane phospholipids requires an additional step to generate lyso-PAF first (43) and is catalyzed by members of the phospholipase A2 (PLA2) family (see **Figure- 2.1** (44)). This pathway of PAF synthesis is known as "remodeling" pathway. More than 20 different PLA2s have been identified in mammals, out of which six cytosolic PLA2s (cPLA2s, also known as Group IV

PLA2s) play a major role in the biosynthesis of PAF (45, 46). All cPLA2s are activated by increase in cytosolic calcium levels or by phosphorylation (46). Interestingly, similar to microsomal localization of lyso-PAF acetyltransferase, cPLA2s are also associated with various intracellular membranes and cPLA2α (~ molecular weight 85 kDa), in particular, gets translocated to the nuclear envelope upon its activation in endothelial cells (47). Therefore, enzymatic biosynthesis of PAF occurs in the perinuclear region in those cells. A second *de novo* PAF synthesis pathway is less well characterized where phosphocholine is directly transferred to 1-alkyl-2-acetyl-sn-glycerols (48). The remodeling pathway is believed to contribute to almost all of PAF during inflammation (19), while *de novo* pathway might be important during embryonic development (49) and in cancer cells (50). Lastly, structural analogs of PAF can be produced by uncontrolled oxidative reactions, including defragmentation of membrane phospholipids, upon exposure to environmental toxins (51, 52). These so called PAF-like lipids mimic biological actions of PAF and have been linked to atherogenesis (53).

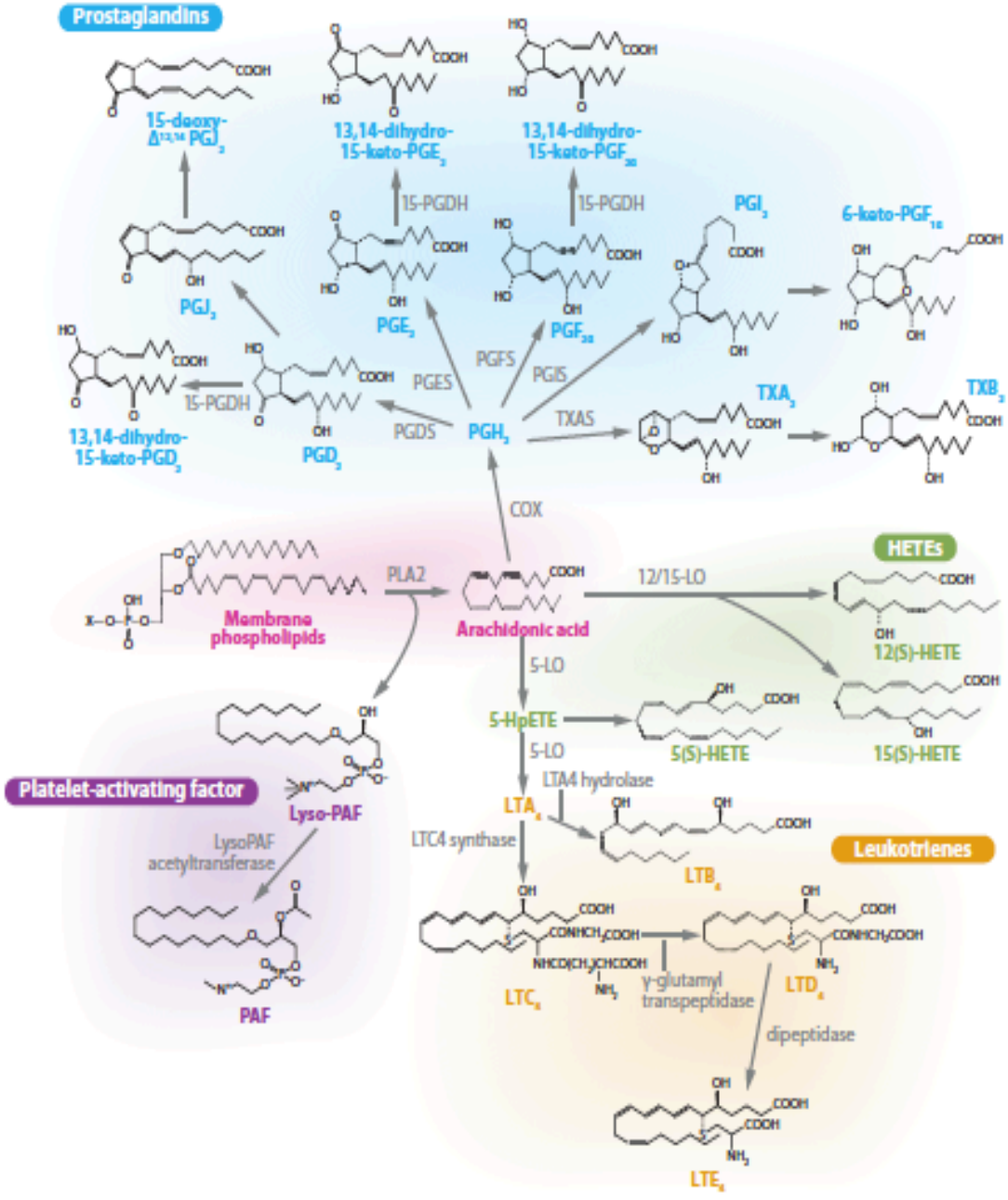

Figure-2.1 Remodeling pathway for biosynthesis of PAF.

It also shows synthesis of eicosanoids (prostaglandins & leukotrienes), derived from membrane phospholipids.

The figure is reproduced, in its original form, from the reference (44).

PAF and PAF-like lipids are inactivated within minutes by deacetylation into lyso-PAF and its analogs, respectively. The reaction is catalyzed by one of the PAF acetylhydrolases (PAF-AHs), which are also part of larger PLA2 superfamily (Group VII and VIII PLA2s). There are three known types of PAF-AHs: two (type- I and II PAF-AH) are cytosolic enzymes (54) and one (plasma PAF-AH) is present in plasma and tissue fluids (55). Type-I PAF-AH is a heterotrimeric protein with α_1, α_2 and β subunits, while other two enzymes (type-II PAF-AH and plasma PAF-AH) are single polypeptides.

Majority of plasma PAF-AH (group VIIA PLA2) is associated with the apo-B100 of low density lipoproteins (LDL) and high-density lipoproteins (HDL) (56). The LDL association might be important for inactivation of circulating oxidized phospholipids, including PAF-like lipids mentioned earlier (57). Biological activity of plasma PAF-AH is significantly lower in patients with fatal anaphylactic reactions than those with non-fatal outcome (58). Several alleles of human gene coding for human plasma PAF-AH (*PLA2G7*) have been linked with its inability to rapidly inactivate circulating PAF (59-61) and are typically associated with increases severity of asthma and other PAF-related respiratory conditions (60, 62, 63). The exact role of plasma PAF-AH in atherosclerosis is highly debated (64) but increasing evidence suggests that enzyme is progressively activated during atherogenesis (65) and has been shown to be an independent predictor of coronary heart disease (66). Type-II PAF-AH (also known as group VIIB PLA2) is an intracellular monomeric protein with 41% sequence identity to plasma PAF-AH (67). The enzyme can also transfer acetyl group (transacetylase activity) from PAF to other acceptor lipids, such as lysophospholipids and sphingosine in a CoA-independent manner (68). Growing evidence suggests that type-II PAF-AH plays a role in cellular defenses against oxidative stress (69, 70). Moreover, transgenic mice overexpressing the

enzyme are refractory to ischemic injury, suggesting its protective role in cerebrovascular insults (71). The heterotrimeric type-I PAF-AH is different from other two counterparts. Its catalytic subunits (α1 and α2, also known as group VIIIA and VIIIB PLA2s) show substrate specificity for PAF but not other oxidized phospholipids (72). The regulatory β subunit of the enzyme is identical to product of human LIS-1 gene, a causal gene for Miller-Dieker syndrome with abnormal neuronal migration (73). This feature, together with the facts that PAF can mediate long-term neuronal potentiation (74) and that switching of catalytic subunits of type-I PAF-AH occurs during brain development (75), hint that this enzyme may be linked to normal neuronal migration.

2.1.3 PAF receptor (Ptafr)

The presence of specific binding sites for PAF was initially reported in platelets and leukocytes from different species. PAF receptor (official symbol- Ptafr) was the first receptor with a bioactive lipid ligand to be successfully cloned; initially from guinea pig (76) and soon after from human (77) cDNA. The human *PTAFR* gene is located on chromosome 1 and lacks introns (78, 79). The human PTAFR protein contains 342 amino acids, and the sequence is more than 80% identical to that of pig and rat PAF receptors (**see appendix-A for sequence alignment**). The PAF receptor is highly expressed in platelets, neutrophils, endothelial cells, monocytes, macrophages and various smooth muscle cells. (80). The protein expression is regulated by various pro-inflammatory factors, including PAF itself (81).

The initial characterization of PAF receptor signaling revealed that the receptor can increase phospholipid turnover in the cells via multiple pathways (phospholipase A2, C and D) and also activate protein kinase C (82). It also increases intracellular calcium levels in many cell types (82, 83), including in vascular endothelial cells (84). Thus, Ptafr signaling, by augmentation of cellular calcium levels, stimulates the enzymatic synthesis of PAF itself (see section- 2.1.2) (85). Ptafr can bind to two different heterotrimeric G-proteins, viz. pertussis toxin –sensitive (Gi) and –insensitive (mostly Gq) to activate various downstream mitogen-activated protein (MAP) kinases (7, 86-88). Ptafr signaling has been also shown to induce nuclear factor (NF)- kappa B via G-protein coupling (7, 89) and this pathway has been implicated in the production of pro-angiogenic factors by macrophages (90). Lastly, like other GPCRs, Ptafr can also signal via G-protein independent mechanisms, partly through activation of Janus activated kinase (JAK)/STAT pathway (91-93).

2.1.4 Ptafr motifs

Site-directed mutagenesis approaches have helped to identify several motifs in PTAFR, which are required for its normal function and trafficking of the receptor. For example, two point mutations: A230E (94) and N100A (95), situated in 3rd intracellular and 3rd transmembrane domain respectively, result in constitutively active PTAFR. The N100A mutant receptor is also activated by lyso-PAF (95). Three histidine residues at positions 188, 248 and 249 are essential for the ligand binding (95). The C-terminal of PTAFR is particularly rich serine and threonine residues, which are required for agonist-induced desensitization of the receptor by phosphorylation (96).

Several potential internalization motifs have been reported in PTAFR. The disruption of a conserved N/DPxxY motif (which is required for activation and internalization of some GPCRs (97, 98)), present in 7th TM domain of PTAFR, produces different effects depending on the site of mutation. Both D289A and Y293A mutants fail to couple to G-proteins but only former shows diminished of internalization as compared to the wild-type PTAFR in transfected cells (99). The C-terminus contains putative monopartite nuclear localization signal (NLS (100)) (KKFRKH$^{298\text{-}302}$) but its functional significance remains unknown to date (7).

The efficient targeting of human PTAFR to the plasma membrane requires N-linked glycosylation at second extracellular loop and point mutation N169A was found to reduce surface expression of the receptor by ~ 70% (101). On the other hand, last 26 amino acid residues (317-342) of PTAFR are essential for receptor endocytosis from the cell surface (99) as well as for its coupling to aforementioned Janus activated kinases (92). The agonist-induced internalization of plasma membrane PTAFR is regulated by two small GTPases, Rab5 (for clathrin-dependent endocytosis) and Rab7 (for

degradation of the receptor) both in endogenous (102) and in transfected (102, 103) cells . The functions of aforementioned rab GTPases are discussed in section-2.2.5.

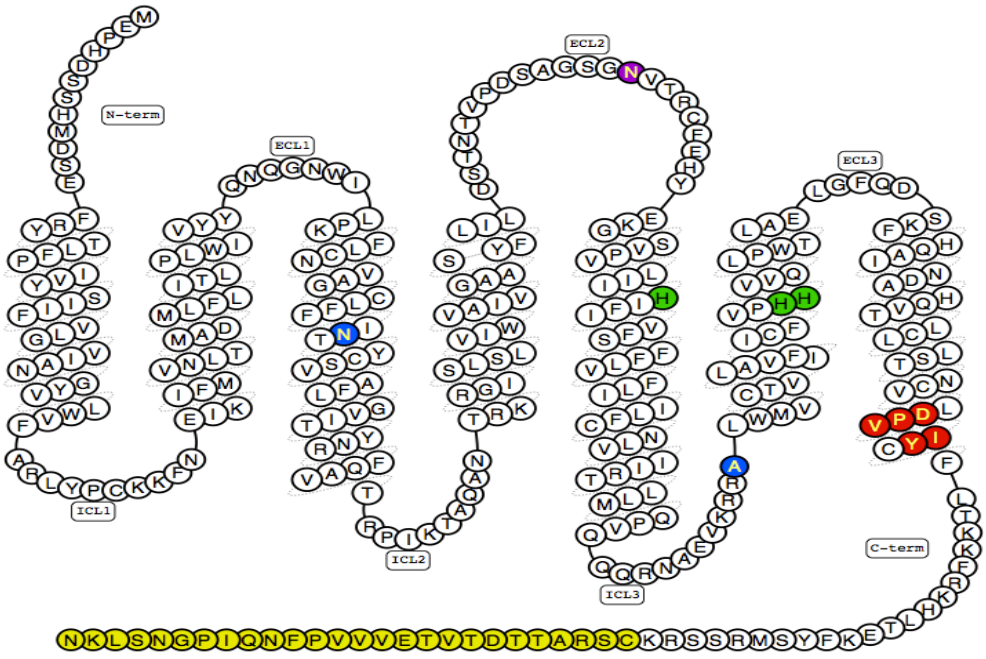

Figure- 2.2 Schematic representation of important PTAFR motifs with known function. The figure was prepared using www.gpcrdb.org

1. N^{100} and A^{230} (in blue) are important for receptor activity.

2. N^{169} (in purple) - Glycosylation is required for targeting of PTAFR to the cell surface.

3. $^{289}DPVIY^{292}$ (in red) – The motif is necessary for G-protein binding but not for internalization of the receptor.

4. Histidine (H) residues at 188, 248 and 249 positions (in green) are essential for ligand binding to the receptor.

5. Last 26 amino acid residues (317- 342 in yellow) – are required for agonist-induced internalization of plasma membrane PTAFR.

Section- 2.2 Nuclear G-protein coupled receptors

2.2.1 Signaling environment at the Nucleus

The nuclear envelope (NE) is made up of two bilayered phospholipid membranes (low in cholesterol content as compared to plasma membrane) (104) named outer and inner nuclear membranes respectively, pierced by giant multiprotein assemblies, nuclear pore complexes (NPCs), which control trafficking of most biomolecules in and out of the nucleus. The outer nuclear membrane (ONM) is contiguous with outer membrane of the endoplasmic reticulum (ER) and the space between outer and inner nuclear membranes is connected to the ER lumen. The basic structure of NPC is evolutionarily conserved in most eukaryotes and is comprised of ~30 nucleoporins forming a ring-shaped central channel with eight identical subunits (105, 106). The ultrastructural analysis of NPC reveals that it is made up of distinct classes of nucleoporins; each with specific localization and function within the NPC (107). Of interest, many scaffold nucleoporins, which form the NPC framework (outer and inner rings), share structural similarities with components of COP-II vesicles, which are part of endomembrane trafficking pathway (108). Both outer (ONM) and inner (INM) nuclear membranes are known to express different sets of transmembrane (TM) proteins, including receptors, ion channels, and linker (e.g., nesprins) proteins (109). The proteomic analysis of NE shows that it varies in composition between tissues (110). Recent evidence also indicates that various protein and non-protein components of NE play diverse roles in regulation of gene expression (111).

Until recently, GPCRs, which form the largest family of transmembrane receptors with more than 800 members in the human genome, were thought to be almost exclusively expressed at the plasma membrane (PM) (112). The canonical GPCR signaling

involves heterotrimeric G-proteins, which act as a connecting link between the receptor at PM and intracellular second messengers. Some of the earliest experimental evidence for nuclear localization of heterotrimeric G-proteins was provided in 1990s (3, 113). It has also been reported that G-proteins can translocate to the nucleus in response to specific stimuli (114-116). GPCRs are also well-known to signal via heterotrimeric G-protein independent pathways (117, 118). Many components of both signaling pathways (G-protein dependent and independent) are found (or are translocated) at the NE or within the nucleus. These include enzymes such as adenylyl cyclase (119), protein kinase A (120), Akt (also known as protein kinase B) (121), protein kinase C (122), some GPCR kinases (e.g., GRK-5 (123)), cyclic nucleotide phosphodiesterases (PDE) (124), phospholipase C-β1 (PLCβ1) (125), phospholipase D1 (126), diacylglycerol kinase (DGK)-ζ (127), NOS (128), and various members of extracellular signal-regulated kinase (Erk/MAPK) family (129-133). The nuclear arrestins (e.g., β-arrestin-1) are discussed in the section of nuclear GPCR trafficking.

Nuclear calcium signaling has attracted increasing attention in the last decade as one of the highly proficient ways of regulating gene expression and is well-studied in neurons (134). The inositol-1,4,5-trisphosphate (IP_3R) and ryanodine (RyR) receptors (135-138) and R-type calcium channels (139) present in the NE are known to expedite the entry of calcium from cytosol as well as from intracellular stores such as ER into the nucleoplasm . Many nuclear GPCRs, including angiotensin II type-1 receptor (AT1 receptor), prostaglandin E2 receptor (EP3 receptor), Ptafr and lysophosphatidic acid receptor type-1 (LPA1 receptor) have been shown to increase nucleoplasmic calcium levels in various tissues (133, 139). In neurons, a pool of 185 genes was identified to be induced by glutaminergic synaptic activity via transient nuclear calcium currents (140). The major molecular targets of nuclear calcium signaling include cAMP responsive

element binding protein (CREB) (141, 142), its co-activator CREB-binding protein (CBP) (143), predominantly nuclear calcium/calmodulin dependent protein kinase-IV (CaMKIV) (143), and de novo DNA methyltransferase (Dnmt3a2) (144). More recently, it has been proposed that nuclear calcium activated potassium channels (nBK) play a role in regulation of nuclear calcium induced gene expression (145). Nuclear calcium signaling is also linked to formation and breakdown of nuclear envelope during cell cycle (146). It is important to note that, in addition to the transmembrane ion channels mentioned above, the NE is reported to harbour Na^{+}-H^{+} exchanger isoform type-I (NHE-1) (147), Na^{+}-K^{+} ATPase (148), Na^{+}-Ca^{++} exchanger (149, 150), and Cl^{-} channels (151).

In addition to GPCRs, transmembrane proteins of the receptor tyrosine kinase (RTK) family have also been found at the nucleus (9). Most of them show agonist-induced internalization from the cell surface. Of note, a member of EGFR super-family, ErB4, has been shown to partly translocate (its soluble intracellular domain "sE4ICD") to the nucleus following proteolytic cleavage (152). Therefore, interactions between various nuclear receptors, including cross-talk between second messengers, might play a role the regulation of nuclear signaling cascade.

The lipid signaling inside the nucleus has been a major focus of biomedical research due to it's proposed roles in transcriptional regulation, mRNA splicing, and DNA repair (153-155). It is now clear that entire nucleus, including the NE, is highly organized into lipid microdomains; each with distinct functions. The roles of nuclear-membrane associated and intranuclear inositide (156) and sphingolipid signaling (157) in various pathophysiologic conditions have been recently reviewed. In this context, it should also be pointed out that membrane phospholipids can also serve as a source of ligands for some of the nuclear GPCRs (see below).

In summary, complex interactions between various transmembranous and intranuclear components are needed to regulate nuclear signaling events, including assembly/disassembly of NE, gene expression, RNA processing, and chromatin modifications.

Based on their primary endogenous ligand, nuclear GPCRs can be broadly divided into three groups.

1. GPCRs with lipid ligands- e.g., Lysophosphatidic acid receptor, PAF receptor
2. GPCRs with non-peptide, non-lipid ligands- e.g., Adrenergic receptors
3. GPCRs with peptide ligands- e.g., Proteinase activated receptor 2, Oxytocin receptor

The first two groups share similarities in enzymatic ligand synthesis and signaling pathways and will be reviewed in more detail as they are relevant to topic of this thesis. At the end, I will summarize all nuclear GPCRs, including those with peptide ligands, in **Table-2.1**.

2.2.2 Nuclear Lipid signaling

Many bioactive lipid entities are known to exert their cellular effects by binding to a specific GPCR (158). In addition, lipids, such as phosphoinositides (PIs), have been recently shown to regulate transmembrane ion channels (159). With accumulating evidence for nuclear localization of many such GPCRs and ion channels in the last two decades, transmembrane-protein mediated lipid signaling is thought to play an important role in nuclear events, most notably in gene expression and nucleocytoplasmic transport.

Nuclear Prostanoid signaling

The sub-family of prostanoid receptors includes G-protein coupled receptors for chemically-related endogenous ligands such as prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$ and PGH_2), prostacyclin (PGI_2), and thromboxane (TXA_2). All prostanoid receptors are members of class-A (Rhodopsin-like) GPCRs (160). The prostanoid receptors have been known to produce diverse physiological and pharmacological responses in cardiovascular, endocrine, immune, skeletal, nervous, renal, and reproductive systems (161, 162). To date, PGE_2 and TXA_2 receptors have been localized in perinuclear and nuclear regions (5, 129, 130, 163).

PGE_2 primarily acts via prostaglandin E (EP) receptors but has lesser affinity for other prostanoid receptors. There are four known subtypes of EP receptors, named EP_1 to EP_4 receptors. Albeit expressed in most human tissues, EP receptors have been implicated in regulation of brain functions especially during developmental stage (164, 165). Bhattacharya M *et al*. first reported presence of EP receptors in porcine newborn cerebral microvascular endothelial cells (EP1 EP3α and EP4 subtypes) and rat brain cortex endothelial cells (EP_1 subtype) using immuno-fluorescence and immuno-electron microscopy approaches (5, 129). They also showed that stimulation of freshly isolated nuclei with pharmacological agonists of EP1 and EP3 agonists results in nuclear calcium current, which is independent of cAMP and IP3 production (5, 129). Gobeil F Jr. *et al.* reported that perinuclear EP3 receptors induce expression of a constitutive gene, endothelial nitric oxide synthase (eNOS), in brain microvascular endothelial cells (ECs), which is inhibited by pre-treatment with pertussis toxin (PTX), calcium channel blockers (e.g., SK&F 96365) as well as with iberiotoxin (which specifically inhibits calcium regulated K+ channels) (130). The last finding is particularly interesting in light of recently reported nuclear localization of BK channels and their proposed role in

regulation of nuclear calcium induced gene expression (145). The eNOS gene has also been shown to be a popular target of other nuclear GPCR signaling pathways in various cardiovascular cells (for e.g., LPA1 receptor (166), β3 adrenergic receptors (167), described below). The enzyme, eNOS, upon induction, can translocate to the nucleus to activate its target genes such as inducible nitric-oxide synthase (iNOS) and microsomal prostaglandin E synthase-1 (mPGES-1), therefore creating a vicious circle of prostaglandin signaling (168). It is also important to note that another inducible enzyme, cyclooxygenase (COX)-2 (but not COX-1), which is required for prostaglandin biosynthesis, is also reported to be present at the nucleus in brain (169). Together, these results uncover a novel mechanism for intracellular prostaglandin signaling, in the regulation of vascular tone and the local blood flow in the central nervous system (CNS) (130). Thromboxane A2 receptor (TP receptor) was shown to exhibit nuclear localization in mature rat oligodendrocytes (OLGs) but not in their precursor cells, using confocal microscopy and cell fractionation approaches (163). The nuclear localization of the receptor was correlated with increasing TXA2 (agonist) production and expression of myelin basic protein (MBP) by OLGs (163).

Nuclear Platelet Activating Factor (PAF) signaling

PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a bioactive phospholipid with regulatory roles in physiopathological processes such as inflammation, homeostasis, reproduction (170, 171). Its actions are mediated via a single class-A GPCR (PTAFR) through G-protein dependent (coupling to Gi and Gq proteins) (7, 172) and G-protein independent (173) pathways. It is also well known that vascular endothelial cells can synthesize PAF from membrane phospholipids via enzymatic pathway and majority of the newly synthesized PAF is retained intracellularly within these cells (21). Using multidisciplinary approach including immunogold labeling, Marrache AM et al. showed

that Ptafr is expressed at the nuclear membrane and inside the nucleus, both in primary cells (porcine brain microvascular endothelial cells) as well as stably transfected Chinese hamster ovary (CHO) cells (7). Moreover, plasma membrane and nuclear Ptafr seem to be coupling to different heterotrimeric G-proteins (Gq and Gi respectively) and nuclear Ptafr induced COX-2 expression by activation of nuclear calcium currents in vascular endothelial cells (7). Next, using chimeric peptide approach, inhibitor peptide of Ptafr (THG315) was fused to the internalization sequence of TAT-protein-transduction domain (YGRKKRRQRRR). Pre-treatment of endothelial cells with fusion peptide (THG315-TAT) resulted in selective inhibition of intracellular Ptafr without affecting their counterpart at plasma membrane and prevented PAF-induced expression of COX-2 (133). These findings, together, provide a strong evidence for existence of nuclear Ptafr with functions distinct from those of plasma membrane Ptafr.

Nuclear Lysophosholipid signaling

Lysophosphatidic acid (LPA) and Sphingosine-1-phosphate (S1P) are the most well-studied members of lysophospholipids family, both of which signal via distinct class-A GPCRs. Currently, 6 different LPA receptors (LPA_1 to LPA_6) and 5 different S1P receptors ($S1P_1$ to $S1P_5$) are known (174). In addition, LPA is described to be an agonist for nuclear hormone PPARγ receptors (175). The LPA_1 receptor is found throughout the body with high expression in the nervous system, gastrointestinal system, heart, and kidney. It is thought to be involved in processes such as neuronal differentiation, neuropathic pain, immune cell regulation, and ischemic stroke (176, 177). $S1P_1$ receptor, on the other hand, has been implicated in neurogenesis, angiogenesis, lymphocyte trafficking, tumor metastasis, and pathogenesis of multiple sclerosis (178).

Gobeil F Jr. *et al.* first reported existence of functional LPA_1 receptors in unstimulated primary porcine cerebral microvascular endothelial cells (pCMVECs) and purified rat liver nuclei using multiple approaches, including radioligand binding, cell fractionation, and immunoelectron microscopy (166). Stimulation of freshly isolated nuclei from the aforementioned tissues with LPA resulted in induction of iNOS and COX-2 RNA and protein, which was blocked by pre-treatment with wortmannin (PI-3-kinase inhibitor) and EGTA (calcium chelator) (166). More recently, nuclear localization of various types S1P receptors has been reported in T lymphocytes ($S1P_1$ receptor) (179), native human umbilical vein endothelial cells ($S1P_1$ receptor) (180) as well as in benign and malignant tissues (using Immunohistochemistry (IHC), authors reported all types S1P receptors except $S1P_2$ at the nucleus) (181). Nuclear S1P1 receptor has been shown to induce expression of Cyr61 (Cysteine-rich angiogenic inducer 61) and CTGF (Connective tissue growth factor) mRNA in isolated nuclei using RT-PCR (180). Also, sphingosine kinase 2 (SphK2), one of the enzymes involved in biosynthesis of S1P, has been shown to a nuclear regulator of histone acetylation (182). It is currently unclear whether and how the latter (SphK2) contributes to nuclear S1P receptor signaling (183).

Some other nuclear GPCRs with lipid-associated signaling include cysteinyl leukotriene receptor 1 (nuclear CysLT1 receptor was detected in colorectal adenocarcinoma cells) (184), and a class-C orphan GPCR, GPR158, whose expression is recently been reported to be regulated by glucocorticoids in cultured ocular trabecular meshwork (TBM) cells (185).

Biosynthesis of lipid ligands at the nucleus

For a GPCR to be functional at the nucleus, it must have access to the ligand. Irrespective of the exact localization of the receptor at outer (ONM) or inner (INM)

nuclear membrane, the ligand binding N-terminal region of the receptor will be present in the space between the membranes. Many lipid ligands, including PAF, prostaglandins, LPA, can be synthesized locally from membrane phospholipids (170, 186, 187). As mentioned earlier, many enzymes required for the biosynthesis, including phospholipase A2 (intracellular cytosolic as well as soluble forms) (188, 189), COX (also known as prostaglandin endoperoxide H synthase) (169, 190), prostaglandin E-synthase (mPGES-1) (169), have been previously detected at the nucleus or in the perinuclear region (191). Moreover, cPLA2 is also known to undergo calcium dependent nuclear translocation (192, 193). Once synthesized, lipid ligands can transverse across phospholipid membranes because of their small molecular size and hydrophobic nature.

2.2.3 Nuclear GPCRs of non-peptidic, non-lipid ligands

This section reviews nuclear GPCRs of non-peptidic, non-lipid ligands such as small-molecules (e.g., acetylcholine), amino acids (e.g., glutamate), and amino acid derivatives (e.g., catecholamines).

Nuclear muscarinic acetylcholine receptors

Acetylcholine (Ach), often described as a small-molecule neurotransmitter, is synthesized from acetyl coenzyme A (acetyl CoA) and choline by a reaction catalyzed by choline acetyltransferase (CAT). Increasing evidence suggests that this process is also active in various non-neuronal tissues (194). Ach exerts its biologic actions by binding to muscarinic or nicotinic cholinergic receptors; the latter being ion-gated channels. All neuronal and extra-neuronal muscarinic receptors belong to class-A of GPCRs (rhodopsin-like) and can be divided into 5 molecular subtypes (m_1 to m_5) (195, 196).

Colley AM *et al.* first reported that stimulation of nuclear preparations from rabbit corneal epithelial cells with Ach or carbachol (non-specific cholinergic agonist for both muscarinic and nicotinic receptors) resulted in increased activity of RNA II and DNA polymerases (197). Later, the presence of nuclear binding sites for a radioligand, [^{3}H]-propylbenzilycholine mustard (PrBCM) , which covalently binds to muscarinic cholinergic receptors (198), was confirmed in cultured rabbit corneal epithelial and endothelial cells as well as in transfected Chinese hamster ovary (CHO) cells (4, 199). Based on molecular size of the PrBCM-bound proteins, nuclear muscarinic receptors were postulated to be of m3, m4 and m5 subtypes (4). It is interesting to note that vesicular acetylcholine transporter (VAChT), a member of vesicular amine transporters (VAT) family of integral membrane proteins which are responsible for transfer of biologic amines across phospholipid membranes (200), has been reported to present at the nucleus in other cell types of epidermal origin (201). The CAT immunoreactivities have also been detected at various nuclear layers of endogenous retinal neurons (202) as well as at the nuclei of heterologous expression cells of neural origin (203). However, exact functions of the nuclear CAT and VAChT remain to be elucidated. In addition, in rabbit coronary endothelial cells, Ach induced prostacyclin synthesis requires translocation of another membrane bound enzyme "cPLA$_2$" to the nuclear envelope, suggesting that different nuclear GPCR signaling pathways might be connected at the level of intracrine ligand synthesis (204).

Nuclear metabotrophic glutamate receptors

Glutamate, an excitatory amino acid, is known to exert its actions by binding to either ion channels (ionotropic glutamate receptors) or members of class-B GPCRs (metabotropic glutamate receptors, or mGluRs). The latter are widely distributed in the nervous system and carry out diverse actions (205, 206). The mGluRs can be divided

into 8 molecular subtypes (mGluR1 to mGluR8) which can be further grouped together (group- I, II, III metabotrophic receptors) based on their pharmacologic properties (207).

O'Malley KL *et al.* reported the presence of mGluR5 receptors (member of group-I metabotropic receptors along with mGluR1) at the nucleus in transiently transfected human embryonic kidney (HEK) cells (208). Furthermore, using differential permeabilizing properties of digitonin and Triton-X-100, authors concluded that the mGluR5s were mostly associated with outer nuclear membrane (ONM) and co-localized with ryanodine receptor 2 (RyR2) (208), a type of intracellular calcium channel known to be present at the nuclear envelope (209). The endogenous (non-transfected) mGluR5s were also localized in the nuclear fractions from mouse forebrain samples (208). In recent years, the same group (O'Malley KL et al.) has reported that nuclear mGluR5s can activate phosphoinositide signaling leading to intranuclear calcium currents in mouse striated neurons (210). The neuronal intracrine mGluR5 signaling involves calcium/calmodulin-dependent protein kinase II (CaMKII) – phosphoERK1/2 – phosphoElk-1 cascade and appears to be independent of their plasma membrane counterparts (211).

Nuclear adrenoreceptors

Adrenoreceptors, which bind to endogenous catecholamines, adrenaline and noradrenaline, to transduce cellular signaling, form one of the most well studied family of GPCRs. Based on pharmacological properties and evidence from molecular cloning, they are divided into three groups viz. alpha1 (α_1), alpha2 (α_2), and beta (β) adrenoreceptors. Each group is further classified into at least three sub-types (212). The adrenoreceptors are distributed in almost all tissues and are known to exert important

pathophysiologic actions in cardiovascular, nervous and metabolic systems. The various subtypes of α and β adrenoreceptors have been detected in heart and blood vessels (213-215) and endogenous α1A, β1, and β3 receptors have been localized at the nucleus in cardiomyocytes to date using biochemical and microscopic methods (216-220).

Nuclear α1 adrenoreceptors

Buu *et al.* provided initial experimental evidence that radiolabelled noradrenaline is taken up by intact neonatal rat ventricular myocytes and is first detected in nuclear fractions following 60 minutes after treatment. Using selective pharmacologic agents, authors showed that isolated nuclei of ventricular myocytes exhibit binding sites similar to α1 and β1 adrenoreceptors (216).

In recent years, the group of O'Connell has provided biochemical evidence for nuclear localization of α_{1A} and α_{1B} subtypes of adrenoreceptors in adult mouse cardiac myocytes (218-221). To overcome the lack of α1 adrenoreceptor specific antibody, the authors used fluorescent-tagged prazosin (non-subtype-selective α1 antagonist) to detect the presence of α1 receptors in isolated nuclei from endogenous adult mouse cardiac myocytes. Moreover, the results were replicated using a "in-vitro reconstitution" model of cardiac myocytes isolated from α_{1A} and α_{1B} double-knockout mice (α1ABKO) for heterologous expression of GFP tagged α1 receptors (alone or together) (218-221). Furthermore, the majority of α1 receptors in cardiac myocytes appeared to be present at the nucleus as compared to plasma membrane and they co-localized with signaling effectors, Gαq and PLCβ1, only at the NE (218). Functional NLSs were identified in carboxyl-terminal domain of both α1A (bipartite type) and α1B (glycine-arginine rich repeat) adrenoreceptors by mutational analysis (219). Functionally, GFP-tagged nuclear α1 receptors were shown to be responsible for phosphorylation of ERK in the "in-vitro

reconstitution" model (218), a signaling pathway previously demonstrated to be responsible for cardiomyocyte survival in cultures (221). The most recent experimental evidence suggests that nuclear localization of α1 adrenoreceptors is required for their positive ionotropic effect through phosphorylation of cardiac troponin I (cTnl) which plays a role in regulation of calcium-mediated interaction between actin and myosin in cardiomyocytes (220).

Nuclear β_1 and β_3 adrenoreceptors

Boivin *et al.* reported the presence of nuclear β_1 and β_3, but not β_2, adrenoreceptors in isolated rat ventricular myocytes using sub-cellular fractionation and immunocytochemistry approaches (167, 217). Using pharmacologic approaches, they also showed that β_3 receptors were responsible for initiation of de novo transcription, measured by increased [^{32}P]UTP incorporation and increased expression of 18S ribosomal RNA, via PTX-sensitive G ("likely Gi") protein-mediated signaling pathway in isolated nuclei from cardiac myocytes (167, 217). Vaniotis *et al.* (2011) provided biochemical evidence for the regulatory role various intracellular kinases (ERK1/2, JNK and p38) in β -adrenoreceptor mediated transcriptional activity in isolated nuclei; however exact molecular mechanisms responsible for this regulation are still unknown (167). Later, the same group (see Vaniotis *et al.* 2013) reported that the stimulation of nuclear β3 but not β1 adrenoreceptors resulted in increased NO synthesis, which was inhibited by L-NAME (L-NG-Nitroarginine methyl ester which inhibits activity of two constitutive isoforms viz. nNOS and eNOS). The NO production, along with cGMP dependent protein kinase G (PKG) activity, were essential for the aforementioned transcriptional activity of the β3 adrenoreceptors (12). The authors also confirmed the intracrine adrenoreceptor signaling in whole-cell cardiomyocytes, pre-treated with membrane-impermeable adrenoreceptor blocking agent (EEDQ) to nullify the effect of

cell-surface receptors and then using a caged derivative of non-selective β -agonist isoproterenol (ZCS-1-67). The caging allows for the hydrophilic agonist molecules to cross plasma membrane, followed by un-caging the intact pharmacologic agent inside the cell by exposure to the ultraviolet light (12).

Intracellular Catecholamine transporters

As mentioned before, Buu *et al.* reported that exogenously administered noradrenaline slowly appears in nuclear fractions of rat ventricular cardiomyocytes (216). All catecholamines, including adrenaline and noradrenaline, are hydrophilic molecules and require vesicular transport mechanisms for transfer across phospholipid membranes. The classical noradrenaline transporters (e.g., norepinephrine transporter, NET) belong to SLC6 family of neurotransmitter transporters and are primarily expressed on cell-surface of neurons (222, 223). In cardiac myocytes, however, the noradrenaline uptake is regulated by extraneuronal, nonselective monoamine transporters belonging to SLC22A family (e.g., organic cation transporter 3, OCT3) (224, 225). Interestingly, Wright *et al.* localized OCT3 at both plasma and nuclear membranes in cardiac myocytes using immunocytochemistry approach. Also, pharmacologic inhibition of the said transporter using cortisone abolished α1 adrenoreceptor mediated ERK1/2 phosphorylation (218).

Table 2.1 Nuclear GPCR table

	GPCR family	Nuclear receptor subtype	Principal ligand(s)	Family of ligand(s)	Endogenous (non-transfected) cells/tissues	Ref
	Class-A					
1-3	Prostanoid receptors	PGE2 receptors (Ptger1, Ptger3 and Ptger4)	Prostaglandin E2 (PGE_2)	lipid	Piglet brain microvascular endothelial cells (PBMEC)	(5, 129, 130)
4	Prostanoid receptors	Thromboxane A2 receptor (Tbxa2r)	Thromboxane A2 (TXA_2)	lipid	Mature rat oligodendrocytes	(163)
5	Platelet-activating factor (PAF) receptor	PAF receptor (Ptafr)	PAF and PAF-like lipids	lipid	Isolated rat liver nuclei, PBMEC	(6, 7)
6	Lysophosphatidic acid (LPA) receptors	LPA receptor 1 (Lpar1)	LPA	lipid	PBMEC, rat pheochromocytoma cell line (PC-12)	(166, 226)
7	Sphingosine-1-phosphate (S1P) receptors	S1P receptor 1 (S1PR1)	S1P	lipid	Human umbilical vein vascular endothelial cells (HUVEC)	(180)
8	Leukotriene receptors	Cysteinyl leukotriene receptor 1 (CYSLTR1)	Leukotriene D4 (LTD_4)	lipid	Human intestinal endothelial cell line (Int-407) and human epithelial colorectal carcinoma cell line (Caco-2)	(184)
9-10	Angiotensin (AT) receptors	Angiotensin II receptor, type 1 and 2 (Agtr1a and Agtr2)	Angiotensin II	peptide	Primary rat neurons (hypothalamus and brain stem), vascular smooth muscle cells (VSMC), rat adult ventricular cardiomyocytes	(3, 227-231)
11	Proteinase-activated receptors (PAR)	Coagulation factor II receptor-like 1 (F2rl1)	Trypsin, tryptase, TF/VIIa, Xa	peptide	Rat and mouse neurons- retinal ganglion cells (RGC)	(13)

	GPCR family	Nuclear receptor subtype	Principal ligand(s)	Family of ligand(s)	Endogenous (non-transfected) cells/tissues	Ref
	Class-A					
12	Bradykinin receptors	Bradykinin receptor B2 (Bdkrb2)	Bradykinin, Kallidin	peptide	Rat hepatocytes	(232)
13	Endothelin receptors	Endothelin receptor type B (Ednrb and EDNRB)	Endothelin-1	peptide	Rat liver cells (ET-1 binding sites), human aortic vascular smooth cells, human ventricular endocardial endothelial cells (EECs), rat ventricular cardiomyocytes	(12, 115, 233-235)
14	Chemokine receptors	X-X-C chemokine receptor 4 (CXCR4)	Chemokine (C-X-C motif) ligand 12 (CXCL12) also known as stromal cell-derived factor 1β (SDF-1β)	peptide	Human malignant hepatocellular carcinoma cell lines and human non-small-cell lung cancer cells	(236, 237)
15	Melatonin receptors	Melatonin receptor 1B (MTNR1B)	Melatonin	peptide	Human placental choriocarcinoma cell-lines JEG-3 and BeWo	(238)
16	Opioid receptors	Opioid receptor κ (kappa) 1 (Oprk1)	Big dynorphin and dynorphin-A	peptide	Hamster ventricular cardiomyocytes (in animals with cardiomyopathy)	(239)
17	Gonadotrophin releasing hormone (GnRH) receptors	GnRH receptor (GNRHR) orthologue	GnRH like peptide	peptide	C. elegans germline, intestine and larynx	(240)
18	Tachykinin receptors	Tachykinin receptor 1 (Tacr1)	Substance P (Tachykinin, precursor 1, Tac1), neurokinin A and B (Tac1 & Tac3)	Peptide	Rat dorsal root ganglion neurons	(241)
19	Melanocortin receptors	Melanocortin 2 receptor (MC2R)	Adrenocorticotrophin (ACTH)	peptide	Human adrenocortical epithelial cell line (H295R)	(242)

	GPCR family	Nuclear receptor subtype	Principal ligand(s)	Family of ligand(s)	Endogenous (non-transfected) cells/tissues	Ref
	Class-A					
20	Vasopressin and oxytocin receptors	Oxytocin receptor (Oxtr)	Oxytocin (Oxt) and arginine vasopressin (Avp)	peptide	Primary mouse osteoblasts and MC3T3.E1 preosteoclastic cells	(14)
21	Apelin receptor (previously PAJ receptor)	Apelin receptor (APLNR)	Apelin (APLN) -13, 17 and 36	peptide	Human cerebellar and hypothalamic neurons	(243)
22	Neuropeptide Y (NPY) receptors	Neuropeptide Y receptor type Y1 (NPY1R)	Neuropeptide Y (NPY), Peptide YY (PYY)	peptide	Human fetal EECs	(244)
23-24	Adrenoreceptors (alpha)	Alpha (α) 1A and 1B adrenoreceptors (Adra1a and Adra1b)	Adrenaline and noradrenaline	amine	Neonatal rat ventricular cardiomyocytes, adult mouse cardiomyocytes	(216, 218-221)
25-26	Adrenoreceptors (beta)	Beta (β) adrenoreceptors 1 and 3 (Adrb1 and Adrb3)	Adrenaline and noradrenaline	amine	Rat and mouse ventricular cardiomyocytes	(12, 167, 217)
27	Acetylcholine receptors (muscarinic)	Suggested M3, M4, M5 and M1 subtypes based on molecular size (Chrm1, Chrm3, Chrm4 and Chrm5)	Acetylcholine	quaternary ammonium compound	Rabbit corneal epithelial and endothelial cells	(4, 199)
	Class-B					
28	Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) receptors	VIP and PACAP receptor 1 (VPAC1) – nuclear localization. VPAC2 – perinuclear localization	VIP, PACAP-38, GHRH, secretin	peptide	Human colon adenocarcinoma cell line (HT29) and human breast cancer cell lines (T47D and MDA-MB-468)	(2, 245)

	GPCR family	Nuclear receptor subtype	Principal ligand(s)	Family of ligand(s)	Endogenous (non-transfected) cells/tissues	Ref
	Class-B					
29	Parathyroid hormone (PTH) receptors	Parathyroid hormone (PTH) 1 receptor (PTH1R)	PTH, PTHLH (previously known as PTH related peptide)	peptide	Rat kidney, liver, intestine, uterus tissues, rat osteosarcoma cell line (ROS 17/2.8), mouse nontransformed osteoblasts (MC3T3-E1) and human osteosarcoma cells (SaOS-2)	(246-248)
	Class-C					
30	Metabotropic glutamate receptors (mGluRs)	Metabotropic glutamate receptor 5 (Grm5)	Glutamine	amine	Adult mouse mesencephalic neurons and Rat pup striatal neurons	(208, 210, 211)
31	Orphan GPCR	GPR158	-	-	Human ocular trabecular meshwork (TBM) cells & immortalized human cell-line TM-1	(185)

Little is currently known about the molecular mechanisms responsible for nuclear localization of GPCRs. Based on topology, every GPCR has seven highly hydrophobic transmembrane (TM) domains, which entails use of vesicular transport mechanisms for its transfer between membranous intracellular organelles through mostly aqueous environment. After the vesicle (the process of vesicle formation is reviewed in ref. (249)) containing a transmembrane receptor reaches its final destination, it has to fuse to the membrane of the target organelle. Increasing evidence suggests that members of small GTPases of Ras superfamily (**summarized in appendix-A table**), especially Rab and Arf families, are involved in regulation of various stages of vesicular transport as well as in the process of membrane fusion (15, 16, 250).

2.2.4 Mechanism of Nuclear localization of GPCRs

The direct experimental evidence for involvement of specific Rab/Arf GTPases in localization of nuclear membrane proteins is currently lacking. However, it is interesting to note that the vesicular fusion has been the conserved mechanism necessary for reassembly of nuclear envelope after mitosis in various eukaryotes and the activity of Ran GTPase is required for the process (251-253).

Ran is a highly conserved member of the Ras superfamily in eukaryotes but lacks – CAAX membrane anchoring motif at its C-terminus which is found in other Ras GTPases. It is primarily involved in nucleocytoplasmic transport (both import and export) of macromolecules (greater than ~ 40 kDa) through the nuclear pore complex (NPC) (254-256). There is high concentration of GTP bound Ran (Ran-GTP; active form) inside the nucleus and Ran-GDP (inactive form) is almost exclusively found in the cytoplasm (257, 258). In the most well-studied pathway of nucleocytoplasmic import, a protein cargo containing nuclear localization signal (NLS; see below for

details) binds to importin-α.β heterodimer (also known as nuclear import receptor) in the cytoplasm and the heterotrimeric complex (α.β.cargo) transverses across the NPC. Inside the nucleus, Ran-GTP tightly binds to importin-β which results in conformational change in the complex causing release of the nuclear protein cargo from importin-α (255, 256). In addition to the aforementioned functions (nucleocytoplasmic transport and NE assembly), Ran GTPase also plays an important role in regulation of cell cycle (253) and in trafficking of inner nuclear membrane (INM) resident proteins (258). Of note, many INM resident proteins contain NLS (17, 18, 259).

2.2.4.1 GPCRs and the Nuclear Localization Signal (NLS)

The classical monopartite NLS consists of small cluster of basic amino acids (at least 3 of them are consecutive) and was originally discovered in the simian virus (SV) 40 large-T antigen (260, 261). The bipartite NLS, on the other hand, consists of two clusters of basic amino acids which are separated by 10-12 residues (262, 263). Both monopartite and bipartite NLSs are recognized by heterodimeric nuclear import receptor. More recently, additional classes of NLSs, binding to different regions of importin-α, have been identified (100).

Some of the earliest evidence for the presence of functional NLS in a GPCR was provided by Lu *et al.* for agonist-induced internalization of Angiotensin-II type-1 (AT1) receptor (but not AT2 receptor which lacks the NLS) in neurons (228). In 2003, Lee *et al.* reported that 17 additional GPCRs (all belonging to Rhodopsin family) contain putative NLS which is located just after seventh transmembrane domain, including AT1 receptor (243). One exception to the general rule was the Apelin receptor (previously known as APJ receptor) which contains a functional NLS in its 3rd intracellular loop (243). Joyal *et al.* first indentified presence of multiple putative NLS motifs (in first and

third intracellular loops, respectively) in coagulation factor II receptor-like 1 (F2rl1, previously known as Par2 receptor) which is a member of protease activated receptor sub-family of class-A GPCRs (13). The mutational disruption of the motifs revealed that both NLSs are necessary for agonist-induced nuclear translocation of the receptor from plasma membrane (13). It is interesting to note that some GPCR ligands can also contain NLS (e.g., Parathyroid hormone-related protein (PTHrP) which is a class-B GPCR ligand) (264). More recently, functional NLS has been characterized as being responsible for nuclear localization of non-GPCRs such as Death receptor-5 (DR5) (265) and ErbB-2 receptor (266).

The NLS and Nuclear importins

As described earlier, classical NLS (monopartite and bipartite) is recognized by the importin-α.β heterodimer. Both importins are members of Karyopherin (Kap) family of proteins. Out of nineteen human Kap-βs (Kapβ), eleven are involved in nuclear import (267). Currently, consensus NLSs have been identified only for importin-α.β (classical NLS) (268) and transportin (Kapβ2 or Kap140p in yeast) (PY-NLS) (269, 270) pathways. PY-NLS consists of weak consensus motifs and physical rules such as structural disorder, overall positive charge, which together identify transportin-mediated nuclear import cargos (269, 270). The role in importins in nuclear translocation of F2rl1 is further evidenced by siRNA mediated silencing of impβ1 (and impα3 and impα5) affecting nuclear localization of the GPCR (13). To date, three GPCRs, two of which belong to chemokine-receptor family (CCR2 and CXCR4 receptors) (271, 272) and oxytocin (Oxtr) receptor (14) have been reported to undergo transportin mediated nuclear translocation.

The role of endosomal sorting proteins in nuclear localization of GPCRs

The sortin-nexins (SNXs) form another class of evolutionarily conserved eukaryotic proteins which contain Bin/Amphiphysin/Rvs (BAR) and phox homology domains. These domains are essential for interaction of SNX proteins with biological membranes (273, 274). SNX1 is known to associate with C-terminal cytosolic domain of many GPCRs (275). SNXs were first identified regulators of retromer-dependent endosomal trafficking (276) but current evidence suggests for more diverse roles (277, 278). Snx11, which lacks BAR domain but contains an extended PX domain (279), has been recently shown to regulate endosomal sorting of F2rl1 (along with aforementioned importins) to the nucleus via trafficking through microtubule network (13).

2.2.4.2 β-arrestins and GPCR trafficking

Many GPCRs have C-terminal β-arrestin interaction motifs and roles of the latter in regulation of trafficking of agonist-bound phosphorylated GPCRs is reviewed elsewhere (280, 281). In addition, they can also act as adapter molecules in non-canonical GPCR signaling (282). Out of two non-visual arrestins, only β-arrestin1 (arrestin-2) shows nuclear localization. β-arrestin2 (arrestin-3) is constitutively exported out of the nucleus as it contains leucine-rich nuclear export signal (NES) (283). The exact role of β-arrestin1 in nuclear translocation of GPCRs is currently unknown. Nonetheless, mutational disruption of β-arrestin interaction motif does not affect agonist-induced nuclear localization of F2rl1 (13). On the other hand, siRNA mediated silencing of β-arrestin1/2 in osteoblasts shows that β-arrestins are required for expression of differentiation-inducing genes in the cells, function reported to be mediated by nuclear oxytocin receptor (14). Whether β-arrestins play a role in the nuclear translocation of oxytocin receptor or the signaling or both is still unknown.

2.2.4.3 Where do nuclear GPCRs come from?

Once synthesized, GPCRs are folded (and in some cases of assembled into oligomeric protein complexes) in endoplasmic reticulum (ER) (284). The process of exit from ER requires passing resident ER quality control mechanisms and may involve specific motifs present within GPCRs (285-287). Because the ONM is contiguous with the membrane of ER, it is hypothesized that resident ONM proteins can reach there by lateral diffusion from the ER membrane (288). Of note, GPCRs undergo post-translational modifications in both ER and trans-golgi network (TGN), including glycosylation which has been implicated in the plasma-membrane expression of various GPCRs (289). Based on immunoblot analysis, plasma membrane and nuclear GPCRs often have similar molecular weight which is an indirect evidence for glycosylation of nuclear GPCR. This, however, does not rule out the presence of alternative, yet unidentified, pathway of glycosylation at the nucleus. Once processed, GPCR (in some cases along with its signaling machinery (290)) is transported from TGN to its final destination via vesicular transport mechanisms, as described earlier. Iborra *et al.* proposed that translation of some NE proteins could take place at the nucleus itself (291). More research is needed to delineate between nuclear trafficking pathways of GPCRs possibly originating from TGN from those coming from the plasma membrane.

Agonist dependent vs. Agonist independent nuclear localization of GPCR

Many GPCRs with peptide ligands need agonist-induced internalization via one of the endocytic pathways for their nuclear translocation (13, 14, 228). This makes sense, physiologically, because most peptide ligands do not have access to the intermembrane space where ligand-binding N-terminal domain would be located (for both ONM and INM resident receptors). The possible exception to the ligand-rule could be importin-β

mediated nuclear translocation of PTHrP, described earlier (264). However, how PTHrP reaches intermembrane space is a mystery. Another GPCR with peptide ligand, apelin receptor (or APJ receptor), has been previously reported to show agonist-independent nuclear localization in endogenous expression in neuronal cells (243). However, there have been recent reports of autocrine apelin signaling in other cellular systems (292). How endogenous Apelin-APJ pathway affects nuclear translocation of the receptor is still unclear. Similar to PTHrP, how apelin gains access to the intermembrane space needs to resolved.

Some GPCRs of bioactive lipids, like Ptafr and LPA1 receptor seem to show agonist-independent nuclear localization (7, 166). Others like S1PR1 is reported to show exogenous S1P-induced nuclear localization in vascular endothelial cells and heterologous expression systems (180). As discussed before, the issue is further complicated by the fact that most lipidic ligands can be synthesized locally at the nucleus and these ligands are capable of transversing across phospholipid membrane. Therefore, one needs to be careful to distinguish between effects of exogenous vs endogenous ligands while characterizing agonist-dependent and agonist-independent nuclear localization of GPCRs.

2.2.5 Rab GTPases and vesicular trafficking of GPCRs

This final section on nuclear GPCRs trafficking focuses on the regulatory roles of small GTPases of rab family in vesicular transport of GPCRs. More than 60 different rab GTPases are found in mammals and they constitute the largest family of the ras-like small G proteins (16). First rab gene (YPT1) was identified in yeast in 1983 (293) and its mammalian orthologs were discovered in 1987 and called rab (ras-like in rat brain) (294). One of the unique features of rabs is that individual members are localized at discrete membrane-bound organelles (295) and in some cases, to distinct microdomains on these organelles. For example, Rab5 interacts with its two effector proteins, APPL or EEA1, in two separate microdomains of early endosomes (296). It is also important to note that same protein cargo can interact with more than one rab GTPases.

2.2.5.1 General rab cycle

Similar to other ras GTPases, the rab proteins cycle between an active (GTP-bound), and an inactive (GDP-bound) states. The active/inactive status of the rabs is regulated by the GTPase activating proteins (GAPs) that accelerate the intrinsic rate of hydrolysis of GTP to GDP and by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP, and that inhibit GDP dissociation (GDIs). Each rab protein has its own set of GAPs, GEFs and GDIs, with some overlap between related rabs (16) (**for schematic representation, see figure- 2.3**). Inactive rabs are bound by the chaperone GDI in the cytosol. When rab-GDP-GDI complex reaches a donor membrane (with that rab's cognate GEF), nucleotide exchange (GDP ➔ GTP) takes place and GDI is released. Alternatively, specific transmembrane protein might also recruit a rab to the location (e.g., GLUT4 localizes Rab11 to plasma membrane in response to insulin (297)). The now active rab accumulates at the membrane and interacts with its effectors

(e.g., a receptor or arrestin). The accumulation of wrong rab is prevented by GAPs (298). Each rab GEF, alone or in conjunction with rab effectors, interacts with different coat proteins which initiate vesicle formation (249). Once the vesicle is formed, it is transported to another location by sub-cellular motors. In some but not all cases, rab GTPases can directly interact with their motor (299). The intra-vesicular exchange of rabs is needed in other situations and is carried out by recruitment of another set of GEFs (250). Lastly, upon delivery at its final destination, vesicle is docked and fused to the target membrane. Rab GTPases can also regulate this step by interacting with SNARE proteins (300).

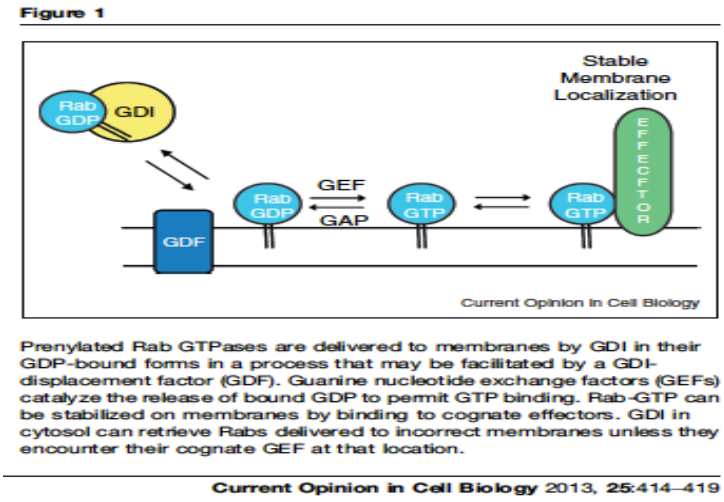

Figure-2.3 Schematic representation of general Rab cycle.

GDI- GDP dissociation inhibitor, GEF- Guanine exchange factor, GAP- GTPase activating protein

Note- The figure is reprinted from Current Opinion in Cell Biology, 25(4), Pfeffer SR, Rab GTPase regulation of membrane identity, pp 414-419, © 2013 (301), with permission from Elsevier .

2.2.5.2 Rabs and GPCRs

There are growing examples of rab GTPases regulating GPCR signaling and trafficking in scientific literature (302). This sub-section focuses on roles of Rab5, Rab7, and Rab11 sub-families in regulation of GPCR endocytosis and trafficking.

Rab5 is primarily localized to the plasma membrane, clathrin-coated vesicles, and early endosomes (303, 304). Some examples of GPCRs endocytosed by Rab5 include β2 adrenergic receptor (305), endothelin receptors A and B (306), and Ptafr (103). The importance of Rab5 in cargo sorting is evident by the fact that replacement of hypervariable region of Rab5 with that of Rab7 (see below) results in mislocalization of internalized cell surface proteins to late endosomes instead of early endosomes (307). Rab5 is also important for phosphoinositide regulation through its effector, phosphatidylinositol 3-kinase (308) and this action is essential for uncoating of clathrin-coated vesicles in the cytosol (16). Additionally, sequential actions of Rab5 and Rab7 are required for recruitment of Vps26-Vps29-Vps35 trimer, which is part of the retromer complex (309).

Current evidence suggests that Rab7 plays important role in transfer of late endosomal cargo to lysosomes (310) by direct interaction with Rab-interacting lysosomal protein (RILP) to recruit dynein-dynactin motor complex to transport late endosomes towards the lysosome (311). Examples of GPCRs trafficked by Rab7 include κ-opioid receptor (312), CXC chemokine receptor 2 (313), as well as Ptafr (103). The transfer of endocytic cargo, including GPCRs, between Rab5 and Rab7 vesicles is also documented (16).

Rab11 is localized to perinuclear recycling endosomes, as well as to the TGN and is considered to control slow endosomal recycling (314), as well as protein trafficking to

and from the TGN (315). It has been described that Rab11 is present at the junction of endocytic and exocytic compartments of the cell (316). Some examples of GPCRs trafficked by Rab11 GTPase include chemokine receptor CXCR2 (317), prostacyclin receptor (318) and vasopressin V2 receptor (319). In addition to recycling, Rab11 also plays a role in targeted delivery of vesicles to the cleavage furrow/midbody during cell division (320). Recently, Rab11 (along with Rab5) has been proposed to regulate activity of another small GTPases, Rac during cell migration (321). There are three members of Rab11 sub-family in mammals, viz. Rab11a, Rab11b, and Rab25. The first two are ubiquitously expressed, while Rab25 is only expressed in epithelial cells (322).

The effectors of Rab11 sub-family of GTPases are called Rab11-family interacting proteins (Rab11-FIPs) and are sub-divided into two classes (class I and II Rab-11 FIPs). Some examples of class-I Rab11-FIPs include Rab coupling protein (RCP), Rip11 and Rab11-FIP2. All of them are involved in slow recycling of cell surface proteins and share a conserved motif at their c-termini (323). On the other hand, class-II FIPs (e.g., Rab11-FIP3/arfophilin-1 and Rab11-FIP4/arfophilin-2) also lack conserved motif found in class-I members (324). It is hypothesized that class-II Rab-11 FIPs are involved in non-recycling functions of Rab11 (320).

Section- 2.3 Retinal vasculature

2.3.1 Blood supply of retina and vascular development

2.3.1.1 Blood supply of the retina

The retina has the highest metabolic demands of any tissues in the body (325). Branches of central retinal artery, which arises directly from ophthalmic artery, supply oxygen and nutrients to the inner two-thirds of retina (326). Retinal capillaries are typically organized in an interconnecting two-layer network (superficial and deep plexus), with exception of peripapillary region of the retina where there is an additional layer of the radial capillary vessels (327). The superficial capillary plexus is located in the nerve fiber and ganglion cell layers and a second deep layer lies in the inner nuclear and outer plexiform layers (326). Similar to microvasculature forming the BBB, capillary networks of the retina are lined by endothelial cells with abundant tight junctions, making them highly selective in permeability of biomolecules. The retinal microvascular endothelial cells form the inner blood retina barrier (BRB), while the outer BRB is made up of retinal pigment epithelial cells and the Bruch membrane (328). The outer third of retina is avascular and is nourished by adjacent choroidal circulation. Thus, choriocapillaries are the primary source of nutrients and oxygen to the metabolically active retinal pigment epithelium and photoreceptors (329, 330).

The retinal (331, 332) and choroidal (333, 334) vessels in adults maintain constant blood flow over a fixed range of perfusion pressure and blood oxygen tension by a process known as autoregulation (335). However, autoregulatory response mechanisms are significantly underdeveloped in chroidal vessels of the newborn (336, 337). This is partly responsible for increased delivery of oxygen to retina in preterm infants (**see**

section 2.4). The perinatal period is also associated with elevated levels of prostaglandins, notably PGD_2 and PGE_2, which in turn upregulate NO production in various vascular beds, including those in the eye (338, 339). The latter is also responsible for changes in autoregulation in ocular vessels of the newborns (336).

2.3.1.2 Development of retinal blood vessels

The blood vessels can be formed by one of two mechanisms. Vasculogenesis refers to *de novo* formation of blood vessels from endothelial precursor cells. Angiogenesis, on the other hand, is formation of new blood vessels by proliferation and spreading of vascular endothelial cells from existing vessels to vascularize neighboring regions (340). In human and other primate retinas, vasculogenesis begins with migration of spindle-shaped vascular precursor cells to inner retina and can be detected as early as 8-10 weeks of gestation (341). These cells are responsible for formation of primitive blood vessels, radiating from optic disk (342). The primitive vessels typically have low capillary density and are unable to meet the metabolic demands of developing retina. The resulting physiological hypoxia stimulates angiogenesis, which is responsible for increase in retinal vascular density as well as spreading of blood vessels to peripheral retina (343).

In human retina, astrocytes migrate and cover the developing retina around 16 weeks of gestation. They secrete various factors including vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (344). These factors, in turn, trigger development of retinal vascular network, beginning at the optic nerve and spreading peripherally. By 36 weeks, the vascular network reaches nasal edge of the retina and the process is completed by reaching the temporal edge at 40 weeks of gestation (345). In rodents (mice and rats), development of retinal vasculature (both superficial and deep

capillary plexuses) takes place after birth and is complete around post-natal day 21 (P21) (346, 347). Therefore, they are commonly used as experimental models to study mechanisms involved retinal vasculogenesis and angiogenesis.

2.3.2 Angiogenesis: major molecular mechanisms

The experimental evidence for presence of diffusible factors which are responsible for blood vessel formation was first provided in tumors in 1970s (348, 349). This lead to identification of a novel, angiogenic peptide secreted by tumor cells in 1983 (350), which was later purified and renamed as VEGF (351). The sequence of human VEGF cDNA suggested presence of multiple molecular species (352), which were characterized as products of alternative mRNA splicing (353). With isolation of second cDNA homologous to VEGF from human and mouse tissues in 1990s, the new gene was called VEGF-B (354) (previous name- VEGF related factor or VRF (355)) and the original gene was renamed as VEGF-A. Soon after, two additional homologs, VEGF-C (356) (previous name- VEGF related protein or VRP (357)) and VEGF-D (358) (also known as c-fos induced growth factor or FIGF (359)) were identified.

2.3.2.1 VEGF family of ligands

All four VGEF homologs (A-D) have unique expression patterns during embryonic and post-embryonic development as well as in adult life (360, 361) and VEGF-A is an important mediator of both vasculogenesis and angiogenesis (362, 363). The human VEGF-A is secreted in three major isoforms, viz. VEGF121, VEGF165, and VEGF189 (corresponding mouse isoforms are Vegf120, Vegf164, and Vegf188 respectively). VEGF121 is freely diffusible, while other two isoforms can bind to cell-surface and extracellular proteoglycans by c-terminal heparin-binding motifs and the binding is strongest for VEGF189 (364, 365). Mice lacking Vegf164 and Vegf188 isoforms (i.e.

only expressing diffusible Vegf120) have major defects in angiogenesis leading to development of ischemic cardiomyopathy (366). On the other hand, transgenic mice expressing only Vegf164 isoform have no visible vascular abnormalities, possibly due to its intermediate diffusion properties (367).

2.3.2.2 VEGF receptors

The primary receptors for VEGF ligands belong to receptor tyrosine kinase family (see **Figure- 2.4** (368)). VEGF-A can bind to VEGFR-1 (official name- fms-related tyrosine kinase 1; FLT1) (369) and VEGFR-2 (official name- kinase insert domain receptor; KDR) (370). VEGFR-2 seems to mediate major actions of VEGF-A, while VEGFR-1 has been proposed to act as a decoy receptor (371) or by suppressing VEGF-A signaling through VEGFR-2. VEGFR-1 KO mice have excess of endothelial cells and abnormal tubule formation during angiogenesis (372) but VEGFR-2 KO mice fail to develop any vasculature (homozygous embryos die in utero between 8.5 and 9.5 days after conception) (373). The third member of the receptor sub-family, VEGFR-3 (official name- fms-related tyrosine kinase 4, FLT4), is essential for formation of lymphatic vessels (374). Based on recent evidence using truncated version of VEGF-C which lacks binding to VEGFR-3, its signaling might be important for vascular sprouting (375).

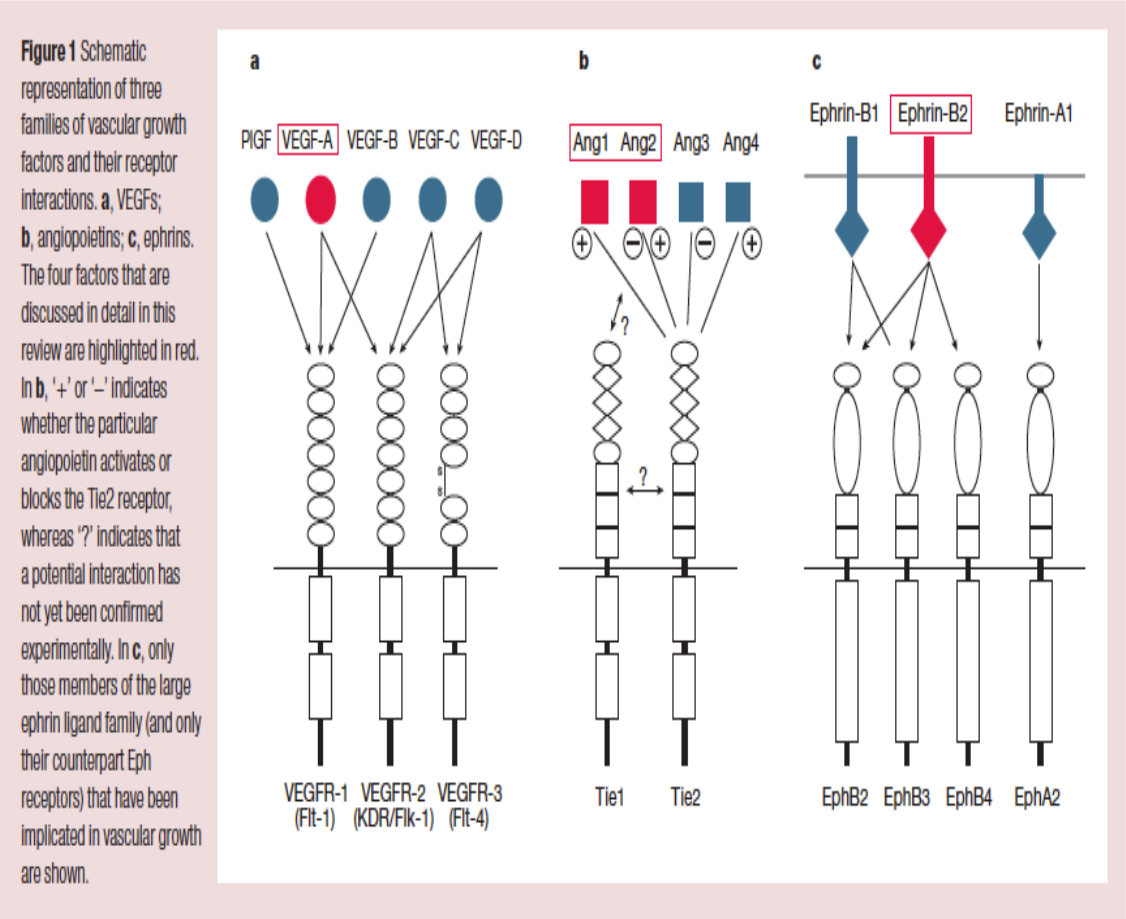

Figure 1 Schematic representation of three families of vascular growth factors and their receptor interactions. **a**, VEGFs; **b**, angiopoietins; **c**, ephrins. The four factors that are discussed in detail in this review are highlighted in red. In **b**, '+' or '–' indicates whether the particular angiopoietin activates or blocks the Tie2 receptor, whereas '?' indicates that a potential interaction has not yet been confirmed experimentally. In **c**, only those members of the large ephrin ligand family (and only their counterpart Eph receptors) that have been implicated in vascular growth are shown.

Figure-2.4 Schematic representation of VEGF, Ang (angiopoietin) and ephrin receptor sub-families and their respective ligands. All receptors belong to receptor tyrosine kinase (RTK) superfamily.

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2.3.2.3 VEGF signaling and neovascularization

According to the current working model, VEGF-A/VEGFR-2 signaling is critical for *de novo* formation of blood vessels (vasculogenesis), evidenced by disruption or deletion of a single allele of VEGF-A resulting in early embryonic lethality (376, 377). Once primitive vessels are formed, angiopoietin signaling plays important roles in vascular remodeling and maturation. Following vessel maturation, angiopoietin continues to act to maintain stability of the vasculature (discussed below in section- 2.3.2.4). The sprouting of new vessels from an existing one in embryos as well as in adults (e.g., in tumors), under the influence of various pro-angiogenic stimuli, also requires VEGF. Two specialized types of vascular endothelial cells, tip and stalk cells, participate in the latter process (378). Tip cells, when exposed to VEGF-A gradient, get localized at the leading edge of the growing vessels (379). Notch signaling in tip cells, in turn, induces transactivation of neighboring stalk cells, which play a role in stabilization of newly formed vessels (380).

In retina, VEGF is essential for physiological as well as pathological (e.g., in proliferative retinopathies) vascularization (381, 382). Insulin-like growth factor 1 (IGF-1, also known as somatomedin C) acts as a permissive factor for VEGF-A dependent retinal vascular endothelial cell proliferation and survival (383-385). Specific roles of VEGF and IGF-1 signaling during different phases of retinopathy of prematurity (ROP) are discussed in section-2.4.

2.3.2.4 Angiopoietin signaling

Angiopoietins are four small-peptide ligands (Ang1-4) which mediate their biological effects by binding to two tyrosine kinase receptors with immunoglobulin-like and EGF-like domains (Tie1 and Tie2) expressed on vascular endothelial cells (386-388). Little is

known of roles of Ang3/4 as well as Tie1 signaling in angiogenesis. Ang1 (Angpt1) is an endogenous agonist of Tie2 (official name- TEK tyrosine kinase, Tek) receptor, while Ang2 (Angpt2) is a natural antagonist of the same receptor (387). Growing evidence suggests that Ang1 is involved in vascular maturation by recruiting pericytes which support endothelial cells (389) and in maintaining stability (quiescence) of the formed blood vessels after maturation is complete (390). Ang2, on the other hand, is predominantly produced in the regions of vascular remodeling and destabilizes quiescent vasculature to trigger formation of new vessels (i.e. angiogenesis) (391, 392), including in the retina (393).

2.3.2.5 PAF: a probable link between Inflammation and Angiogenesis

Inflammation and angiogenesis can co-exist in wide range of diseases (394-396), including ischemic retinopathies (discussed in section-2.4). PAF, a pleiotropic phospholipid mediator, is also known to exert pro-inflammatory as well as pro-angiogenic effects on the vasculature (397). Both VEGF (398, 399) and Ang(1-2) (400) are well-known to stimulate PAF production by vascular endothelial cells. PAF, in turn, promotes synthesis of various pro-angiogenic factors, including VEGF itself (401). Unsparingly, administration of Ptafr antagonists reduces angiogenesis in experimental models of cancer (402, 403) and other diseases (404). PAF directly activates macrophages to augment pro-angiogenic factors' production via NF-κB dependent pathway (90). Such a signaling mechanism remains to be elucidated in vascular endothelial cells, however potential role of NO synthesis has been proposed in the process (405).

Section-2.4 Ischemic retinopathies

Ischemic retinopathies (IR) are a group of diseases where blood supply fails to meet the metabolic demands of the retina, resulting in retinal cell death leading to blindness in most severe form. Common causes of IR include diabetic retinopathy (DR), retinopathy of prematurity (ROP), hypertensive retinopathy and central retinal artery occlusion. Many of these conditions, including ROP and DR, share common features such as proliferation of abnormal blood vessels induced by rise in vaso-proliferative factors (406). In this section, I will review major mechanisms involved in the pathogenesis of ROP.

2.4.1 Retinopathy of Prematurity (ROP)

2.4.1.1 Historical perspectives and epidemiology of ROP

In 1942, Terry described an ocular condition called retrolental fibroplasia, later termed as ROP, which was characterized by retinal neovascularization in premature infants (407). In 1950s, high oxygen treatment received by premature infants was identified as a possible cause of ROP (408, 409). The subsequent clinical recommendations to reduce percentage of inhaled oxygen resulted in an expected decrease in the incidence of ROP in 1960s and 70s but there was corresponding increase in number of cases of cerebral palsy and neonatal death (410, 411). There was an increase in the ROP incidence in the 1980s, which was attributed to better neonatal intensive care and increased survival of the premature infants (412). Today, ROP is the most common cause of preventable blindness in children in middle-income countries, including India and China (413, 414). While ideal oxygen treatment protocols at different stages of neonatal development and at different phases of ROP (see below) remain unknown, animal models of ROP has

helped to understand etiopathology of the disease better. Both human ROP and its animal models follow a biphasic course. The **phase I** (**vaso-obliteration**) is characterized by failure of normal growth of retinal vasculature, while **phase II** (**neovascularization**) exhibits growth of abnormal blood vessels (415).

2.4.2 Animal models of ROP

Some of the earliest animal experiments to study ROP were carried out in cats (416), mice (417) and rats (418), all in 1950s. These models were based on same basic principal of oxygen-induced retinopathy (OIR) which produces ocular changes similar to those seen in human ROP. The current model of OIR is based on works by Penn (419) and Smith (420) on rats and mice, respectively. The development of rat model of OIR is reviewed in brief, as it is relevant to this thesis.

Rat model of OIR

In rat, the development of retinal vasculature is complete by post-natal day 21 (P21) (346). In other words, retinal vessels of newborn rat pup resemble those of a preterm infant and are vulnerable to damage by oxidative stress (see below). The initial OIR experiments in rats produced somewhat inconsistent results, due to differences in oxygen treatment protocols and variable methods of assessment of retinal neovascularization (421-423). Today, two protocols of oxygen treatment are commonly used to assess retinovascular changes associated with two distinct phases of OIR.

In the **hyperoxia protocol** of OIR, rat pups are constantly exposed to 80% oxygen (O_2) during first week of life which produces marked retinal vaso-obliteration (27, 424-426), which is a hallmark of **phase I** of human ROP (415). As recently demonstrated by our group, maximum vaso-obliteration can be obtained as early as post-natal day 11 (P11) with five-day hyperoxia treatment from P5 to P10 followed by return to room air for

one day (427). While the hyperoxia protocol produces reliable experimental vaso-obliteration, extent of retinal neovascularization (NV) is not uniform (428).

The **cycling** (alternating hyperoxia-hypoxia) **protocol** of OIR is supported by clinical observations of fluctuating tissue oxygen levels (measured by partial pressure of O_2) which have been positively correlated with severity of ROP in preterm infants (429-431). Based on original work done by the group of Penn, the severity of retinal NV in rats also depends on the range of oxygen fluctuation (432, 433). The current cycling OIR protocol comprises of exposure of rat pups to alternating 50% and 10% O_2 for 24 hours each for first two weeks (P1 to P14) followed by exposure to room air (21% O_2) for 4 days (P15 to P18) and it produces abnormal retinal NV changes (427, 433) comparable to those seen in **phase II** of human ROP (see below) (415).

2.4.3 Two phases of ROP/OIR

Phase-I vaso-obliteration

Hyperoxia and increase in the reactive oxygen species (ROS) in retina are considered to be major risk factors for development of phase I of ROP. (434, 435). The premature infants have decreased antioxidants in their tissues including in the retina to neutralize the ROS (436), the latter being responsible for vascular endothelial cell damage and apoptosis. The retina is also a rich source of polyunsaturated fatty acids (PUFA) (437) which are particularly vulnerable to ROS-induced lipid peroxidation (415, 438). In addition, ROS are known to activate enzymes of PLA2 family (439, 440). Activation of PLA2 enzymes in turn results in biosynthesis of pro-inflammatory lipid mediators, most notably prostanoids, PAF, and TXA_2, from membrane phospholipids. Finally, phase-I of ROP is characterized by hyperoxia-induced suppression of pro-angiogenic growth factors such as VEGF (382) and by low levels of IGF-1 in preterm infants (384),

leading to a state of decreased angiogenesis. Together, all these factors contribute to degeneration of existing microvasculature as well as to failure of new vascular growth, ultimately leading to retinal ischemia.

PAF and vaso-obliteration

Choline phosphoglycerides are major precursors of PAF and PAF-like lipids in the retina. In addition to PAF generated by enzymatic remodeling of membrane phospholipids (as described in **section- 2.1**), oxidative fragmentation products of PUFA can also exert their biological effects by binding to the Ptafr, due to their resemblance in structure with PAF (441, 442). The latter might contribute significantly to vascular changes during phase I of ROP. PAF, in turn, can also stimulate more production of ROS (443, 444). Synthetic PAF derivative, C-PAF (carbamyl-PAF), is not only cytotoxic to cultured microvascular endothelial cells but also to freshly isolated retinal microvessels and this effect is successfully reversed by different Ptafr antagonists, including BN-52021 (27).

Some of the actions of PAF can be mediated indirectly by activation of prostanoid biosynthesis via COX pathway (see below). In this regard, our group previously reported that Ptafr can upregulate expression of COX-2 in isolated nuclei, latter being a central enzyme is PG and TXA_2 synthesis (see **Figure- 2.1**) (44). Both vaso-obliteration and vasoconstriction actions of PAF seem to be mediated, to a large extent, by TXA_2 *in vivo* (27, 30, 426, 445).

Thromboxane A_2 and vaso-obliteration

Similar to PAF, TXA_2 is also a potent stimulator of platelet aggregation (446), vasoconstriction (447, 448), and as mentioned earlier, vaso-obliteration (426). The dual

inhibition of TXA_2 synthesis and signaling has been shown to prevent retinovascular changes in the experimental model of diabetic retinopathy (449).

The biosynthesis of TXA_2 shares a common first step with that of other PGs viz. production of PGH_2 from released arachidonic acid (AA) and is catalyzed by prostaglandin-endoperoxide synthase (Ptgs) enzymes (two isoforms Ptgs1 and Ptags2). Then, PGH_2 can be enzymatically converted to either one of five prostanoid derivatives (TXA_2, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2) (see **Figure- 2.1**) (44). The Ptgs is known to contribute to generation of ROS and is also activated in turn by them (337, 450). The second step of TXA_2 synthesis from PGH_2 is catalyzed by thromboxane A synthase 1 (Tbxas1). The ROS also favor TXA_2 synthesis over that of other prostanoids (448, 450). The enzymes Ptgs (both isoforms) and Tbxas1 are anchored to perinuclear endoplasmic reticulum (ER) membrane (451, 452). Finally, biological actions of TXA_2 are mediated by binding to TP receptor (Tbxa2r) which is a GPCR and is highly expressed in microvascular endothelial cells (453, 454). The cytotoxic effect of TXA_2 on retinal and brain microvasculature is mediated in large part by cell necrosis as a result of calcium-dependent activation of calpain protease (455).

Prostaglandins (PGs) and retinal circulation

The effect of PGs on vasculature depends on type of blood vessel, its developmental stage, and presence of specific prostanoid receptor sub-types. For example, the retinal vessels in newborn animals respond to PGI_2 (vasodilator action) to a greater extent than to PGE_2 and $PGF_{2\alpha}$ (vasoconstrictor action) (456) as compared to their effects on adult retinal vessels (457). As mentioned earlier, during **phase I** of ROP, elevated ROS favor TXA_2 synthesis over that of other prostanoids, which is responsible for vaso-obliteration (426). During subsequent neovascularization (**phase II**, see below for more

detailed discussion), however, this preference is shifted towards PGE_2 synthesis, in part, mediated by preferential coupling between Ptgs2 and microsomal prostaglandin E synthase (Ptges). PGE_2 then contributes to intravitreal neovascularization in ROP, via its prostaglandin E receptor 3 (Ptger3, previously known as EP3) (458).

Proinflammatory cytokines and ROP

Cytokines are small secreted molecules (~ 5-20 kDa) which participate in cell-cell communications between immune cells as well as those between non-immune cells. Proinflammatory cytokines, such as IL-1β and TNF-α, are significantly elevated in newborn infants with perinatal infection who are at higher risk of developing ROP (459, 460). The aforementioned proinflammatory cytokines are known to have a synergistic action with PAF in pathogenesis of sepsis (461, 462). Even though high plasma TNF-α levels are positively correlated with increased risk of development of ROP in very low birth weight preterm infants (463), its mechanism of action still remains a mystery. Our group recently demonstrated that, early during **phase I** of OIR, microglia derived IL-1β acts on adjacent retinal ganglion cell (RGC) neurons to promote secretion of semaphorin 3A (Sema3A). The latter is responsible for triggering apoptosis in retinal microvascular endothelial cells (427). Increased IL-1β also augments expression of coagulation factor 2 receptor-like 1, F2rl1 (previously known as proteinase-activated receptor-2 PAR2), which acts as negative feedback for IL-1β signaling in RGCs during **phase-II** of OIR (see below) (464).

NO and ROP

NO is produced from L-arginine by enzymatic action of one of the members of NOS family. There are three distinct members, out of which Nos1 (previous name- nNOS) is predominantly expressed in photoreceptors and some retinal neurons (465), Nos3

(previous name- eNOS) is mainly expressed in vascular endothelial cells and müller cells (466), and Nos2 (previous name- iNOS) expression is detected in retinal glia and circulating neutrophils (467). NO, mainly via Nos3 signaling, is known to promote endothelial cell survival by inhibition of apoptosis induced by proinflammatory cytokines (468) as well as to promote angiogenesis following tissue ischemia (469). During **phase-I** of OIR, ROS (along with other proinflammatory mediators including PAF) are known to promote expression and activity of Nos3 in retina (470). NO can, in turn, interact with ROS species to produce peroxynitrite and other nitrite- related compounds (471). This state of elevated cellular ROS and NO derivatives is known as nitro-oxidative stress. Pharmacological inhibition of Nos3 activity (472) as well as use of Nos3 deficient animals (473) show reduced retinal microvascular degeneration during phase-I of OIR. The role of NO during phase-II of OIR remains poorly understood.

Together, all these factors contribute to produce significant retinal ischemia during phase-I of ROP.

Phase-II neovascularization

The retinal ischemia during phase-I results in stabilization of hypoxia-inducible factor-1 (HIF-1), which in turn activates transcription of various pro-angiogenic factors, such as VEGF, erythropoietin and Ang2 ultimately resulting in neovascularization (415, 474). Even though aforementioned pro-angiogenic factors are produced by ischemic retina, the newly formed vessels grow aberrantly towards the vitreous during **phase II** of human ROP as well as of its rodent models (OIR). VEGF, in particular, is required for the survival of the pathologic blood vessels (475). The VEGF signaling also contributes,

in part, to the anatomy (e.g. tortuosity) of newly formed blood vessels during OIR (476).

Neuronal signaling in ROP

Neurons govern vascular growth by two distinct mechanisms, directly by secreting molecules which act on neighboring vasculature, and indirectly by activating glial cells and pericytes (477). Both neurons and blood vessels are also guided by similar chemo-attractive and repulsive cues (478). Of interest, ischemic retinal neurons when stimulated by IL-1β secrete semaphorin-3A (Sema3a) which acts via neuropilin-1 (Nrp1) expressed on endothelial cells and repels blood vessels away from ischemic zone (and towards the vitreous). Silencing of Sema3a expression, as expected, reverses aberrant neovascularization in ROP (479).

Our group has demonstrated that knockdown of succinate receptor (previously known as GPR91) in retina not only interferes with normal vasculogenesis but also successfully attenuates intravitreal neovascularization during phase II of ROP (480). Succinate, a Krebs cycle intermediate, rapidly accumulates in ischemic retina and acts on its cognate receptor (GPR91) which is predominantly expressed on neurons. Succinate receptor signaling in RGCs results in up-regulation of pro-angiogenic VEGF and down-regulation of anti-angiogenic thrombospondin-1 (480). We recently reported the differential action of F2rl1 based on its subcellular localization in retinal neurons. F2rl1 signaling at the plasma membrane of RGCs induces *Ang1*, while that at the nucleus is responsible for *Vegfa* expression (13). Vegfa causes differentiation, proliferation and migration of vascular endothelium, whereas Ang1 stimulates the maturation of newly formed blood vessels (481), thus producing different phenotypes during phase II of ROP (13). Lastly, F2rl1 signaling, which is triggered at the end of phase I characterized

by hyperoxia-induced retinal inflammation, attenuates neuronal Sema3A production by down-regulation of IL-1 receptor type I (Il1r1) to suppress cytotoxic effects of IL-1β on the adjacent vascular endothelium (464).

In summary, ROP is a complex disease with dynamic interaction between various pro- and anti- angiogenic factors during its two phases. Current treatment options for ROP are limited and only target pathological neovascularization seen in phase-II (25). Pleiotropic agents, such as PAF, can act during both phases of ROP and can be an attractive pharmacological target.

In the following **chapter- 3** , we describe several techniques commonly used for immuno-detection of nuclear GPCRs focusing on subcellular fractionation of proteins based on their localization and transmission electron microscopy (TEM) using primary cultured cells as well as tissue sections. We also describe the use of confocal microscopy to study nuclear calcium currents, which can further affect downstream events such as gene transcription, nuclear envelope breakdown, or its reconstruction and nucleocytoplasmic protein transport.

In **chapter- 4**, we present experimental evidence for the nuclear localization of PTAFR in primary human retinal microvascular endothelial cells and further investigate the mechanism of localization and the function of nuclear PTAFR in detail using the methods described in the chapter- 3.

CHAPTER 3

METHODS (PAPER- 1)

Chapter- 3 Title- High Resolution Imaging and Function of Nuclear G Protein-Coupled Receptors (GPCRs)

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Abstract

The traditional view of G protein-coupled receptors (GPCRs) being inactivated upon their internalization has been repeatedly challenged in recent years. GPCRs, in addition to forming the largest family of cell surface receptors, can also be found on intracellular membranes such as nuclear membranes. Since the first experimental evidence of GPCRs at the nucleus in the early 1990s, approximately 30 different GPCRs have been localized at the nucleus by independent research groups, including ours. In this chapter, we describe several techniques commonly used for immuno-detection of nuclear GPCRs focusing on subcellular fractionation of proteins based on their localization and transmission electron microscopy (TEM) using primary cultured cells as well as tissue sections. We also describe the use of confocal microscopy to study nuclear calcium currents, which can further affect downstream events such as gene transcription, nuclear envelope breakdown, or its reconstruction and nucleocytoplasmic protein transport.

Key words- Nucleus, G protein-coupled receptor, Protein fractionation, Electron microscopy, Nuclear calcium currents

3.1 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors (1). Although an oversimplification, heterotrimeric G proteins have been classically viewed as a link between GPCRs at plasma membrane (PM) and intracellular signaling events (2). Rubins et al. (3) first identified G proteins in the nuclear envelop of isolated rat liver nuclei. It has been also reported that G proteins can translocate to the nucleus in response to the receptor stimulation by specific growth factors and can control cellular events such as mitosis (4).

Some of the earliest evidence for a nuclear localization of a GPCR was provided by Lind and Cavanagh (5, 6) using radioligand binding studies, which detected the presence of muscarinic cholinergic receptors in isolated nuclei from rabbit corneal and Chinese Hamster Ovary (CHO-K1) cells. Bhattacharya et al. (7) first reported the presence of functional prostaglandin E (EP) receptors in isolated nuclei from a variety of tissues. Different subtypes of EP receptors (EP_1, EP_2, and EP_4) were detected in nuclear membranes using multiple approaches from primary cells, including piglet brain microvascular endothelial cells (PBMEC). Later, Gobeil et al. (8) reported that stimulation of isolated nuclei expressing the aforementioned EP receptors by prostaglandin analogs resulted in pertussis toxin-sensitive, G protein-mediated nuclear calcium signals regulating specific transcription of genes such as endothelial nitric oxide synthase (eNOS). These results provided a plausible mechanism for the contribution of prostaglandins (PGs), acting intracellularly, in local control of cerebral blood flow and oxygen delivery to neuronal tissues, critical during development (8).

Over the last decade, approximately 30 different GPCRs have been detected at nuclear membranes by independent research groups, including ours (9-11). In some cases, the cellular machinery required for production of the GPCR agonist is already present at the

nucleus. For example, enzymes such as cytosolic phospholipase A_2 ($cPLA_2$) (12), cyclooxygenase-1/2 (COX-1/2) (13), and some types of prostaglandin E synthases (PGE synthases) (13) have been detected at the nucleus. More and more GPCRs have been found to be functional at the nucleus via signaling mechanisms which are independent from those of cognate plasma membrane receptors (8, 14, 15). More recently, receptors of tyrosine kinase (RTK) family (e.g., vascular endothelial growth factor receptor type-2; VEGFR2) have also been shown to translocate to the nucleus (16, 17). VEGFR2 directly interacts with several transcription factors including Sp1 to regulate gene expression (16). On the other hand, nuclear GPCRs like the type-1 lysophosphatidic acid receptor (LPA1) (18) and β-adrenergic receptor (βAR) (19) can regulate gene expression by modulating cardiovascular nitric oxide signaling. Possible mechanisms for delivery of GPCRs and other receptors to the nuclear membrane are shown in Fig. 1.

Diverse types of primary cells (e.g., hepatocytes, cardiomyocytes, vascular endothelial cells), cancer cell lines, and stably transfected cell lines (e.g., human embryonic kidney cells) have been used to demonstrate nuclear localization of GPCRs. Common experimental approaches to localize receptors include protein fractionation according to subcellular localization, transmission electron microscopy (TEM) using primary cultured cells as well as using tissue sections and confocal microscopy (7, 8, 15, 20). The latter can be utilized to confirm nuclear localization as well as to study nuclear calcium signaling. A brief rationale for each method is given below.

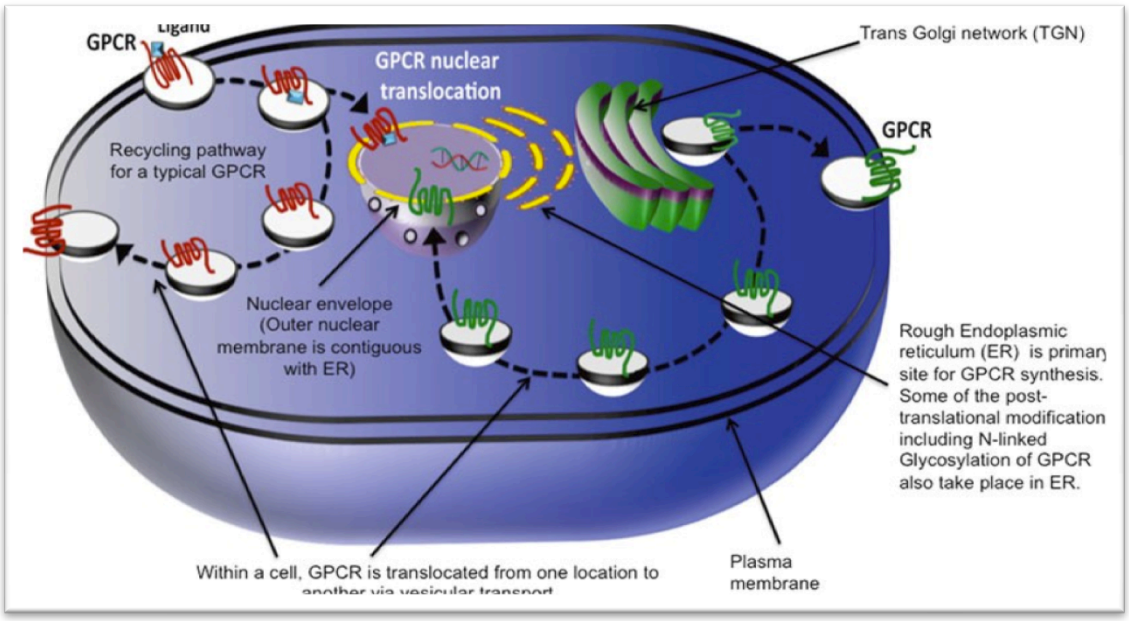

Fig. 1 Simplified schematic to show different pathways for nuclear translocation of a G protein-coupled receptor (GPCR). Little is currently known about mechanism of nuclear GPCR delivery. Different hypotheses are proposed (9-11). Some propose that specific sequence motifs such as nuclear localization signal (NLS) might be responsible. In other cases, nuclear translocation might take place following agonist-induced internalization of the receptor from plasma membrane. The latter was recently reported to be the case (16) for a receptor tyrosine kinase (RTK) "vascular endothelial growth factor receptor type-2 (VEGFR2)". This mechanism might also be relevant for nuclear translocation of some, if not all, GPCRs.

3.1.1 Subcellular Fractionation of Proteins from Cultured Cells to Study Nuclear Localization of GPCRs

Subcellular fractionation of cellular proteins, using either detergents (21) or ultracentrifugation (22), is commonly used to confirm their localization in different cellular compartments. However, isolation of nuclear membrane proteins without contamination from peri-nuclear organelles (e.g., endoplasmic reticulum, mitochondria) still remains a practical challenge (23). We describe a simple protocol to isolate nuclear fractions with reasonable purity (see Note 1a, b). The method described in Subheading 3.1 is based on the use of a hypotonic/Nonidet P-40 lysis buffer, which was originally described in ref. (24) and modified in refs. (8, 15) for CHO-K1 cells and piglet brain microvascular endothelial cells (PBMEC).

3.1.2 Using Transmission Electron Microscopy (TEM) to Confirm Nuclear Localization of a GPCR in Primary Cultured Cells

Subcellular fractionation is relatively simple but the approach is not without limitations. Impurities (from surrounding organelles) in pelleted fractions (including the nuclear fraction) are often present. The homogenization step (see Subheading 3.1, step 6) requires special care to preserve nuclear integrity without compromising purity. Transmission electron microscopy provides a practical alternative to confirm nuclear localization for GPCRs of interest (7, 15, 18). However, there is limited literature available on preparation of primary cell culture samples for TEM (25, 26). The protocol described below (Subheading 3.2) is a modified version of the protocol originally described in ref. (18) using instructions supplied by manufacturer of the Nanogold® antibody conjugate (Nanoprobes Inc.) (27, 28).

3.1.3 Using TEM to Study the Nuclear Localization of a GPCR in Tissue Sections

The ultrastructural demonstration of nuclear GPCR localization in intact cells under normal physiological conditions is particularly important. We performed such a study to demonstrate nuclear localization of platelet-activating factor (PAF) receptor in nuclear membranes of microvascular endothelial cells and in porcine brain sections (15). The protocol below (Subheading 3.3) represents an updated version of the protocol used in the aforementioned publication and can be adapted with minor modifications to other tissues and animal species. It has been used by one of us to study the nuclear localization of another GPCR (29). The advantage of the pre-embedding silver-intensified immunogold protocol we propose is that it is very easy to reproduce.

3.1.4 Identification of Functions and Signaling of Nuclear GPCR in Cell-Free Nuclei

The functional relevance of GPCR immunoreactivity identified at nuclear sites can be evaluated in terms of modulatory role of nuclear GPCRs on nucleoplasmic calcium homeostasis and ensuing effects on gene transcription, as highlighted by several studies including ours (7, 8, 10, 18, 30). The details for determination of gene transcripts elicited upon stimulation of nuclei with GPCR ligands, for which nuclei contain respective receptors, are presented in a sister chapter by Vaniotis et al. Ligand occupation of plasma membrane GPCRs, which are coupled mainly to heterotrimeric Gi and Gq proteins, results in activation of one or more members of phospho- lipase C (PLC) class of enzymes, and subsequently, in transient augmentation of Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) production. This is followed by transient elevations in the concentration of free intracellular calcium. All of these signal transducers also reside in the nucleus (9-11).

The protocol described in Subheading 3.4 is based on the use of laser scanning confocal microscopy to monitor spatiotemporal movements of nuclear Ca2+ within a single nucleus upon GPCR stimulation by diverse ligands including lipids (e.g., PGs, LPA) (8, 30), amino acids (e.g., glutamate) (14), and peptides (e.g., bradykinin) (31). This protocol has been tested in isolated nuclei from rat hepatocytes.

3.2 Materials

3.2.1 Subcellular Fractionation of Proteins from Cultured Cells to Study Nuclear Localization of GPCRs

1. Culture medium for Chinese Hamster Ovary (CHO-K1) cells - Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with fetal bovine serum (FBS) (final concentration: 10 %) and penicillin-streptomycin (P/S) (final concentration: 100 I.U./mL penicillin and 100 μg/mL streptomycin) added.

2. Culture medium for piglet brain microvascular endothelial cells (PBMEC) - Endothelial Cell Medium (ECM) with endothelial cell growth supplement (ECGS) (ScienceCell™ Research Laboratories), FBS (final concentration: 5 %), and P/S (final concentration: 100 I.U./mL penicillin and 100 μg/mL streptomycin) added.

3. Lysis buffer - 10 mM Trizma-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2 in distilled water (dH_2O). Store at 4 °C for up to 1 month.

4. Complete, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics) (see Note 1d). Store at 4 °C.

5. Nonidet P-40 alternative (EMD biosciences, Inc). Store at room temperature.

6. Dounce tissue grinder set (volume: 7 mL).

7. Primary antibodies for immunoblot analysis in (Fig. 2).

(a) Anti-calnexin antibody (Abcam®).

(b) Anti-pan-cadherin antibody (EMD Millipore Corp).

(c) Anti-lamin-B receptor antibody (Epitomics Inc., An Abcam® Company).

(d) Anti-platelet activating factor receptor antibody (Cayman Chemical Company).

(e) Anti-β-actin antibody (Santa Cruz Biotechnology Inc.).

3.2.2 Using Transmission Electron Microscopy to Confirm Nuclear GPCR Localization in Primary Cultured Cells

1. Lab-Tek® chamber slides (Thermo Fischer Scientific) - It is important to use slides chambers with plastic bottoms (Permanox®), not glass, for electron microscopy.

2. Culture medium for piglet brain microvascular endothelial cells (PBMEC) - Endothelial Cell Medium (ECM) with endothelial cell growth supplement (ECGS) (ScienceCell™ Research Laboratories), FBS (final concentration: 5 %), and P/S (final concentration: 100 I.U./mL penicillin and 100 μg/mL streptomycin) added.

3. The recipe to make 100 mM (0.1 M) sodium phosphate buffer (PB) (32):

Add 3.1 g of $NaH_2PO_4 \cdot H_2O$ and 10.9 g of Na_2HPO_4 (anhydrous) to distilled H_2O to make final volume of 1 L. The pH of the final solution will be 7.4 at room temperature.

4. Fixation Buffer for first fixation (Subheading 3.2, step 4) - 4 % paraformaldehyde, 0.25 % glutaraldehyde, 50 mM sucrose, 0.4 mM $CaCl_2$ in 0.1 M sodium phosphate buffer (pH 7.4).

5. Use heat shock-treated bovine serum albumin (fraction V) (Thermo Fischer Scientific) to prepare all solutions containing bovine serum albumin (BSA).

6. Washing Buffer (Subheading 3.2, steps 14, 15, and 18) - To prepare the washing buffer, mix 100 mL of 0.01 M PBS with 0.5 g fish gelatin and 0.8 g of BSA and adjust final pH to 7.4.

7. The secondary antibody used in this protocol is the Nanogold®- Fab' (Nanoprobes Inc.) fragment of goat anti-rabbit (host for primary antibody) IgG. The final Fab' concentration is 80 μg/ mL. Store Nanogold®-Fab' at 4 °C (do not freeze). The concentrations provided for primary and secondary antibodies are for reference only. Final concentrations must be determined for each experiment.

8. Nanoprobes HQ SILVER™ kit (Nanoprobes Inc.) for silver intensification (28). Store at − 20 °C.

9. Use a digital camera and imaging software to acquire the images.

3.2.3 Using TEM to Study the Nuclear GPCR Localization in Tissue Sections

1. Perfusion mixture (Subheading 3.3, step 1) - 3 % paraformaldehyde, 0.1 % glutaraldehyde, and 15 % picric acid (v/v) in 0.1 M sodium phosphate buffer (PB) pH 7.4.

2. Use heat shock-treated bovine serum albumin (fraction V) (Thermo Fischer Scientific) to prepare all solutions containing bovine serum albumin (BSA).

3. Washing Buffer (Subheading 3.3, steps 9–11) - To prepare the washing buffer, mix 100 mL of 0.01 M PBS with 0.5 g of fish gelatin and 0.8 g of BSA and adjust final pH to 7.4.

4. The secondary antibody used in this protocol is Nanogold®- Fab' (Nanoprobes Inc.) fragment of goat anti-rabbit (host for primary antibody) IgG. The final Fab' concentration

is 80 μg/mL. Store Fab'-Nanogold® at 4 °C (do not freeze). The concentrations provided for primary and secondary antibodies are for reference. Final concentrations must be determined for each experiment.

5. Nanoprobes HQ SILVER™ kit (Nanoprobes Inc.) for silver intensification (28). Store at −20 °C.

6. Use a digital camera and imaging software to acquire the images.

3.2.4 Identification of Functions and Signaling of Nuclear GPCR in Cell-Free Nuclei

1. The fluorescent calcium indicator Fluo-4AM (Molecular Probes; now part of Life Technologies) and fluorescent green nucleic acid dye SYTO-11 (Molecular Probes).

2. Glass coverslips for confocal fluorescence microscopy (Circles No. 1, 25 mm diameter, 0.13 - 0.17 mm thickness, Thermo Fischer Scientific).

3. Incubation buffer - 25 mM HEPES pH 7.2, 125 mM KCl, 4 mM MgCl2, 2 mM K2HPO4, 400 nM CaCl2, and 0.5 mM ATP.

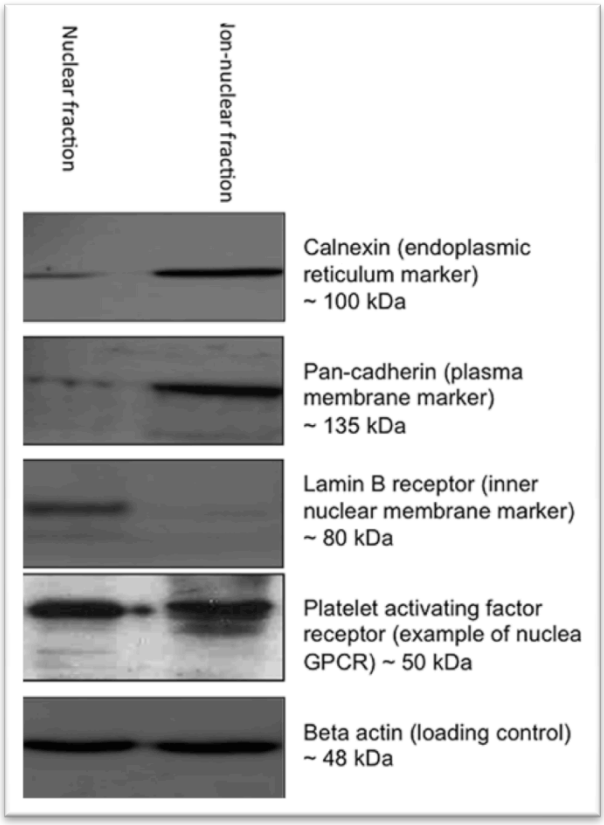

Fig. 2 Purity of nuclear and non-nuclear fractions was studied in subcellular fractions of piglet brain microvascular endothelial cells (PBMECs) analyzed for organelle-specific protein markers. Both nuclear and non-nuclear fractions were subjected to SDS-PAGE (9% acrylamide) electrophoresis followed by immunoblot analysis. Lane-1 corresponds to the nuclear fraction and lane-2 to the non-nuclear fraction, which includes plasma membrane and cytosolic proteins. **(1)** Calnexin is a marker associated with endoplasmic reticulum (ER) membrane. **(2)** Cadherins are single chain glycoprotein receptors expressed at plasma membrane in tissue-specific manner. **(3)** Lamin B receptor (LBR) is an integral membrane protein associated with inner nuclear membrane. **(4)** Platelet activating factor receptor (PAFR) is an example of a GPCR found at the plasma membrane as well as at the nuclear membrane in piglet brain microvascular endothelial cells (PBMEC) (15). **(5)** β-actin was used as loading control since it is found in both cytoplasm as well as nucleoplasm (33).

3.3 Methods

3.3.1 Subcellular Fractionation of Proteins from Cultured Cells to Study Nuclear Localization of GPCRs

1. Cell Culture - Seed cells of interest at initial density of 100,000 cells per 10 cm plate (see Note 1c). Grow them to an approximately 80 % confluence. The protocol below uses ≈ 50 × 106 cells as a starting material. Starve the cells for 4 h in the appropriate basal medium lacking in growth supplements and fetal bovine serum (FBS).

2. All the following steps are carried on ice unless mentioned otherwise.

3. Wash cells with ice-cold PBS and gently scrape them into 15 mL tubes.

4. Pellet the cells at 500 × g for 5 min at 4 °C.

5. Resuspend the cell pellet in 2 mL Lysis buffer with complete (EDTA-free) protease inhibitors (see Note 1d).

6. Homogenize the suspension with approximately 100 gentle strokes (or for 20 min) with Dounce tissue grinder on ice (see Note 1e).

7. Centrifuge the homogenized suspension from Subheading 3.1, step 6 at 600 × g for 10 min at 4 °C. Store the supernatant (non-nuclear fraction) at − 20 °C or refer to Note 1f for further processing.

8. Resuspend the nuclear pellet from Subheading 3.1, step 7 in 2 mL Lysis buffer containing 0.1 % (v/v) Nonidet P-40 Alternative (see Note 1g) and protease inhibitors.

9. Leave suspension from Subheading 3.1, step 8 on ice for 10 min and sediment the pellet at 600 × g for 10 min at 4 °C.

10. Wash nuclear pellet with 10 mL lysis buffer containing 0.1 % (v/v) NP-40 Alternative and protease inhibitors for three times.

11. Leave suspension from Subheading 3.1, step 10 on ice for 10 min, followed by sedimentation of nuclear pellet at 600 × g for 10 min at 4 °C after each wash.

12. At the end of the washes, resuspend final nuclear pellet in 500 μL of lysis buffer with 0.1 % (v/v) Nonidet P-40 Alternative and protease inhibitors.

13. Store both protein fractions (nuclear and non-nuclear) at − 80 °C for long term.

3.3.2 Using Transmission Electron Microscopy to Confirm Nuclear GPCR Localization in Primary Cultured Cells

1. Grow cells in Lab-Tek® chamber slides until they are about 80 % confluent (see Note 2a).

2. Wash cells twice with pre-warmed PBS (5 min per wash).

3. Following steps are done on a shaker (low speed).

4. First Fixation - Fix cells at room temperature (RT) for 30 min in fixation buffer (see Note 2b, c).

5. Wash cells twice with 1 % BSA in PBS (5 min per wash).

6. Treat with 1 % sodium borohydride in PBS for 30 min (see Note 2d).

7. Wash with PBS until bubbles completely disappear (usually four or five 10 min washes).

8. Block for 1 h with 1 % BSA in PBS.

9. Wash with PBS twice for 5 min each.

10. Permeabilize the cells for 15 min with 0.2 % Triton X-100 and 1 % BSA in PBS at RT.

11. Prepare primary antibody dilutions (10× more concentrated than used for confocal microscopy) in 1 % BSA in PBS.

12. Incubate primary antibody overnight at 4 °C in a humid chamber (see Note 2e). Do not forget negative control (no primary antibody) for each condition.

13. Wash with PBS three times for 10 min each.

14. Block for 15 min with Washing buffer.

15. Prepare gold-conjugated IgG dilution (1:500 as starting dilution) in Washing buffer.

16. Incubate chamber slides with the secondary antibody over- night at 4 °C in humid chamber. Avoid contact of chamber slides with any metal following secondary antibody incubation, as it will interfere with silver intensification (Subheading 3.2, step 22).

17. Remove Silver intensification kit (Nanoprobes HQ Silver™) from the freezer. It has to be at room temperature before use.

18. Wash chamber slides with Washing buffer for three times (1 min per wash).

19. Second Fixation—Fix cells at RT for 10 min in 1 % glutaraldehyde in PBS.

20. Wash with distilled water (dH_2O) twice (1 min per wash).

21. Silver Intensification (with Nanoprobes HQ Silver™): combine reagents A (initiator) + B (moderator) (mix well, viscous) then add C (activator) at a ratio of 1:1:1 just prior to use (see Note 2f).

22. Put 500 μL of reagent mixture into each chamber in the dark room.

23. Incubate for 8 min. Lightly shake the chamber slides during the incubation.

24. As the solution is very viscous, at the end of 8 min, try to take some solution out and then flush with dH2O. Remove all the water immediately.

25. Wash twice with dH2O for 5 min each.

26. Wash once with 0.1 M phosphate buffer, pH 7.4 for 5 min.

27. Incubate for 1 h in 1 % osmium tetroxide in 0.1 M phosphate buffer.

28. To dehydrate the cells:

    10 min wash with 30 % ethanol.

    10 min wash with 50 % ethanol.

    10 min wash with 70 % ethanol.

    10 min wash with 90 % ethanol.

    10 min wash with 95 % ethanol.

    10 min wash with 100 % ethanol (three times).

29. Place in Epon: 100 % ethanol mixture in 1:1 ratio for 1 h.

30. Place in Epon: 100 % ethanol mixture in 2:1 ratio for 1 h.

31. Place in Epon: 100 % ethanol mixture in 3:1 ratio for 1 h.

32. Embed in pure Epon for 1 h.

33. Cure in oven at 60 °C for 48 h.

34. Remove the embedded cells from the Lab-Tek® chamber slide by peeling off the slide and then breaking off the sides of the chamber.

35. Obtain ultrathin sections in an ultramicrotome equipped with a diamond knife. Collect them on single-slot Formvar coated grids, contrast stain them with 4 % uranyl acetate in water and Reynold's lead citrate, and observe under the TEM.

3.3.3 Using TEM to Study the Nuclear Localization of a GPCR in Tissue Sections

1. Animals are anesthetized with sodium pentobarbital (100 mg/kg) and then perfused through the left ventricle briefly with saline followed by with the perfusion mixture (refer to Subheading 2.3, item 1 for preparation) for 30 min, followed by with the perfusion mixture without glutaraldehyde for further 30 min and, finally, with 10 % sucrose in 0.1 M phosphate buffer for 30 min as well (see Note 3a).

2. The tissue of interest (e.g., cerebral cortex) is removed from the animal. The region of interest trimmed with a knife in thick slices (or blocks) with a thickness of 5 mm or less and placed overnight in 30 % sucrose in 0.1 M phosphate buffer at 4 °C.

3. Freeze-thaw the tissue by direct immersion in liquid nitrogen for 30 s and quickly thawing it in 0.1 M phosphate buffer at room temperature. For this, the tissue is placed in a customized small ladle with tiny holes drilled in it.

4. Cut 50 μm thick sections using a vibratome and collect them into a tissue culture multi-well plate (24 wells, BD Falcon). The wells should be pre-filled with PBS (do not use Triton) (see Note 3b).

5. Replace PBS in the wells by a solution of 1 % sodium borohydride ($NaBH_4$) in PBS. After 30 min, wash extensively in PBS until all bubbling disappears (usually four to five 10 min washes).

6. Place sections for 30 min in PBS with 0.5 % BSA.

7. Incubate sections overnight at 4 °C in the primary antibody diluted in PBS with 0.1 % BSA, on a shaker (gentle shaking).

8. Wash sections 3 × 10 min each with PBS.

9. Block with washing buffer for 1 h.

10. Incubate sections overnight at 4 °C, with gentle shaking, in a gold-conjugated Goat anti-rabbit antibody, diluted 1:200 in Washing buffer (see Note 3c).

11. Wash sections thrice (1 min each) with Washing buffer, followed by three washes (1 min each) with PBS.

12. Place in each well 250 μL of 1 % glutaraldehyde for 10 min.

13. Wash once for 1 min in dH2O.

14. Move sections to properly labelled glass scintillation vials containing dH2O.

15. After a 1 min rinse, it is time to carry out the silver intensification. Use Nanoprobes HQ Silver™ kit: mix reagents A (initiator) + B (moderator) (mix well, viscous) then + C (activator) in a 1:1:1 ratio just prior to use (at the end of the glutaraldehyde incubation is a good time). Put 750–900 μL of mixture in each vial with 1 mL syringe (caution: this reagent is light sensitive, carry it in a room lit only by dim incandescent light). Lightly shake the vials to move sections around or put the vials in a rack on a nutating mixer covered with foil. Total incubation time: 8 min. As this solution is very viscous, at the end of the

8 min try to take some solution out, flush the vials with water, and remove it immediately.

16. Wash 2× (5 min each) with dH2O and then for 5 min with 0.1 M PB.

17. Incubate for 1 h in 1 % osmium tetroxide in 0.1 M PB.

18. Start dehydration of the sections:

5 min wash in 50 % ethanol.

5 min wash in 70 % ethanol.

5 min wash in 90 % ethanol.

5 min wash in 95 % ethanol.

2 × 10 min wash in 100 % ethanol.

10 min wash in propylene oxide.

2 h in Epon: propylene oxide mixture in 1:1 ratio.

2 h in Epon: propylene oxide mixture in 2:1 ratio. Immerse in pure Epon for 2 h.

19. Flat embed sections in pure Epon on thick acetate foil taped to a plastic or metal plate, using plastic coverslips. Use wood applicator sticks to remove sections from vials and do not apply pressure on the coverslip to avoid damaging the tissue.

20. Cure sections in oven at 60 °C overnight.

21. Remove material from oven, label coverslips (while still attached to the acetate sheet) with a permanent pen on the coverslip side (not on the Epon side), and detach the coverslips (with the Epon and sections attached) from the acetate foil using a razor blade. Store them in small cardboard boxes.

22. Select relevant fields for EM study with a light microscope. Photograph as needed.

23. Re-embed selected fields in Epon. For that, trim the plastic coverslip/Epon to the inside diameter the plastic covers on the base of plastic capsules whose tops have been cut out. Close the covers to hold the plastic coverslip/Epon containing the relevant field in

place. For best results the plastic coverslip should not be removed and should face the plastic on the bottom of the capsule (see Note 3d).

24. Fill the capsule with Epon and cure at 60 °C for 48 h (do not forget to insert a label).

25. Trim the blocks and obtain ultrathin sections in an ultramicrotome equipped with a diamond knife, collect them on single- slot Formvar coated grids, contrast stain them with 4 % uranyl acetate in water and Reynold's lead citrate, and observe under the TEM.

3.3.4 Identification of Functions and Signaling of Nuclear GPCR in Cell-Free Nuclei

1. Load suspension of nuclei with Fluo-4AM (30 μM) for 45 min at room temperature. Wrap tubes in aluminum foil to protect from light.

2. Dilute the nuclear suspension with (20×) excess volume of incubation buffer (see Subheading 2.4, item 3).

3. Spin down at 700 × g for 5 min at room temperature.

4. Discard the supernatant and resuspend in the same buffer.

5. Gently place 10 μL aliquot of nuclear suspension (about 250,000 nuclei/assay) into custom-made chamber (designed for imaging in aqueous solutions) containing 500 μL of incubation buffer.

6. Allow 5–10 min to sediment nuclei onto the glass coverslips forming the bottom of the chamber.

7. Analyze nucleoplasmic calcium signals in single isolated nuclei in a rapid scan mode by using a multi-probe laser scanning confocal system (Bio-Rad) equipped with an inverse phase epifluorescence microscope (Nikon Eclipse TE300) and a 60× Nikon Oil Plan achromat objective (see Note 4a).

8. Use an injection volume of 10 μL of agonists (or other agents) to limit fluid disturbance so as to avoid displacement of nuclei during the confocal analysis (see Note 4b).

9. Identification and delineation of the nucleus can be accomplished at the end of experiments with a nucleic acid fluorescent dye (i.e., Syto-11, 100 nM) (see Note 4c, d).

3.4 Notes

3.4.1 Subcellular Fractionation of Proteins from Cultured Cells to Study Nuclear Localization of GPCRs

(a) The purity of nuclear fraction can be confirmed by light microscopy using Trypan blue staining (>90 % intact nuclei) or by electron microscopy.

(b) Western blotting results of nuclear and non-nuclear fractions using organelle-specific protein markers are shown in Fig. 2.

(c) Use at least five to eight 10 cm plates per condition to obtain enough amount of nuclear protein.

(d) One protease inhibitor complete cocktail tablet is used to prepare 50 mL of lysis buffer. Alternatively, to prepare aliquots (25×), dissolve one tablet in 2 mL of distilled water, aliquot, and store at −20 °C.

(e) Alternatively, the suspension from Subheading 3.1, step 5 can be homogenized using a 2 mL syringe and 23 G needle (with approximately 50 strokes) on ice.

(f) The supernatant from Subheading 3.1, step 6 is the non- nuclear fraction and can be subjected to ultracentrifugation to obtain mitochondrial and microsomal fractions as described in ref. (21).

(g) Nonidet P-40 Alternative is a nonionic, non-denaturing detergent used for isolation of functional protein complexes from nuclei. The optimal concentration of Nonidet P-40 Alternative might need to be adjusted (range - 0.1 – 0.5 % v/v) depending on the cell type (8, 15, 24).

3.4.2 Using Transmission Electron Microscopy to Confirm Nuclear GPCR Localization in Primary Cultured Cells

(a) It is important to make sure cells are not more than 80 % confluent; otherwise they will detach during subsequent treatment.

(b) First fixation step is critical to ensure cellular ultrastructure (including integrity of biomembranes) is maintained during immunolabeling and embedding.

(c) Occasionally, we have used 4 % paraformaldehyde without glutaraldehyde, but the fixation quality is less satisfactory. In this case only, Subheading 3.2, steps 6 and 7 can be omitted.

(d) Sodium borohydride ($NaBH_4$) is used to quench the remaining free aldehyde groups following glutaraldehyde fixation. Ammonium chloride (50 mM) or glycine (50 mM) in PBS (pH 7.4) can be used in place of $NaBH_4$.

(e) Wrap paraffin film around Lab-Tek® chamber slides before placing them in humid chamber. A conventional slide box with wet brown papers placed inside can be used as humid chamber.

(f) The reagents used for silver intensification are light sensitive. Mix them in dark room with a small incandescent light just prior to use. At the end of second fixation is a good time.

3.4.3 Using Transmission Electron Microscopy to Study the Nuclear GPCR Localization in Tissue Sections

(a) For better morphological preservation, animals can be perfused instead with a mixture of 4 % paraformaldehyde and 0.5 % glutaraldehyde in 0.1 M PB, followed by the same mixture without glutaraldehyde and finally with 10 % sucrose in 0.1 M PB (each perfusion step lasting for 30 min). However, some antibodies against GPCRs may not give sufficient signal with this second fixation protocol (Fig. 3).

(b) The number of sections placed in each well should not be excessive to prevent section overlap that will compromise exposure to reagents.

(c) Avoid contact of sections with metal following secondary antibody incubation, as it will interfere with silver intensification step.

(d) Do not re-embed with the plastic coverslip facing up because the specimen can detach easily from the block during pyramid trimming or ultramicrotomy.

3.4.4 Identification of Function and Signaling of Nuclear GPCRs in Cell-Free Nuclei

(a) Keep image acquisition settings constant (e.g., laser line intensity, photometric gain, filter attenuation) throughout the experiment.

(b) Ensure that the baseline fluorescence signal is relatively stable prior to the stimulation of nuclei. This can be done by performing short scans. There should be no probe leakage during the experiment.

(c) Intensity of fluorescence of the calcium-Fluo-4 complex can be converted into absolute calcium concentration as described in ref. (34).

(d) Application of ionomycin (20 μM) and calcium (1 mM) mixture to the chamber can be done as a positive control at the end of experiments to measure maximal fluorescence intensity attainable corresponding to the levels of active (de-esterified) form of the Fluo-4AM calcium indicator.

(e) Activation of mitogen-activated protein kinases (MAPK) by nuclear GPCR can be evaluated as previously described in refs. (18, 30). In brief, rat-liver derived nuclei (50 μg of proteins) are resuspended in aforementioned buffer (see Subheading 2.4, item 3) for calcium signal assay. In concomitant experiments, nuclear suspensions are pretreated with or without various pharmacological inhibitors of phosphatidylinositide 3-kinases (PI3K) prior to agonist- induced stimulation of GPCR of interest. Collected protein samples can be subjected to SDS-PAGE electrophoresis on a 9 % gel and immunoblotted for phospho-ERK1/2 (extracellular-signal-regulated kinases).

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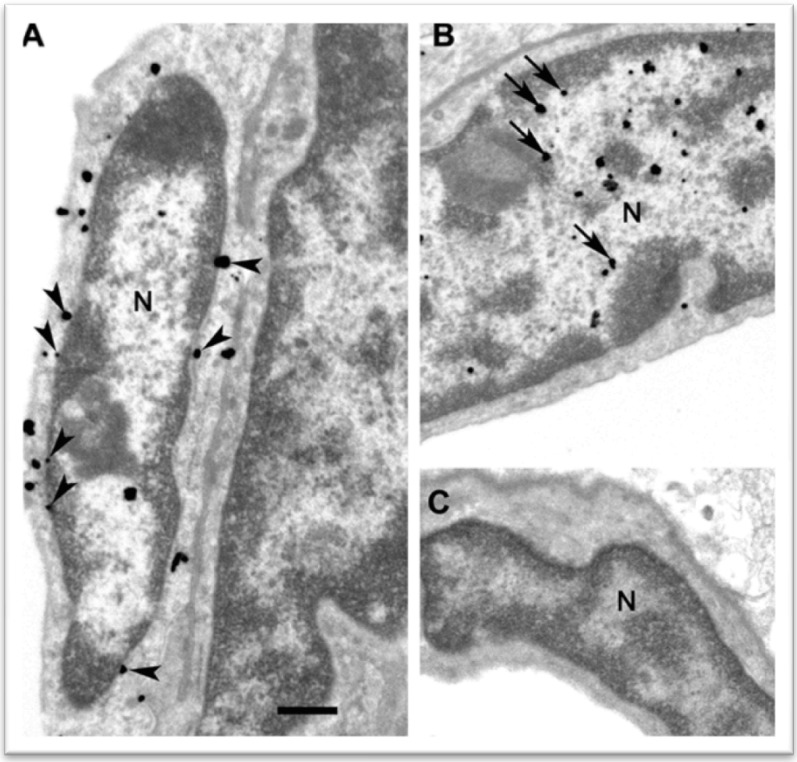

Fig. 3 Immunogold labeling for PAFR in microvascular endothelial cells from porcine cerebral cortex sections, obtained using a protocol similar to the one described in Subheading 3.3. In panel (a), note the localization of silver-gold grains over the nuclear membrane (arrowheads), which represent PAFR antigenic sites. In panel (b), note the predominantly intranuclear labeling in this endothelial cell (arrows indicate silver-gold grains). In panel (c), from material stained with the anti-PAFR antibody pre-adsorbed with the cognate peptide; note the complete absence of immunostaining. N-endothelial cell nucleus. Scale bar for all images = 0.5 μm

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CHAPTER 4

RESULTS (PAPER- 2)

Chapter- 4 Nuclear localization of platelet-activating factor receptor controls retinal neovascularization

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Abstract:

Platelet-activating factor (PAF) is a pleiotropic phospholipid with pro-inflammatory, pro-coagulant, and angiogenic actions on the vasculature. We and others have reported presence of PAF receptor (Ptafr) at intracellular sites such as nucleus. However, mechanism of localization and physiologic functions of intracellular Ptafr remain poorly understood. We hereby identify importance of c-terminus of the receptor and uncover novel roles of Rab11a GTPase and importin-5 in nuclear translocation of Ptafr in primary human microvascular endothelial cells. Nuclear localization of Ptafr is independent of PAF stimulation and biosynthesis. Moreover, nuclear Ptafr is responsible for upregulation of unique set of growth factors, including vascular endothelial growth factor, *in vitro* and *ex vivo*. We further corroborate intracrine PAF signalling, resulting in angiogenesis *in vivo*, using Ptafr antagonists with distinct membrane permeability. Collectively, our findings show that nuclear Ptafr translocates in an agonist-independent manner, and distinctive functions of Ptafr based on its cellular localization point to another dimension needed for pharmacologic selectivity of drugs.

4.1 Introduction:

PAF (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine), a pro-inflammatory phospholipid, was first discovered in 1960s for its role in platelet aggregation and release of histamine by the activated platelets (1). Since then, PAF and PAF-like lipids have been shown to perform diverse functions in allergic and inflammatory processes, including airway hyper-responsiveness (2, 3), bacterial septicemia (4, 5), and anaphylaxis (6) through their actions on cardiovascular and immune systems (7). At a cellular level, both PAF and PAF-like lipids (collectively referred to as "PAF" in rest of the paper) activate a single G-protein coupled receptor (GPCR) viz. platelet-activating factor receptor (Ptafr) which is abundantly expressed in endothelial cells (ECs), platelets, neutrophils, monocytes, macrophages and various smooth muscle cells (8). The biosynthesis of PAF in ECs is triggered by various stimuli like thrombin, angiotensin-II, vascular endothelial growth factor (VEGF), vasopressin, and interleukin-1 (IL-1) (9). Some newly synthesized PAF by ECs is released into the circulation but most remains intracellular, which hints towards its possible role as an intracrine signaling molecule (10). The biological actions of PAF are short-lived (plasma half-life < 15 minutes (6)) as it is rapidly inactivated by different circulating and intracellular forms of PAF acetylhydrolase (11, 12). Nonetheless, PAF is known to produce potent vasoconstrictor, vasodilator (13, 14), hyper-permeability (15), vaso-obliterative (16) and conversely angiogenic (17, 18) effects on the vasculature.

The GPCRs, such as Ptafr, were long believed to signal by binding to their ligands exclusively at the cell surface. This notion has been challenged in recent years by discovery of functional, intracellular GPCRs in the endosomes (19), at the mitochondria

(20) and at the nucleus (21, 22). So far, more than 30 different GPCRs have been detected at nuclei of endogenous cells and tissues (23, 24). Our group first reported presence of Ptafr at the nuclei of piglet brain microvascular endothelial cells using transmission electron microscopy (TEM) and further demonstrated that the receptor is functional *in vitro* using purified EC nuclei (25). But the mechanism of nuclear translocation of Ptafr, like most nuclear GPCRs, remains an unsolved mystery so far. All members of GPCR family contain seven hydrophobic transmembrane domains, which necessitates the use of vesicular transport for their translocation between membranous subcellular organelles through mostly aqueous intracellular environment. After the vesicle containing a GPCR reaches its final destination, it has to fuse to the membrane of target organelle. The current evidence suggests that members of Ras superfamily of small GTPases, especially those in Rab and Arf families, are involved in regulation of various stages of the vesicular transport as well as in the process of membrane fusion (26, 27). Another conserved eukaryotic protein family, Importin (part of karyopherin superfamily), has been recently proposed to play a role in nuclear translocation of GPCRs based on the evidence from RNA interference studies (21, 22). Interestingly, importins recognize their cargo by presence of nuclear localization signal (NLS) (28) and many GPCRs, including Ptafr, contain putative NLS (29). Therefore, we hypothesized that nuclear translocation of Ptafr in vascular ECs is governed by specific small GTPase and importin interaction.

The GPCRs, including Ptafr, are known to signal via heterotrimeric G-protein dependent (30) or independent (31) pathways (25, 32). Many components of both signaling pathways such as G-proteins (33), β-arrestin1 (34), several GPCR kinases (35) have already been detected at the nucleus. Additionally, in the last decade, increasing studies have shown that nuclear GPCRs can perform specific functions in cultured cells

(21, 22, 36, 37). However, *in vivo* proof to substantiate these claims remains sparse. It was only recently uncovered that a GPCR, F2rl1 (previously known as Par2), has opposing actions depending on its localization in rat retinal ganglion cells (21); in this case nuclear F2rl1 originates from the plasma membrane, whereas the origin of nuclear Ptafr is not established but does not seem to augment upon cell surface stimulation with PAF (38). We therefore proceeded to elucidate (1) the cellular mechanisms implicated in nuclear localization of Ptafr, (2) the motifs on the receptor essential for this latter function, and (3) to determine if Ptafr at different sub-cellular locations results in regulation of distinct genes which in turn translate into the different *in vivo* vascular functions of PAF using a model of proliefrative ischemic retinopathy.

4.2 Results:

4.2.1 Localization of Ptafr at the nucleus is cell-type specific

Cellular localization of Ptafr was studied in endogenous and stable-transfected cells using multiple approaches, specifically subcellular fractionation, confocal microscopy, and transmission electron microscopy (TEM) (39). The specificities of primary anti-Ptafr and secondary nanogold antibodies were confirmed in endogenous human retinal microvascular endothelial cells (hRMEC) as well as in transfected human embryonic kidney (HEK293T) cells by comparable staining for second antibody against c-terminal myc tag labelled PTAFR; native HEK cells lack endogenous PTAFR (Figure 1A and Supplementary information, Figure S1A). The purity of subcellular fractions was evaluated by immunoblotting for organelle-specific marker proteins for endoplasmic reticulum (ER), plasma membrane (PM) and the nucleus (Figure 1B) (39). TEM revealed presence of nuclear PTAFR in hRMEC (Figure 1A), which was confirmed on immunoreactivity of isolated nuclei by subcellular fractionation (Figure 1B), consistent

with previous reports in porcine neuro-microvascular endothelial cells (25). Similar to HEK293T cells, Chinese hamster ovary (CHO-K1) cells showed negligible native Ptafr (Supplementary information, Figure S1B). But, upon stable transfection with PTAFR-myc, CHO-K1 displayed nuclear localization of the receptor, whereas HEK293T cells did not (Figure 1C and Supplementary information, Figure S1C). This cell-type specific difference in the subcellular localization of PTAFR inferred possible differences in the levels of nuclear transport proteins between different cell-lines.

4.2.2 The nuclear translocation of Ptafr does not arise from the plasma membrane and is not agonist-dependent

To assess whether nuclear Ptafr arises from the plasma-membrane we tagged cell surface proteins with (cell impermeable) biotin (sulpho-NHS-SS biotin) (40). Biotin-tagged PTAFR was not detected at the cell nucleus before and after cell stimulation with exogenous PAF C-16 (41), as the nuclear marker LBR was not detected on biotin-tagged PTAFR (Figure 2A). There are two distinct sources of endogenous PAF in vascular ECs, specifically circulating (extracellular) and intracellular (retained) PAF. To remove extracellular source of PAF, we serum-starved ECs for 8 hours in growth factor-devoid media. Under these conditions, stimulation of cells with PAF C-16 again did not affect cellular distribution of PTFAR (Supplementary information, S1D). Next to evaluate the effect of endocytosis of cell-surface PTAFR on its nuclear localzation, we used dynasore hydrate (42) and nystatin (43) to inhibit clathrin-dependent and clathrin-independent pathways, respectively. However, the inhibition of endocytosis had no effect on nuclear localization of PTAFR in hRMEC (Supplementary information, S1E). Correspondingly, inhibition of cytosolic phospholipase A2α (cPLA2α), the most abundant PAF producing enzyme in vascular ECs (44), also had no effect on nuclear localization of PTAFR in hRMEC (Figure 2B and Supplementary information, S1F).

Hence, endogenous and exogenous agonist PAF do not affect nuclear localization of PTAFR, and nuclear PTAFR does not originate from the plasma membrane.

4.2.3 The c-terminus region of Ptafr governs its nuclear localization

Many class-A GPCRs, including Ptafr, contain putative nuclear localization signal (NLS) (29). Upon bioinformatic analysis, we identified three potential internalization motifs in the C-terminus of human PTAFR (Table- 1), including a 'classical' NLS (45). We analyzed the function of the following putative motifs by mutagenesis (^{298}NNFRKH^{302}, 311stop and 330stop) (Supplementary information, S2A, S2B and S2C). All three mutant receptors showed similar activity compared to the wild type PTAFR in transfected HEK293T cells, based on calcium mobilization assayed fluorometrically (Supplementary information, S2D). The 311stop PTAFR mutant did not localize to the cell nucleus, as shown on subcellular fractionation as well as on confocal microscopy (Figures 2C and 2D); the other mutations did not affect nuclear localization of the PTAFR receptor (Supplementary information, S2E and S2F). Hence, the C-terminal motif in PTAFR is essential for its nuclear localization.

4.2.4 Perinuclear Rab11a and Ipo5 govern nuclear localization of Ptafr

Small GTPases of the Rab family predominantly coordinate GPCR trafficking in cells (27, 46). They are specific for cargo selection and their subcellular localization (27). In this context, the endocytic trafficking of human Ptafr from the cell surface is known to be regulated by Rab5a (early endosomes) and Rab7a (late endosomes/lysosomes) (47, 48). The members of Rab11 sub-family (Rab11a, Rab11b and Rab25) regulate endocytic trafficking via slow recycling (49). We co-immunoprecipitated PTAFR with aforementioned rabs (RAB5, RAB7 and RAB11A) at different time-points following

stimulation of hRMEC with PAF C-16. As anticipated, RAB5A and RAB7A co-immunoprecipitated with PTAFR only after agonist-stimulation, whereas RAB11A did so independent of PAF C-16 stimulation (Figure 3A). We examined if RAB11A contributed to trafficking of PTAFR to nucleus (agonist independently). hRMEC knocked down of RAB11A (using siRNA [Supplementary information, S3A]) resulted in decreased nuclear localization of PTAFR by approximately 80% (Figure 3B) on cell fractions; this observation was confirmed on TEM (Figure 3C). Further evidence to support a role for RAB11A in localizing PTAFR at the nucleus was revealed using constitutively RAB11A-Q70L and inactive RAB11A-S25N mutants transiently transfected (50) in hRMEC. Nuclear PTAFR localization was increased in hRMEC over-expressing the constitutively active RAB11A-Q70L mutant, and decreased by the inactive RAB11A-S25N mutant (Figure 3D) in heterogeneous expression system.

Importins play an important role in nuclear transport of proteins (28, 51), including of GPCR. So far, importin β1 (Kpnb1) and transportin (Tnpo1) have been reported to be responsible for nuclear translocation of full-length GPCRs or their partial fragments (21, 22, 52). We investigated the role of importins in nuclear localization of PTAFR. Neither PTAFR nor RAB11A co-immunoprecipitated with importin β1 in hRMEC (Supplementary information, S3B); as for TNPO1, based on analyzed cNLS motif prediction PTAFR lacks the PY-NLS motif required for its transport (53). On the other hand, a lesser known member of the same family, importin-5 (Ipo5, also known as RanBP5) (54), did co-immunoprecipitate with wild-type PTAFR; but not with the 311stop mutant of PTAFR (Supplementary information, S3C). Moreover, siRNA-induced knockdown of Ipo5 significantly reduced PTAFR expression in nuclei of hRMEC (Figure 3E and Supplementary information, S3D). Correspondingly, transfection of HEK293T cells with human wild-type PTAFR (which does not localize

at the nucleus in these cells [Supplementary information, S1C]) with IPO5 did result in nuclear presence of PTAFR (Figure 3G); concordantly, PTAFR-transfected CHO-K1 cells (revealing PTAFR at the nucleus) express IPO5 (Figure 3F). Collectively, these findings indicate that localization of PTAFR to the cell nucleus requires RAB11A and IPO5.

4.2.5 Nuclear Ptafr conveys functions distinct from Ptafr at the plasma membrane

Distinct functions for PTAFR depending on its general localization have been proposed, as stimulation of vascular ECs with carbamyl-PAF (synthetic PAF analog) has been shown to activate intracellular endothelial nitric oxide synthase (NOS3) but not that at the cell surface (15). Stimulation of freshly isolated nuclei from hRMEC with PAF C-16 elicited an increase in *NOS3* and *VEGFA* but not *IL1B* mRNA expression (Figure 4A). Consistent with these observations, pre-treatment of hRMEC with plasma membrane-impermeable PTAFR antagonist BN-52021 (55, 56) did not affect PAF-induced *VEGF-A* mRNA expression, but inhibited that of the inflammatory cytokine IL-1β (Figure 4B). Whereas membrane-permeable PTAFR antagonist WEB-2086 (57, 58), which acts on cell surface and intracellular PTAFR, prevented PAF-induced *VEGFA* and IL-1β mRNA expression (Figure 4B); a similar profile was observed *ex vivo* on freshly isolated rodent brain microvessels (Supplementary information, S3E, S3F and S3G). Correspondingly, PAF-induced NOS3 and VEGFA mRNA expression was abrogated upon specific knockdown of IPO5, which impairs nuclear PTAFR localization (Figure 4C); whereas IL-1β was normally generated. Along these lines, chemical cross-linking with plasma membrane-impermeable small BS^3 linker (59, 60), only affected PAF-induced IL-1β expression but not that of NOS3 and VEGFA (Figure

4D). Collectively, nuclear and cell surface Ptafr convey distinct functions, such that nuclear Ptafr stimulation induces VEGF-A and NOS3, while cell surface Ptafr stimulation elicits IL-1β mRNA expression.

4.2.6 Intracellular Ptafr regulates in vivo angiogenesis in oxygen-induced retinopathy

Finally, we used an *in vivo* model of oxygen-induced retinopathy (OIR) in the rat (61) to show distinct functions of surface and intracellular Ptafr utilizing BN-52021 and WEB-2086. PAF has been shown to induce both a (inflammatory TXA_2- and cytokine-dependent) vasoobliteration (16, 62) as well as a (VEGF-dependent) neovascularization in the ocular tissue (63). In line with these findings, systemic administration of either Ptafr antagonists (BN-52021 and WEB-2086) significantly reduced thromboxane A2 synthase (*Tbxas1*) and interleukin-1β expression (Supplemental information, Figures S4C and S4D) and corresponding retinal vasoobliteration (Figure 4E) of rats subjected to hyperoxia from P6 to P10; retinas were collected at P11 (Supplementary information, S4A).

The role of intracellular PTAFR in OIR-triggered neovascularization was studied by exposing rat pups to cycling O_2 concentrations (as described in Methods section) between P1 and P14; retinas were collected at P18 (Supplementary information, S4B). Membrane-permeable WEB-2086, but not membrane-impermeable BN-52021 (treatment during neovascularization phase), reduced pathological neovascularization (Figure 4F) and corresponding *Vegfa* and *Nos3* expression (Supplementary information, S4E and S4F). Together, the *in vivo* findings are consistent with *in vitro* and *ex vivo* observations on endothelial cells and microvessels showing distinct functions for the cell surface and intracellular nuclear PTAFR, such that the cell surface receptor is

mostly implicated in (inflammatory-dependent) vasoobliteration while the nuclear-comprising intracellular PTAFR is foremost involved in (VEGF-dependent) neovascularization in OIR.

4.3 Discussion:

PAF-induced angiogenesis is well-characterized in various experimental models of tumorogenesis (64-66). However, cellular mechanisms governing the process in vascular ECs are poorly understood. In this study, we provide *in vitro*, *ex vivo* and *in vivo* lines of evidence for nuclear localization of Ptafr and its intracrine signaling in microvascular ECs resulting in retinal angiogenesis. We further show that Ptafr translocates to nucleus in an agonist-independent manner via a process involving Rab11a and Ipo5. The intracellular Ptafr has functions distinct from its cognate cell surface receptor, as evidenced by impaired localization to the nucleus and effects of PAF antagonists with different membrane permeability, and corroborated by stimulation of isolated cell nuclei; a schematic diagram depicting our observations is presented in Figure 5. The nuclear trafficking mechanism is likely to differ among different GPCRs and needs to investigated speartely in each case.

The nuclear envelope is made up of two bi-layered phospholipid membranes (outer and inner nuclear membranes) perforated by multiprotein assemblies known as nuclear pore complexes (NPC). The outer nuclear membrane is contiguous with the ER membrane (67). The localization of GPCRs at the nucleus has been reported for numerous receptors (23). Yet the mechanisms for this intracellular localization has been for the most part not well understood. Even though lateral diffusion is suggested to be the primary mechanism of localization for some resident nuclear-membrane proteins like

lamin-B receptor (Lbr) (68), it is unlikely to be the major pathway for translocation of GPCRs which require post-translational (e.g., N-glycosylation) modification in the Golgi network (69). Unlike GPCRs, F2rl1 and Oxtr, which get translocated to the nucleus following agonist-stimulation at the cell surface (21, 22), we show that Ptafr is localized at the nucleus in an agonist- and endocytosis-independent manner, contrary to $cPLA_2$ the key enzyme for synthesis of Ptafr ligand PAF within the cell which does translocate to the nucleus upon activation (70), and acts on phosphatidylcholine-rich nuclear envelope (71) facilitating PAF generation (72), most of which is retained within endothelial cells (10). Hence, the nuclear envelope appears an ideal locus for intracrine PAF signaling. Along these lines, we previously reported that Ptafr is able to activate pertussis-toxin sensitive heterotrimeric G-proteins in purified nuclei and is responsible for gene induction of prostaglandin-endoperoxide synthase 2 (*Ptgs2*) which is a central enzyme in biosynthesis of prostanoids under inflammatory conditions (25), particularly relevant for nuclear prostaglandin E2 signaling in microvascular ECs (73-75). Consistent with this study, we found that cell surface Ptafr controls upregulation of pro-inflammatory vaso-degenerative cytokines (*Il1b*), while intracellular Ptafr augments expression of pro-angiogenic vascular factors (*Vegfa* and *Nos3*) in isolated microvessels (76, 77). This claim is based on *Vegfa* and *Nos3* mRNA generation upon isolated nuclear stimulation, and interference with the latter in conditions whereby Ptafr does not localize to the nucleus such as in a) cells devoid of endogenous Ipo5 (HEK293 cells), and b) hRMEC knocked down of Ipo5, as well as by membrane-permeable Ptafr antagonists.

To unravel mechanism of translocation of Ptafr, we focused on the potential role of rab family of small GTPases. Even though members of rab11 sub-family are well-known regulators of slow endocytic recycling of cell surface proteins (78), they also perform

other functions such as protein trafficking to and from trans-golgi network (79, 80), as well as during cellular processes like phagocytosis (81) and cytokinesis (82). Little is currently known about regulatory proteins involved in non-recycling functions of Rab11a, partly due to significant overlap between various small GTPase-mediated trafficking pathways (46, 83). We found that both siRNA-mediated knockdown as well as transient transfection with dominant mutant form of Rab11a (S25N) significantly reduced nuclear translocation of Ptafr. Given that karyopherins act as molecular chaperones for nuclear translocation, we examined their roles in Ptafr localization at the nucleus. Out of 18 known human karyopherin-beta family proteins, 11 are involved in the nuclear import (together known as importins) (28). The most studied pathway of nucleocytoplasmic import is a protein cargo containing a classical NLS is recognized by importin αβ heterodimer in the cytoplasm (84). In the case of Ptafr however, mutational disruption of the putative monopartite NLS had no effect on its nuclear localization, and well-established importin β1 did not interact with Ptafr; whereas Ipo5 did, and silencing of Ipo5 interfered with nuclear localization of Ptafr and corresponding nuclear Ptafr function (notably herein, VEGF and NOS3 induction); Ipo5 is known to be involved in nuclear import of ribosomal proteins (85) and some viral proteins (86) but there is no consensus NLS among its cargos (28). Interestingly, we identified a new motif present at the C-terminus of Ptafr (between amino acids 311 and 330) which is not known to be involved in endocytosis (87), but is essential for its nuclear localization and ensuing local Ptafr function.

To translate our *in vitro* and *ex vivo* findings revealing distinct functions for cell surface and nuclear Ptafr into *in vivo* functions we used an ischemic retinopathy model, involving a significant contribution by PAF (16). In the corresponding oxygen-induced retinopathy model inflammation-dependent vasoobliteration (88) precedes an aberrant

pre-retinal (VEGF-driven) neovascular phase. Our *in vivo* findings are consistent with cellular observations, whereby the cell surface Ptafr triggers endothelial cytotoxic IL-1 (and TXS) and vasoobliteration, while the intracellular (nuclear harboring) Ptafr elicits upregulation of *Vegfa* and corresponding aberrant pre-retinal neovascularization. Hence, different (and opposing) functions for Ptafr are observed, consistent with its distinct localization.

The exact orientation of nuclear GPCRs, including Ptafr, at the nuclear envelope is still unknown. It is hypothesized that G-protein interacting C-terminus would face cytoplasm or nucleoplasm for a receptor located at outer and inner nuclear membrane respectively; while ligand-binding domain(s) would face the inter-membrane space (23). One could argue about access of the ligand to its activating site, but this should not be a limitation as the phospholipid ligand PAF can transverse across bio-membranes and is generated intracellularly. Other than at the nuclear envelope, an abundance of Ptafr is also present within the nucleus as reported for other GPCRs (25).

In summary, our findings uncover a new mechanism for nuclear localization of a GPCR, specifically involving Rab11a (and Ipo5). We hereby demonstrate for the first time that Ptafr at the cell surface and nucleus convey distinct functions not only *in vitro* but more relevantly *in vivo*. These observations provide a new dimension to the concept of biased signaling of GPCRs, and point to the need for specific targeting of a GPCR based on its cellular localization.

4.4 Materials and Methods

4.4.1 Plasmids, siRNAs, chemical reagents and antibodies

All chemicals were obtained from Sigma-Aldrich Canada Co., Oakville, ON, unless mentioned otherwise.

PAF C-16, ginkgolide B (BN-52021), and apafant (WEB-2086) were purchased from Cayman Chemical Company, Ann Arbor, Michigan. The (EZ-Link™) Sulpho-NHS-SS-Biotin and BS^3 reagent were purchased from Thermo Fisher Scientific Inc. (distributed by Life Technologies Inc., Burlington, ON). The Strepatavidin Sepharose™ was bought from GE Healthcare Bio-Sciences, Pittsburgh, PA. The RIPA buffer was purchased from Cell Signaling Technology®, Danvers, MA. The complete (EDTA-free) protease inhibitor cocktail tablets for lysis buffers were bought from Roche Diagnostics GmBH, Germany. Protein A/G PLUS-Agarose Immunoprecipitation reagent was obtained from Santa Cruz Biotechnology, Inc., Dallas, TX. iScript™ Reverse Transcription Supermix for RT-qPCR and iQ SYBR® Green supermix were bought from Bio-Rad laboratories, Inc., Hercules, CA.

The antibodies were purchased from following companies (dilutions in brackets indicate those used for the immunoprecipitation experiments, unless noted otherwise)- 1. Rabbit polyclonal anti-PAF receptor antibody (1:500) – Cayman Chemical Company; 2. Rabbit polyclonal anti-calnexin (1:200) and monoclonal anti-lamin-B receptor (1:200) antibodies – Abcam Inc., Cambridge, MA; 3. Rabbit polyclonal anti-pan-cadherin antibody (1:100) – EMD Millipore Corp, MA; 4. Rabbit polyclonal anti-importin-5 (1:100) – Acris Antibodies, Inc., San Diego, CA; 5. Mouse monoclonal anti-rab-11 (1:250) – BD Biosciences, San Jose, CA; 6. The secondary antibodies (conjugated to

horseradish peroxidase) for western blot (1:5000) were purchased from EMD Millipore Corp; 7. The secondary antibodies for immunofluroscence (1:1000) were bought from Molecular probes™ (distributed by Life Technologies Inc., Burlington, ON); 8. All other antibodies (1:200) were obtained from Santa Cruz Biotechnology, Inc., Dallas, TX.

The pcDNA3.1+ (Invitrogen) plasmid with cDNA clone for wild-type human PTAFR was obtained from the cDNA Resource Center, Rolla, MO (www.cdna.org). The site-directed mutagenesis on PTAFR (summary is provided at the end of methods section in Table-2) was done by TOP Gene Technologies Ltd., Montréal (http://www.topgenetech.com/) . All mutant sequences were confirmed at the Genomics core facility, Institute for Research in Immunology and Cancer (IRIC), University of Montréal. The RAB11A constructs GFP-rab11 DN (S25N) and EGFP-Rab11A Q70L were gifts from Richard Pagano (Addgene plasmid # 12678) and Marci Scidmore (Addgene plasmid # 49553), respectively (89, 90).

All siRNAs (Silencer® Select Validated siRNAs) were purchased from Ambion Inc. (part of Thermo Fisher Scientific Inc.). The siRNA IDs were s16704 targeting human RAB11A, s7937 targeting human IPO5 and catalog # AM4611 for scrambled siRNA (negative control). The transient transfection of siRNA (final siRNA concentration - 10 nM) was done using Lipofectamine® 3000 reagent from Invitrogen Inc. (distributed by Life Technologies Inc., Burlington, ON). The RNA or protein were harvested 72 hours after transfection.

4.4.2 Cell culture

Human embryonic kidney (HEK293T) and Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection; Manassas, VA) were cultured in Dulbecco's

Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% of penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells reached 80% confluence before passage. Cells were transfected with appropriate plasmid DNA using linear polyethylenimine (linear PEI, molecular weight ~ 20000) with PEI:DNA ratio of 4:1 (91). The stable selection was done by addition of G418 sulfate (92) in a final concentration of 500 µg/mL for 2 weeks.

Primary hRMEC (originally from Cell Biologics, Inc; Chicago, IL) were a gift from Dr. Pierre Hardy and were cultured in fibronectin-coated (from Roche Diagnostics, Laval, QC) flasks in endothelial cell medium with endothelial cell growth supplement (ECGS) (ScienceCell™ Research Laboratories; Carlsbad, CA), FBS (final concentration: 5%), and P/S (final concentration: 100 I.U./mL penicillin and 100 µg/mL streptomycin) added. The cells were used for experiments between passage 6 and 8.

4.4.3 Subcellular fractionation of cultured cells

The protocol for subcellular fractionation utilized is identical to that we described (39) and described in chapter-3.

4.4.4 Immunoprecipitation and Western-blotting

Total cellular proteins were extracted on ice with RIPA lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM PMSF, 1 mM β -glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin. The cells were incubated on ice for 5 minutes and collected by scraping. Following brief sonication (3 seconds), supernatants were collected for use by centrifuging at 14,000 x g for 10 minutes at 4 °C. The final protein concentration was determined by Bradford method (93). The protein

samples were mixed with 4x reducing Laemmlli sample buffer (250 mM Tris-HCl, 8% SDS, 40% Glycerol, 8% β-mercaptoethanol, and 0.02% Bromophenol blue) in 3:1 ratio (Boston BioProducts Inc., Ashland, MA) (94). The western-blotting was done using SDS-PAGE electrophoresis (9% acrylamide for all proteins, except rab GTPases for which 15% acrylamide was used) followed by immunoblot analysis. We analyzed Western blots densitometrically using the NIH ImageJ program (95).

For immunoprecipitation (IP), the cell lysates (500 μg) were centrifuged at 10,000 × g for 10 minutes at 4 °C and supernatant was pre-cleared with 1 μg of appropriate control IgG (corresponding to the host species of primary antibody used for IP), together with 20 μl of Protein A/G PLUS Agarose for 1 hour at 4 °C with shaking. The beads were pelleted by centrifuging at 1000 x g for 5 minutes at 4 °C and incubated with 2 μg of primary antibody and 20 μl of protein A/G-agarose overnight at 4 °C on a shaker. On next day, samples were centrifuged at 1000 × g for 5 minutes, washed four times with lysis buffer, and final pellet was resuspended in 40 μl of 1x electrophoresis sample buffer (Bio-rad laboratories).

4.4.5 Confocal microscopy

Cells were cultured in 6-wells plate containing coverslips (VWR International, Canada). They were fixed and permeabilized with paraformaldehyde 4%, taxol (5 μM) and Triton-X 100 (0.2%) for 30 minutes. Blocking solution [5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] was added for one hour and was also used to dilute appropriate primary and secondary antibodies. The concentration of anti-PATFR antibody used was 1:200. Nuclei labeling was done with DAPI (1:3000 for 10 minutes) in permeabilized cells. Confocal microscopy imaging was performed on LSM 510 meta

confocal microscope (Zeiss, Toronto, Canada) at Sainte Justine Hospital Research Centre, Montréal, QC, Canada.

4.4.6 Transmission electron microscopy (TEM) on cultured cells

The protocol for TEM is that we previously reported (39) and described in chapter-3.

4.4.7 Cell surface biotinylation

Prior to biotinylation, hRMEC were stimulated with PAF C-16 as described below for indicated time points. The biotinylation protocol was slightly modified from the original version (40). In brief, cells were washed with ice-cold PBS/CM (1x PBS, 0.9 mM $CaCl_2$, 0.33 mM $MgCl_2$) and incubated with 0.5 mg/ml Sulpho-NHS-SS-Biotin in PBS/CM for 30 minutes at 4ºC. After quenching excess (unbound) biotin with 50 mM NH_4Cl_2 in PBS/CM for 15 minutes at 4ºC, cells were lysed and biotinylated proteins (i.e. those present at cell surface at the time of biotin treatment) were precipitated using strepatavidin beads. The non-biotinylated proteins were collected in supernatant and were further sub-fractionated into nuclear and non-nuclear parts, as described in (39).

4.4.8 cPLA2 activity assay

The cPLA2 activity kit was purchased from Cayman Chemical. Following $AACOCF_3$ (96) treatment at the indicated concentrations for 30 minutes, cells were collected and homogenized. Supernatant was collected for the assay by centrifuging at 10,000 g for 15 minutes at 4ºC. The assay (97) and analysis was performed exactly as per manufacturer's instructions (98). After the assay, the absorbance was read at 405 nm.

4.4.9 Fluro-4 NW Calcium Assay

Fluo-4 NW (No-Wash) Calcium Assay Kit was purchased from Molecular Probes™. The assay is used to study GPCR function by measuring intracellular rise in Ca^{++} levels (99) (typically mediated by GPCR coupling to heterotrimeric Gq proteins, although not exclusively (100)). One day before the experiment, ~ 40,000 cells/ 100 µl per well were seeded in a 96-well microplate. The following day, the dye loading solution was prepared by adding 10 ml of assay buffer and 100 µl probenecid stock solution to one bottle of Fluo-4 NW dye mix (component-A). The final concentration of probenecid was 2.5 mM.

Briefly, the medium was removed and replaced with 100 µl of the dye loading solution in each well of the plate. The plate was incubated at 37ºC for 30 minutes and at room temperature for additional 30 minutes. The cells were stimulated with 1 µM PAF C-16 (i.e. 10x normal concentration) in Assay buffer (provided in the kit, final volume of 10 µl per well) for 4 hours at room temperature. The fluorescence was measured by microplate reading fluorescence reader (EnVision® Multilabel Reader, PerkinElmer, MA) for excitation at 494 nm and emission at 516 nm.

4.4.10 Isolation of intact nuclei from cultured cells

The protocol is described in (74). hRMEC were washed twice with ice-cold PBS, scraped, and pelleted at 500g for 5 minutes at 4ºC. Pellets were resuspended in lysis buffer (10 mM Tris-HCL, 10 mM NaCl, 3 mM $MgCl_2$ and complete protease inhibitor added, final pH 7.4). The resulting mixture was homogenized in a Dounce tissue grinder (tight pestle, Bellco glass Inc, NJ) with 100 strokes on ice. The homogenate was centrifuged at 700g for 10 minutes at 4ºC. The pellet contained the nuclei and was collected in 7.5 ml lysis buffer containing 0.1% alternative to NP-40 and was left on ice

for 5 minutes. The mixture was centrifuged at 800g for 10 minutes at 4ºC and was washed twice with lysis buffer with NP-40 alternative. After the final wash, functional nuclei were resuspended in an incubation buffer containing 25 mM HEPES, pH 7.2, 125 mM KCl, 4 mM $MgCl_2$, 2 mM K2HPO4, 400 nM $CaCl_2$, and 0.5 mM ATP.

4.4.11 Stimulation of cells, nuclei, microvessels with PAF C-16 and endocytosis assay

All stock solutions were prepared in DMSO and stored at -20º C and the final dilutions were freshly prepared in basal medium just prior to their use.

All biological samples (cells, tissues and microvessels) were starved in serum- and growth factor- free endothelial-cell basal medium for 8 hours prior to the agonist stimulation. The microvessels were used for experiments within 12 hours of isolation. The agonist stimulation was carried out in a basal medium with added PAF C-16 (concentration- 100 nM) for 30 minutes. The RNA ad/or proteins wee isolated at the indicated time points. In experiments involving pre-treatment with Ptafr antagonists, BN-52021 and WEB-2086, were used in concentrations of 10 μM and100 nM for 30 minutes prior to the PAF C-16 stimulation, respectively (101-103). The endocytosis inhibitors, Dynasore hydrate (80 μM) (42) and nystatin (50 μM) (43) in 1% DMSO, were used for pre-treatment of hRMEC for 30 minutes prior to PAF C-16 stimulation, where indicated.

4.4.12 Chemical cross-linking with BS^3

The protocol was modified from (59, 60). For chemical cross-linking, prior to stimulation with PAF C-16, hRMEC were treated with 1 mM BS^3 in reaction buffer (20 mM sodium phosphate, 150 mM NaCl, pH 8.0) for 30 minutes at room temperature.

The reaction was stopped by treatment with 1 M Tris pH, 7.5 for 15 minutes, followed by washing with basal medium twice.

4.4.13 RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

We extracted mRNA with Trizol (Invitrogen), synthesized cDNA and amplified it using Taq DNA polymerase (Invitrogen, Molecular Probes™). All primers were synthesized by Alpha DNA (Montréal, Canada). All new primers were designed using NCBI's primer-BLAST program (104). The primer sequences are provided in the Supplemmental Tables- 1 and 2.

Prior to RNA isolation, retina samples in Trizol were homogenized by using Precellys®24 tissue homogenizer (two runs of 5000 rpm for 60 seconds each with gap of 90 seconds between the cycles). Then, samples were incubated at room temperature (RT) for 5 minutes. 100 µl $CHCl_3$ was added to each tube and was mixed for 15 sec by shaking and incubated for 3 min at RT. The, they were centrifuged at 12,000 rpm 15 min at 4ºC and the clear liquid phase was transferred to a new tube. 250 µl isopropanol was added to each tube and incubated for 10 minutes at RT. Then, they were centrifuged at 12,000 rpm for 10 minutes at 4ºC, followed by washing twice with cold 70% ethanol and centrifuged for 5 minutes at 8000 rpm at 4ºC. Lastly, pellet was air-dried for 15 minutes and re-suspended in 20 µl of nuclease-free sterile water. The nucleic acid quantification was done using NanoDrop™ 2000 Spectrophotometer from Thermo Fischer Scientific.

The iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc.) was used for reverse transcription of RNA into cDNA (20 µl per reaction) with the cycle setting of 5 minutes at 25ºC (priming), 30 minutes at 42ºC (reverse transcription)

and 5 minutes at 85ºC (stopping the reaction). The DNA concentration was measured using NanoDrop™ 2000 and samples were stored at -20ºC.

For comparative quantitative PCR (qPCR), 2x iQ SYBR® Green supermix (Bio-Rad Laboratories, Inc.) was used. Total volume of each reaction was 25 µl (12.5 µl 2x supermix, 100 nM each of forward and reverse primers, 500 ng of cDNA and distilled water). All PCR reactions were run in triplicate. The qPCR was carried out using Applied Biosystems® 7500 Real-Time PCR. The thermal cycle was 50ºC for 2 minutes and 95º for 2 minutes (holding stage), followed by 35 cycles each of 95ºC for 15 seconds, 58ºC for 1 minute, 72ºC for 15 seconds (cycling stage). The analysis was performed by ΔΔCt method. We normalized cDNA concentrations against GAPDH levels in each sample.

Animal Care (for *ex vivo* and *in vivo* protocols)

All experimental procedures were approved by the Animal Care Committee of the Hôpital Sainte-Justine, Montréal, Québec, Canada in accordance with guidelines established by the Canadian Council on Animal Care.

4.4.14 Isolation of intact microvessels from rat brain

The protocol is a modified version of the one described (105).

Briefly, after euthanizing the animal (4 week old rats), brain was perfused with PBS to eliminate blood elements. The brain was removed and immediately kept in ice-cold Krebs buffer (120 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 27 mM $NaHCO_3$, 1 mM KH_2PO_4, 0.01 mM sodium edetate, and 10 mM glucose) and was cut into fine (< 5 mm) pieces. The cut tissue was spun at 1000 rpm for 10 minutes at 4ºC and then homogenized gently (5–6 strokes) in ice-cold PBS containing 20% Ficoll-400

using a glass homogenizer with tight fitting glass pestle. The homogenate was centrifuged at 12000 rpm for 15 min at 4°C. The pellet containing the microvessels was washed 3 or 4 times with 25 ml of ice-cold PBS to eliminate Ficoll and centrifuged at 2000 rpm for 20 minutes at 4ºC after each wash. Lastly, microvessels were filtered through 70 and then 40 μm filters (Millipore) to remove bigger vessels. The final filtrate was transferred to 6-well plate containing endothelial growth medium. The microvessel preparations were assessed for purity by light microscopy and γ-glutamyl transpeptidase activity as described in (106). The fresh microvessels were used for PAF stimulation within 12 hours of isolation. The RNA isolation from microvessels was done by the method, as described previously.

4.4.15 In vivo Oxygen-induced retinopathy (OIR) models

Retinal vaso-obliteration model

To investigate PAF-induced vasoobliteration, we used a well-established model of human ROP – the vaso-obliteration (VO) model in rats, characterized by exposing the pups to 80% O2 during the first week of life, when vasculature is still underdeveloped, and susceptible to damage caused by various inflammatory mediators, including PAF, leading to central loss of retinal microvasculature (16, 88), a hallmark of the first phase of ischemic retinopathies in humans (107). Briefly, Sprague-Dawley albino rat pups (Charles River, St. Constant, Québec, Canada) were placed with their mothers in an 80% oxygen environment from P5 to P10, when normal retinal vasculature reaches the periphery. The ability of BN-52021 or WEB-2086 to curb vaso-obliteration was tested in four different VO groups (n= 8 animals per group) treated from P5 to P10 as followed; VO group 1, did not receive any treatment; VO groups 2, 3 and 4, were randomly selected to receive daily i.p. vehicle (DMSO), or 10 mg/kg/day of BN-52021

or WEB-2086, respectively in a final volume of 50 µl. Control animals (group 5) were maintained in room air (21% O2) throughout the 5 days. The pups were euthanized on P10. Eyes were enucleated and retinas dissected. 50% of the retinas were used for RT-qPCR. In the other half, vasoobliteration was evaluated in retinal flat-mounts stained with lectins by using the Image J software (Universal Imaging, Sunnyvale, CA). Vaso-obliterated areas were assessed as the retinal area devoid of vasculature over the total retina.. All other conditions (e.g., light exposure, temperature, feeding, etc.) were similar for all the treatment groups.

Retinal neovascularization model

Within 4 hours after birth, litters of Sprague-Dawley albino rats were placed with their mothers in an oxygen regulated environment (OxyCycler A820CV; BioSpherix Ltd., Redfield, NY) adjusted to alternate between 50% and 10% of oxygen every 24 hours for 14 days. At postnatal day 14 (P14), pups were transferred to room air (21% O2) for 4 days. The cycling O2 conditions produce the peripheral retinal neovascularization which closely resembles the second phase of ROP in humans (61). The ability of BN-52021 or WEB-2086 to curb vaso-obliteration and a subsequent decrease in pathological neovascularization was tested in four different oxygen-induced retinopathy (OIR) groups (n= 8 animals per group) treated from P14 to P18 (during neovascularization) as follows; OIR group 1, did not receive any treatment; OIR groups 2, 3 and 4, were randomly selected to receive daily i.p. vehicle (DMSO), or 10 mg/kg/day of BN-52021 or WEB-2086, respectively in a final volume of 50 µl. Control animals (group 5) were maintained in room air (21% O2) throughout the 18 days. On day P18, rats were anaesthetized with isoflurane (2%) and sacrificed by decapitation. Eyes were enucleated and retinas dissected. Neovascularization was evaluated in retinal flat-mounts stained with lectins. 50% of the retinas were used for RT-qPCR. All other

conditions (e.g., light exposure, temperature, feeding, etc.) were similar for all treatment groups.

Retinal Flat-Mounts

In all cases the eyes were enucleated and fixed in 4% paraformaldehyde for one hour at room temperature and then stored in PBS at 4ºC until used. The cornea and lens were removed and the retina was gently separated from the underlying choroid and sclera under a dissecting microscope. Then, the retinas were stained overnight at 4ºC with fluorescein-labeled GSL 1, isolectin B4 (Vector Labs; 1:100). Lectin-stained retinas were whole-mounted onto Superfrost/Plus microscope slides (Fisher Scientific) with the photoreceptor side down and imbedded in Fluoro-gel (Electron Microscopy Sciences) and imaged at 10x using a Zeiss AxioObserver.Z1. Images were merged into a single file using the MosiaX option in the AxioVision 4.6.5 software (Zeiss). Quantification of neovascularization was assessed using the SWIFT-NV method (108), that consists of a set of macros that was developed to quantify all the pixels represented by neovascular tufts and clusters, but not normal vessels in lectin-stained retinal whole mounts.

4.4.16 Statistical analyses

All data are presented as means ± s. d. (standard deviations). We made comparisons between groups by indepdent Student's T test (for two groups) or one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's test for comparison between more than two groups. $P < 0.05$ was considered statistically significant.

Figure-1

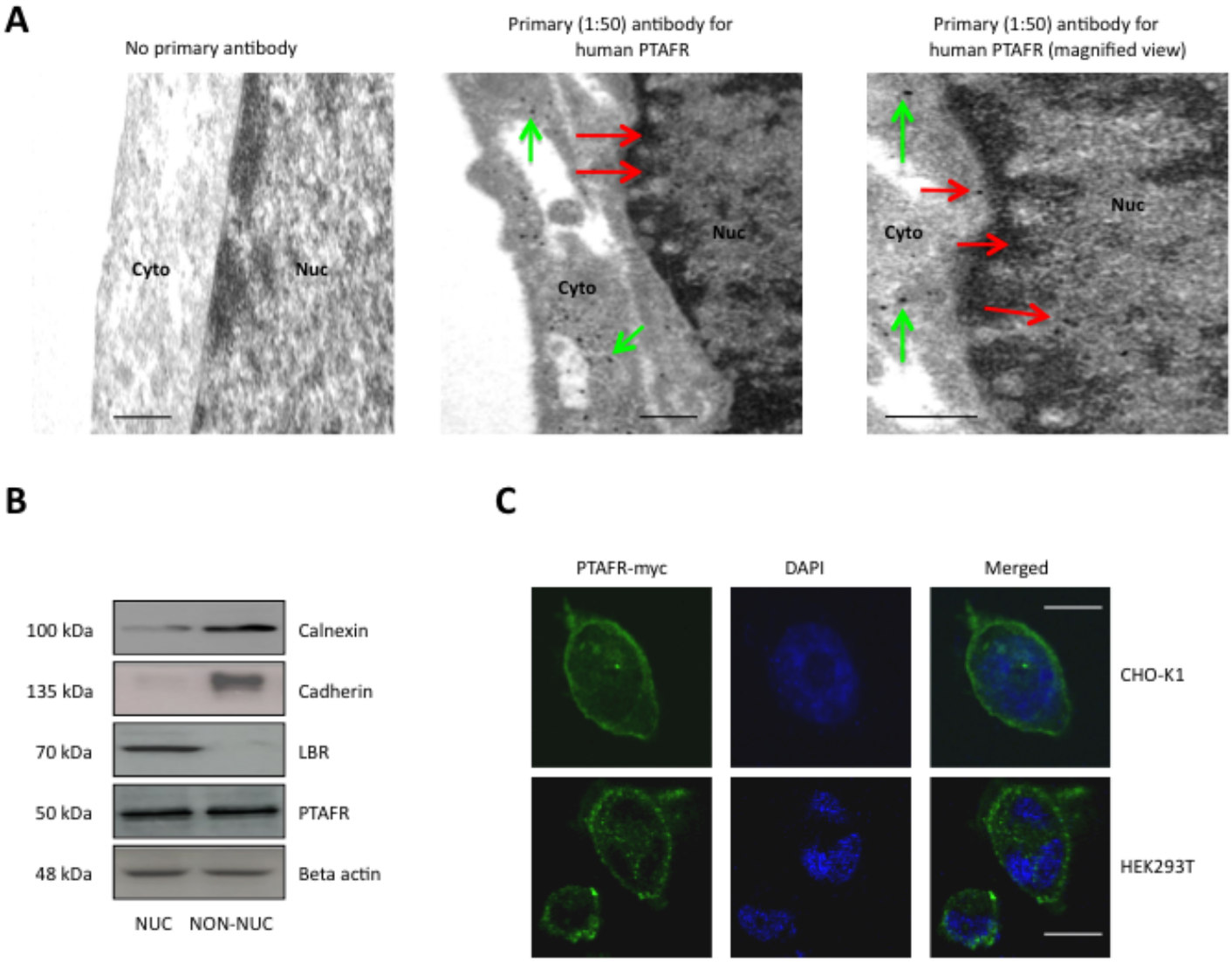

A
No primary antibody
Primary (1:50) antibody for human PTAFR
Primary (1:50) antibody for human PTAFR (magnified view)
Cyto
Nuc
Cyto
Nuc
Cyto
Nuc
B
100 kDa
Calnexin
135 kDa
Cadherin
70 kDa
LBR
50 kDa
PTAFR
48 kDa
Beta actin
NUC
NON-NUC
C
PTAFR-myc
DAPI
Merged
CHO-K1
HEK293T

Figure-2

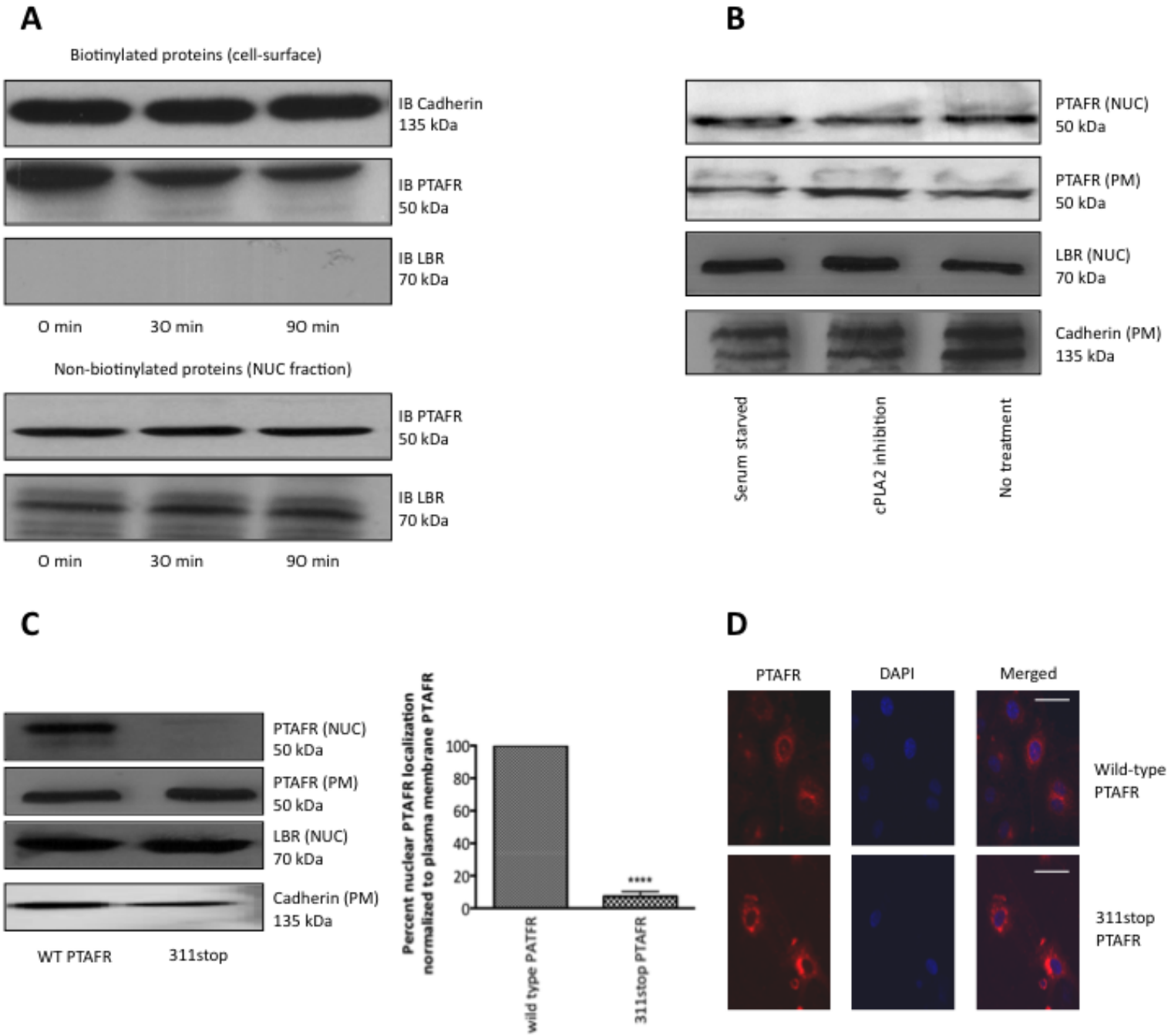

A
Biotinylated proteins (cell-surface)
IB Cadherin
135 kDa
IB PTAFR
50 kDa
IB LBR
70 kDa
O min
3O min
9O min
Non-biotinylated proteins (NUC fraction)
IB PTAFR
50 kDa
IB LBR
70 kDa
O min
3O min
9O min
B
PTAFR (NUC)
50 kDa
PTAFR (PM)
50 kDa
LBR (NUC)
70 kDa
Cadherin (PM)
135 kDa
Serum starved
cPLA2 inhibition
No treatment
C
PTAFR (NUC)
50 kDa
PTAFR (PM)
50 kDa
LBR (NUC)
70 kDa
Cadherin (PM)
135 kDa
WT PTAFR
311stop
Percent nuclear PTAFR localization normalized to plasma membrane PTAFR
100
80
60
40
20
0
****
wild type PATFR
311stop PTAFR
D
PTAFR
DAPI
Merged
Wild-type PTAFR
311stop PTAFR

Figure-3

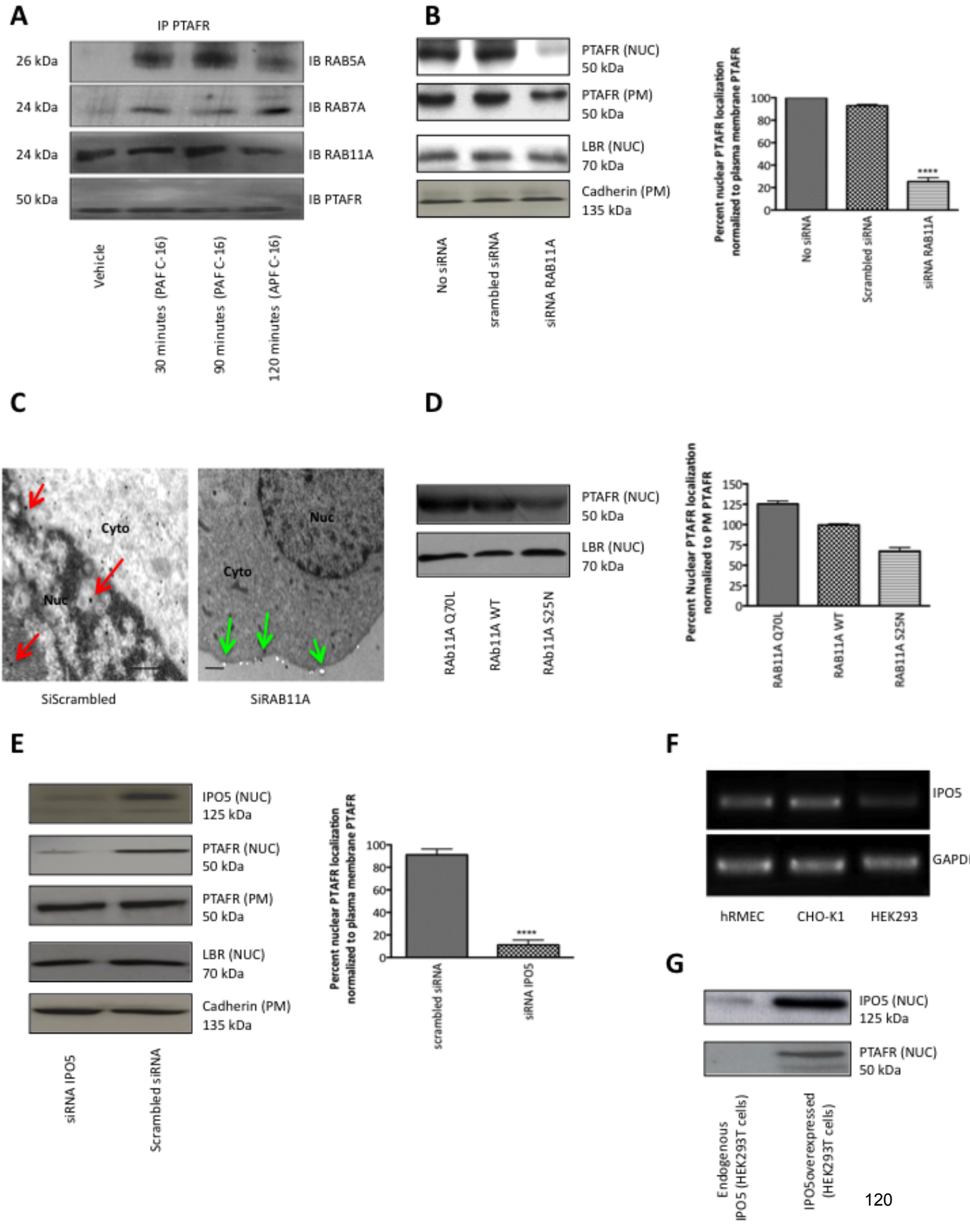

A
IP PTAFR
26 kDa
IB RAB5A
24 kDa
IB RAB7A
24 kDa
IB RAB11A
50 kDa
IB PTAFR
Vehicle
30 minutes (PAF C-16)
90 minutes (PAF C-16)
120 minutes (APF C-16)
B
PTAFR (NUC)
50 kDa
PTAFR (PM)
50 kDa
LBR (NUC)
70 kDa
Cadherin (PM)
135 kDa
No siRNA
srambled siRNA
siRNA RAB11A
Percent nuclear PTAFR localization normalized to plasma membrane PTAFR
100
80
60
40
20
0
****
No siRNA
Scrambled siRNA
siRNA RAB11A
C
Cyto
Nuc
Nuc
Cyto
SiScrambled
SiRAB11A
D
PTAFR (NUC)
50 kDa
LBR (NUC)
70 kDa
RAb11A Q70L
RAb11A WT
RAb11A S25N
Percent Nuclear PTAFR localization normalized to PM PTAFR
150
125
100
75
50
25
0
RAB11A Q70L
RAB11A WT
RAB11A S25N
E
IPO5 (NUC)
125 kDa
PTAFR (NUC)
50 kDa
PTAFR (PM)
50 kDa
LBR (NUC)
70 kDa
Cadherin (PM)
135 kDa
siRNA IPO5
Scrambled siRNA
Percent nuclear PTAFR localization normalized to plasma membrane PTAFR
100
80
60
40
20
0
****
scrambled siRNA
siRNA IPO5
F
IPO5
GAPDH
hRMEC
CHO-K1
HEK293
G
IPO5 (NUC)
125 kDa
PTAFR (NUC)
50 kDa
Endogenous IPO5 (HEK293T cells)
IPO5overexpressed (HEK293T cells)

Figure-4

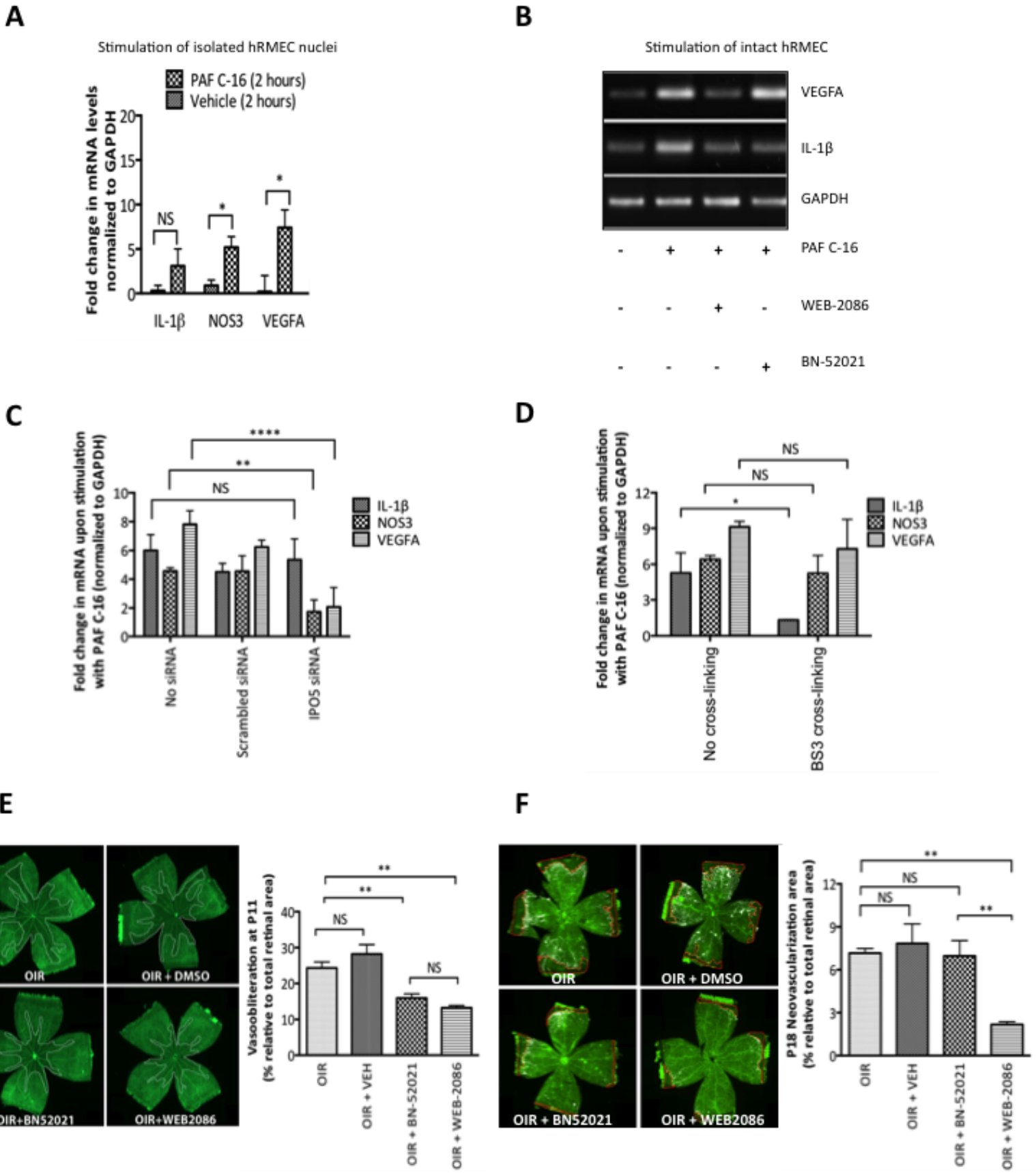
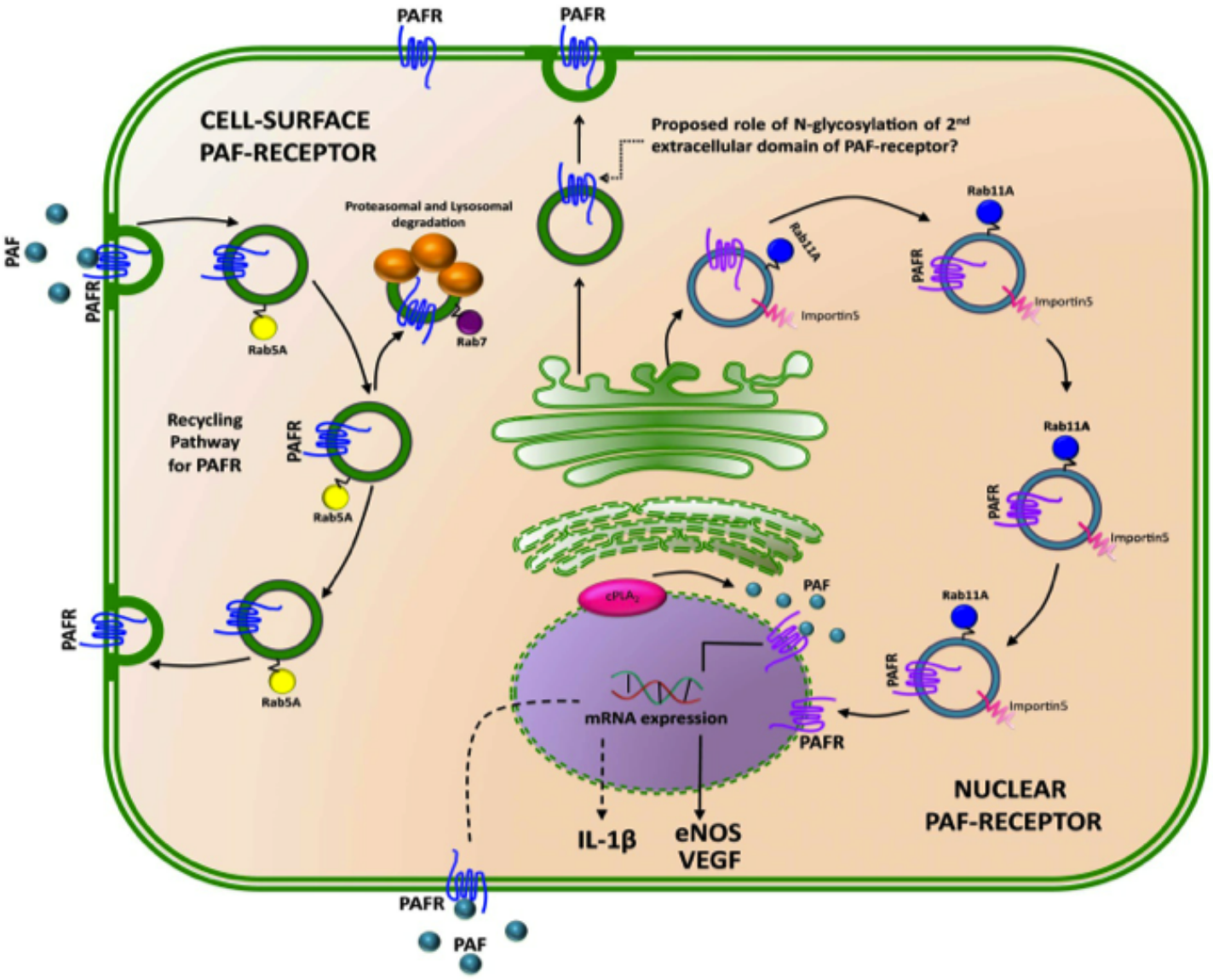
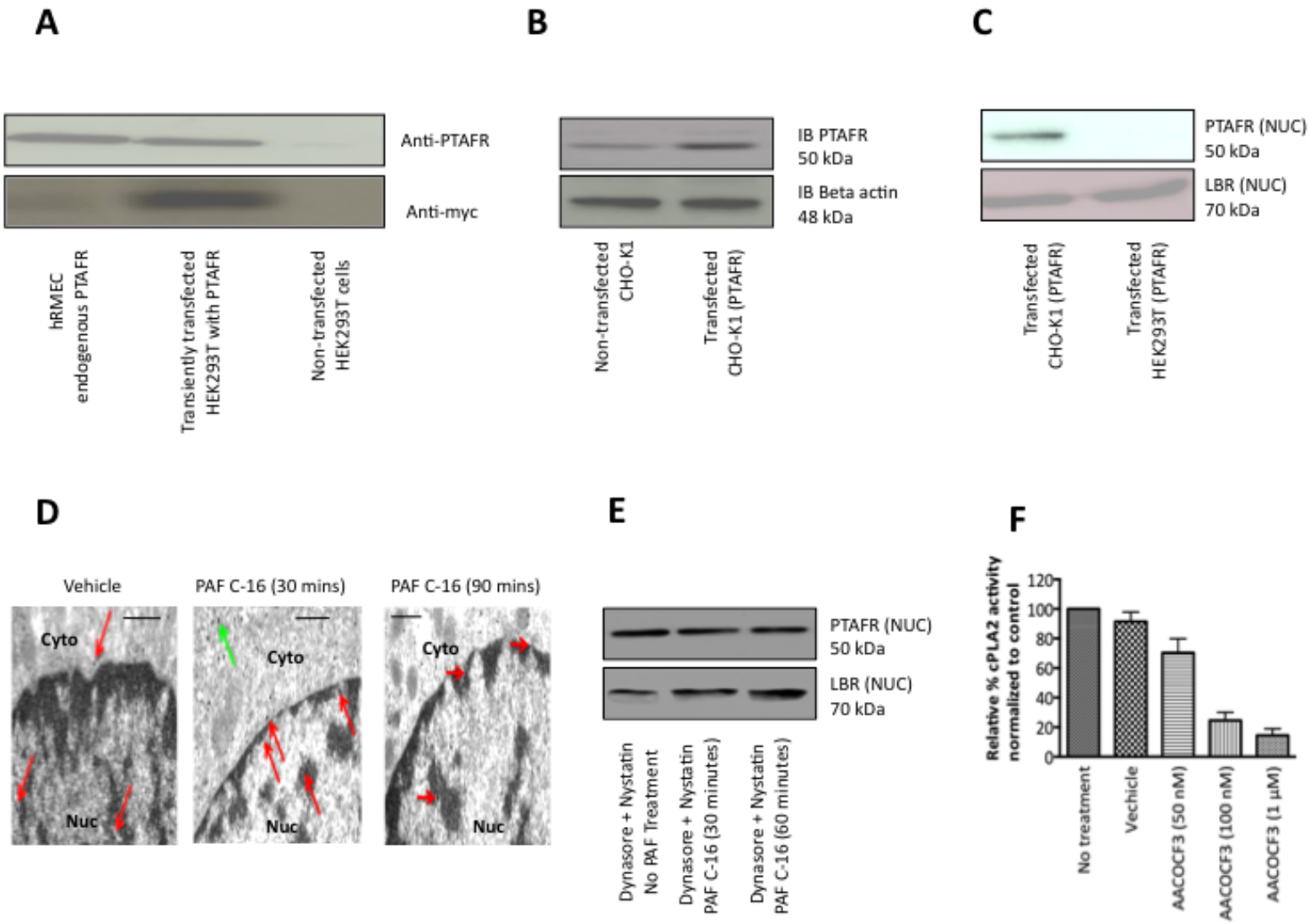

A
Stimulation of isolated hRMEC nuclei
PAF C-16 (2 hours)
Vehicle (2 hours)
Fold change in mRNA levels normalized to GAPDH
NS
*
*
IL-1β
NOS3
VEGFA
B
Stimulation of intact hRMEC
VEGFA
IL-1β
GAPDH
- + + + PAF C-16
- - + - WEB-2086
- - - + BN-52021
C
Fold change in mRNA upon stimulation with PAF C-16 (normalized to GAPDH)
****
**
NS
IL-1β
NOS3
VEGFA
No siRNA
Scrambled siRNA
IPO5 siRNA
D
Fold change in mRNA upon stimulation with PAF C-16 (normalized to GAPDH)
NS
NS
*
IL-1β
NOS3
VEGFA
No cross-linking
BS3 cross-linking
E
OIR
OIR + DMSO
OIR+BN52021
OIR+WEB2086
Vasoobliteration at P11 (% relative to total retinal area)
**
**
NS
NS
OIR
OIR + VEH
OIR + BN-52021
OIR + WEB-2086
F
OIR
OIR + DMSO
OIR + BN52021
OIR + WEB2086
P18 Neovascularization area (% relative to total retinal area)
**
NS
NS
**
OIR
OIR + VEH
OIR + BN-52021
OIR + WEB-2086

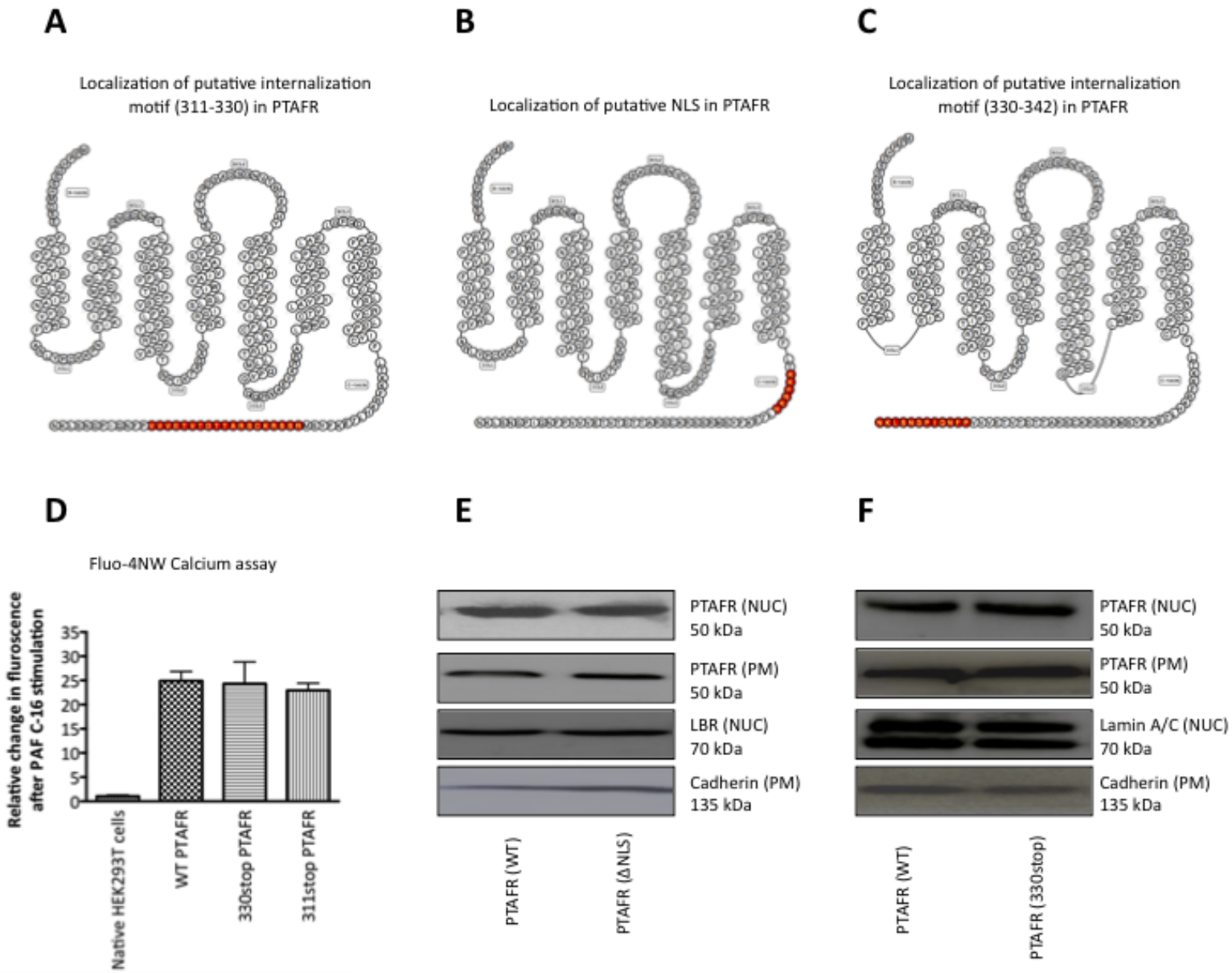
Figure-5 Schematic representation of subcellular trafficking of PTAFR



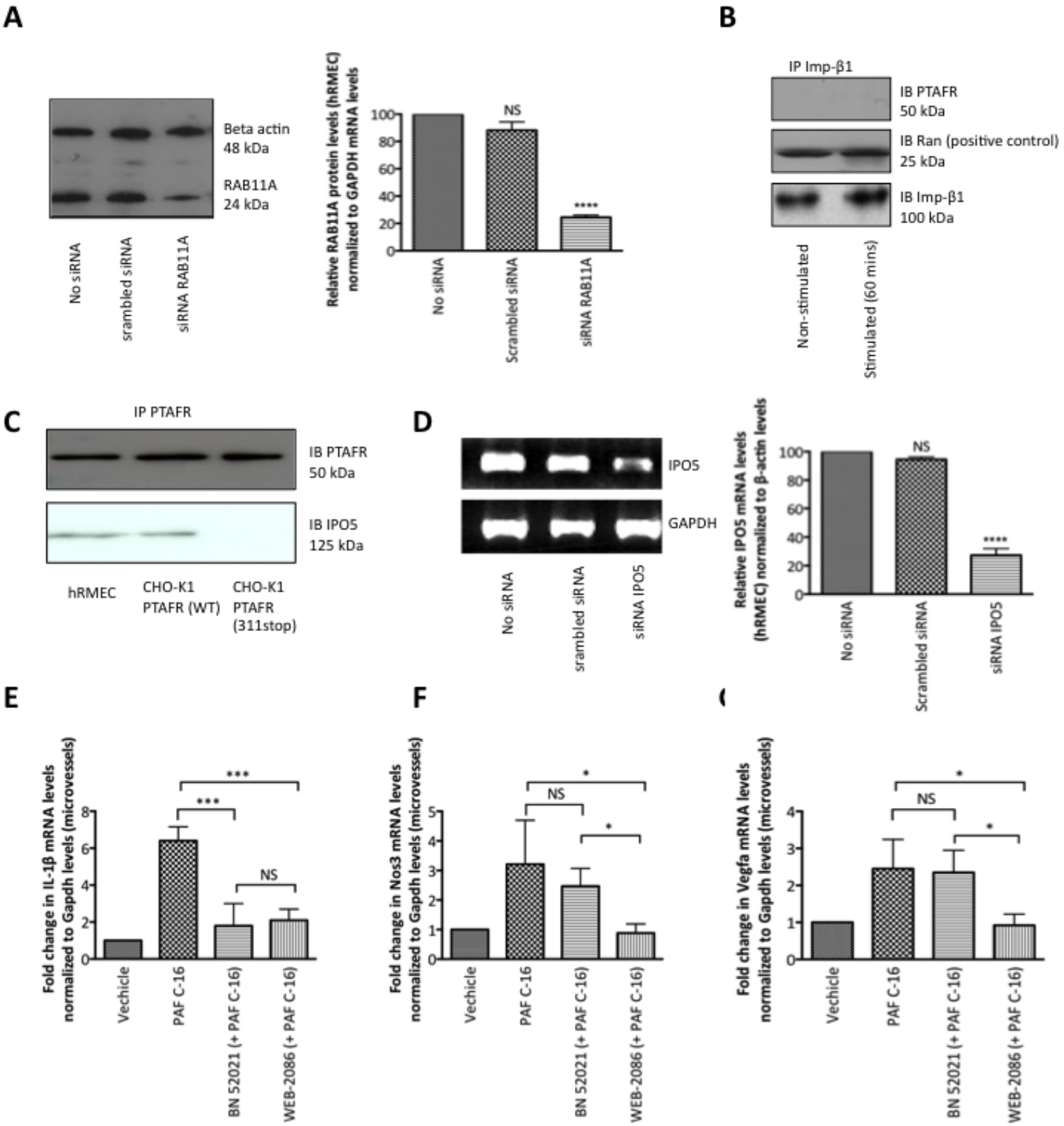
Supplemental figure-1



Supplemental figure-2



Supplemental figure-3



Supplemental figure-4

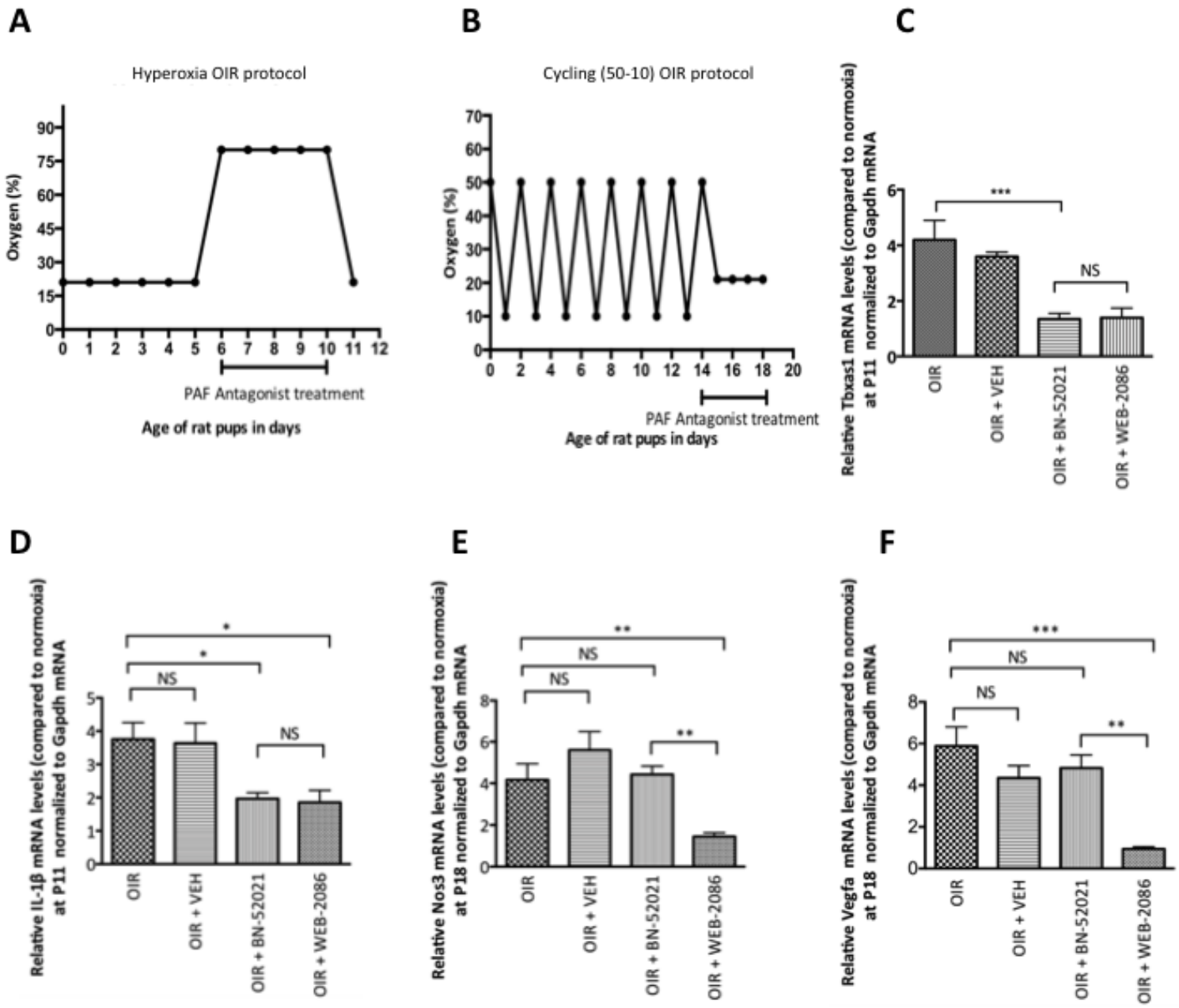


Figure legends:

Figure-1 Nuclear localization of PTAFR is cell-type specific. (**A**) Transmission electron microscopy (TEM) on cultured hRMEC. The left panel indicates no primary antibody (negative control) and secondary anti-rabbit nanogold (1:500) showing lack of non-specific labeling. The other two panels were treated with respective primary (1:50) and secondary (1:500) antibodies. They show specific immunogold labeling, corresponding to PTAFR in perinuclear (green arrows), nuclear envelope and intranuclear regions (red arrows). The right panel is magnified to show intranuclear localization of the receptor. The TEM images are representative of 3 independent experiments. Scale bar = 0.5 μm**.** (**B**) Purity of subcellular (NUC = nuclear, NON-NUC = non-nuclear) fractions of hRMEC. The images are representative of 3 western blots. Organelle specific markers used are calnexin for endoplasmic reticulum (ER), cadherin for plasma membrane (PM) and lamin-B receptor (LBR) for nuclear envelope. Beta actin is found in both cytoplasm and nucleoplasm and was used as loading control (109). PTAFR is also detected in both fractions of hRMEC. (**C**) Confocal microscopy on HEK293T and CHO-K1 cells stably transfected with PTAFR-myc receptor. Nuclei are stained with DAPI. PTAFR shows nuclear localization in CHO-K1 cells, but not in HEK293T cells. The images are representative of 3 replicates. Scale bar = 20 μm.

Figure-2 Nuclear PTAFR does not arise from plasma membrane, and requires C-terminal internalization motif. (**A**) Biotinylation of hRMEC using PM impermeable sulpho-NHS-SS-Biotin tag. Following stimulation of with PAF C-16 for indicated time-points, biotinylated proteins were separated by binding to strepatavidin beads, while supernatant containing non-biotinylated intracellular proteins was further sub-

fractionated into nuclear and non-nuclear parts. IB = immunoblot. The top two panels correspond to plasma-membrane cadherin and PTAFR, respectively. Nuclear protein, LBR, is absent in biotinylated fraction. The last two panels confirm presence of LBR and PTAFR in the nuclear fraction. All western blots are representative of 3 independent experiments. (**B**) Effect of AACOCF3 (cPLA2 inhibitor (96)) on subcellular localization of PTAFR in hRMEC. There is no difference in localization of PM or nuclear PTAFR following AACOCF3 treatment. LBR and cadherin are used as loading controls for nuclear (NUC) and plasma-membrane (PM) fractions, respectively. (**C**) Subcellular fractionation of CHO-K1 cells transfected with either wild-type (WT) or 311stop PTAFR. The quantification of three independent western blots (representative blot is shown) using NCBI's image J software indicates approximately 90% reduction in nuclear signal as compared to that at plasma-membrane (normalized using LBR and cadherin as loading controls for the respective fractions). *** $p < 0.001$ (**D**) Confocal microscopy on CHO-K1 cells transfected with WT or 311stop PTAFR. The 311stop PTAFR is present at cell-surface and perinuclear regions, but not at the nucleus. The figures are representative of 3 replicates. Scale bar = 50 μm.

Figure-3 RAB11A and IPO5 govern nuclear localization of PTAFR in hRMEC. (**A**) Co-immunoprecipitation (co-IP) of PTAFR with 3 major rabs controlling GPCR trafficking at indicated time-points following PAF C-16 stimulation. RAB5A (top row) co-IPs with only PTAFR following stimulation (early endocytosis (110)). RAB7A (second row) also co-IPs following stimulation and peaks at around 2hours (receptor targeted for degradation (111)). RAB11A (third row) co-IPs with PTAFR at all tested time points, even in absence of PAF C-16 stimulation (first column). The last row shows PTAFR as loading control. (**B**) Knockdown of RAB11A using specific siRNA in hRMEC. The quantification of three westerns using image J software reveals

approximately 75% reduction in nuclear immunoreactivity, as compared to that at PM. Plasma membrane (PM) PTAFR is slightly affected (to much lesser extent); possibly due to recycling function of RAB11A. *** $p < 0.001$ (**C**) TEM on hRMEC transfected with either scrambled or specific RAB11A siRNAs. RAB11A knockdown specifically affects nuclear localization of PTAFR. Red arrows indicate nuclear labeling, while green arrows point labeling at plasma membrane. The TEM images are representative of three replicates. Scale bar = 0.5 μm. (**D**) Overexpression of constitutively active (Q70L) or dominant negative (S25N) RAB11A mutants in hRMEC (heterogeneous expression). The Q70L and S25N mutants resulted in ~ 125% and ~70% nuclear localization of PTAFR respectively, as compared to non-transfected hRMEC with endogenous RAB11A levels (set at 100%) and normalized against LBR levels in all nuclear fractions. (**E**) siRNA mediated knockdown of IPO5 in hRMEC. Plasma membrane (PM) PTAFR is unaffected. The quantification of western blots reveals >90% reduction in nuclear immunoreactivity, as compared to that at PM. The values were normalized against LBR and cadherin in NUC and PM fractions, respectively. *** $p < 0.001$ (**F**) Endogenous levels of IPO5 mRNA in hRMEC, CHO-K1 and HEK293T cells. HEK293T show negligible endogenous expression of IPO5. (**G**) Co-transfection of HEK293T cells with PTAFR and IPO5. The over-expression of both proteins results in nuclear localization of PTAFR in HEK293T. All western blots are representative of 3 independent experiments.

Figure-4 Nuclear PTAFR has functions distinct from its cell surface counterpart and former affects retinal neovascularization in oxygen-induced retinopathy (OIR). (**A**) Stimulation of freshly isolated nuclei from hRMEC with PAF C-16 (100 nM for 30 minutes). PAF C-16 causes significant augmentation of *NOS3* and *VEGFA* but not *IL1B* levels. * $p < 0.05$; all values are represented as mean +/- s.d. (standard

deviation). The gene expression was analyzed using four independent replicates. RNA was isolated 120 minutes after stimulation in all conditions and gene expression analyzed by qRT-PCR. (**B**) Stimulation of cultured hRMEC with PAF C-16 (100 nM for 30 minutes) with or without pre-treatment using indicated PTAFR antagonist. The pre-treatment with membrane-permeable antagonist (WEB-2086) inhibits PAF-induced IL-1β and *VEGFA* expression. The non-permeable antagonist (BN-52021), on the other hand, only attenuates PAF-induced IL-1β but not VEGFA mRNA levels. RNA was isolated 120 minutes after stimulation and analyzed by RT-PCR. (**C**) Effect of IPO5 knockdown on PAF-induced gene expression. IPO5 siRNA treatment significantly reduces PAF-induced augmentation of NOS3 and VEGFA levels but has no effect on IL-1β levels. ** $p < 0.01$ and **** $p < 0.0001$. (**D**) Effect of chemical cross-linking on PAF-induced gene expression. The cross-linking prevents upregulation of PAF-induced IL-1β (* $p < 0.05$) but has no significant difference on NOS3 and VEGFA levels. (**E**) Retinal vaso-obliteration using hyperoxia model of oxygen-induced retinopathy (OIR) in rats. The OIR resulted in central retinal vaso-obliteration at P11, as delineated by white margins in lectin-stained retinal flat mounts (top left panel), and this was prevented by systemic administration of either PAF antagonists (5 injections from P6 to P10) BN-52021 and WEB-2086 (two bottom panels) but not by the vehicle treatment (top right panel); there was no statistically significant difference between the two antagonists. The Y-axis in graph represents % of avascular retina at P11 relative to the total area. (n= 5-8 retinas per group, NS = not significant). ** $p < 0.01$. (**F**) Retinal neovascularization using cycling model of OIR in rats. OIR-induced retinal neovascularization (top left panel), is reduced by administration of membrane permeable WEB-2086 (bottom right panel) but not that of either impermeable BN-52021 (bottom left panel) or the vehicle (top right panel). The Y-axis in graph

represents % of retina with neovascularization (quantified with SWIFT-NV (108)) at P18 relative to the total area (n= 5-8 retinas per group, NS = not significant). ** $p < 0.01$.

Figure-5 Schematic diagram showing intra-cellular trafficking of PTAFR.

Once synthesized in ER and glycosylated in trans-golgi network (TGN), Rab11a and Importin-5 together control (pathway highlighted by blue vesicles) nuclear localization of Ptafr, possibly directly from TGN. The nuclear PTAFR can be activated by production of local PAF from membrane phospholipids by nuclear cPLA2α (70) and microsomal Lyso-PAF-acetyltransferase (112). The nuclear Ptafr, in turn, activates expression of pro-angiogenic genes such as *Nos3*, *Vegfa*; while cell-surface Ptafr regulated expression of pro-inflammatory cytokines such as IL-1β. It is known that Rab5a controls agonist-induced internalization of PTAFR from the plasma membrane, while Rab7a controls proteasomal degradation of the receptor (47). The role of N-glycosylation in 2nd extracellular domain of the receptor has been proposed in the cell-surface targeting of Ptafr (113).

Supplemental figure-1

(**A**) Specificity of primary PTAFR antibody against its N-terminus (endogenous) or C-terminus (for myc tagged receptor). The N-terminus antibody (top panel) detects both native (in hRMEC) and transfected PTAFR (HEK293T cells). The non-transfected HEK293T cells don't express the receptor. (**B**) Expression of Ptafr in CHO-K1 cells. Non-transfected CHO-K1 cells express very little Ptafr. Beta actin was used as loading control. (**C**) Subcellular fractionation of CHO-K1 and HEK-293T cells stably transfected with PTAFR-myc. PTAFR is only found in nuclear fraction in the transfected CHO-K1 cells but not in transfected HEK293T cells. (**D**) TEM on cultured hRMEC following agonist stimulation with PAF C-16 at the indicated time-points. All tested time-points show nuclear localization of PTAFR (red arrows), while perinuclear nanogold labeling gradually increases following PAF C-16 stimulation (receptor present in recycling endosomes). TEM images are indicative of three replicates. Scale bar = 0.5 μm. (**E**) Effect of endocytosis inhibitors on nuclear localization of PTAFR. hRMEC were pre-treated with 80 μM Dynasore hydrate and 50 μM nystatin for 30 minutes to inhibit clathrin-dependent and clathrin-independent endocytosis respectively, followed by stimulation with 100 nM PAF C-16 for indicated times. The inhibition of endocytosis with or without PAF C-16 stimulation had no effect on nuclear localization of PTAFR in hRMEC. (**F**) Effect of AACOCF3 on cPLA2 activity in hRMEC. The treatment with 100 nM AACOCF3 for 30 minutes resulted in more than 80% reduction of cPLA2 activity and was used for the experiment in figure- 2B. All western blots are representative of three independent experiments.

Supplemental figure-2

(**A**) Schematics of Ptafr sequence to show the putative internalization motif of PTAFR between amino acid positions 311 and 330 (87). (**B**) Localization of putative nuclear

localization signal in PTAFR at positions 298 to 302 (KKFRKH). (**C**) Putative ER retention motif at the end of C-terminus of PTAFR (25). (**D**) Calcium mobilization assay using Fluo-4NW indicator. The stimulation of HEK293T cells transfected with wild-type, 311stop and 330stop PTAFR results in intracellular rise in calcium levels, indicating that all receptors are functional. (**E**) Subcellular fractionation of CHO-K1 cells transfected with PTAFR. The mutational disruption of putative NLS from KKFRKH to NNFRKH (ΔNLS PTAFR) does not affect localization of the receptor at the cell surface or at the nucleus. (**F**) Subcellular fractionation of CHO-K1 cells transfected with PTAFR. The removal of putative ER retention motif (330stop PTAFR) also does not affect either PM or nuclear localization of PTAFR. All western blots are representative of three replicates.

Supplemental figure-3

(**A**) RAB11A knockdown in hRMEC. There is ~ 65% reduction in RAB11A protein levels 72 hours following the specific siRNA treatment. The graph is average of 3 separate western blots and the representative blot is shown here. *** $p < 0.001$ (**B**) Co-immunoprecipitation of importin β1 interacting partners in hRMEC under non-stimulated and stimulated with PAF C-16 (after 60 minutes) conditions. It shows that former does not interact with PTAFR. Ran GTPase, a known interacting partner Imp β1 was used as positive control. (**C**) Co-immunoprecipation with IPO5 in hRMEC as well as CHO-K1 cells transfected with the receptor. IPO5 co-IPs with wild type PTAFR but not with 311stop mutant receptor. (**D**) IPO5 knockdown in hRMEC. There is ~ 80% reduction in IPO5 mRNA levels 72 hours following the specific siRNA treatment. The graph is average of 3 separate agarose gels and the representative gels is shown. *** $p < 0.001$ (**E**), (**F**) and (**G**) represent PAF-induced expression *Il1b*, *Nos3*, and *Vegfa* (analyzed by qRT-PCR) respectively in freshly isolated microvessels in presence or

absence of PAF antagonists. The *Il1b* expression was attenuated by both PAF antagonists, while that of other two genes was only reduced by membrane permeable antagonist (WEB-2086) acting on surface as well as intracellular Ptafr. The data are represented as mean +/- s.d. (average of 3 independent qPCR measurements). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NS = not significant. All blots on the figure are representative of 3 independent experiments.

Supplemental figure-4 *In vivo* gene expression by intracellular PTAFR during two phases of OIR.

(**A**) Schematic representation of oxygen-induced retinopathy (OIR) model. (**B**) Schematic representation of cycling model of OIR. (**C**) Hyperoxia-induced upregulation of *Tbxas1 at P11. Tbxas1* mRNA levels are significantly elevated in OIR (hyperoxia) and this upregulation is prevented by administration of either PAF antagonists (from P5 to P10). (**D**) IL-1β mRNA expression at P11 during hyperoxia model of OIR. The OIR-induced IL-1β expression is attenuated by once daily treatment with either PAF antagonists but not that of the vehicle (DMSO). (**E**), and (**F**) Induction of pro-angiogenic genes, Nos3 and Vegfa respectively, at P18 during cycling model of OIR. The OIR-induced Nos3 and Vegfa levels are significantly reduced by WEB-2086 but not by BN-52021 or the vehicle treatment from P14 to P18. For sub-panels (C) to (F), the data are represented as mean +/- s.d (n= 5-8 retinas per group, NS = not significant). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table-1 PTAFR Motifs and Mutagenesis

	PTAFR motif (all motifs are present in C-terminus)	Ref. for the motif	Mutagenesis	Ref. for the mutation
1.	Monopartite nuclear localization signal (NLS) ^{298}KKFRKH^{302}	*(25)*	^{298}NNFRKH^{302} i.e. first two lysine (K) residues were mutated to asparagine (N) residues.	(114)
2.	Putative internalization motif (last 26 amino acid residues) i.e. 317 to 342 amino acid residues	(87)	311stop PTAFR	(115)
3.	Putative ER retention motif ^{338}NLSK^{341}	(25)	330stop PTAFR	

Supplemental table-1 Table of primer sequences (human genes)

	Human Gene name	symbol	Primer sequence (5'-3')	Ref
1.	glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Forward- 5'-CGGAGTCAACGGATTTGGTCGTAT-3' Reverse- 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	(116)
2.	nitric oxide synthase 3 (endothelial nitric oxide synthase)	*NOS3*	Forward- 5'-CCAGCTAGCCAAAGTCACCAT-3' Reverse- 5'-GTCTCGGAGCCATACAGGATT-3'	(117)
3.	RAB11A, member RAS oncogene family	*RAB11A*	Forward- 5'-TTTTGCAGAGAAGAATGGTTTGTC-3' Reverse- 5'-CCTTTGGCTTGTTCTCAGTGGT-3'	(118)
4.	importin 5 (karyopherin beta 3 & Ran binding protein 5)	*IPO5*	Forward- 5'-CTTTCCAGGACCCATGTGTAG-3' Reverse- 5'-CTTTCCTCTGTTGAGTGCCG-3'	
5.	vascular endothelial growth factor A	*VEGFA*	Forward- 5'-ACGAAAGCGCAAGAAATCC-3' Reverse- 5'-GGAGGCTCCAGGGCATTAG-3'	
6.	interleukin 1, beta	*IL1B*	Forward- 5'-AAACAGATGAAGTGCTCCTTCCAGG-3' Reverse- 5'-TGGAGAACACCACTTGTTGCTCCA-3'	(119)

Supplemental table-2 Table of primer sequences (rat genes)

	Rat Gene name	symbol	Primer sequence (5'-3')	Ref
1.	glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	Forward- 5'-CCAGGGCTGCCTTCTCTTGT-3' Reverse- 5'-CCAGCCTTCTCCATGGTGGT-3'	
2.	vascular endothelial growth factor A	*Vegfa*	Forward- 5'-CAATGATGAAGCCCTGGAGT-3' Reverse- 5'-AATGCTTTCTCCGCTCTGAA-3'	(88)
3.	thromboxane A synthase 1	*Tbxas1*	Forward- 5'-TCCTATAAGGGCAGGACACG-3' Reverse- 5'-GCCAAGAGAACCACGGACAG-3'	
4.	interleukin 1, beta	*Il1b*	Forward- 5'-CATCTTTGAAGAAGAGCCCG-3' Reverse- 5'-GGGATTTTGTCGTTGCTTGT-3'	(88)
5.	nitric oxide synthase 3	*Nos3*	Forward- 5'-ATTGGCATGAGGGACCTGTG-3' Reverse- 5'-CCGGGTGTCTAGATCCATGC-3'	

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CHAPTER 5

CONCLUSION AND DISCUSSION

Section- 5.1 Conclusion (summary of findings)

Following original contributions have been presented in this thesis:

1) Primary hRMEC exhibit nuclear localization of endogenous PTAFR.

2) Nuclear translocation of PTAFR is independent of PAF stimulation as well as internal PAF biosynthesis by hRMEC. In other words, the nuclear localization is agonist-independent.

3) The putative NLS motif present in PTAFR is non-functional, while c-terminal internalization motif present between 311 and 330 amino acid positions of PTAFR is essential its nuclear localization.

4) Rab11a GTPase and Ipo5 act as molecular chaperones during nuclear localization of the receptor both in endogenous and transfected (heterogeneous) cell-systems. GTP-bound (active) state of Rab11a favors this process. To our knowledge, this is a first report unraveling a interaction between rab GTPase and an importin.

5) The cell-surface and intracellular Ptafr perform distinct functions as evident from *in vitro*, *ex vivo* and *in vivo* lines of evidence.

6) The cell Ptafr controls up-regulation of pro-inflammatory cytokines (IL-1β) and vaso-obliterative factors (e.g., *Tabxas1*, an enzyme essential for TXA2 synthesis).

7) On the other hand, nuclear Ptafr primarily governs pro-angiogenic genes, like *Nos3*, and *Vegfa*.

8) In phase-I of oxygen-induced retinopathy (OIR), an animal model of human retinopathy of prematurity (ROP), pharmacological inhibition of cell-surface Ptafr alone successfully prevents pathological vasoobliteration and decreases total area of avascular retina.

9) In phase-II of OIR, antagonism of cell-surface and intracellular Ptafr together significantly reduces retinal neovascularization but not that of the cell-surface receptor alone. (There is no pharmacologic inhibitor currently available to target only intracellular Ptafr).

10) Together (8 and 9), these findings highlight different roles of cell-surface and intracellular Ptafr in controlling retinal vascular endothelial cell phenotypes to produce microvascular changes during ROP.

Section- 5.2 Discussion and future perspectives

5.2.1 Subcellular GPCR trafficking: unanswered questions

While recent work by independent laboratories has highlighted intracrine GPCR signaling in early endosomes (10), mitochondria (11) and nucleus (13, 14), there are important unanswered questions regarding the intracellular trafficking. The agonist-induced internalization of GPCRs with peptide ligands (e.g., oxytocin receptor) would require trafficking of their respective ligands (together or separately from the receptor). The latter process is poorly understood. The orientation of GPCRs at the membranes of subcellular organelles, which is critical for ligand access, is also highly-debated due to lack of direct evidence. Better biochemical tools are needed to answer these questions.

In case of Ptafr, ligands (PAF and PAF-like lipids) can be locally produced at the intracellular membranes (as discussed in chapter-2) and they can freely permeate through phospholipid bilayer. With highly regulated synthesis as well as degradation mechanisms (section-2.1) in place, PAF needs to act rapidly (within minutes). The agonist-independent nuclear localization of Ptafr, along with intracellular retention of newly synthesized ligand, seems to be the most efficient way to augment PAF signaling in vascular endothelial cells. Our results indicate that cell-surface and nuclear Ptafr perform distinct functions. This might be explained, in part, based on the previous observation that cell-surface Ptafr preferentially couples to heterotrimeric Gq, while nuclear Ptafr binds to Gi protein (7). However, it needs to be interpreted with caution as there is often crosstalk between pathways downstream of G-protein coupling and G-protein independent pathways might also be involved. Even though experiments done with stimulation of isolated organelles provide valuable clues, specific intracellular

modulators of GPCR signaling (see below) are needed to better understand their intracrine modes of action.

Lastly, current findings as well as recent papers (13, 14) underscore the importance of spatio-temporal regulation of GPCR transport proteins (e.g., arrestins, importins, and small GTPase) in determining intracrine signaling outcomes. It is also well-known that expression of these proteins is altered in diseases like cancer. This might be important for creation of GPCR signaling bias in certain types of cancer by favoring one subcellular location over another. The vesicular transport mechanism for nuclear-membrane resident proteins also remains poorly understood. Recently, first evidence for presence of vesicles in the nuclear envelope (in space between ONM and INM) was provided for nuclear export of herpes viral nucleocaspid (482) and it has been suggested that such a pathway might be existing for endogenous transmembrane nuclear proteins as well (17). More research is needed in coming years to better understand nuclear translocation of GPCRs and their signaling partners.

5.2.2 Targeting intracellular (nuclear) GPCRs

The GPCRs, like Ptafr, are popular drug targets to treat diseases. Our findings highlight the significance of subcellular localization of GPCR in determination of its functional selectivity. Majority of the canonical GPCR antagonists are cell membrane impermeable but two useful approaches to target intracellular, transmembrane receptors viz. cell-penetrating peptides (CPPs) (483) and lipid-coated nanoparticles (484) are promising to overcome this problem. They can also be delivered to specific, subcellular organelle by tagging with small localization signal peptides (e.g., nuclear localization signal or mitochondria targeting signal) (485, 486).

Most of current modulators acting on cell-surface receptors do so by binding at extracellular domain(s). However, there is growing interest in development of novel class of CPPs called pepducins, which act on intracellular domain(s) of GPCRs (487). This strategy might be a particularly valuable tool for targeting subcellular GPCRs, based on their orientation at intracellular membrane. For example, for a GPCR located at ONM with N-terminus facing intermembrane space and C-terminus facing cytoplasm, the latter will be more easily accessible to pepducins.

5.2.3 Pharmacological modulation of PAF signaling

Over the years, different direct and indirect strategies have been used to modulate PAF signaling.

1. **PLA2 inhibitors** non-specifically inhibit biosynthesis of PAF, prostanoids, and other phospholipid mediators from membrane phospholipids. Though they produce promising results in cultured cells as well as animal models of diverse inflammatory conditions, their clinical trials are yet to yield successful outcome (488, 489). The high incidence of off-target side effects with PLA2 inhibitors can be partly explained by their lack of isoform-specificity as well as their diverse roles in non-inflammatory physiological processes (488).

2. **Ptafr antagonists**- 1980s and 1990s saw rising interest in research community to develop potent Ptafr antagonists. Many of these compounds were evaluated in human trials but failed due to poor efficacy. Of note, a dual inhibitor of Ptafr and histamine receptors, rupatadine, is presently approved for treatment of allergic conditions.

Our current findings highlight the importance of membrane permeability of Ptafr modulators to act on cell surface and/or intracellular population of the receptor. This

information is valuable to choose right antagonist to treat a specific condition and minimize side-effects. For example, in metastatic cancers, where pro-angiogenic action of PAF on blood vessels is a desired target for inhibition, membrane permeable Ptafr antagonist like WEB-2086 would be the choice of treatment. As mentioned earlier, there is no known modulator (agonist or antagonist) acting exclusively on the intracellular Ptafr so far. Nonetheless, a new class of Ptafr antagonists, which neutralize circulating PAF and PAF-like lipids, has been described recently. These biotinylated heptapeptides with d-amino acids are highly stable in plasma (490). The oligopeptides, with some modifications, could be an useful tool to target intracellular PAF (retained by vascular endothelial cells) in future.

5.2.4 concluding remarks

In summary, the present work and growing number of reports on functional intracellular GPCRs from recent years (11, 13, 14, 491) open new doors to study bias signaling. Better understanding of subcellular trafficking of GPCRs and their *in vivo* roles in health and disease are of paramount importance for development of tools to target the intracrine receptors and their signaling partners. The rapidly developing field of nanomedicine seems most promising in the latter regard (484, 485). As a result, in the not-so distant future, targeting a receptor for specific functions based on its cellular localization must be considered in the process of drug discovery, and development.

CHAPTER 6

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APPENDIX- A

Appendix A

Appendix-A consists of

1. A summary of functions of Ras superfamily of small GTPases
2. Sequence alignment of human, rat, mouse and pig PAF receptors

Summary of functions of Ras superfamily of small GTPases:

Ras superfamily of small GTPases can be divided into nine distinct sub-families. The table, on the next page, summarizes their main functions (1, 2). Different members of Ras superfamily are found at the cell nucleus (3), some by virtue of presence of conserved NLS motifs (4). However, only Ran GTPase has been linked to the nucleocytoplasmic protein transport to date (5). Out of nine sub-families, we decided to focus our attention to Rab GTPases for following reasons (6):

1. The family is conserved in all eukaryotes.
2. Rabs primarily control vesicular trafficking between subcellular compartments and directly interact with various membranes components.
3. Many rabs exhibit specific intracellular localization as well as cargo selectivity for vesicular transport.
4. GPCRs require vesicular transport to reach subcellular destinations because of presence of seven hydrophobic transmembrane domains.

	Subfamily	Main function(s) / physiological roles	Approx. # of members in mammalian genome
1	Ras	Cell proliferation	32
2	Rho	Cytoskeletal reorganization, and gene expression	20
3	**Rab**	**Vesicular trafficking between membrane compartments**	**70**
4	Sar1/Arf (ADP ribosylation factor related proteins)	Vesicular trafficking (often overlapping functions with rabs mainly in intra-golgi transport)	30
5	Ran	Nucleocytoplasmic protein transport	1
6	Rheb	mTOR pathway	2
7	Rad	Exact function not yet known. (RRAD- Ras related associated with diabetes)	1
8	Rit	Exact function not yet known (proposed role in neuronal differentiation)	2
9	Miro	Mitochondrial transport	2

Sequence alignment of human, rat, mouse and pig PAF receptors

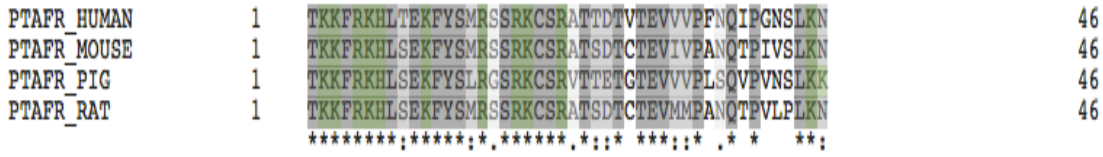
Both human and pig PAF receptors consist of 342 amino acids, while rat and mouse PAF receptors lack one amino acid in 3rd intracellular loop. The predicted topology of human PTAFR is as follows (amino acid positions in brackets):

1. N-terminus: (1-16)
2. First transmembrane domain: (17-38)
3. First intracellular domain: (39-54)
4. Second transmembrane domain: (55-74)
5. First extracellular domain: (75-91)
6. Third transmembrane domain: (92-113)
7. Second intracellular domain: (114-133)
8. Fourth transmembrane domain: (134-155)
9. Second extracellular domain: (156-184)
10. Fifth transmembrane domain: (185-205)
11. Third intracellular domain: (206-233)
12. Sixth transmembrane domain: (234-254)
13. Third extracellular domain: (255-276)
14. Seventh transmembrane domain: (277-296)
15. C-terminus: (297-342)

The main goal of sequence alignment was to identify regions of similarity (conserved sequence), with focus on C-terminal region because it contains multiple structural and functional motifs in Ptafr (discussed in section-2.1). The sequence alignment was done using NCBI's Cobalt Constraint-based Multiple Protein Alignment Tool (7).

1. Human (Homo sapiens) PTAFR- NCBI Reference Sequence: NP_001158195.1
2. Pig (Sus scrofa) Ptafr- GenBank: AAD28739.2
3. Rat (Rattus norvegicus) Ptafr- NCBI Reference Sequence: NP_445773.1
4. Mouse (Mus musculus) Ptafr- NCBI Reference Sequence: NP_001074680.1

Only alignment of C-termini of the four PAF receptors is shown below.



Summary of findings:

1. 30 out of 46 amino acids (65.2%) in the C-terminus are conserved in all four species.
2. The C-terminus of human PTAFR is ~ 78%, 76% and 82% identical with that of rat, pig and mouse Ptafr, respectively.
3. The C-termini of PAF receptor in all four species contain small clusters of basic amino acid residues (highlighted in green), which have been predicted to play a role in intracellular localization of other class-A GPCRs (8).

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APPENDIX- B

Appendix- B Subcellular G-protein coupled receptor signaling hints at greater therapeutic selectivity

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Abstract

G-protein coupled receptors (GPCRs) evolved as specialized sensors of the extracellular environment. Comprising the largest family of cell surface receptors, GPCRs are common therapeutic targets. Over the last 25 years, several GPCRs have been observed at the cell nucleus, suggesting the presence of intracrine GPCR signaling beyond the plasma membrane. Yet specific physiological functions of nuclear GPCRs had not been reported, until lately. We recently uncovered distinct but complementary angiogenic roles of *F2rl1* (formerly known as PAR2) depending on its subcellular localization at the plasma membrane or at the nucleus. Targeting subcellular compartments to improve drug selectivity may therefore inspire novel therapeutic strategies for transmembrane receptors.

Key words- angiogenesis, cell nucleus, F2rl1, G-protein coupled receptor, intracrine signaling

1. Intracrine G-protein coupled receptor signaling

The phrase 'intracrine signaling' was initially coined to describe the action of peptide hormones on intracellular receptors (1). Increasing evidence suggests that G-protein coupled receptors (GPCRs) can also signal inside the cell (2-4). At the plasma membrane, GPCRs bind heterotimeric G-proteins and b-arrestins to form a signaling platform for second messenger cascades. However, alternative modes of transcriptional regulation have expanded GPCRs' signaling repertoire. In response to delta-opioid receptor activation, b-arrestin1 translocates to the cell nucleus where it directly regulates histone acetylation and gene transcription, bypassing traditional second messengers (5). An example of targeting transcriptional events even more directly involves a fragment of Frizzled2 C-terminus, which is able to migrate to the nucleus upon activation (6), with a unique signaling signature in neurons (7). Full-length GPCRs also internalize following activation and redistribute to early endosomal compartments where cyclic AMP is produced for extended period of time (2, 8). Similarly mitochondrial cannabinoid receptor 1 (Cnr1) triggers cAMP production and protein kinase-A activation in murine hippocampal neurons (3). GPCRs and their signaling partners have also been detected at the trans- golgi network (e.g., Gpr107) (9) and melanosomes (e.g., Gpr143) (10) in recent years. Hence, the plasma membrane can no longer be considered the exclusive signaling locus of GPCRs. However, the trafficking and signaling pathways of intracellular non-nuclear GPCRs remain largely unknown.

Since 1987 (11), more than 30 GPCRs have been detected at the nuclei of endogenous (non-transfected) cells or tissues (4, 12-16). The presence of phospholipids in the nucleoplasm (17) and nuclear bodies (18), as well as electron microscopy evidence of nuclear membrane invaginations (19) support the existence of intranuclear network of membranes and hydrophobic regions, that could harbor nuclear GPCRs. As GPCRs, tyrosine kinase receptors previously believed to be solely expressed at the plasma membrane have now been uncovered

at the nucleus, having distinct signaling signatures (20). Although the presence of transmembrane receptors at the nucleus is accepted, the mechanisms governing their nuclear localization remain actively investigated.

2. Journey to the center of the cell

Subcellular localization sequence motifs and structural domains dictate the trafficking fate of GPCRs. Following synthesis, GPCRs undergo post-translational modifications in the ER-Golgi network. From the trans-golgi, receptors are delivered to their final destination using the vesicular transport pathway that is tightly regulated by a network of intracellular small GTPases (21). Interestingly, many GPCRs contain putative nuclear localization signal (NLS) motifs (14) recognized by importins, which are carrier proteins of the karyopherin superfamily. Sorting-nexins (SNX) are also involved in the intracellular sorting of cargo proteins, albeit discovered more recently (22). In addition, cytoskeletal tracks (microtubules, micro- and intermediate filaments) and carrier motors (dynein and kinesin) drive the vesicular transport of many receptors. Ultimately, the spatio-temporal distribution of the endocytic cargos is determined by the timely interaction of these players, highlighting once more the complexity of intra- cellular trafficking and ensuing signals. In the case of F2rl1, we found two NLS motifs required for agonist-induced trans- location of the receptor mediated by importin a/b hetero-dimer and Snx11, using retrograde motor dynein via microtubules to reach the nucleus (4). The oxytocin receptor also translocates to the nucleus upon agonist stimulation and the process is regulated by transportin (12). Conversely, bradykinin (B2R) and apelin receptors that contain putative NLS motifs are constitutively expressed at the nuclear membrane (14). To date, mechanisms that dictate constitutive or agonist-induced nuclear localization of GPCRs remain unknown. Choosing the right subcellular therapeutic targeting strategy will require knowledge of the cellular origin of the nuclear GPCR.

3. Location, location, location: why signaling from the nucleus matters to GPCRs?

The presence of GPCRs at the cell nucleus suggests unique subcellular functions. Accordingly, an elaborate GPCR signaling machinery is found at the nucleus, including heterotrimeric G-proteins (23), beta-arrestin1 (24), G-protein--receptor kinases (25), regulators of G protein signaling (26) and nuclear membrane channels (27) involved in the regulation of nuclear calcium currents, the latter affecting gene expression (28). Indeed, direct stimulation of pure isolated nuclei can also result in calcium transients and distinct gene expression profiles (16). In heart myocardium, nuclear b-adrenergic and endothelin receptors regulate nitric oxide production, thereby, indirectly modulating gene expression (13). Finally, we recently showed that proximity to the genome allows F2rl1 to form a transcriptional complex that directly binds DNA. Nuclear F2rl1 chromatin immuno-precipitation and sequencing of DNA fragments (ChiP-Seq) confirmed a unique transcriptional program at the nucleus (4). Hence, nuclear localization of GPCRs may favor in situ interaction with transcription factors and DNA creating a subcellular geographic signaling bias.

To confirm that distinct *in vivo* phenotypes are conferred by location-specific signaling events, we used F2rl1 deficient mice in which mutant F2rl1 receptors, either preventing nuclear translocation or remaining at the cell nucleus, were introduced in the developing retina. Plasma membrane F2rl1 caused early vascular maturation concordant with increased angiopoeitin-1 (*Ang1*) and reduced vascular endothelial growth factor-a (*Vegfa*) synthesis. Nuclear F2rl1 instead promoted a proliferative vascular phenotype, producing more *Vegfa* and less *Ang1*. Thus, the same GPCR (F2rl1) in different subcellular locations orchestrates distinct yet complementary angiogenic functions important to retinal vascular development (Figure 1) (4). Along similar lines, the nuclear oxytocin receptor was recently shown to promote osteoblastic gene expression, required *in vivo* for bone formation (12). Dissecting the relative

physiological significance of distinct subcellular signals remains a practical challenge but it is the ultimate readout of our efforts to achieve greater therapeutic signaling selectivity.

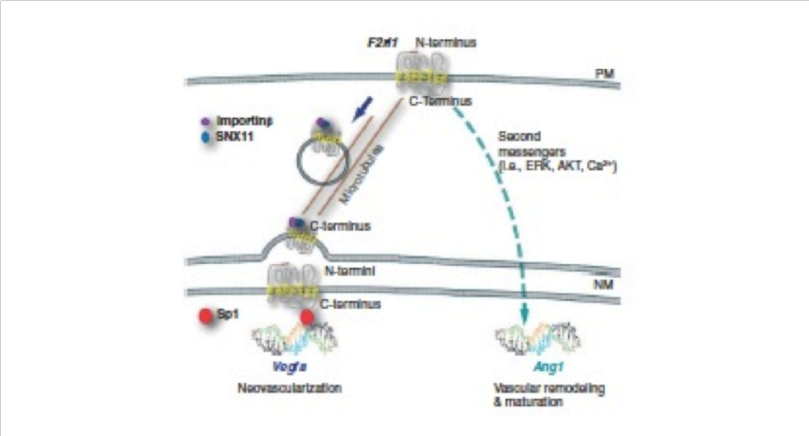

Figure 1. Subcellular localization of F2rll governs angiogenesis. Schematic representation of F2rll translocation from the plasma membrane to the cell nucleus, requiring Snxll and importin-beta1 on microtubules; ensuing *Vegfa* expression involves recruitment of transcription factor (Sp1), whereas signaling from F2rll at the plasma membrane results in *Angl* expression.

Putative orientation of nuclear G-protein coupled receptor (GPCR) with N-termini buried within the nuclear GPCR with n-terminal buried within the nuclear envelope, exposing the C-terminus to the cytoplasm (outer NM) or nucleoplasm (inner NM).

Adapted from Ref (4). NM: Nuclear membrane; NM: Plasma membrane.

4. Expert opinion: targeting GPCR subcellular localization to achieve greater therapeutic specificity

Understanding GPCRs signaling bias at the plasma membrane may help to improve drug selectivity. To identify bias signaling, conformational changes in receptor's quaternary structure induced by orthosteric or allosteric modulators are assessed against their functional impact. This iterative exploratory approach will likely be necessary to uncover the means that govern the subcellular distribution of nuclear GPCRs, such as F2rl1. Upon activation at the plasma membrane, F2rl1 simultaneously triggers traditional second messenger cascades and dispatches a pool of receptors to the cell nucleus (and late endosomes); the presence of F2rl1 at the nucleus is negligible prior to stimulation (4). Hence, pharmacologically tilting the balance between the plasma membrane and nuclear translocation signals will require greater understanding of F2rl1 quaternary structure and the conformational changes that expose the NLS motifs at the expanse of the b-arrestin binding site, for example. Alternatively, interfering with the assembly of carrier proteins (importins, Snx11, dynein) on the receptor may prevent its nuclear translocation, preserving only the plasma membrane signals. Therefore, targeting agonist- induced nuclear translocation mechanisms will involve strategies to modulate signaling at the plasma membrane and relies on pharmacological selectivity and allosteric modulation. Specifically targeting GPCRs, which are constitutively distributed inside the cell, raises a unique set of delivery challenges. Ligands would first need to cross the plasma membrane and bypass their cell-surface counterparts, in order to reach intracellular targets. Many endogenous as well as synthetic GPCR ligands are hydrophilic and, thus, impermeable to the plasma membrane. 'Caged ligands' with hydrophobic protective groups that permeate the cell membrane have recently been used to study intracrine GPCR signaling in intact cells, instead of isolated organelles (13, 29). The ligands are activated intracellularly by exposing the

cells to UV light, which removes caging groups without any change to the parent compound. So far, caged ligands have been reported for endothelin, beta-adrenergic and angiotensin-II receptors (13, 29). Hence, these ligands offer a solution for targeting intracellular receptors, but are not specific for nuclear GPCRs. Moreover, the application of this approach *in vivo* is not readily amenable.

The double-membrane anatomy of the nuclear envelope poses a signaling dilemma. At the cell-surface, GPCRs' N-termini face the extracellular matrix and their ligands bind to one or more of the extracellular domains, whereas inside the cell heterotrimeric G-proteins interact with the C-termini. However, the exact orientation of a GPCR at the nuclear envelope is currently unknown. It is hypothesized that the C-terminus would face either cytoplasm or nucleoplasm for a GPCR located at the outer or the inner nuclear membrane respectively, to allow the interaction with afore- mentioned signaling messengers (Figure 1). Accordingly, the ligand-binding domain(s) of GPCRs would be buried inside the nuclear envelope, between outer and inner nuclear membranes. This is not a concern for lipid agonists that can be synthesized locally from nuclear membrane phospholipids, such as the platelet activating factor (PAF) and its nuclear receptor (30). Other hydrophilic ligands such as norepinephrine use selective membrane transporters (OCT3) at the nuclear envelope and activate nuclear α1-adrenergic receptors in cardiomyocytes (15). Presently, two promising intracellular drug-delivery systems include lipid-coated nanoparticles and cell-penetrating peptides (CPPs) (31, 32). Some of them use organelle-specific localizing tags, such as NLS for nuclear localization (31, 32) and cleavable mitochondria targeting signal (MTS) for mitochondrial drug delivery (33). However, to date, no drug-delivery strategies have been designed to specifically target nuclear membrane proteins. In our quest to achieve greater therapeutic selectivity, we may expand the therapeutic repertoire of current drugs by developing new intracellular delivery strategies that will harness the functional significance of subcellular GPCR signaling.

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J-S Joyal and VK Bhosle contributed comparably to this work.

Declaration of interest

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APPENDIX- C

Appendix- C List of publications

A. Part of the thesis:

1. High-resolution imaging and function of Nuclear G-protein coupled receptors (GPCRs)

   Bhosle V. K., Gobeil F., Jr., Rivera J. C., Ribeiro-da-Silva A., Chemtob S. (2015) ***Methods Mol Biol*** 1234: 81-97. PMID: 25304350.

2. Nuclear localization of platelet-activating factor receptor controls retinal neovascularization

   Bhosle V. K., Rivera J. C., Zhou T. E., Omri S., Sanchez M., Hamel D., Zhu T., Rouget R., Al Rabea A., Hou X., Lahaie I., Ribeiro-da-Silva A., Chemtob S. (Submitted to ***Cell Discovery*** in 2016/03, Manuscript # CELLDISC-00121-T)

3. Subcellular GPCR signaling hints at greater therapeutic selectivity

   Joyal J. S.*, **Bhosle V. K.***, Chemtob S. (2015) ***Expert Opin Ther Targets*** 19: 717-21. PMID: 25976229. The article is included in appendix-B.

   Note- *- These authors contributed equally to the respective works.

B. Not part of the thesis:

1. Subcellular localization of coagulation factor II receptor-like 1 in neurons governs angiogenesis

   Joyal J. S., Nim S., Zhu T., Sitaras N., Rivera J. C., Shao Z., Sapieha P., Hamel D., Sanchez M., Zaniolo K., St-Louis M., Ouellette J., Montoya-Zavala M., Zabeida A., Picard E., Hardy P., **Bhosle V.**, Varma D. R., Gobeil F., Jr.,

Beausejour C., Boileau C., Klein W., Hollenberg M., Ribeiro-da-Silva A., Andelfinger G., Chemtob S. (2014) ***Nat Med*** 20: 1165-73. PMID: 25216639.

2. Developmental Pharmacology and Pharmacokinetics
   Bhosle V. K., Altit G., Autmizguine J., Chemtob S.
   A pharmacology chapter in "***Fetal and Neonatal Physiology***" by Richard A. Polin, MD and Steven H. Abman, MD. 5th edition, Expert Consult - Online and Print, 2-Volume Set. (Accepted and in press for 2016/07)

3. Tailoring myopic CNV treatment based on pre-existing ophthalmic condition

   Zhou T. E., **Bhosle V. K.**, Chemtob S. Letter to the editor. (2015) ***Invest Ophthalmol Vis Sci*** 56 (13): 8362-3. PMID: 26747765.

4. SNX30 negatively regulates Wnt/β-catenin signaling

   Cameron M., Leclerc S., **Bhosle V. K.***, Sanchez M.*, Rouget R., Beauséjour C., Parker A., Dubois N., Gagnon C., Wünnemann F., Chemtob S., Andelfinger G. (Submitted to ***Traffic***, currently in 2nd round of revision)

5. Choroidal involution is associated with a progressive degeneration of the outer retinal function: early role for interleukin-1β

   Zhou T. E., Rivera J. C., **Bhosle V. K.**, Lahaie I., Shao Z., Tahiri H., Zhu T., Lachapelle P., Chemtob S. (Submitted to ***Am J Pathol***, currently in revision)

6. The succinate receptor GPR91 signals from the Endoplasmic Reticulum

   Sanchez M.*, Hamel D.*, **Bhosle V. K.**, Duhamel F., Rouget R., Roy O., Nadeau-Vallée M., Zheng S., Julien L. A., Zhu T., Rivera J. C., Sitaras N., Omri S., Hou X., Sapieha P., Mitchell G., Ribeiro-da-Silva A., Chemtob S. (Manuscript in preparation)

   Note- *- These authors contributed equally to the respective works.

7. Nuclear GPCRs: Journey to the Center of the Cell

   Bhosle V. K., Chemtob S., Joyal J. S. (Review, manuscript in preparation)