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Mechanism and Function of Nuclear HCAR1

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Mechanism and Function of Nuclear HCAR1

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Résumé:

Les récepteurs couplés aux protéines G (RCPG) sont une famille de protéines hautement conservée chez les eucaryotes et constituent la plus grande famille de récepteurs. Ces récepteurs sont impliqués dans presque tous les processus physiologiques, mais leur capacité à réguler un vaste éventail de processus biologiques différents fait l'objet de recherches intenses. Bien qu'ils soient classiquement considérés comme des récepteurs de la membrane plasmique, les RCPG sont présents dans tous les organites membranaires intracellulaires et certains d'entre eux ont la capacité de transduire des signaux à partir de ces organites. La signalisation d'un RCPG à partir de ces organelles intracellulaires est appelée signalisation biaisée par la localisation et cette signalisation peut avoir un résultat fonctionnel différent de celui des événements de signalisation du récepteur localisé dans la membrane plasmique. La signalisation biaisée par la localisation est un concept émergent en biologie des RCPG et peut ajouter une couche supplémentaire à la fonction du récepteur. D'autre part, avec la détection de certains RCPG à l'intérieur de différents organites, y compris le noyau, une modalité fonctionnelle non réceptrice pour les RCPG pourrait être postulée et pourrait également expliquer les divers rôles de cette famille. Cependant, cet aspect est presque entièrement inexploré.

HCAR1 (GPR81), en tant que RCPG, est activé de manière endogène par le lactate et il a été démontré qu'il favorise la malignité du cancer en favorisant un niveau plus élevé de glycolyse dû à l'effet Warburg et cela par différentes voies. Son niveau d'expression est très élevé dans de nombreux cancers et présente une corrélation négative avec le pronostic du patient. Cependant, son mécanisme d'action n'est pas bien compris. Dans cette thèse, nous avons étudié la localisation nucléaire et les rôles potentiels du HCAR1 et nous avons découvert que ce récepteur est localisé à la membrane nucléaire et à l'intérieur du noyau, en plus de sa localisation à la membrane plasmique. Le HCAR1 nucléaire (N-HCAR1) est capable d'induire une signalisation intranucléaire basée sur la localisation pour induire la phosphorylation de ERK et d'AKT dans le noyau. En utilisant des approches protéomiques et génomiques, nous avons découvert que N-HCAR1 est impliqué dans plusieurs fonctions non réceptrices régulant différents processus à travers ses interactomes nucléaires. Ce regroupement nucléaire de HCAR1, en fonction de ses facteurs de liaison, favorise la

traduction des protéines, la biogenèse ribosomale et la réparation des dommages à l'ADN. De manière intéressante, N-HCAR1 interagit également avec des facteurs de remodelage de la chromatine et régule directement l'expression des gènes d'après notre séquençage ChIP à l'échelle du génome. Nous avons également effectué un séquençage de l'ARN et les résultats montrent que N-HCAR1 régule l'expression d'un réseau de gènes plus large que son homologue de la membrane plasmique. Notamment, l'exclusion nucléaire de HCAR1 s'est avérée avoir le même effet que son knockdown complet sur la croissance tumorale et les métastases *in vivo*. Nos données révèlent une signalisation basée sur la localisation et des fonctions non canoniques pour un RCPG dans le noyau par lesquelles HCAR1 peut réguler différents processus cellulaires.

Mots-clés: Fonction non réceptrice, RCPG, Biais de localisation, HCAR1, Lactate, Effet Warburg.

Abstract:

G Protein-Coupled Receptors (GPCR) are a highly conserved protein family in eukaryotes through evolution and they are the largest receptor family. These receptors are virtually involved in every physiological processes, but their ability to regulate such a vast array of different biological processes is under intense investigation. Although classically considered a plasma membrane receptor, GPCRs are found in every intracellular membranous organelle and some of them are shown to have the capacity for signal transduction from those organelles. The signaling of a GPCR from these intracellular organelles is called location-biased signaling and this signaling could have a different functional output than the signaling events from the plasma membrane-localized receptor. Location-biased signaling is an emerging concept in the GPCR biology and can add an extra layer to the receptor function. On the other hand, with the detection of some GPCRs inside different organelles including the nucleus, a non-receptor functional modality for GPCRs could be postulated and could also account for the diverse roles of this family. However, this aspect is almost entirely unexplored.

HCAR1 (GPR81), as a GPCR, is endogenously activated by lactate and has been shown to promote cancer malignancy via a higher level of glycolysis due to the Warburg effect, through different pathways. Its expression level is highly elevated in many cancers and negatively correlates with the patient's prognosis. However, its mechanism of action is not well understood. In this thesis, we investigated the nuclear localization and potential roles of HCAR1 therein and we found this receptor is localized to the nuclear membrane and inside the nucleus, besides its plasma membrane localization. The Nuclear HCAR1 (N-HCAR1) is capable of inducing location-biased signaling intranuclearly to induce nuclear-ERK and AKT phosphorylation. Using proteomics and genomics approaches, we discovered that N-HCAR1 is involved in several different non-receptor functions regulating different processes through its nuclear interactomes. This nuclear pool of HCAR1, depending on its binding factors, promotes protein translation, ribosomal biogenesis, and DNA-damage repair. Interestingly, N-HCAR1 also interacts with chromatin remodeling factors and directly regulates gene expression based on our genome-wide ChIP-sequencing. We also performed RNA-seq, and the results show N-HCAR1

regulates the expression of a broader gene network than its plasma membrane counterpart. Notably, nuclear exclusion of HCAR1 proved to have the same effect as its complete knockdown on tumor growth and metastasis *in vivo*. Our data reveal location-biased signaling and non-canonical functions for a GPCR in the nucleus by which HCAR1 can regulate different cellular processes.

Keywords: Non-receptor function, GPCR, Location bias, HCAR1, Lactate, Warburg effect.

Preface:

This thesis is written in a hybrid manuscript format and it is divided into four chapters. The first chapter is the introduction containing the literature review and research hypothesis. The second chapter is in the format of a full-length original research manuscript; this manuscript is under revision in *Nature Communications*. The third chapter is in the format of a brief communication manuscript covering an extension to some of the data from the main article (chapter two); this manuscript is under revision in *Cell Communication and Signaling*. The final chapter is the discussion of the thesis. The thesis also, contains three manuscripts as annexures for more extensive elaboration. Two of these manuscripts are review papers, one published in *iScience* and one submitted; and one is a published commentary article in *Frontiers in Pharmacology*. There are also three data sets from our high-throughput experiments in the format of excel files. They are provided separate from the thesis file as they are not compatible in the format.

Acknowledgement:

Foremost, my highest gratitude goes to Dr. Chemtob, whose support was beyond what I could have imagined. Dr. Chemtob made me regain my forgotten enthusiasm and passion for academia and research after changing my previous PhD lab. His encouragements, mentorship, and support were the turning points in my future career and life goals; and his trust is the reason that I truly enjoyed *the exploration* in science and had a great time doing my PhD. I owe him my personal and professional growth in these impactful years.

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I had the opportunity to work with some of the best lab members who constantly helped me through my PhD. Dear Christiane, the best lab manager I have seen; Gael, who did all the bioinformatics and I really bugged him during all my PhD by constantly asking him for more analysis. Emmanuel, one of the nicest and warmest people, and one of the most knowledgeable people that I had the pleasure to work with and always was there to give me the best advice; he become a dear friend. I owe the joy of lab life to all my great lab members (Prabhas, Monir, Ali, France, and Tiffany); especially France for her kind French translation of my abstract.

I want to thank all the supporting agencies who funded me and my project, literally making it possible for me to do both this project and my PhD; all the Canadian tax payers, CIHR, Fonds Suzanne Véronneau-Troutman et Fonds de recherche en ophtalmologie de l'Université de Montréal, Molecular Biology program and faculty of Medicine scholarships.

On a personal note, I am in eternal debt to my wife, the joy and motivation of my life. Her kind and supportive heart and her love are a glimpse of hope in my world. She turned out to be the incentive of life in my nihilistic worldview. I am in eternal debt to my family, they are the reason I am here; the endless sacrifices of my mom in raising us with her infinite devotion, and my stepdad who loved and supported us beyond any dad could. My lovely sisters (Nehzat and Behjat) taught me to pursue my dreams and grow as the person I am today. My little brother (Eshragh); whose presence is such a joy that inspired me to push myself to become a better man, so I might be a role model for him. My dad taught me life is tough and how to deal with it. Many thanks to my dear friends (Mahsa, Amin, Hamed, and Saeideh) who became my family in the foreign country and made the life here a bliss, who were there for me in these years through happiness and sadness.

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List of abbreviations:

3-OBA: 3-hydroxy-butyrate acid

AC: Adenylyl cyclase

AT1aR: Angiotensin 1A receptor

cAMP: Cyclic adenosine monophosphate

DAG: Diacylglycerol

ECL: Extracellular loop

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor, a receptor tyrosine kinase

EMT: Epithelial-mesenchymal transition

ER: Endoplasmic reticulum

ERK: Extracellular-signal Regulated Kinase

Erα: Estrogen receptor α

GAIN: GPCR-autoproteolysis inducing domain

GEF: Guanine nucleotide exchange factors

GDP: Guanosine diphosphate

GLUT: Glucose Transporters

GPCR: G protein-coupled receptor

GPR81: G protein-coupled receptor 81

GRK: GPCR kinases

GTP: Guanosine-5'-triphosphate

HCAR1: Hydrocarboxylic acid receptor 1

ICL: Intracellular loop

IHC: immunohistochemistry

IP3: Inositol triphosphate

KO: knockout

KD: Knockdown

MAPK: Mitogen-activated protein kinase

MCT: Monocarboxylate Transporters

MET: Mesenchymal-epithelial transition

N-HCAR1: Nuclear HCAR1

PI3K: Phosphoinositide 3-kinases

PIP2: phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PM: Plasma membrane

PPP: pentose phosphate pathway

PTM: post-translational modification

SSP: serine/glycine synthetic pathway

VEGF: Vascular endothelial growth factor

Chapter 1

Introduction:

Life is a very complex phenomenon and could encompass a wide range of definitions and criteria. But probably, most of these definitions and criteria would include the capacity to grow, reproduce, and interact with the environment. These few main factors are determinants in the distinction of life from non-live matter. Among these three, the ability to interact with the environment could directly dictate the first two. The abundance of food in the environment could direct an organism to grow, and food plus security probably could direct an organism to reproduce. Thus, every life form needs to interact with its environment. These interactions are the primary foundations of life and a better understanding of the nature of these interactions would help us to better understand our own nature as well as life.

Basically, everything happening at the biological level requires an environmental input at a direct or indirect level to generate an output. These environmental inputs could be sensed by various modes in an organism, but probably the most common mechanism adopted for this sensation is cellular receptors. Receptor as the name suggests, are modules that can receive the environmental input and translate it to biological language in cells to produce the desired output. These biological languages are called "*signaling*", a process by which the receptor signals the cell what to do based on those inputs.

Depending on the input, these receptors could be anywhere in the cell: on its membrane, on its organelles, or inside it. We generally have looked for these receptors at the cell surface since it is the first line separating the cell from the outside world and potentially the first place getting in contact with the environmental inputs. The largest receptor family in the higher forms of life, *eukaryotes*, is called G Protein-Coupled Receptors (GPCRs). We tremendously rely on this family to interact with the environment; we see the light through these receptors (e.g., Rhodopsin), we smell through these receptors (e.g., olfactory receptors), and we feel through these receptors, whether it is a feeling for danger (e.g., adrenergic receptors) or love (e.g., oxytocin receptor), we feel through these receptors. As you can see, understanding this family would help us to understand our nature.

1. **G Protein-Coupled Receptors (GPCRs):**

GPCRs have the highest number of members among all receptor families in eukaryotes. The GPCR family has approximately 831 members and almost 4% of the human protein-coding genome is allocated to this family which is a large number for just one protein family[1]. GPCRs are highly conserved through evolution in all eukaryotes and they are descendants of prokaryotic proteins which have similar structures and molecular mechanisms such as microbial rhodopsins[2]. The majority of human GPCRs are olfactory and around 350 of them are non-olfactory receptors which are virtually involved in every biological processes[1,3]. From 350 non-olfactory GPCRs, around one-third of them are not de-orphanized so far and have no identified ligand[4]. Besides the olfactory GPCRs which respond to odor molecules, the other two third of non-olfactory GPCRs have a diverse ligand repertoire including lipid molecules, nucleotides, carbohydrates, peptides, and even photons[5]. Their significance in human physiology is attested by the fact that around 40% of all FDA-approved drugs target this family[6].

1.1. GPCR structure:

GPCRs have a distinct structural feature known as 7-transmembrane receptors. This structure consists of 5 different domains: 1) an N-terminus, 2) 7 helical transmembrane domains, 3) 3 extra-cellular loop domains (ECL), 4) 3 intra-cellular loop domains (ICL), and 5) a C-terminus (Fig. 1). The N-terminus of the protein is facing the extracellular environment, while the C-terminus is towards the inside of the cell. The 7-helical transmembrane domains are highly hydrophobic and according are incorporated into the lipidic plasma membrane. These 7 transmembrane domains are connected to each other via the ECL and ICL domains[7]. These 7-transmembrane domains plus the ECL and ICL parts of the receptor are spatially organized in a cylindrical shape and binding to a ligand enables conformational changes in the cylinder as well as the C-terminus of the receptor leading to the receptor activation[8].

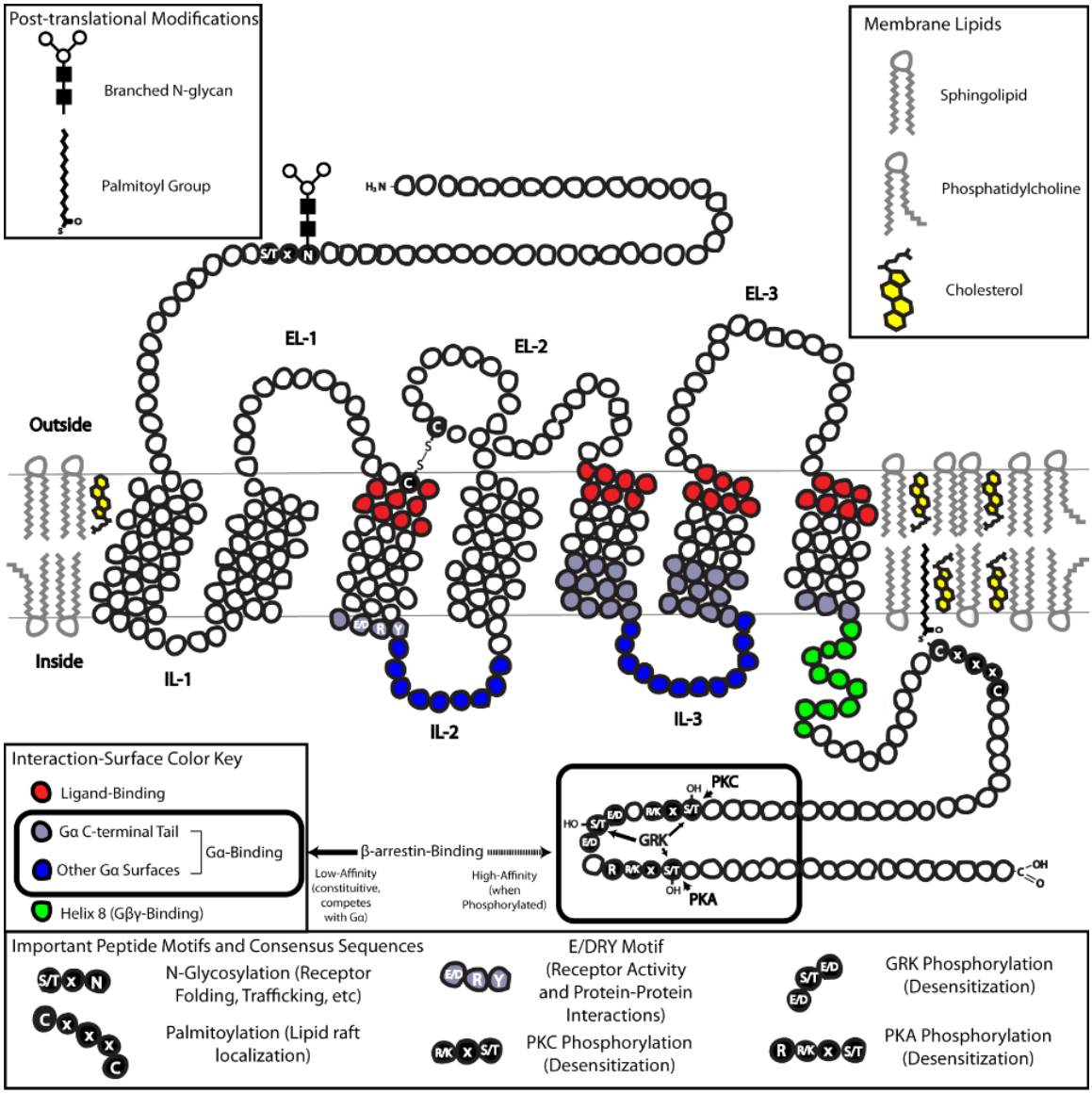

Figure 1: Generic GPCR 2D structure schematics (adopted from Wikipedia)

1.2.GPCR classification:

The most accepted system for GPCR classification is called the "*A-F system*" which is based on the receptor homology and amino acid sequence similarity as well as functional similarities[9]. These groups are as below[10]:

Class A) Rhodopsin-like receptors: This is the largest group and as the name suggests, they are homologically related to Rhodopsin GPCR, and are activated by light,

neurotransmitters, and hormones such as thyroid stimulating hormone, follicle-stimulating hormone, and luteinizing hormone[11].

Class B) Secretin Receptors: With around 70 receptors in this family, they are recognized by their long N-terminal domain containing about 120 amino acids which are usually stabilized by disulfide bonds[12]. High molecular weight molecules such as calcitonin and glucagon are among their ligands[13].

Class C) Metabotropic glutamate/pheromone receptors: This receptor class has an even larger N-terminus reaching up to 600 residues. These amino acids are involved in ligand binding. The class contains conserved cysteine-rich domains enabling the binding of the N-terminus to the ECL1. GABA receptors, taste receptors, calcium-sensing receptors are members of this class [14].

Class D) Fungal mating pheromone receptors: This class contains 2 receptors: STE2 and STE3. They are required for pheromone sensing and mating of haploid *Saccharomyces cerevisiae* and signal through the mitogen-activated protein kinase (MAPK) pathway to ultimately induce diploid formation[15,16].

Class E) cAMP receptors: These receptors distinctly control the development of *Dictyostelium discoideum*. Their signaling leads to aggregation of individual cells in order to form multicellular organism[17,18].

Class F) Frizzled/Smoothened receptors: This class is considered an atypical GPCR class which are the target of Wnt ligands. They are involved in various cell and physiological processes such as cell polarity, cell proliferation, embryonic and neural development. Their signaling is mediated through canonical Wnt/β-catenin pathway and non-canonical Wnt signaling[19,20].

Although this is the most common classification system, there are other systems in place as well. One of those is called GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin), and it is widely known for the separate classification of adhesion GPCRs, which are included in class B in the A-F system[21,9]. The N-terminus of adhesion GPCRs are exceptionally large and they contain various domains including a GPCR-autoproteolysis inducing domain (GAIN)[22]. Their N-terminus can act both as ligand or

inhibitor of their own receptor, depending on the receptor[5]. This class is known for its involvement in cellular adhesion and migration[23], but they have different functions as well (e.g., differentiation and development[24]).

1.3. GPCR signaling

As the name of the receptors suggests, they are mostly known for their association with G-proteins. Their signaling vastly relies on the G-proteins activation, but it is not limited to this pathway[25] (Fig. 2).

1.3.1. G-protein dependent signaling

The 7-helical transmembrane domains of the GPCR plus the ICL domain inside the cytoplasm provide a docking site for unstimulated heterotrimeric G-proteins[26]. The Gα and Gγ subunits are anchored to the plasma membrane and the Gβ subunit strongly remains bound to Gγ[27]. In the inactive state of the receptor, the heterotrimeric G-proteins are bound with the GPCR, however, upon the ligand-induced conformational change in the receptor, the G-proteins get activated and dissociate from the receptor and are able to actively participate in downstream signal transduction. Active conformation of a GPCR acts as a guanine nucleotide exchange factors (GEFs) and switched the GDP in the Gα subunit with a GTP[28].

1.3.1.1. Gα signaling

Gα is a guanine nucleotide-binding protein and is able to hydrolyze GTP with its innate GTPase activity. It has four main subfamilies which define the downstream signaling of a GPCR. $G\alpha_s$ stimulates the accumulation of cAMP through adenylyl cyclase (AC), while $G\alpha_i$ inhibits this function. cAMP in turn can regulate some GEFs, protein kinase A (PKA), and other proteins depending on the cell-context. $G\alpha_{q/11}$ as the third member of this family is able to activate Phospholipase C-β leading to the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG), with their subsequent intracellular signaling (e.g., calcium signaling). $G\alpha_{12/13}$ is the last member of this family and it mediates its signaling through modulation of Rho, Ras, cadherins, and ion channels[29]. GTP gets hydrolyzed into GDP by the innate GTPase activity of Gα and the GDP-bound Gα circles back to bind to a GPCR[30].

1.3.1.2. Gβγ signaling

The conformational change induced by ligand in the GPCR leads to the binding of GTP to Gα and this subsequently results in the release of Gβγ subunit. The released Gβγ has a diverse and broader downstream effect than Gα and is able to activate various second effectors depending on the isoforms of their subunits in a cell-type specific manner and can include Phosphoinositide 3-kinases (PI3K), PLC, AC, ion channels, etc. GDP-bound Gα reassembles with the Gβγ to reassociate it with a GPCR[31,32].

1.3.2. G-protein independent signaling

After activation of GPCRs by their ligand, they are recognized by GPCR kinases (GRK) and receive phosphorylation in their c-terminus and ICL domain which in turn attracts β-arrestins. β-arrestins are primarily known for GPCR endosomal integration and subsequent recycling or degradation[33]. However, despite this classical model, β-arrestins are now known as another route of GPCR signaling. Angiotensin 1A receptor (AT1R) is an example of this signaling mechanism, which induces phosphorylation of the Extracellular-signal Regulated Kinase (ERK) pathway through β-arrestin binding[34], separately from G Protein-mediated signaling. Another extraordinary example of β-arrestin-medicated signaling of a GPCR was first documented on opioid receptors, where its activation induces translocation of β-arrestin 1 to the nucleus and its subsequent involvement in chromatin acetylation and gene expression[35].

1.4. Biased signaling

Biased signaling was a major advancement in our understanding of cellular signal transduction, specifically, it allowed us to exploit this feature in order to design more precise drugs with either higher efficacy or lower side effects. Biased signaling (i.e., functional selectivity) points to the ligand-dependent preference for one or more signaling pathways over others from the same receptor[36]. An example could be referred to as above mentioned G-protein-dependent and β-arrestin-dependent signaling of GPCRs in AT1aR by an agonist called TRV120027 which has a preference for inducing β-arrestin-dependent signaling[37].

1.5. GPCR cycle

Active GPCR conformation has a higher affinity to GRKs, and thus gets phosphorylated. The phosphorylated GPCRs go under homologous desensitization, while the long exposure to ligands leads to the phosphorylation of a wider range of GPCRs by PKA and PKC and initiates heterologous desensitization. The phosphorylated GPCRs have a higher binding affinity to arrestins and arrestin-bound GPCRs recruit clathrin and AP2 proteins which organize the endosomal formation and initiate receptor internalization with the help of other accessory proteins such as dynamin. The endosomal GPCR either goes through lysosomal degradation or its ligand is digested and the GPCR gets dephosphorylated in the endosome and the inactive GPCR recycles back to the plasma membrane, ready for new ligand and signaling transduction[38,39].

2. Location-biased signaling

GPCRs are classically considered plasma membrane receptors. They are reported in the endoplasmic reticulum and Golgi as part of their synthesis and post-translational modification (PTM) to translocate them to the plasma membrane for functional activity, but they were not thought to be active in those organelles. However, the first report on the intracellular GPCR appeared in 1998 when Lu et al, showed that the AT1R is translocated to the nucleus upon ligand stimulation[40]. Later on, our lab showed that EP1 is present in the nucleus without the requirement of ligand-dependent translocation, and its activation leads to intranuclear calcium accumulation[41].

The differential signaling activity of a receptor from the intracellular organelles is called location bias[42] (Fig. 2). This signaling activity could be the same or different from their counterpart on the plasma membrane. Functional GPCRs are now detected in the nucleus, mitochondria, Golgi apparatus, and endoplasmic reticulum (ER). Some GPCRs are the primary resident in these organelles, some translocate to these sites upon stimulation, and some have distinct pools of localization independent of ligand binding [42]. Also, it has been shown that some part of GPCR signaling continues while it is internalized and is in the endosomes. Interestingly, the transcriptional output of a signaling activity from the plasma

membrane is different than from the endosomes, entailing this process for location-biased signaling[43].

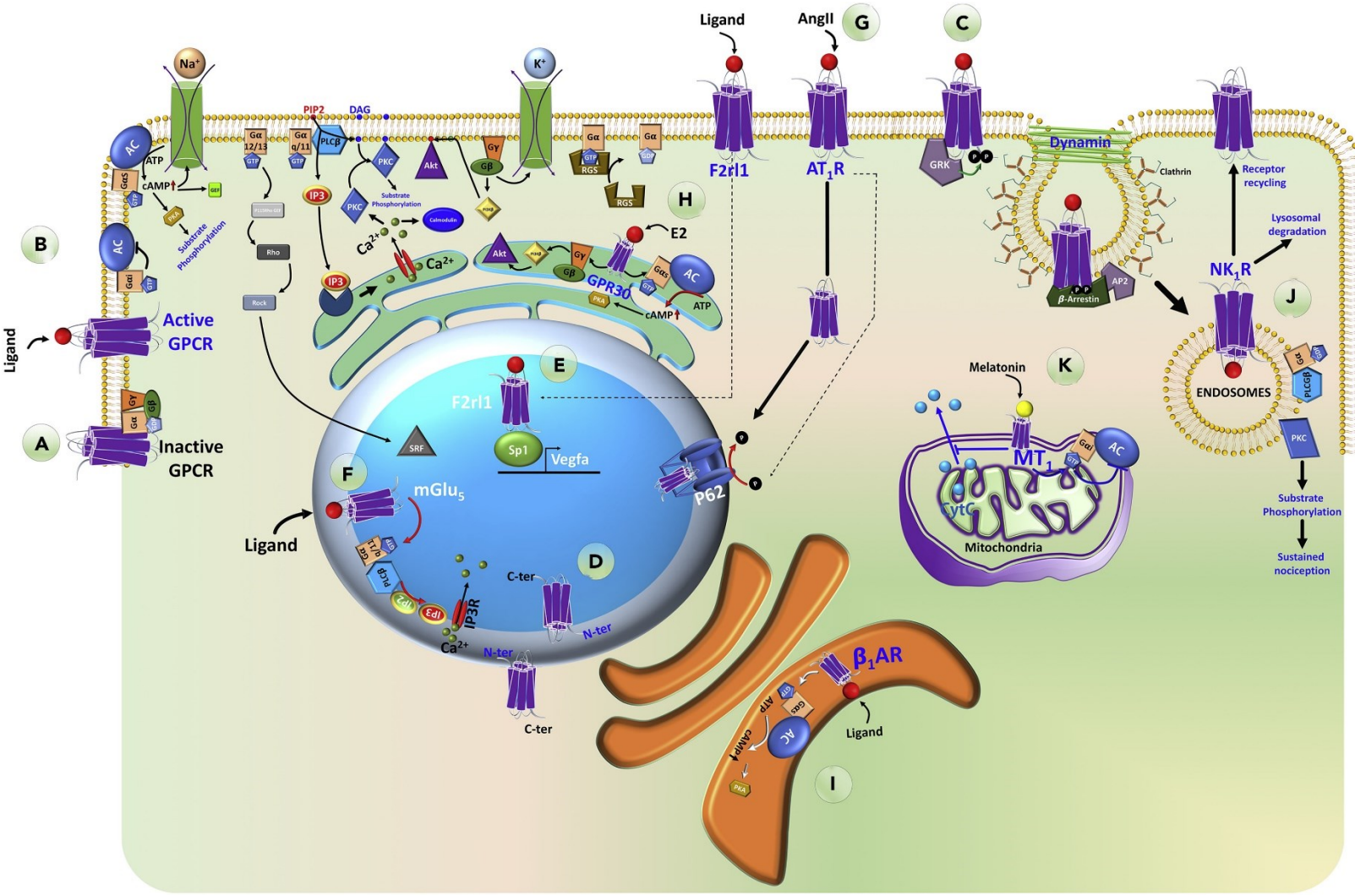

Figure 2: GPCR downstream signaling and Location-biased signaling (Mohammad Nezhady, Chemtob; iScence2020)

2.1. Nuclear GPCRs

Nuclear-GPCRs are the most studied receptor in location bias, and interestingly besides their ability to induce intranuclear signaling, they can directly interact with transcription factors and induce gene expression[44]. There are more than 40 GPCR detected in the nucleus, with evidences for intranuclear cAMP modulation[45], calcium modulation[46], and

phosphorylation of secondary effectors[47]. Additionally, it has been shown these intranuclear signaling effects can induce transcriptional initiation[48].

2.2. Mitochondrial GPCRs

So far, around 8 GPCRs have been found in the mitochondria with functional receptor activities. Interestingly, some of them like AT_1R and AT_2R are also found in the nucleus[42,40,49]. Mitochondrial GPCRs are able to induce or inhibit mitochondrial calcium uptake[50], modulate cAMP level in this organelle[51], and regulate its respiration[52].

2.3. ER and Golgi GPCRS

In contrast to the nuclear GPCRs, so far only two functional GPCRs are reported in the endoplasmic reticulum[53,54]. GPR30, the first functional ER GPCR is an estrogen receptor with predominant localization only in the ER and able to induce intracellular calcium modulation from there[53]. SUCNR1 is the second known functional GPCR in the ER in normoxic condition which translocates to the plasma membrane upon hypoxia[54]. Very few functional GPCRs are also found in the Golgi apparatus; they are mostly opioid receptors and detected in the neuronal Golgi system[55].

Please for the full literature overview of location-biased signaling refer to our published review paper in annex 1.

3. HCAR1

Hydroxycarboxylic acid receptor 1 (HCAR1) is a class A GPCR and is widely known as GPR81. Lactate is the only identified endogenous ligand for this receptor with an EC_{50} of 1.30 mM. HCAR1 is coupled with the $G\alpha_i$ subunit, thus reduces cAMP level in the cell[56], but its $G\beta\gamma$[57] and arrestin-mediated signaling[58] are reported as well. Its highest expression is found in adipocytes where it inhibits lipolysis[59], but it is detected in almost every tissue.

Many reports have shown that HCAR1 suppresses inflammation, decreases neural activity and is involved in retinal, cardiovascular, renal, intestinal, and muscle cell function. HCAR1 is also highly expressed in many different cancers and promotes various features of cancer progression and metastasis.

3.1. HCAR1 in lipolysis

Using [^{35}S]GTPγS binding assay and brain extracts coupled with fractionation and chromatography, Liu et al, found that lactate is the endogenous ligand of HCAR1 and its stimulation with this ligand leads to inhibition of glycerol and fatty acid release indicating HCAR1 inhibits lipolysis. Their results were verified by using HCAR1 KO mouse model as well[60]. The EC_{50} of HCAR1 in this study was 5mM.

3.2. HCAR1 in inflammation

HCAR1 depletion in the liver of mice with LPS-induced inflammation shows 100% mortality due to progressive liver injury[61]. Similar observations are reported in the intestinal inflammation mouse model as well, where HCAR1 Knock Out (KO) mice had severe inflammation and consequent diminished health effects in the colitis model[62]. We also have shown that HCAR1 activation during labor can inhibit the expression of several proinflammatory genes and prevent preterm birth induced with endotoxin model[63]. Overall, many studies have shown that increasing the concentration of lactate in the site of inflammation through HCAR1 activation acts as a feedback mechanism to reduce the inflammation.

3.3. HCAR1 in neurons and brain

Lactate-mediated activation of HCAR1 is able to reduce neural excitability[64], and its activation in the brain can reduce the excitatory presynaptic current frequency, firing and spiking frequency as well as their excitability[57]. Lactate, whether injected exogenously or produced endogenously by exercise, is able to induce angiogenesis in the cortex and hippocampus by inducing expression of VEGF in an HCAR1-dependent manner[65]. Our laboratory also showed that HCAR1 regulates brain vascular development during post-natal pup development by inducing the expression of several angiogenic factors. Additionally, we showed that HCAR1 activation is able to protect the brain from hypoxia-induced infarcts in the brain[66].

3.4. HCAR1 in retina

HCAR1 expression is detected both in Muller and ganglion cells in the retina[67]. Our laboratory showed that HCAR1 induces inner retinal vasculature development through Norrin/Wnt pathway in normal development as well as its revascularization after ischemic insults in the retina[68]. According, lactate through HCAR1 regulates neuro-visual development[69].

3.5. HCAR1 in muscles

HCAR1 activation by lactate-induced accumulation of triglycerides in myotubes which can be used as an energy source. This pathway is also able to enhance mitochondrial function and maintenance by regulation mitochondrial proteins[70]. Additionally, HCAR1 activation leads to induction of the MEK pathway leading to an increase in myotube diameter[71].

3.6. HCAR1 in cancer

HCAR1 is highly expressed in many cancer cell lines as well as different tumors derived from patient samples. Depletion of HCAR1 in cancer cells reduces their proliferation, survival, and ability to metastasize[72,73]. Additionally, HCAR1 Knock Down (KD) in cancer cells leads to lower angiogenic capacity and metastasis in tumors[74]. The effects of HCAR on cancer progression have been attributed to different mechanisms including enhancing DNA damage repair[75], increased mitochondrial function[76], inhibition of ferroptosis[77], enhancing tumor immuneescape[78] as well as classical cancer promoting AKT and ERK pathways[74].

For the detailed literature overview of HCAR1, please refer to our submitted review on HCAR1 in annex 2.

4. Lactate

Lactate or lactic acid discovery dates back to 1780 by a Swedish scientist named Cark Wilhelm Scheele. He isolated the lactate from milk and the name is a reflection of this fact; in Latin “Lac” means milk. Later in 1808, another Swedish chemist, Jöns Jacob Berzelius, showed that lactate is produced during physical exercise in muscles. Louis Pasteur discovered the role of Gram-positive bacteria Lactobacillus in lactate production in 1856 and its structure with the molecular formula of CH3CH(OH)COOH was established by

Johannes Wislicenus in 1873 (Fig. 3). In the early 1900s, lactate was recognized as an energy donor molecule used in muscles for their contractility, and in the 1940s the glycolytic pathway was fully depicted by Embden and Meyerhof[79,80,81,82,83].

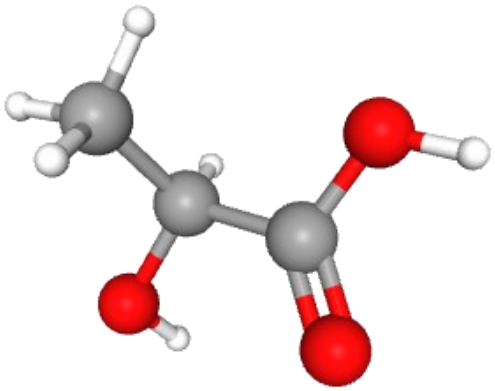

Figure 3: Lactic acid structure (adopted from pubchem)

4.1. Lactate homeostasis

After the discovery of the glycolytic pathway, lactate was considered a waste product of glucose metabolism for more than 40 years. However, increasing evidences afterward showed that lactate could be an energy donor, regulate various physiological processes, participate in pathological conditions, and act as a signaling molecule for different cellular functions.

More than 99% of lactic acid is dissociated into lactate and proton (H^+) at the physiological pH (~7.4) and in humans, around 1500mM lactic acid is produced and released into blood circulation on a daily basis. This amount is primarily produced by muscle, skin, and brain. The blood lactate mostly is metabolized in the liver and used for gluconeogenesis and oxidative phosphorylation and ATP generation[84]. Lactate concentration in resting normal muscles and blood is about 1mM and it can reach up to ~20mM in muscles during intensive exercise[85]. The physiological concentration of lactate in different tissues varies, for example in the brain it is in the range of 1-2 mM[86].

4.2. Lactate transport

Lactate is transported across the plasma membrane through Monocarboxylate Transporters (MCTs). There are 14 MCTs identified with the gene names of SLC16, but the four first ones (MCT1 to MTC4) are the most studied ones so far. These proteins co-transport H+ along with mono carboxylates such as lactate, and pyruvate depending on the substrate and H+ concentrations[84]. While MCT1 is a ubiquitously expressed protein, MCT2 is mostly expressed in tissues using lactate as an energy source such as the heart, kidney, brain, liver, and muscle[87], and accordingly, MCT2 is mostly an importer of lactate with a high affinity for it ($K_{m\ Lactate}$= 0.5mM)[88]. On the other hand, MCT4 is mostly known as an exporter of lactate, but it has a very low affinity for it with a $K_{m\ Lactate}$ of 22mM; it is expressed in cells such as astrocytes and white blood cells[89].

4.3. Lactate energetics

Lactate seems to be the primary energy source in some tissues compared to glucose and pyruvate, including cardiac muscles and the brain[85,90]. This seems to happen in resting condition in these tissues, however, in skeletal muscle, the switch in metabolic preference seems to happen relative to the exercise intensity level[91]. Exercise-induced increase in glycolysis leads to a higher concentration of lactate, which in turn inhibits lipolysis through HCAR1 activation[92]. This additionally helps the transition of fat utilization as an energy source in skeletal muscle cells to carbohydrate oxidation[93], where lactate enters mitochondria via the mitochondrial MCTs and through mitochondrial LDH is oxidized to pyruvate and enters into the Krebs cycle to generate ATP for energy consumption[94].

4.4. Lactate signaling

Lactate has long been known to modulate different physiological processes. Lactate's ability to inhibit lipolysis was known long before the discovery of HCAR1[95]. Its various effects on immunity and inflammation[96], angiogenesis[97], cellular migration[98], and neurons[99] were discovered and studied extensively irrespective of HCAR1. However as briefly mentioned in section 3 (HCAR1) and detailed in annex 2, later on, many of the various effects of lactate were discovered to be mediated through HCAR1-dependent signaling. Part of the lactic acid-signaling effects are mediated through the acidic nature of

this molecule and receptors mediating H^+ effects[100], but it seems that the majority of these effects are mediated via HCAR1 activity.

4.5. Lactate in wound healing

Due to both inflammation and rapid proliferation, the glycolysis rate is higher in the wound site and consequently, lactate concentration increases therein[97]. Lactate concentration in the wound site reaches up to 15mM (compared to ~1mM blood concentration) and remains high during the healing processes[101]. Endogenous or exogenous lactate on the wound site can promote angiogenesis and accelerate the healing processes[97]. It can also enhance collagen expression and deposition in the wound site as well as recruitment of progenitor endothelial cells[102,103].

4.6. Lactate GPCR other than HCAR1

HCAR1 is the only established receptor for lactate, however, there is a single report in PNAS showing GPR132 could be also a receptor for lactate. This study shows lactate through GPR132 in macrophages contribute to activation of the M2 phenotype and aid cancer migration and invasion[104]. Although GPR132 is long known as a proton sensing GPCR[105], this paper shows lactate could be a potential ligand for GPR132 rather than its proton. They pull down GPR132 and identify lactate by liquid chromatography-mass spectrometry. Although this could indicate lactate is bound to GPR132, it does not necessarily establish the receptor-ligand relationship because it is long known that GPCRs have a high tendency for heterodimerization[106]. Their findings could simply be because of heterodimerization of GPR132 with HCAR1 as this was not considered in their investigation. Noteworthy, there is no report reproducing or further validating their results in more detail.

4.7. Lactic acidosis

The most common form of metabolic acidosis is lactic acidosis and is characterized by a high level of lactate in the blood concurrent with a decrease in blood pH level. Lactate acidosis is usually considered as a sign of an underlying cause and can indicate poor prognosis factor[107]. The underlying cause of lactic acidosis is either increased production of lactate or decreased clearance of lactate. Although usually both of them are present,

frequently one of these factors is predominant. This imbalance could be due to hypoxia, sepsis, intoxication (e.g., ethanol), malignancy, etc. Less frequently, lactic acidosis could be congenital due to mutations in any of the genes involved in the lactate metabolism and transport[108]. Increased blood lactate above 2mM is considered hyperlactatemia and above 5mM indicates a severe condition[109]. There are two types of lactic acidosis: Type A is due to hypoperfusion and tissue hypoxia such as cardiogenic shock, while in type B hypoxia and hypoperfusion are not determinants such as liver disease. The treatment type depends on the type of lactic acidosis[110].

5. Cancer:

As mentioned above, different pathologies can lead to lactic acidosis. Malignancy and cancer also can cause systemic lactic acidosis and it is among the type B[110]. It has long been known that cancer cells prefer using glucose as a source of energy and producing lactate regardless of available oxygen level[100].

5.1. Cancer

Cancer is an umbrella term for a plethora of diseases in various tissues all characterized by the uncontrollable growth of a population of cells that could acquire the potential to spread to other organs. Cells have several steps to control their growth, also several mechanisms to halt overgrowth. In a multi-step process, some cell(s) acquires features enabling them to divide without restriction. The cancerous cells overburden the body and by several mechanisms result in the lethality of the patient. Cancer patients succumb to this malignant disease either by organ failure in the affected organ by primary cancer or metastatic invasion, or they fail to overcome several secondary clinical syndromes[111,112]. For example, brain tumor death in most cases is due to failure in this organ. The clinical syndromes depend on the site of cancer. Cachexia is one of these syndromes and is the main cause of death in pancreatic and gastric cancers[113]. Almost 20% of all cancer death is related to cachexia which happens when the patients lose around 30% of their body weight[114]. Thrombotic syndrome and coagulopathy are other clinical symptoms that leads to death in around 10% of patients but is present in more than 50% of patients before their death[115]. Dyspnea as the result of cancer is another syndrome that could be lethal and this can stem

from direct lung involvement or obstructive pulmonary disease or systemic cytokine production[116,111].

More than 100 different types of cancer are defined so far, however, all share common mechanisms to develop and overcome the internal control systems. These mechanisms are elegantly illustrated by the seminal review of Hanahan and Weinberg; hallmarks of cancer. Initially, in 2000, they proposed 6 distinct common mechanisms that are acquired by cancer cells: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion from apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis[117]. A decade later, they revisited their hallmarks and added two new major established hallmarks for cancer: 7) reprograming energy metabolism and 8) evasion from immune destruction. They also recognized genome instability and tumor microenvironment with tumor-promoting inflammation as enabling characteristics of these hallmarks[118]. Recently, early this year, Hanahan added a new dimension to their original hallmark with 9) phenotypic plasticity and disrupted differentiation; and non-mutational epigenetic reprogramming and polymorphic microbiome as new enabling characteristics[119].

5.2. Metastasis

The spread of the primary tumor and its re-establishment in another organ distant from its original site is called “metastasis”. Metastasis was one of the original hallmarks of cancer as mentioned above and it is responsible for more than 90% of cancer-related deaths and therapy failure[120].

Although metastatic-capable cells are less than 0.01% in a given cancer, the evolutionary pressure on the heterogenous population of cancer cells imposes progression into a random selection of cells that are able to spread and colonize at distant sites. The enabling characteristic of genomic instability and epigenomic reprogramming allows cells to randomly go through these processes and form a heterogenous population that some are selected evolutionary to invade the surrounding tissues and enter into circulation. Among those entering into circulation, some again evolutionary are selected that are capable to exit and colonize in the secondary site[121]. Indeed, there are at least 5 major steps for metastasis

that cancer cells need to go through evolutionary selection to be able to spread: 1) dissemination and invasion, 2) intravasation, 3) circulation, 4) extravasation and 5) colonization and homing. The dissemination of cancer cells is recognized by epithelial-mesenchymal transition (EMT). During this transition, epithelial cancer cells which are tightly bound to each other and the neighboring extracellular matrix (ECM), go through a process to acquire mesenchymal properties enabling them to dissociate and migrate. This is done by modifying the adhesion molecules and cell polarity in the cancer cells. Noteworthy, the EMT is not a binary switch, it rather is a spectrum of transitional steps that are gained by genomic instability and epigenetic reprogramming that ultimately induces the expression of certain transcription factors such as Snail and Twist and certain miRNAs[122]. During the angiogenesis for tumors, these newly formed vessels have weaker cell junctions and mesenchymal cancer cells further express proteins that enable them to compromise endothelial vessels cells' integrity (e.g., metalloproteinases). After entering the bloodstream, circulating tumor cells resists various stresses such as interaction with immune cells and mechanical forces[123]. When the circulating tumor cells reach capillaries, they are trapped there due to size constraints and attach to the endothelial and start the extravasation process[124]. Interestingly, the reverse of EMT happens after the colonization in the secondary site for the homing and seeding of the cancer cells. Cells go through a process that is called mesenchymal-epithelial transition (MET) which is concurrent with re-acquiring non-migratory phenotype, re-expression of junctional and adhesional proteins as well as cell polarization[122,125].

5.3. Cancer and GPCRs

As mentioned in the first section, GPCRs are virtually involved in every physiological process and it could be inferred that their misregulation is involved in most, if not all, pathologies. This is attested by more than 35% of marketed therapeutics that are targeting GPCRs. Considering the various roles of many different GPCRs in cellular proliferation, differentiation, metabolism, angiogenesis, immunoregulation, etc, easily one could see the crucial roles of GPCRs in cancer (Fig. 4). Given they are the most druggable proteins, this family is really under-appreciated in cancer studies evident by having only 8 approved anti-cancer drugs targeting GPCRs[126].

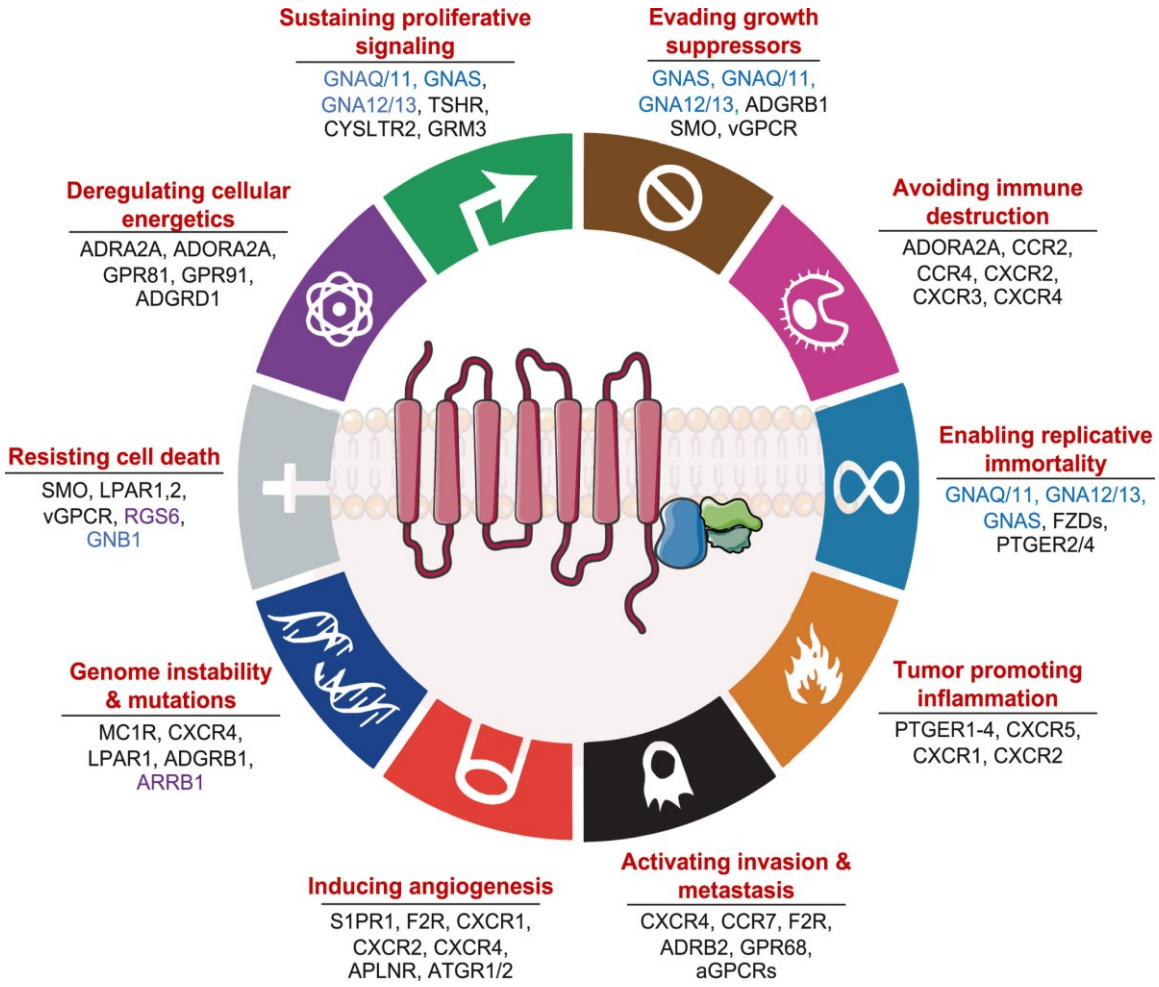

Figure 4: Role of different GPCRs in hallmarks of cancer (Arang, Gutkind; FEBS letters 2020)

5.3.1. GPCRs in cancer cell proliferation and survival

GPCRs can become oncogenic upon excessive production of their ligands (e.g., mAChR, LPAs)[127,128]. In contrast to this case where a WT GPCR becomes tumor-promoting, some mutations in a GPCR could lead to their constitutive activation resulting in aberrant proliferation even in the absence of their ligand (e.g., α_{1B}ADR)[129]. On the other hand, some GPCRs act as tumor suppressors and their loss of function can lead to uncontrolled cellular proliferation. These tumor suppressive GPCRs could play a role in a cell-type specific manner or not (e.g., MC1R, P2RY8)[130,131].

In the opposite cases, GPCRs are involved in cell survival and resistance to therapy as well. Two different studies on the resistance-driving genes in melanoma found that GPCRs are among the top-ranked genes (e.g., GPR35, LPAR1)[132,133].

5.3.2. GPCRs in angiogenesis and metastasis

Cancer cells hijack the GPCRs in the endothelial cells to invoke their proliferation and angiogenesis into the tumor bed. Many of the pro-angiogenic factors that are produced by the cancer cells, act on GPCRs that are expressed in the endothelial cells. Additionally, some of these secreted factors act on leukocytes and macrophages in the tumor niche and induce the expression of VEGF[134]. On the other hand, secretion of some factors such as thrombin and $S1P_1$ from tumor cells, in an auto and paracrine fashion through GPCRs can induce expression of various factors such as metalloproteinases and facilitating the migration and invasion of both cancer cells and sprouting of vessels into tumor bed[126].

Interestingly, GPCRs are the reason why some organs are more prone to be a metastatic site. Chemokines and the chemokine GPCRs in the cancer cells are the major players in this system. For example, CXCL12 is a chemokine for CXCR4 and this chemokine is mostly secreted by organs such as the lungs, liver, and bone marrow. CXCR4 is expressed by many metastasizing cancer cells and attracts circulating tumor cells to these common secondary sites due to secretion of their ligands from these organs. Another example is the preference of melanoma, ovarian, and breast cancers to metastasize into the small intestine. These cancer types express CCR9, a chemokine receptor for CCL25 which is expressed by the small intestine[135,136,126].

5.3.3. GPCRs in immune-escape of cancer

Prostaglandins are major regulators of immunity and PGE2 as a prostaglandin is highly produced by the cancer cells. PGE2 targets different GPCRs in different immune cells; The PGE2-GPCRs (EP1-EP4) signaling in Treg cells leads to an immune-suppressive phenotype, in CD8 T cell leads to decreased activation, and overexpression of PD-1, a well-known immune-escape axis[137].

5.3.4. GPCRs in genome instability

GPCR signaling regulates many of the mechanisms that cells cope with stress. CXCR4 and its ligand CXCL12 are not only involved in cancer metastasis, but they also decrease mitochondrial reactive oxidative species and protect cells from their subsequent genotoxic effects. Interestingly, inhibition of CXCR4 leads to mitotic failure in cancer cells[138].

Inhibition of P53, the major tumor suppressor, can lead to the accumulation of DNA damage and subsequent transformation of cancer cells. β2-adrenoreceptors downstream signaling via β-arrestin-1 can sequester P53 via MDM2 and lead to its degradation[139]. In contrast, ADGRB1 as an adhesion GPCR is known to stabilize P53 by preventing its MDM2-mediated degradation and has a significant role in the prevention of brain tumors[140]. Polymorphisms of MC1R, a GPCR expressed in skin cells, is associated with melanoma. Melanomas harboring these polymorphisms have a higher mutational burden. The downstream signaling of this receptor leads to stabilization of P53 by phosphorylation, in addition to higher expression of DNA damage repair enzymes[141,142].

5.3.5. GPCRs and cancer metabolites

Cancer cells have distinct metabolic reprogramming and produce many metabolites. Lactate is among these metabolites and its link to HCAR1 in promoting cancer has been discussed earlier and extensively in annex 2. Another metabolite and its cognate GPCR that play some role in cancer are succinate and SUCNR1. Its signaling in tumors can induce angiogenesis by expressing VEGF, regulate cancer-immune interaction, and promote their metastasis[143,142]. Many metabolites such as amino acids, nucleotides, and their derivatives are recognized by GPCRs, and they can be involved in different stages of cancer because of the aberrant production of these metabolites in cancers. For example, $A_{2A}R$ and $A_{2B}R$ are adenosine binding GPCRs and can mediate the immune-escape of cancer cells[144,142].

5.3.6 Drugs targeting GPCR in cancer

As mentioned before, compared to all drugs targeting GPCRs that is occupying more than 35% of the market, only 8 anti-cancer drugs targeting GPCRs are approved by FDA. These GPCRs are: 1) Dopamine 1 receptor (1 inhibitor), 2) Smoothened, the receptor for sonic hedgehog signaling (2 antagonists), 3) Somatostatin receptors (1 agonist), 4) gonadotropin-

releasing hormone receptor (1 antagonist), 5) CXCR4 (1 antagonist), 6) GPR30 (1 agonist), and 7) CCR4 chemokine receptor (1 humanized monoclonal antibody)[126].

The involvement of GPCR goes beyond what we have discussed here. Many mediators of GPCR are involved in many types of cancers, including G-Proteins, GRK, Arrestins, and downstream effectors of GPCRs. G-Proteins are among highly mutated cancer-associated genes in pan-cancer studies[142,137,145].

6. Warburg effect in cancer

It is almost a century since the discovery of the Warburg effect in 1924. Otto Warburg explored a similar phenomenon in mammalian cells as Louis Pasteur did for the fermentation of glucose to ethanol in yeast. He discovered that cancer cells ferment glucose into lactate in an oxygen-sufficient environment, unlike normal cells which catabolize glucose into pyruvate and utilize it for oxidative phosphorylation[146,147]. This rather paradoxical metabolic switch to a lower energy-producing pathway in cancer cells was named after him as the Warburg effect. Disappointingly, my efforts to retrieve the original seminal paper of 1924 were not fruitful. Only citations of this influential paper are circulating in the literature. Warburg received the Nobel prize in 1931 for his studies on respiratory enzymes.

Non-proliferating differentiated cells in the presence of oxygen utilize glucose to produce pyruvate for the Krebs cycle in the mitochondria. This process has shaped the commonly accepted model of energy production as it yields high levels of ATP, but usually, the requirement for non-proliferating cells is ignored. Krebs cycle in the mitochondria generates 36 ATPs while the lactate fermentation generates only 2 ATPs[148]. Non-proliferating differentiated cells switch their metabolism to glycolysis with lactate production upon lack of oxygen. However, cancer cells are proliferating cells, and oxygen level does not dictate their metabolic decision. In general, even in the presence of oxygen, proliferative cells such as regenerating cells, embryonic and activated-immune cells prefer lactate fermentation. This is most likely because lipids and nucleotides are the building blocks of rapidly growing cells and their synthesis relies on the molecules that are produced

during glycolysis and initiate the pentose phosphate pathway (PPP) and the serine/glycine synthetic pathway (SSP)[149].

Besides the anabolic demand for lipid and nucleotides for the proliferative cells, the produced lactate act as an energy source. The fermented lactate goes to the liver where it is converted back into glucose by gluconeogenesis. The resulting glucose is used for the production of glycogen and stored as an energy source[150,151]. When this energy is required again for example in the brain, the glycogen breaks down in the liver to glucose which goes and feeds the neurons for energy production[152]. This cycle is known as lactic acid or Cori cycle after its discoverers who received the Nobel prize for this.

6.1. Warburg effect in diagnostics

There is a fierce glucose uptake by the cancer cells due to the Warburg effect. This rapid glucose intake by the tumor compared to surrounding tissue has made the basis for one of the most used diagnostic tools in oncology for cancer detection and remission. 18-fluorodeoxyglucse (FDG) is an analog of glucose with radiolabeled flour. This analog is taken up by the cancer cells similar to glucose via Glucose Transporters (GLUTs)[153]. Interestingly, GLUT1 is highly upregulated in most cancers[154]. Once in the cancer cell, FDG enters the glycolysis and in the first step gets phosphorylated by the Hexokinase into FDG-6-phosphate. Due to the lack of 2-OH in the FDG-6-phosphate, this molecule is not able to be processed further in the cell, leading to its cytosolic accumulation. The high concentration of radio-labeled FDG in the tumor is detected with Positron emission tomography (PET) and is a reflection of tumor size and distribution[153].

6.2. Warburg effect promoting cancer

Energy-wise, although the numerical comparison of ATP production in lactate fermentation is lower than oxidative phosphorylation, this paradigm does not take the kinetics into account. Interestingly, lactate fermentation is 10-100 times faster in glycolysis compared to the full Krebs cycle in the mitochondria. This fact results in a similar amount of ATP production that is produced for a given period of time for both glycolysis and Krebs cycle. On the other hand, the energy from ATP that is required for cell division usually is below the threshold to limit cancer cell proliferation[155,156].

Besides resolving the energy paradox of the Warburg effect in cancer cells, the over-production of lactate provides several advantages for the tumor. The secreted lactic acid into the tumor microenvironment leads to acidification of the tumor niche ranging from pH level of 5.5 to 7[157]. This acidity helps cancer cells' invasion and migration. This acidity can aid in extracellular matrix remodeling and easier dissociation of matrix proteins enabling cellular migration and invasion. Additionally, lactate has been known to promote cellular migration[158,159]. On the other hand, both the acidic microenvironment and lactate are known to promote tumor immune escape. The proton sensors in macrophages suppress their activity and promote their noninflammatory phenotype[149]. Lactate itself, can also reduce cytokine production of T cells and suppress the cytotoxic activity of both T and natural killer cells[100,160]. On top of all these, lactate is known to induce expression of VEGF in a dose-dependent manner both in normal and cancer contexts, thus promoting tumor angiogenesis[100].

6.3. Warburg effect and clinical relevance

As a result of the Warburg effect in cancer cells, lactate concentration in the tumor microenvironment raises up to 50mM. Comparing this value to blood lactate level during high-intensity workouts ranging around 10-15mM is striking. Lactate concentration in tumors is negatively correlated with the patient's survival and disease-free survival. In line with this, primary tumors with a high-lactate niche have a higher incidence of cancer metastasis, compared to their counterparts with a lower-lactate niche. Not surprisingly, high-lactate tumors are also more resistant to therapy[149].

7. Research Hypothesis and Objectives

Due to Warburg effect and ensuing overload of lactate in the tumor microenvironment, HCAR1 received many attentions to explore its role in various features of cancer progression. Lactate role in diverse functions that could be pro-cancer was re-evaluated through HCAR1 including tumor growth, angiogenesis, immune escape and metastasis. However, there was a lack of mechanistical insight into how these different cellular processes are all coordinated via a single receptor. On the other hand, our preliminary results and images from published data indicated that HCAR1 has a significant intracellular

localization, prompting us to consider the possibility of location bias. To this end, I hypothesized that HCAR1 has a nuclear localization and it regulates different biological processes with this localization pattern, specifically in the cancer context. Thus, I first tried to thoroughly investigate nuclear localization pattern of HCAR1 in cancer cells. Afterward I set to explore if HCAR1 in the nucleus is a functionally active receptor capable of signal transduction intranuclearly, as well as exploring the role of HCAR1 in the nucleus in an unbiased manner through high-throughput omics studies. Finally, I aimed to validate those results in an *in vivo* model to better understand the significance of HCAR1 nuclear localization at a system level.

Chapter 2

Unconventional Receptor Functions and Location-Biased Signaling of the Lactate GPCR in the Nucleus:

Unconventional Receptor Functions and Location-Biased Signaling of the Lactate GPCR in the Nucleus

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Abstract:

G-Protein-Coupled Receptors (GPCR) form the largest receptor family virtually involved in every physiological process. However, mechanisms for their ability to regulate a vast array of different biological processes remains elusive. An unconventional functional modality for GPCRs could at least in part account for such diverse involvements but has yet to be well explored. We investigated the regulatory role of HCAR1, a multi-functional lactate receptor. We found this receptor to localize at the nucleus and therein capable of initiating location-biased signaling notably nuclear-ERK and AKT phosphorylation. Using HCAR1 mutants that avoid the nucleus and a multi-omics approach, we discovered that nuclear HCAR1 (N-HCAR1) is directly involved in regulating diverse processes through non-traditional receptor functions. Specifically, N-HCAR1 binds to protein complexes that are involved in promoting protein translation, ribosomal biogenesis, and DNA-damage repair. N-HCAR1 also interacts with chromatin remodelers to directly regulate gene expression. We hereby show that N-HCAR1 displays a broader transcriptomic signature than its plasma membrane counterpart. Interestingly, exclusion of HCAR1 from the nucleus has the same effect as its complete cellular depletion on tumor growth and metastasis *in vivo*. These results reveal non-canonical functions for a cell nucleus-localized GPCR that are distinct from traditional receptor modalities and through which HCAR1 can participate in regulating various cellular processes.

Keywords: Unconventional receptor function, GPCR, Signaling, Location bias, HCAR1, Lactate, Warburg effect, Cancer.

Introduction:

GPCRs are considered the forefront of cellular communication, yet are largely reduced to the role of ligand signal conveyors from the plasma membrane. Meanwhile, there is a surge in discovery of functional intracellular GPCRs. Every membranous organelle has been shown to harbor active GPCRs, either as a primary site of localization or as a result of plasma membrane translocation upon ligand binding[1,2]. The differential signaling activity of a GPCR from these intracellular organelles as opposed to their signaling output from plasma membrane is generally referred to as location-biased signaling[2]. In this context, spatiotemporal coordination of GPCR signaling is determinant[3] and can lead to different outputs even though downstream effectors remain constant[4]. For example, antagonism of NK_1R in endosomes is more effective with longer effect in pain relief than targeting this receptor at the plasma membrane[5]. Despite showing clinical and translational relevance[5], location-biased signaling remains an understudied concept. Besides location-biased signaling, it has recently been proposed that GPCRs might possess non-signaling activities. Indeed, a population of PAR2 receptor localizes to nucleus and was shown to interact with SP1 transcription factor and regulates gene expression[6]. Thus, it appears that the location bias of GPCRs permits a diversification of the regulatory roles of GPCRs, either through downstream signaling or through other interactions. However, the latter concept remains unexplored.

Hydroxycarboxylic acid receptor 1 (HCAR1), a GPCR also known as GPR81, is the receptor for lactate[7], which is a glycolysis metabolite present at high concentrations in most tumors as a result of the Warburg effect[8]. Accordingly, a major focus for this receptor has been placed on cancer studies[9,10,11]. Although the Warburg effect associated with marked elevations in lactate concentrations (up to ~50mM within the tumor microenvironment)[12,13] has been linked to different processes in promoting cancer progression, this feature remains enigmatic due to the paradoxical metabolic switch[14]. Remarkably *HCAR1* is overexpressed in numerous cancer cell lines and resected tumors from patients[15,16,17], and promotes tumor

proliferation, metastasis, angiogenesis, survival and immune evasion *in vivo*[18,15,16]. Lactate through HCAR1 promotes DNA damage repair[19,20] in cancer cells and abolishes IFN-α production in immune cells[21]. Interestingly, although HCAR1 is considered to date a cell surface receptor, its actions were limited when intracellular lactate uptake was inhibited[19,20,21], suggesting that cell surface HCAR1 signaling was not determinant for its functions. Thus, mechanisms to explain such multidimensional involvement of HCAR1 in cancer biology is lacking; in this context an intracellular mode of action along with possible non-traditional signaling activities of the receptor should be accounted for.

In the present study we show that HCAR1 has a nuclear localization and decipher its topology on the nuclear membranes. We show that nuclear HCAR1 (N-HCAR1) is capable of initiating G_{α} and $G_{\beta\gamma}$- mediated intranuclear signaling, and using bottom-up high-throughput omics studies demonstrate that N-HCAR1 promotes various processes through different non-traditional receptor mechanisms involving formation of protein complexes inside the nucleus that promote protein translation and DNA damage repair. N-HCAR1 is found to regulate a broader transcriptomic signature than its plasma membrane counterpart, emphasizing that N-HCAR1 functional output is larger than its plasma membrane localized counterpart. Cellular effects of N-HCAR1 which translate into cell proliferation, survival and migration *in vivo* unveil importance of N-HCAR1 in promoting a variety of roles in cancer malignancy.

Results:

HCAR1 displays a nuclear localization pattern dependent upon the 3rd intracellular loop domain and S305 phosphorylation site

We generated stable HeLa cell lines expressing either C-terminal or N-terminal Flag-tagged HCAR1 enabling to utilize various methods to ascertain its subcellular localization and ensuing functions. Complete nuclear isolation upon biochemical cell fractionation revealed abundant HCAR1 at the nucleus, as well as in the cytoplasm (Fig. 1a). Immunofluorescent staining with confocal microscopy using Lamin B1 as inner nuclear membrane marker exhibited clear HCAR1 colocalization with Lamin B1 in intact cells and

isolated nuclei. Strikingly, HCAR1 was also detectable inside the nucleus (Fig. 1b,c; Extended Fig. 1a,d; Fig. 2a). 3D rendering of z-stacked confocal images clearly showed HCAR1 is present inside the nucleus (Fig. 1d). Moreover, electron microscopy using immunogold staining of HCAR1 confirmed nuclear envelope and intranuclear HCAR1 distribution (Fig. 1e; control experiments at Extended Fig. 1e-g). Quantification of electron microscopy showed nearly one-third of cellular HCAR1 localized at the nucleus in unstimulated cells (Fig. 1f). Nuclear localization of HCAR1 was also detected in U251MG and A549 HCAR1-expressing cells (Extended Fig. 2a,d).

Treatment of cells with lactate did not alter the nuclear ratio of HCAR1, indicating that ligand stimulation does not lead to translocation from plasma membrane to the nucleus (Fig. 1f; Extended Fig. 1h) as occurs for some other GPCRs[6]. To ascertain this biologic process, we devised a pulse chase experiment using Fluorogen Activating Peptide (FAP) technology utilizing cell impermeable fluorogen[22]. Activation of the receptor with lactate, triggered HCAR1 internalization within 5 min; HCAR1-containing endosomes were tracked in the cytoplasm for up to 40 min, after which they were no longer detected (either because of recycling to the plasma membrane or endosomal degradation) (Extended Fig. 3a-d). While nuclear localization of HCAR1 from plasma membrane in our FAP system was not observed, the chimeric receptor was hitherto present in the nucleus prior to lactate stimulation (Extended Fig. 3e). Hence HCAR1 does not translocate from plasma membrane to the nucleus upon ligand stimulation, and is *de facto* localized at the nucleus.

We analyzed the sequence and 3D model of the receptor in an attempt to determine HCAR1 domains necessary for nuclear localization (Fig. 1g) and found that although there is no classical nuclear localization signal (NLS) in HCAR1 sequence, there are predicated bipartite NLS in intracellular loop 3 (ICL3), and the C-terminus of the receptor[23]. Corresponding truncation in ICL3 completely abolished nuclear localization as well as cytoplasmic staining (Fig. 1h; Extended Fig. 2b,e). A phosphorylation site in the NLS of the C-terminus (Supp Fig. 1b)[24], prompted us to determine its potential role in localization. Single point substitution of S305 to alanine at the C-terminus led to nuclear exclusion of HCAR1 (but retained cytoplasmic staining; Fig. 1i; Extended Fig. 2c,f). These findings

suggest a scaffolding role of ICL3 and post translational phosphorylation of S305 are required for HCAR1 nuclear localization.

Since nuclear localization of HCAR1 is not observed in HCAR1 cells containing a single amino acid substitution in δS305A, one cannot attribute the localization pattern to an artifact of overexpression. To further ascertain this inference, we knocked down (KD) the endogenous HCAR1 (shHCAR1) and ectopically expressed an RNAi resistant *HCAR1* in HeLa cells; this led to expression of HCAR1 to a level comparable to cells with wildtype (WT) HCAR1, and to cells subjected to a scrambled shRNA (Supp Fig. 2a-d). Importantly these cells also exhibited a similar pattern of nuclear localization (Supp Fig. 1c). Altogether, our data establishes proper nuclear localization pattern of HCAR1.

Topology of nuclear HCAR1

Understanding the topology of HCAR1 in nuclear membranes enables better comprehending the cellular location of signaling domains. We first determined the orientation of HCAR1 on both outer and inner nuclear membranes (ONM, INM, respectively) of the nuclear envelope. Immunofluorescence staining (of Flag) of intact (non-permeabilized) nuclei[6] isolated from HCAR1 C-terminus Flag-tagged expressing cells indicates that the C-terminus of the receptor is oriented towards the cytoplasm on the ONM (Fig. 2aI); whereas N-terminus Flag-tagged HCAR1 in intact nuclei did not reveal staining, consistent with the suggestion that the N-terminus of the receptor resides within the nuclear envelope (Fig. 2aI). This orientation was ascertained by devising a protocol which selectively permeabilizes the ONM, while keeping the INM intact. For this purpose we used a combination of 3 proteins as markers located in different parts of the nuclear envelope: *a)* NUP98 - detectable across the nuclear membrane[25]; *b)* the C-terminus of SUN2 - a luminal marker[26]; *c)* Lamin B1 - located on the nuclear side of the INM. Selective permeabilization of the ONM allowing antibody access and retaining intact INM (Extended Fig. 4a) using (mild detergent) 0.0008% digitonin[27] allowed us to detect the N-terminus Flag-tagged HCAR1, consistent with its luminal nuclear envelope localization (Fig. 2aII). Additionally, treatment of the intact nuclei with proteinase K (PK) to remove the cytoplasm-facing C-terminus of HCAR1, followed by permeabilization of the ONM, revealed the absence of the C-terminus in the lumen while preserving the signal for the

nuclear envelope lumen-localized N-terminus (Fig. 2aIII). We then permeabilized the ONM, followed by sequential treatment of nuclei with PK and permeabilization of the INM. Under these conditions, we could again observe HCAR1 C-terminus staining co-localized with Lamin B1 (Fig. 2aIV), while the N-terminus in this condition was only detected inside the nucleus. Altogether, these experiments reveal that the C-terminus of HCAR1 at the ONM orients within the cytoplasm, while at the INM it has analogous conformation to that at the plasma membrane to putatively initiate signaling cascade into the nucleus, as nuclear envelope membranes are known to contain conventional GPCR signaling machinery[28].

Endogenous Nuclear HCAR1 has a pro-proliferative and survival nuclear location-biased signaling

To elucidate nuclear location-biased signaling of endogenous HCAR1, we isolated intact nuclei, stimulated them with lactate and measured nuclear cAMP levels. Lactate treatment (10 mM for 10 min) of nuclei isolated from WT HCAR1-expressing HeLa cells significantly decreased cAMP levels (Fig. 2b). On the other hand, nuclei isolated from cells KD of HCAR1 (using two distinct shRNAs; Supp Fig. 2a-d) did not respond to lactate (Fig. 2b), validating that HCAR1 at the nucleus is coupled to $G_{\alpha i}$. Lactate also induced ERK1/2 and AKT phosphorylation in isolated nuclei from WT cells expressing endogenous HCAR1, but not in nuclei of cells knocked-down of HCAR1 (Fig. 2c,d; Extended Fig. 4b). ERK1/2 and AKT phosphorylation were respectively inhibited by pertussis toxin and gallein which accordingly inhibit $G_{\alpha i}$ and $G_{\beta\gamma}$ (Fig. 2c,d; Extended Fig. 4c,d), consistent with report of downstream effectors at the nucleus[1,2]. Together, these findings confirm functional G-protein-coupling ($G_{\alpha i}$ & $G_{\beta\gamma}$) of endogenous HCAR1 receptor at the nucleus.

Since ERK1/2 and AKT modulate proliferation and survival in cancer cells[29,30], we measured homeostatic cell proliferation rate and cell survival upon 5-Fluorouracil (5-FU) challenge in cells containing or not HCAR1 at the nucleus. Nuclear HCAR1 (N-HCAR1) containing cells include WT endogenously HCAR1-expressing cells and HCAR1 KD cells rescued with RNAi-resistant WT *HCAR1* (referred to as "WT rescue" cells; Supp Fig. 2a-d). Cells depleted of N-HCAR1 are HCAR1 KD cells rescued with RNAi-resistant *HCAR1* constructs containing δICL3 or δS305A mutations (referred to as N-HCAR1 KD cells;

Supp Fig. 2a-d). While all ectopically HCAR1-expressing cells had the same expression level as the endogenous HCAR1 (Supp Fig. 2b-d), HeLa cells harboring WT HCAR1 exhibited higher proliferation and survival rate compared to total HCAR1 KD (un-rescued) and N-HCAR1 KD cells (Fig. 2e,f). Interestingly, the magnitude of cell proliferation and survival observed upon exclusion of HCAR1 from the nucleus (as seen with the δICL3 and the S305A rescues) was similar to that of cells totally depleted of HCAR1. Importantly, the mutant versions of the HCAR1 preserved their signaling activity (Extended Fig. 4e). Nucleus-excluded HCAR1 mutations caused similar effects in U251MG and A549 cancer cells (Extended Fig. 5a,b; Supp Fig. 2e). Hence nuclear location-biased signaling of HCAR1 promotes proliferation and survival in cancer cells.

N-HCAR1 interactome discloses unconventional receptor functions in protein translation and DNA damage repair

Presence of GPCRs inside the nucleus has been reported including by us[6,31,32,33], yet their roles independent of membrane-bound G proteins are not known. Hence detection of HCAR1 inside the nucleus prompted us to investigate spatiotemporal interactome of N-HCAR1. We used the Bio-ID system[31] to construct HCAR1-Bio-ID fusion protein which again revealed the expected nuclear localization of HA-tagged HCAR1 (Extended Fig. 6a,b). Cells were treated or not with lactate followed by nuclear isolation. We purified the biotinylated proteome of isolated nuclei and subjected them to mass spectrometry (Fig. 3a). Surprisingly, different proteins found in the interactome of N-HCAR1 are not classical GPCR signaling modulators (Fig. 3b; Extended Fig. 6c,d), suggesting potential involvement of N-HCAR1 in functions other than canonical receptor-mediated signaling. There was a clear distinction in the interactome of N-HCAR1 stimulated or not with lactate (Fig. 3b; Extended Fig. 6c,d), suggesting that different conformations of N-HCAR1 participate in separate protein complexes (Fig. 3b). The protein interactome of N-HCAR1 after lactate treatment was enriched for ribosomal regulatory processes (Fig. 3c; Extended Fig. 6f). Experiments using sucrose gradient ribosomal profiling further revealed that HCAR1 total KD and N-HCAR1 KD cells have a lower content of non-polysomal ribosomes (Fig. 3d). The interactome of N-HCAR1 isolated from cells untreated with lactate was particularly enriched for proteins mediating tRNA aminoacylation involved in

protein translation (Fig. 3c; Extended Fig. 6e). Concordantly, quantification of methionine incorporation rate revealed that protein translation was decreased in HCAR1 KD and N-HCAR1 KD HeLa cells compared to cells with intact HCAR1 (Fig. 3e). Similar observations on protein translation were made in U251MG and A549 cells (Extended Fig. 7a,b).

Strikingly, the interactome of N-HCAR1 with and without lactate also revealed components of the DNA damage repair machinery, including the dominant DNA damage marker H2AX (Fig. 4a), consistent with the proposed role of HCAR1 in DNA damage response (DDR)[20]. We validated the interaction of N-HCAR1 with H2AX from our BioID mass spectrometry data with co-immunoprecipitation (Fig. 4b). We thus proceeded to irradiate cultured HeLa cells and measured ensuing γH2AX foci number as a proxy for DDR (Fig. 4c). WT and WT-HCAR1-rescued cells displayed lower number of γH2AX foci compared to HCAR1 KD and N-HCAR1 KD cells, suggesting nuclei devoid of HCAR1 have limited DNA damage repair capacity (Fig. 4c). Thus, the functional effects of N-HCAR1 activity identified from the BioID data were corroborated based on the functional assays in our system. Together these data show that N-HCAR1 interacts with non-classical GPCR effectors in the nucleus to promote protein translation and DNA damage repair.

N-HCAR1 is involved in direct gene regulatory function by interacting with chromatin remodelers

Since several chromatin remodeling factors were also detected in the interactome of N-HCAR1 (Fig. 3b), the potential for direct gene/chromatin regulation (rather than signaling for downstream gene regulation) by HCAR1 prompted us to perform ChIP-sequencing of HCAR1 to identify genes that interact with the receptor. N-HCAR1 interacted with chromatin and approximately 260 genes were found to bind to HCAR1 upon stimulation with lactate, while the number of genes associated with the unstimulated receptor was higher (~600) (Fig. 5a). Less than 8% of the genes were shared between vehicle and lactate treatment (Fig. 5a), inferring that a conformational change in the N-HCAR1 caused a genomic redistribution. We verified the interaction of a selected gene panel (such as *SERPINE1*, *HCAR*, *PTGER4*) that are interacting with N-HCAR1 with or without lactate treatment or the shared genes with ChIP-qPCR along with extra controls (Supp Fig. 4a-c).

Unstimulated N-HCAR1 mostly localized to gene deserts, while upon lactate stimulation it occupied gene segments with considerable increase in promoter occupancy (Fig. 5b; Extended Fig. 8a). A similar trend can be observed at a smaller scale within individual genes, where unstimulated N-HCAR1 distributes in an unorganized pattern around transcription start sites, contrasting with a precise reorientation at transcription start sites upon lactate stimulation (Fig. 5c; Extended Fig. 8b). Consistently, the putative binding motifs enriched in the unstimulated and in the ligand-activated conditions are completely different (Extended Fig. 8 c,d). Further computational analysis revealed that the promoters of HCAR1-bound genes are co-enriched with positive regulatory epigenetic markers upon lactate treatment including H3K9ac, H3K27ac and H3K4me3 [35, 36], but are devoid of compact chromatin marker H3K27me3 (Fig. 5e). Accordingly, gene expression analysis for some of the genes highly enriched with HCAR1 based on our ChIP-seq analysis showed an HCAR1-dependent expression profile, abrogated by N-HCAR1 KD (Fig. 5d). Altogether data provide convincing evidence for direct gene regulatory function of N-HCAR1.

Ontological and gene set enrichment analysis revealed that while the enriched genes for unstimulated N-HCAR1 are mainly involved in general homeostatic processes, ligand activated N-HCAR1 binds to genes that regulate various features of cell migration (Fig. 5f). However, a complementary analysis using the Reactome feature of both gene sets concentrate on pathways related to different migratory phenotype (Extended Fig. 8g,h). We performed cell migration assay to validate the role of the nuclear population of HCAR1 in cell migration (Fig 5). Correspondingly HeLa cells devoid of total or N-HCAR1 exhibited defective migration (Fig. 5g); similar observations were made on U251MG and A549 cells (Extended Fig. 7c,d). Thus N-HCAR1, distinct from its canonical signaling capacity, is able to directly interact and regulate gene expression, particularly those involved in cell movement.

HCAR1 at the nucleus regulates a larger gene network than its plasma membrane counterpart

Our observations suggested that N-HCAR1 regulates gene expression through location-biased signaling and interactions with nuclear proteins and genes. We elucidated the

transcriptomic network regulated by N-HCAR1 by performing RNA-seq (Fig. 6a; Supp Fig. 4d). Approximately 35% of all differentially regulated genes by HCAR1 were governed solely by N-HCAR1 and ~26% through plasma membrane/cytoplasmic HCAR1 (Fig. 6b,c). Although two thirds of HCAR1 reside extra-nuclear, this higher level of gene regulation by N-HCAR1(Fig. 1f), highlights the importance of nuclear localization in this process. Stimulated and unstimulated conditions disclosed different transcriptomic profiles (Fig. 6a,b); only ~34% of genes were shared for stimulated and unstimulated conditions (Fig. 6b). Interestingly, unstimulated N-HCAR1 regulates a larger gene network than other counterparts (Fig. 6c), consistent with the ChIP-seq data.

In an attempt to determine if the N-HCAR1-gene complex based on ChIP-seq results culminates in gene expression or suppression, we aligned RNA-seq on the ChIP-seq data. Analysis revealed that most of the genes bound to N-HCAR1 (lactate stimulated or not [based on CHIP-seq]) were upregulated by the N-HCAR1 (based on RNA-seq) (Fig. 6e). Overall, findings suggest an unconventional function for N-HCAR1 in directly regulating gene expression through interactions involving protein/chromatin complexes, which are notably independent of lactate stimulation.

Ontological analysis of lactate-stimulated N-HCAR1-dependent transcriptome related to the migration pathways including anchoring junctions, network-forming collagen trimer and extracellular matrix organization (Fig. 6d), consistent with ChIP-seq data (Fig. 5f). Transcriptomic signature of unstimulated N-HCAR1 revealed other aspects of migration such as cell-substrate adhesion, collagen fibril organization and lamellipodium (Fig. 6d). Gene Set Enrichment Analysis (GSEA) relative to migration ascertained N-HCAR1-dependent induction of genes involved in migration (Supp Fig. 4e). Hence, stimulated and unstimulated N-HCAR1 coordinately promote expression of genes involved in different features of cell movement resulting in migration (as per Fig. 5g).

N-HCAR1 promotes cancer growth and metastasis *in vivo*

HCAR1 has been shown to enhance cancer progression and metastasis *in vivo*[15,16, 17]; and N-HCAR1 mediates proliferation, survival and migration of cancer cells *in vitro* (as shown in Fig. 2e,f; Fig. 5g). We validated the role of N-HCAR1 *in vivo* by injecting luciferase-

expressing HeLa cells subcutaneously in NOD/SCID/IL2Rγ null (NSG) mice. Proliferation of tumors was monitored by bioluminescent live imaging (Extended Fig. 9a). Tumor volume and mass markedly increased in mice injected with HCAR1-expressing WT rescue cells compared to tumors silenced for HCAR1 and N-HCAR1 KD cells (δS305A rescue) (Extended Fig. 9b,c). Coherently, resected tumors expressing HCAR1 at the nucleus exhibited higher proliferation index (Ki-67) and endothelial density (CD31 positivity) consistent with angiogenesis, and less apoptosis (TUNEL staining), compared to tumors devoid of N- HCAR1 (Extended Fig. 9d; Supp Fig. 5). To assess metastatic spread, HeLa cells were injected in the tail vein and metastatic tumor spread was monitored by bioluminescence. As seen with tumor volume, metastatic spread was observed only in HeLa cells expressing nuclear-intact HCAR1 (Extended Fig. 9e,f); no metastatic spread was detected in HCAR1 KD and N-HCAR1 KD cells. These data support the notion that the effects of nucleus-specific localized HCAR1 on different functions translate into promoting cancer growth and propagation *in vivo*.

Discussion

GPCRs are involved in essentially every pathophysiological process; this has largely been thought to be based on their plasma membrane location and the receptor modality. Mounting evidence points to intracellular location of GPCRs and their downstream effectors, such that location-biased signaling is arising as a major concept in the field. On one hand, subcellular GPCRs and especially the nuclear ones are readily detected inside these organelles, other than on lipid membrane of these organelles[2]. However, there is no report on the function of these GPCRs other than through their classical reliance on membrane-associated receptor-function. Non-conventional activity of GPCRs would introduce the new concept of “location-biased activity” next to the location-biased signaling. While the latter concept remains in its infancy, the former is a totally new one that has yet to be introduced, to the best of our knowledge. Accordingly, these understudied aspects of GPCR biology provide new avenues for therapeutic exploitation of this highly druggable receptor family; location bias (signaling and activity) expands on the physiologic effects of GPCRs which could explain enigmatic features of their involvement in a number of roles.

The requirement for intracellular lactate is observed for a number of HCAR1-dependent functions and the receptor's mechanism of action is unexplained in these conditions[19,20,21]. Herein, we demonstrate several unprecedented molecular functions for a GPCR at the nucleus, which promote cancer malignancy, independent of conventional signaling activity. While the nucleus contains one third of the cellular reservoir of HCAR1, we provide unparalleled evidence that combined nuclear location-biased activity and signaling of a nuclear GPCR on gene regulation surpasses that exerted through its plasma membrane counterpart, underlining in this case the importance of nuclear HCAR1. Essentially, the nuclear HCAR1, other than its signaling activity, directly governs gene regulation via its interaction with the genome for various important functions including migration; the receptor also modulates critical processes such as protein translation and DNA damage repair through protein-protein interactions. All these processes were validated by functional assays at endogenously expressed level of HCAR1 (i.e. using scrambled shRNA and WT rescue); these effects were not observed in the mutant rescues excluded from the nucleus. Overall, these two points ascertain the validity of our high-throughput analysis and the specificity of N-HCAR1 involvement in these processes.

HCAR1 was found at the INM with analogous conformation to its plasma membrane counterpart and comparably capable of triggering classical $G_{\alpha i}$ and $G_{\beta\gamma}$ protein-coupled signaling bursts of ERK and AKT activation in the nucleus. Additionally, based on the interactome data for the N-HCAR1, it binds to transcriptional factors of different ATP-dependent chromatin remodeling complexes INO80, SWI/SNF and ISWI (e.g., INO80b, SMARCC1 and BPTF, respectively)[37]; these interactions were observed with the stimulated and unstimulated receptor, suggesting a potential constitutive role of HCAR1 in modulating their activity. While these chromatin remodelers are significantly misregulated in many cancers[38,39,40], N-HCAR1 activated by the higher concentration of lactate seen in tumors (Warburg effect) could alter their activity in favor of cancer promotion. Interestingly, N-HCAR1 also interacted with NSD1, a histone methyltransferase known to bind to different nuclear receptors (including estrogen, thyroid, retinoic acid, and retinoid receptors)[41]. Since NSD1 is frequently mis-regulated in cancers[41], it is tempting to speculate that metabolic rewiring could cause epigenomic alterations in favor of cancer malignancy. Concordantly, our genome-wide association

study suggests that N-HCAR1 could directly promote expression of genes involved in migration, potentially through such epigenetic modulations. We found that several genes such as *WNT3, SERPINE1,* and *CDH5* previously reported to be regulated through HCAR1[42,43,16,44], are indeed immunoprecipitated with N-HCAR1, suggestive of direct gene regulation. Direct gene regulation also seems to apply for *HCAR1* gene itself as well through lactate-stimulated N-HCAR1, consistent with the reported auto-induction of HCAR1[9,7,45]. These wide-ranging properties of this GPCR are reminiscent of non-GPCR classical nuclear receptors, such as estrogen receptor (ERα)[46]; ERα has recently been reported to possess RNA-binding capacity while regulating post-transcriptional expression and splicing of specific sets of genes. Although nuclear receptors are essentially transcriptional factors (compared to GPCRs), uncovering their non-transcriptional roles is a major discovery with potential therapeutic implications[46]. Accordingly, emphasis on unconventional receptor functions of GPCRs could capitalize on development of inhibitors and allosteric modulators rather than solely focus on antagonists/agonists for therapeutic discovery.

GPCRs for ligands such as metabolites that are constantly present within the cell, are stochastically in either active and inactive states at any given time; the ratio of active to inactive state depends on the cellular concentration of the ligand[47]. On the other hand, a single molecule GPCR stoichiometrically can simultaneously bind to Gα, Gβ, GRKs, and arrestin[48]. These interactions occur through the intracellular domains of a GPCR, and are distinct from their ability to form homo/heterodimers via their hydrophobic transmembrane domains[49], thus providing other docking sites for protein-protein interactions. These intricacies expose the abilities of GPCRs to form various protein complexes. Along these lines, one could envisage non-cylindrical conformations for a GPCR inside the nucleus with its hydrophobic domains deeply buried in protein complexes interacting with hydrophobic domains of other proteins[2]; such interactions could explain transcriptional and translational control of N-HCAR1 from within the nucleus.

The present study highlights the multifaceted functionality of GPCRs through its nuclear location and direct interaction with the genome. Nuclear HCAR1 provides an adaptive fitness of cells to respond to metabolic tweaks through intracellular ligands, as is the case

for lactate which augments survival, proliferation and propagation of cancer cells, by acting via N-HCAR1. These myriad of roles for nuclear-resident HCAR1 might not be determinant for individual cellular processes it participates in, however its collective functions on various processes convey a significant adaptation for cancer progression and malignancy, while providing an unprecedented dimension for GPCR biology.

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Contribution:

MA.MN conceived and designed the study, performed and analyzed the experiments and drafted the manuscript. G.C performed the bioinformatic analysis under supervision of S.J. E.B & P.C contributed to the analysis of the data. M.M helped with performing animal study. D. M provided the Luciferase virus and A549 cells and helped with the analysis of some data under the supervision of C.B. S.C supervised the whole project and oversaw the conception, experiments, analysis and drafting of the work.

Declaration of Interests: The authors declare no competing interests.

Material and Methods:

Cell lines and treatments:

HeLa (CCL-2 ATCC) and A549 (CCL-185 ATCC) were purchased from commercial vendors and maintained according to the manufacturers protocol in DMEM, 10% FBS and 1% Pen/Strep, U-251MG cells were a kind gift from Dr. Hardy's lab and maintained in EMEM + 2 mM Glutamine + 1% non-essential amino acids + 1 mM Sodium Pyruvate + 10% FBS and 1% Pen/Strep in a humidified incubator with 5% CO2 at 37 °C. Stable cells were generated using appropriate drug selection (G-418, Puromycine) after plasmid transfection or viral transduction, and were maintained in these antibiotic instead of Pen/Strep. A stock concentration of 500 mM lactate (in PBS and pH adjusted to 7.4) was used for cell stimulation and similar volume of PBS as vehicle was used as control. The data for end point phenotypic effects in HeLa cell are presented in the main figures and the data for end point phenotypic effects in A549 and U-251MG cells are presented in the extended figures.

Cell replication and survival were determined by enumerating live and dead cells using automatic countess cell counter (Thermofisher) using trypan blue exclusion assay (Thermofisher). Cells were treated with 20μM 5FU or starved for 24h for survival assay, and cell numbers were calculated before and after the treatments. Trypan blue was added in 1X ratio to the media containing cells and each replicate was performed in quadruplicates to determine the number of live and dead cells. Each experiment was conducted at least in triplicates.

Plasmids, RNAi and CRISPR:

Cells were transfected with human HCAR1 because of enhanced immunoreactivity to exogenous tag (such as Flag), enabling superior localization resolution and for immunoprecipitation, as well as for FAP and Bio-ID construct preparation. The cDNA encoding HCAR1 was PCR-amplified with encompassing appropriate restriction enzymes sites at both ends of the amplicon. The final product was gel-purified (ThermoFisher GeneJET Gel Extraction Kit), digested with the restriction enzymes and cloned into each vector. pCMV-Tag 2A (Agilent) was used for N-terminal flag tagging using EcoRI and HindIII flanking sites, pCDNA3.1-HCAR1-flag (Genscript) was used for C-terminal flag

tagging. ICL3 and S305A mutations were generated using back-to-back primers on pCDNA3.1-HCAR1-Flag vector by Q5 Site-Directed Mutagenesis Kit (NEB). Fluorogen activating peptide fusion to HCAR1 was synthesized with insertion of HCAR1 using BsmI site into pMFAP-β1 vector (Spectragenetics), and BioID fusion was generated with HCAR1 insertion into flanking sites AccIII and AflII in MCS-13X Linker-BioID2-HA (Addgene 80899) vector. All plasmids were sequenced to verify the correct insertion. Vectors were transfected into the cells using TranIT-X2 reagent (Mirus) according to the manufacturers protocol and grown on appropriate antibiotics to generate stable cell lines.

Lentiviral shRNA against HCAR1 targeting 3'UTR regions of the gene (shHCAR1a: GCTTTATTTCAGGCCGAATGA; shHCAR1b: GCTCTGACCTTCTTCAAATCT) and the scrambled shRNA were purchased from GeneCopoeia (Cat# LPP-HSH007585-LVRU6MP-100). Targeting the 3'UTR regions allowed us to use our previous plasmid constructs for rescue experiments.

RNA isolation and quantitative PCR:

RNA was isolated using either RiboZol (VWR) or RNeasy mini kit (Qiagen) then was converted to cDNA using iScript (Bio-Rad) following manufacturer's instructions. qRT-PCR was performed using SYBR green master mix (Bio-Rad) on Roche light cycler. HRP and 18S were used for normalization of the results (normalization to 18S is reported in the manuscript).

Immunoblot and ELISA:

Cells were lysed in RIPA buffer (Cell Signaling) and a cocktail of protease inhibitors (Roche) and protein concentration was measured with Bradford assay (Bio-Rad). Proteins were heated in reducing Laemmli sample buffer at 95°C and resolved in SDS-PAGE protein gel and transferred to PVDF membrane (Bio-Rad). Membranes were blocked using 5% BSA (Sigma) for 1 h and then incubated for overnight with the primary antibodies. Afterward membranes were washed 3X with TBST and incubated with HRP-conjugated secondary antibodies for 1h and then were washed again and revealed by ECL (VWR) chemiluminescence.

ERK1/2 and AKT phosphorylation levels were measured with both western blot and ELISA kits (Abcam). Cells were treated overnight with PTX (300ng/ml) or Gallein (20μM) and then nuclei were isolated and suspended in 10mM lactate or vehicle with rotation at 37°C for 15min, washed with PBS 2X and then were lysed in either Laemmeli buffer for western blot or treated according to the manufacturers protocol for ELISA.

Immunofluorescence (IF) staining:

Cells were seeded on Poly-L-Lysine coated cover slips in a 6 well plates for over night in incubator. For DNA damage, cells were irradiated with 1Gy intensity using Faxitron CP-160 irradiator and let to recover for 4 hours at 37°C in incubator, and then IF was performed on them. Wells were rinsed three times with PBS, fixed in 4% formaldehyde (Sigma) for 10 min at RT, and then washed three times 5min with PBS. Subsequently, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT and blocked in 1% BSA in PBS/ 0.1% Tween-20 for 1h. Cover slips were incubated in a humid chamber with primary antibody for overnight at 4°C diluted in new blocking buffer. The antibody solution was washed with PBST (3x, 5min) and samples were incubated with secondary antibody (Alexa-fluor conjugated secondaries) for 1h at RT in dark. Cells were washed again, stained with DAPI, washed and then mounted. Slides were imaged with Leica confocal microscopy (SP8 or SP8-STED) with appropriate channels and 60X objectives. Fluorescent lights were gated to avoid any overlap between channels. Images were analysed using LAS-X (Leica microscopy licenced software) and ImageJ software. Imaris 9.9 software was used for 3D rendering of z-stacked confocal images.

TEM:

Cells were fixed in 4% paraformaldehyde + 0.5% Glutaraldehyde in cacodylate buffer (0.1M, pH7.2). After fixation, cells were washed 2 times in cacodylate buffer (5 min) and then in PBS. Permeabilization was performed with 0.2% Triton X-100 for 15 min, and then cells were blocked with PBST with 10%FBS for 1h. Samples were incubated ON with primary antibody in blocking buffer at 4°C, and delivered to electron microscopy facility of faculty of medicine (Universite de Montreal) for further processing and imaging. Primary antibody was detected with nanogold conjugated secondaries and silver

enhancement, and imaged with transmission electron microscope (Philips CM120) equipped with a Gatan digital camera.

Fluorogen Activating Peptide (FAP) pulse chase:

HCAR1 gene was cloned into pMFAP-β1 vector (Spectragenetics) and pulse chase experiments were performed after generation of stable cell lines. Cell were treated with 100nM βGREEN-np membrane impermeant fluorogen (Spectragenetics), which can not enter the cell unless bound to FAP, and one minute later cells were treated with 10 mM lactate (or PBS) for indicated time points. Afterward, cells were briefly washed with PBS and fixed with 4% paraformaldehyde, and the nuclei was stained with Hoechst 33342. βGREEN-np was excited and imaged using Alexa fluor 514 channel with Leica confocal microscopy. No nuclei with βGREEN-np were observed in the experiments. IF staining of stable cell lines without lactate treatment were performed as mentioned previously with Myc primary antibody against the Myc-tag in the N-terminus of HCAR1 and C-terminus of FAP.

Nuclei isolation & staining:

Isolating nuclei was performed as described previously[50]. Briefly, cells were washed and resuspended in PBS plus protease inhibitor cocktail (Roche) and 1mM PMSF. They were centrifuged (10000rpm for 10sec) and resuspended in PBS + protease inhibitor cocktail + 0.1% NP-40 and triturated for 7 times with P1000 micropipette tip. Supernatant was collected (or removed) after centrifugation as cytoplasmic fraction. The pellet (containing nuclei) was subjected to second time trituration (5 times) and centrifuged again to obtain pure nuclei fraction. After last centrifugation, supernatant was removed and nuclei was collected in the desired solution. The purity of nuclear fraction was checked under the microscope and was validate by western blotting.

Intact non-permeabilized nuclei were resuspended in PBS and mounted on Poly-L Lysine coated cover slips for IF staining. For ONM permeabilization, nuclei were resuspended in 0.0008% Digitonin and rotated for 5min at RT in microfuge tubes. Permeabilized nuclei were centrifuged, washed and resuspended in PBS + PI cocktail. For proteinase K digestion, nuclei were incubated at 37°C with rotation in 100µg/ml proteinase K (Sigma) solution before or after ONM permeabilization with Digitonin. Nuclei were then washed

3x in 1%BSA + 5mM PMSF solution and then further permeabilized with Digitonin for ONM permeabilization or 0.1% triton for INM permeabilization and then were subjected to IF staining.

HCAR1 3D modeling:

We analyzed the structure of HCAR1 in both active and inactive forms as described elsewhere[51,52], using the web service: https://gpcrm.biomodellab.eu/. Also we examined the inactive structure using recent AlphaFold[53], and there was minimal differences between both models only in low confidence regions of the free c-terminus. Post-translation phosphorylation site analysis was done using: https://www.phosphosite.org/, and described in[24]. Only phosphorylation residues with validated mass spectrometry data (HTP) were used for mutagenesis analysis.

cAMP measurement in isolated nuclei:

Isolated nuclei were resuspended in PBS with 10mM lactate with rotation at 37°C for 10min, nuclei were then counted using hemocytometer and were subjected to immunoassay cAMP Direct kit (Abcam) according to manufacturer's protocol. Protein G-coated plated were used for the ELISA provided in the kit and measurements were performed with HRP development by measuring its OD at 450nm with Clariostar plate reader.

Bio-ID & Mass Spectrometry:

HCAR1 gene was cloned into MCS-13X Linker-BioID2-HA (Addgene 80899) vector. After validating the fusion protein has same localization pattern as the HCAR1 itself, stable cells were generated by antibiotic selection. Control samples were transfected stable cell lines with the empty vector. Cells were treated with Biotin (50µM) and 10mM lactate (or PBS) and incubated for ~16 hours in incubator. Nuclei were isolated and their purity was validated. Isolated nuclei were lysed with non-denaturing lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 (NP-40), 2 mM EDTA) plus PI cocktail[54]. Both experimental and control samples were analyzed in triplicates. The lysate was incubated with magnetic streptavidin MyOne Dynabeads (ThermoFisher) at 4°C for ON with rotation. Beads were washed 5X in the lysis buffer and delivered to LC-MS/MS at IRIC Center for Advanced Proteomics Analyses, a Node of the Canadian Genomic Innovation Network that is supported by the Canadian Government through Genome Canada. Peptides

were prepared with on-bead tryptic digestion based on previously established protocol[55,56]. Beads were washed 10 times with 50mM Tris (pH 7.2), and afterward, were reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP [Tris(2-carboxyethyl) phosphine hydrochloride; Thermo Fisher Scientific], and vortexed for 1 h at 37°C. Chloroacetamide (Sigma-Aldrich) was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. One microgram of trypsin was added, and digestion was performed for 8 h at 37°C. Samples were dried down and solubilized in 4% formic acid (FA). Peptides were loaded and separated on a home-made reversed-phase column (150-μm i.d. by 200 mm) with a 56-min gradient from 10 to 30% ACN-0.2% FA and a 600-nl/min flow rate on an Easy nLC-1000 connected to an Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA). Each full MS spectrum acquired at a resolution of 60,000 was followed by tandem-MS (MS-MS) spectra acquisition on the most abundant multiply charged precursor ions for a maximum of 3s. Tandem-MS experiments were performed using collision-induced dissociation (CID) at a collision energy of 30%. The data were processed using PEAKS X (Bioinformatics Solutions, Waterloo, ON) and the Uniprot human database (20349 entries). Mass tolerances on precursor and fragment ions were 10 ppm and 0.3 Da, respectively. Fixed modification was carbamidomethyl (C). The data were visualized with Scaffold 4.0 (protein threshold, 99%, with at least 2 peptides identified and a false-discovery rate [FDR] of 1% for peptides)[57,58].

BioID data were analyzed first with Scaffold (Proteome Software Inc. Portland OR) to produce quantitative values from normalized total spectra (Top 3 area based on Total Ion Count, TIC) for the amino acid sequences detected by mass spectrometry. Quantitative values for annotated proteins were then batch corrected using an empirical bayes framework (ComBat, SVA, https://rdocumentation.org/packages/sva/versions/3.20.0) and differential protein abundance between condition was assessed using MetaboAnalyst after quantile normalization, log transformation and autoscaling[59]. Statistical analysis using t-test was performed to determine the proteins enriched in HCAR1 BioID cells compared to cells containing empty vectors (i.e., only the biotin ligase), in PBS and lactate treatment separately. Visualization of normalized data was done in R (version 4.1.0, 2021 The R Foundation for Statistical Computing) using gplots/heatmap.2 (https://CRAN.R-project.org/package=gplots), ggplot2 (https://CRAN.R-project.org/package=ggplot2) and

EnhancedVolcano (Blighe K, Rana S, Lewis M (2022). EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.14.0). Pathway analysis of enriched proteins was performed on EnrichR[60] and Panther[61].

Ribosomal profiling:

Ribosome profiling was performed by sucrose gradient fractionation as described previously[62]. Briefly, cells were treated with 10µg/ml of cycloheximide (CHX) for 15min at 37°C in the incubator to install ribosome disassembly. Cells were washed and resuspended in cold PBS containing CHX and PI cocktail, and then lysed in lysis buffer (20 mM Tris-HCl,100 mM KCl, 5 mM MgCl2, 0.5% Nonidet P-40) containing CHX, RNase inhibitor and PI cocktail. Lysate was cleared by centrifugation and equal amounts were layered on top of a cold sucrose gradient (10 to 60 % gradient containing CHX, RNase inhibitor and PI cocktail). Gradients were centrifuged in Hitachi swinging ultracentrifuge (CP90NX) at 190,000g for 1.5h at 4°C. Gradients were fractionated by piercing the bottom of sucrose gradient tube and the OD of collected fractions were measured at 254nm spectrum. Ribosomal profile was plotted and area under the curve of each monosome subunit (40S, 60S and 80S) and polysomes were measured for quantification of the ribosomal content.

Protein translation rate measurement:

Nascent protein synthesis rate was measured using Click-iT AHA Alexa Flour 488 protein synthesis HCS assay kit (Invitrogen) according to the manufacturer's protocol. Equal number of cells were plated ON in a 96-well plate and the media was washed out the next day and replaced with a methionine-free media containing L-azidohomoalanine (AHA) as the methionine analog, and incubated for 30min. AHA is incorporated into proteins during protein synthesis in the methionine-free media. The amount of incorporated AHA is detected with a click chemical reaction by Alexa flour 488. The intensity of Alexa fluor 488 is adjusted with the intensity of DNA counterstain Hoechst 33342 and directly corresponds to the nascent protein synthesis rate.

Co-IP:

Cells were fractionated and cytoplasmic and nuclear fractions were lysed with non-denaturing lysis buffer plus PI cocktail. The lysates were then pre-cleared with equilibrated

protein G magnetic beads (Cell Signaling) for 1h at RT with rotation. The pre-cleared lysate was incubated with primary antibody O/N at 4°C. Pre-washed magnetic beads were added to the immunocomplexes and incubated for 1h at RT with rotation. Afterward, beads were isolated with magnetic separation rack and washed 5x with lysis buffer. Finally, beads were resuspended in 3x SDS sample buffer and incubated at 95°C for 5min to elute the immunocomplexes. Elutes were analyzed by western blotting.

ChIP-Seq:

Chromatin immunoprecipitation (ChIP) was performed using SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling) based on manufacturer's protocol. Briefly, DNA-protein complexes were crosslinked using 1% final concentration of formaldehyde (Sigma) for 10min at RT, and quenched with Glycine (final concentration of 125mM). Cells were then washed with cold PBS + PI cocktail, scraped into conical tubes and centrifuged to remove the supernatant. After isolation, nuclei were treated with micrococcal nuclease to digest the DNA, and then sonicated. Digested chromatin was analyzed by agarose gel. Chromatins were then incubated with immunoprecipitating antibody O/N at 4°C with rotation. Control samples were incubated with IgG antibody. ChIP-grade protein G magnetic beads were added to the IP reactions and incubated for 2h at 4°C with rotation. Beads were then washed with low- to high-salt wash buffers and chromatin was eluted in elution buffer for 30min at 65°C. Chromatins were reverse-crosslinked with NaCl and proteinase K and incubation at 65°C for 2h. DNA was purified with spin columns, and analyzed by qPCR or sent to Next Generation Sequencing. Samples were analyzed with bioanalyzer for quality control and single-end NGS was performed at IRIC genomic platform with Nextseq 500 illumina system. Samples were sequenced with a depth of ~35M per sample with 75 cycles. Both C- and N-terminus flag tagged cells were used for ChIP-seq studies. Two samples from each terminus tagged cells were used for either lactate or vehicle (PBS); in total 4 samples per treatment group, and only shared genes in each group was used for further bioinformatic analysis.

ChipSeq fastq files were processed with default parameter using the ChipSeq pipeline from GenPipe[63]. BAM files were visualized with IGV[64] (A public access version is also available: PMC3346182). Peak files were analyzed using the ComputeMatrix function from deeptool[65] to determine distance relative to histone modification marks based on

Broad Histone Helas Chip-seq data from the Encode project[66]. ChIPseeker[67] was used to annotated the data and profile the binding peaks.

Migration assay:

Cells were cultured to reach a density of 90-100% confluency and then a wound was made by scratching the monolayer cells with sterile p200 pipette tips. Cells were washed and new media was added. Cells were imaged by phase contract microscopy right after scratch to measure the initial distance (t0), and later in indicated time points. Reduction of the scratched area due to the migration of the cells were measured as the rate of migration. Each experiment was conducted in triplicates and each time in multiple wells of the plates.

Transcriptomic:

Equal number of cells were seeded in 10cm dish and let to grow a density of 70-80% confluency. Lactate was added to the final concentration of 10mM (or equal volume of PBS) and incubated to 6 hours. RNA was extracted with RNeasy mini Kit (Qiagen). Samples were sent to IRIC genomic platform for analysis and sequencing. RNA integrity and quantity was validated with Bioanalyzer and then used for sequencing. Samples were sequenced with Nextseq 500 illumina system with a depth of ~35M per sample with single-end 75 cycles. Each sample was sequenced at least in triplicates.

RNAseq data were pre-processed using the RNAseq next-flow pipeline[68] with the star_salmon aligner and the salmon pseudo-aligner (reference genome GRCh38). Gene counts were normalized and scaled to perform differential gene expression analysis between groups using Seurat after regressing for batch effect[69]. Differentially expressed genes were further analyzed using fastGSEA[70] and enrichR[60].

Sequencing:

500 ng of total DNA for ChIP-sequencing or RNA was used for library preparation. DNA/RNA quality control was assessed with the Bioanalyzer Nano assay on the 2100 Bioanalyzer system (Agilent technologies) and all samples had a RIN above 9,5. For RNA, PolyA selection was done using Dyna Beads Oligo(dT) (Thermo Fisher). Library preparation was done with the KAPA DNA or RNA Hyperprep kit (Roche). Ligation was made with Illumina dual-index UMI (IDT). All libraries were diluted and normalized by qPCR using the KAPA library quantification kit (KAPA; Cat no. KK4973). Libraries were

pooled to equimolar concentration. Sequencing was performed with the Illumina Nextseq500 using the Nextseq High Output 75 (1x75bp) cycles kit. Around 30M single-end PF reads were generated per sample for ChIP-sequencing and around 35M for RNA sequencing. Library preparation and sequencing was made at the Institute for Research in Immunology and Cancer's Genomics Platform (IRIC).

Raw base calls were converted to FASTQ files using bcl2fastq version 2.20 and allowing 0 mismatches in the multiplexing barcode. Prior to that, base calls had been obtained from the Illumina NextSeq 500 sequencer that runs RTA 2.11.3.0.

Animal experiments:

All animal procedures were approved by institutional ethic committee of CR Sainte-Justine Hospital. NOD/SCID/IL2Rγ null (NSG) mice were obtained from Humanized mouse platform of CR-CHU Saint-Justine. Mice were housed in the sterile animal facility under pathogen-free conditions. 5-week-old animals were separated for acclimatization and were injected with cells at 6 weeks of age. HeLa cells (shScrambled, shHCAR1b and KD+δS305A) were transduced with Renilla-Luciferase viral vectors and GFP-positive cells were sorted. Passage 3 of sorted cells were counted and 1 million cells were injected into animals. Mice were anesthetized with 2.5% isoflurane and cells were injected subcutaneously in the right flank after shaving and sterilizing the area for tumor growth monitoring, or injected into the tail vein for metastatic analysis of the cells. Two male and two female mice were used for each cell line. Animals were imaged once every 3 days for 5 weeks. Mice were anesthetized and injected with D-Luciferin (150mg/kg) 10 min before imaging. In vivo whole-body imaging was performed using Epi-Fluorescence and Trans-Fluorescence imaging system (OiS300, LabeoTech) and signal intensities were normalized and measured in radiance integrated density (photons | s^{-1} | sr^{-1} | cm^{-2}) using Fiji Macros. Animals were sacrificed after 5 weeks or at the study cut-off points (extreme abscess or 30% weight loss and morbid condition) and tumor and organs were harvested for histological analysis.

Immunohistochemistry:

Tissue samples were fixed in 10% Formalin O/N at RT, and then embedded in paraffine. Paraffin embedded blocks were cut to 5µm sections and deparaffinized in Xylene and decreasing concentrations of ethanol. Antigen retrieval was done with Sodium Citrate

buffer (10mM Sodium Citrate, 0.05% Tween, pH6) for 10 min with pressure cooker. Slides were washed with TBS / Triton X-100, and then blocked with 10% normal serum, 1% BSA in TBS for 2h at RT. Incubation with primary antibody was done ON in 4°C, and endogenous peroxidase activity was blocked with 0.3% H_2O_2. Secondary HRP-conjugated antibody was used for detection. Slides were developed with DAB reagent and counterstained with DAPI. Samples were dehydrated, mounted and visualized with Leica DMi8 wide-field microscope with monochrome color camera.

Statistics:

The number of samples per group, number of replicates and details of error bars are provided in the figure legends. Statistical tests were performed using GraphPad Prism 9.0 (GraphPad Software). For comparisons between two experimental groups, unpaired two-tailed t-tests were used, and for comparison of three and more groups Analysis of Variance (ANOVA) was used followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, ***$P < 0.0001$ significance levels. Data are shown as the mean ± s.d, except data in panel fig. 7b, c & f which are mean ± s.e.m. Every dataset is composed of at least n≥3 independent experiments. List of genes from high-throughput experiments were compared with Venny[71].

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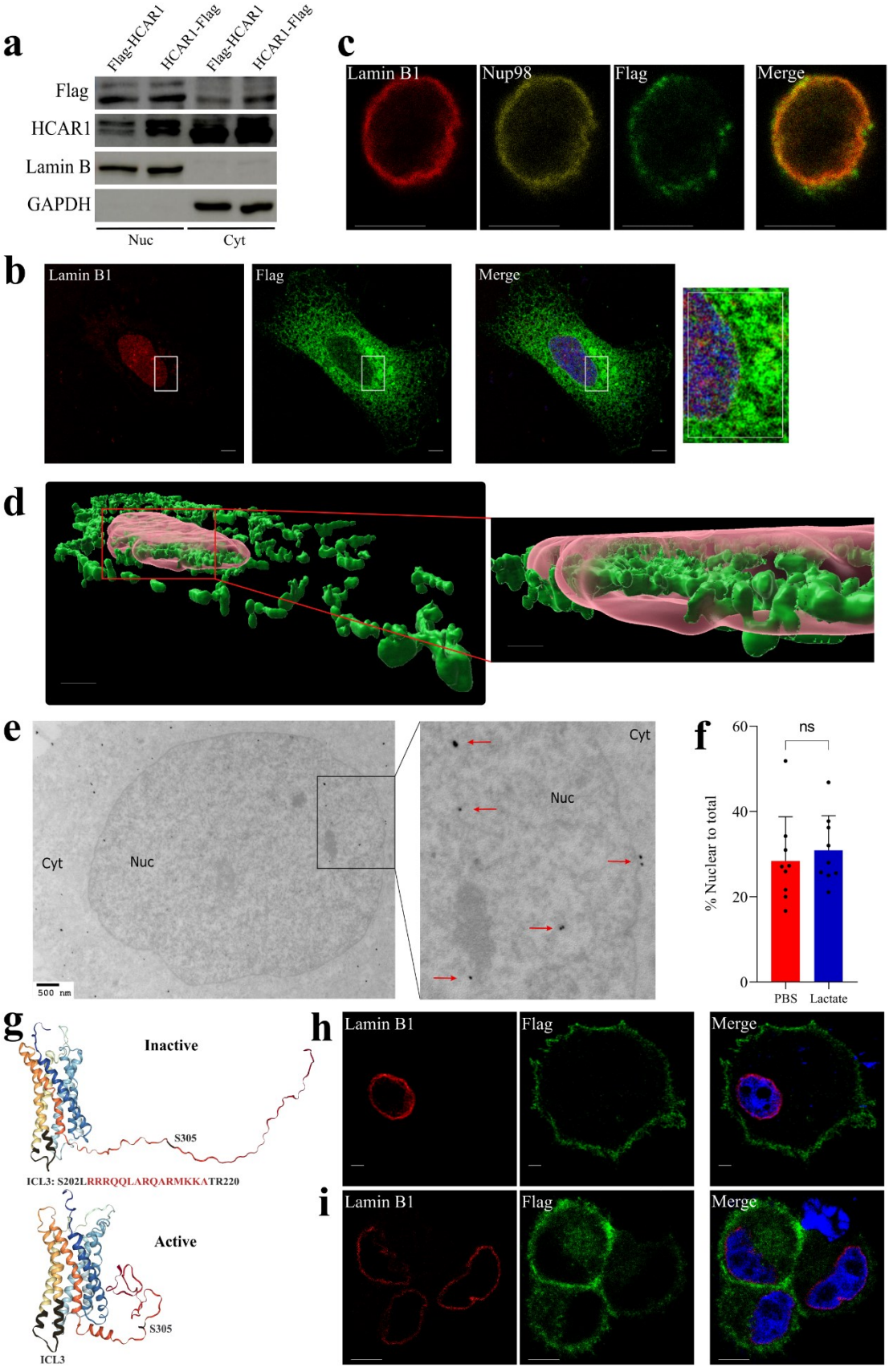
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Fig. 1: HCAR1 is present in the nucleus and ICL3 and S305 phosphorylation are responsible for this localization pattern.

a) Western blot analysis of fractionated cells transfected with C & N-terminally Flag-tagged HCAR1. Lamin B and GAPDH were used to confirm pure isolation of nuclei. **b-c)** Confocal imaging of C-terminally flag-tagged HCAR1 whole cells **(b)** or isolated nuclei **(c)**. **d)** 3D image of z-stacked confocal images of C-terminally flag-tagged HCAR1 whole cells. Transparent red is Lamin B and green is anti-flag. Z-stack are 200nm layers. **e)** TEM graphs from C-terminally flag-tagged HCAR1. **f)** Quantification of HCAR1 from TEM images of PBS and Lactate treated cells (10mM for 1h). **g)** 3D modeling of HCAR1 in inactive and active conformations by GPCRM. The black highlights indicate the spanning regions for ICL3 domain and S305. **h-i)** Confocal imaging of C-terminally flag-tagged HCAR1 with ICL3 deletion **(h)** and S305A mutation **(i)**. Notice the cytoplasmic signal of HCAR1 in S305A. Scale bars are 5μm.

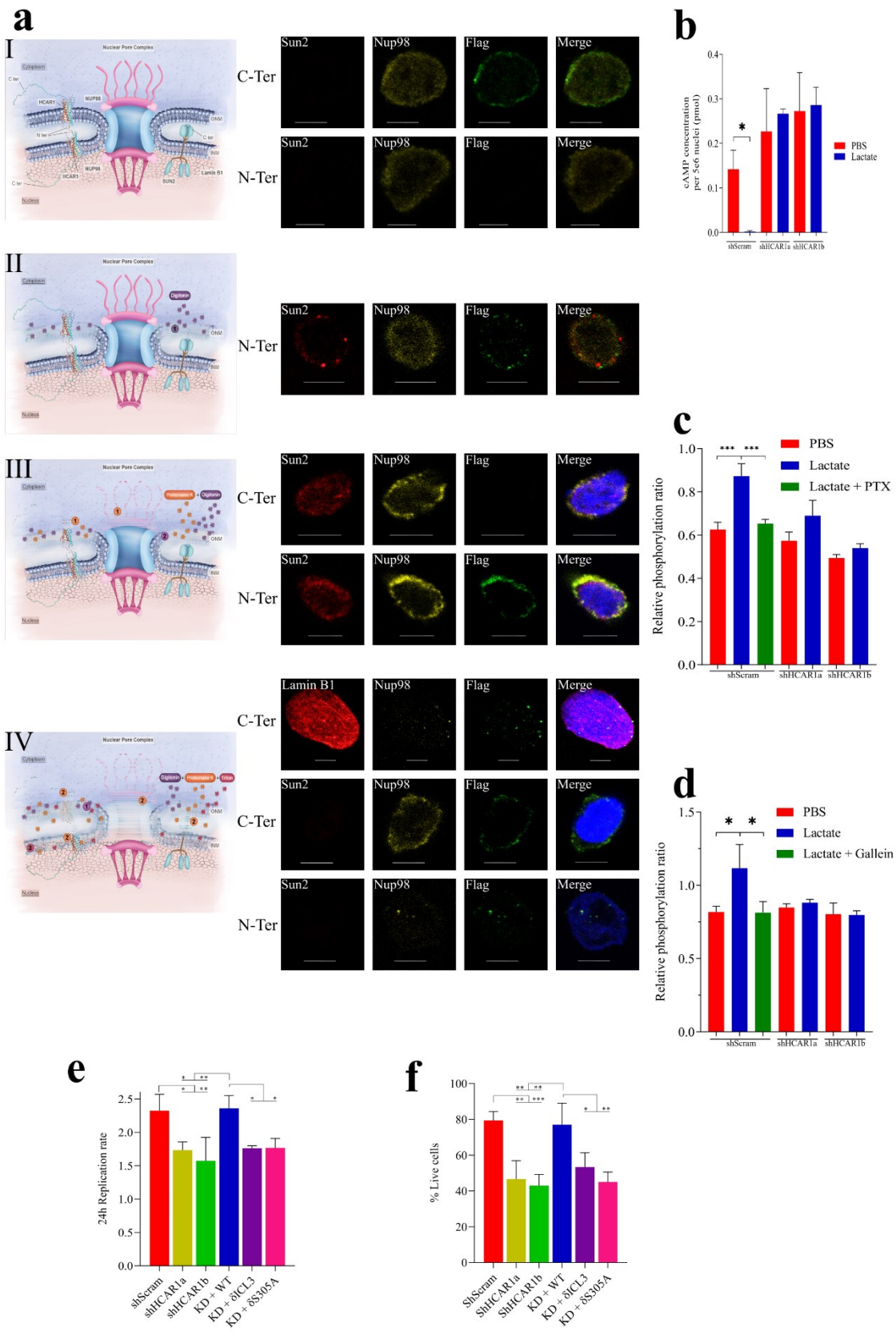

a
I
II
III
IV
C-Ter
N-Ter
Sun2
Nup98
Flag
Merge
Lamin B1
b
cAMP concentration per 5e6 nuclei (pmol)
PBS
Lactate
shScram
shHCAR1a
shHCAR1b
c
Relative phosphorylation ratio
Lactate + PTX
d
Lactate + Gallein
e
24h Replication rate
KD + δICL3
KD + δS305A
f
% Live cells

Fig. 2: Intranuclear signaling of HCAR1 activates nuclear ERK and AKT effectors leading to cellular proliferation and survival.

a) Confocal images of nuclei isolated from cells expressing C-ter or N-ter flag-tagged HCAR1. **(I)** intact nuclei, **(II)** ONM permeabilized nuclei with intact INM, **(III)** surface protein digested nuclei with ONM permeabilization and intact INM, **(IV)** ONM permeabilized nuclei with intact INM was treated with PK to digest proteins on the ONM and nuclear lumen, and after washing PK, nuclei were treated with triton to permeabilize INM. Notice loss of Sun2 indicating digestion of luminal proteins. **b)** cAMP level in isolated nuclei from scrambled shRNA or two different HCAR1 KD cells with PBS or lactate treatment (10mM for 10min). The cAMP concentration is presented in picomole per 5 million nuclei. **c-d)** ELISA analysis of ERK **(c)** and AKT **(d)** phosphorylation rates in isolated nuclei from scrambled shRNA or two different HCAR1 KD cells with PBS or lactate treatment (10mM for 15min). PTX or Gallein treatment of scrambled cells were performed prior to nuclei isolation. **e)** Cell proliferation rate in scrambled shRNA, two different HCAR1 KD cells, WT-rescue and nuclear KD cell lines. **f)** Cellular survival rate in 5FU treated cells. Data are mean ± s.d. from n≥3 biological replicates. Analysis of Variance (ANOVA) was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ significance levels. Scale bars are 5µm.

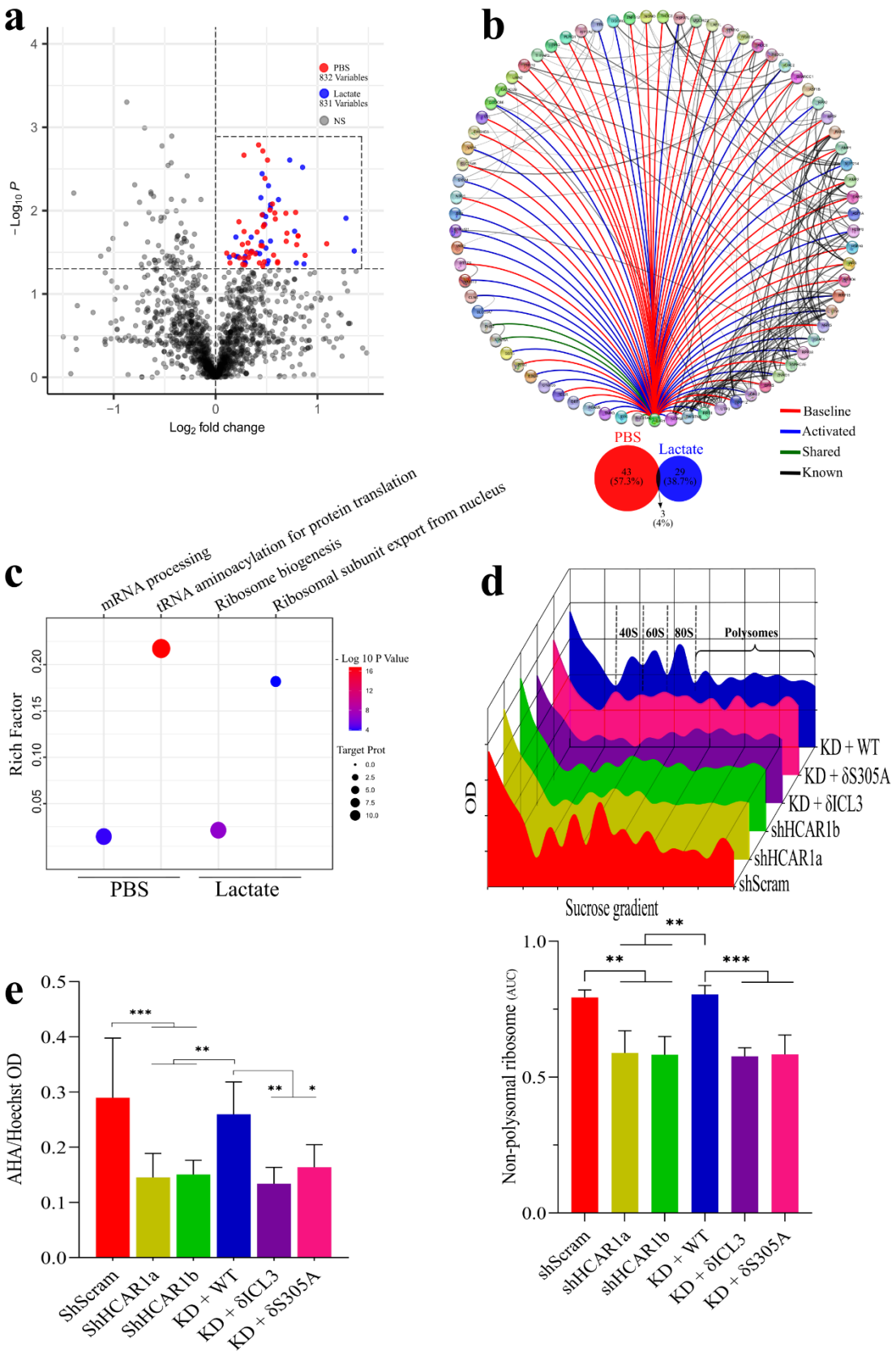

a
PBS
832 Variables
Lactate
831 Variables
NS
−Log10 P
Log2 fold change
b
Baseline
Activated
Shared
Known
PBS
Lactate
43
(57.3%)
29
(38.7%)
3
(4%)
c
mRNA processing
tRNA aminoacylation for protein translation
Ribosome biogenesis
Ribosomal subunit export from nucleus
Rich Factor
- Log 10 P Value
Target Prot
PBS
Lactate
d
40S
60S
80S
Polysomes
OD
KD + WT
KD + δS305A
KD + δICL3
shHCAR1b
shHCAR1a
shScram
Sucrose gradient
e
AHA/Hoechst OD
ShScram
ShHCAR1a
ShHCAR1b
KD + WT
KD + δICL3
KD + δS305A
Non-polysomal ribosome (AUC)
shScram
shHCAR1a
shHCAR1b
KD + WT
KD + δICL3
KD + δS305A

Fig. 3: N-HCAR1 interactome is enriched for protein translational processes and it promotes protein translation rate.

a) Volcano plot representing significantly interacting proteins with N-HCAR1. Plot shows protein abundance (log_2 fold change) versus significance ($-log_{10}$ *P* value) in isolated nuclei of HCAR-BirA expressing cells relative to BirA alone. Significantly enriched proteins in the upper right quadrant (proteins within the dashed square) in both PBS and lactate treated (10mM for 24h) samples are selected for subsequent analysis. **b)** Interactome map of N-HCAR1 in both PBS or lactate treated cells. Red lines indicate interaction of enriched proteins with HCAR1 when treated with PBS, blue lines indicate interactions with HCAR1 when treated with lactate, green lines indicate interaction in both cases, and black lines represents already established interactions based on STRING. The bottom Venn diagram shows differential and overlapping significantly enriched proteins in PBS and lactate treated samples. **c)** Enrichment dot plot of proteins in panel b based on gene ontology molecular functions (Panther). **d)** Upper panel: Representative sucrose gradient ribosomal profiling for Scrambled shRNA, total and nuclear HCAR1 KD, and WT rescue cells. Lower panel: Normalized measurement of the upper panel for Area Under the Curve (AUC) of the monosomes (40S, 60S and 80S subunits). **e)** Protein translation rate with methionine incorporation rate measurement. Methionine incorporation rate (L-azidohomoalanine; AHA) was adjusted to the number of cells (Hoechst). Data are mean ± s.d. from n≥3 biological replicates. ANOVA was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ significance levels.

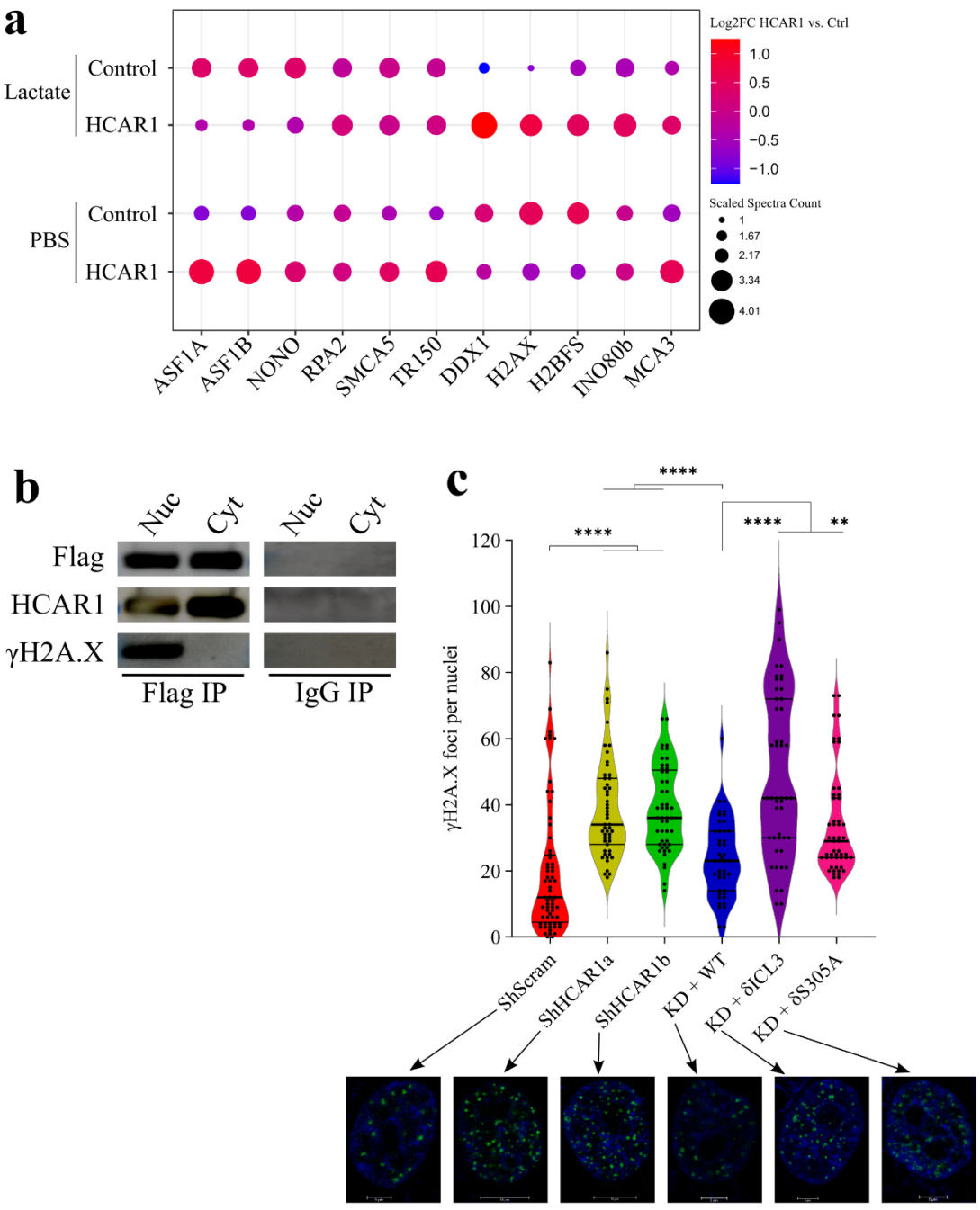

a
Lactate
Control
HCAR1
PBS
Control
HCAR1
ASF1A
ASF1B
NONO
RPA2
SMCA5
TR150
DDX1
H2AX
H2BFS
INO80b
MCA3
Log2FC HCAR1 vs. Ctrl
1.0
0.5
0.0
−0.5
−1.0
Scaled Spectra Count
1
1.67
2.17
3.34
4.01
b
Nuc
Cyt
Nuc
Cyt
Flag
HCAR1
γH2A.X
Flag IP
IgG IP
c
γH2A.X foci per nuclei
0
20
40
60
80
100
120
****
****
****
**
ShScram
ShHCAR1a
ShHCAR1b
KD + WT
KD + δICL3
KD + δS305A

Fig. 4: N-HCAR1 with its interactome promotes DNA damage repair.

a) Dot plot of enriched proteins with HCAR1 which are involved in DNA damage repair. **b)** Validation of BioID mass spectrometry for interaction of HCAR1 and H2AX (from Fig. 3b). Co-immunoprecipitation of γH2AX with HCAR1 or IgG in fractionated cells. **c)** Irradiated cells were let to recover for 4h and the amount of DNA damage was measured with γH2AX foci. Each dot represents the number of γH2AX foci per nucleus, for 4 separate experiments. Underneath are the representative nuclei of irradiated cells with confocal imaging of γH2AX staining. Data are mean ± s.d. from n=4 biological replicates. ANOVA was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ significance levels.

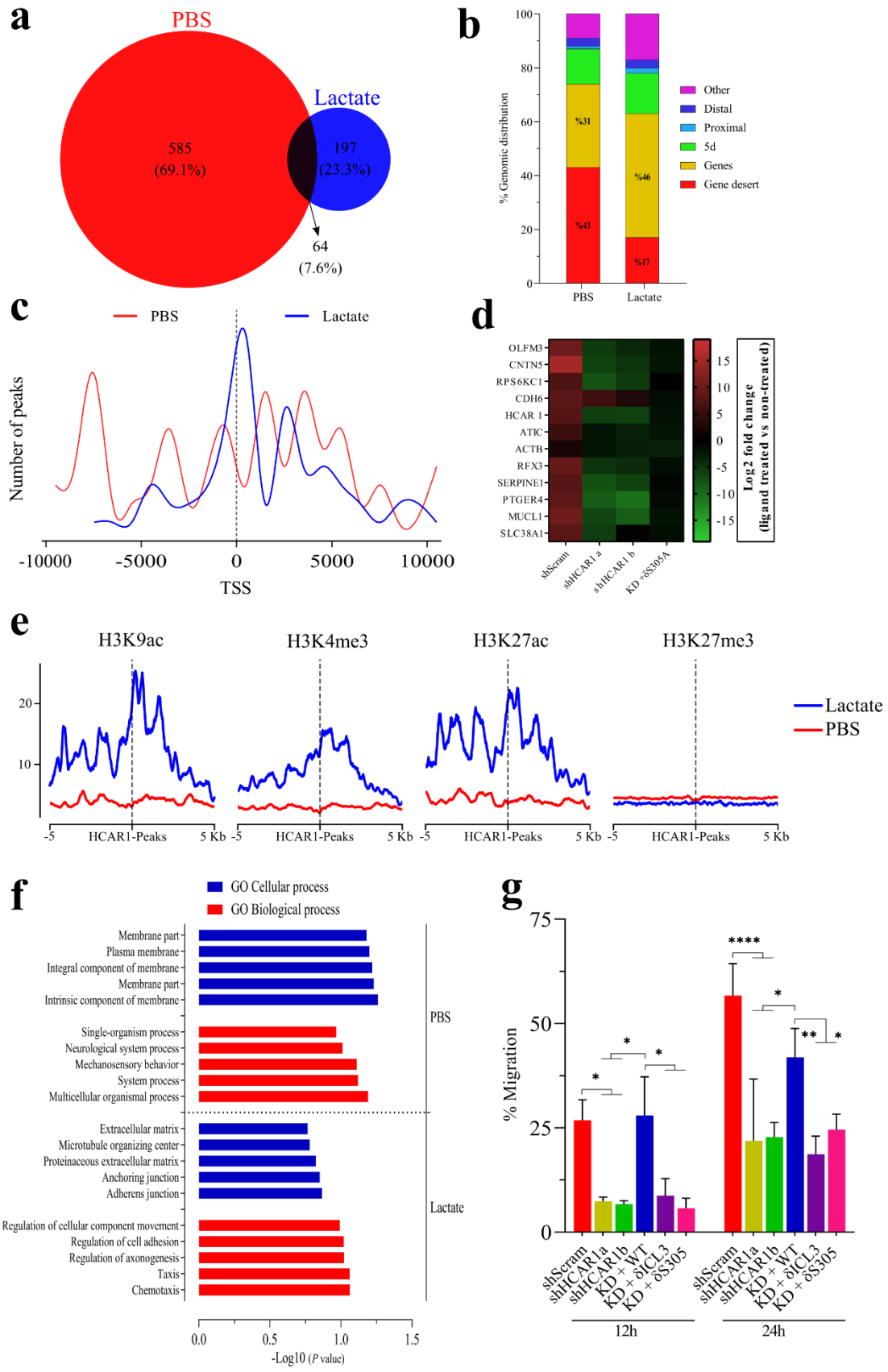

a
PBS
Lactate
585
(69.1%)
197
(23.3%)
64
(7.6%)
b
% Genomic distribution
Other
Distal
Proximal
5d
Genes
Gene desert
%31
%43
%46
%17
PBS
Lactate
c
PBS
Lactate
Number of peaks
-10000
-5000
0
5000
10000
TSS
d
OLFM3
CNTN5
RPS6KC1
CDH6
HCAR 1
ATIC
ACTB
RFX3
SERPINE1
PTGER4
MUCL1
SLC38A1
Log2 fold change
(ligand treated vs non-treated)
shScram
shHCAR1 a
shHCAR1 b
KD +δS305A
e
H3K9ac
H3K4me3
H3K27ac
H3K27me3
Lactate
PBS
HCAR1-Peaks
5 Kb
f
GO Cellular process
GO Biological process
Membrane part
Plasma membrane
Integral component of membrane
Membrane part
Intrinsic component of membrane
Single-organism process
Neurological system process
Mechanosensory behavior
System process
Multicellular organismal process
Extracellular matrix
Microtubule organizing center
Proteinaceous extracellular matrix
Anchoring junction
Adherens junction
Regulation of cellular component movement
Regulation of cell adhesion
Regulation of axonogenesis
Taxis
Chemotaxis
PBS
Lactate
-Log10 (P value)
g
% Migration
shScram
shHCAR1a
shHCAR1b
KD + WT
KD + δICL3
KD + δS305
12h
24h

Fig. 5: HCAR1 genome-wide interactions show enrichment for genes promoting migration.

a-c) ChIP-seq of HCAR1 from PBS or Lactate-treated (10mM for 1h) cells from quadruplicate samples. For controls and validations see Supp Fig. 4. **a)** Venn diagram representing the number of genes associated with HCAR1 in each treatment. **b)** Genomic distribution of HCAR1 in each treatment. Genes (exon or intron), proximal (2kb upstream of TSS), distal (between 2 and 10kb upstream of TSS), 5d (between 10 and 100kb upstream of TSS), Gene desert (≥100kb up or down stream of TSS), Others (anything else). **c)** Normalized number of HCAR1 peaks around TSS of genes. **d)** qRT-PCR for the top 4 genes in each section of the Venn diagram (panel a). Expression levels are presented as Log_2 fold changes of lactate treated (10mM for 6h) cells over PBS treatment (n=4). **e)** Co-alignment of histone marks from encode project from HeLa cells over HCAR1 peaks. **f)** Ontological analysis of HCAR1-bound genes in PBS- and lactate-treated samples. **g)** Scratch assay to measure the migration rate of cells (n=3). Data in panel d) & g) are mean ± s.d. from biological replicates. Their ANOVA was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, ***$P < 0.0001$ significance levels. TSS: Transcription Start Sites.

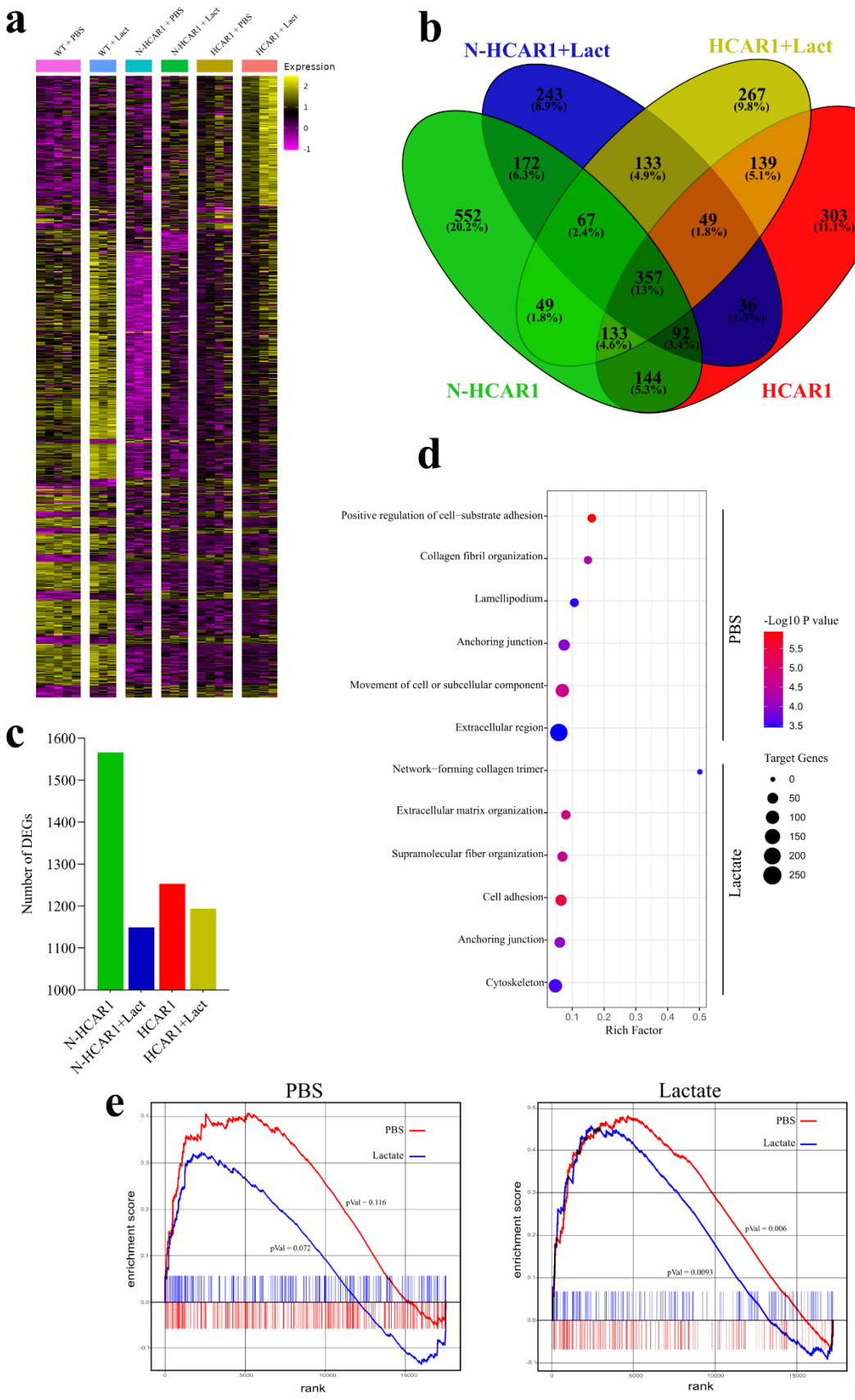
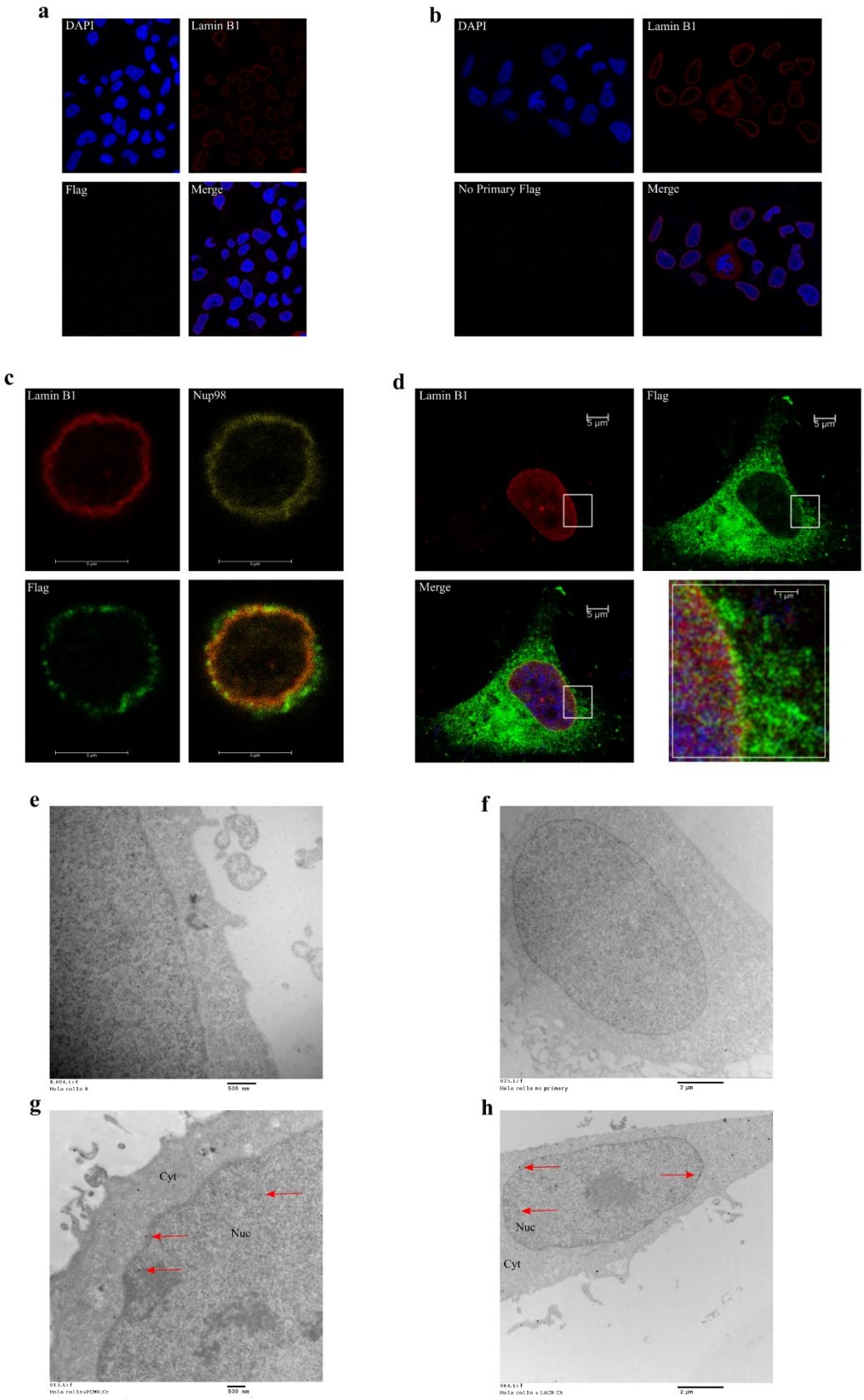

a
WT + PBS
WT + Lact
N-HCAR1 + PBS
N-HCAR1 + Lact
HCAR1 + PBS
HCAR1 + Lact
Expression
2
1
0
-1
b
N-HCAR1+Lact
HCAR1+Lact
243 (8.9%)
267 (9.8%)
172 (6.3%)
133 (4.9%)
139 (5.1%)
552 (20.2%)
67 (2.4%)
49 (1.8%)
303 (11.1%)
357 (13%)
49 (1.8%)
36 (1.3%)
133 (4.6%)
92 (3.4%)
144 (5.3%)
N-HCAR1
HCAR1
c
Number of DEGs
1600
1500
1400
1300
1200
1100
1000
N-HCAR1
N-HCAR1+Lact
HCAR1
HCAR1+Lact
d
Positive regulation of cell−substrate adhesion
Collagen fibril organization
Lamellipodium
Anchoring junction
Movement of cell or subcellular component
Extracellular region
Network−forming collagen trimer
Extracellular matrix organization
Supramolecular fiber organization
Cell adhesion
Anchoring junction
Cytoskeleton
PBS
Lactate
-Log10 P value
5.5
5.0
4.5
4.0
3.5
Target Genes
0
50
100
150
200
250
0.1
0.2
0.3
0.4
0.5
Rich Factor
e
PBS
Lactate
PBS
Lactate
pVal = 0.116
pVal = 0.072
pVal = 0.006
pVal = 0.0093
enrichment score
rank
0
5000
10000
15000

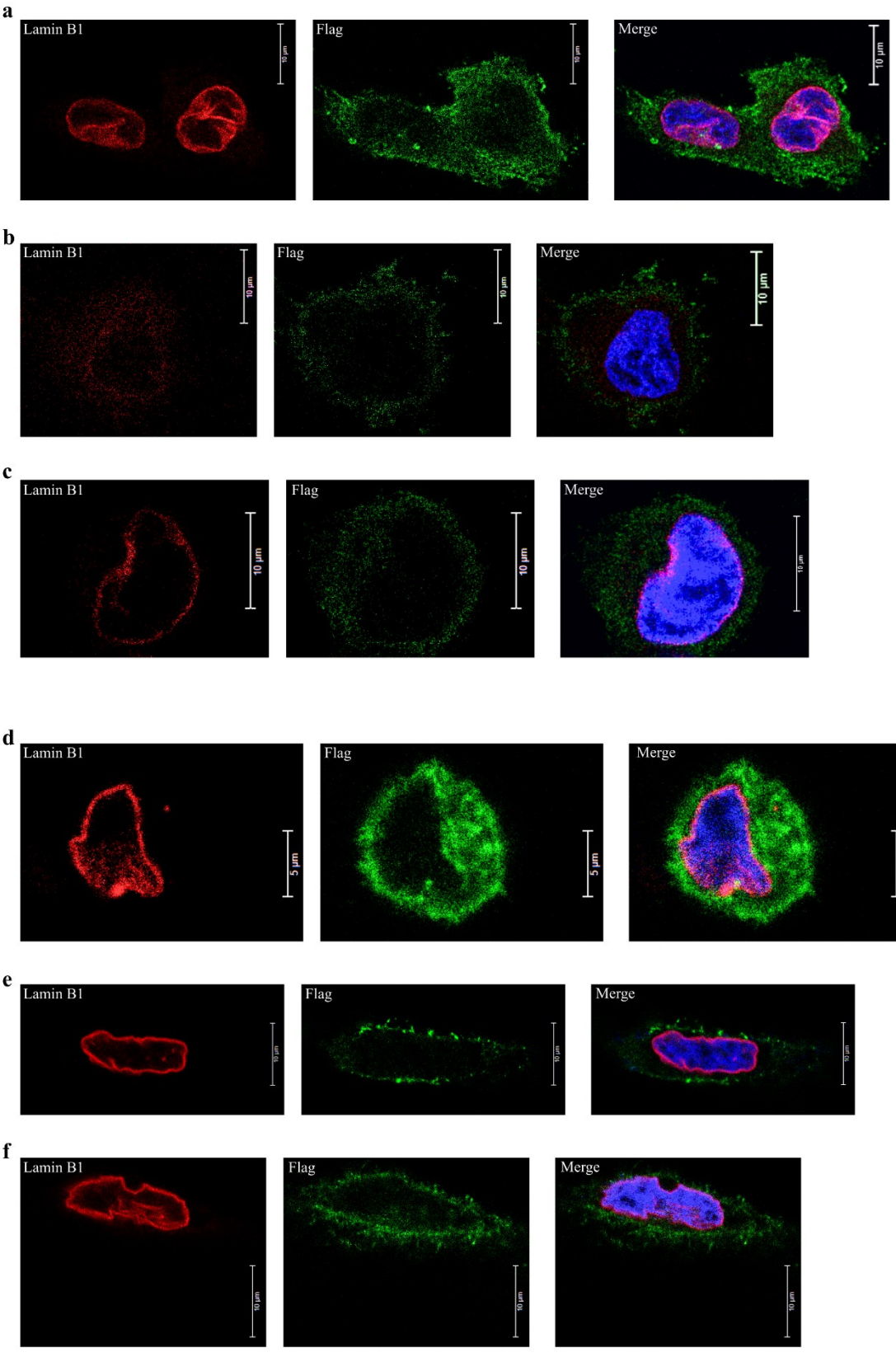
Fig. 6: N-HCAR1 regulates a larger gene network than its plasma membrane/cytoplasmic counterpart

a-c) RNA-seq of PBS and lactate treated (10mM for 6h) samples from Scrambled shRNA, shHCAR1b and shHCAR1b+RNAi δS305A HCAR1 cells. For validation of ChIP-seq by qRT-PCR see Fig. 5d. **a)** Heatmap of significantly DEGs. **b)** Venn diagram representing all DEGs in each line compared to shScrambled with their corresponding treatment. **c)** Bar graph representing total number of all DEGs in each line compared to shScrambled with their corresponding treatment. **d)** Ontological analysis of genes that were uniquely downregulated only in HCAR1 nuclear KD cells with PBS or lactate treatments. **e)** Waterfall plots representing overall general positive regulatory function of N-HCAR1 on gene transcription in N-HCAR1-bound genes (linking ChIP-seq and RNA-seq data). The expression values are extracted from RNA-seq data of HCAR1 nuclear KD cells with PBS and lactate treatments. The expression values represent WT condition to indicate expression level of genes regulated through N-HCAR1. The gene list is extracted from PBS-treated (left panel) and Lactate-treated (right panel) HCAR1 ChIP-seq data. shScrambled PBS (n=5), shScrambled Lactate (n=3), shHCAR1b PBS (n=4), shHCAR1b Lacate (n=4), shHCAR1b+ RNAi δS305A HCAR1 PBS & Lacate (n=3). DEG: Differentially Expressed Genes.


a
DAPI
Lamin B1
Flag
Merge
b
DAPI
Lamin B1
No Primary Flag
Merge
c
Lamin B1
Nup98
Flag
d
Lamin B1
5 µm
Flag
5 µm
Merge
5 µm
1 µm
e
500 nm
f
2 µm
g
Cyt
Nuc
500 nm
h
Nuc
Cyt
2 µm

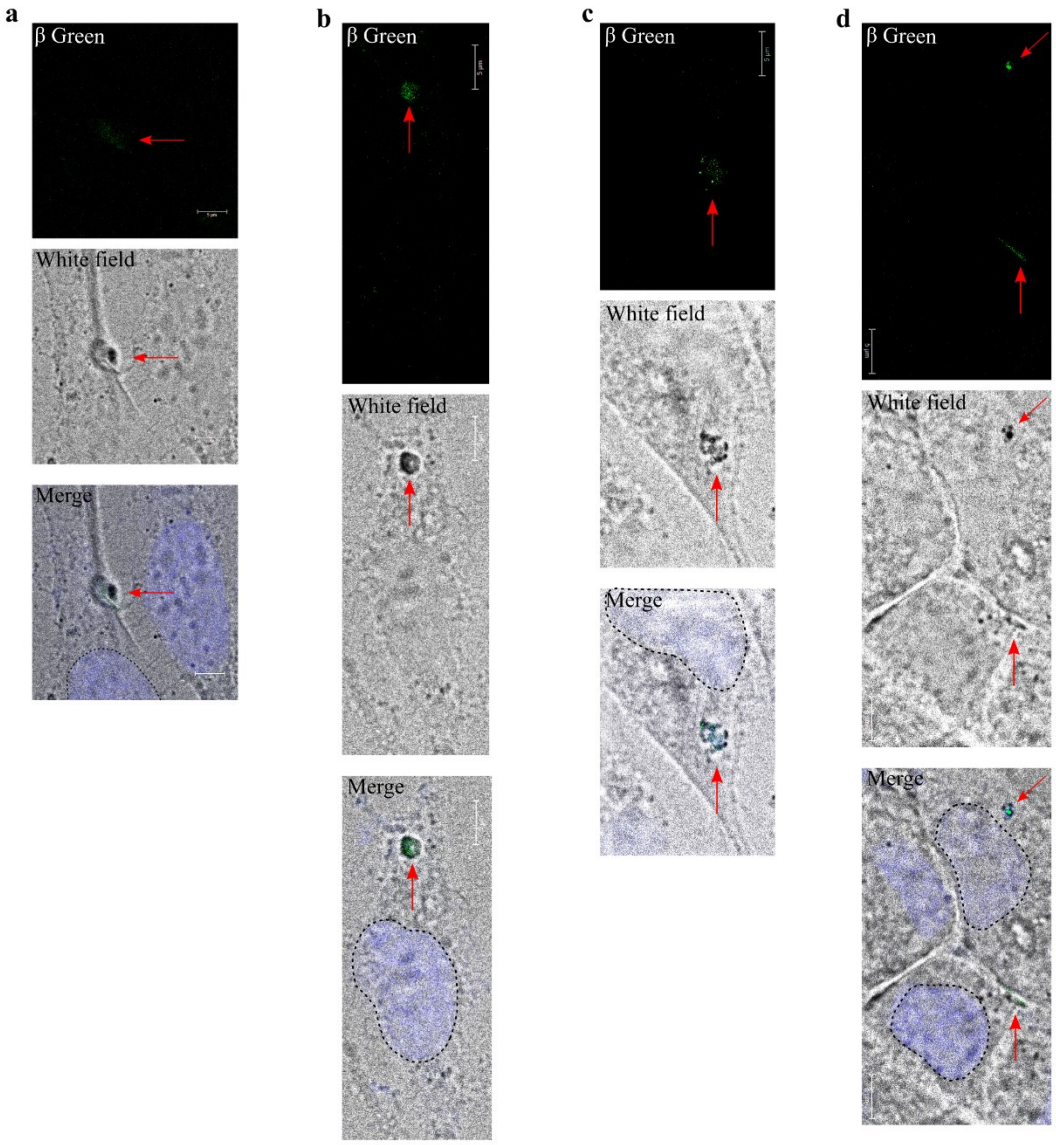
Extended Fig. 1: Controls and extra validations of Fig. 1

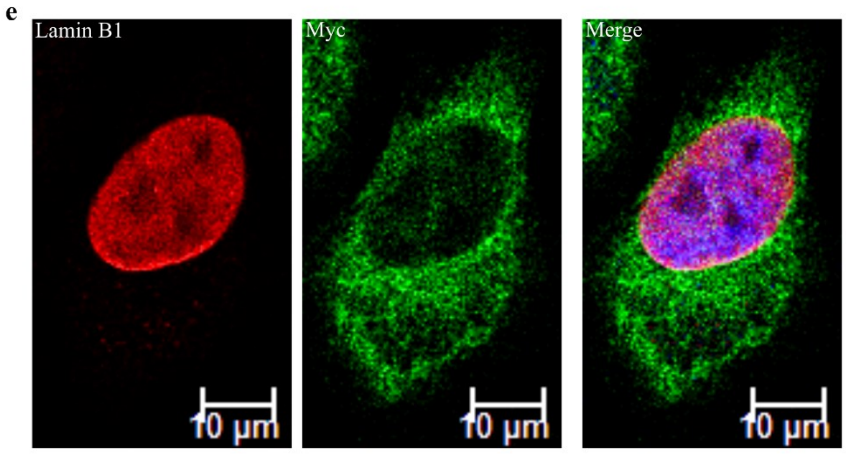
a-b) Control experiments for HCAR1 localization with empty vector containing Flag tag **(a)**, and no primary antibody staining control **(b)** in immunofluorescence confocal imaging. **c-d)** Immunofluorescence confocal imaging of N-terminally Flag tagged HCAR1 in isolated nuclei **(c)** and whole cell **(d)**. **e-f)** Control experiments for HCAR1 localization with empty vector containing Flag tag **(e)**, and no primary antibody staining control **(f)** in TEM images. **g-h)** TEM images of N-terminally Flag-tagged HCAR1 with PBS **(g)** and Lactate treated (10mM for 1h) cells **(h)**. All images are in HeLa cells.


a
Lamin B1
Flag
Merge
10 μm
b
Lamin B1
Flag
Merge
10 μm
c
Lamin B1
Flag
Merge
10 μm
d
Lamin B1
Flag
Merge
5 μm
e
Lamin B1
Flag
Merge
f
Lamin B1
Flag
Merge

Extended Fig. 2: HCAR1 localization in other cell lines

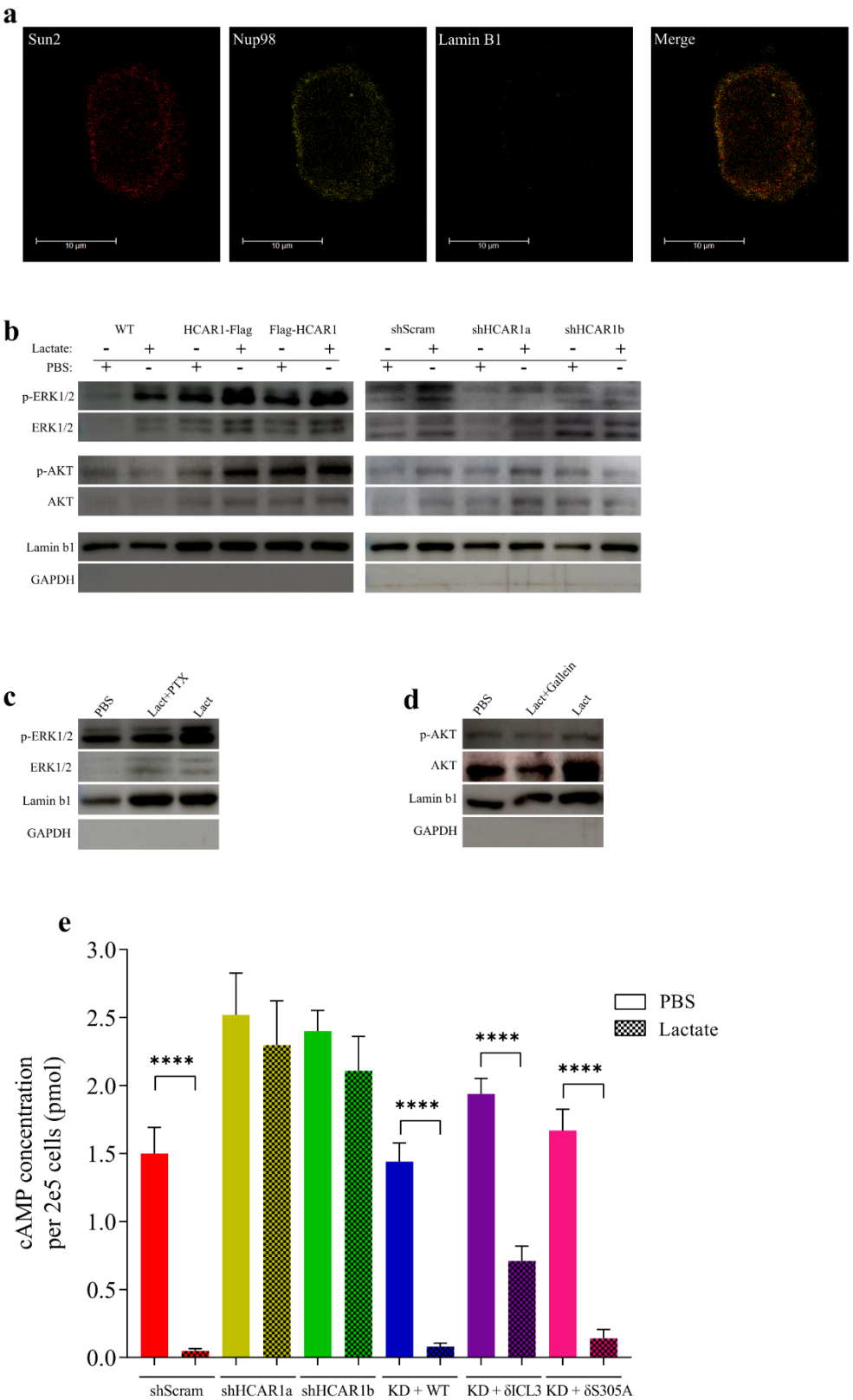
a-c) Immunofluorescence confocal imaging of C-terminally Flag tagged HCAR1 in U251MG cells with WT HCAR1 **(a)**, δICL3 HCAR1**(b)** and δS305A HCAR1 **(c)** cells. **d-f)** Immunofluorescence confocal imaging of C-terminally Flag tagged HCAR1 in A549 cells with WT HCAR1 **(d)**, δICL3 HCAR1 **(e)** and δS305A HCAR1 **(f)** cells.


a
β Green
White field
Merge
b
β Green
White field
Merge
c
β Green
White field
Merge
d
β Green
White field
Merge


e
Lamin B1
Myc
Merge
10 μm
10 μm
10 μm

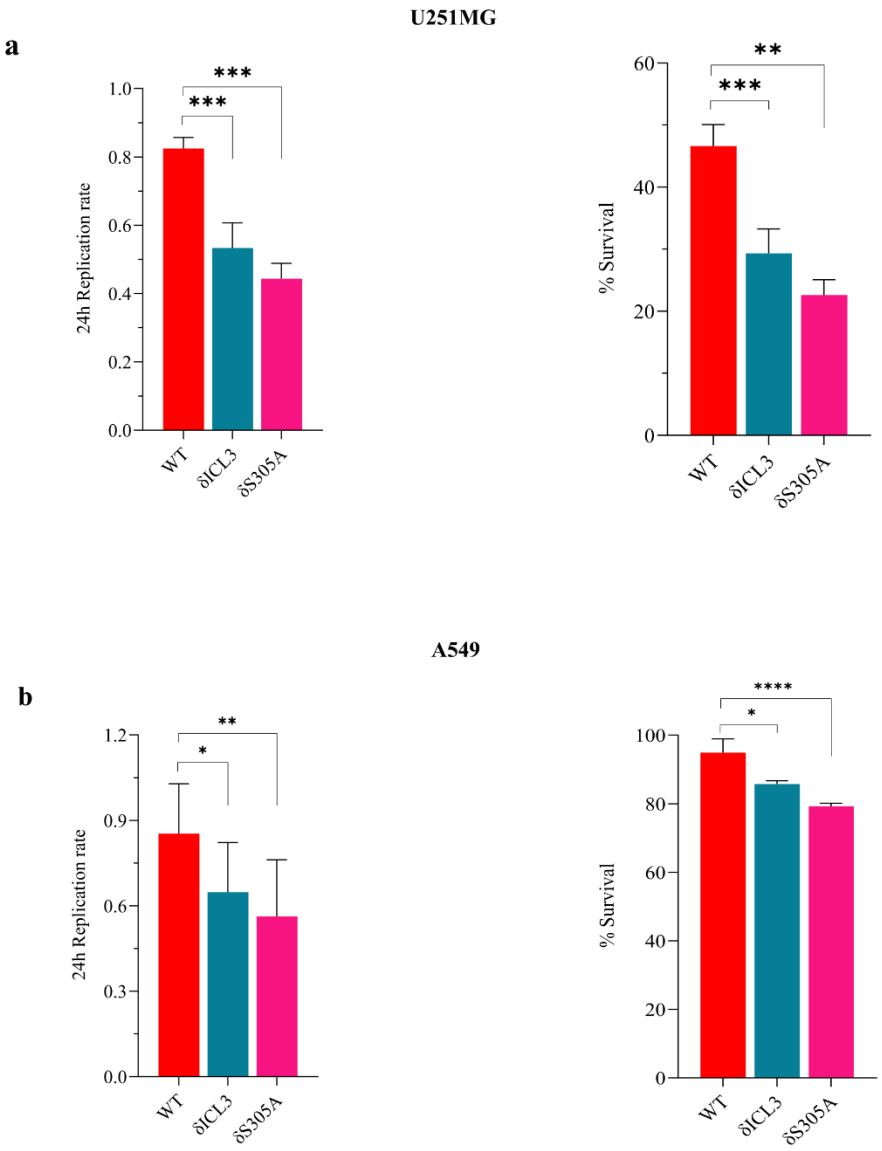
Extended Fig. 3: Pulse chase assay with FAP for HCAR1 shows no translocation from PM

a-d) Confocal imaging of pulse-chase FAP system with HCAR1 in HeLa cells using impermeant green fluorogen followed by lactate treatment (10mM) for 5 to 40min. **e)** Immunofluorescence confocal imaging pMFAP-β1 FAP construct with C-terminally Myc-tagged HCAR1 in HeLa cells.


a
Sun2
Nup98
Lamin B1
Merge
10 μm
b
WT
HCAR1-Flag
Flag-HCAR1
shScram
shHCAR1a
shHCAR1b
Lactate:
PBS:
p-ERK1/2
ERK1/2
p-AKT
AKT
Lamin b1
GAPDH
c
PBS
Lact+PTX
Lact
p-ERK1/2
ERK1/2
Lamin b1
GAPDH
d
PBS
Lact+Gallein
Lact
p-AKT
AKT
Lamin b1
GAPDH
e
cAMP concentration per 2e5 cells (pmol)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Lactate
****
shScram
shHCAR1a
shHCAR1b
KD + WT
KD + δICL3
KD + δS305A

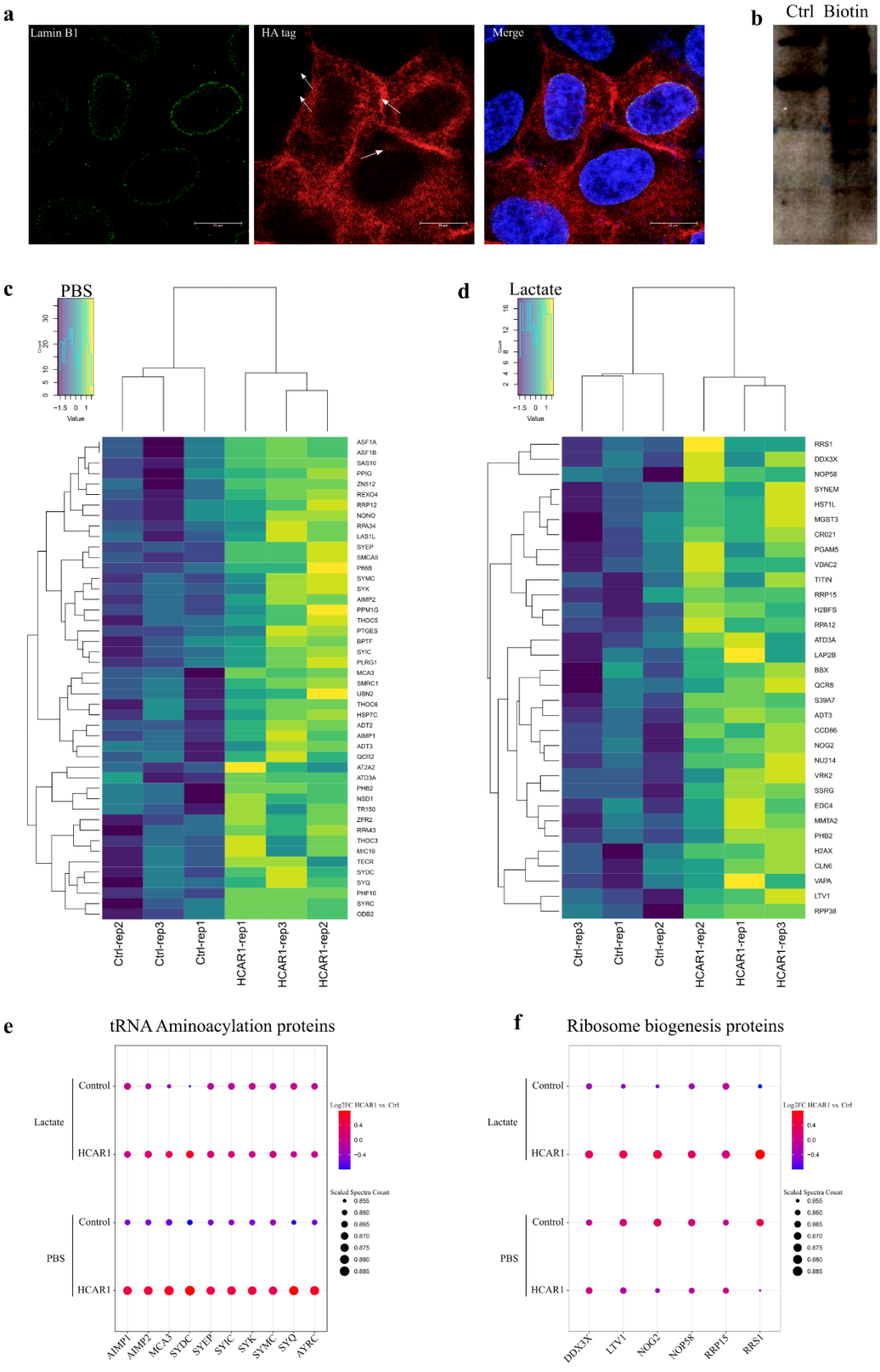
Extended Fig. 4: Validations for Fig. 2

a) Immunofluorescence confocal imaging of isolated nuclei with selective ONM permeabilization with intact INM. Detection of Sun2 C-terminus indicates INM permeabilization and the absence of Lamin B1 signal indicates intact non-permeabilized INM. **b)** Western-blot analysis on isolated nuclei from WT cells, cells overexpressing C- & N-terminally tagged HCAR1, shScrambled or two HCAR1 KD HeLa cells. Isolated nuclei were stimulated with PBS or lactate (10mM for 15min). **c-d)** Western-blot analysis on isolated nuclei from shScrambled HeLa cells from different treatments. **e)** cAMP level in whole cells with PBS or lactate treatment (10mM for 10min). The cAMP concentration is presented in picomole per 2e5 cells. The decrease in the cAMP level with the mutant rescues show that the signaling activity of the mutant HCAR1 from the plasma membrane is largely intact.


a
U251MG
24h Replication rate
1.0
0.8
0.6
0.4
0.2
0.0
***
***
WT
δICL3
δS305A
% Survival
60
40
20
0
**
***
WT
δICL3
δS305A
b
A549
24h Replication rate
1.2
0.9
0.6
0.3
0.0
**
*
WT
δICL3
δS305A
% Survival
100
80
60
40
20
0
****
*
WT
δICL3
δS305A

Extended Fig. 5: N-HCAR1 promotes proliferation and survival in other cell lines

Homeostatic proliferation rate (left panel) and survival rate in 5FU-treated cells (right panel) in U251MG **(a)** and A549 **(b)** cell lines. Both U251MG and A549 cell lines are expressing lower levels of endogenous *HCAR1* (see Supp Fig. 2d), so we generated stable cell lines over-expressing either WT HCAR1, or nuclear-excluded δICL3 HCAR1 and δS305A HCAR1 in these two cell lines.


a
Lamin B1
HA tag
Merge
b
Ctrl Biotin
c
PBS
Ctrl-rep2
Ctrl-rep3
Ctrl-rep1
HCAR1-rep1
HCAR1-rep3
HCAR1-rep2
d
Lactate
Ctrl-rep3
Ctrl-rep1
Ctrl-rep2
HCAR1-rep2
HCAR1-rep1
HCAR1-rep3
e
tRNA Aminoacylation proteins
Control
HCAR1
Lactate
PBS
AIMP1
AIMP2
MCA3
SYDC
SYEP
SYIC
SYK
SYMC
SYQ
AYRC
f
Ribosome biogenesis proteins
DDX3X
LTV1
NOG2
NOP58
RRP15
RRS1

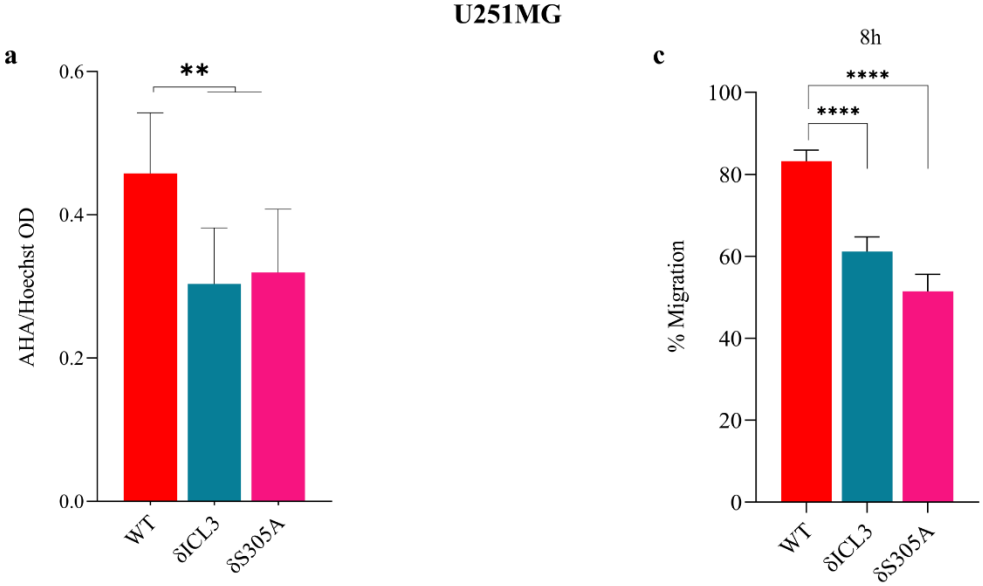
Extended Fig. 6: Controls and extra validations of Fig. 3

a) Immunofluorescence confocal imaging of HA-tagged HCAR1-BirA construct shows same localization pattern (on the nuclear membrane and in inside the nucleus) for HCAR1-BirA fusion protein as the WT HCAR1 in HeLa cells. **b)** Western blot analysis with streptavidin-HRP on biotinylated whole cell lysate from PBS or Biotin-treated cells. **c-d)** Heatmaps showing enrichment of proteins with HCAR1 based on Log_2 Fold change and *p* value in isolated nuclei of PBS **(c)** or Lactate-treated **(d)** cells with biotin. Control samples are from stable cell lines expressing empty Bio-ID vector, expressing only BirA. **e)** Enrichment dot plot graph showing proteins enriched in tRNA aminoacylation pathway in PBS and lactate-treated cells compared to control cells. **f)** Enrichment dot plot graph showing proteins enriched in ribosome biogenesis pathway in PBS and lactate treated cells compared to control cells.

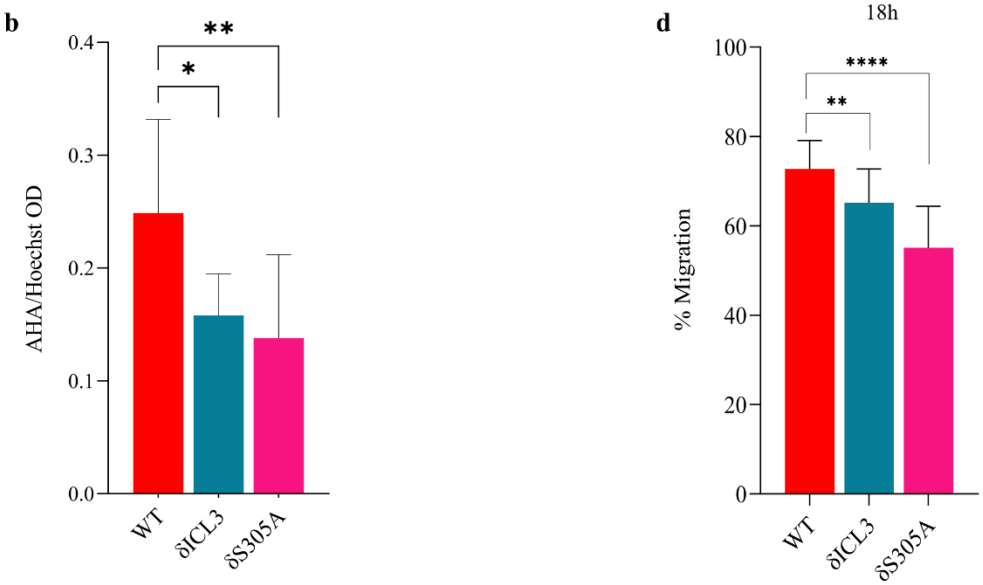
Protein translation rate

Migration rate

U251MG



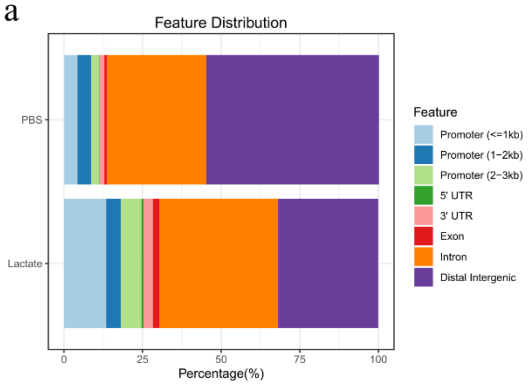
A549



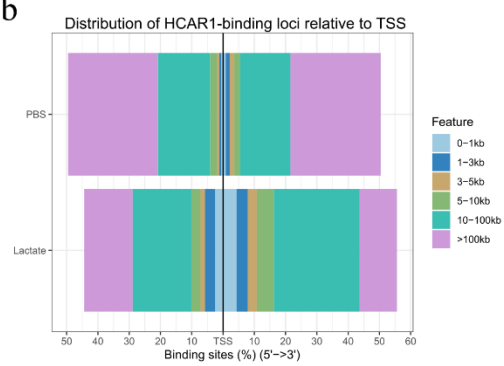
Extended Fig. 7: N-HCAR1 promotes protein translation and migration rates in other cell lines

Protein translation rate with methionine incorporation rate measurement in U251MG **(a)** and A549 **(b)** cell lines. Methionine incorporation rate (AHA) was adjusted to the number of cells (Hoechst). Scratch assay to measure the migration rate in U251MG **(c)** and A549 **(d)** cell lines. Migration rate was measured 8h post-scratch in U251MG cell lines and 18h post-scratch in A549 cell lines.

a



b



c

PBS

p-value:	1.00E-14
Number of Target Sequences with motif	49
Percentage of Target Sequences with motif	5.50%
Percentage of Background Sequences with motif	1.39%

d

Lactate

p-value:	1.00E-10
Number of Target Sequences with motif	29
Percentage of Target Sequences with motif	10.39%
Percentage of Background Sequences with motif	2.44%

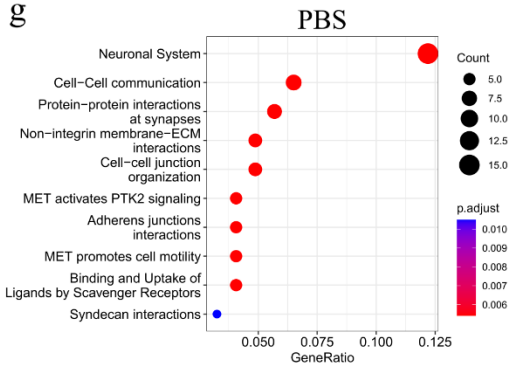
e

TF	Score	Sequence
Mafb	0.7	
NFIA	0.67	
NF1	0.65	

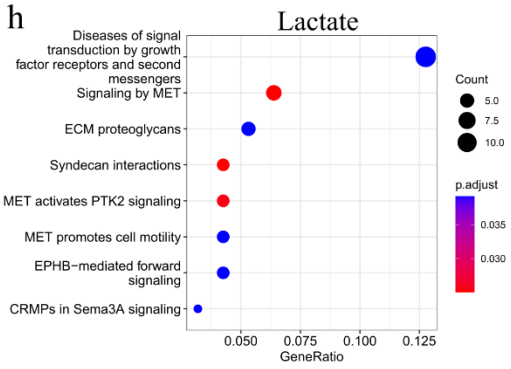
f

TF	Score	Sequence
SPI1	0.66	
SOX8	0.61	
SPIC	0.61	

g

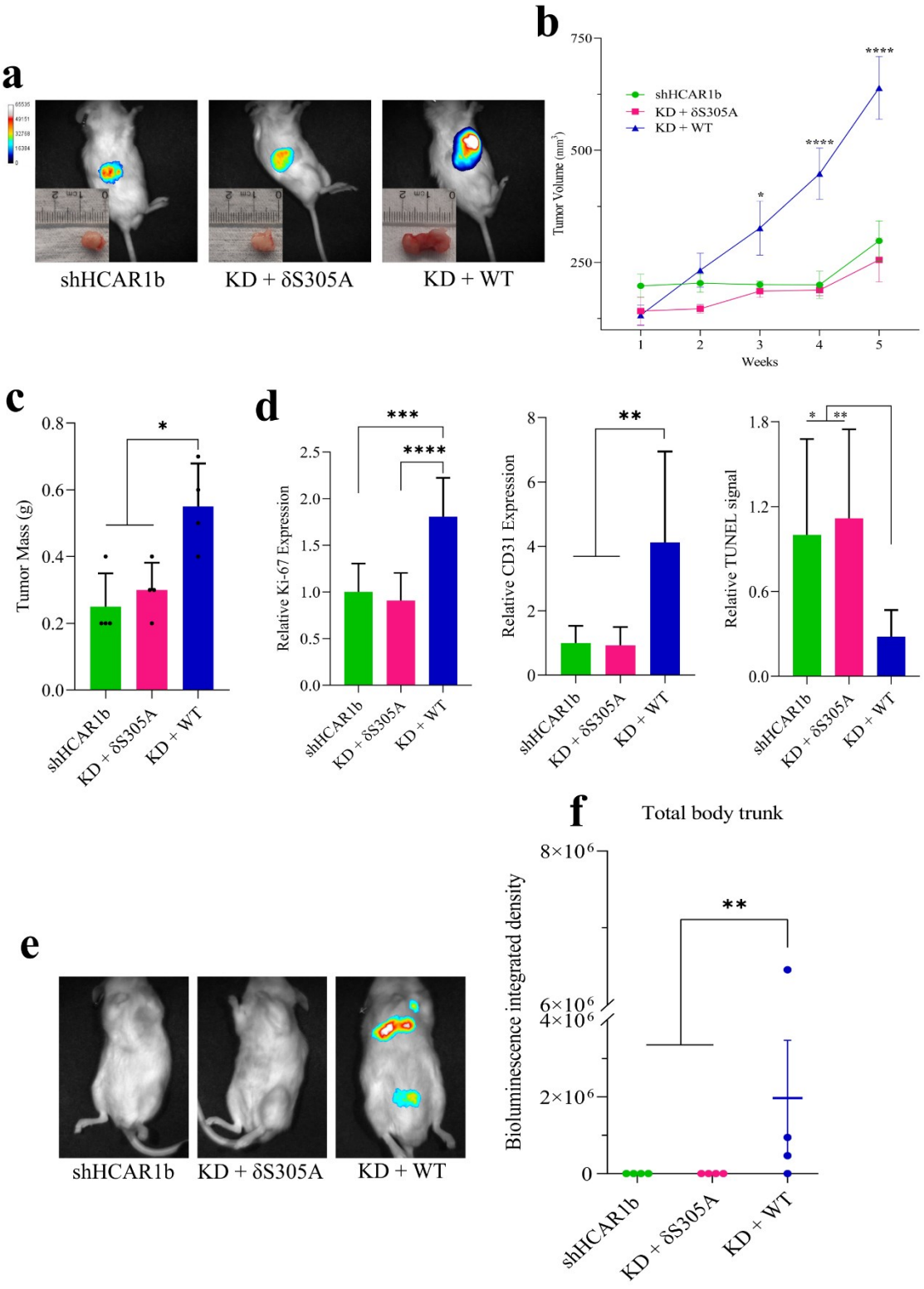


h



Extended Fig. 8: Extra analysis of ChIP-seq data

a) Detailed feature distribution of HCAR1 occupancy on the genome. **b)** Distribution of HCAR1 around TSS. **c-d)** The most enriched binding motifs for HCAR1 in PBS and lactate-treated conditions. **e-f)** The top three match to known motifs for transcription factors with the binding motifs of HCAR1 in PBS and lactate conditions (c-d). **g-h)** The Reactome pathway analysis for HCAR1-bound genes with PBS and lactate-treatment.


a
shHCAR1b
KD + δS305A
KD + WT
b
Tumor Volume (mm³)
Weeks
shHCAR1b
KD + δS305A
KD + WT
c
Tumor Mass (g)
d
Relative Ki-67 Expression
Relative CD31 Expression
Relative TUNEL signal
e
f
Total body trunk
Bioluminescence integrated density

Extended Fig. 9: N-HCAR1 promotes cancer malignancy *in vivo*

a-d) Subcutaneous injection of luciferase expressing shHCAR1b cells, rescued or not with RNAi-resistant constructs δS305A or WT HCAR1, in NSG mice. **a)** Representative images of *in vivo* luciferase signal and corresponding dissected tumors 5 weeks after injection. **b)** Tumor volume measurement. **c)** Weight of dissected tumors 5 weeks after injection. **d)** Immunohistochemistry staining analysis from dissected tumors indicating relative expression levels of Ki-67 and CD31 and relative cell death (TUNEL assay). **e-f)** Tail vein injection of the same cell lines as above in NSG mice. **e)** Representative luciferase *in vivo* images indicating metastasis of the cells. **f)** Bioluminescence intensity from body trunk of mice indicating metastasis. Each dot represents one mouse. Data in panel b, c & f are mean ± s.e.m. and in panel are mean ± s.d., from n=4 biological replicates. The ANOVA was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, ***$P < 0.0001$ significance levels.

a

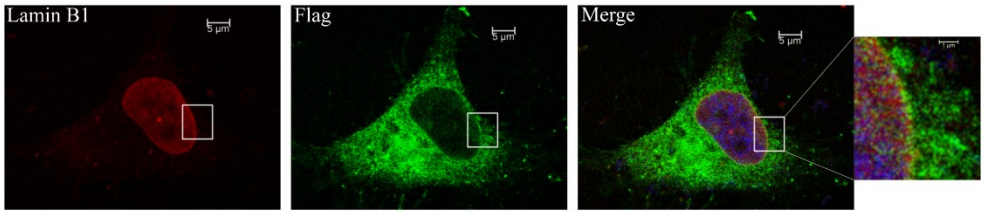
b

Modification Sites in Parent Protein, Orthologs, and Isoforms

Show Multiple Sequence Alignment

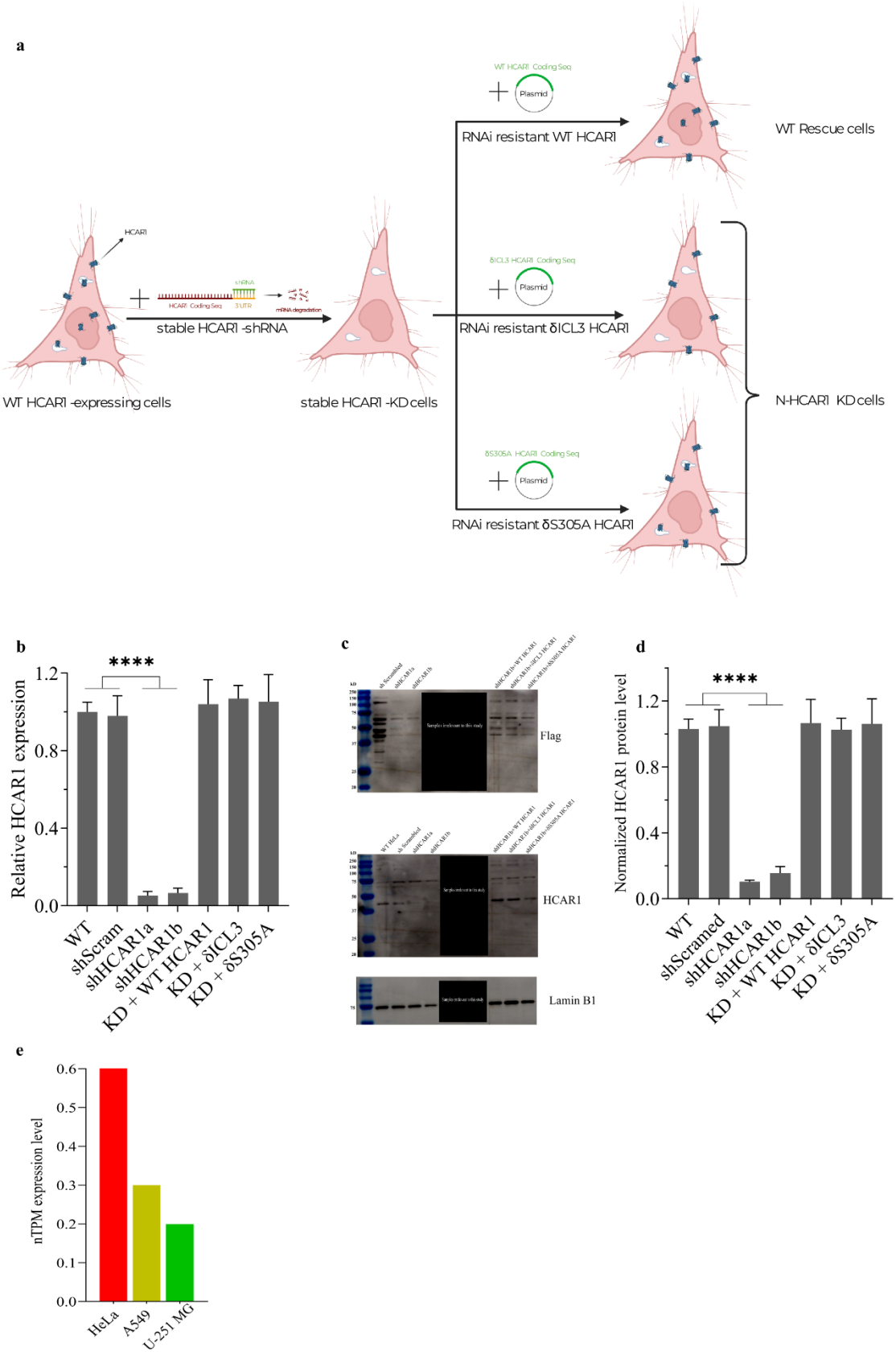
LTP	HTP	human		mouse	
0	1	T219	QARMKKATRFIMVVA	T219-p	QARMRRAtRFIMVVA
0	1	I227	RFIMVVAIVFITCYL	S227-p	RFIMVVAsVFITCyL
0	1	Y233	AIVFITCYLPSVSAR	Y233-p	AsVFITCyLPSVLAR
0	1	S305	KPKQPGHSKTQRPEE	T305-p	KPKRPGRtKTRRSEE

c



Supplementary Fig. 1:

a) Full blot images for Fig. 1a. **b)** Phosphorylation sites in HCAR1 from "Phosphosite.org" (HTP: high throughput papers, LTP: low throughput papers). **c)** HCAR1 expressed at the endogenous level shows same nuclear localization pattern. The endogenous HCAR1 was knocked down and RNAi resistant HCAR1 was ectopically expressed to the similar level as WT HeLa cells (see Supp Fig. 2b-d).

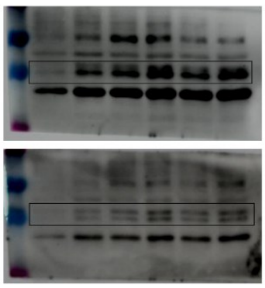

a
WT HCAR1 Coding Seq
Plasmid
RNAi resistant WT HCAR1
WT Rescue cells
δICL3 HCAR1 Coding Seq
Plasmid
RNAi resistant δICL3 HCAR1
δS305A HCAR1 Coding Seq
Plasmid
RNAi resistant δS305A HCAR1
N-HCAR1 KD cells
HCAR1
shRNA
HCAR1 Coding Seq
3'UTR
mRNA degradation
stable HCAR1 -shRNA
WT HCAR1 -expressing cells
stable HCAR1 -KD cells
b
****
Relative HCAR1 expression
1.2
0.8
0.4
0.0
WT
shScram
shHCAR1a
shHCAR1b
KD + WT HCAR1
KD + δICL3
KD + δS305A
c
Flag
HCAR1
Lamin B1
d
****
Normalized HCAR1 protein level
1.2
0.8
0.4
0.0
WT
shScramed
shHCAR1a
shHCAR1b
KD + WT HCAR1
KD + δICL3
KD + δS305A
e
nTPM expression level
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HeLa
A549
U-251 MG

Supplementary Fig. 2:

a) Schematic of the modifications in the HeLa cell lines used in this study. WT HCAR1-expressing HeLa cells were stably transduced with shRNA against 3'UTR of HCAR1 (and scrambled control of shRNA) to stably KD the HCAR1. Then, these cells were transfected with RNAi resistant plasmid expressing either the coding sequence of WT or the mutant versions of the HCAR1, to generate stably expressing cells that either harbor WT or nuclear excluded HCAR1. **b)** qRT-PCR analysis of *HCAR1* expression normalized to WT validating KD and rescue efficiencies. **c)** Western blot analysis of HCAR1 expression validation KD and rescue efficiencies. **d)** Quantitation of western blots in b. (notice similar expression levels of HCAR1 rescues with WT cells) **e)** *HCAR1* expression level in 3 cell lines used in this study; nTPM: normalized protein-coding transcripts per million. (proteinatlas.org)

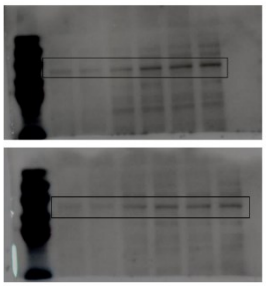
HCAR1 cDNA ERKPhospho and total

a



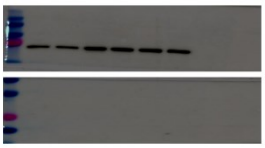
HCAR1 cDNA AKT phospho and total

b



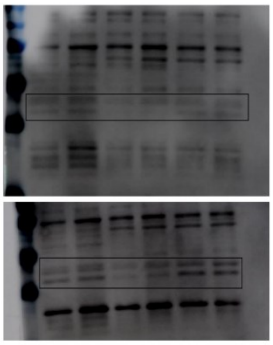
Lamin and gapdh of the images in the right

c



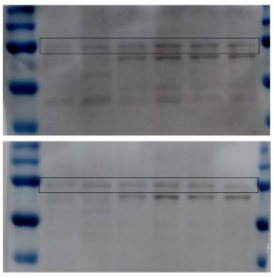
HCAR1 KD ERKPhospho and total

d



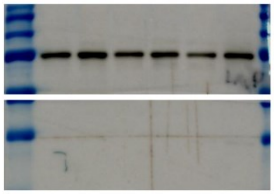
HCAR1 KD AKT phospho and total

e



Lamin and gapdh of the images in the right

f

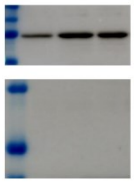


shScram ERK Phospho and total

d

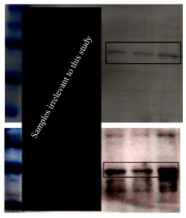
Lamin and gapdh of the images in the right

e



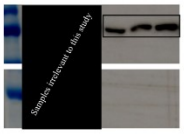
shScram AKT phospho and total

f



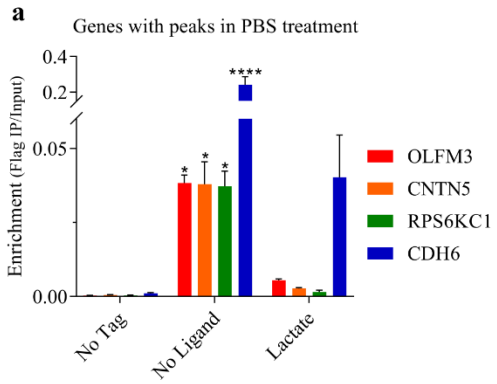
Lamin and gapdh of the images in the right

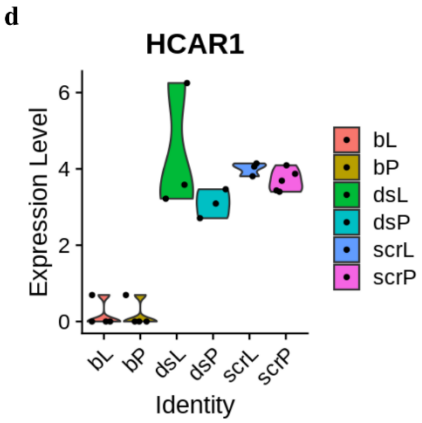
g

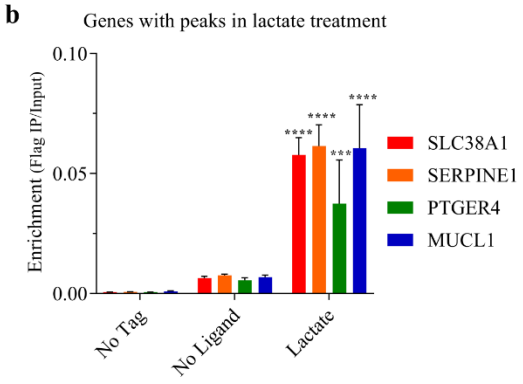


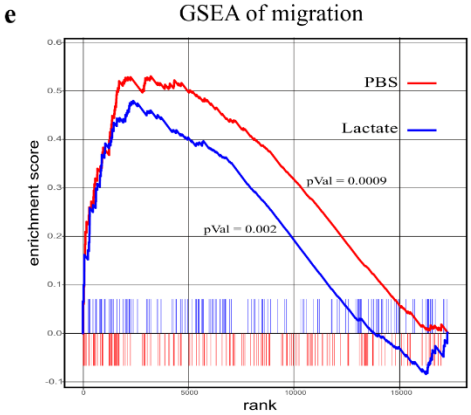
Supplementary Fig. 3:

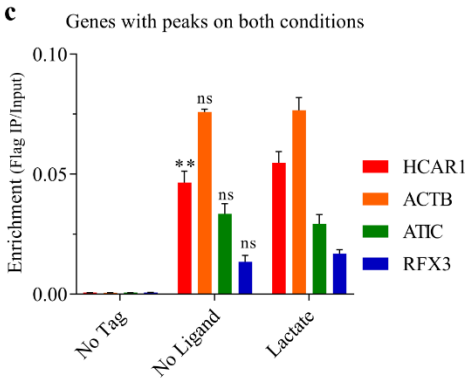
a-f) Full blot images of Extended figure 4d. **d-e)** Full blot images of Extended figure 4e. **f-g)** Full blot images of extended figure 4f.


a
Genes with peaks in PBS treatment
Enrichment (Flag IP/Input)
0.4
0.2
0.05
0.00
****
*
*
*
OLFM3
CNTN5
RPS6KC1
CDH6
No Tag
No Ligand
Lactate


d
HCAR1
Expression Level
6
4
2
0
bL
bP
dsL
dsP
scrL
scrP
Identity

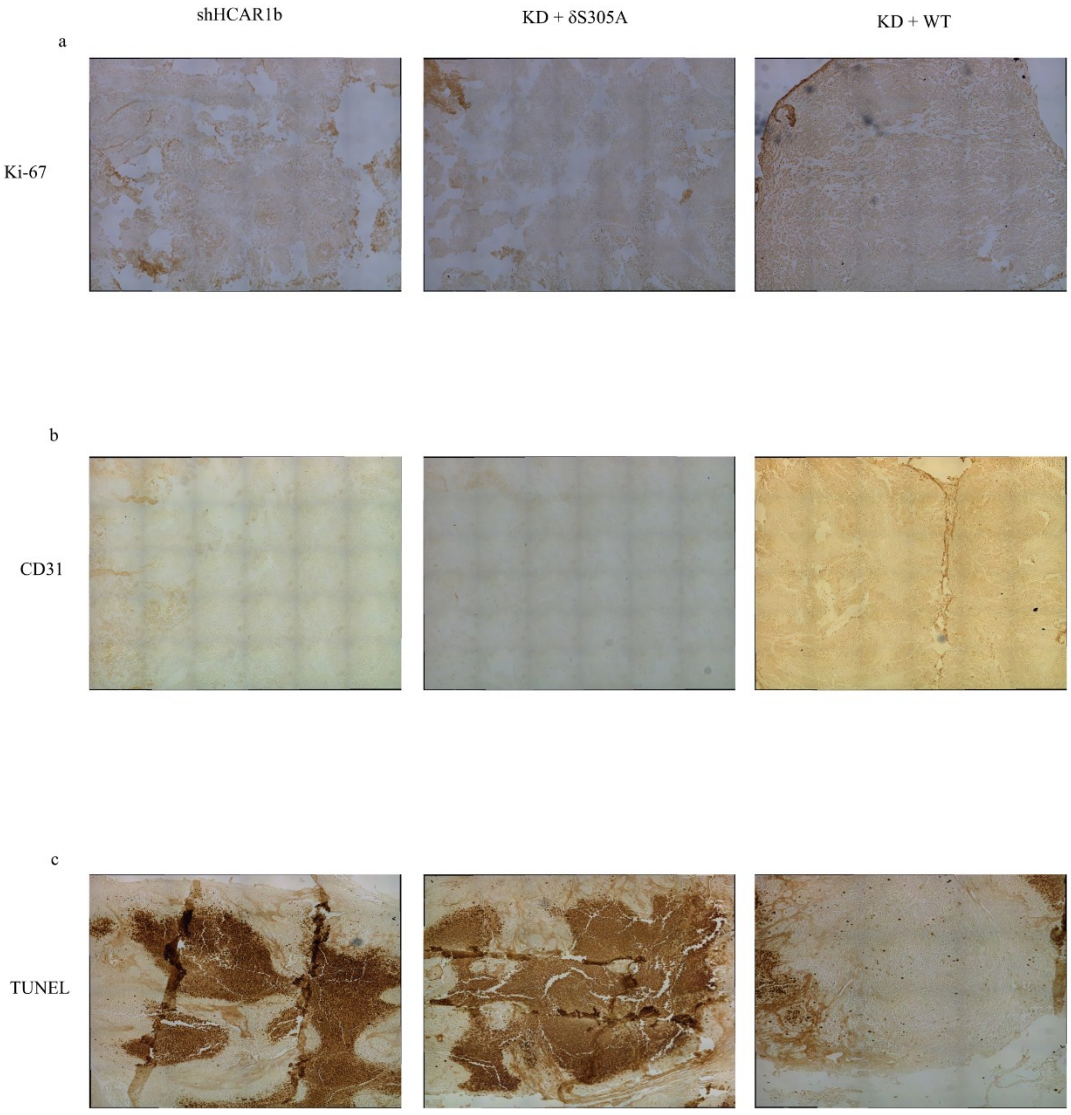

b
Genes with peaks in lactate treatment
Enrichment (Flag IP/Input)
0.10
0.05
0.00
****
****
****
***
SLC38A1
SERPINE1
PTGER4
MUCL1
No Tag
No Ligand
Lactate


e
GSEA of migration
enrichment score
PBS
Lactate
pVal = 0.0009
pVal = 0.002
rank


c
Genes with peaks on both conditions
Enrichment (Flag IP/Input)
0.10
0.05
0.00
**
ns
ns
ns
HCAR1
ACTB
ATIC
RFX3
No Tag
No Ligand
Lactate

Supplementary Fig. 4:

a-c) ChIP-qPCR confirmation for ChIP-seq. Selected genes are the top 4 enriched loci in each PBS-treated only **(a)**, Lactate-treated only **(b)**, and shared genes **(c)**. **d)** Expression level of *HCAR1* in RNA-seq data validating the RNA-seq as it shows its low expression in KD (bL: shHCAR1b+ Lactate; bP: shHCAR1b+PBS), but higher expression in scrambled shRNA and rescue of KD with δS305A HCAR1 (dsL, dsP). **e)** GSEA waterfall plot of migration. Genes list is extracted from those that are uniquely downregulated only in HCAR1 nuclear KD cells with PBS or lactate treatments. The "rank" is based on higher expression values in WT compared to HCAR1 nuclear KD from left to right.


shHCAR1b
KD + δS305A
KD + WT
a
Ki-67
b
CD31
c
TUNEL

Supplementary Fig. 5:

a-c) Representative immunohistochemistry images corresponding to Figure 7.d. Images are tiled to give a complete picture over a large portion of the sections.

Table 1: Materials used in this study

Reagent	Source	Identifier	Information
5-Flurouracil	Sigma-Aldrich	F6627	
AKT ELISA Kit	Abcam	ab126433	pS473+total
Anti DYKDDDDK tag	Cell Signaling Technology	14793	Rabbit mAB
Anti- Lamin B1	Abcam	ab8982	Mouse mAb
Anti-CD31	Abcam	ab28364	Rabbit pAb
Anti-DDDDK tag	Abcam	ab49763	Mouse mAB
Anti-GAPDH	Santa Cruz Biotechnology	sc-47724	Mouse mAb
Anti-Goat IgG-Alexa Flour 594	Thermo Fisher Scientific	A-21468	Chicken pAb 2^{ndry}
Anti-GPR81 (s296)	Sigma-Aldrich	SAB1300089	Rabbit pAb
Anti-HA tag	Santa Cruz Biotechnology	sc-7392	Mouse mAb
Anti-Ki-67	Cell Signaling Technology	9449	Mouse mAb
Anti-Lamin B receptor	Abcam	Ab201349	Recombinant Rabbit mAb
Anti-Mouse IgG-Alexa Flour 488	Abcam	ab150113	Goat pAb 2^{ndry}
Anti-Mouse IgG-Alexa Flour 647	Thermo Fisher Scientific	A-21235	Goat pAb 2^{ndry}
Anti-Mouse IgG-HRP	BioRad	1721011	Goat 2^{ndry}
Anti-Myc tag	Cell Signaling Technology	2278	Rabbit mAb
Anti-Nup98	Santa Cruz Biotechnology	sc-14155	Goat pAb
Anti-Phospho-H2A.X (Ser139)	Sigma-Aldrich	05-636	Mouse mAb
Anti-Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling Technology	4377	Thr202/Tyr204; Rabbit mAb
Anti-Rabbit IgG-Alexa Flour 594	Thermo Fisher Scientific	A-21442	Chicken pAb 2^{ndry}
Anti-Rabbit IgG-Alexa Flour 594	Thermo Fisher Scientific	A-11012	Goat pAb 2^{ndry}
Anti-Rabbit IgG-Alexa Flour 647	Thermo Fisher Scientific	A-21245	Goat pAb 2^{ndry}
Anti-Rabbit IgG-Alexa Flour 680	Abcam	ab175773	Goat pAb 2^{ndry}

Anti-Sun2	Abcam	Ab124916	Recombinant Rabbit mAb to C-terminus
Bio-Rad Protein Assay Dye Reagent Concentrate	BioRad	5000006	
Biotin	Sigma-Aldrich	B4501	
Bovine Serum Albumin	Sigma-Aldrich	A9647	
cAMP Assay kit	Abcam	ab65355	ELISA kit
Click-iT AHA Alexa Flour 488 protein synthesis HCS assay kit	Thermo Fisher Scientific	C10289	
cOmplete EDTA-free Protease Inhibitor Cocktail	Roche	1.1874E+10	
Cycloheximide	Sigma-Aldrich	C7698	
DAB Substrate kit	Thermo Fisher Scientific	34002	
DAPI	Sigma-Aldrich	D9542	
Digitonin	Sigma-Aldrich	D141	
D-Luciferin	PerkinElmer	122799	
Dynabeads MyOne Streptavidin C1	Thermo Fisher Scientific	65001	
ERK1/2 ELISA kit	Abcam	ab176660	pT202/Y204 +total
FasrDigest EcoRI	Thermo Fisher Scientific	FD0274	
FastDigest BpiI	Thermo Fisher Scientific	FD1014	
FastDigest BshTI	Thermo Fisher Scientific	FD1464	
FastDigest BspTI	Thermo Fisher Scientific	FD0834	
FastDigest Eco31I	Thermo Fisher Scientific	FD0293	
FastDigest HindIII	Thermo Fisher Scientific	FD0504	
FastDigest Kpn2I	Thermo Fisher Scientific	FD0534	
FastDigest Mva1269I	Thermo Fisher Scientific	FD0964	
FastDigest Mva1269I	Thermo Fisher Scientific	FD0964	
FastDigest NheI	Thermo Fisher Scientific	FD0974	

FastDigest NotI	Thermo Fisher Scientific	FD0593	
FastDigest XhoI	Thermo Fisher Scientific	FD0694	
Fluoro -Gel	Electron Microscopy Sciences	17985-10	mounting media
Formaldehyde	Thermo Fisher Scientific	BP531500	0.37
Formalin	Thermo Fisher Scientific	SF1004	Buffered 10%
Gallein	Santa Cruz Biotechnology	sc-202631	
GeneJET Gel extraction & DNA cleanup kit	Thermo Fisher Scientific	K0832	
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	K0503	
Hoechst 33342	Thermo Fisher Scientific	H3570	
IGEPAL	Sigma-Aldrich	I8896	NP-40
ImageJ			
Immun-Star Anti-Rabbit-HRP	BioRad	1705046	Goat 2^{ndry}
iScript Reverse Transcription Supermix	BioRad	1708841	
iTaq Universal SYBR Green Supermix	BioRad	1725124	
Laemmli SDS-Sample Buffer	Boston BioProducts	BP-110R	
Lentivurs shRNA	GeneCopoeia Inc.	LPP-HSH007585-LVRU6MP-100	
L-Lactic acid	Sigma-Aldrich	L1750	
MCS-13X Linker-BioID2-HA	Addgene	80899	
Mounting media	Thermo Fisher Scientific	23245691	Xylene
Normal Mouse IgG	Santa Cruz Biotechnology	sc-2025	
p-AKT	Cell Signaling Technology	9271	Ser473; Rabbit pAb
Paraformaldehyde	Sigma-Aldrich	P6148	
pCDNA3.1-HCAR1-flag	Genscript Biotech		
p-CMV-Tag 2A	Agilent	211172	

Pertussis Toxin	Santa Cruz Biotechnology	sc-200837	
pFETCH Donor	Addgene	63934	
pMFAPβ1 Tagging Vector	Spectragenetics		
pMFAP-β1 Tagging Vector	Spectragenetics		Plasmid
PMSF	Sigma-Aldrich	P7626	
Poly-L-Lysine	Sigma-Aldrich	A-005-M	Solution 0.01%
Protein G Magnetic beads	Cell Signaling Technology	70024	
Proteinase K	Sigma-Aldrich	P2308	
pU6-(BbsI)_CBh-Cas9-T2A-mCherry	Addgene	64324	
PureLink HiPure Plasmid Maxiprep Kit	Thermo Fisher Scientific	K210007	
Q5 site-directed mutagenesis kit	New England Biolabs	E0552S	
QuantumRNA Universal 18S Internal Standard	Thermo Fisher Scientific	AM1718	
RiboZol RNA extraction Reagent	VWR	N-580	
RIPA buffer	Cell Signaling Technology	9806	
RNaseOUT	Thermo Fisher Scientific	10777019	Ribonuclease inhibitor
RNeasy Mini Kit	Qiagen	74104	
SimpleChIP Enzymatic Chromatin IP Kit	Cell Signaling Technology	9003	Magnetic beads
Streptavidin (HRP)	Abcam	Ab7403	
Streptavidin Alexa Flour 488 conjugate	Thermo Fisher Scientific	S11223	
Sucrose	Thermo Fisher Scientific	S5	
Total AKT	Cell Signaling Technology	9272	Rabbit pAb
Total p44-42 (Erk1/2)	Cell Signaling Technology	4695	Rabbit mAb
TransIT-X2	Mirus Bio	MIR6000	
Triton X-100	Sigma-Aldrich	X100	
Trypan Blue Stain	Thermo Fisher Scientific	T10282	
TUNEL assay Kit	Abcam	Ab206386	

Tween-20	Thermo Fisher Scientific	BP337500	
Western Lightning Plus-ECL	PerkinElmer	NEL103E001EA	
Xylene	Thermo Fisher Scientific	HC7001GAL	
βGREEN-np fluorogen	Spectragenetics		Membrane impermeant flurogen

Table 2: List and sequence of oligos used in this study

Target	**Oligo**
HCAR1 F q&RT-PCR	CGCCTCAGGCTCCAAACAA
HCAR1 R q&RT-PCR	TGCTGGAGAACCATCTCTGC
ACTB F q&RT-PCR	TGAACTTTGGGGGATGCTCG
ACTB R q&RT-PCR	ACCTAACTTGCGCAGAAAACA
ATIC F q-PCR	GCTGGGTCTGCTAACATCACT
ATIC R q-PCR	CCCAATTGAGTGCTTCATGCC
ATIC F RT-PCR	CTTTCAGCCTTATTTAGTGTCTCTG
ATIC R RT-PCR	CCTTACCTGACTGCCAGACC
RFX3 F Ligand q-PCR	CCATGTTACCCTTCCCTCCCT
RFX3 R Ligand q -PCR	TGGCTAGCTTTAGGAGGTGG
RFX3 F No Ligand q-PCR	CTTGGGTCAAAAATGGTCCTGT
RFX3 R No Ligand q-PCR	ACAAATGCACTGGTATCCAACAAC
RFX3 F RT-PCR	CCCGAGTTGTGTGTGTCACT
RFX3 R RT-PCR	ACTACCGTGGTCTCATCCCA
SLC38A1 F q-PCR	CAAAACACAGAGATTGGCAAAATGG
SLC38A1 R q-PCR	AGTCATCCCAGCTCTCTTCTG
SLC38A1 F RT-PCR	CGCAACCATGGCTTGTGATG
SLC38A1 R RT-PCR	CAGAGAGCTGCAAGAGGGAG
SERPINE1 F q&RT-PCR	CTGGAGATGCATCGGGAAAG
SERPINE1 F q&RT-PCR	CTCAGGTGGAGACTAGGGAGT
PTGER4 F q-PCR	AGGTGCAGCATTTCTAGTGT
PTGER4 R q-PCR	GCCTTATCACCTAGGATTGTACCT
PTGER4 F RT-PCR	GGTGATGTTCATCTTCGGGGT
PTGER4 R RT-PCR	ACCAACAAAGTGCCCAACAG
MUCL1 F q-PCR	TTCATTTCCCCAGAGCACAG
MUCL1 R q-PCR	CATTGAGCTTGGATATAAAGGAGG

MUCL1 F RT-PCR	TCCTGCTGATGATGAAGCCC
MUCL1 R RT-PCR	GGAATGTCTTTACGAGCAGTGG
OLFM3 F q-PCR	ATCTAGATGACCTTTGGTTTGGCA
OLFM3 R q-PCR	TTTCTCTCAGCCTGTGGATTG
OLFM3 F RT-PCR	CAGCATACTCCAGGCTTCGT
OLFM3 R RT-PCR	ACCTTCCTTTCAAGTGGGCA
CNTN5 F q-PCR	AAATCAGACTCCCCTGGGCTA
CNTN5 R q-PCR	CAAGGAAAGAAGTCACAGGGTGT
CNTN5 F RT-PCR	TGGCTTTCAGACCCAATGGA
CNTN5 R RT-PCR	TGACTAAAAGGCCCATCTCCT
RPS6KC1 F q-PCR	TTGTGTTGTCCTTGTCCCGT
RPS6KC1 R q-PCR	CCAAGCATTCAAAGAACAGAAACT
RPS6KC1 F RT-PCR	TGATGTGGATTCTCTTGCTGAGT
RPS6KC1 R RT-PCR	GGCTACGACTTTCCCGTTCT
CDH6 F q-PCR	TAACAACATTTGGGGTGCCCT
CDH6 R q-PCR	AAAATCATCTCAGGTGTTCAAGGT
CDH6 F RT-PCR	GCCCTACCCAACTCTCTCAAC
CDH6 R RT-PCR	TCGGATCCTGTGTATTCCTCCA
HCAR1 F RT-PCR	GTGGTTTCTGCTTCCACATGAAG
HCAR1 R RT-PCR	CCGTCCAGAGGAAATAGAGTCTAGC
HCAR1 δICL3 R	CAGGCTCCAAACAATCTTGAAGGAGC
HCAR1 δICL3 F	ACCCGGTTCATCATGGTGGTG
HCAR1 δS305A F	GCAAAAACACAAAGGCCGGAAGAGATGC
HCAR1 δS305A R	GTGTCCTGGCTGCTTGGGTTTCAGACTG

Chapter 3

Lack of HCAR1, the lactate GPCR signaling promotes autistic like behavior:

Lack of HCAR1, the lactate GPCR signaling promotes autistic-like behavior

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[2] Centre de Recherche du CHU Sainte-Justine, Montreal, QC H3T 1C5, Canada

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Abstract:

The GPCR HCAR is known to be the sole receptor for lactate, which modulates its metabolic effects. Despite its significant role in many processes, mice deficient in HCAR1 exhibit no visible phenotype and are healthy and fertile. We performed transcriptomic analysis on HCAR1 deficient cells, in combination with lactate, to explore pathophysiologically altered processes. Processes such as immune regulation, various cancers, and neurodegenerative diseases were significantly enriched for HCAR1 transcriptomic signature. However, the most affected process of all was autism spectrum disorder. We performed behavioral tests on HCAR1 KO mice and observed that these mice manifest autistic-like behavior. Our data opens new avenues for research on HCAR1 and lactate effect at a pathological level.

Keywords: Lactate, HCAR1, GPCR, Signaling, Autism Spectrum Disorder, Autistic-like behavior, Anxiety,

Main Text:

Lactate has been extensively studied for its various effects, including cell migration, immune modulation, angiogenesis, cytoprotection, and many others. Its mechanism of action remained unknown until the discovery of its receptor HCAR1(1). Initially identified as GPR81, HCAR1 was discovered in 2001(2) but remained an orphan GPCR until 2009, when lactate was found to be its endogenous ligand(3). Studies on HCAR1-deficient mice demonstrated that lactate inhibits lipolysis in an insulin-dependent, para-autocrine manner through HCAR1 activation(4). Although the highest expression level of HCAR1 is detected in adipocytes, it is expressed in almost every tissue tested(3). Accordingly, a variety of physiologic roles of HCAR1 have been observed.

Lactate produced during labor in the uterine tissue reduces inflammation via HCAR1 activation(5); lactate also reduces inflammation by modulating Toll-Like receptor signaling through HCAR1(6). HCAR1 activation by lactate produced in intestinal microbiota promotes intestinal stem cell proliferation and epithelial development by activation of Wnt/β-catenin pathway(7). HCAR1 activation using different agonists leads to hypertension by regulating the endothelin vasopressor system in the kidneys(8). In brain HCAR1 signaling downregulates neural basal activity and firing frequency(9), while separately enhances angiogenesis by inducing VGEF(10). Notably, HCAR1 is predominantly expressed in neurons of the cerebral cortex and hippocampus, and participates in postnatal microvascular development(11). Whereas in astrocytes, HCAR1 promotes the expression of neurotrophic factors (12) and neurogenesis in the ventricular-subventricular zone in the brain(13).

Despite these wide-ranging roles of HCAR1, mice lacking this receptor are devoid of visible phenotypic changes, and are healthy and fertile. In order to explore specific phenotypic features of HCAR1 deficiency, we performed whole transcriptomic analysis of the HCAR1 signaling signature. We treated HCAR1-expressing and knocked-down HeLa cells (as a model cell line) with lactate (10 mM for 6 h) or PBS, and performed RNA-sequencing to identify potential pathophysiologic alterations in HCAR1-deficient animals (Fig. 1a-c). While more than 1200 genes were differentially regulated in HCAR1 knocked down cells, a smaller number of genes were regulated by the receptor stimulated with

lactate (Fig 1b). Interestingly, approximately half of the genes in both lactate stimulated and unstimulated states were shared, while the other half of the genes were unique to either PBS or lactate treatments (Fig. 1c). This infers that HCAR1-regulated genes are not solely modulated through lactate signaling, but that basal activity of HCAR1 may also exert some form of gene regulation.

Using gene ontological analysis, we focused on pathological/physiological processes that could be affected by HCAR1 signaling, rather than cellular or molecular processes regulated by this receptor. Various diseases were associated with transcriptomic signature of HCAR1, whether stimulated or not with lactate. Under basal HCAR1 transcriptomic activity (PBS treatment), we observed associations with different cancers, type 2 diabetes, certain immunological disorders and the blood coagulation pathway (Fig. 1d). In addition, HCAR1-dependent differentially-regulated genes were significantly shared with the top 500 genes that are altered in SARS-CoV-2 infection (Fig. 1d). Notably, the lactate-treated group also showed a strong correlation with gene expression changes observed in SARS-CoV-2 infection (Fig. 1e). Interestingly, a variety of neurologic disorders such as Alzheimer's disease, bipolar disorder and neuromyelitis optica stood out (Fig. 1d). Upon lactate stimulation, additional neurologic disorders were also linked to HCAR1-mediated transcriptional network, such as Huntington's disease and adrenoleukodystrophy (ALD) (Fig. 1e). However, among neurologic ailments, Autism Spectrum Disorder (ASD) was the most significantly process affected by lactate signaling through HCAR1 (Fig. 1e,f); lactate was found to down regulate many of the genes involved in the autism syndrome (Fig. 1g).

We therefore proceeded to explore if HCAR1-deficient mice exhibited features of ASD given the presence of endogenous lactate in the brain. We performed 3-chamber social test on HCAR1 KO and WT mice. The 3-chamber social test measures animal sociability and social novelty seeking behaviors, which are among the main characteristic behavioral deficits in ASD(14),(15). HCAR1-deficient mice spent significantly less time with either of the object (empty cage) or the novel mouse in the sociability phase indicative of lower interest in exploring novel stimuli (i.e., curiosity) (Fig. 2d). While the HCAR1-KO animal exhibited altered behavior in this phase, other criteria for sociability were not significantly affected by the deficiency of HCAR1 (Fig. 2b,c,e). However, during the social novelty

phase, HCAR1-KO mice visited the familiar mouse more often than the new mouse, and generally scored lower for social novelty behavior compared to WT mice (Fig. 2g,i). In addition, HCAR1-KO mice spent more time spinning on the spot - an indicator of repetitive behavior as seen in ASD (Fig. 2j,k).

We next evaluated the anxiety level of HCAR1 deficient animals, as a strongly associated co-morbid symptom of ASD, by performing elevated plus maze test(16). HCAR1 KO animals exhibited aversion to the open arms of the maze (Fig. 3a). Accordingly, they traveled shorter distances in the open arms of the maze while preferring to move along the closed arms (Fig. 3b,c,d). The KO mice spent shorter times in the open arms compared to WT animals and mostly stayed longer in the closed arms (Fig. 3c). Even the resting time was significantly shorter in the open arms for KO mice (Fig. 3d). Overall, these features of HCAR1-deficient animals display anxiety-like behavior, pointing to a significant role for HCAR1 in regulating genes implicated in behavior which otherwise leads to autistic-like behavior. Of relevance, HCAR1 KO mice presented no locomotor deficiencies, further attesting to altered behavioral patterns (Fig Supp. 1, 2). There was also no sex differences for sociability, social novelty, and anxiety tests. Altogether, HCAR1-deficient animals exhibit reduced social behavior with increased repetitive and anxiety-like behaviors, pointing to a significant role for HCAR1 signaling in neural modulation of activities involved in regulating autistic spectrum phenotypes.

The role of HCAR1 in neurons and brain tissue has been explored(9),(10),(12),(13),(11). The metabolism of astrocytes is to a large extent based on glycolysis resulting in accumulation of lactate (as end product), which seems to enhance their plasticity(12). In neurons, HCAR1 reduces cell excitability(17); conversely, neurons of HCAR1-deficient mice display higher basal activity(18). Our data reveal that the absence of HCAR1 signaling axis along with the augmented brain activity could promote autistic-like behavior. Increased neural excitability in brain regions controlling sensory, social and emotional behavior have been linked to autism(19). It would be tempting to speculate that lack or disruption of HCAR1 signaling could participate in hyperactive neural firings that contribute to manifestations of autism. Depending on the brain region, this disruptive signaling could be displayed through different features and degree of autistic-like

behaviors. Consistent with this notion, although our data fails to reveal changes in some parameters of sociability feature in mice lacking HCAR1, brain regions controlling social novelty behavior could exhibit altered function due to silencing of HCAR1 signaling pathway. Accordingly, modulation of HCAR1 could potentially serve as a therapeutic target for autism, opening new avenues for investigation in this context.

As expected, given the involvement of HCAR1 in both neural and immune cell functions, many diseases associated with the HCAR1-regulated transcriptome were neurological and immunological disorders (Fig. 1d, e). An interesting observation was the similarity of HCAR1-deficient transcriptomic signature with altered transcriptome of SARS-CoV-2 infection, which could at least partially arise from immune related functions of HCAR1, and the metabolic switch of immune cells upon activation with the ensuing increased lactate production(6). Similarly, the association of HCAR1 with various cancer types aligns with previous findings(20). While HCAR1 may not be a determinant factor in these diverse diseases, modulating its signaling could potentially offer a safe adjunct treatment for a wide range of conditions. HCAR1 is thus an attractive therapeutic target with broad applicability.

Declarations

Ethical Approval

All procedures on animal were approved by institutional ethic committee at CR Sainte-Justine Hospital.

Competing interests

Authors declare no competing interest.

Authors' contributions

MA.MN conceived and designed the study, performed and analyzed the experiments and drafted the manuscript. G.C performed the bioinformatic analysis. JS.J supervised the

bioinformatic analysis. S.C supervised the whole project and oversaw the conception, experiments, analysis and drafting of the work.

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Availability of data and materials

The dataset supporting the conclusions of this article will be available in a public repository upon acceptance.

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Material & Method:

Transcriptomic:

Equal number of cells were plated in 10cm Petry dish and were grown to reach a density of 70-80% confluency. A volume lactate for a final concentration of 10mM for experimental groups and equal volume of PBS for control group were added to the cell media and incubated to 6 hours. We extracted RNA with RNeasy mini Kit (Qiagen Cat#74104). Sample analysis and sequencing was performed at IRIC genomic platform. Before sequencing, RNA quantity and integrity was validated with Bioanalyzer. 500 ng of RNA was used for library preparation. RNA quality control was assessed using the Bioanalyzer Nano assay on the 2100 Bioanalyzer system (Agilent technologies) with all samples having a RIN above 9,5. Dyna Beads Oligo(dT) (Thermo Fisher) was used for PolyA selection, RNA Hyperprep kit (Roche) for Library preparation, and Illumina dual-index UMI (IDT) for Ligation. Normalization of all diluted libraries were done by qPCR using the KAPA library quantification kit (KAPA; Cat no. KK4973). Sample libraries were pooled to equimolar concentration. Library preparation and sequencing was made at the Institute for Research in Immunology and Cancer's Genomics Platform (IRIC).

Nextseq 500 illumina system was used for sequencing with a depth of ~35M per sample with single-end 75 cycles. Sample for each group was sequenced at least in triplicates. Base calls were obtained from the Illumina NextSeq 500 sequencer that runs RTA 2.11.3.0 and raw base calls were converted to FASTQ files using bcl2fastq version 2.20 and allowing 0 mismatches in the multiplexing barcode.

RNA-seq analysis:

Next-flow pipeline was used for pre-processing the data[1] with the salmon pseudo-aligner and the star_salmon aligner (reference genome GRCh38). After regressing for batch effect, we used Seuart[2] to perform differential gene expression analysis between groups with normalized and scaled gene counts. Genes with more or less than 0.5-fold expression change and $p < 0.05$ were selected for further analysis. fastGSEA[3] and enrichR[4] were used to analyze differentially expressed genes. Final gene list was analyzed by Enrichr for diseases association that are significantly enriched in either of the groups.

Animal experiments:

All procedures on animal were approved by institutional ethic committee at CR Sainte-Justine Hospital. One month old male and female C57BL/6J background mice with HCAR1 knock out and wild type genotypes were used in this study. *Hcar1*$^{-/-}$ mice were generated in Lexicon Pharmaceuticals (The Hoodlands, TX, USA) by a 4-kb IRES-LacZ-Neo cassette insertion in the transmembrane domain 2 coding sequence of *Hcar1* in C57BL/6J mice. Animals were housed in separately 1 week prior to experiments and maintained on standard feeding protocol with 12 hours light and dark cycles with free access to water and food.

The behavioral tests were monitored and assessed using a camera on top of the test instruments. The animal behavior including their movement, resting time, distance traveled, number of entries to each section and etc were video recoded and analyzed with the SMART video tracking software (v#.0, Harvard Apparatus).

3-Chamber social test:

The 3-Chamber social test was performed in 3 phases of 10 minutes each with the subject mouse being able to freely move and explore all three chambers. In the first phase, animals were placed in the empty chambered arena without any object to acclimatize with the environment. Then the subject mouse was removed from the arena between the phases and placed back after the new set-up. In the second phase, an empty wire cage was placed in the either of left or right chamber (alternating for every new subject mouse) and another wire cage with an unfamiliar mouse was placed in the opposing chamber. The sociability of mice was assessed in the 2nd phase of this test. In the third phase, a new unfamiliar mouse was placed in the empty cage from the 2nd phase before bringing the subject mouse back. At this phase, the social novelty behavior of animal was assessed. The sociability and social novelty parameters (1st latency entrance, number of entries, resting time, sociability, and social novelty) were measured by the presence of mouse in the interaction zone only (the circled area surrounding the wire cages, depicted in the Fig. 1d). The subject mice were always put in the middle chamber in all phases. Both caged mice were wild type background with same age and sex as the subject mouse but from different home cages and

had no prior contact with each other nor the subject mice. The sociability and social novelty behavior were evaluated by quantifying the time that subject mouse spent with the object or each caged mice in their surrounding designated area[5,6].

Elevated plus maze:

We performed elevated plus maze to measure the anxiety-like behavior of mice[7]. Mice were placed in the center of the maze with two opposing open arms and two perpendicular opposing closed arms. Their movement was monitored for 5 minutes by video recording.

Statistics:

Statistical analyses were performed using Prism 9.0 (GraphPad Software). Differences between groups were assessed with Analysis of variance (ANOVA) followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$, ***$P < 0.0001$ significance levels.

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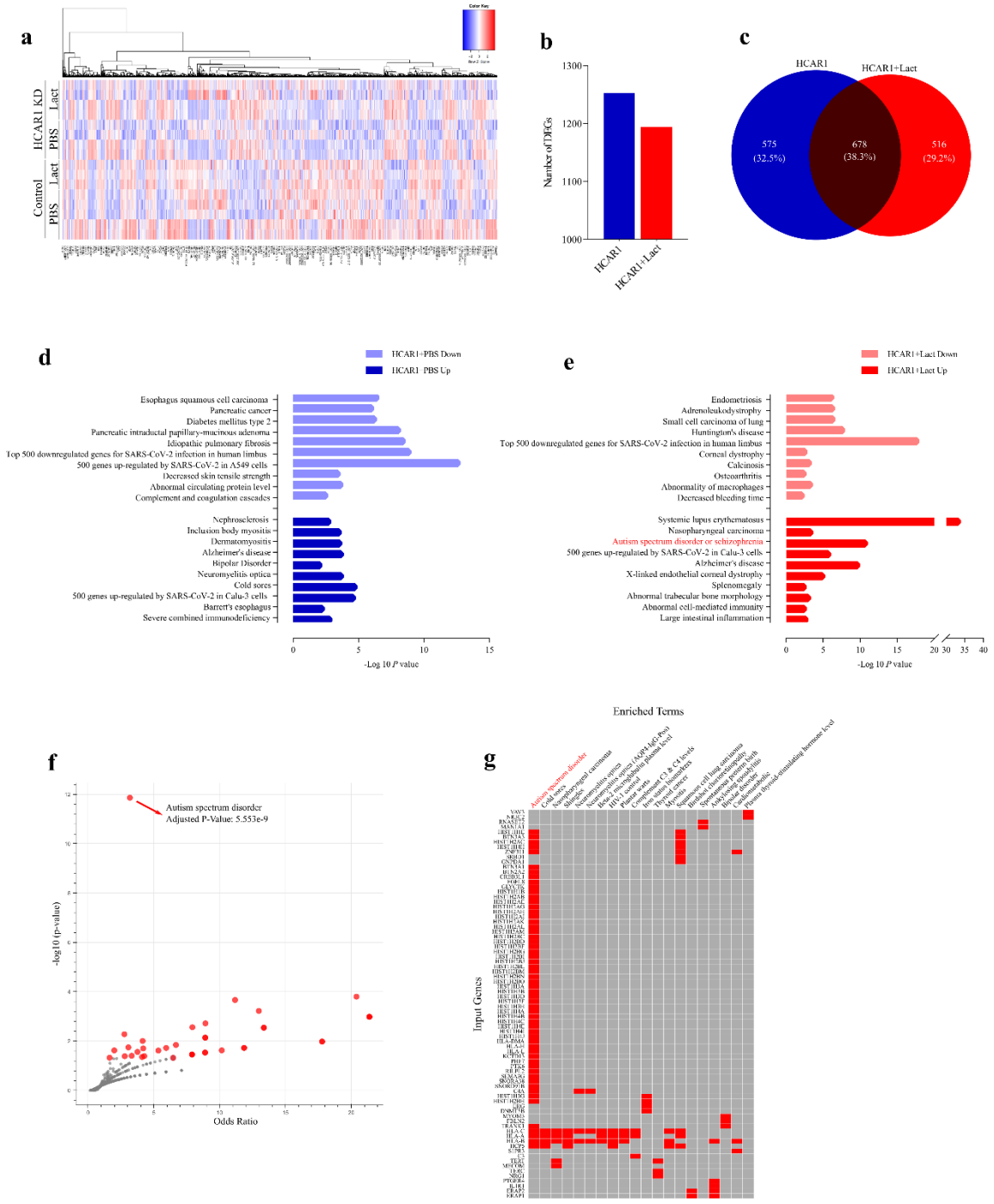

Fig. 1: HCAR1 transcriptomic signature modulates wide range of pathologies.

a) Heatmap of HCAR1 RNA-seq in HeLa cell lines. KD and scrambled shRNA sells were treated with PBS or lactate and subjected to RNA-sequencing. b) number of DEGs through

HCAR1 with and without lactate treatment. c) Venn diagram showing the overlap of DEGs through HCAR1 with or without lactate. d) disease association with DEGs in HCAR1 PBS treated groups. HCAR1+PBS down: downregulated genes upon HCAR1 KD in PBS treated cells. HCAR1+PBS up: upregulated genes upon HCAR1 KD in PBS treated cells. e) disease association with DEGs in HCAR1 lactate treated groups. HCAR1+Lact down: downregulated genes upon HCAR1 KD in PBS treated cells. HCAR1+Lact up: upregulated genes upon HCAR1 KD in PBS treated cells. f) volcano plot for DEGs specific to HCAR1+lact group (the 516 genes in c). g) clustergram showing DEGs associated with each term. DEG: differentially-expressed genes; KD: knockdown.

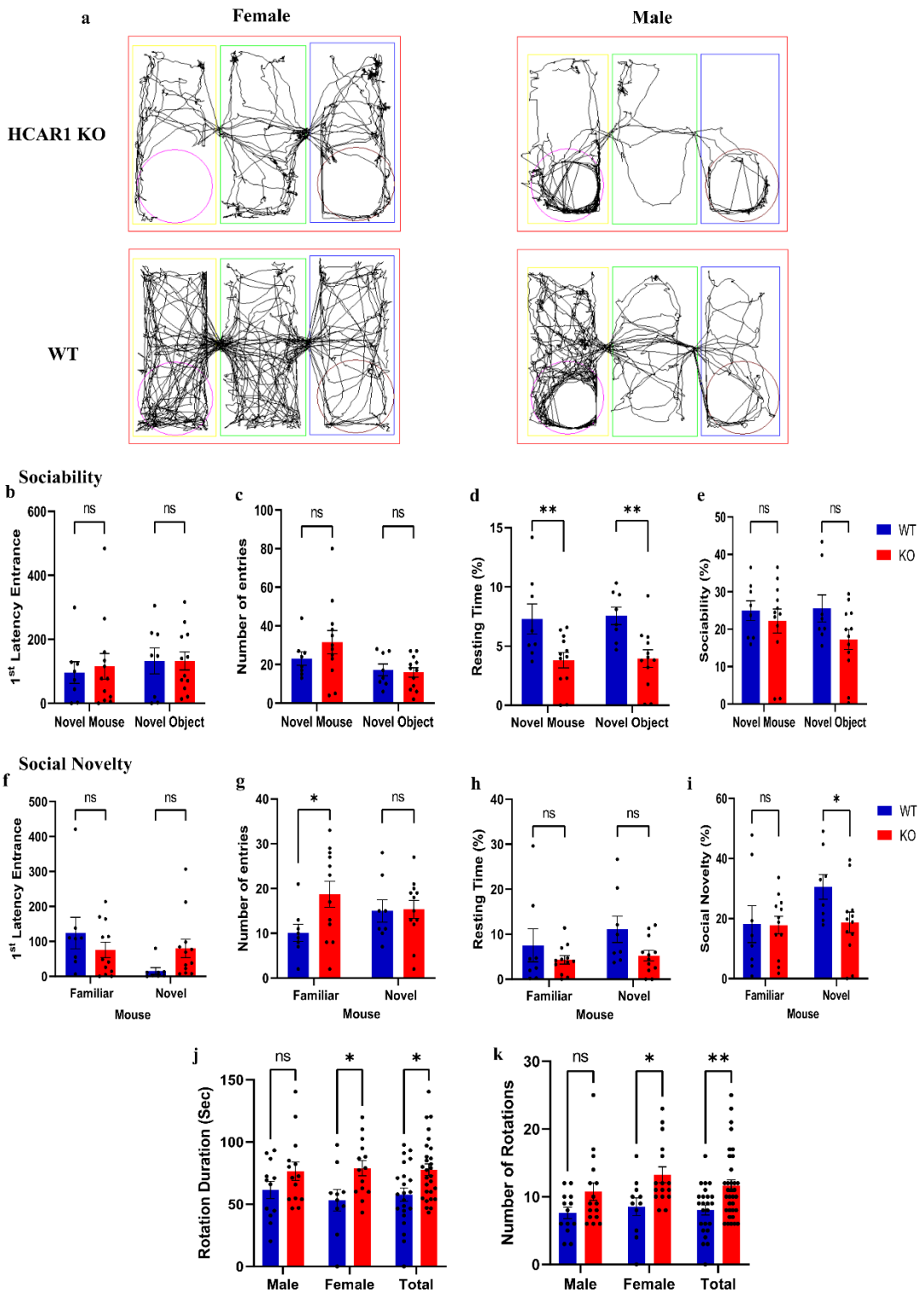
a
Female
Male
HCAR1 KO
WT
Sociability
b
1st Latency Entrance
ns
Novel Mouse
Novel Object
c
Number of entries
d
Resting Time (%)
**
e
Sociability (%)
WT
KO
Social Novelty
f
g
*
h
i
Social Novelty (%)
Familiar
Novel
Mouse
j
Rotation Duration (Sec)
Male
Female
Total
k
Number of Rotations

Fig. 2: HCAR1-deficient mice exhibit altered social behavior

3-chamber social test for social behavior: a) Representative images showing subject mouse movement from social novelty phase of the test (3rd phase). b-e) Quantitative behavioral measure for sociability phase of the test (2nd phase); 1st latency to enter (b) and the number of entries (c) in the interaction zones and the sociability did not show any changes between HCAR1 KO and WT mice. However, KO mice spent less time in the chambers with novel mice or the chamber with novel object (d) indicating lower curiosity level in KO animals. f-i) Quantitative behavioral measure for social novelty phase of the test (3rd phase); 1st latency to enter (f) and the resting time (h) in the interaction zones in the social novelty phase of the test di not show any changes between HCAR1 KO and WT mice. However, KO mice spent significantly more time in the interaction zone with the familiar mice (g) and had an overall lower social novelty score (i). j-k) Total time that mice spent rotating clock or counter clockwise (j) and total number of clock or counter clockwise rotations (k) in the whole 3 phases of the test indicate that KO mice exhibit a higher rate of repetitive behavior. Data are mean ± s.d. from n=12 for KO animal and n=8 for WT animal. Analysis of Variance (ANOVA) was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$ significance levels.

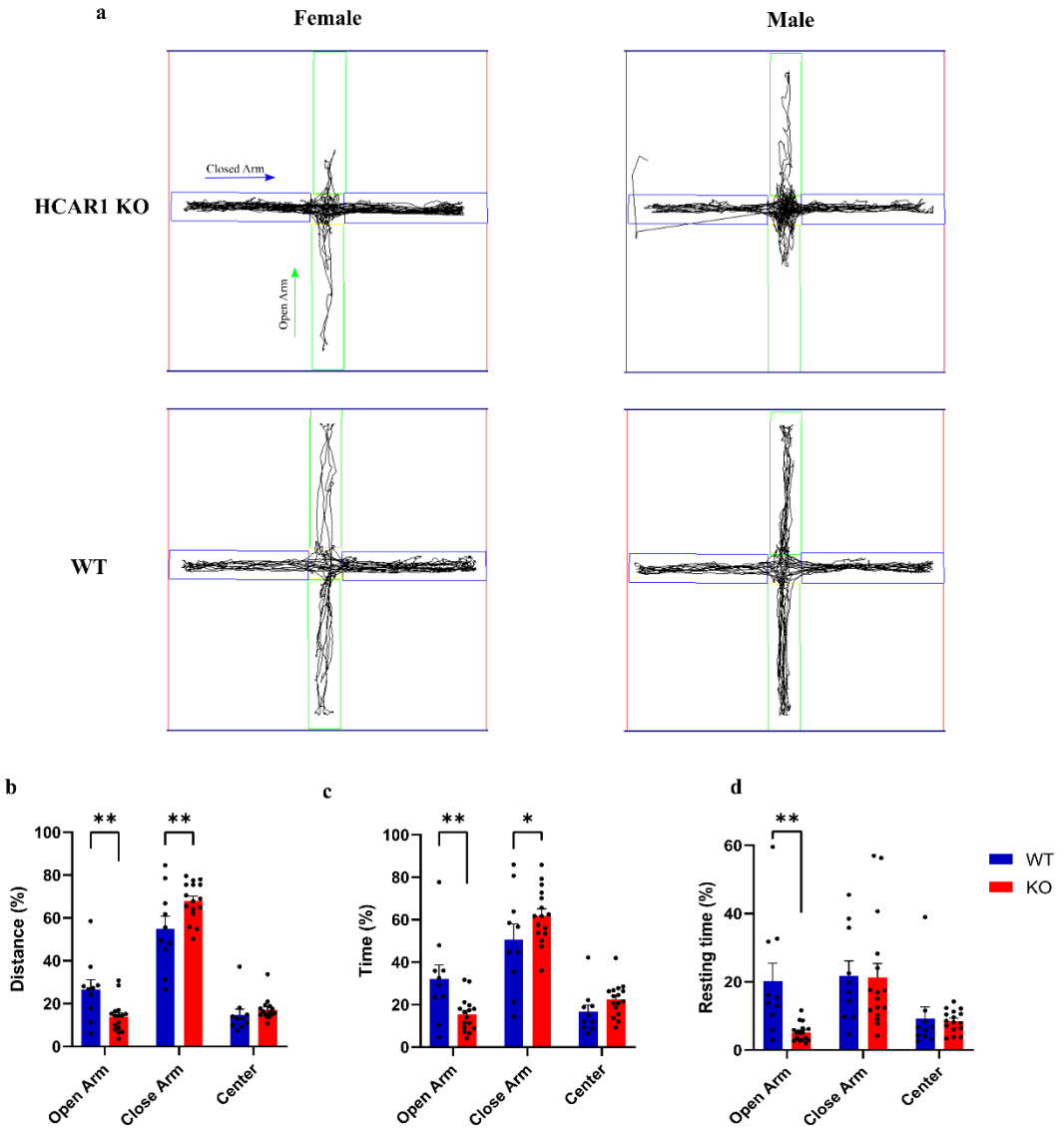
a
Female
Male
HCAR1 KO
WT
Closed Arm
Open Arm
b
Distance (%)
c
Time (%)
d
Resting time (%)
Open Arm
Close Arm
Center
WT
KO

Fig. 3: HCAR1-deficient mice exhibit anxiety-like behavior

Elevated plus maze test for anxiety-like behavior: a) Representative images showing subject mouse movement in open and closed arms of the maze. b-d) Quantitative behavioral measures for anxiety-like behaviors. HCAR1 KO mice traveled less distance (b), spent less time (c) and rested less time (d) in the open arm of the maze compared to close arm, indicating KO mice have more anxiety than WT. Data are mean ± s.d. from n=16 for KO animal and n=10 for WT animal. Analysis of Variance (ANOVA) was followed by Bonferroni post hoc correction test with * $P < 0.05$, ** $P < 0.01$ significance levels.

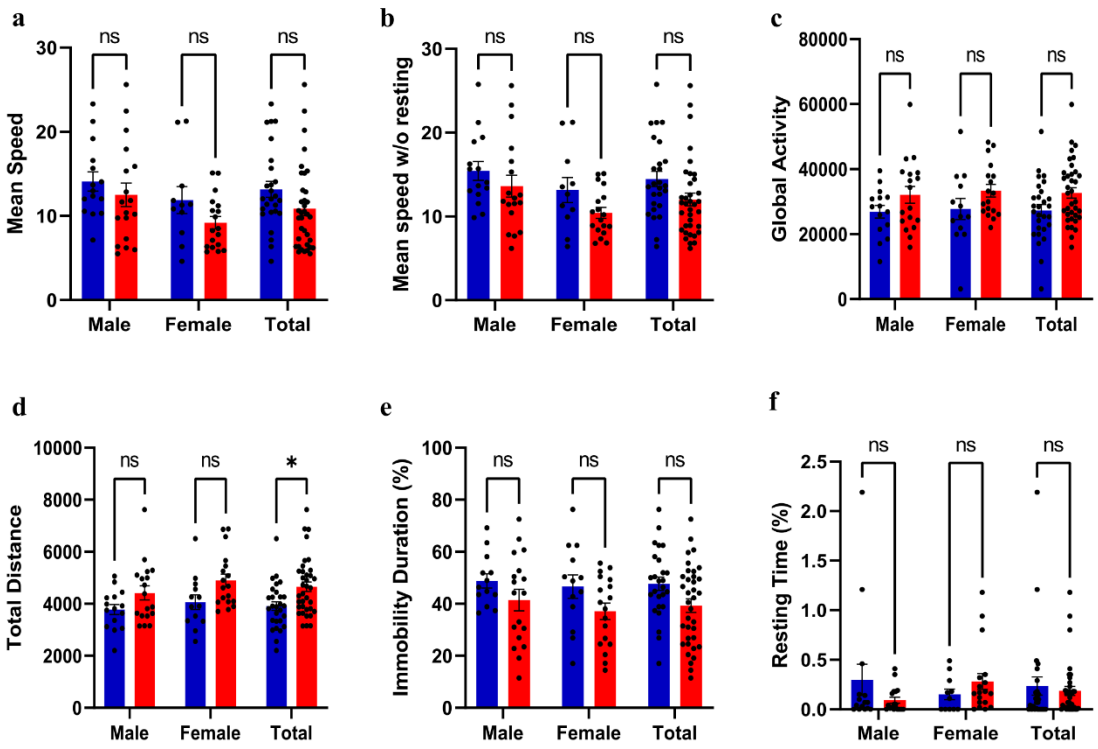

Fig. Supplementary 1: 3-Chamber social behavior test

a-f) Different parameters of motor behavior does not show any deficiency in locomotion. The quantifications are the cumulative score of each behavior during all 3 phases of the test from all 3 chambers.

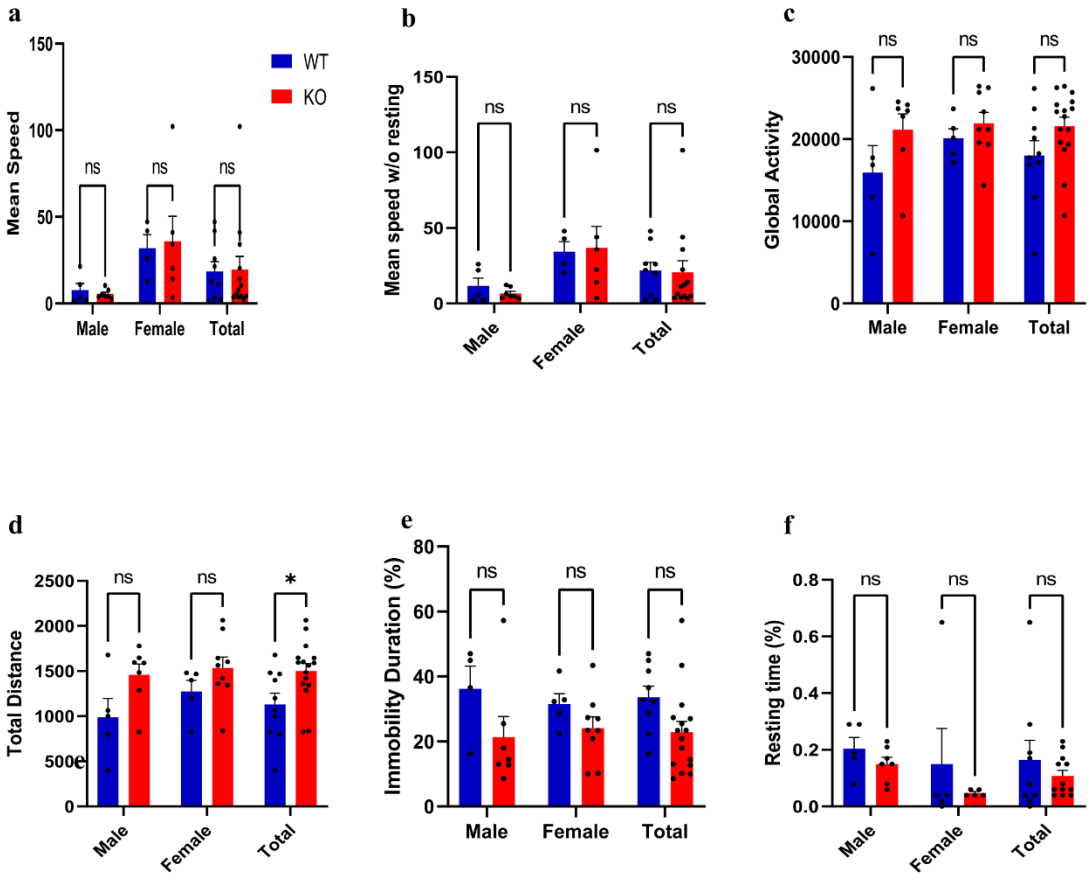

Fig. Supplementary 2: Elevated plus maze test

a-f) Different parameters of motor behavior does not show any deficiency in locomotion. The quantifications are the cumulative score of each behavior during the whole test.

Chapter 4

Discussion and Conclusion:

GPCRs are the foundation of cellular signaling and capable of regulating every biological processes. While most of them are used in odor detection, the remaining ~400 non-olfactory GPCRs[222] are the forefront of cell communication whether its in an auto, paracrine or endocrine fashion. Their ability to regulate such a vast array of different biological processes is a key question to understanding human physiology and consequently many pathologies. This ability could rely on several so far well-established features of this receptor family. One of these features is probably is their structure[223].

The GPCR activation/function is not a simple matter of on and off switch, but rather its structure enables the receptor to possess a wide spectrum conformational state each exerting different levels of engagement with their potential downstream signaling effectors[223]. This could easily be elucidated with various types of receptor agonists which all bind the same ligand binding pocket. While full agonists are able to stimulate the receptor to the highest activity level, partial agonists only induce a fraction of this activity level. Neutral agonists on the other hand do not have any effect on the receptor activity compared to its basal level, but occupy the binding pocket and compete with other agonists. On the opposing side, inverse agonists completely lower the basal energy level of the receptor to induce their signaling cascade. Then we have allosteric ligands which change the conformation of the receptor into a state that favors activation of a particular downstream pathway (or pathways) over the alternative potential pathways of that receptor[224]. All these structural flexibilities directly result in the functional regulation of the receptor and consequently the underlying physiological response. An extreme example of this situation is β_2AR; there is agonists for this receptor (ICI118551) that induce the signaling through arrestin and MAP kinase pathway, meanwhile this agonist acts as an inverse agonist to suppress the $G_{\alpha s}$ – cAMP- PKA pathway[225]. This exemplifies how even binding to the same site could elicit distinct conformations in the structure of a GPCR that not only it favors a particular pathway, at the same time it diminishes the threshold for activating another pathway.

Another important feature in the GPCR biology to regulate diverse functions is their ability to form homo-heterodimers and oligomers. This similarly has a multitude of regulatory level depending on the homo or hetero dimer/oligomer. While monomeric GPCR could

elicit signaling, some are obligate heterodimers (e.g., GABAB R1 &2) to function[226]. But different outcomes could come out from homo and heterodimerization[227], such as synergistic potentiation of the signaling by M2 and M3 receptors[228] and A2aR and mGluR5 receptors[229]. On the other hand, heterodimerization can lead to signal attenuation, or activation of an alternative downstream pathway on the ligand-free dimer[230]. The later could be due to switch in selective coupling of GPCRs upon heterodimerization with different G protein species[231]. On top of the GPCR dimerization regulatory effects, we have the cross dimerization and cross talk of GPCRs with receptors tyrosine kinase. A well-known example of this cross talk is endothelin-mediated endothelin receptor transactivation of EGFR (epidermal growth factor receptor, a receptor tyrosine kinase)[232]. GPCR isoforms are also involved in the regulation of cellular communication through this family. GPCR isoforms show unique signaling properties leading to tissue-specific signaling. Additionally, even the co-expression of different isoforms of a specific receptor changes the dynamics of their signaling depending on the combination of those isoforms[233]. The other feature of GPCR regulating their function, similar to most proteins, is their post-translational modifications (PTM). GPCRs undergo PTM to receive phosphorylation, ubiquitination, glycosylation, palmitoylation, SUMOylation, *S*-nitrosylation, tyrosine sulfation, and methylation. These PTMs regulate many different aspects of the GPCR biology, including their trafficking and life cycle as well as controlling the spatiotemporal signaling states of these receptors. Controlling the signaling states via these PTMS is not limited to their initiation or termination, rather a vast dynamic range of signaling activity including biased signaling different signaling intensities or adjusting the receptor potentiation level. Additionally, the crosstalk of these PTMs with each other is also involved in the regulation of GPCR biology and signaling[234].

As discussed in the introduction, location bias is another layer in regulating GPCR signaling[42]. In this study, we employed several methods and demonstrated that HCAR1 has a nuclear localization in addition to classical plasma membrane localization. Our data showed that not only the receptor is present on the nuclear membrane, it is also present inside the nucleus as well. To investigate the receptor functionality and direction of the signaling cascade, we further dissected the topological orientation of the HCAR1 on INM and ONM. We devised a unique method for the first time to selectively permeabilize the

ONM while maintaining the integrity of INM by using the mild detergent digitonin. Combination of this method with surface protein digestion using proteinase K, allowed us to show that the orientation of the HCAR1 on the INM is analogous to that of the plasma membrane, which would warrant the intranuclear signaling. We confirmed the nuclear location-biased signaling of HCAR1 and identified its individual Gα and Gβγ mediated downstream effectors in this intranuclear signaling events. These data establish the fact that a complete classical G-protein mediated signaling is taking place at the nuclear level for HCAR1; a full example of location biased signaling.

To our knowledge, our study is the first study to completely show the topological orientation of a GPCR on both ONM and INM. Although this was a long-lasting question in the field, only the presence of both N and C-terminus of a GPCR in a fully permeabilized nucleus was demonstrated without proving the exact location of these domains[46]. The intranuclear signaling of GPCRs is well-established, but our finding proves the required orientation for this intranuclear signaling which was only speculated in the field.

Another aspect of our findings that was not addressed is the HCAR1 on the ONM. With the detection of C-terminus on the cytoplasmic side of the ONM and N-terminus in the lumine of nuclear membrane, the receptor on the ONM has an orientation to inwardly signal into the cytoplasm. To our knowledge, no study has investigated this possibility. We are not even sure if this is possible as this signaling would require the assembly signaling machinery on the cytoplasmic side of the ONM. However, if there is such a signaling event, that could also provide a further layer to the complexity of location-biased signaling. This potential signaling could detect the ligand in the luminal space of the nucleus and emit signaling cascades into the cytoplasm. Subtle changes in the concentration of the ligand in the nucleus, in this case lactate, could rapidly induce a response via this signaling and propagate into the cytoplasm. Alternatively, the produced ligand inside the cytoplasm, could reach this luminal space and activate the intra-cytoplasmic signal transduction. This could serve as a mode for autocrine signaling without the need to secrete the ligand to the extracellular space, an intracrine pathway. Investigating this possibility is an interesting avenue for future discoveries in this field. Live signaling biosensors could be used for such a study.

Beside these features that could partly explain the diverse functions of GPCRs, our study uncovers a completely new feature; the non-receptor functions of the GPCRs. GPCRs are considered membranous receptors which are engulfed by the phospholipidic bilayer. Especially for the plasma membranous GPCRs, this phenomenon puts them in a restricted position that only enables them to convey the signaling message from the cellular microenvironment into the cytoplasm/cell. All these receptor functions of GPCRs are exerted through the interactions with other proteins known as the signaling effectors. However, the interaction of GPCRs with other molecular than non-signaling effector proteins has not been investigated. These potential interactions become more highlighted for intracellular and particularly for nuclear/intranuclear GPCRs. To dive into this discussion, one should account two pools of nuclear GPCR: 1) The pool of a GPCR that is in the nuclear membrane and has the analogous conformation on the INM to that in the plasma membrane; and 2) The pool of that GPCR inside the nucleus.

1) INM GPCR: This pool is potentially the main intranuclear signal-emitting pool. It probably has all the same features as those in the plasma membrane and interacts with downstream signaling effectors. However, the potential higher concentration of proteins inside the nucleus could both change the interaction dynamics and provide new downstream effectors. The nucleus occupies roughly 10% of the cell, but it harbors about up to 20% of the cellular proteome[235]. Adding the genome into this, the nucleus is a very dense place and potentially many other proteins could also interact with the INM localized GPCRs. The proteins could be other than the classically known signaling effectors such as G-proteins. However, one still might consider this as part of the receptor function since it conveys the message from a membranous location. Additionally, both heterochromatin and euchromatin are interacting with nuclear membrane scaffolds[236] and DNA itself is also capable of such interactions[237]. So accordingly, there is a possibility of GPCR interaction with the chromatin and DNA from the INM. This possible interaction could be a completely a new function for GPCRs in regulating cell biology. This potential function could be involved in either genome conformation to tether chromatin to the nuclear membrane, epigenetic regulation or direct transcriptional regulation.

2) The inner-nuclear GPCR: The complexity of GPCR biology inside the nucleus is even more unknown than the INM resident GPCRs. The first question in this regard is how

the GPCR is inside the nucleus? This family is known to be only membranous with its typical cylindric shape. Now inside the nucleus, it is completely unknown how its topology is organized. There are a few hypotheses but no proven example is reported so far. Micro-lipidic domains have been observed inside the nucleus, and this could be a mechanism by which GPCRs are still maintaining their structure via these lipidic domains inside the nucleus[42]. This could be reminiscent of plasma membranous GPCRs after internalization in their endosomal path. It could also be completely different than this. But in case of the former, this could be a mechanism for attenuating the receptor signaling. However, such a mechanism could also indicate a potential analogous location-biased signaling to that of endosomal location-biased signaling while inside the nucleus; a sub location-biased signaling within a nuclear location-biased signaling!!! In case of the latter, the inner-nuclear GPCRs are separate entities than the INM resident ones. These GPCRs could function in a totally different fashion.

Another mode of GPCR presence inside the nucleus is protein-protein interaction in a manner that its hydrophobic transmembrane domains are buried in the interfaces of interaction with other proteins' hydrophobic domains. While this could save the cylindrical shape of the receptor, these interactions also could organize in a manner that the cylindrical shape is no longer the case. This further adds to the dilemma. However, this protein-protein interaction could regulate different non-receptor functions for the GPCRs depending on the protein complex it is involved with.

In this project, we showed this possibility and identified the potential interactome of the HCAR1 inside the nucleus. N-HCAR1 interaction was dependent on the ligand abundance, indicating the ligand-stimulated conformational change is a determinant in the N-HCAR1 interactome selection. These interactions are with non-effector proteins pointing to the non-receptor functions of the N-HCAR1. Our data, showed these interactomes were enriched for processes regulating protein translation and ribosomal biogenesis and we further proved N-HCAR1 involvement in these processes. Additionally, some of DNA damage repair component were among the interactome data with N-HCAR1. Previously, HCAR1 signaling has been linked to DNA damage repair[168,75], but the exact mechanism for this was unknown. Our data shows this action is probably is regulated by direct involvement of N-HCAR1 with the DNA repair machinery. HCAR1 and/or N-HCAR1 signaling could

also be a part of this response, but we confirmed the interaction of N-HCAR1 with phosphorylated H2A.x (γH2AX) which indicates the direct involvement in this process. The molecular mechanism of this involvement and the role of lactate in this context could be an interesting avenue to pursue.

Among our interactome data, there were several chromatin remodeling factors including NSD1 (a histone methyltransferase), INO80b, SMARCA5, SMARCC1 and BPTF which some are components of INO80, SWI/SNF and ISWI ATP-dependent chromatin remodeling complexes and interestingly their roles in cancer is well-established[182]. The interaction of N-HCAR1 with histone (H2B & H2A) and these chromatin remodelers suggests the involvement of this receptor in direct epigenic/gene regulation. Our ChIP-seq experiment confirmed this interaction and proved similar to the proteome data, the ligand-stimulated verse non-stimulated N-HCAR1 is interacting with different set of genes. While these genes were mostly enriched for cellular migration pathway, the lack of nuclear HCAR1 proved this involvement. Although, one should account the potential contribution of N-HCAR1 location-biased signaling, specifically that of ERK activation since this is known to promote migration[238,239]. But with the ChIP-seq experiment and its coupling with our transcriptomic analysis, we can show at least some of the genes involved in migration are directly upregulated with N-HCAR1 binding to the genome. Coupling our ChIP-seq and RNA-seq data, actually showed that N-HCAR1 acts as a generally positive regulatory factor for gene expression. This positive regulatory action is regardless of ligand abundance (both of binding and transcription), indicating the two conformations of the receptor are separately upregulating different genes potentially by associating with different chromatin modifiers. This is probably mainly due to its interaction with chromatin modifier rather than direct interaction with the naked DNA, but this possibility should not be ignored as well.

These functions of the N-HCAR1 resembles those of nuclear receptors such as estrogen or thyroid receptors. Basically, our data so far shows, the N-HCAR1 besides its GPCR activity, also acts similar to nuclear receptors too. Interestingly, at least in case of estrogen receptor α (ERα), it has been shown this receptor has other functions than its transcriptional regulation. Xu et al, showed that ERα also has a RNA-binding capacity and through this binding it regulates mRNA splicing and translation for stress response genes[190]. Similarly,

this shows that ERα has other functions (i.e., non-receptor functions) that its classical transcriptional regulation. This further establishes the potential involvement of receptors in various roles.

Just for clarification, obviously the term "non-receptor functions" here is pointing to the contrary of the classical G-protein-coupled receptor function. Because at the end, these functions seem to be regulated with the ligand-stimulated conformational changes and similarly would still fall withing the receptor mode. The true non-receptor functions would be discovering something like an enzymatic activity for the receptor. However, since the function of GPCRs so far is believed to be exerted via known signaling effectors from a the lipidic membranes, our discoveries contradict this and we used this terminology to point to this fact.

Our culminative results was showing that the effect of nuclear exclusion of HCAR1 on the cellular processes is similar to the total KD of the HCAR1 and its cellular depletion; this was suggesting all these processes are either completely or to a large part are regulated solely via the N-HCAR1 (rather than the plasma membrane/cytoplasmic HCAR1). The cellular processes we observed, all were pointing toward either tumor growth or metastasis. The nuclear location-biased signaling, DNA damage repair and protein translation are involved in cellular growth, and the chromatin binding and gene regulation for migration are involved in metastasis. As mentioned in the introduction, both of these cancer hallmarks are already discovered for HCAR1, but through rather ill-understood mechanisms. We conducted *in vivo* experiments, to see if the magnitude of our results from *in vitro* studies matches the magnitude of effects observed by total HCAR1 KD in tumor growth and metastasis. Both tumor growth (subcutaneous) and metastasis (tail vein) xenograft assays in immune-incompetent mice proved that the overall effect of HCAR1 nuclear exclusion is similar to complete cellular depletion of this receptor. This further demonstrates that all these nuclear functions of HCAR1 are the main determinants in the roles of this receptor in cancer malignancy.

One rather striking observation in our data was the impact of HCAR1 on the investigated cellular processes. The complete or nuclear KD of HCAR1 did not completely abrogate any of these cellular processes; proliferation, survival, protein translation, DNA damage repair and migration, all were statistically significantly decreased but the reduction was not

more than 50% in either of cases. This is mostly in agreement with previous reports on these processes[168,74,72,189]. The role of HCAR1 on each of these individual processes is not determinant, but it seems that the collective effect of all these pathways is providing a great advantage in favor of cancer progression. This is evident by the *in vivo* studies on the general effect of HCAR1 on tumor growth and metastasis which are showing a profound reduction in both of these main cancer malignancy features[72,74,189]. So, in the big picture, it seems our findings is in agreement with previous reports.

To further expand our findings toward a translational view, we tried to develop peptide inhibitors for HCAR1. However, these peptides should infiltrate into the nucleus in line with our data to see the effect of nuclear HCAR1 inhibition. Unfortunately, despite adding classical nuclear localization signal tags to these peptides, we did not observe a significant nuclear localization for these putative inhibitors. We tested a few different combinations, but were not successful in this front. Further in-depth investigations are required for this approach.

Unfortunately, no established antagonist/inhibitor has been developed/discovered for HCAR1, further limiting our potential for these kinds of translational studies. Several studies have reported the use of 3-hydroxy-butyrate acid (3-OBA) to inhibit HCAR1 activity. 3-OBA is the ligand of HCAR2 which complicates the use of this chemical for HCAR1 studies. But most importantly, there is no single experiment proving that HCAR1 is inhibited by this chemical. For the detailed discussion on this issue, please refer to our published commentary in annex 3.

The lack of chemical inhibitor for HCAR1 was one of the limitations in our projects. Another limitation is the exogenous expression of HCAR1 and its mutant versions in our investigations. The available marketed antibodies against HCAR1 are not completely specific, as we and others[220] have observed staining patterns in KO animals. We had to use tagged receptor to tackle this problem for precise cellular and molecular studies that we have conducted in this project. The most attainable approach for this, was exogenous expression of the tagged receptor. However, our scrambled shRNA control, as well as re-expression of RNAi resistant HCAR1 (WT and mutants) showed similar levels of expression to that of WT HCAR1; both at mRNA level and protein level attested by qRT-

PCR and western blot analysis respectively. This clarifies the possible artifacts from overexpression of the receptor (caveat: overexpression verse exogenous expression). Although, one upon meticulous evaluation could see the possibility of any artifacts from the exogenous expression is remote in our experimental design. The sole comparison of HCAR1 KD with control cell line (scrambled shRNA) proves the involvement of HCAR1 in these processes. But in the cases of exogenous re-expression of HCAR1 for rescue experiments (WT and mutants), it is obvious the mutants behave differently with the WT HCAR1. If there were a possible artifact associate with the exogenous expression for this protein, we should not have seen any significant difference between the WT and mutant RNAi resistant HCAR1 rescues. The remote possibility in this scenario is the artifact from exogenous expression is particularly associated with the mutant versions. On the other hand, we have used Flag tag (both at C & N terminus of the HCAR1), Myc tag and HA tag in different constructs during our experimentations and did not observe any difference in the localization pattern of the receptor. This further largely clarifies the possible artifacts of tag-effect on the receptor localization. Nevertheless, the most ideal approach is the closest to the natural situation and deviations from this situation creates limitations in the study, even though there are justifications and rationales behind those deviations.

The Plato's cave (Allegory of the Cave) illustrates an analogy to a possible scenario in which we live. In this scenario the shadows are the reality of the cave's prisoners, but they are not the most accurate representation of the true reality; they are just merely a shadow for an outsider to the cave, a fragment of the reality[240]. The chains are the limitations of the prisoners in comprehending and accessing the true reality. Similarly, the limitations of our study (including the time and budget as well) are the chains on us, but we are trying to reflect on the shadows and find explanations to the true reality despite these chains. Socrates believed this is the philosopher's job to reflect on the shadows and find the true reality[240]. With the advancement of the modern science, I believe now this mostly is the responsibility of scientists to reflect on the shadows despite these limitations and explain the potential realities in an effort to discover the true reality. Scientists basing their approach on formulating a falsifiable hypothesis, experimental reasoning, statistical analysis and probabilities and reproducibility, provide a more comprehensive and non-ideologic path to discovering the truth rather than the approaches taken by philosophers. I

do not mean that the philosophical approach is not useful, but the history of science has strongly shown that relying on the modern scientific approach has led to a better and faster understanding of nature and the true reality and consequently using these understandings has led to human and society flourishing. The scientific approach also does not hold the devastating pitfalls of the philosophy including the ideological attachments. However, in line with my efforts in writing this thesis to obtain a "doctorate of philosophy", I think every scientist must utilize and engage in philosophy next to their scientific abilities. Scientists should be the first ones to own their discoveries and data and philosophically analyze it; they should be the ones to provide the implications of their findings in a global context and do not just leave it for the philosophers and politicians to explain the masses what these discoveries mean and how these should be utilized.

Socrates believed that the most honorable people are those who follow the endeavors in finding the reality behind the shadows to enlighten the truth, but these people must share their enlightenments with the other prisoners. I completely agree with his view on this part, and think scientist should be the primary individuals to share their endeavors, discoveries and its political and philosophical implications rather than philosophers and politicians. This might prevent the ideological attachments propagated by these people to control the masses, and this might overthrow the *opium of the masses* sooner than the later as it has taken and is taking many lives.

In light with the Iranian "*WOMEN, LIFE, FREEDOM*" Revolution to overcome centuries of brutal oppression by religious ideologist, so called philosophers,

I dedicate this work to IRANIAN WOMEN, May The Freedom Be With Them

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Annexures

Annex 1:

Mohammad Ali Mohammad Nezhady, Jose Carlos Rivera, and Sylvain Chemtob. Location Bias as Emerging Paradigm in GPCR Biology and Drug Discovery. iScience 23, 101643, October 23, 2020.

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Review

Location Bias as Emerging Paradigm in GPCR Biology and Drug Discovery

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SUMMARY

GPCRs are the largest receptor family that are involved in virtually all biological processes. Pharmacologically, they are highly druggable targets, as they cover more than 40% of all drugs in the market. Our knowledge of biased signaling provided insight into pharmacology vastly improving drug design to avoid unwanted effects and achieve higher efficacy and selectivity. However, yet another feature of GPCR biology is left largely unexplored, location bias. Recent developments in this field show promising avenues for evolution of new class of pharmaceuticals with greater potential for higher level of precision medicine. Further consideration and understanding of this phenomenon with deep biochemical and molecular insights would pave the road to success. In this review, we critically analyze this perspective and discuss new avenues of investigation.

INTRODUCTION

G-protein-coupled receptors (GPCRs) are the largest family of receptors in eukaryotes to signal for cellular adaptation in response to environmental cues and are involved in virtually all biological processes. They are expressed from approximately 800 genes covering almost 4% of human coding genome and are the target of more than 40% of pharmaceutical agents, validating their significance in pathophysiology (Dupré et al., 2009). They possess a diverse array of ligands spanning from odor molecules, lipid, nucleotide, and carbohydrate metabolites to peptide neurotransmitters and photons. Activation in adhesion GPCRs (adGPCR) is even more complicated; in some cases their basic conformation as active form is inhibited by their own N terminus, whereas in other adGPCRs, a segment of N terminus serves as ligand for their respective receptor (Purcell and Hall, 2018). GPCR regulation and signaling are subject of intensive research. Their subcellular (mostly nuclear) localization and ensuing intra-organelle signaling as well as non-signaling actions, such as co-transcriptional activation, adds a plethora of complexity to GPCR biology.

GPCRs are integral membrane proteins in the plasma membrane (PM) consisting of seven transmembrane receptors with an extracellular N terminus and cytoplasmic C terminus. Unstimulated GPCRs are associated with heterotrimeric G-proteins: Gα and Gγ subunits are membrane anchored and a Gβ subunit that tightly interacts and remains bound to Gγ (Figure 1A). Gα is a guanin nucleotide-binding protein with GTPase activity. There are four sub-families of Gα ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$) bound to GPCRs in cell and context-dependent manner, and they dictate primary GPCR signaling depending on their subfamily and sub-type. Unstimulated GPCR is in complex with G-proteins, whereas Gα is loaded with GDP. Upon ligand binding to GPCR, inactivated Gα (GDP bound) exchanges GDP with GTP leading to conformational change resulting in release of G-proteins from GPCR. GTP-bound Gα is also separated from Gβγ and diffuses along the PM to signal for downstream effectors (Figure 1B) (Katritch et al., 2013)-(Sato et al., 2006).

$G\alpha_s$ stimulates activation of Adenylyl Cyclase (AC), a membrane-bound enzyme converting ATP to cAMP. Increased cAMP level leads to activation of protein kinase A (PKA) and subsequent phosphorylation of many targets and cellular response. cAMP can also modulate activity of some of Guanine Exchange Factors (GEFs) and ion channels. On the contrary, $G\alpha_{i/o}$ sub-family inhibits the activity of AC when it is bound to GTP and lowers cellular cAMP level. $G\alpha_{q/11}$ sub-family can activate Phospholipase C-β (PLCβ) in the membrane, which in turn catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 acts on endoplasmic reticulum (ER) triggering efflux of

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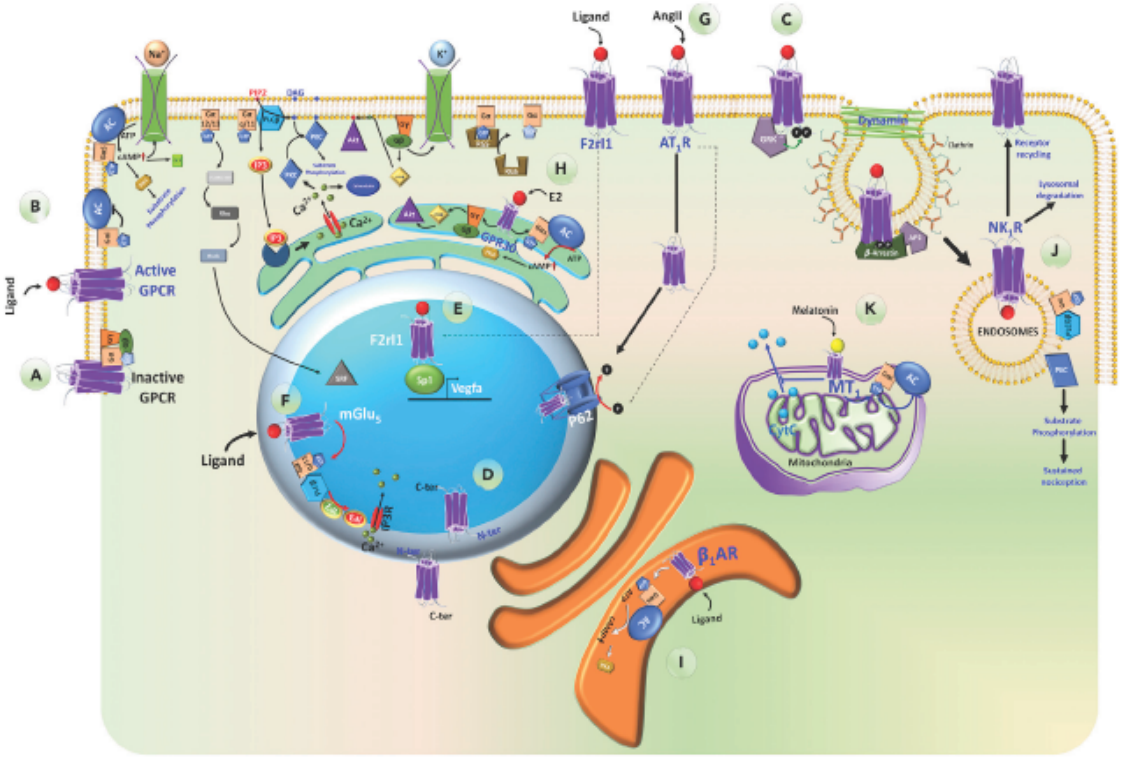

Figure 1. PM and Subcellular Organelle Localization and Signaling of GPCRs

A schematic representation of GPCR cycle and different GPCRs in various cellular organelles.

(A) A GPCR in its inactive confirmation coupled with heterotrimeric G proteins.

(B) Ligand binding to GPCR induces conformational changes releasing heterotrimeric G proteins. Various downstream signaling cascades are activated depending on the specific subfamily of each G protein.

(C) Ligand-bound GPCR attracts GRKs, which phosphorylate the receptor initiating their signal termination process through interaction with β-arrestins and endosomal entry.

(D) The proposed model for conformation of GPCR in the outer and inner nuclear membranes.

(E) F2rl1 is an example of a nuclear GPCR that translocates to the nucleus upon activation and induces *Vegfa* transcription through interaction with Sp1 transcriptional factor.

(F) $mGlu_5$, another example of a nuclear GPCR initiates downstream signaling inside the nucleus.

(G) Activation of PM AT_1R leads to phosphorylation of nuclear pore, which in turn facilitates its nuclear translocation.

(H) GPR30 is an example of ER resident GPCR, which initiates downstream signaling within the ER network.

(I) β_1AR is an example of Golgi apparatus GPCR initiates downstream signaling inside the Golgi lumen.

(J) NK_1R is an example of endosome-located active GPCR which displays physiological output location bias from endosomal signaling. The receptor could also go through lysosomal degradation or recycle to PM as part of GPCR life cycle.

(K) MT_1 is an example of mitochondrial resident GPCR, which initiates downstream signaling inside the mitochondria.

Ca^{2+} to cytoplasm and its subsequent cellular effects such as activation of Ca^{2+}-dependent proteins (e.g., calmodulin, transcription factors). DAG, in parallel with increased Ca^{2+} level, activates protein kinase C (PKC) in the membrane followed by phosphorylation of its target proteins. Lastly, $G\alpha_{12/13}$ interacts with various proteins and exerts its effects mainly by modulation of Rho and Ras-GEFs, cadherins, and ion channels. Released Gβ and Gγ subunits function together in a complex and can signal for various downstream effectors concomitant with Gα activity. Gβγ targets partially depend on the isoforms of their sub-units and span from ion channels to PI3K, AC, and PLC. Gα has inherent GTPase activity, although with different rates depending on its sub-family and isoforms. Regulators of G-protein signaling (RGS) and some downstream effectors of GPCR have GTPase-activating capacity and are able to increase the GTP hydrolysis rate of Gα subunit. Once GTP is hydrolyzed, GDP-bound Gα is able to assemble heterotrimeric Gαβγ and re-associate with GPCR, ready for new stimuli and activation (Ritter and Hall, 2009)(Hilger et al., 2018).

Conformational changes in GPCR after ligand binding increases its affinity to G protein-coupled receptor kinases (GRKs) and their mediated phosphorylation of GPCRs. Ligand-bound and phosphorylated GPCRs are targeted by arrestins leading to signal attenuation (Figure 1C). GRK-mediated phosphorylation of GPCRs initiates homologous desensitization, whereas heterologous desensitization is the result of longer exposure of ligand to receptor and phosphorylation of wider range of GPCRs via signaling activated PKA and PKC. Arrestin bound to the GPCR recruits clathrin and AP2 to initiate receptor internalization. The forming vesicle is pinched off from PM with aid of accessory proteins such as dynamin. The endosomal GPCR has two fates depending on receptor type and accessory proteins available in cell-type specific manner: (1) recycling of receptor and (2) lysosomal degradation. Once the GPCR is destined for recycling, ligand is released and degraded in endosome and the GPCR is dephosphorylated and the vesicle fuses to the PM leading to receptor resensitization and recycling. When the GPCR is targeted for degradation, endosome is sorted to lysosome and the GPCR is subjected to degradation (Marchese et al., 2008)(Peterson and Luttrell, 2017).

One of the emerging concepts in GPCR biology is their signaling from subcellular organelles, demonstrating another level of specificity and regulation in signal transduction. GPCRs are found in all membranous organelles within cells, acting as a functional receptor modulating signal transduction. Interestingly, some GPCRs are primarily resident to these compartments rather than to the PM. Some signaling cascades are sent to downstream effectors during endosomal entry of activated GPCRs. Mitochondria, Golgi apparatus, and ER have been shown to harbor GPCRs exerting classical signaling cascades, either potentially into the cytoplasm or into the organelle. Unfortunately, conformation of GPCRs in organelles remains largely unresolved resulting in uncertainty as to signaling directionality (intra-organelle versus into cytoplasm). Nuclear GPCRs, on the other hand, are shown to have more dynamic functions. Some are translocated from PM to the nucleus upon ligand binding, whereas others have a ligand-independent nuclear pool. Other than their participation in classical GPCR intra-nuclear signaling, nuclear GPCRs have been shown to interact with transcription factors and regulate gene expression directly (Jong et al., 2018a)(Joyal et al., 2015). In this review, we outline advances in intracellular GPCRs as a means of localizing signaling bias and address some of the challenges that remain unsolved in the field, and their potential therapeutic consequences.

NUCLEAR GPCRS

Nuclear GPCRs are the most studied permanent intracellular GPCRs in cellular organelles (compared with endosomes). The first report of nuclear GPCR localization was published in 1998 (Lu et al., 1998) showing translocation of AT1 receptor to the nucleus upon ligand stimulation potentially mediated through a classical nuclear localization signal (NLS). Later that year, the second report confirmed using immunogold labeling the presence of EP1 receptor in the cell nucleus independent of ligand binding, with functional consequences resulting in increased intranuclear calcium concentration and gene transcription (Bhattacharya et al., 1998). These studies paved the road for identification of intracellular GPCRs, re-shaping the classical exclusive model of PM receptor for GPCRs.

So far more than 40 GPCRs are shown to be either translocated to or localized in the nucleus (Table 1). These GPCRS have a variety of functional outputs in the nucleus. Most experimental efforts reveal classical GPCR signaling through modulation of downstream effectors such as phosphorylation of different intermittent signaling modules (Cattaneo et al., 2016), or calcium (O'Malley et al., 2003) and cAMP (Valdehita et al., 2010) level fluctuations. Since these signaling activities are known to take place inside the nucleus, it has been assumed that nuclear GPCR conveys signals via inner nuclear membrane (Tadevosyan et al., 2012). This would warrant an inward signaling cascade inside the nucleus upon activation of nuclear GPCRs similar to the cytoplasmic events (Figure 1F). Based on this model, the N terminus of GPCR in inner and outer nuclear membranes would be presumed to locate inside the lumen of nuclear membrane, whereas the C terminus would either be inside the nucleus or in the cytoplasm (Figure 1D); this GPCR orientation remains, however, speculative. Although this model would explain intra-nuclear signaling, it also provides a mode of signal transduction into the cytoplasm from activation of GPCRs on the outer nuclear membrane, but this has not been studied.

Our expanded knowledge of nuclear GPCRs compared with other intracellular GPCRs is based on feasible methods of intact nuclear isolation. Isolated nuclei have the ability to behave as functional units, providing reliable examination of GPCR effects in the nucleus. However, we are unable to scrutinize the signal transduction cascade of outer nuclear membrane-localized GPCRs into the cytoplasm. Cells receive external

GPCR	Signaling/Function	Reference
AT_1R	PKC-NOX4-mediated ROS stimulation, NO production	(Lu et al., 1998)* (Gwathmey et al., 2009)* (Pendergrass et al., 2009)* (Morinelli et al., 2007)
AT_2R	RNA synthesis via IP3R- and NO-dependent pathways	(Tadevosyan et al., 2017)
$\alpha_{1A\ \&\ B}$-AR	ERK and PKC phosphorylation	(Wright et al., 2008)* (Wu et al., 2014)
$\beta_{1\&3}$AR	G_s-mediated RNA synthesis, AC stimulation	(Vaniotis et al., 2011)
Apelin R	–	(Lee et al., 2004)
Bradykinin B_2 R	Interaction with Lamin C	(Lee et al., 2004)* (Takano et al., 2014)
CCR2	–	(Favre et al., 2008)
CXCR4	G_i-mediated intra-nuclear Calcium release	(Wang et al., 2005)* (Don-Salu-Hewage et al., 2013)
$ET_{A\&B}R$	NO production, IP3-dependent increase of calcium	(Boivin et al., 2003)* (Vaniotis et al., 2013)* (Merlen et al., 2013)
FPR2	ERK2, c-Jun, c-Myc phosphorylation	(Cattaneo et al., 2016)
GRLN-R	–	(Leung et al., 2007)
GnRH-R	Acetylation and phosphorylation of histone H3	(Re et al., 2010)
$CysLT_{1\&2}$	ERK1/2 phosphorylation, nuclear calcium signaling	(Nielsen et al., 2005)* (Eaton et al., 2012)* (Dvash et al., 2015)
LPA_1R	G_i, PI3K, and AKT-mediated calcium transit and iNOS expression	(Gobeil et al., 2003)
$S1P_1$	ERK and c-Jun dephosphorylation, transcriptional initiation	(Liao et al., 2007)* (Estrada et al., 2009)
MC2R	–	(Doufexis et al., 2007)
MT2	–	(Lanoix et al., 2006)
mAChR	–	(Lind and Cavanagh, 1993)
Y1R	–	(Jacques et al., 2003)
NTS1	–	(Toy-Miou-Leong et al., 2004)
MOR_1	–	(Khorram-Manesh et al., 2009)
Oxtr	Induction of gene transcription	(Kinsey et al., 2007)* (Di Benedetto et al., 2014)
Ptafr	cAMP reduction, G_i-mediated calcium increase, ERK 1/2 phosphorylation, NF-κB DNA binding, induction of gene transcription	(Marrache et al., 2002)* (Bhosle et al., 2016)
$EP_{2,3\&4}$	G_s-mediated calcium increase, ERK1/2 &AKT phosphorylation, induction of gene transcription	(Bhattacharya et al., 1999)* (Gobeil et al., 2002)
TPR	Coupling to G_q, CREB phosphorylation, induction of gene transcription	(Ramamurthy et al., 2006)* (Mir and Le Breton, 2008)

Table 1. List of GPCRs detected on nuclear membrane and/or inside the nucleus

(Continued on next page)

GPCR	Signaling/Function	Reference
F2rl1	Sp1-mediated gene transcription	(Joyal et al., 2014)
$NK_{1\&3}$	–	(Aline Boer and Gontijo, 2006)* (Lessard et al., 2009)
UTR	Transcriptional initiation	(Nguyen et al., 2012)* (Doan et al., 2012)
PTH_1R	–	(Watson et al., 2000)
$VPAC_1R$	cAMP production	(Valdehita et al., 2010)*
$mGlu_5$	Calcium transit, ERK1/2, Arc/Arg3.1, c-fos & CREB phosphorylation	(O'Malley et al., 2003)* (Jong et al., 2005)* (Kumar et al., 2012)* (Vincent et al., 2016)
GPR158	Cyclin D1-mediated cell proliferation	(Patel et al., 2013)

Table 1. Continued

cues from cellular niche and respond to environmental changes in the classical GPCR signaling model. On the contrary, outer nuclear membrane-localized GPCRs would enable cells to respond to the internal nuclear cues and adopt to nuclear changes. Evidence for intra-nuclear mRNA translation (Iborra et al., 2001), as well as presence of various metabolites (Campbell and Wellen, 2018) and lipids (Cascianelli et al., 2008) that can stimulate GPCRs at the nucleus, suggest that GPCR ligands are readily available inside the nucleus to act on their corresponding receptors. Alternatively, GPCR activation and signaling can be delayed until ligands reach the targeted intracellular organelle; in this case, these ligands are either transported or diffuse into the cytoplasm, to activate first PM GPCRs and subsequently nuclear GPCRs.

Alongside GPCRs, growing evidence shows presence of almost every GPCR signaling effectors in the cell nucleus. The classical partners of GPCRs, Gα and Gβγ and their different isoforms, have readily been detected in the nucleus (Zhang et al., 2001) (Boivin et al., 2005) (Dahl et al., 2018) (Sato et al., 2011). This enables formation of a fully functional coupled receptor on the nuclear membrane. In addition to the G-proteins, their immediate effectors such as AC (Yamamoto et al., 1998), PLC isoforms (Schievella et al., 1995) (Freyberg et al., 2001), Ca^{2+} and K channels (Bootman et al., 2009) (Quesada et al., 2002), as well as their corresponding second messengers including cAMP (Haj Slimane et al., 2014), DAG, and IP3 (Kumar et al., 2008) are observed in the cell nucleus and their activities are reliant on ligand-dependent stimulation of their respective GPCRs. Major components of GPCR signaling downstream cascades are also present and functional at the nuclear level. Various isoforms of ERK, JNK, p38 (Turjanski et al., 2007) (Plotnikov et al., 2011), AKT (Sang et al., 2008), and PKC (Martelli et al., 2003) are resident in the nuclear compartment and/or trafficked as part of their signaling translocation system. On the other hand, GPCR regulating proteins are found in the nucleus as well, including β-Arrestin (Wang et al., 2003), GRK (Johnson et al., 2004) (Jiang et al., 2007), and RGS (Burchett, 2003) (Panicker et al., 2010). The nucleus even harbors proteins such as clathrin (Ybe et al., 2013) required for endosomal entry and recycling of GPCRs. The existence of all major components of GPCR signaling cascades indicate the potential for a fully functional signaling system that could operate independently at the nuclear level.

Initiation of transcription upon stimulation of isolated nuclei provides another feature of nuclear GPCR signaling output (Vaniotis et al., 2011). However, it is not clear these transcriptional activities are the result of anything other than signaling cascades initiated inside the nucleus. A study of F2rl1, also referred to as PAR2, provided for the first time another potential mechanism of action for nuclear GPCRs, direct transcriptional regulation (Joyal et al., 2014). F2rl1 is translocated to the nucleus (from PM) upon ligand stimulation and then binds to various transcription factors and genes inside the nucleus; specifically, F2rl1 interacts with Sp1 transcription factor leading to expression of *Vegfa* (Figure 1E). Besides being present on the nuclear membrane, GPCRs have been detected inside the nucleus; in this case, nuclear F2rl1 is an example for potential distinct mechanisms and functions.

Given that GPCRs contain seven hydrophobic (transmembrane) domains, it is unlikely they are freely "floating" inside the nucleus. There are a number of potential mechanisms to accommodate such hydrophobic proteins inside the nucleus. Lipids are abundantly present inside the nucleus, and more specifically,

they can form lipid microdomains inside the interphase nucleus reported to be co-localized with ribonucleoproteins and involved in transcription and transport of transcripts in and out of the nucleus (Maraldi et al., 1992) (Cascianelli et al., 2008). These lipid microdomains could harbor GPCRs, probably through invaginations taking place inward from nuclear envelope (Fricker et al., 1997). Besides lipid microdomains, nuclear vacuoles and some other nuclear bodies have hydrophobic constituents suitable for interactions with lipophilic proteins such as GPCRs (Zimber et al., 2004) (Gobeil et al., 2006).

Other potential explanation for intranuclear GPCR organization is their possible involvement in phase separation. Emergence of colloidal biochemistry enabled identifying many membrane-less organelles capable of having hydrophobic and hydrophilic regions (Rabouille and Alberti, 2017) (Li et al., 2012). Nucleus also has been shown to organize phase separation in order to facilitate function and formation of ribonucleoproteins and RNP bodies for RNA processing (Feric et al., 2016). Interestingly, many of the phase-separating proteins are largely hydrophobic, with multi-domains and linkers (Alberti, 2017). By these criteria, GPCRs are among top candidates for phase separating proteins based on their seven hydrophobic domains, intra and extracellular loop domains, and C- and N-terminal domains as linkers. These multidomain proteins (GPCRs) could be buried with different multivalent proteins covering their lipophilic domains, meanwhile concentrating different protein complex machineries for various cellular functions such as transcription. GPCRs inside the nucleus could operate in different nuclear functions via these mechanisms. The caveat here would be avoiding restriction of functions of GPCRs based on their classical cylindrical 3D structures, as they might adopt different shapes depending on the interacting protein complexes. In this case, their sequential linker-hydrophobic domains would provide them with huge flexibility in modulating a phase-separating function.

Although the number of nuclear GPCRs is increasing rapidly, and we are starting to differentiate their cellular and physiological functions compared with their cell surface counterparts, the field lacks deep non-signaling biochemical and biomolecular analysis of their features and functions specially as it applies to the ones inside the nucleus.

MITOCHONDRIAL GPCRS

Mitochondria also harbors GPCRs (Figure 1K). A large and increasing number of these receptors can be found on this organelle (Table 2) and opens new insights into the GPCR localization and signaling bias.

Interestingly, at least two GPCRs have been shown to be present both in the nucleus and on the mitochondria, specifically AT_1R and AT_2R (Jong et al., 2018b). Their multi-compartmental presence vastly increases the role of a single GPCR in regulation of cell physiologic functions as it adapts to environmental signals at large. Although the role of mitochondrial AT_1R remains elusive, in the case of mitochondrial AT_2R, its activation leads to increased mitochondrial nitric oxide (NO) production as reported for nuclear AT_2R stimulation resulting in formation of nuclear NO (Abadir et al., 2011) (Gwathmey et al., 2009). This is an example of similar downstream output of subcellular GPCR activation regardless of its location. However, increased AT_2R-dependent NO in both nucleus and mitochondria is likely to elicit distinct physiological functions. Interestingly, AT_1R and AT_2R do not have a unique nuclear or mitochondrial localization signal (Abadir et al., 2012). Yet their subcellular localization varies in different tissues and cell types (Jong et al., 2018b). The most plausible mechanism to explain differential localization pattern of GPCRs applies to post-translation modifications (PTMs). Most PTMs information on GPCRs focuses on their conformational and signaling modalities rather than other potential functions, including localization. The fact that there is a difference in localization pattern among various tissues indicates other PTM are tightly regulated to enable tissue-specific function of these GPCRs by their concentrated localization in different subcellular compartments.

The increasing number of mitochondrial GPCRs comes from our technical ability to isolate mitochondria and use them as separate functional units to induce mitochondrial functions, similar to the nucleus. But the same hurdles regarding nuclear GPCRs apply here as well. Specifically, the exact location of GPCRs in outer and inner mitochondrial membranes is not known, as is the case for their topographical orientation. As is the case with nuclear GPCRs, some mitochondrial GPCRs are detected inside the mitochondria as well (Suofu et al., 2017) (Belous et al., 2004), but the relative roles of intra-mitochondrial and mitochondrial membrane-localized GPCRs are not known.

GPCR	Signaling/Function	Reference
$P2Y_1$	Stimulation of mitochondrial Ca^{2+} uptake	(Belous et al., 2004)
$P2Y_2$	Inhibition of mitochondrial Ca^{2+} uptake	(Belous et al., 2004)
AT_1R	Regulation of superoxide production and increase in mitochondrial respiration	(Valenzuela et al., 2016); (Abadir et al., 2011)
AT_2R	Nitric oxide formation and decrease in mitochondrial respiration	(Valenzuela et al., 2016); (Abadir et al., 2011)
$5\text{-}HTR_3$	Increase in Ca^{2+} uptake (in hypoxia)	(Wang et al., 2016)
$5\text{-}HTR_4$	decreases Ca^{2+} uptake	(Wang et al., 2016)
MT_1	Inhibition of adenylyl cyclase through Gαi	(Suofu et al., 2017)
CB_1	Inhibition of adenylyl cyclase through Gαi	(Bénard et al., 2012); (Hebert-Chatelain et al., 2016)

Table 2. List of GPCRs detected on and/or inside the mitochondria

Similar to the nucleus, many receptor-interacting proteins and their downstream effectors are observed to have mitochondrial localization as well. Gα (Suofu et al., 2017) and Gβγ (Fishburn et al., 2000), their effectors such as AC (Yamamoto et al., 1998), PLD1 (Freyberg et al., 2001), ERK (Rasola et al., 2010), AKT (Bijur and Jope, 2003), PKD, DAG (Cowell et al., 2009), C^{2+} channels (Belous et al., 2004), and even β-arrestins (Suofu et al., 2017) are detected in the mitochondria. Accumulation of major components of GPCR signaling in this organelle ascertains a complete functional system withing the mitochondria.

ER AND GOLGI GPCRS

ER and Golgi apparatus participate in endogenous route of GPCR trafficking, where they get translated, matured, and post translationally modified and sorted to their destination. It is therefore complicated to ascertain functionally active resident GPCRs in ER and Golgi, since they are readily detectable in both organelles by way of translation and PTM.

One of the best methods to differentiate transient receptor trafficking from these compartments versus functionally active residents is utilizing nanobodies. Nanobodies, also called single-domain antibodies, are able to selectively bind to only active conformation (or ligand occupied) of the receptor and stabilize them in such a conformation providing a versatile tool for structural studies and crystallography of active states of receptor (Steyaert and Kobilka, 2011). Nanobodies have successfully been utilized to detect active receptor pool of β_2-adrenoceptor as prototypical GPCR in both endosomes and Golgi apparatus demonstrating that activation of this receptor in these compartments contribute significantly to the overall cellular cAMP response besides the PM localized receptor activation (Irannejad et al., 2017) (Irannejad et al., 2013). Using engineered cells to express nanobodies as active opioid receptor sensors (MOR and DOR), it is as well shown that they localized to both endosomes and Golgi apparatus in neurons (Stoeber et al., 2018). Interestingly, active ORs localize to Golgi outposts throughout the dendrite too, and they contribute to cellular response to membrane permeable agonists such as morphine. This might explain longer and stronger activation of ORs and pain suppression to some agents through cell penetration and receptor activation on both endosomes and Golgi apparatus. Thus, taking advantage of these nanobodies as biosensors they provide an easy and rapid tool to simultaneously interrogate activation of intracellular GPCR in various organelles without requirement for subcellular fractionation.

An abundance of a particular GPCR in these compartments compared with other organelles and PM is an indication to distinguish between resident GPCRs and trafficking ones. This was the case of the first functional ER GPCR: GPR30. GPR30 is predominantly resident of ER with very low levels in other compartments. This made it possible to determine its functionality and signaling in the cells (Figure 1H); GPR30 binds to estrogen and initiates intracellular calcium mobilization (Revankar et al., 2005). If GPR30 would have been present on other intracellular compartments, it would be very difficult to measure its functionality and activation in ER solely. This is the case for $mGlu_5$, which has been detected abundantly in the nucleus and ER, in

addition to its PM localization. Caged ligand and nanobodies provide a means to differentiate between activation of intracellular $mGlu_5$ versus PM $mGlu_5$. Uncaging the $mGlu_5$ ligand (Glutamate) near the ER with laser results in sustained intracellular increase of calcium (Purgert et al., 2014). However, these results are not fully conclusive in differentiating between the effect of nuclear and ER $mGlu_5$; one can only infer the overall effects of intracellular $mGlu_5$.

ER and Golgi cannot be readily isolated and treated as functional organelles; this obscures the ability to study GPCR signaling in them. Caged ligands provide an efficient method for this hurdle only if the GPCR is largely localized in one intracellular organelle (Audet et al., 2018). Alternatively, one can use FRET-BRET biosensors coupled with various techniques such as caged ligands and cAMP biosensors (Salahpour et al., 2012) conjugated to specific organelle localization signals to feasibly dissect intra-organelle signaling initiated by their respective GPCRs. However, the most reliable method would be to discover the mechanisms of GPCR subcellular localization. For instance, identifying compartmentalization-specific PTMs in GPCRs would allow us to abrogate their ER or Golgi localization (by substitutional mutations), which would be similar to GPCR knockdown in organelle-specific manner. This would enable us to precisely observe the organelle-specific function of GPCRs.

However, receptor localization to these organelles might not necessarily mean a location bias and translocation of a GPCR to these compartments might have different consequences rather than intra-organelle signaling. Unlike classical pathway of β-arrestins-mediated signal termination by endo-lysosomal pathway, it has been shown that cAMP production in response to PTHR activation is further prolonged with β-arrestin interaction. The signal termination of PTHR is initiated with retromer binding and translocation of the endosomal PTHR to the Golgi apparatus (Feinstein et al., 2011). So, in case of PTHR, Golgi translocation serves as a mode of signal termination rather than location biased signaling. Thus, different processes in signal transduction and GPCR biology should be considered for organelle localized/translocated receptors.

Receptor-coupled proteins and their effector modulators are observed in both ER and Golgi. However, detection of these proteins suffers from the same dilemma as GPCRs regarding their residence verse trafficking as part of their translation and PTM routes. Notwithstanding consideration of this obstacle, functional Gα (Godbole et al., 2017), Gβγ(Jamora et al., 1999) (Klayman and Wedegaertner, 2017), and their effector proteins including AC (Yamamoto et al., 1998), PKA (Godbole et al., 2017), PKC (Jamora et al., 1999), PLD1 (Freyberg et al., 2001), PLA2 (Schievella et al., 1995) and ERK (Wainstein and Seger, 2016) are present in the ER and/or Golgi apparatus. This enables a closed system capable of classical GPCR signaling in these two organelles.

In the case of Golgi localized GPCRs, β_1AR has been shown to be present in this compartment and does not translocate from PM upon ligand binding. Activation of Golgi β_1AR triggers Golgi internal G_s-mediated cAMP response contributing to overall cellular β_1AR-mediated cAMP level changes (Figure 1I) (Irannejad et al., 2017). This effect seems to be physiologically relevant since it is recently shown that blocking of Golgi β_1AR inhibits norepinephrine-induced cardiac myocyte hypertrophy (Nash et al., 2019).

ENDOSOMAL GPCRS

GPCR internalization and endosomal integration is a typical consequence of GPCR activation. After ligand binding and initiation of signal, usually the GPCR starts to internalize through endosomes, where it can either go through re-sensitization process and recycle back to the PM, or combine with lysosomes to degrade. This mechanism provides a mode of signal attenuation and re-sensitization (Bahouth and Nooh, 2017).

Increasing evidence shows most GPCRs continue to signal while they are in endosomes (Table 3) (Irannejad and Von Zastrow, 2014). One of the consequences of GRK-mediated GPCR phosphorylation (leading to internalization) is recruitment of β-arrestins. β-Arrestin-mediated GPCR signaling is a widely known G-protein-independent signaling mode for GPCRs (Shenoy and Lefkowitz, 2005). It has been shown that endosomal signaling of GPCRs can occur via the classical G-protein-mediated pathways in line with their initial signal (Irannejad et al., 2013); this has a location bias in the control of downstream moieties leading to different transcriptional programs induced by the endosomal GPCR (Tsvetanova and von Zastrow, 2014). The physiological consequence of endosomal GPCR signaling seems to be in line with their initial signal. For example, NK_1R signaling in endosomes results in sustained nociception and chronic pain contrary to the initial acute pain sensation (Figure 1J) (Jensen et al., 2017).

GPCR	Signaling/Function	Reference
β_2-AR	Differential transcriptional program from PM receptor via Gαs	(Tsvetanova and von Zastrow, 2014)
PTHR	Sustained activation of adenylyl cyclase through Gαs	(Ferrandon et al., 2009)
CaSR	Sustained Gαq-mediated signaling	(Gorvin et al., 2018)
D_1R	Sustained activation of adenylyl cyclase through Gαs	(Kotowski et al., 2011)
LHR	Sustained activation of adenylyl cyclase through Gαs	(Godbole et al., 2017)
NK_1R	Sustained Gαq-mediated signaling	(Jensen et al., 2017)
IGF_1R	β-Arrestin1-mediated ERK phosphorylation	(Lin et al., 1998)
AT_1R	β-Arrestin2-mediated JNK3 activation	(McDonald, 2000)
CXCR4	β-Arrestin2-mediated p38 activation for chemotaxis	(Sun et al., 2002)
F2rl1	Gαq-mediated ERK signaling for hyperexcitability of nociceptors in IBS	(Jimenez-Vargas et al., 2018)

Table 3. Examples of GPCRs with endosomal signaling

Although the location bias of endosomal signaling yields to a different extent GPCR activation compared with that at the PM, this could still be considered a continuation of the same initial signal. Given the different functional output of these two location biases, after ligand binding and receptor activation, there is no separate receptor stimulation or conformational dependent activity. The process takes places linearly, and signaling is sustained through the GPCR life cycle.

However, endosomal GPCR signaling could elicit distinct consequences from its PM signaling (Tsvetanova et al., 2015), a true example of location bias signaling output. A great and elegantly verified example is the case of β_2-AR signaling, in which its activation results in different transcriptional signature when cAMP is generated from PM versus endosomal-generated cAMP (Tsvetanova and von Zastrow, 2014). Authors used optogenetics to induce cAMP generation specifically either from PM or endosomes (and cytoplasm) and proved that location difference in the cAMP generation leads to a biased signaling output and in differential transcriptional signature. Although endosomal-generated cAMP results mainly in CREB phosphorylation and CREB-target gene induction, PM-generated cAMP has little effect on CREB phosphorylation and its related gene expressions (Tsvetanova and von Zastrow, 2014). As such, different agonists of β_2-AR (epinephrine and dopamine) produce signaling bias based on their ability to induce higher endocytosis (epinephrine is strong stimulant of β_2-AR endocytosis).

MECHANISMS OF CELLULAR LOCALIZATION/TRANSLOCATION OF GPCRS

Some GPCRs are primarily localized in intracellular organelles without substantial PM expression, others are simultaneous residents in numerous organelles, whereas others are translocated from PM to these organelles upon ligand binding. Different mechanisms of GPCR subcellular localization/translocation are studied for nuclear GPCRs.

Endogenous nucleus-localized GPCRs such as $\alpha_{1A\ \&\ B}$-AR (Wright et al., 2008), MT2 (Lanoix et al., 2006) and MOR_1 (Khorram-Manesh et al., 2009) are directly targeted to this compartment without appearing at the PM. These GPCRs could originate via two main sources. First one is canonical protein synthesis pathway via translation and maturation in ER and further processing in Golgi apparatus. Terminally mature proteins in ER are able to diffuse laterally to the nuclear membrane and inside the nucleus since the ONM is contiguous with the ER membrane. These proteins are diffused to the ONM where they can get translocated to the INM or inside the nucleus via a diffusion-retention mechanism mostly involving nuclear pore complexes

(Ungricht et al., 2015). Nucleus-targeted proteins which traffic to the Golgi apparatus can proceed via retrograde transport to the ER or be directly transported to the nucleus via nuclear-targeted vesicles (Liu et al., 2018). Since glycosylation is a common PTM occurring in GPCR, which mainly takes place in the Golgi apparatus (Goth et al., 2020), this route is highly probable. Processed proteins in the Golgi can directly travel to the nucleus via nuclear destined vesicles or they are retrograded to the ER network and, as explained above, diffuse to the ONM and subsequently to the INM and inside the nucleus. Either of these routes are the most likely localization mechanism of endogenous nuclear GPCRs. The second possibility for endogenous nuclear GPCR localization mechanism is their nuclear translation directly in the nucleus, similar to reported transcription-coupled translation inside the nucleus (Iborra et al., 2001).

Many GPCRs that are translocated from the PM have nuclear localization signals. Both classical monopartite NLS consisting of several consecutive basic amino acids and bipartite NLS, which contains two separate clusters of basic amino acids, are shown as functional mechanisms for nuclear translocation of GPCRs (Bhosle et al., 2019). These NLS sequences are located in cytosolic portion of GPCRs, either in the intracellular loop (ICL) domains or C terminus of the receptor. In case of F2rl1, a single monopartite NLS in the ICL3 as well as C terminus of the receptor was coordinately involved in nuclear translocation as deletion of either of them resulted in aberrant nuclear transport from PM upon ligand stimulation (Joyal et al., 2014). However, in other cases such as PTAFR, the C terminus of the receptor is strictly required for the nuclear translocation despite presence of monopartite NLS sequence elsewhere, the deletion of which did not result in any nuclear translocation defects (Bhosle et al., 2016), suggesting more complex means of translocations is necessary for the nuclear GPCRs rather than simple regulation via a single NLS (mono or bipartite) (Figure 1G).

NLS sequences in GPCRs follow the common path to the nucleus through their interaction with nuclear transport machinery, mainly importins. Different members of the importin family, including Impα1, Impα3, Impα5, Impβ1, and Imp5, have been shown to mediate nuclear translocation of different GPCRs, and occasionally more than one is required for this process (Bhosle et al., 2019). Other members of the Karyopherin family are also involved in nuclear translocation of GPCRs such as transportin 1 (Favre et al., 2008) (Don-Salu-Hewage et al., 2013) (Di Benedetto et al., 2014).

Other modulators of nuclear transport machinery such as Rab GTPases are also shown to regulate GPCR nuclear localization/translocation. For instance, Rab11 in conjugation with Imp5 is required for nuclear localization of PTAFR (Bhosle et al., 2016). On the other hand, endosomal translocation machinery such as SNX family (sortin nexins) that is essential for endosomal trafficking are major players of nuclear translocation of GPCRs from PM, thus providing the first step in the nuclear translocation from PM by their endosomal sorting. Snx11 is such an example for translocation of F2rl1 (Joyal et al., 2014). Additionally, proteins regulating cytoskeletal rearrangement and their associated signaling such as integrins and Rho kinases are reported to play some roles in GPCR nuclear trafficking (Waters et al., 2006).

Other modulators of GPCR endosomal targeting and mediators of ligand-induced GPCR PTM such as β-arrestins are also involved in this process. *Arrb1* knockdown or substitutional mutations that abrogate Arrb1 binding to oxytocin receptor inhibits its nuclear translocation (Di Benedetto et al., 2014). It is likely that arrestins are involved in the regulation of GPCRs PTM (post ligand-binding) leading to recruitment of factors initiating their nuclear translocation, rather than their direct involvement as a trafficking modulator.

Although modulators and NLS motifs mediate translocation of nuclear GPCRs from PM, they do not explain how they participate in initiating such an action. Most studies focus on deletions in the NLS regions but lack the mechanisms showing how these NLSs are activated; this is probably through ligand-induced PTMs on other regions. These PTMs could either directly or indirectly recruit importins for nuclear translocation or unfold different regions of the receptor exposing the NLS sequences for recognition by importins.

Unfortunately, there is a dearth of data on the mechanisms of localization/translocation to other subcellular organelles; however, bioinformatics can provide clues. ER GPCRs translated in this compartment remain in the ER by lacking necessary domains for PM localization. This could also be the case for Golgi resident GPCRs, which are trafficked there as part of their maturation, however, do not receive further localization signal and reside in the Golgi. On the other hand, mitochondrial GPCRs need a localization signal to target this organelle. Identifying the underlying mechanism of GPCR subcellular localization/translocation would

not only reveal different aspects of their regulation and biology but also would provide a tool to manipulate the system for better understanding of differential effects of these location biases by targeted depletion or accumulation of the receptor in specific compartments.

IMPLICATIONS AND CONCLUSION

Although GPCRs are among the best studied receptors, cell compartment localization can extend biased signaling and adds another layer of complexity in their biology and ensuing cellular and physiological effects. Current efforts in the field try to elucidate potential functions of intracellular GPCRs, without addressing the concept of bias that is enforced by these differential localizations. Investigations into differentially induced signaling cascades in various subcellular organelles by a unique GPCR or the endpoint differential physiological output is required to disseminate the location bias concept. Expanding our understanding of this bias and differentiating it from PM receptors would greatly advance the ability to recognize and design new therapeutics with greater precision. From a translational point of view, it is logical to envisage that targeting a drug to an undesired compartmentalized GPCR can lead to unwanted effects; precise targeting of a specifically localized GPCR would prevent such undesired effects.

Directing a drug to a specific cell location can also enhance efficacy. For example, β-blockers that avoid the β_1AR localized to intracellular compartments elicits attenuated efficacy (Nash et al., 2019); rational design of cell-penetrant blockers would alter the treatment potential. The same scenario applies to NK_1R (Jensen et al., 2017), by which its endosomal targeting can prolong the antinociceptive effect of pain medications. Targeting intracellular Kinin B_1 receptor also shows significant anticancer activity, whereas cell impermeant antagonist do not possess the same effect (Dubuc et al., 2019). The case of Kinin B_1 receptor proves that only cell surface study of GPCRs might result in escaping valid therapeutic targets whose intracellular functions can be pathophysiologically relevant.

Besides the implications of such location bias, we have almost no information regarding general GPCR biology in subcellular organelles including processes such as desensitization and resensitization, potential signaling biases integrated with ing different organelles, and GPCRs post-translation modifications in these compartments. This calls for extensive research in these areas to elucidate the differences in the biology of differentially localized GPCRs.

All in all, the increasing evidence in the importance of intracellular GPCRs and their location bias calls for serious consideration of this concept. A combination of deeper biochemical and biomolecular studies at basic and clinical levels could improve rational design and precision of pharmacologic agents.

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AUTHOR CONTRIBUTIONS

M.A.M.N. conceptualized, outlined, and drafted the manuscript and figure. S.C. reviewed, outlined, edited, and supervised the manuscript. J.C.R. visualized and prepared the figure. All authors approved the final version of the manuscript.

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Annex 2:

Mohammad Ali Mohammad Nezhady, Monir Modaresinejad, Aliabbas Zia, Sylvain Chemtob. HCAR1: Emerging from Lactate, a Tale for a GPCR. In submission process.

HCAR1: Emerging from Lactate, a Tale for a GPCR

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Abstract:

G-coupled protein receptors (GPCR) are the ultimate refuge of pharmacology and medicine as more than 40% of all marketed drugs are directly targeting these receptors. Through cell surface expression, they are the forefront of cellular communication with the outside world. Metabolites amongst the conveyors of this communication are becoming more prominent with the recognition of them as ligands for GPCRs. HCAR1 is a GPCR conveyor of lactate. It is a class A GPCR coupled to $G_{\alpha i}$ which reduces cellular cAMP along with the downstream $G_{\beta\gamma}$ signaling. It was first found to inhibit lipolysis, and lately has been implicated in diverse cellular processes, including neural activities, angiogenesis, inflammation, vision, cardiovascular function, stem cell proliferation, and involved in promoting pathogenesis for different conditions, such as cancer. Other than signaling from the plasma membrane, HCAR1 shows nuclear localization with multiple functions therein, including intra-nuclear signaling, protein translation, DNA damage repair, etc. Although different functions for HCAR1 are being discovered, its cell and molecular mechanisms are yet ill understood. Here we provide a comprehensive review on HCAR1, which covers the literature on the subject, and discussing its importance and relevance in various biological phenomena.

Keywords: HCAR1, Lactate, GPCR, signal transduction, location bias

Introduction:

GPCRs are the largest class of receptors involved in the regulation of essentially every biological process. Their vast array of ligands from different molecules and photons to mechanical and thermal changes enable eukaryotic cells to interact with the environment[42]. Recently, several secondary metabolites which were considered byproducts of metabolism are found to be the ligands of GPCRs, thus regulating cellular physiological processes rather than simply being mere waste of metabolism. Of these we will focus herein on the lactate receptor, HCAR1.

Hydrocarboxylic acid receptors (HCARs) belong to a family of GPCRs activated by intermediate metabolites of energy production pathways[241] (Table 1). HCAR1 is the first member of this cluster, and its gene was first discovered in 2001 through an investigation into the GeneBank database based on previously known GPCR sequences. Its expression was confirmed in pituitary[211]. HCAR1 was initially and still is widely known as GPR81, however, the former nomenclature is the most appropriate based on both the official gene and protein names as well as the International Union of Basic and Clinical Pharmacology (IUPHAR)-recommended nomenclature[242]. Two years after its discovery, *HCAR1* was found to be highly expressed in human adipose tissue; its ligand at that time remained unidentified[59]. Later on, its expression in fetus heart, liver, and placenta was found to be high[243]. Expression-wise, *HCAR1* transcription is itself regulated to a significant extent by metabolic regulators, specifically peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor transcription factor involved in adipocytes differentiation and maintenance[244]; the proximal promoter of *HCAR1* contains a PPAR and retinoid receptor binding site, enabling transcriptional induction of this receptor via PPARγ[244]. STAT3 can also bind the *HCAR1* promoter and activate it through a complex involving Snail/EZH2. This activation is elicited by lactate (the ligand of HCAR1), produced in cancer cells to promote in a positive feedback loop the expression of its own receptor[245].

Using chimeric HCAR1 receptors, *Ge. et al*, showed that HCAR1 is coupled to $G_{\alpha i}$ and inhibits lipolysis in adipocytes[246]. Shortly afterward, lactate was reported to be the endogenous ligand of HCAR1 with an EC_{50} of 1.30 mM, consistent with normal physiologic levels of lactate; in this context, the anti-lipolytic effect of lactate was confirmed to be mediated through HCAR1 signaling[56]. Through structural modeling of the receptor, 4 residues: 1) Arg71 in the transmembrane domain (TM) 2, 2) Arg99 in TM3, 3) Glu166 extracellular domain (EC) 2, and 4) Arg240 in TM6, were found to be responsible for ligand binding of the receptor and critical for its function[196]. Further mutational analysis of R71L, R71A, R71K, R240L, E166A, E166S, and E166I residues abolished the EC_{50} and E_{max} of HCAR1 while mutation of E166 to Asp with a similar negative polarity retained the EC_{50} and E_{max} of HCAR1[196,60]; this amino acid analysis uncovered their specific roles in HCAR1 function, and assisted in identifying 3,5-dihydroxybenzoic acid (3,5-DHBA) as an exogenous specific ligand for HCAR1 with an EC_{50} of 150 μM[247]. Along these lines 3,5-DHBA inhibited lipolysis of adipocytes from wildtype (WT) mice but not from HCAR1 knock-out (KO) mice[247].

HCAR1 in lipolysis

To identify the potential endogenous ligands of HCAR1, *Liu. et al* used extracts from different tissues of rat and porcine to measure the [^{35}S]GTPγS binding in HCAR1 transfected cells and identify its potential ligands. The highest [^{35}S]GTPγS binding activity was observed when using brain extracts. Further fractionation and iterative chromatography followed by NMR structural analysis identified lactate as the endogenous ligand of HCAR1 and stimulation of cells expressing *HCAR1* with lactate resulted in internalization of HCAR1, a well-known feature of GPCR activation. The EC_{50} of lactate in this study was found to be ~5 mM. Pertussis toxin, an inhibitor of $G_{\alpha i}$ was able to inhibit HCAR1 stimulation by lactate consolidating that this GPCR is a $G_{\alpha i}$-dependent receptor (Fig. 1). Lactate also was able to phosphorylate ERK in a HCAR1-dependent manner as well. In this study the highest expression of *HCAR1* was observed in brown and white adipose tissues, which led the authors to examine the effect of this receptor on lipolysis. HCAR1 stimulation in adipose cell lines (3T3-L1), primary human subcutaneous

adipocytes, primary mature adipocytes isolated from rat epididymis, and mature adipocytes from subcutaneous fat of mouse, inhibited glycerol and fatty acid release; this effect was not observed in HCAR1 KO mice[60].

The discrepancy regarding the EC_{50} of HCAR1 continues in another report by *Ahmed., et al.* They found the EC_{50} of HCAR1 around 1.5 mM with GTPγS binding assay and around 7 mM with Ca^{2+}-aequorin assay. Authors found that the injection of sufficient lactate to reach a plasma concentration of 15 mM is enough to reduce plasma Free Fatty Acids (FFA) by more than 50% in WT mice, but not in HCAR1 KO animals. Oddly enough, plasma FFA and glycerol levels did not differ between WT and HCAR1 KO mice upon intensive exercise which increased plasma concentrations of lactate to 10 mM, suggesting that HCAR1 might not be critical in regulating lipolysis; however, the injection of glucose converted to lactate through the glycolysis pathway, did inhibit lipolysis in WT mice but not in the HCAR1 KO mice; this observation was consistent with a reduction in the cAMP level in WT mice following addition of insulin to the 300 mg/dl glucose. Data show that insulin induces cellular uptake of glucose in adipocytes, which is in turn converted to lactate and in an autocrine/paracrine manner inhibits lipolysis through HCAR1 activation. Interestingly, high-fat diet-fed KO mice displayed a reduced weight gain compared to WT animals, inferring that the inhibition of lipolysis through the lactate-HCAR1 axis is at least partially responsible for hypercaloric weight gain[92].

Since HCAR1 was found to inhibit lipolysis, it was suggested as a therapeutic target for dyslipidemia. Niacin as a commonly used drug for dyslipidemia targets HCAR2, but its use is limited due to the cutaneous flushing side-effect[248]. This made HCAR1 even more attractive since its highest expression is detected in adipocytes, suggestive of a potential lower side effect for flushing. Using high-throughput screening *Sakurai. et al,* uncovered a new selective agonist of HCAR1 (Compound 2: (4-methyl-N-(5-(2-(4-methyl piperazin-1-yl)-2-oxoethyl)-4-(2-thienyl)-1,3-thiazol-2-yl)cyclohexane carboxamide), which enabling suppression of lipolysis without the undesired cutaneous flushing (in mice)[249].

HCAR1 in inflammation:

The first link of HCAR1 to inflammation revealed the expression of *HCAR1* during inflammation[250]. In this case a variety of TLR2 and TLR4 stimulants mimicking bacterial and fungal infections were found to inhibit the expression of *Hcar1* in adipose tissues; TLR4 silencing prevented changes in *Hcar1*-expression. Diabetic obese ob/ob mice which also display inflammation, have lower expression of *Hcar1* in their adipose tissues.

In return, HCAR1 was found to impact TLR activity. HCAR1 signaling in mouse and human mononuclear phagocytes was essential in reducing TLR4-induced *IL1β*, *NLRP3* and *CASP1* expression, NF-κB activation, IL1β release, and CASP1 cleavage[61]; moreover, immune regulatory factors such as Aldh1, IDO1 and IL-10 have low expression in HCAR1-KO subjects[62]. This inflammatory amplification resulted in 100% mortality. Likewise, *Hcar1* knock-down in pancreas resulted in severe pancreatitis. Hence, HCAR1 is critical in various tissues to dampen damaging effects of inflammation.

HCAR1 has also an essential role in dendritic cells and macrophages in the colon. HCAR1 regulates colon inflammatory response. Compared to other immune cells, antigen-presenting cells reveal highest expression of HCAR1. HCAR1 KO mice with intestinal inflammation caused by dextran sulfate sodium or by $CD45RB^{hi}CD4^{+}$ T cell transfer show enhanced inflammation. Conversely, silencing of HCAR1 causes increased Th1/Th17 cell differentiation and diminishes the differentiation of regulatory T cells, which intensifies inflammation.

Our lab showed that increased lactate produced by uterine smooth muscle during labor induces HCAR1, highly expressed in myometrium. Interestingly, HCAR1 expression is highest when labor is eminent and its activation can inhibit the expression of key proinflammatory factors such as *Il1b*, *Il6*, *Ccl2*, and *Pghs2*; these changes were not detected in HCAR1-KO animals. Correspondingly, the HCAR1 agonist 3,5-DHBA lowered uterine inflammation and ensuing preterm birth and neonatal mortality[63].

HCAR1 has also been found to participate in vascular inflammation associated with atherosclerosis and oscillatory shear stress (OSS). Lactate preserves HCAR1 expression, and suppresses pro-inflammatory mediators; this process involves vascular cellular adhesion molecule (VCAM)-1 and endothelial-selectin (E-selectin) downregulation[251].

HCAR1 has been shown to regulate endothelial cell-cell interactions. *Yang. et al*, also showed that this VE-Cadherin reduction happens in different organs including liver, lung, and kidney upon bacterial-induced sepsis leading to their vascular permeability; mice lacking HCAR1 have attenuated organ failure during sepsis and survive better in response to bacterial infection[252]; vascular changes were found to be VE-Cadherin-dependent[188]. Overall, this suggests that elimination of HCAR1 during sepsis allows the innate immune system to combat infection. However, if excessive inflammation takes place as reported by *Hoque. et al,* [61], survival is compromised. Overall, compelling evidence indicates that HCAR1 protects organs from excessive and potentially destructive inflammation by suppressing inflammation.

HCAR1 on neural processes

HCAR1 is present in brain and involved in neural processes[253]. Immunohistochemical staining of HCAR1 in mouse brain shows clear reactivity throughout the cerebral neocortex and hippocampus having the highest expression, specifically in pyramidal cells, cerebellar Purkinje cells and astrocytes[253]. HCAR1 in the brain is coupled to Gαi in reducing neural excitability by modulating calcium transition in a dose-dependent manner[64]. Using a mouse model with mRFP expression under HCAR1 promoter, approximately 80% of *Hcar*1-expressing primary neurons are NeuN positive cells[213]. Once again, HCAR1 activation reduces by ~50% excitatory presynaptic current frequency and a similar decrease in firing and spiking frequency; this effect in not seen in HCAR1-KO animals; conversely, HCAR1 KO neurons had around 100% higher basal activity compared to WT neurons indicating this receptor is involved in the inhibition of spontaneous neural activity.

Lactate generation during exercise can also promote brain angiogenesis in a HCAR1-dependent manner[65]. This effect was mediated via ERK1/2 and AKT phosphorylation resulting in VEGF overexpression, and was not observed in other tissues such as skeletal muscle. Several other neurotrophic factors such as *Arc/arg3.1*, *Ngf*, *Bdnf* and *Gdnf*, are induced by HCAR1, and reduces calcium influx in astrocytes upon glutamate damage to protect them from apoptosis[58]. Moreover, in the ventricular-subventricular zone of the

brain and more specifically in the fibroblasts and ependymal cells of the choroid plexus at the dorsal part of the third ventricle[254], lactate treatment or exercise enhances neurogenesis[255].

Our lab showed that HCAR1 is expressed in NeuN-positive neurons of the cerebral cortex and hippocampus in developing mouse brain, with a gradual increase in its expression from post-natal days 5 to 9 where it reached a peak and then showed decreased expression. Interestingly, developing HCAR1-KO pups had delayed brain vascular development in these stages. Concordantly, angiogenic factors including *Vegf-a*, *Ang-1*, *Ang-2,* and *Pdgfbb* showed a similar expression pattern to that of *Hcar1* during these stages, while the anti-angiogenic factor TSP-1 had the reverse expression pattern. The expression pattern of these factors was dependent on HCAR1 signaling by lactate stimulation. We also found that lactate acting through HCAR1 is able to protect the brain and reduce infarct size upon hypoxia-ischemic insult[256]; comparable compromised neural progenitor repair processes were found in HCAR1-KO[257].

HCAR1 in retina

Expression of HCAR1 in retina exceeds that in the hippocampus and cortex[67]. The lactate receptor is present in Müller and retinal ganglion cells (RGC). We showed that HCAR1 in Müller cells regulates intra-retinal vasculature formation during development and pathophysiologic conditions. HCAR1 regulates expression of various angiogenic factors, including Wnt3, Wnt7, Wnt10 and Norrin, in Müller cells. Norrin in turn regulates intra-retinal vasculature development by interacting with co-receptors Fz4, Lrp5 and Tspan12 resulting in downstream Wnt/B-catenin pathway activation[258]. Hence, HCAR1 activation of Müller cells causes Norrin secretion essential for deep retinal vascular network development in normal and conditions resulting in ischemic retinopathy[68]. Consistent with the neovascular effects of HCAR1 activation, the latter raises glucose metabolism and mitochondrial respiration in Müller cells[259].

HCAR1 also participates in neuro-visual development. Essentially, HCAR1 activation by lactate or 3,5-DHBA enhances RGC axonal length and filopodia during embryonic

development[69]; these effects are not detected in HCAR1-KO mice. Accordingly, HCAR1 KO mice have fewer RGC projections in the dorsal lateral geniculate nucleus which contributes to the development of the retino-thalamic pathway[69].

HCAR1 on renal and cardiovascular systems

Using newly generated HCAR1 agonists, labelled AZ1 and AZ2, *Wallenius. et al* studied their effects on lipolysis and the cardiovascular system[212]. Both agonists were anti-lipolytic in WT mice but not in HCAR1 KO mice. AZ1 exerts insulin-sensitizing and antidiabetic effects, which in diet-induced obese (DIO) mice leads to decreased insulin levels. Surprisingly, AZ2 and the potent 3,5-DHBA analog 3-chloro-5- hydroxybenzoic acid (CHBA) cause HCAR1-dependent hypertension in mice and dogs. This hypertension was associated with an increase in the renal vascular resistance, a reduction in renal blood flow and a rise in femoral perfusion, in part due to an increase in endothelin[212]. Increased blood pressure and concomitant rise in endothelin-1 in response to AZ2 was confirmed by others[260].

HCAR1 in the gut

HCAR1 is highly expressed in ghrelin-producing cells in the gastric mucosa[261,262]. Stimulation of HCAR1 with lactate or CHBA in primary gastric mucosal cells inhibits ghrelin secretion. During exercise, as lactate rises ghrelin secretion decreases,[262] potentially suppressing the sense of hunger.

HCAR1 in muscles

Lactate is the notorious source of muscle soreness during intensive exercise. It was mainly believed that high oxygen demand leads to higher glycolysis resulting in lactate over-production as a waste metabolite[263]. But the discovery of HCAR1 sheds some light on its benefits for muscle cells. Lactate induces the accumulation of triglycerides in myotubes in a dose-dependent manner, which could be used as an energy source. Lactate potentially acting through HCAR1 inhibits the cAMP-PKA pathway and consequently reduces

pCREB levels[70]. MCAT is a mitochondrial protein that has a role in maintaining the mitochondrial function and stimulating the synthesis of α-lipoic acid under the control of lipoylation of PDH and αKDH in the mitochondria[264]. Lactate or 3,5-DHBA are able to induce MCAT protein expression. This in turn increases lipoylation and activity of PDH. Since the MCAT level is regulated post-transcriptionally, this implies that lactate promotes the MCAT level at the translational level[70]. These findings suggest that HCAR1 activation by lactate and inhibition of cAMP in myotubes can induce triglyceride accumulation and mitochondrial maintenance in myotubes[70].

The effect of lactate on myotube size was studied in the process of investigating if exercise-generated lactate increases muscle volume through HCAR1[71]. Indeed, HCAR1 was found to be highly expressed in myotubes (compared to myoblasts), and lactate as well as 3,5-DHBA significantly increased myotube diameter, along with HCAR1 downstream phosphorylation of MEK1/2, ERK1/2, and p90RSK.

HCAR1 and microbiota-produced lactate

A significant source of exogenous lactate arises from lactate-producing microbiota, particularly those residents in the intestinal system such as Lactobacillus[265]. Microbiota-generated lactate provides a communication mode between the host and its flora. This symbiotic relationship reveals that lactate from intestinal microbiota play an important role in intestinal stem cell (ISCs) mediated epithelial regeneration via HCAR1 signaling. The Paneth cells which support ISC proliferation and stromal cells express HCAR1. Specifically, probiotics such as Bifidobacterium and Lactobacillus spp., or alternatively feeding lactate orally to mice increases number of ISCs, Paneth cells, goblet cells, and crypt height in the small intestine, through a Lgr5-coupled Wnt/β-catenin pathway[265]. These effects could not be observed in HCAR1 KO mice. Similarly, lactate exposure or probiotic usage during chemo- or radiation therapy protected mice from damage to gut[187]. Collectively lactate from microbiota is shown to lead to development, differentiation, and proliferation of the ISCs through the Wnt pathways by activating HCAR1 signaling.

Other than intestinal stem cells, lactate-producing microbiota can regulate function of mesenchymal stem cells (MSC) and affect hematopoiesis and erythropoiesis through

HCAR1 signaling; along these lines, HCAR1-KO mice exhibit lower density of bone marrow-derived MSC[266]. Moreover, hematopoiesis in general is positively regulated by HCAR1; this effect is partly dependent upon MSC-derived stem cell factor[266].

HCAR1 in cancer

The Warburg effect which ensues in increased lactate concentration, is regularly observed in various cancer cells and suggested to participate in tumor progression[267]. Lactate concentration in the tumor niche can increase up to 50 times its physiological concentration in circulation[268]. It has been shown that *HCAR1* is highly expressed in many cancer cell lines and 94% of tumors resected from pancreatic cancer patients. When lactate is the primary energy source pancreatic cancer cells growth is largely HCAR1-dependent, such that depletion of HCAR1 curtails tumor growth rate and metastasis. Interestingly, as cancer cell growth progresses HCAR1 expression rises concomitantly[72]. HCAR1 overexpression is also seen in tissue samples of breast cancer patients as well as primary breast cancer cells. Silencing of HCAR1 in breast cancer cells reduces their viability[73]. Proliferation and survival of cancer cells is dependent upon activation of the PI3K/Akt-CREB axis leading to increased expression of angiogenic factors including AREG, PDGF-AA, SERPIN E1, SERPIN F1, uPA, and VEGF. HCAR1 depletion of tumor cells limits their proliferation and metastasis[74].

HCAR1 regulates a number of pathways involved in cancer progression and malignancy. HCAR1 signaling enhances DNA repair in cervical cancer cells treated with chemotherapeutic agents, increasing their survival[75]. HCAR1 stimulation leads to PKC activation and increased BRCA1 and NBS1 expression concomitant with their translocation to the nucleus along with DNA-PKcs, all of which are major players in DNA repair[168]. In squamous cell tumors, HCAR1-depletion limits cell proliferation and tissue invasion as it increases apoptosis; these changes occur concomitant to decreased Phosphofructokinase 1 (PFK-1) potentially reducing glycolysis rate and increasing expression of Translocase of the outer mitochondrial membrane 20 (TOMM20) thus likely inducing oxidative phosphorylation[76]. Besides PFK-1, HCAR1 depletion in breast cancer

cell line downregulates other glycolytic enzymes including Hexokinase 2 (HK2) and Lactate dehydrogenase A (LDHA)[189], which leads to reduced ATP production.

HCAR1 has also been shown to confer resistance of hepatocellular carcinoma cells to ferroptosis-inducing agent, namely Sorafenib[77]. Ferroptosis is a form of programmed cell death characterized by membrane damage due to lipid reactive oxygen species (ROS)[269]. HCAR1 knock-down leads to stearoyl-coenzyme A (CoA) desaturase-1 (SCD1) down-regulation and increases intracellular ROS levels, which ultimately induces ferroptosis[77].

Lactate via HCAR1 also regulates immune checkpoints in cancer progression. HCAR1-induced inhibition of PKA activity leads in turn to suppression of TAZ-TEAD transcriptional factor activation which induces the expression of PD-L1 - a known immunosuppressor involved in tumor immune escape[78]. Lactate stimulation of HCAR1 also reduces IFNα production in plasmacytoid dendritic cells which induces an anti-tumor immune response, and further assists the immunescape of tumors[270]. Accordingly, HCAR1 KO animals have higher tumor-infiltrating T cells and high-expressing MHC II-dendritic cells, indicating that HCAR1 signaling in immune cells suppresses their anti-tumor activity[164]. Beside these mechanisms, HCAR1 also assists radiotherapy-induced immunosuppression through actions on myeloid-derived suppressor cells (MDSC) in pancreatic cancers. In this case, HCAR1 stimulation in MDSCs promotes Akt/mTOR/HIF-1α/STAT3 pathway resulting in augmented immunosuppressive phenotype of MDSC cells. In addition to these effects of lactate on immune suppression, HCAR1 in MDSCs cells upregulates *S100a8*, *S100a9*, *Arg1*, and *Mmps*, *Nos2,* and *Vegf* which in turn promote tumor progression[271].

The detailed mechanistic involvement of HCAR1 through all these diverse activities in cancer progression was elusive, especially number of studies have reported that inhibition of lactate cellular uptake, abrogates HCAR1 signaling[75,168,169], and this adds to the conundrum. We recently showed that HCAR1 has a location-biased activity, through which its modulates different aspects of its cancer-promoting features[272]. GPCRs are not necessarily only a plasma membrane-localized receptors, despite the portraited classical model of the receptor family. GPCRs are found in all the intracellular membranous organelles, and even inside the nucleus, and location bias in GPCR biology refers to the

differential activity of the receptor from within those organelles[42]. We showed that one-third of the cellular HCAR1 pool resides on the nuclear membrane and inside the nucleus as well. HCAR1 phosphorylation at its c-terminus mediates its nuclear localization by involving the ICL3 domain of the receptor. HCAR1 from the inner nuclear membrane is able to induce canonical $G_{\alpha i}$ and $G_{\beta\gamma}$ intranuclear signaling leading to the nuclear EKR1/2 and AKT activation. Additionally, HCAR1 inside the nucleus forms different protein complexes regulating various aspects of cellular function. It interacts with proteins involved in ribosomal biogenesis and protein translation and promotes their rate. Interestingly, nuclear HCAR1 interacts with different proteins involved in DNA damage, including H2AX; and cells lacking the nuclear HCAR1 (but harboring the plasma membrane HCAR1) were deficient in DNA damage repair[272], a previously reported properties of HCAR1 with unclear mechanism. Additionally, nuclear HCAR1 interacts with chromatin remodeling complexes and directly regulates gene transcription as a transcriptional co-regulator, and in line with cancer malignancy, it directly binds and promotes the expression of genes involved in migration which are needed for metastasis. Our study showed how the nuclear localization of HCAR1 is able to exacerbate different cancer features through various mechanisms. Overall, HCAR1 is able to mediate several different processes both in cancer cells and immune cells to enable cancer growth, metastasis, immune escape, and therapy resistance (Fig. 2).

Discussion:

For decades lactate has been the topic not only of metabolic end product but also a factor causing a variety of physiologic functions (Fig. 3). HCAR1 opened widely this topic, by conveying many effects of lactate[84]; HCAR1 seems to account for the majority of lactate's physiological and cellular roles. Lactate is produced by virtually every cell through glycolysis and is present in every tissue; immune cells convert to anaerobic respiration upon activation to produce lactate;[273] astrocytes by default rely heavily on glycolysis and lactate for energy production[274]. Phenomena like these further point to the potential role of HCAR1 in regulating various aspects of cell biology and making it an attractive receptor for metabolic-related regulations.

While observing HCAR1 in inhibiting lipolysis in adipose tissue, hopes for targeting this receptor to treat dyslipidemia has been triggered. Currently, HCAR2 is the main therapeutic target of dyslipidemia since it can also inhibit lipolysis in the adipose tissue. However, HCAR2 agonism has a major side effect of "flushing" due to its expression in the Langerhans cells and production of prostaglandin D2 therein; this side effect is not observed upon HCAR1 activation. Pre-clinical work is ongoing in this direction.

HCAR1 expression in tissues other than adipose is relatively low. Consistent with this observation, HCAR1-KO animals are viable, fertile under normal conditions and do not display visible anomalies. A number of transient minor developmental delays have been reported in HCAR1 KO mice, but over time these are compensated by unknown mechanisms. Along these lines, the role of HCAR1 in regulating neural function is undeniable, yet KO animals in isolation do not display significant neural dysfunction. This may suggest that HCAR1 is not a major player during healthy conditions, but becomes a significant factor under pathological processes such as inflammation, oxidative stress and cancer. In this context, the unexplored role of HCAR1 in neurodegenerative disease and aging could be an interesting avenue to investigate.

Another hurdle related to exploration of this receptor applies to the lack of antagonist, which would provide useful tools to study HCAR1 functions. Unfortunately, several studies wrongfully used 3-hydroxy-butyrate acid (3-OBA) as an antagonist of HCAR1 without any prior experimental validation[275]. Importantly, 3-OBA is the ligand of HCAR2 and although in a number of experiments administration of 3-OBA had a similar effect to that of HCAR1 KD or KO, this could be due to the potential counteracting signaling mechanism of HCAR2[275]. Regrettably reports that have used 3-OBA erroneously conclude a role for HCAR1.

Finally, an interesting aspect regarding HCAR1 biology applies to mystic crosstalk with MCT transporters, in which inhibition of lactate cellular uptake, reduces HCAR1 effects[75,168,169]. This begs the question on how intracellular lactate affect signaling of a GPCR presumably at the cell surface? Our study on the nuclear location bias of HCAR1 could easily explain this crosstalk with MCT. We showed a larger part of HCAR1 effects comes from its nuclear pool by its involvement in different processes rather than just its

function as a signaling module. These effects are modulated by the intracellular lactate level, which are either produced within the cell through the glycolysis or they are imported inside the cell by the MCTs, and this further elucidates this crosstalk. MCTs on the nuclear membrane[276,277] could further import this metabolite inside the nucleus and act on the inner-nuclear pool of HCAR1. Yet there are many different aspects of HCAR1 function and interactions in the nucleus that needs further investigation.

In summary, discovery of HCAR1 has opened a vast understanding on lactate function. The body has utilized lactate to compensate for events that compromise cell survival. Lactate has been coined as the 'fulcrum of metabolism'[278]. Lactate is an energy-derived metabolic intermediate, but it is also a signaling ligand[279].

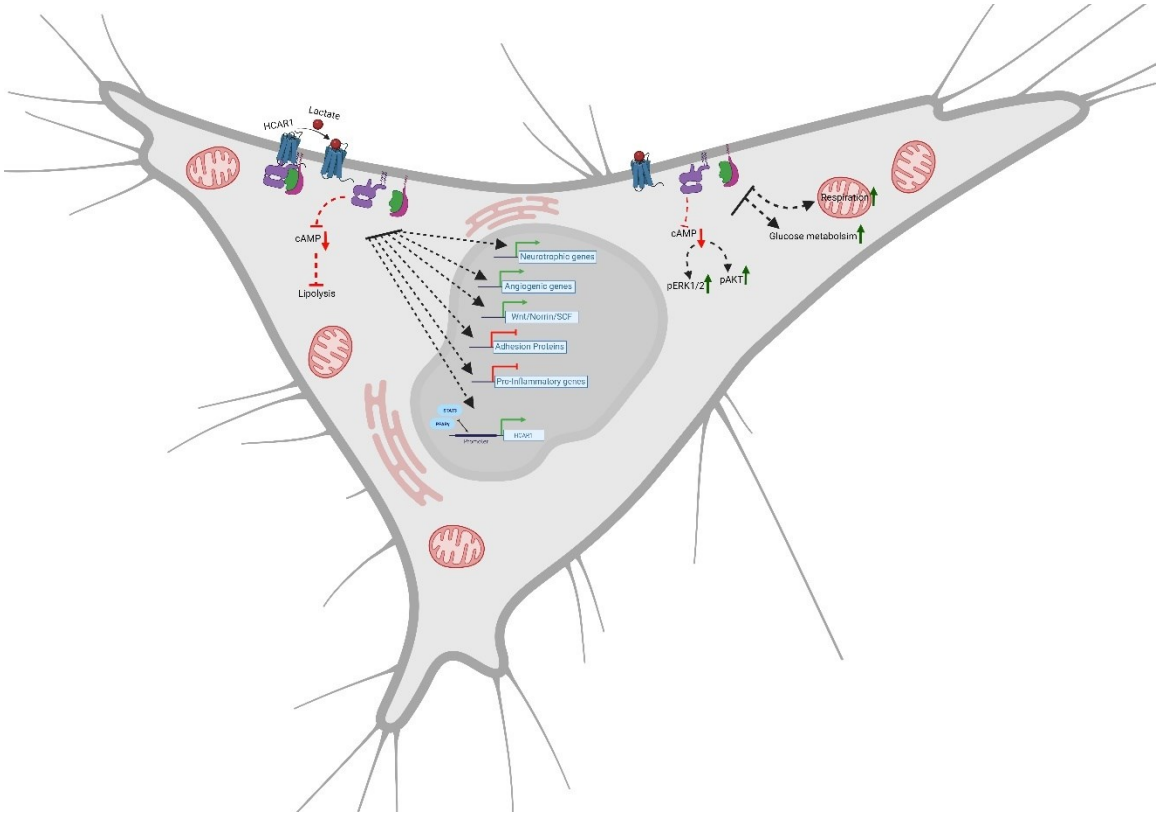

Fig. 1:

HCAR1 signal transduction in the cell. HCAR1 downstream signaling and consequences of its activation are depicted in this graph. HCAR1 is $G_{\alpha i}$ and $G_{\beta\gamma}$ coupled receptor and its activation reduces cellular cAMP level. ERK1/2 and AKT are known downstream effectors of HCAR1. Lactate-mediated activation of HCAR1 triggers glucose metabolism and mitochondrial respiration and inhibit lipolysis. This signaling can also induce or inhibit the expression of genes illustrated in the nucleus (largely via unknown downstream signaling).

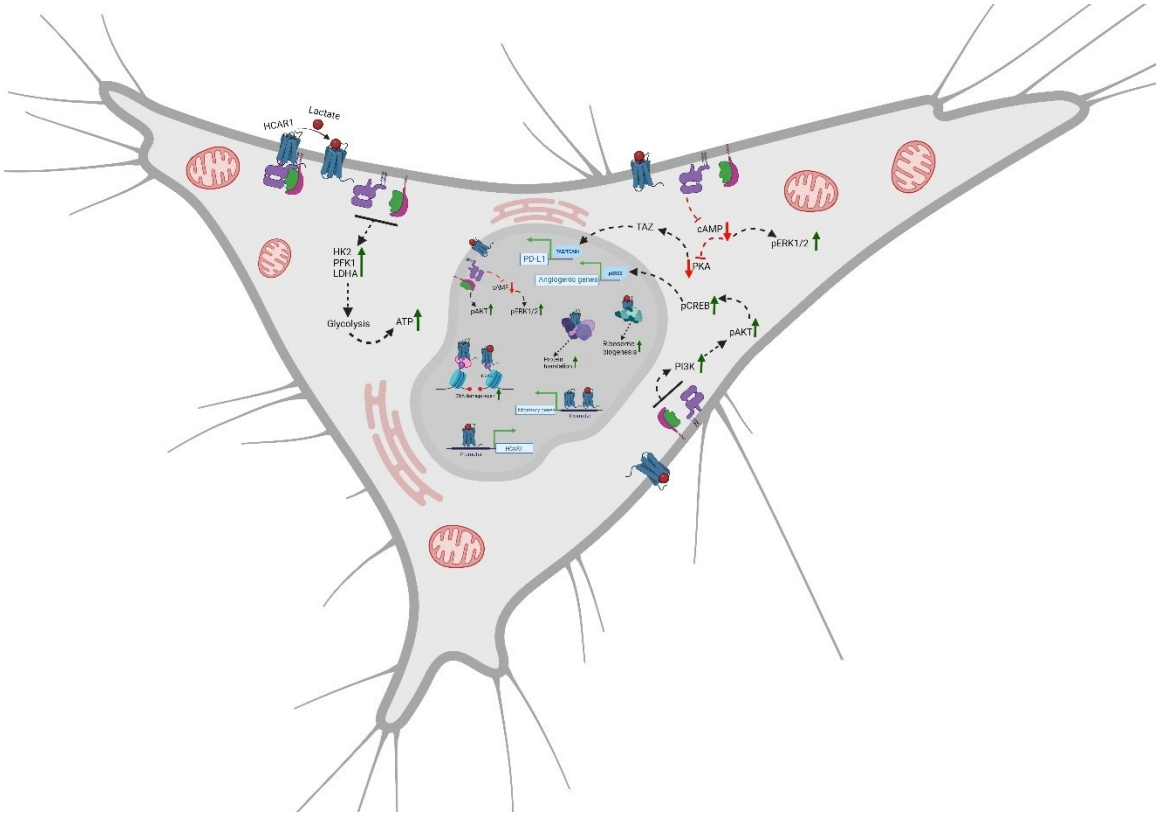

Fig. 2:

HCAR1 activity in cancer cells. HCAR1 signaling in cancer cells leads to activation of different downstream effectors known to promote cancer progression and malignancy. Furthermore, it can promote these features via nuclear location bias as well, both by intra-nuclear signal transduction and additional functions including protein-protein interaction to enhance translation, DNA damage and etc.

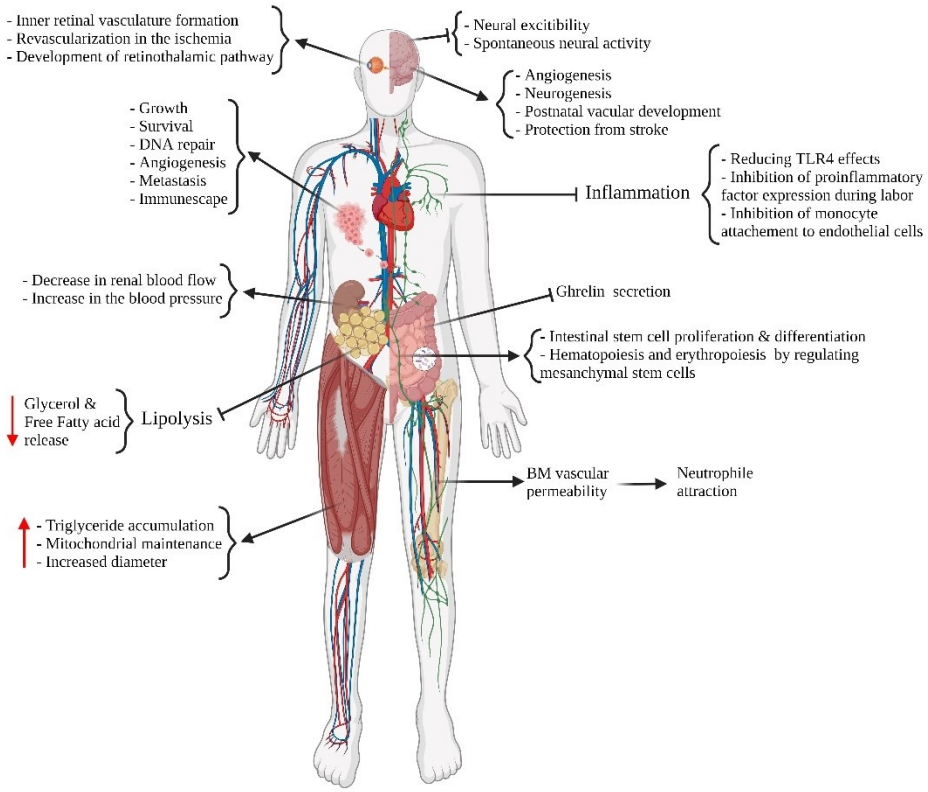

Fig. 3:

Physiological effects of HCAR1 signaling. HCAR1 has various roles in many organs and tissues. The effects of HCAR1 signaling in physiology and pathology at different organs/tissues are depicted in this figure.

Table 1: Metabolite sensing GPCRs

Receptors	Endogenous Ligand	Transducer	Tissue, Cells expression & Key Functions	References
GPR81/HCAR1	Lactate	*Gαi*	Adipocytes: inhibition of lipolysis Ghrelin cells: inhibition of ghrelin secretion Immune cells: anti-inflammatory	([247]) Liu et al., 2012 ([280]) Koyama et al., 2016 ([256]) Chaudhari et al, 2022. ([68]) Madaan et al, 2019.
GPR109A/HCAR2	β-hydroxybutyrate Butyrate	*Gαi*	Adipocytes: inhibition of lipolysis Immune cells: anti-inflammatory Langerhans cells (LCs): Induction of prostaglandin secretion Gastrointestinal tract: maintains the intestinal integrity	([281]) Plaisance et al., 2009 ([282]) Gambhir et al., 2012 ([283]) Maciejewski-Lenoir et al., 2006 ([284]) Gong et al., 2021
GPR109b/ HCAR3	β-hydroxyoctanoate	*Gαi*	Adipocytes: inhibition of lipolysis	([285])Ahmed et al., 2009
GPR84	Decanoic acid, undecanoic acid, lauric acid	*Gαi*	Adipocytes: inhibition of adiponectin secretion Immune cells: Amplifies LPS-stimulated *IL-12 production in* macrophages	([286]) Nagasaki et al., 2012 ([287])Suzuki et al., 2013
GPR91/Sucnr1	Succinate	*Gαi/ Gαq*	Adipocytes: inhibition of lipolysis Dendritic cells (DCs); regulation of proinflammatory function of DCs Liver: migration of Langerhans cells to draining lymph node	([288]) McCreath et al., 2015 ([289]) Rubic et al., 2008 ([54]) Sanchez et al, 2022
GPR40/FFAR1	Unsaturated fatty acids, omega-3 fatty acids, omega-6 fatty acids	*Gαq*	Endocrine pancreas: induction of insulin secretion from pancreatic β cells Enteroendocrine cells: Induction of GLP-1 and GIP secretion	([290]) Kebede et al., 2008 ([291]) Hauge et al., 2015
GPR43/FFA2	Butyrate, Acetate, Propionate,	*Gαi/ Gαq*	Immune cells: Anti-inflammatory Adipocytes: inhibition of lipolysis Enteroendocrine cells: Induction of GLP-1 secretion Endocrine pancreas: Induction of insulin secretion from pancreatic β cells gut epithelium: gut homeostasis	([292]) Maslowski et al., 2009 ([293]) Park et al., 2016 ([294]) McNelis et al., 2015
GPR41/FFA3	Butyrate, Acetate, Propionate,	*Gαi*	Immune cells: anti-inflammatory Enteroendocrine cells: Induction of GLP-1 secretion Endocrine pancreas: Inhibition of insulin secretion Dendritic cells (DCs); DC maturation	([295]) Park et al., 2019 ([296])Tolhurst et al., 2012 ([297]) Veprik et al., 2016

GPR120/FFAR4	Unsaturated fatty acids, omega-3 fatty acids, omega-6 fatty acids	*Gαi/ Gαq*	Immune cells: anti-inflammatory Endocrine pancreas: Inhibition of SST secretion Stomach: inhibition of ghrelin and SST secretion	([298]) Ren et al., 2019 ([299]) Oh et al., 2010 ([300]) Stone et al., 2014 ([301]) Gong et al., 2014
GPR119	N-oleoylethanolamide, N-palmitoylethanolamine	*Gαs*	Endocrine pancreas: Induction of insulin and glucagon secretion Enteroendocrine cells: Induction of GLP-1 and GIP secretion	([302]) Flock et al., 2011 ([303]) Lan et al., 2012
TGR5/GPBAR/GPR131	Lithocholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid	*Gαs*	Immune cells: suppression of macrophage functions by bile acids Enteroendocrine cells: Induction of gut hormone secretion	([304]) Fiorucci et al., 2018 ([305]) Goldspink et al., 2018
GPR142	L-Tryptophan, L-Phenylalanine	*Gαi/ Gαq*	Endocrine pancreas: induction of insulin secretion from pancreatic β cells Enteroendocrine cells: Induction of gut hormone secretion	([306]) Wang et al., 2016 ([307]) Rudenko et al., 2019
GPR35	kynurenic acid, 2-oleoyl-LPA, cGMP, DHICA, Reverse T3, CXCL17	*Gαi/ Gα13*	Immune cells: anti-inflammatory Enteroendocrine cells: Induction of CCK secretion CNS: neuronal excitability and nociception	([308]) Sharmin et al., 2020 ([309]) Quon et al., 2020 ([310]) Guo et al., 2008
FPR1-2	N-formyl-methionine, N-formyl-Met-oligopeptides	*Gαi/ Gαq*	Immune cells: Induction of immune-cell chemotaxis and cytokine release	([311]) Chen et al., 2017
CaSR/ GPRC2A	L-phenylalanine, L-tryptophan, L-histidine, L-alanine, L-serine, L-proline, L-glutamic acid, L-aspartic acid, Gd^{3+}, Ca^{2+}, Mg^{2+}, S-methylglutathione, γGlu-Val-Gly, glutathione, γGlu-Cys, spermine, spermidine, putrescine, PO_4^{3-} and SO_4^{2-}	*Gαi/ Gαq Gα12/13*	Immune cells: anti-inflammatory Parathyroid: synthesis and secretion of PTH	([312]) Iamartino et al., 2018 ([313]) Centeno et al., 2019
GPRC6A	Arg, Lys	*Gαi/ Gαq*	Endocrine pancreas: induction of insulin secretion from pancreatic β cells	([314]) Pi et al., 2011
TA1R/TAAR1	Tyramine, β-phenylethylamine, octopamine, dopamine, 3-iodothyronamine	*Gαi/ Gαq*	CNS: regulating neurotransmission in dopamine, norepinephrine, and serotonin neurons	([315]) Revel et al., 2011
GPR65/PSYR	Protons	*Gαs*	Immune cells: phagocytosis-mediated intracellular bacteria clearance Gastrointestinal tract: promotes intestinal mucosal Th1 and Th17 cell differentiation and gut inflammation	([316]) Lee et al., 2021 ([317]) Lin et al., 2022

GPR68/PGR1	Protons	*Gαi/ Gαq*	Bone: Osteoclast differentiation Brain: neuroprotective role	([318]) Yang et al., 2006 ([319]) Wang et al., 2020
GPR4	Protons	*Gαi/ Gαq* *Gα12/13*	Endothelial cells: Increases Endothelial Cell Adhesion, promotes acid-mediated angiogenic capacity of endothelial progenitor cells	([320]) Krewson et al., 2020 ([321]) Ouyang et al., 2021
GPR132	Protons	*Gαs*	Immune cells: influence migration of macrophages and modulates inflammation	([322]) Osthues et al., 2020

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Annex 3:

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OPINION
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3-OBA Is Not an Antagonist of GPR81

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HCAR1, commonly known as GPR81, is a G-Protein Coupled Receptor (GPCR) and has been deorphanized more than a decade ago. Lactate is the endogenous ligand of GPR81, and many high-potential pharmacological agonists have been developed for its activation. Although some reports mention using 3-hydroxy-butyrate acid (3-OBA) as an antagonist of GPR81 thus inferring GPR81-mediated signaling mechanisms for their observed effects, there is no evidence for such an antagonistic activity in 3-OBA against GPR81. In fact, to this date, there is no report for an antagonist or an inhibitor of GPR81 at all, whereas 3-OBA is a ligand for HCAR2 (GPR109A) (Blad et al., 2011).

In a recent paper, Chen et al. used 3-OBA as the antagonist of GPR81 in combination with metformin and PD-1/PD-L1 blockade to demonstrate enhanced antitumor efficacy of later compounds (Chen et al., 2021). Their whole hypothesis is based on inhibition of GPR81 signaling that would increase the efficacy of metformin and PD-1/PD-L1 inhibition. The only method they used is inhibition of GPR81 signaling by 3-OBA to test their hypothesis. They attributed all the observed effects such as cell growth, metabolism, and T cell activation to GPR81 signaling. All of their conclusions are scientifically unfounded as 3-OBA is not a proven antagonist of GPR81 and since they have not used any other experiments to validate the GPR81-mediated effects (e.g., RNAi, knockout/knockdown). In another recent paper by Yang et al. (2021), authors used 3-OBA as an antagonist for GPR81 to investigate the role of this receptor in lactate-induced HMGB1 acetylation. Initially, they show that lactate is able to promote HMGB1 acetylation. They also show that this acetylation is independent of the lactate acidity since there is a similar HMGB1 acetylation when cells are treated with sodium lactate. GPR81 as the main known receptor for lactate signaling is their first guess to induce HMGB1 acetylation, and to this end they used 3-OBA as an antagonist for GPR81. They observed that prior treatment of cells with this putative antagonist reduces lactate-mediated HMGB1 acetylation. Authors have used this assumption in a previous publication (Yang et al., 2020) as well and mistakenly draw conclusion that TNFα production upon lactate treatment in LPS-stimulated macrophages is mediated by GPR81 signaling. However, based on GPR81 knockdown used in their previous study, findings using 3-OBA are incongruent. Moreover, these authors do not provide a reference for their rationale on using 3-OBA as GPR81 antagonist in both papers. Importantly, the use of 3-OBA as an antagonist of GPR81 is not limited to these authors. Khatib-Massalha et al. also used 3-OBA to inhibit GPR81 and indicated its pharmacological inhibition decreases the effect of lactate on neutrophil mobilization from bone marrow (Khatib-Massalha et al., 2020). However, alongside their so-called pharmacological inhibition of GPR81, they used GPR81 knockout animals to further prove their points which keeps their conclusion intact. Lee et al. as well used 3-OBA as the inhibitor of GPR81 and suggested various factors are expressed through GPR81-mediated signaling which are important in promoting intestinal stem cell-mediated epithelial development (Lee et al., 2018). Although they too used gene knockout mice to ascertain their conclusion, findings applying to other experiments inconsistently relied on 3-OBA as an inhibitor. The latter two papers refer to Shen et al. for their use of 3-OBA as a GPR81 antagonist (Shen et al., 2015). But Shen et al. in turn refer to a review paper for their claim on 3-OBA being the antagonist of GPR81 (Blad et al., 2011). Importantly, it should be underlined that there is no suggestion in the entire review paper to indicate that 3-OBA inhibits GPR81 (HCAR1). As a matter of fact, the review

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paper clearly indicates that no antagonist is known for HCAR1 to date. Notably, it should be emphasized that the review paper points to signaling activity of a different HCAR, precisely HCAR2 (GPR109A) for which 3-OBA is an agonist.

As mentioned above, some of the minor effects seen by 3-OBA are consistent with GPR81 knockdown or knockout experiments; however, this does not establish 3-OBA as an antagonist of GPR81. 3-OBA could simply act as a modulator of GPR81. However, current experimental evidence does not support such an activity.

Altogether, there is no experimental proof that 3-OBA acts as an antagonist of GPR81; to date, we are not aware of a specific antagonist of GPR81. Accordingly, this compound should not be used to investigate GPR81-selective signaling. Otherwise, one can be misled by the role of HCAR1 (GPR81) versus that of HCAR2 (GPR109A) for which 3-OBA is an agonist ligand (Offermanns et al., 2011).

AUTHOR CONTRIBUTIONS

MM conceptualized and drafted the manuscript; SC conceptualized and supervised.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annex 4:

Data Sets

Data Set I: BioID- mass spectrometry data for proteins interacting with N-HCAR1

Data Set II: ChIP-seq data for genes interacting with N-HCAR1

Data Set III: RNA-seq data for genes regulated by HCAR1 and N-HCAR1

- Data sets are excel files, provided separately from the thesis file.