FUNCTIONAL CHARACTERIZATION OF *RRAS2* MUTATIONS AND ROLE OF *RRAS2*^{Q72L} IN OVARIAN CANCER

Ph.D. Thesis

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CERTIFICAN

Que el trabajo de tesis titulado **"Functional characterization of *RRAS2* mutations and role of *RRAS2*^{Q72L} in ovarian cancer"**, presentado por **Dª LAURA CLAVAÍN MATEO** para optar al Grado de Doctor por la Universidad de Salamanca, ha sido realizado bajo mi 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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Vuelve hacia atrás la vista, caminante,
verás lo que te queda de camino[...]

Miguel de Unamuno

ABSTRACT

R-RAS2 is a small GTPase with high structural proximity to classical RAS proteins. *RRAS2* gain-of-function mutations have been identified at low frequency in recent PanCancer studies. However, the cancer driver and pathobiological roles of this GTPase remain poorly characterized. In this thesis we have used in vitro and in vivo models to tackle those issues. We have demonstrated that tumor-found *RRAS2* mutations targeting residues involved in the GTP-binding are able to induce cell transformation in vitro. Analyses of R-RAS2^{Q72L}-expressing cancer cell lines have shown that this protein is required for the tumoral fitness of the cells. In these cell models, R-RAS2^{Q72L} modulates pathways involved in cell proliferation and survival and regulates basic processes such as polysomal translation and cell metabolism. However, these effects are not driven by R-RAS2^{WT} expression. This work also exposes a driver role for R-Ras2^{Q72L} in tumorigenesis. The R-Ras2^{Q72L}-driven tumors exhibit differential sensitivity to mTORC1 and/or PI3Kα/δ pharmacological inhibition, with some tumors showing resistance to all the inhibitors tested. The characterization of the R-Ras2^{Q72L}-triggered ovarian cystadenomas has revealed a rete ovarii origin and a sex-reversal phenotype in these tumors. Beyond tumorigenesis, R-Ras2^{Q72L} also triggers follicular atresia- and absent spermatogenesis-induced infertility in mice. Altogether, our findings unveil novel pathological functions of R-RAS2^{Q72L} and expand the current knowledge of cellular functions of the wild-type and the oncogenic R-RAS2 versions.

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Introduction

1 R-RAS2

1.1 RAS SUPERFAMILY

Since the discovery of p21 H-RAS and K-RAS proteins more than 50 years ago [1, 2], many other small GTPases have been characterized and enlisted in the RAS superfamily of proteins. To date, this superfamily is composed of more than 160 proteins [3], that can be classified into five major families: ARF, RAB, RAN, RAS and RHO proteins. The ARF and RAB proteins are involved in the vesicular transport within the cell [4, 5]. RAN GTPases participate in several steps of the cell mitosis by regulating mitotic spindle and nuclear envelope assembly [6], as well as in the transport of RNA and proteins between the nucleus and the cytoplasm [7]. Proteins of the RAS family respond to a variety of extracellular stimuli to regulate cytoplasmic signaling networks linked to cell proliferation, differentiation, and survival. RHO proteins regulate actin organization, cell cycle progression, cell survival, and cell-type-specific responses such as angiogenesis or neurogenesis, among others [8, 9]. Although the RAS family was believed to be the founding members of the RAS superfamily [10], more recent phylogenetic analyses have marked the ARF family of proteins as the most ancient members of this superfamily [3].

The proteins of the RAS superfamily act as modulators of a complex combination of cellular processes. However, most of them share a common feature: they are able to hydrolyze GTP and operate as binary molecular switches. In this mechanism, on and off states are modulated by guanosine nucleotide (guanosine triphosphate, GTP, or guanosine diphosphate, GDP) binding, which affects the conformation of the protein. In the GTP-bound state, they display a binding surface with high affinity for effector proteins, allowing stimulation or attenuation of the downstream signaling elements. Activation of GTPases is promoted by guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP and the subsequent activation of the protein by GTP entry in the guanosine-nucleotide-binding pocket. RAS proteins can be governed by several GEFs, which allows a sophisticated regulation of divergent RAS downstream

pathways in response to diverse upstream stimuli. The activated state is shut off when GTPase-activating proteins (GAPs) accelerate the intrinsic low GTPase activity of RAS proteins, thus favoring the transition back to the inactive, GDP-bound state [10] (Figure 1). Importantly, most oncogenic mutations in GTPases impair their GTP hydrolysis capacity, leading to the GEF-independent constitutive activation of these proteins [11].

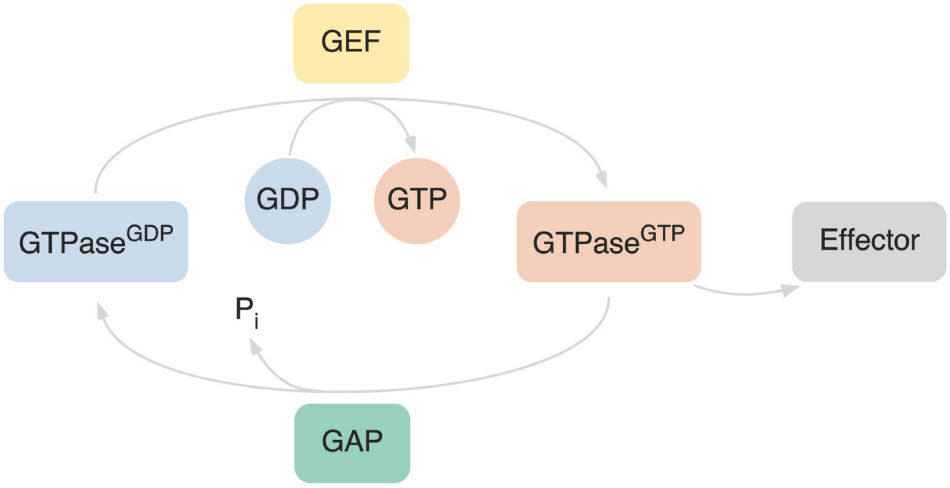

Figure 1. GTPase activation cycle. Illustration of the mechanism of activation and deactivation of GTPases and the agents driving such process.

1.2 R-RAS SUBFAMILY

Several major subfamilies constitute the RAS family of proteins: RAS, R-RAS, RAL, RAP, RAD, RHEB and RIT [12]. Among them, R-RAS subfamily members (R-RAS1, R-RAS2/TC21 and R-RAS3/M-RAS) are the closest to classical RAS proteins (H-, K- and N-RAS) from the structural point of view. Despite such a similarity, the function of the R-RAS subfamily members might be divergent. Thus, R-RAS1 localizes to focal adhesions where it promotes cell adhesion and activates integrins [13, 14]. R-RAS2 physiological role will be reviewed in the following sections. M-RAS has been demonstrated to be involved in cell migration and many physiological functions such as neurons and osteoblasts differentiation [15, 16]. All R-RAS members have been described to promote growth transformation and alter cellular differentiation [17-19]. However, only R-RAS2 has transforming activity comparable to those of classical

RAS. Furthermore, unlike R-RAS1 and M-RAS, mutational activation of R-RAS2 has been reported in human tumors [12, 20, 21].

1.3 R-RAS2

R-RAS2 was discovered more than thirty years ago, when screening for proteins containing the RAS conserved domains using a cDNA library from a teratocarcinoma. The protein was named after the number of the clone: TC21 [22]. High homology to R-RAS1 led to the renaming of the TC21 protein to R-RAS2 [23].

1.3.1 Structure

R-RAS2 is a 204-amino-acid protein that has 65.2% homology to classical RAS, a 71.8% homology to R-RAS1 and a 68.5% homology to M-RAS. R-RAS2 protein sequence contains evolutionary conserved regions involved in the GTP binding and hydrolysis: the "G box" sequences. These regions, present in all RAS superfamily members, are directly engaged in the binding of the guanine nucleotides (G1), the reorientation of the molecule to provide a proper interaction surface with the effector (Switch I or G2), the binding to the nucleotide-associated Mg^{2+} ion (Switch II or G3), the stabilization of the nucleotide and of the molecule itself (G4), and the generation of indirect associations with the guanine nucleotides (G5) [13]. Within these well-established domains, R-RAS2 shares 84.28% of amino acid sequence with H-RAS. Interestingly, the Switch I and Switch II regions show a 100% identity to classical RAS sequences (Figure 2).

Figure 2. Structural homology between human RAS family members. Scheme depicting the skeleton of RAS proteins. Sequence of G domains (G1, SwitchI, SwitchII, G4 and G5) are shown in green boxes. Levels of domain homology compared to H-RAS is displayed under the boxes as a percentage. Within these green boxes, non-homologous residues are highlighted in blue color. Light blue boxes illustrate the polybasic region. Within this region, uppercase letters point out basic residues. Sequence of CAAX box is exhibited in light orange boxes. In the case of K-RAS, the polybasic region of isoform 4A is shown.

An additional sequence with functional relevance for both RAS proteins and R-RAS2 is the CAAX box. Full processing of the CAAX and palmitoylation (at the cysteine residue close to the CAAX box) are necessary for the attachment of R-RAS2 to the plasma membrane [12, 24-26] (Figure 2). Upstream this terminal sequence, R-RAS2 contains a conserved proline-rich motif (designated as R-RAS box) that is present in R-RAS1 but not in M-RAS [27]. In R-RAS1, such region contains a focal adhesion-targeting signal that is responsible for the subcellular location of this GTPase in those cytoskeletal structures [28] (Figure 3). Variability in protein length is observed among the RAS family of proteins: a short fragment of 11 amino acids in the

N-terminal part of R-RAS2 predominantly accounts for length variability compared to other RAS family members.

Protein	Sequence	Homology
R-RAS1	175 RLNVDEAFEQLVRAVRKYQEQELPPSP-PSA-PRKKGGGCP----CVLL 218	-
R-RAS2	161 RMNVDQAFHELVRVIRKFQEQECPPSPEPTR-KEKDKKGCH----CVIF 204	72.7 %
M-RAS	161 PLNVDKAFHDLVRVIRQQIPEKSQKKKKKTKWRGDRATGTHKLQ-CVIL 208	46.2 %
H-RAS	149 RQGVEDAFYTLVREIRQHKLRKLNP-------PDESGPGCMSCK-CVLS 189	56.5 %
K-RAS4A	149 RQRVEDAFYTLVREIRQYRLKKIS--------KEEKTPGCVKIKKCIIM 189	55.3 %
N-RAS	149 RQGVEDAFYTLVREIRQYRMKKLNS-------SDDGTQQCMGLP-CVVM 189	54.3 %

Figure 3. Sequence homology in the R-RAS box sequence. Comparison between the final sequence of the indicated proteins. In the case of R-RAS1 and R-RAS2, this sequence is designated as R-RAS box. The percentage of homology compared to R-RAS1 sequence is indicated. Green dots above the sequences indicate R-RAS1 homology with R-RAS2 and other proteins. Red dots indicate exclusive homology in R-RAS2.

Both R-RAS2 and classical RAS undergo prenylation, but the latter are only farnesylated in physiological conditions while R-RAS2 is substrate for both geranylgeranyl- and farnesyltransferase [29-31]. However, in the presence of farnesyl transferase inhibitors, K-RAS and N-RAS (although to a lesser extent) can undergo geranylation [32, 33]. Other post-translational modifications for R-RAS2 are

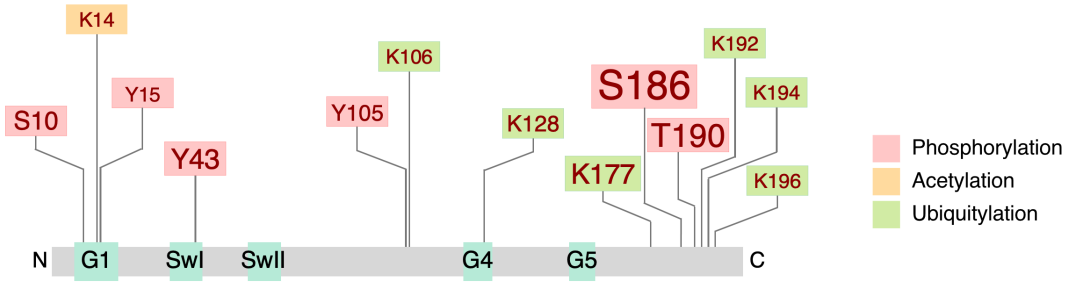

Figure 4. Post-translational modifications in R-RAS2. Scheme showing R-RAS2 residues subjected to phosphorylation, acetylation and ubiquitylation. Letter size is proportional to the number of references describing each modification. Information was obtained from Phosphosite database (www.phosphosite.org).

phosphorylation, ubiquitylation and acetylation [34] (Figure 4). Of note, some of those modifications are conserved among the RAS proteins in the G boxes.

1.3.2 Phylogenetic evolution

R-RAS subfamily divergence from classical Ras seems to have occurred about 750 years ago, as precursor R-RAS relatives have been found in cnidarians [35] (Figure 5). From this first R-RAS precursor, a primitive M-RAS seems to have emerged in Platyhelminthes. Thereafter, two primitive R-RAS members are present: a precursor of M-RAS and a common ancestor of both R-RAS and R-RAS2. This suggests an

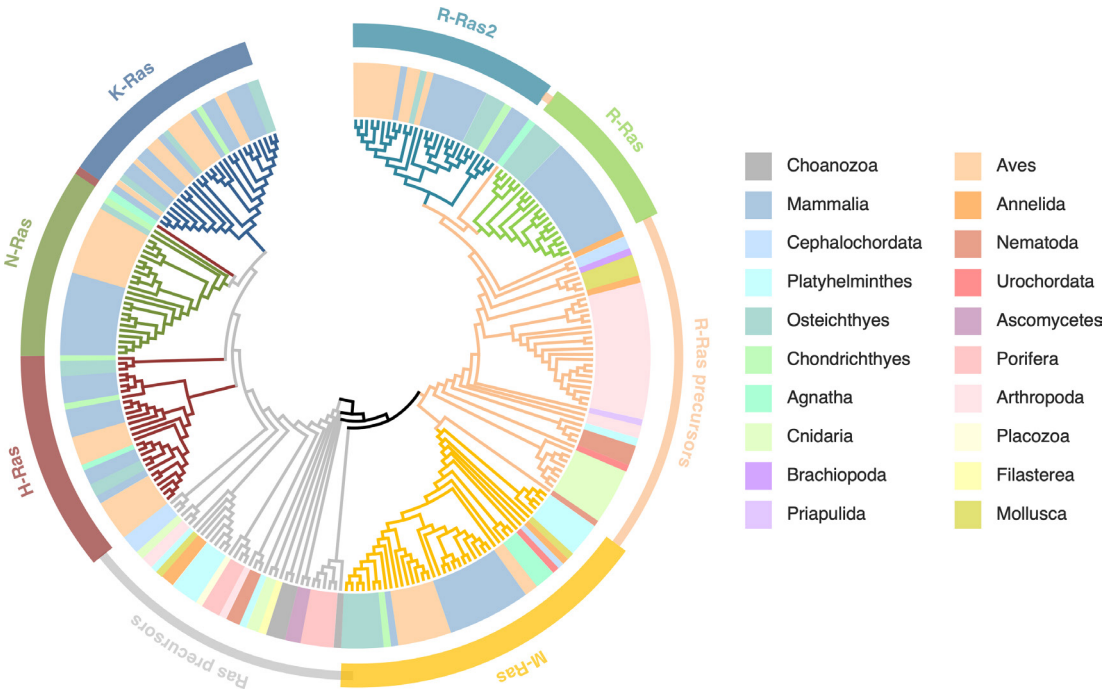

Figure 5. Phylogenetic tree of the RAS family members. Evolutionary tree of RAS family proteins (left). Protein sequences were obtained from the Uniprot, SMART and NCBI Protein databases and aligned with MUSCLE using the Neighbor Joining approach in MEGA X software. Phylogenetic tree construction was performed in MEGA X using the Maximum Likelihood method. Proteins are distributed in eight groups depending on homology criteria. Nodes belonging to each group are indicated by the same color (Ras precursors in gray, H-Ras in dark red, N-Ras in dark green, K-Ras in dark blue, R-Ras precursors in light orange, R-Ras in light green, R-Ras2 in light blue and M-Ras in yellow). Phylogenetic clades are represented by the color code indicated on the right.

independent evolution of M-RAS and R-RAS/R-RAS2, according to previous analyses [36]. Still, it is not until the vertebrate clade when the three R-RAS members emerge (Figure 5).

R-RAS2 homologs are highly similar in terms of protein length and sequence homology in the core of the protein (Figure 6). Switches I and II are the most conserved domains among all the homologs, as they are crucial for nucleotide binding and GTPase activity. CAAX sequence is highly conserved in vertebrates and, interestingly, the cysteine residue that undergoes palmitoylation is only present in vertebrates and urochordates. The distinctive N-terminal 11 amino acid head that discriminates R-RAS2 from classical RAS in humans is vastly variable among the homologs studied

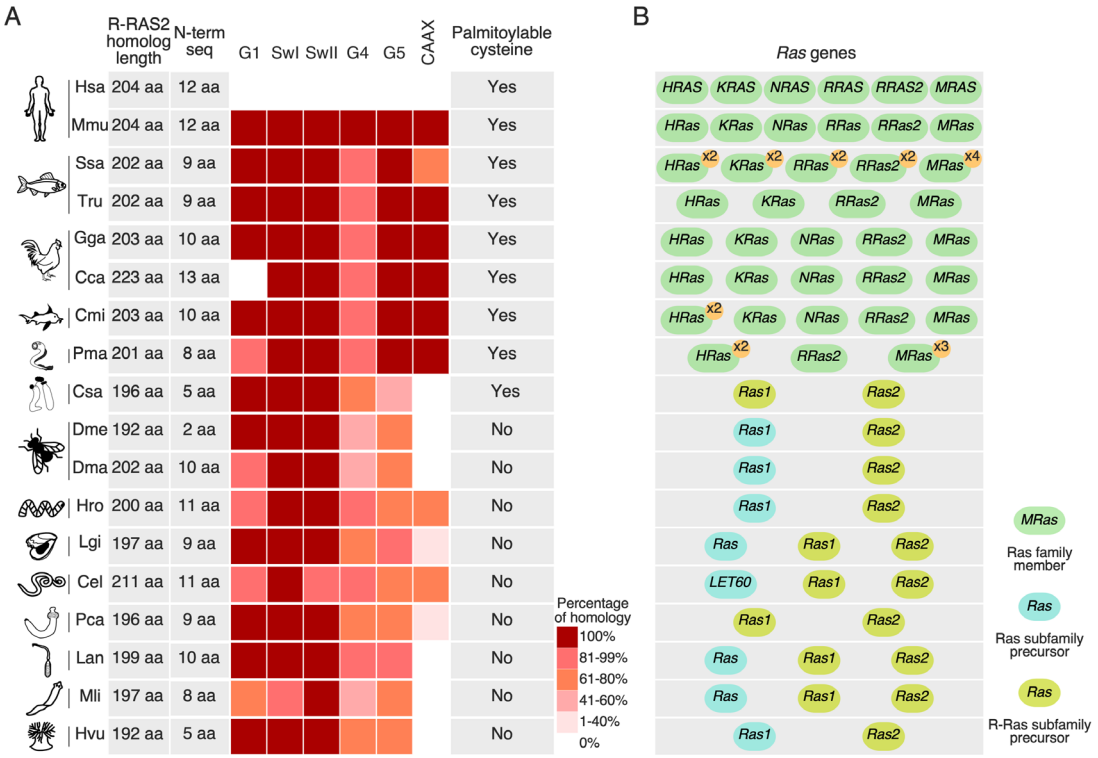

Figure 6. Conservation of R-RAS2 homologs among species. A Protein sequence comparison between human R-RAS2 and homologs in representative species in different taxa. Species with high-coverage sequenced genomes were selected. **B** Number of RAS genes. In case of RAS gene amplifications, number of genes present are indicated in a yellow circle. Vertebrata: Homo sapiens (Hsa), Mus musculus (Mmu), Salmo salar (Ssa), Takifugu rubripes (Tru), Gallus gallus (Gga), Cuculus canorus (Cca), Callorhinchus milii (Cmi), Petromyzon marinus (Pma); Urochordata: Ciona savignyi (Csa); Arthropoda: Drosophila melanogaster (Dme), Daphia magna (Dma); Annelida: Helobdella robusta (Hro); Mollusca: Lottia gigantea (Lgi); Nematoda: Caenorhabditis elegans (Cel); Priapulida: Priapulus caudatus (Pca); Brachiopoda: Lingula natine (Lan); Plathyhelminthe: Macrostomum lignano (Mli); Cnidaria: Hydra vulgaris (Hvu); aa: amino acid; N-term seq: R-Ras2 characteristic N-terminal fragment.

in terms of sequence (data not shown). Genetic amplification of both *RAS* and *RRAS* genes is observed in atlantic salmon (*Salmo salar*, Ssa in Figure 6) and sea lamprey (*Petromyzon marinus*, Pma in Figure 6).

1.3.3 Cellular localization

Studies using ectopically expressed R-RAS2 preferentially localized this protein at the plasma membrane, the Golgi Aparatus, recycling endosomes, the endoplasmic reticulum and, to a lower extent, in focal adhesions, microtubules and intermediate filaments [25, 37-39].

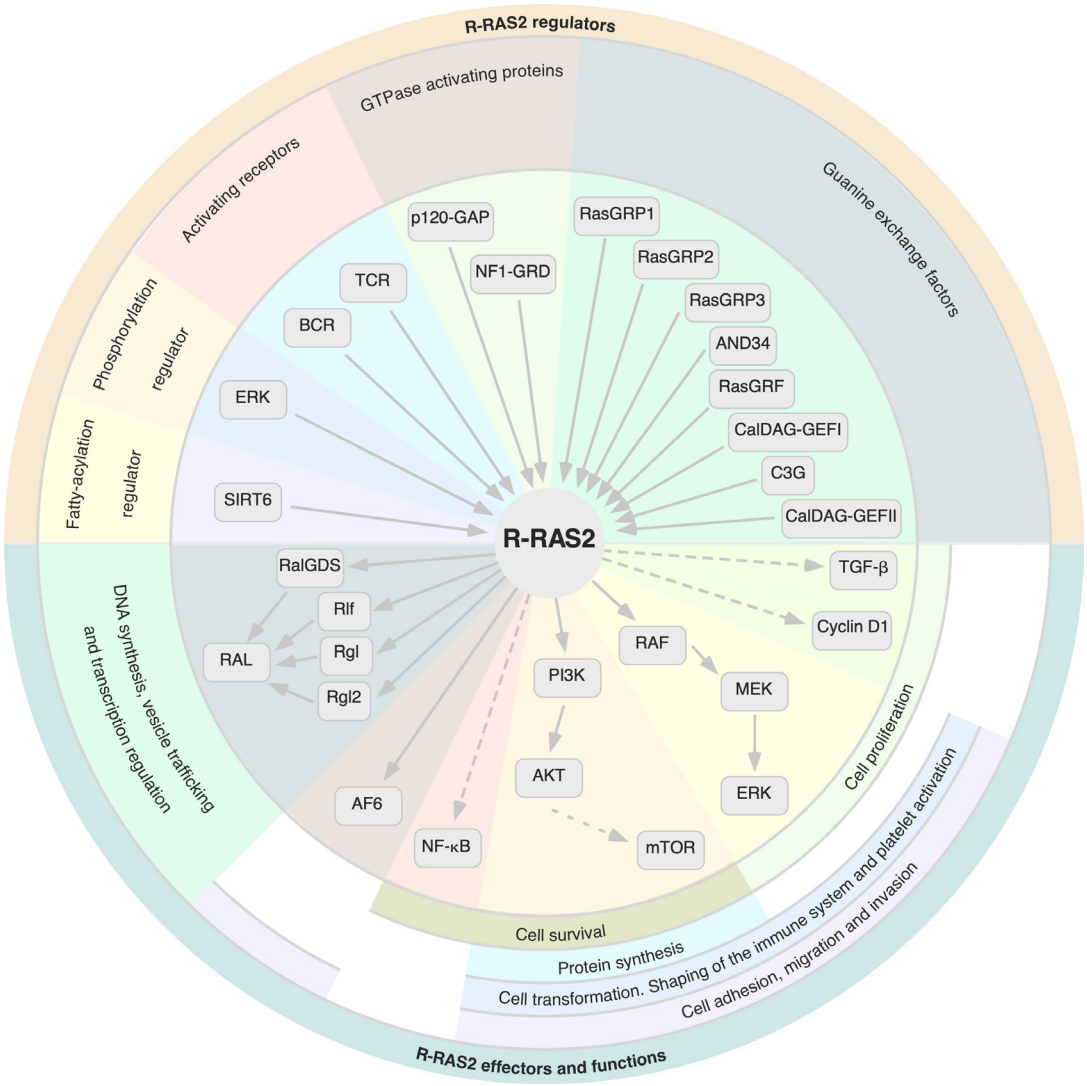

Figure 7. Schematic summary of the known interactions and functions of R-RAS2. Upper semi-

circle represents R-RAS2 regulators. Regulators are categorized based on their function, and proteins interacting with R-RAS2 are indicated in grey boxes. Lower semicircle shows the effectors and functions described for R-RAS2 so far. Some signaling pathways overlap several functions. Dashed arrows indicate that direct interaction has not been demonstrated or intermediate proteins are not shown in this scheme.

1.3.4 Interactors

In vitro studies in human and murine cell lines have identified a considerable amount of R-RAS2 molecular partners, many of them are shared with other RAS family members. (Figure 7).

Several RAS known GEFs and GAPs were found to interact with R-RAS2 (enlisted in Table 1). However, not all of them activated this GTPase to the same extent. Interaction of R-RAS2 with GEFs activating RAL proteins takes place when R-RAS2 is GTP-loaded, meaning that RalGEFs are effectors of R-RAS2 instead of regulators.

Table 1. Regulators RRAs2

Protein	Interaction with other GTPases	Type of regulator	Reference
GFR/CDC25	Rap1	GEF	[18]
RasGRF1	H-Ras, M-Ras, R-Ras	GEF	[27, 41, 37]
RasGRP1	Ras, M-Ras, R-Ras	GEF	[27]
RasGRP3	H-Ras, M-Ras, R-Ras, Rap	GEF	[27]
CalDAG-GEFI	R-Ras, Rap1	GEF	[27, 37, 80]
C3G	R-Ras, Rap1	GEF	[80]
AND-34	R-Ras, Rap, Ral	GEF	[80]
RasGRP2	N-Ras, K-Ras, R-Ras	GEF	[27]
RalGDS	Ral	GEF	[40, 43]
Rgl	Ral	GEF	[43]
Rgl2	Ral	GEF	[43]
p120-GAP/RASA1	Ras	GAP	[18]
NF1-GRD	Ras	GAP	[18]

R-RAS2 can be further regulated by mechanisms outside this basic regulatory cycle, including posttranslational modifications. ERK1/2 kinase is known to activate R-RAS2 by phosphorylation on its serine 186 [34]. SIRT6 enzyme also regulates R-RAS2 by lysine defatty-acylation [25].[40]

R-RAS2 was first linked to the RAF/MAPKs and PI3K/AKT pathways. R-RAS2 interacts with all the RAF isoforms (RAF1, A-RAF and B-RAF) and with RAC-1 [41-44]. Direct interaction with PI3K and activation of its downstream effectors AKT and mTOR [43, 45-49] are also observed. Moreover, R-RAS2 has been found to interact with both B and T Cell Receptor (BCR and TCR, respectively) [50] and AF-6 [43]. R-RAS2 has shown activation of the NF-κB factor [23, 47, 51], TGF-β, cyclin D1 [25, 51, 52] and PLCε [27, 43]. However, whether this activation is direct or is mediated by other effectors is not known.

Highly conserved switch regions in R-RAS2 are responsible for the interaction with specific effectors and regulators through their RAS Binding (RB) or RAS Association

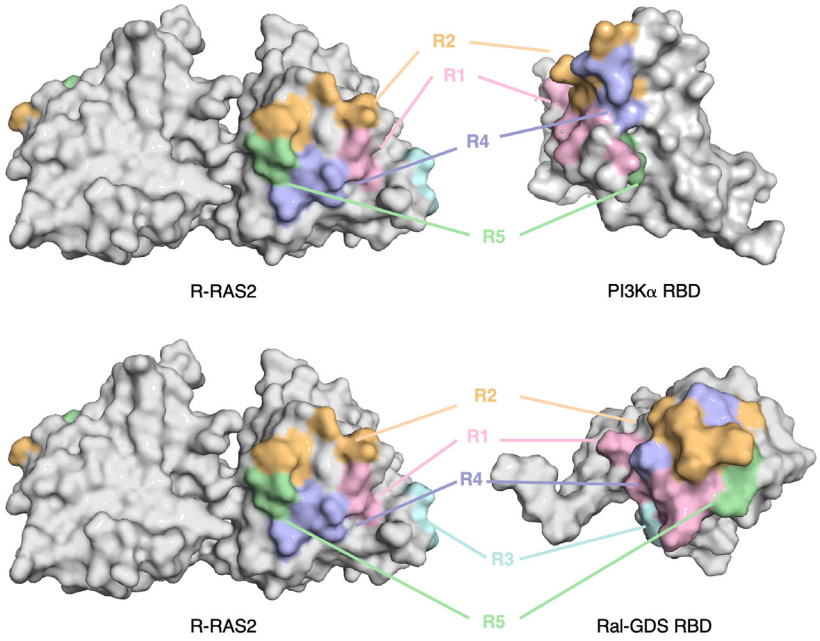

Figure 8. 3D representation of the interaction hotspots of R-RAS2 and its effectors PI3Kα and RalGDS. Residues in the interaction hotspots are represented following the color code: R1, made up of residues 47-52 in R-RAS2, in pale pink; R2, residues 32, 35-36, 38, 40, 42, 44-45 in R-RAS2, in orange; R3, containing amino acids 47-48, 74-75 in R-RAS2, in pale cyan; R4, including residues 49-52 in R-RAS2, in light blue; and R5, residues 35,36 in R-RAS2, in green. The same color code was used

for the corresponding hotspots residues in the effector structure. Protein sequences were obtained from Protein Data Bank (PDB codes: R-RAS2, 2ERY; PI3Ka, 6V07; RalGDS RBD, 2RGF) and structural representations were generated using PyMol software.

(RA) domains. No structural or sequential conserved pattern has been found in the different interactors of R-RAS2. Instead, it seems that the surface provided by GTP-loaded R-RAS2 conformational restructuring enables superposition with the interactor. There are five key known amino acid regions involved in this interaction mechanism (Figure 8). These interaction hotspots happen to be extremely well conserved among the different RAS proteins, which suggests that effector-specificity must come from other non-conserved amino acids residing outside the interaction environment or non-identical amino acids within the interaction hotspots in RAS members [53].

1.3.5 Cellular, physiological and pathological functions

R-RAS2 regulates fundamental cellular processes, such as DNA synthesis, transcription and translation [23, 45, 48]. R-RAS2 has also been associated to cell proliferation and survival [23, 25, 47, 51, 52]. In addition, R-RAS2 has been described to be involved in cell migration through activation of PI3K and MAPK pathways [54]. In keeping with this, mutant R-RAS2 has been linked to invasion in vitro by promoting epithelial to mesenchymal transition and to metastatic processes in vivo [48, 55, 56]. A role for R-RAS2 in the control of the cell metabolism has also been postulated, as alterations in mitochondrial function, number and morphology has been described in B cells and cells from the central nervous system [57, 58].

Furthermore, its capacity for cell transformation made of R-RAS2 a focus of research interest soon after its discovery: it is the only non-classical RAS protein to have demonstrated transforming capacity levels comparable to those of classical RAS [18, 23, 41, 42, 44, 59, 60]. In keeping with the transforming capacity, RRAS2 knock-down cells decreased tumorigenicity when injected orthotopically in mice [48].

Further studies using R-Ras2-deficient mice have revealed essential functions for R-Ras2 at the physiological level. R-Ras2 has a pivotal role in the shaping of the adaptative immune system, by participating in the process of antigen presentation [57]

and the formation of the germinal centers [57]. Moreover, R-Ras2 was found to have a function in platelet activation and thrombus stability [61]. R-Ras2 is also essential for oligodendrocyte differentiation and survival [62] and for proper mammary gland development [63].

Conversely, R-RAS2 is also associated to pathological functions. Recently, *RRAS2* mutations have been reported in individuals diagnosed with Noonan Syndrome [38, 64]. The course of this syndrome includes physical particularities such as short stature and distinctive facial features, congenital heart defects, male infertility and mild intellectual disability, among other symptoms [65].

RRAS2 deregulation has been associated to cancer pathogenesis. In 1994, the first mutation of *RRAS2*, found in a cDNA library from ovarian cancer A2780 cells, was described [59]. The same mutation, a glutamine to leucine substitution in residue 72, was later described in the breast cancer CAL-51 cell line [66], and in juvenile myelomonocytic leukemia [67], germ cell line tumor [68] and central nervous system germinoma [69] patients. More recently, *RRAS2*^{Q72L} mutation was described as a hotspot in 9 tumor types [20, 21]. Along with the *RRAS2*^{Q72L} mutation, other

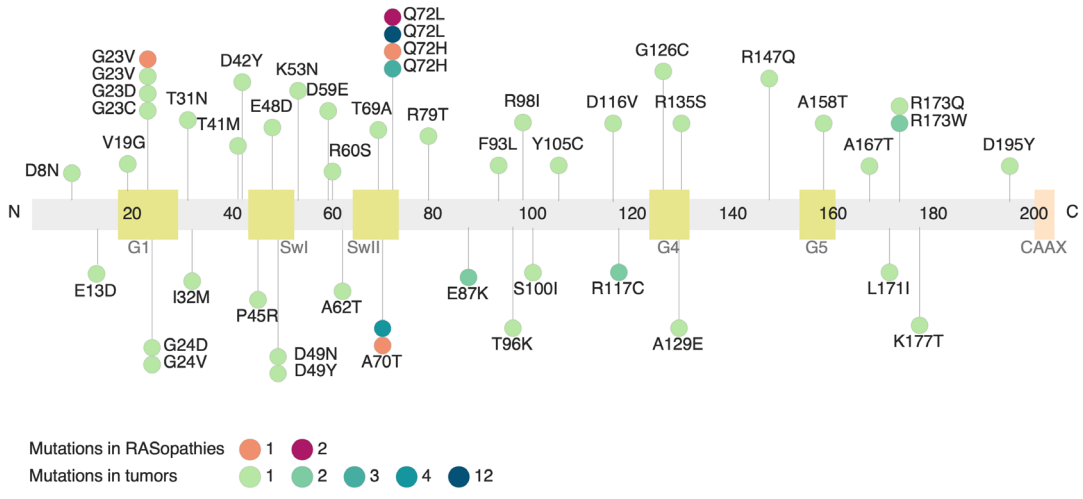

Figure 9. R-RAS2 mutations found in humans. Representation of protein variants affecting different residues in the R-RAS2 sequence. Mutations found in tumor and RASopathies patients are illustrated. Color code indicates the number of reported cases. Yellow boxes represent the G boxes of R-RAS2: G1, switch I, switch II, G4 and G5. Light orange box represents the CAAX sequence. Data was obtained from the COSMIC (cancer.sanger.ac.uk/cosmic) and cBioportal (www.cbioportal.org) genomic databases.

RRAS2 alterations equivalent to classical *RAS* leading mutations (such as the widely studied K-RAS G12C or N-RAS Q61L mutations) have been found in some tumors from the COSMIC database (cancer.sanger.ac.uk/cosmic) [69] (Figure 9). The most frequent mutations are the ones affecting the switch II, but a functional and oncogenic characterization of the mutations is still lacking.

Wild-type *RRAS2* is found upregulated in breast tumor cell lines [60], skin cancer [70-73], lymphoid malignancies [50], hepatocarcinoma [56] and in tumors of the central nervous system [74]. Genetic amplification is also seen in gliomas arising after radiotherapy treatment [75]. By contrast, *RRAS2* downregulation has been described in *KRAS*-mutated colorectal cancer [76].

Despite its promising attributes as a putative novel driver, considerably little research has been done to understand the cancer driver and pathobiological roles of R-RAS2. Recent massive pan-cancer screenings have uncovered gain-of-function mutations at low frequency, suggesting that *RRAS2* could be an autonomous driver of tumorigenesis. Considering the potential diagnostic, clinical and therapeutic interest of *RRAS2* mutations, we decided to make R-RAS2 protein the focus of our work.

2 OVARIAN CANCER

2.1 SEX DETERMINATION AND GONAD FORMATION IN MAMMALS

In mammals, genetic sex determination is established when gonads start to differentiate. Such a differentiation is orchestrated by the key genes such as *Wt1*, *Sry*, *Sox9* and *Amh* in males, and *Foxl2*, *Rspo1*, *Wnt4* and *Ctnnb1* (encoding for β-catenin) in females. Opposite sex genes play antagonistic roles and are involved in mutually exclusive regulatory loops (Figure 10). These molecular networks have been demonstrated to be very similar among mammals, but mice are by far the most studied organisms [77-82]. Therefore, all the genes mentioned in the present section are referred to mice. Beyond sex differentiation, expression of some of these genes is maintained in a cell-specific pattern in the adult differentiated gonad. For instance, *Wt1* is specifically expressed in

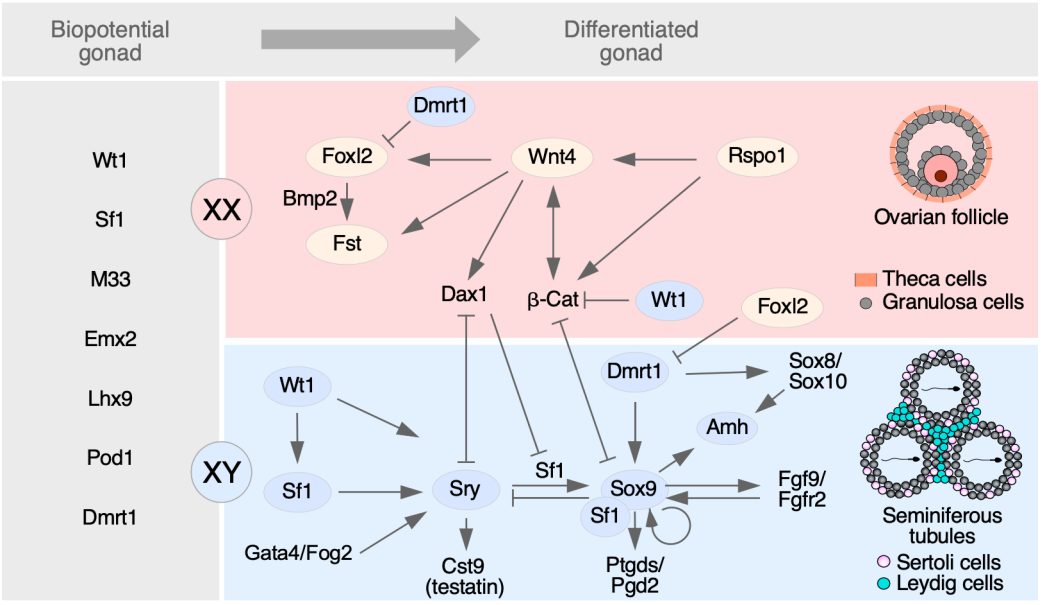

Figure 10. Molecular programs involved in gonad differentiation. Schematic representation of the activation and repression of genes regulating the gonad differentiation process in mice. Bipotential gonads express the same set of genes for XX and XY individuals. Key genes start the differentiation programs giving rise to ovaries or testis.

both granulosa and Sertoli cells in the adult ovary and testis, respectively [83].

Sexual fate must be actively maintained throughout the adult life. Deregulation of sex-determining key genes in the adult gonads can lead to sex reversal [78]. Two genes have a leading role in maintaining sexual phenotype in mice: *Dmrt1* (from doublesex and mab-3 related transcription factor 1) and *Foxl2* (from forkhead box L2) [80]. A sophisticated antagonistic relationship between both genes ensures gonadal phenotypic maintenance throughout adult life [84]. Interaction of *Foxl2* with estrogen receptors-α and -β (ER-α-) directly repress *Sox9* expression, an event needed for ovary phenotypic stability. Deletion of *Foxl2* triggers *Sox9* expression and subsequent granulosa and theca cells transdifferentiation into Sertoli-like and Leydig-like cells, respectively. This molecular reprogramming is accompanied by other aspects of testis differentiation such as the formation of seminiferous tubule-like structures, production, and release of male-like levels of testosterone, upregulation of testis-related genes, and silencing of ovary-promoting genes [78]. *Dmrt1* is the *Foxl2* counterpart in male gonads. Loss of Dmrt1 in adult testes results in transdifferentiation of Sertoli cells into granulosa-like cells expressing *Foxl2* [84]. Additionally, *Dmrt1* expression in adult ovaries represses

Foxl2, leading to the reprogramming of granulosa cells into Sertoli-like cells [85]. Similar to the mode of action of *Foxl2*, *Dmrt1* deregulation exerts other molecular events that lead to sexual phenotypic switch. Together, these genes have shown to be key for the upkeep of sexual fate and deregulation of these genes and their related network can lead to sex reversal [80].

2.2 OVARIAN STRUCTURE AND CELL POPULATIONS

The ovary can be divided in two main components: the ovarian follicles and the ovarian stroma. Ovarian follicles change in morphology and composition as they go through the maturation process that gives rise to the female gametocyte. Mature follicles are composed of three cell types: the oocyte or the female germ cell, the granulosa cells,

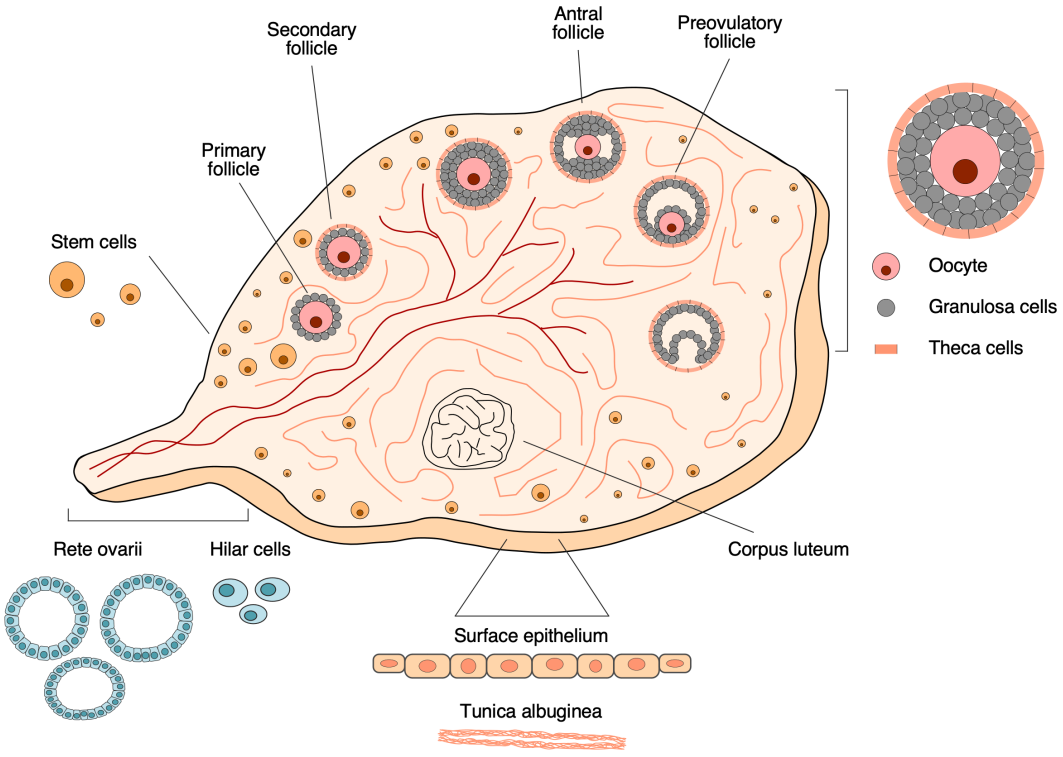

Figure 11. **Ovarian cell components**. Illustration of the human ovary showing the different cell populations. Steps of the follicle maturation process are represented: primary follicle formation, secondary follicle, antral follicle, preovulatory follicle and corpus luteum. Corpus albicans is not represented. Illustration inspired in [90].

and the theca cells (Figure 11). Both granulosa and thecal cells are involved in the control of the ovarian follicle maturation through the sensing of the gonadotropins and the production of steroid hormones [86]. In the follicular growth, however, most follicles undergo atresia, a degenerative process that depends on the apoptosis or autophagy of granulosa cells [87, 88]. This process ensures that only healthy oocytes will ovulate [89]. The ovarian follicles that do not undergo atresia, after ovulation, eventually develop into corpus lutei.

The ovarian stroma plays a fundamental role in ovarian function throughout both its development phase and adult life. Besides non-specific components such as immune cells, blood vessels, nerves and lymphatic vessels, several ovarian cell populations can be found in the stroma. The ovarian surface epithelium is a keratin-rich epithelial cell layer participating in the reparation after the mechanic stress originated by cyclic ovarian changes. The ovarian tunica albuginea is a thin layer of connective tissue that remodels before ovulation with a protective purpose. The rete ovarii are vestiges of the mesonephric ducts with not well understood functions. Hilar cells are known to participate in androgens production upon gonadotropin stimulation, however, their entire function has not been stablished. Moreover, the ovary contains stem cells able to differentiate to a number of ovary-specific somatic cells and also germline cells (Figure 11). Lastly, a mixed population of uncharacterized cells are lodged in the ovarian stroma, known as stromal cells, including fibroblast-like cells (Figure 11) [90]. For the purpose of this thesis, a deeper description of the rete ovarii is required.

This structure, and its male counterpart the rete testis, originate from cells of mesonephric origin which migrate to the developing gonad during embryogenesis. The rete ovarii localizes at the hilus of the ovary [91]. In the rete ovarii of adult mice, three parts can be distinguished based on the location and morphology: (i) the extraovarian rete, surrounded by the parovarian tissues; (ii) the connecting rete, in contact with the ovarian ligament; and (iii) the intraovarian rete, situated within the ovary [92, 93]. Although this ovarian structure is not well understood yet, it is thought to regulate the meiosis of germ cells and the differentiation of granulosa cells, and to have secretory functions in the adult ovary [90, 93].

2.3 OVARIAN CANCER

The term ovarian cancer comprises a complex variety of diseases with unique histopathological features. Several attempts have been made to find out the proper classification system for ovarian cancer since the early 70s. Most recently, the World Health Organization (WHO) established a classification system based on the histological and genomic traits of the different tumor types [94]. However, for the purpose of this thesis, a classification based on the cell of origin is more suitable. Thus, ovarian cancers can be divided into epithelial, sex cord-stromal, germ cell and miscellaneous tumors [95]. Ovarian cancers of epithelial origin account for 95% of all cases [96]. This group was originally classified as epithelial because it was thought to arise from the ovarian surface epithelium [79]. However, this group is constituted by tumors of different subtypes, including serous, endometroid, mucinous and clear cell, being the high grade serous ovarian carcinoma the most prevalent with 70% of the epithelial ovarian cancer cases [96]. Non-epithelial tumors account for 5% of malignant ovarian tumors [97]. Sex cord-stromal tumors include granulosa-theca cell tumors, thecoma-fibromas and Sertoli-Leydig cell tumors. Among them, granulosa cell tumors are the most frequent, accounting for 90% of the sex cord-stromal tumors [97]. Germ cell ovarian tumors arise from primordial germ cells. They include teratomas, dysgerminomas and Yolk sac tumors, among others [79]. Miscellaneous tumors are classified in rete ovarii, mesothelial and soft tissue tumors [79]. Discrepancies in the tumor classification convolute the establishment of accurate frequencies for the different cancer subtypes.

Despite intensive study during many years, the cellular origin and pathogenesis of ovarian cancer is not completely determined. The silent character of this disease complicates its early diagnosis. In fact, most ovarian cancer cases are diagnosed after the tumor has metastasized [98] and spread of the tumor might conceal the cell of origin. Moreover, the rarity of some tumors, together with the lack of understanding of the ovarian tumorigenesis, leads to the frequent misdiagnosis of the most uncommon ovarian cancer subtypes. Indeed, frequency of rete ovarii tumors, the object of study of the present thesis, has been claimed to be misestimated as their histologic phenotype is similar to cystic tumors of ovarian surface origin [99]. For these reasons, there is an

imperative need for finding molecular markers that provide a suitable method for early detection of ovarian cancer, unequivocal subtype diagnosis and specific gene-targeted treatment.

2.3.1 Ovarian cancer epidemiology

Ovarian cancer is the third most frequent gynecologic cancer, following cervical and uterine cancer [100]. Nonetheless, ovarian cancer ranks first in relation to its poor prognosis and high mortality rate, with a 5-year survival rate of approximately 45%. Incidence of ovarian cancer primarily affects 50-60-year-old women [101], who account for more than 70% of the total cases. Lethality of this disease reflects its asymptomatic nature, in addition to the lack of regular screening due to the absence of proper and accurate early detection techniques. Early diagnosis of the disease, namely when tumor is still localized, increases 5-year survival rate up to 92% [102]. Among the known risk factors that impinge on the development of ovarian cancer, family history of the disease is the most significant, accounting for about 20% of ovarian tumors [103]. Mutations in *BRCA1* and *BRCA2* genes are responsible for most (65-85%) of the hereditary cases of ovarian cancer [104]. Other oncogenes and tumor suppressor genes have been found frequently mutated in ovarian cancer, such as *TP53*, *KRAS*, *BRAF*, *ERBB2*, *PTEN*, *CTNNB1* and *PIK3CA*, among others [105]. Additionally, other risk factors might contribute to the appearance of ovarian tumors, such as the presence of gynecologic conditions – polycystic ovarian syndrome, endometriosis, and pelvic inflammatory disease – or high body mass index [102].

2.3.2 Ovarian cancer treatment

Standard ovarian cancer management strategies consist of surgery and platinum-based chemotherapy. In the early stage of the disease, when the malignancy is confined to the ovaries, surgical removal of the uterus (known as hysterectomy), the fallopian tubes and the ovaries (named salpingo-oophorectomy) is generally successful. When ovarian cancer is diagnosed in fertile women, unilateral salpingo-oophorectomy is considered when tumor is affecting one ovary and the remaining is normal [106]. In more advanced stages of the disease, application of surgery followed by chemotherapy is more an attempt to achieve a decent quality of life than an accurate method to cure the disease.

Repeated surgical interventions are usually needed for complete removal of the tumor. Apart from being used as an adjuvant (after surgical management), chemotherapy is also used as a neoadjuvant (before surgery, in order to reduce the tumor volume) [106]. The primary chemotherapeutic approach for ovarian cancer treatment consists of the intravenous administration of a taxane/carboplatin combination. However, use of new treatments in combination with chemotherapy, such as PARP inhibitors, are strengthening ovarian cancer treatments [107].

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Appendix:

Resumen en Castellano

CARACTERIZACIÓN FUNCIONAL DE *RRAS2* MUTATIONS AND ROLE OF *RRAS2*^{Q72L} IN OVARIAN CANCER

TABLA DE CONTENIDOS

RESUMEN DE LA INTRODUCCIÓN

R-RAS2

R-RAS2 es una GTPasa perteneciente a la familia de proteínas RAS. Entre los miembros de la subfamilia de proteínas R-RAS, R-RAS2 es la única que ha mostrado capacidad de transformación comparable a la de las RAS clásicas. Esta GTPasa posee una alta homología estructural con las proteínas RAS, especialmente en los dominios implicados a la unión de GTP y su hidrólisis. Además de estos dominios, la estructura de R-RAS2 también posee otra región conservada entre las proteínas de la familia RAS, como es la caja CAAX, con una relevante función para la localización subcelular de estas proteínas. La alta homología entre R-RAS2 y las proteínas RAS clásicas se mantiene a nivel de modificaciones post-traduccionales y de localización subcelular. Sin embargo, esta última solo ha sido estudiada en modelos de expresión ectópica de R-RAS2.

Entre las moléculas reguladoras y efectoras de R-RAS2 encontramos numerosos conocidos interactores de otras proteínas RAS. De entre los interactores de R-RAS2, las rutas de señalización más ampliamente estudiadas son las vías de PI3K-AKT-mTOR y RAF-MEK-ERK.

Se han descrito numerosas funciones celulares para R-RAS2, que incluyen procesos celulares fundamentales, como la síntesis de ADN, la transcripción y la traducción; proliferación y supervivencia; migración y control del metabolismo mitocondrial. Además, a nivel fisiológico, R-RAS2 ha demostrado ser esencial para el modelado del sistema inmune adaptativo, para la diferenciación y supervivencia de los oligodendrocitos y para el correcto desarrollo de las glándulas mamarias.

También se ha postulado un papel patológico para R-RAS2. Recientemente, se han identificado mutaciones de *RRAS2* en individuos diagnosticados con el Síndrome de Noonan. Por otro lado, la aparición de mutaciones en este gen en tumores humanos ha provocado una asociación de las mutaciones oncogénicas de RRAS2 con distintos

tipos de cáncer. De entre las mutaciones encontradas, las más frecuentes son las que afectan a las regiones implicadas en la unión a GTP, como el residuo Q72, equivalente a la posición Q61 en proteínas RAS clásicas. Aunque *RRAS2*^{Q72L} se ha descrito como un hotspot mutacional, esta mutación solo ha sido encontrada en baja frecuencia en la población estudiada. Más allá de las mutaciones, la versión silvestre de *RRAS2* también se ha visto desregulada en distintos tumores humanos. El aumento o disminución de su expresión en tumores ha demostrado ser específico para cada tejido.

A pesar de sus prometedoras cualidades como impulsor tumorigénico, se ha invertido considerablemente poco esfuerzo científico en tratar de dilucidar las funciones patobiológicas de R-RAS2. El advenimiento de las nuevas técnicas de secuenciación masiva ha permitido desvelar nuevas mutaciones que aparecen en baja frecuencia, manifestando un posible papel de R-RAS2 como impulsor autónomo de la tumorigénesis. Teniendo en cuenta el potencial interés diagnóstico, clínico y terapéutico de las mutaciones de *RRAS2*, esta tesis se ha centrado en el estudio de la proteína R-RAS2.

Cáncer de ovario

La formación del ovario a partir de la gónada bipotencial está regulada a través de un programa molecular específico de hembras, cuyos genes clave son *Foxl2, Rspo1, Wnt4* and *Ctnnb1*. La activación de este programa ocurre de manera paralela a la inhibición de los genes masculinos, entre los que destacan *Wt1*, *Sry, Sox9* and *Amh*.

Esta combinación de expresión de genes específicos de hembras y represión de genes específicos de macho debe mantenerse en el ovario adulto. Siendo la desregulación de estos programas moleculares capaz de inducir una inversión del sexo. Dos genes antagonistas son los responsables principales de este mantenimiento del destino sexual en los adultos: *Dmrt1* en machos y *Foxl2* en hembras. La desregulación de estos genes ha demostrado ser responsable de la transdiferenciación de células especializadas.

El ovario se puede dividir en dos componentes principales: los folículos y el

estroma ováricos. Los ovarios folículos cambian su morfología y composición durante el proceso de maduración que dan lugar al gametocito femenino. Durante el crecimiento folicular, la mayoría de los folículos sufre atresia, un proceso degenerativo que depende de la muerte de las células de la granulosa. De esta forma se asegura que solo los oocitos llegan al proceso de ovulación. El estroma ovárico está formado por componentes no específicos (como las células inmunes, entre otros) y poblaciones celulares ováricas. De entre los componentes específicos de ovario, es de especial interés para el propósito de esta tesis una descripción de la rete ovarii. La rete ovarii es una estructura con origen en el mesonefro que localiza en el hilio ovárico. Aunque su función no es el todo conocida, se piensa que interviene en la regulación de la meiosis de las células germinales y la diferenciación de las células de la granulosa, además de tener funciones secretoras en el ovario adulto.

El cáncer de ovario engloba a una compleja variedad de enfermedades con características histopatológicas particulares. Los tumores de ovario pueden tener distintos orígenes, siendo el cáncer de ovario de origen epitelial el más prevalente (95% de los casos). Aunque se ha estudiado durante muchos años, el origen celular y la patogénesis del cáncer de ovario aún no se conoce. Además, el carácter silencioso de esta enfermedad favorece un diagnóstico frecuentemente tardío que imposibilita la identificación de la célula de origen del tumor. Esto, unido a la falta de conocimiento sobre la tumorigénesis en ovario y la baja frecuencia de ciertos tumores, provoca el frecuente mal diagnóstico de los subtipos tumorales ováricos menos comunes. Esto pone de manifiesto la necesidad de encontrar marcadores moleculares que faciliten la detección temprana de los tumores de ovario, el diagnóstico inequívoco del subtipo tumoral y su tratamiento específico.

El cáncer de ovario es el tercer cáncer ginecológico más frecuente, no obstante, es el de peor prognosis y mayor tasa de mortalidad. Entre los factores de riesgo, la herencia genética supone un 20% de riesgo, con los genes *BRCA1* y *BRCA2* siendo responsables de la mayoría de los casos de cáncer de ovario hereditario (entre un 65 y 85%). Otros factores de riesgo incluyen la presencia de condiciones ginecológicas, como el síndrome del ovario poliquístico o la endometriosis, o el alto índice de masa corporal.

El tratamiento del cáncer de ovario comprende la aplicación de técnicas quirúrgicas muy invasivas (histerectomía o salpingooforectomía) y el uso de quimioterapias basadas en platino. En los casos muy avanzados de la enfermedad, este tratamiento es insuficiente para curar la enfermedad, y se realiza como un intento de conseguir una calidad de vida decente para el paciente. Actualmente, están surgiendo nuevas terapias combinatorias, como el uso de inhibidores de PARP que están fortaleciendo el tratamiento de ciertos cánceres ováricos.