# SIGNALING NETWORKS AND INTRINSIC MECHANISMS THAT REGULATE THE PRODUCTION OF RIBOSOMES IN TRANSFORMED CELLS

## Ph.D. THESIS

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Que el trabajo de tesis titulado **"Signaling networks and intrinsic mechanisms that regulate the production of ribosomes in transformed cells"**, presentado por **Dª NATALIA FERNÁNDEZ PAREJO** para optar al Grado de Doctor por la Universidad de Salamanca, ha sido realizado bajo nuestra dirección en el Centro de Investigación del Cáncer de Salamanca (USAL/CSIC). Considerando que cumple con las condiciones necesarias, autorizo su presentación a fin de que pueda ser defendido ante el tribunal correspondiente.

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# ABSTRACT

Eukaryotic ribosomes contain four rRNAs and ~80 proteins that are assembled in two subunits of unequal sizes, the 40S and 60S subunits. The formation of the two subunits starts with the synthesis by RNA polymerase I of a pre-rRNA transcript, and is followed by the formation of the initial 40S and 60S pre-ribosomes. Those complexes then enter two separate multi-step maturation pathways that happen in the nucleolus, nucleoplasm and cytoplasm. The whole process of ribosome formation is assisted by more than 200 ribosome biogenesis factors (RBFs) that mediate the modification, folding and processing of rRNA precursors, and the incorporation of ribosomal proteins. Because ribosome synthesis is hyperactivated in many cancers there is much interest in understanding how transformed cells deregulate this process. One aspect to ascertain is which signaling pathways activate ribosome synthesis in different cell contexts. Another important aspect is to unveil all the events within the human ribosome synthesis pathway that are subject to regulation. This thesis addressed those two aspects in two separate projects. The first project focused on the study of a VAV2-mediated signaling pathway that activates ribosome production in human keratinocytes. We dissect the elements that participate in the pathway, identify the step of ribosome synthesis that is activated, and provide evidence of its deregulation and amenability to inhibition in squamous cell carcinoma. The second project was devoted to the characterization of a set of so-far undescribed intermediates of 40S subunit maturation in human cells. We define in which steps of the 40S pathway they intervene, identify a group of RBFs associated to them, and perform loss-of-function analyses to characterize their functions. Our results reveal a process that exhibits regulatory features and is required for proper production of 40S ribosomal subunits.

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# FIRST CHAPTER

## VAV2-MEDIATED SIGNALING ACTIVATES RIBOSOME SYNTHESIS

# INTRODUCTION I

# INTRODUCTION I

## 1. RHO PROTEINS

### 1.1. The family of Rho GTPases

Rho GTPases are small G proteins that play central roles in the regulation of cytoskeletal remodeling, cell polarity, migration, proliferation, differentiation, and survival [1,2]. These proteins constitute a large family of over 20 members. Among them, RhoA, Rac1, and Cdc42 GTPases are the most extensively characterized in terms of biochemical properties, molecular regulation, and biological functions.

Rho GTPases behave as molecular switches that cycle between an inactive conformation when bound to GDP and an active conformation when bound to GTP. The switch between both states is coordinated by two types of regulators: guanosine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs promote the activation of GTPases by catalyzing the exchange of GDP for GTP. In contrast, GAPs promote the hydrolysis of GTP to GDP, which returns the GTPase to the inactive state. Rho GTPases in their GTP-bound form interact with proximal effectors to transduce extracellular signals into appropriate cellular responses (Figure 1). Over 60 Rho GTPase proximal effectors have been characterized so far, which include: i) regulators of the cytoskeleton, ii) transcription factors, iii) serine/threonine kinases, iv) tyrosine kinases, and v) phospholipid kinases, among others [3]. Each Rho GTPase binds to a particular set of effectors, which is one of the key determinants of the functional idiosyncrasy of each Rho family member [4].

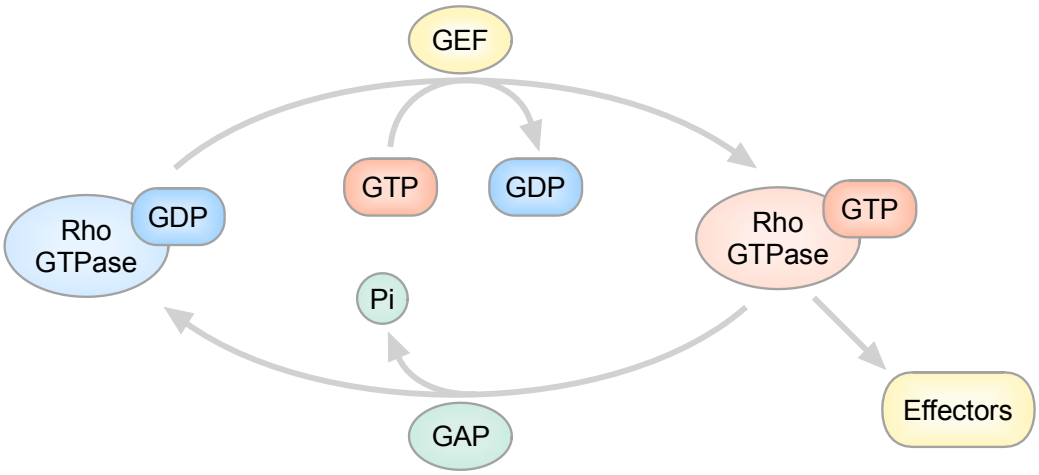



**Figure 1. Rho GTPases function as molecular switches**. Scheme depicting the main elements and mechanisms that regulate the activity of Rho GTPases. Pi, inorganic phosphate.

### 1.2. Rho GDP/GTP exchange factors

Rho GEFs constitute a large and heterogeneous family of more than 70 members that can be classified according to their catalytic domain in Dbl and Dock subfamilies. These proteins exhibit extensive diversity in terms of expression pattern, subcellular location, regulatory mechanism, Rho GTPase specificity, the scaffolding of downstream effectors, and other non-catalytical functions (for a review, see [5]).

Although the function of Rho GEFs is tightly regulated to maintain cell homeostasis, they are frequently found hyperactivated as a consequence of GEF activity upregulation (either by increased expression or mutation) in several pathologies, including cancer [3,5,6]. One group of GEFs that has been described to play relevant roles in cancer is the Vav family.

# 2. THE VAV FAMILY

## 2.1. General characteristics of the Vav GEF family

The Vav family comprises a group of tyrosine-phosphorylation-regulated GEFs (Vav1, Vav2, and Vav3) that catalyze the activation of Rac1 and RhoA subfamily members. While Vav2 is the most ancient protein of the family, Vav1 and Vav3 emerged later in evolution from a common ancestor (Figure 2) [7]. They also exhibit different tissular expression patterns. In humans, Vav1 is primarily detected in hematopoietic cells, whereas Vav2 and Vav3 display ubiquitous expression patterns.

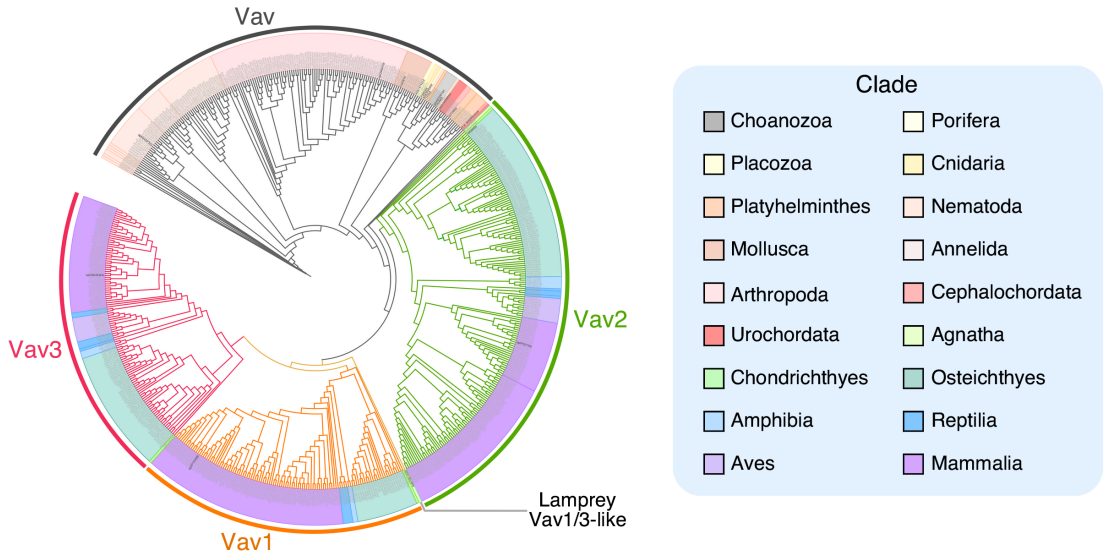



**Figure 2. Evolution of Vav family proteins.** Phylogenetic tree showing the classification of the Vav family members. Node colors indicate if the protein belongs to the single Vav family group (gray color) or the vertebrate Vav1 (orange color), Vav2 (green color) or Vav3 (purple color) branch. The color code used for each phylogenetic clade is shown on the light blue box (right). Figure taken from Rodríguez-Fdez et al., 2019 [7]

## 2.2. Structure and regulation of Vav proteins

Vav family proteins share an evolutionarily conserved structure composed of an N-terminal calponin-homology (CH) domain, an acidic (Ac) region, the catalytic Dbl-homology (DH) domain, a pleckstrin-homology (PH) region, a C1 subtype zinc finger (ZF) domain, a proline-rich region (PRR), and a NSH3-SH2-CSH3 cassette (Figure 3). The DH domain is part of the DH-PH-ZF cassette, which is involved in the catalytic activity of Vav proteins [8]. The activation cycle of GEFs depends on phosphorylation events to promote changes in

protein conformation. In a non-phosphorylated state, the CH-Ac and CSH3 domains establish intramolecular interactions with the catalytic DH-PH-ZF core, sustaining a "closed" and inactive conformation. The phosphorylated state is mediated by protein tyrosine kinases (PTKs), and induces an "open" and active conformation of the Vav protein that allows the catalytic activity of the DH-PH-ZF cassette [8–10].

Modifications to the structural domains of Vav proteins, either by point mutations in phosphosites or deletions, affect the catalytic activity of these proteins [11,12]. For instance, the removal of the N-terminal inhibitory region of Vav2 induces a stable open conformation that leads to constitutive GEF activity [9]. By contrast, point mutations in critical residues of the DH domain, such as E200A in Vav2, impair the GEF activity of this protein [13].

In addition to the catalytic activity, Vav proteins have also been asigned catalysis-independent functions. For example, the CH domain and the NSH3-CSH3 domains of Vav1 elicit the activation of the nuclear factor of activated T cells (NFAT) and the degradation of the intracellular active fragment of Notch1 (ICN1), respectively (Figure 3).

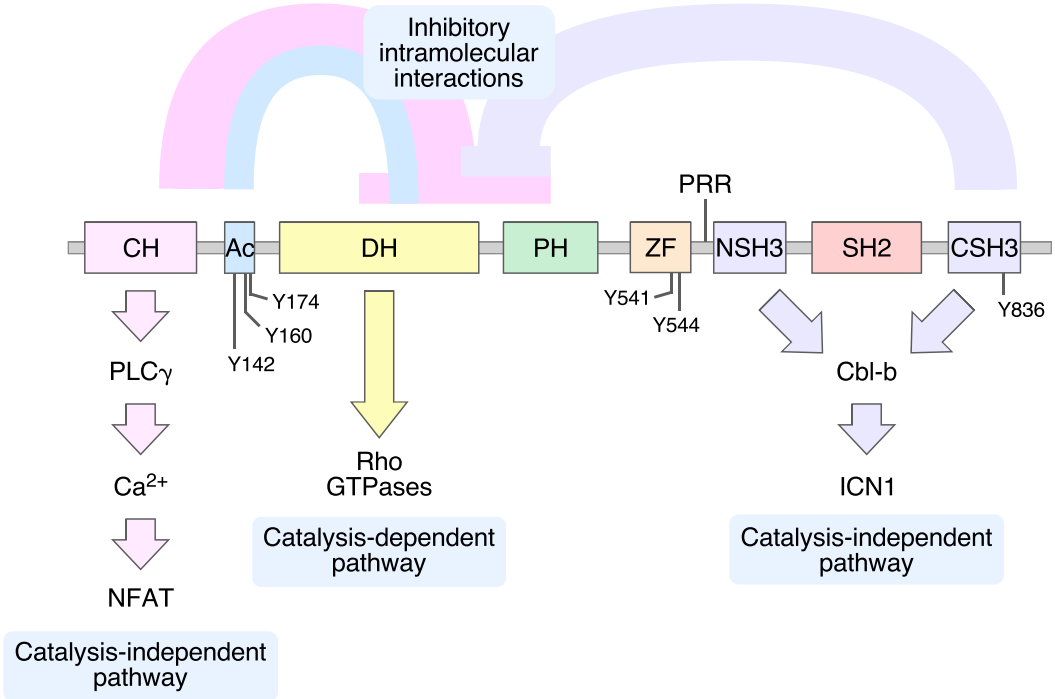



**Figure 3. Structural domains, regulation and function of Vav proteins.** Depiction of the structure, the intramolecular interactions that control the signaling output (top), the main regulatory phosphosites (using the amino acid sequence corresponding to mouse Vav1), and the main downstream pathways (bottom) of Vav proteins. Figure adapted from Rodríguez-Fdez et al., 2019 [7]

### 2.3. Physiological and pathological roles of Vav proteins

The three members of the Vav family play central roles in the homeostasis of different tissues, including the immune (Vav1), the cardiovascular (Vav2), and the nervous (Vav3) system. These proteins have also been linked to pathobiological processes, including immune-related dysfunctions, atherosclerosis, and cancer. [14–17].

VAV1 has been primarily related to blood tumors and is frequently deregulated in peripheral T cell lymphomas [11,18] and acute myeloid leukemia [12,17,19], although the ectopic expression of VAV1 has also been recorded in a minor number of solid tumors [20,21]. VAV2 and VAV3, however, have been associated with various types of solid tumors [22–24], although the only findings currently validated by mouse models are restricted to skin, breast cancer, and lung cancer [18,25,26]. Studies in double *Vav2*$^{-/-}$;*Vav3*$^{-/-}$knockout mice demonstrated that Vav2 and Vav3 cooperate in the skin to control an autocrine and paracrine program that triggers the proliferation of keratinocytes and pro-inflammatory responses, creating an environment that favors cutaneous squamous cell carcinoma (cSCC) development [25]. The topical administration of the skin carcinogens DMBA+TPA on *Vav2*$^{-/-}$;*Vav3*$^{-/-}$ deficient mice elicit fewer, smaller, and more benign tumors than the wild-type mice [25].

# 3. THE SQUAMOUS CELL CARCINOMA

## 3.1. Epidemiology of squamous cell carcinoma

SCC is the most prevalent type of cancer worldwide [27,28], arising from the accumulation of oncogenic events in the squamous epithelium of the human body. Depending on the affected tissue, this cancer can be classified into five main subgroups: cutaneous SCC (cSCC), head and neck SCC (hnSCC), lung SCC (lSCC), cervical SCC (ceSCC), and esophageal SCC (eSCC). Epidemiological data reveal an escalating incidence of these tumors each year (particularly in the case of cSCC), and increasing mortality rate (especially concerning hnSCC and lSCC) (Figure 4).

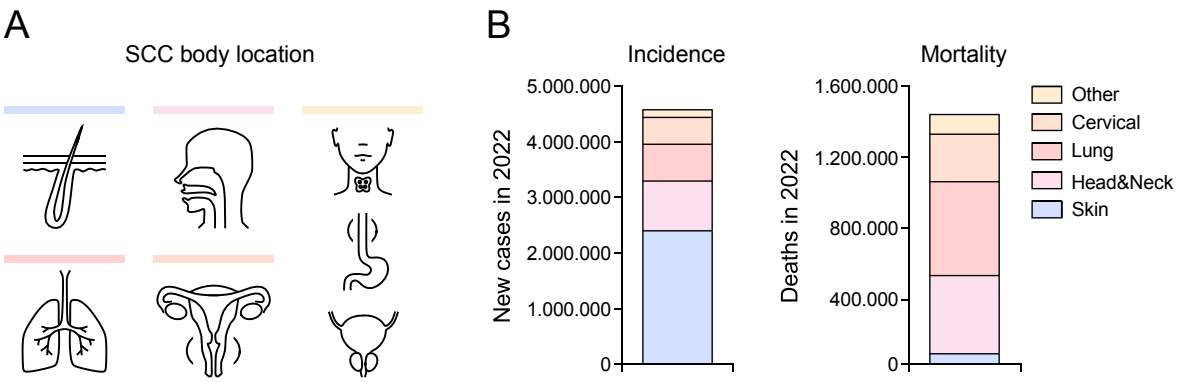



**Figure 4. Global incidence and mortality rates of SCC in 2022. (A)** Representation of the most common subtypes of SCC: cutaneous SCC, head and neck SCC, lung SCC, cervical SCC, thyroid, esophageal, and bladder SCC. **(B)** Bar graph indicating the estimated number of new cases and deaths worldwide recorded for each specific subtype of SCC in 2020. Statistics adapted from the American Cancer Society's publication and the National Cancer Institute website.

## 3.2. Cutaneous squamous cell carcinoma

cSCC is the second most common non-melanoma skin cancer worldwide, accounting for approximately 20% to 50% of all cases [29] (Figure 5). The main risk factor involved in cSCC development includes prolonged exposure to ultraviolet (UV) radiation and other human intrinsic factors such as age and skin color [29,30]. The incidence of cSCC is steadily rising every year [30], with an estimated annual incidence of over 700,000 cases. Despite showing relatively low 5-year mortality rates (below 5%), cSCC is responsible for 20% of all skin cancer-related deaths [27] and 5% of the cases exhibit aggressive features, with high recurrence, elevated metastatic potential, and poor response to available therapeutic options [28,30].

### 3.3. Head and neck squamous cell carcinoma

hnSCC is the sixth most common type of cancer worldwide, representing more than 90% of all head and neck tumors (Figure 5) [28]. It comprises a group of malignancies arising from the mucosal epithelium in the oral cavity, the nasopharynx, and the larynx [28,31]. Diverse risk factors contribute to hnSCC onset: tobacco and alcohol consumption is generally associated with oral cavity and larynx cancers, whilst pharynx cancers are increasingly attributed to infection with human papillomavirus (HPV). Although the number of larynx and hypopharynx cases has decreased over the last decade, the incidence of oral cancers has experienced a notorious rise. Of note, hnSCC are typically aggressive malignancies associated with poor prognosis, with a 5-year survival rate of approximately 50% [32].

Oral SCC (oSCC) is a subtype of hnSCC that arises from the tongue, buccal surfaces, and the hard palate. The incidence of this type of cancer is currently increasing among young individuals, particularly the tongue subtype that is associated with the poorest prognosis [33].

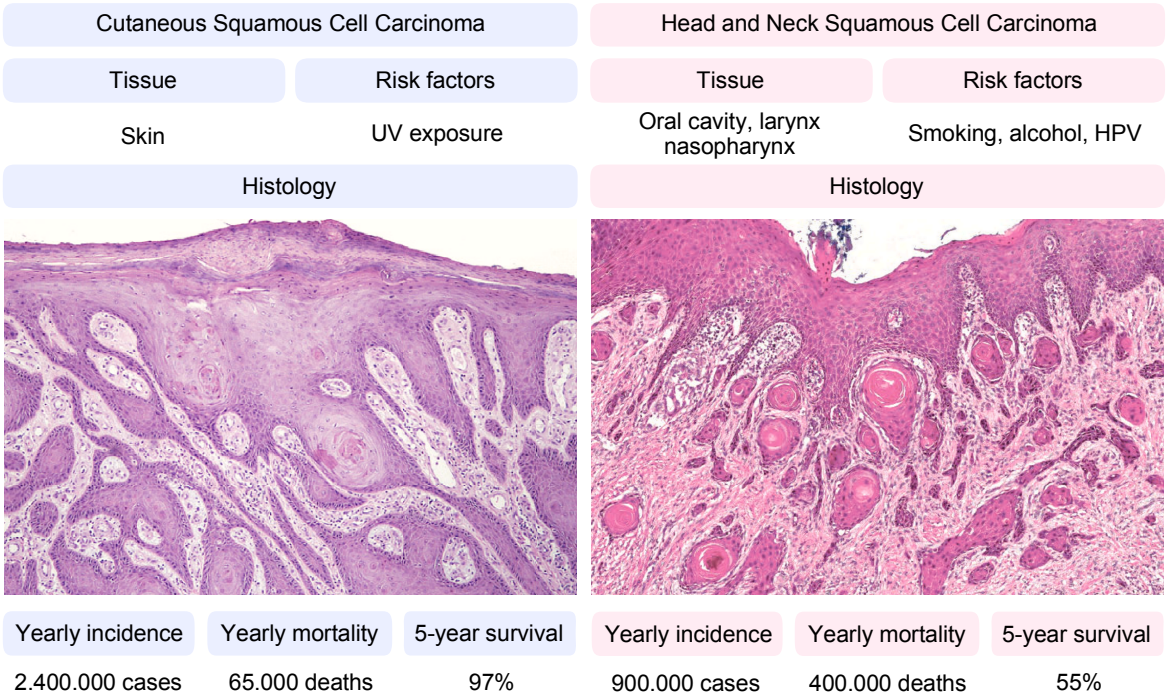



**Figure 5. Epidemiology of cSCC and hnSCC.** Compilation of the most relevant epidemiological data associated with cSCC and hnSCC. Histological images were obtained from the HSP atlas.

### 3.4. Role of Vav2 in squamous cell carcinoma

Previous studies reported that VAV2 trasncript and protein levels are frequently overexpressed in both cSCC and hnSCC. In the case of hnSCC, it was also shown that the levels of Vav2 mRNA correlate with a poor prognosis in hnSCC [13]. These observations prompted many studies on the role of Vav2 in cSCC and hnSCC both in vivo and in vitro [13]. Studies of knockin mice expressing a hyperactive version of Vav2 ($Vav2^{Onc}$) revealed the development of skin hyperplasia, characterized by highly proliferative and immature keratinocytes. This pre-neoplastic state, induced by Vav2, facilitates the formation of skin tumors upon DMBA topic administration [13].

The Vav2-induced hyperplasic phenotype was also observed in primary mouse or human keratinocytes growing in organotypic 3D cultures. Moreover, mechanistic studies have unveiled that the Vav2 signaling pathway is involved in hyperplasia formation. This pathway is activated in a catalysis-dependent manner and engages i) Rho GTPases (Rac1 and RhoA), ii) proximal effectors (Pak and Rock), and iii) distal elements (c-Myc and Yap/Taz) (Figure 6) [13].

The therapeutic potential of VAV2 has been evaluated by knocking down VAV2 in oSCC patient-derived cells. VAV2 knockdown blocks epithelial hyperplasia developed by oSCC tumor cells in 3D cultures and reduces the tumorigenic and metastatic capacity when orthotopically transplanted to $Vav1^{-/-}$;$Vav2^{-/-}$;$Vav3^{-/-}$ mice [13].

Intriguingly, this study also reports that upregulated Vav2 signaling leads to an upregulation of the ribosome biogenesis pathway [13]. Given that many cancer cells rely on this process to maintain high proliferation rates, we decided to explore the connection between Vav2 and the ribogenesis process in SCC.

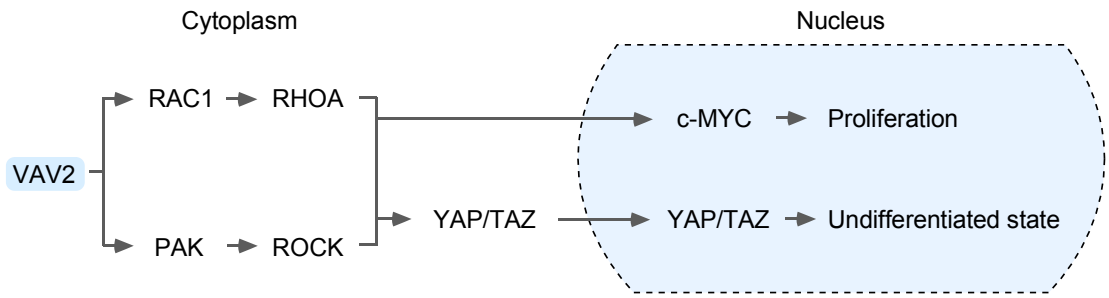



**Figure 6. Vav2 signaling pathway in cSCC and hnSCC.** Upregulated Vav2 catalytic activity promotes cell proliferation and undifferentiation by the activation of the following cascade of elements: Rho GTPases (Rac1 and RhoA), proximal effectors (Pak and Rock), and distal transcription factors (c-Myc and YAP/TAZ).

# 4. THE RIBOSOME BIOGENESIS IN CANCER

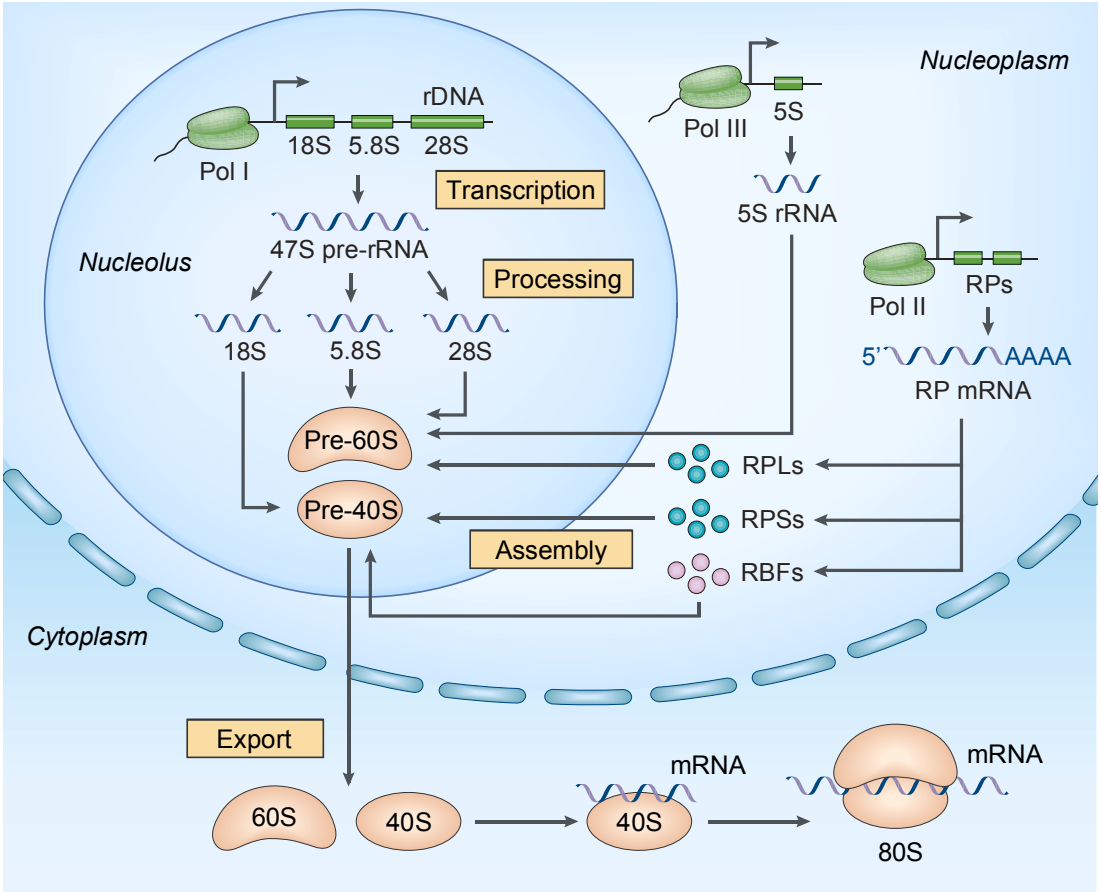
## 4.1. General aspects of the ribosome biogenesis

Ribosomes are macromolecular complexes formed by ribosomal RNA (rRNA) and ribosomal proteins (RPs) that function as translation machines. In humans, ribosomes comprise a small 40S subunit (composed of the 18S rRNA and 33 ribosomal proteins), and a large 60S subunit, (constituted by the 28S, 5.8S, and 5S rRNAs and 47 ribosomal proteins). Together, the 60S and 40S subunits conform the 80S ribosome, responsible for protein synthesis.

Ribosome biogenesis is a highly regulated and energy-demanding process required to sustain cell growth and proliferation. This multistep process begins in the nucleolus and finishes in the cytoplasm (Figure 7A). In the nucleolus, RNA polymerase I (Pol I) transcribes the ribosomal DNA (rDNA) into an initial rRNA precursor (47S pre-RNA) containing the 18S, 28S, and 5.8S rRNAs. This 47S RNA precursor requires numerous processing steps to eliminate external (5'ETS1 and 3'ETS2) and internal (ITS1 and ITS2) transcribed spacers (Figure 7B). Simultaneously, in the nucleoplasm, RNA polymerase III (Pol III) and RNA polymerase II (Pol II) synthesize the 5S pre-rRNA and the mRNAs encoding small (RPS) and large (RPL) ribosomal proteins, respectively. Both 5S rRNA and RPs are then imported into the nucleolus and incorporated into pre-40S and pre-60S ribosomal complexes [34–37]These pre-ribosomal subunits undergo pre-rRNA processing, rRNA modification (methylation or pseudouridylation) and assembly of RPs with the assistance of numerous ribosome biogenesis factors (RBFs). These processes occur in a stepwise manner throughout the nucleolus, nucleoplasm, and cytoplasm. In this latter location, the two ribosomal subunits bind to each other to form functional 80 ribosome [37–40].

Because of its elevated complexity, the ribosome biogenesis pathway involves an enormous energy cost for the cell. As a result, optimal regulation of this pathway is crucial to maintain cell homeostasis. However, cancer cells are capable to manipulate ribosome production to be able to sustain high proliferative rates.

A

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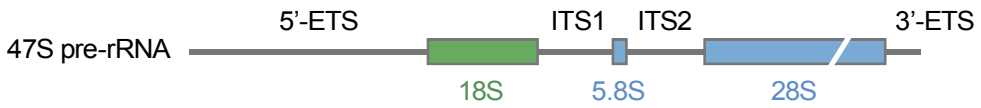


**Figure 7. Ribosome biogenesis pathway in humans. (A)** Ribosome biogenesis predominantly occurs in the nucleolus, where RNA polymerase I (Pol I) transcribes the 47S rRNA precursor (47S pre-rRNAs) from ribosomal DNA (rDNA) genes, which contain the 18S, 5.8S, and 28S rRNAs sequences. The 5S rRNA is transcribed by RNA polymerase III (Pol III) in the nucleoplasm and transported to the nucleus to be part of the pre-60S subunit. RPSs, RPLs, and RBFs mRNAs are transcribed by RNA polymerase II (Pol II) in the nucleus and exported to the cytoplasm for translation into RPs and RBFs, which are subsequently re-imported into the nucleus. The association of rRNA precursors and RPs generates two immature particles, the pre-40S and pre-60S ribosomal subunits, that are subjected to rRNA processing, RPs assembly, and export from the nucleus to the cytoplasm, where they became completely functional. In the cytoplasm, 40S and 60S subunits join together to form the 80S ribosome and start protein synthesis. RPs, ribosomal proteins; RPLs, large ribosomal proteins; RPSs, small ribosomal proteins; RBFs, ribosome biogenesis factors. **(B)** Scheme of the initial rRNA precursor in humans (47S pre-rRNA), the showing mature rRNAs (18S, 5.8S, and 28S), and the external (5'-ETS and 3'-ETS) and internal (ITS1 and ITS2) transcribed regions. Figure adapted from Pelletier et al., 2018 [38]

### 4.2. Ribosome biogenesis in cancer biology

Increasing evidence has revealed that cancer cells hyperactivate the ribosome biogenesis pathway to support enhanced growth and proliferation. In line with this, some studies have determined that cancer cells exploit different steps of the ribogenesis process to promote tumorigenesis [41]. These regulatory steps mainly include Pol I-mediated transcription and pre-rRNA modification.

Cancer cells frequently manipulate Pol I activity to increase the synthesis of 47S pre-rRNA. The MYC oncogene typically engages in this process, as it recruits UBF and SL1 transcription initiation factors to promote rDNA transcription through Pol I (Figure 8) [42]. In addition, Myc also regulates Pol II- and Pol III-mediated transcription, thus facilitating 5 rRNA and RP gene synthesis, respectively [43,44] (Figure 8). Importantly, the deregulation of ERK and mTOR signaling pathways, the maintained phosphorylation of the GEF ECT2, or the loss of the tumor suppressor proteins P53, RB, ARF, and PTEN also stimulate Pol I-dependent transcription [45]. Another regulatory point targeted by cancer cells involves alterations in rRNA modification (methylation and pseudouridylation). For instance, the upregulation of the methyltransferase FBL in cancer cells increases the 2'-O-ribose methylation in the rRNA, which increases ribosome translation efficiency [46,47].

Highly proliferative cancer cells frequently become addicted to ribosome biogenesis to sustain the increased protein synthesis demands [48–50]. Interestingly, recent studies have proposed that cancer cells addicted to ribogenesis are sensitive to drugs targeting Pol I activity [49,50]. Among them, CX-5461 appears to be a promising inhibitor of rDNA transcription. Although this compound induces DNA damage by the stabilization of G4 DNA structures [51], CX-5461 was first described as an inhibitor of rDNA transcription that blocks the binding of the UBF/SL1 pre-initiation complex to rDNA promoters, preventing the recruitment of Pol I (Figure 8) [52,53]. CX-5461 is currently in Phase I clinical trials in patients with BRCA1/2-deficient cancer and has already been recommended for Phase II clinical studies (NCT02719977). Therefore, this drug opens a therapeutic window for targeting tumors characterized by the presence of ribosome-biogenesis-addicted cancer cells.

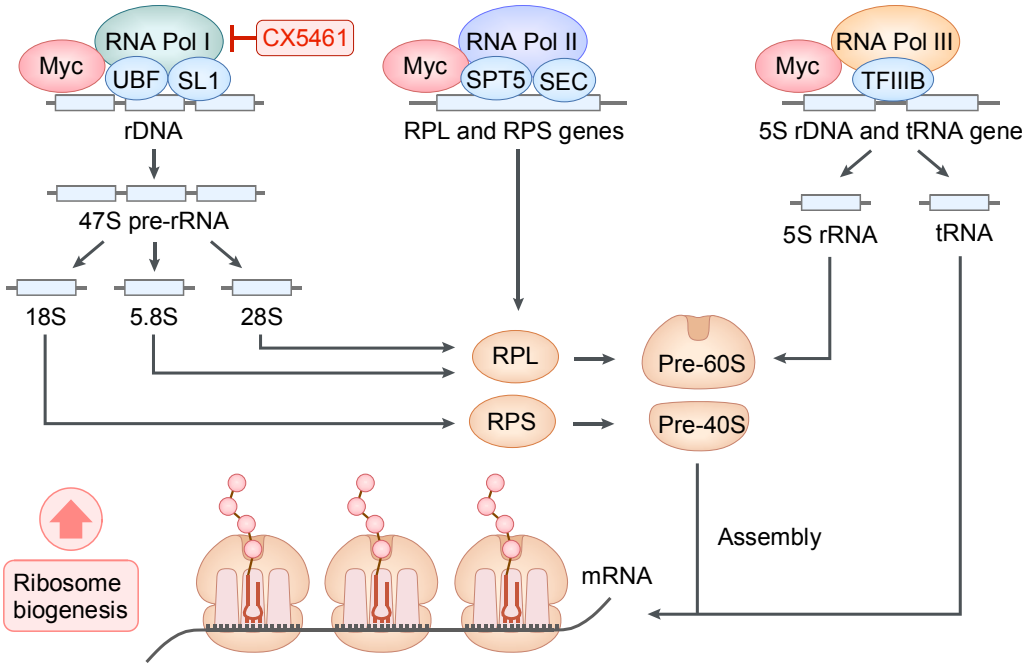


**Figure 8. MYC regulates ribosome biogenesis through the activation of the three RNA polymerases.** MYC is the oncoprotein with the most widespread effects in the ribogenesis pathway. MYC elicits Pol I-mediated rDNA transcription by binding to UBF and SL1 transcription initiation factors and increases the abundance of 18S, 28S, and 5.8S rRNAs. The MYC-STP5/SEC complex stimulates RP-and-RBF mRNA transcription in a Pol II-dependent manner. MYC interacts TFIIIB transcription initiation factor and promotes Pol III-mediated expression of the 5S rRNA and tRNA. An increased MYC activity leads to the upregulation of the ribosome biogenesis process. This deregulation can be inhibited by CX-5461, a drug that disrupts Pol I-driven rRNA synthesis. Figure adapted from Jiao et al., 2023 [54]

# 5. VAV2 IN RIBOSOME BIOGENESIS IN THE CONTEXT OF cSCC AND hnSCC

Recent functional annotation data obtained from transcriptome analysis of the skin of Vav2$^{Onc/Onc}$ mice unveiled the upregulation of a transcriptional program related to ribosome biogenesis (RiBi) (Figure 9) [13]. This finding was surprising, as Vav2 has never been reported to play a role in this biological process. Neither Vav1 nor Vav3 have been described to elicit in this process either. Interestingly, several papers have demonstrated that the GEF ECT2 promotes the activation of the ribogenesis pathway through RAC1 in many types of cancer [55–58]. We reasoned that studying the connection between Vav2 and the ribosome biogenesis might provide novel insight into a signaling pathway activated in cSCC and hnSCC tumors that has been poorly explored to date [13].

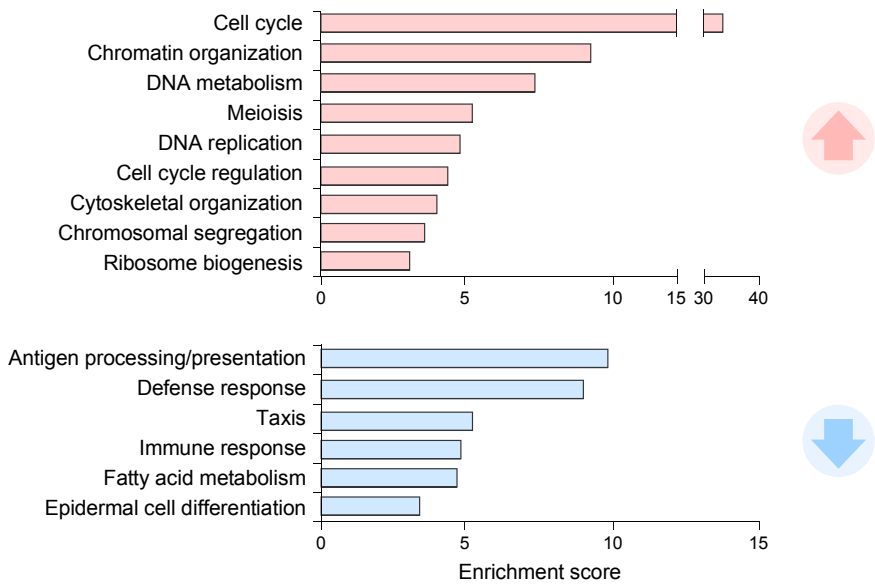


**Figure 9. Vav2 is associated with a ribosome biogenesis transcriptional program.** Main functional categories encoded by the Vav2$^{Onc}$-dependent transcriptome obtained from mice. Red and blue bars indicate genes that are upregulated and downregulated, respectively. Figure taken from Lorenzo et al., 2020 [13]

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# PUBLICATIONS

The research conducted in this thesis will be submitted for publication soon. In addition to this, the author has also contributed to the following publications:

1. Lorenzo-Martín, L.F., **Fernández-Parejo, N.**, Menacho-Márquez, M., Rodríguez-Fdez, S., Robles-Valero, J., Zumalave, S., Fabbiano, S., Pascual, G., García-Pedrero, J.M., Abad, A., García-Macías, M.C., González, N., Lorenzano-Menna, P., Pavón, M.A., González-Sarmiento, R. Segrelles, C., Paramio, J.M., Tubío, J.M.C., Rodrigo, J.P., Benitah, S.A., Cuadrado, M., and Bustelo, X.R. VAV2 signaling promotes regenerative proliferation in both cutaneous and head and neck squamous cell carcinoma. *Nat. Commun.* (2020), 11: 4788.

2. Baltanas, F.C., Mucientes-Valdivieso, C., Lorenzo-Martín, L.F., **Fernández-Parejo, N.**, García-Navas, R., Segrelles, C., Calzada, N., Fuentes-Mateos, R., Paramio, J.M., Bustelo, X.R., and Santos, E. Functional specificity of the members of the Sos family of Ras-GEF activators: novel role of Sos2 in control of epidermal stem cell homeostasis. *Cancers* (2021), 13: 2152

3. Lorenzo-Martín, L. F., Menacho-Márquez, M., **Fernández-Parejo, N.**, Rodríguez-Fdez, S., Pascual, G., Abad, A., Crespo, P., Dosil, M., Benitah, S. A., & Bustelo, X. R. The Rho guanosine nucleotide exchange factors Vav2 and Vav3 modulate epidermal stem cell function. *Oncogene* (2022), 3341:3354.