Transformation by ras oncogenes induces the deregulation of intracellular signalling cascades that are critical elements in cell growth control. Ras genes code for small GTPases that act as GDP/GTP-regulated molecular switches, that mediate cell proliferation, growth and development [1]. The signaling activity of Ras is dictated by a regulated GTPase cycle that modulates the conformation of Ras and its affinity for downstream effectors [2]. Among those, Raf kinase, phosphoinositol-3 kinase and RalGDS are the ones best characterized [3, 4]; however the list continues to grow and more interactors have been reported to belong to the family of Ras effectors [5]. Although there is some structural data on these interactions, the physical characterization of these systems has been hampered by the relative instability and transient nature of such interactions.

1. Ras-Interactors from the RASOMICS database

- Objectives

As mentioned above, the list of Ras effectors has continued to grow and has provided the link between Ras activity and diverse biological responses.

Structural techniques have partly unraveled the Ras:effectors interaction mechanism. However, this set of protein-protein interactions is redundant and does not represent all partners. Possible Ras binding partners detected in vivo and reported in databases also span other protein families. So far, the studies carried out to rationalize the specificity of Ras proteins towards their effectors have been based only on the available set of Ras:effector crystal structures, ignoring the importance of protein flexibility.

- Work performed

In this work we extend the above to a large-scale bioinformatics and computational study, where we have carried out an extensive and massive driven-docking analysis for all the members of the Ras superfamily with those effectors for which no complex structure is available, but biological data supports complex formation. In this way we provide a broader picture of the Ras:effector association, which could then be used for extending the knowledge to other disciplines such as molecular biology or medicinal chemistry.

We are currently in the process of generating an open access knowledge-base resource focused on Ras superfamily and its effectors. This is meant to be an integrative approach in the line of wiki-like initiatives, where external knowledge will be can incorporated by other users as well.

- Results

For the sake of simplicity, we are describing here our findings only for the RasH interactors found through all the Protein-Protein Interaction (PPI) databases, grouped into several different categories according to the existence of specific domains along their sequence, thus creating functionally related groups (see Figure 1).

---

1. Ehrhardt, A., Ehrhardt, G.R., *et al.* (2002) Ras and relatives--job sharing and networking keep an old family together. *Exp Hematol,* **30:**1089-1106.
2. Vetter, I.R. and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions. *Science,* **294:**1299-1304.
3. Marshall, M.S..(1993) The effector interactions of p21ras. *Trends Biochem Sci,* **18:**250-254.
4. Malumbres, M. and Barbacid, M. (2003) RAS oncogenes: the first 30 years. *Nat Rev Cancer,* **3:**459-465.
5. Kiel, C., Foglierini, M., *et al.* (2007) A genome-wide Ras-effector interaction network. *J Mol Biol,* **370:**1020-1032.

Figure 1. Interaction network of RasH (a member of the Ras superfamily) and all the effectors extracted from the protein-protein interaction databases.

☑ Regulatory proteins.

The regulatory proteins include GTPase activating proteins (GAPs) and guanine nucleotide exchange factor proteins (GEFs). Although these proteins are very important for the functioning of Ras, in general, the interest for our project is reduced due to the extensive work already performed on them. Certain noteworthy cases are described below:

- **Protein containing a RasGAP domain:** two distinct GAPs have been identified for Ras; namely RASA1 and NF1. The complex of the p120GAP with Ras has been characterized by X-ray crystallography. The NF1 structure was solved as an isolated moiety and the fact that NF1 shares both sequence similarity and functional specificity with the C-terminal domain of RASA makes its use possible in driven-docking approaches.
- **Protein containing a RasGEF domain:** SOS1 is a very well characterized structure, both isolated and in the complex with Ras, with a high sequence similarity to SOS2.
- **Protein containing Ras Associated (RA) domain**: although they can exert a regulator role, they have been recognized as effectors of Ras as well. Some of them such as GNDS have been reported to function as a link between the Ras and Rho pathways. The structure of the RA domain for the latter is available and it has been used for modeling the interaction with Ras (one of the models in the article that we just published on the topic in TIBS, see attached CV).

☑ RBD:

The representative of this group in association with RasH has been characterized by crystallography (RasH:RBD-RAF). However, we have proposed the ternary model between RasH:RBD/CRD-RAF (in TIBS). Since small differences along the sequences for the isoforms are present, this opens possibilities to model the associations with the other isoforms and explain the

differences in binding affinity and biological functions observed in literature, based on the specificity originated in the interactions through the CRD.

☑ Kinases containing RBD or RA domains:

The interest on this group is quite high. Differences between the two isoforms of PI-3 kinases (PI3CG (γ-isoform) and PI3CA (α-isoform)) have already been revealed using crystallography and driven-docking approaches. They enable the search for specific targeted therapies for one or the other.

MK08, also known as JNK1, is a distant relative of the MAP kinases. This kinase responds to activation by several different factors, among them the activated RasH. This activation and the presence of conserved Thr and Tyr residues in subdomain VIII suggest certain similarities between the mechanisms of activation of ERK and JNK, although most likely the signalling pathway leading to the activation is distinct. It has been reported that oncogenic p21 (V12) binds directly to jun-N terminal kinase (JNK1). There is the possibility of modeling such an association, and thus revealing some interesting biological questions regarding this kinase group.

☑ Novel effectors:

This represents the most interesting group, in order to first assess these interactions and then characterise them. Little is known about them, however they might be playing important roles associated with cancer and other diseases.

GRB2 (Growth factor receptor-bound protein 2): GRB2 is an adaptor protein essential in the RAS signalling pathway, whose main role is to recruit SOS to allow Ras activation and subsequent translocation of MAP kinases into the nucleus and activation of early transcription factors. GRB2 is a modular protein composed of a central SH2 domain flanked by two SH3 domains. The SH2 domain recognizes and interacts with phosphotyrosine residues of activated tyrosine kinases. The SH3 domain has been shown to bind to several proline-rich domain-containing proteins, such as the guanine nucleotide exchange factor SOS1. These two domains are connected through highly flexible linker regions, making possible certain malleability in the interactions with several proteins. It has also been demonstrated that SOS1 associates with RasH. Several authors pointed to the formation of a Grb2-Sos-Ras complex. However, this complex could either be functional or structural. The GRB2:SOS1 complex is recruited to the plasma membrane, into proximity with (membrane-bound) RasH, which will position GRB2 and RasH in a favourable situation for a direct interaction. In case of confirmation of such a ternary complex, this will represent another point of targeting Ras upstream and blocking activated response in certain cases.

BCL2 (B-cell lymphoma 2): Proteins for the Bcl-2 family are important regulators of programmed cells; they can be either pro-apoptotic or anti-apoptotic. In certain conditions, such as for example during apoptosis, the newly synthesized Bcl-2 preferentially distributes in the mitochondrial compartments where it interacts with the mitochondrial Ras, blocking the Ras-mediated apoptotic signal. The possibility of finding inhibitors to abolish this association can help in triggering signals needed for the functioning of the cells.

LEG1: Galectin-1 is a member of an adhesion/growth-regulatory family known to interact for instance with ganglioside GM(1) and also the hydrophobic tail of oncogenic H-Ras. Ras transformation itself requires membrane anchorage and the overexpression of galectin-1 increases membrane-associated Ras. It will then be interestingto model such a complex in order to find ligands to inhibit the association to the membrane.

For every complex we have extracted information regarding the interaction partners through literature searches. For those cases where no interaction was reported at residue-level we have used bionformatics tools for the prediction of the binding interface. All this data is contained in the database. Apart from this information we are providing the number of clusters found in the docking

and which are the putative residues that can be mutated and thus confirm their implication in the association. A big part of such a database is dependent on the user input; either to corroborate the mutations above mentioned and to include them on a new run or simply to provide relevant information that is still not contained within the database. We hope that in such a way we can provide an extensive resource (the type of Ras wiki-like), interesting for all those researchers working in the Ras field.

2. Ras & classical effectors

- Objectives.

Ras proteins are molecular switches with a prolific signalling ability that enables them to recognize and select their binding partners amid a plethora of proteins present in the cell, with a remarkable affinity and specificity, thus securing the correct transfer of information and activating a wide spectrum of effector moleculesthrough more than one signalling pathways [6]. Among those, Raf kinase, phosphoinositol-3 kinases and RalGDS are the ones best characterized [7]. Despite the considerable structural similarity among all Ras-binding domains of these proteins, they have little in common in terms of sequence similarity and biological function.

Although many of these interactions are required for the normal functioning of the cell, Ras-associated aberrations in signal transduction occurring via protein-protein interactions have further implications in many cancer types [8]. One of the most challenging points in the signal transduction field is therefore to dissect these pathways and help define the implications at the protein association level for each of them. The generation of partial loss-of-function Ras variants, harboring mutations in their effector loop region, has been instrumental in understanding these selective protein associations and the implications in their pathways [6,9,10]. These single-point mutations are believed to bind selectively to only one of these effectors, thus only maintaining active the pathway that the particular effector regulates.

Undoubtedly, high specificity is essential for the identification of Ras interacting partners and the biological implications of these associations. However, the question is how do they achieve this specificity and how can the underlying mechanism be explained. With these ideas in mind and we have carried out an analysis at atomic detail of the interactions of Ras with these effectors, with the aim of addressing the following objectives:

1. Give an explanation of the atomistic details of the classical partial loss-of-function mutants of Ras for these systems.
2. Provide insight into the mechanism by which Ras recognizes the different RBDs among its effectors.

Such a mechanistic work facilitates the targeting of these interfaces using small molecules or redesigning them. However, the currently unanswered questions are how, given the considerable structural similarity of the Ras-binding domains of the various effectors, they achieve this structural specificity and how the underlying molecular mechanisms can be explained. To address these issues

---

6. White, M.A., Nicolette, C., *et al.* (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell,* **80:**533-541.
7. Marshall, C.J..(1996) Ras effectors. *Curr Opin Cell Biol,* **8:**197-204.
8. Gupta, S., Ramjaun, A.R., *et al.* (2007) Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell,* **129:**957-968.
9. Khosravi-Far, R., White, M.A., *et al.* (1996) Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol Cell Biol,* **16:**3923-3933.
10. Rodriguez-Viciana, P., Warne, P.H., *et al.* (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell,* **89:**457-467.

we have revisited the available literature on mutations and biochemical assays, and placed this information into a framework of atomistic interaction models of Ras and its effectors.

- Work performed.

Apart of the information concerning the so-called loss-of-function mutants of Ras, these three systems are very well characterized regarding the interaction sites of the effectors in spite of the lack of success in their structural characterization. We have exhaustively extracted information concerning residues involved in the binding and used this data as restraints to drive the docking of the Ras:effectors complexes. All the runs were performed using the driven-data docking algorithm called HADDOCK [11].

- Results.

A comparative structural analysis of all these three models generated permits us to get insight into the molecular mechanism underlying the specificity of these systems as well as providing an explanation on the 3D determinants in the specificity of the loss-of-function mutants.

Specificity in Ras and its Interactors

We have been able to to propose a plausible "recognition" motif on the effector structures and explain it in a semi-quantitative fashion: Ras specifically recognizes these RBDs by inducing a rotation along the interacting β-sheet of this domain with respect to the Ras effector region (Figure 2). The degree of rotation correlates with the length and complexity of the interfacial loop-rich regions just before and after the secondary structures, where the interacting residues are sitting. In such a way, RAF binds to Ras via a "perfect" canonical β-β sheet interaction, that gradually gets distorted for RalGDS and PI3Kα (see sequence alignment in Figure 2). These subtle differences in the orientation cause striking differences in the residues that participate in the different complexes, which can be used to selectively inhibit only one effector.

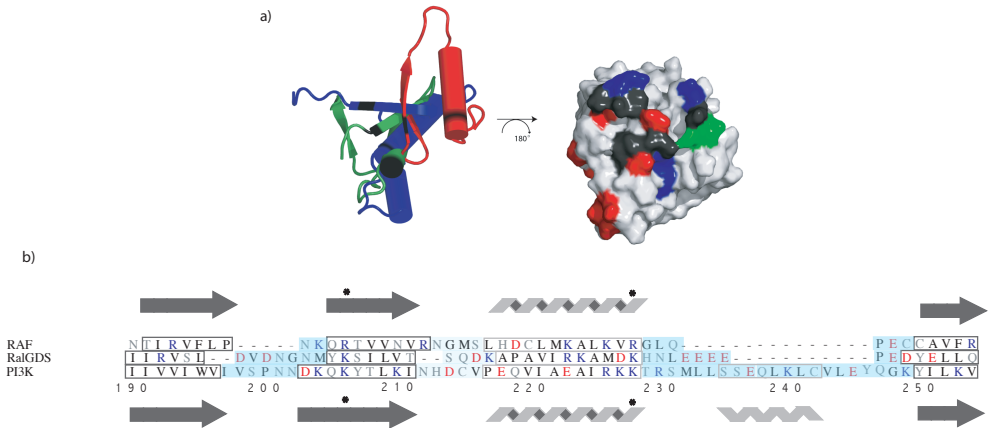

Figure 2. Role of Ras induced-rotation of the RBDs in the discrimination between various effectors. The cartoon in (a) shows an overlay of the structures of the three RBDs (RAF is coloured in red; RalGDS in green and PI3Kα in blue) and their relative orientations when binding to Ras. The surface representation of Ras in (a) depicts the residues proposed to form part of the binding site on Ras shared by all three effectors (grey) as well as those bound by a specific effector. (b) Sequence alignment for the three RBDs, loop-rich regions highlighted in pale blue; beta strands and alpha helices indicated by shapes above and below alignment; residues are color-coded by electrostatic charge.

---

11. Dominguez, C., Boelens, R. and Bonvin, A.M. (2003) HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. *J Am Chem Soc,* **125:**1731-1737.

3D insights into the Ras partial loss-of-function mutants.

We have been able to correlate the information on partial loss-of-function mutants that discriminate between various downstream pathways with the structural characteristics of the models for Ras and their effectors regulating these pathways. All the three effectors studied here have a RBD, although we have used, when available, other domains aside the RBD to mimic the biological event and unify biological data. Although all these domains have considerable structural similarities, they have little sequence identity, which affects slightly the binding mode as it has been previously mentioned. These deviations in the structural arrangements and/or in the 3D structures of the effectors are responsible for the observed differences. This is the case for example for the RBD of the kinases and that of RalGDS. The latter presents a relatively flat surface (Figure 3) that permits accommodate some of the mutations on Ras via a sliding motion without compromising its activity (E37G). However, due to the less partially positive charge for this receptor, a more concerted stabilization is required for the negatively charged residues on the surface of Ras (D38E and Y40C), so mutants of these residues will be highly affected (Figure 4).

In our opinion, the 3D models for the complexes of Ras and its three classical effectors that we have derived for this work provide a suitable structural framework to facilitate the interpretation of the wealth of available experimental information available and, in the long run, aid molecular biologists and medicinal chemists to understand the structural requirements for these different interactions. We hope to see such approaches guiding mutation design and more importantly leading to further progress in the development of anti-Ras drugs that target these interactions.

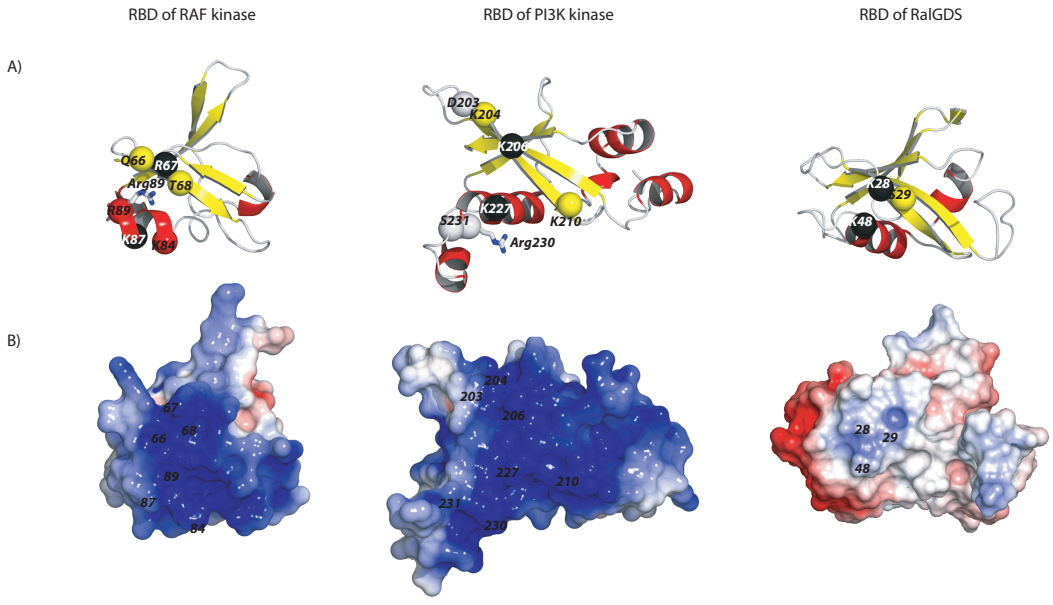

Figure 3. Fold and Surface Representation of the Classical effectors of Ras. In the top row, cartoon representation of the a) the RBD of Raf kinase, b) the RBD of PI3Kα and c) the RBD of RalGDS. In these structures, the residues involved in the binding with Ras have been shown as spheres, and the two positively charged conserved residues have been coloured in gray. In the bottom row, and following the same order, the electrostatics surfaces for these domains is presented. All these domains belong to the same family, however there striking morphological differences that will determine the binding to Ras.

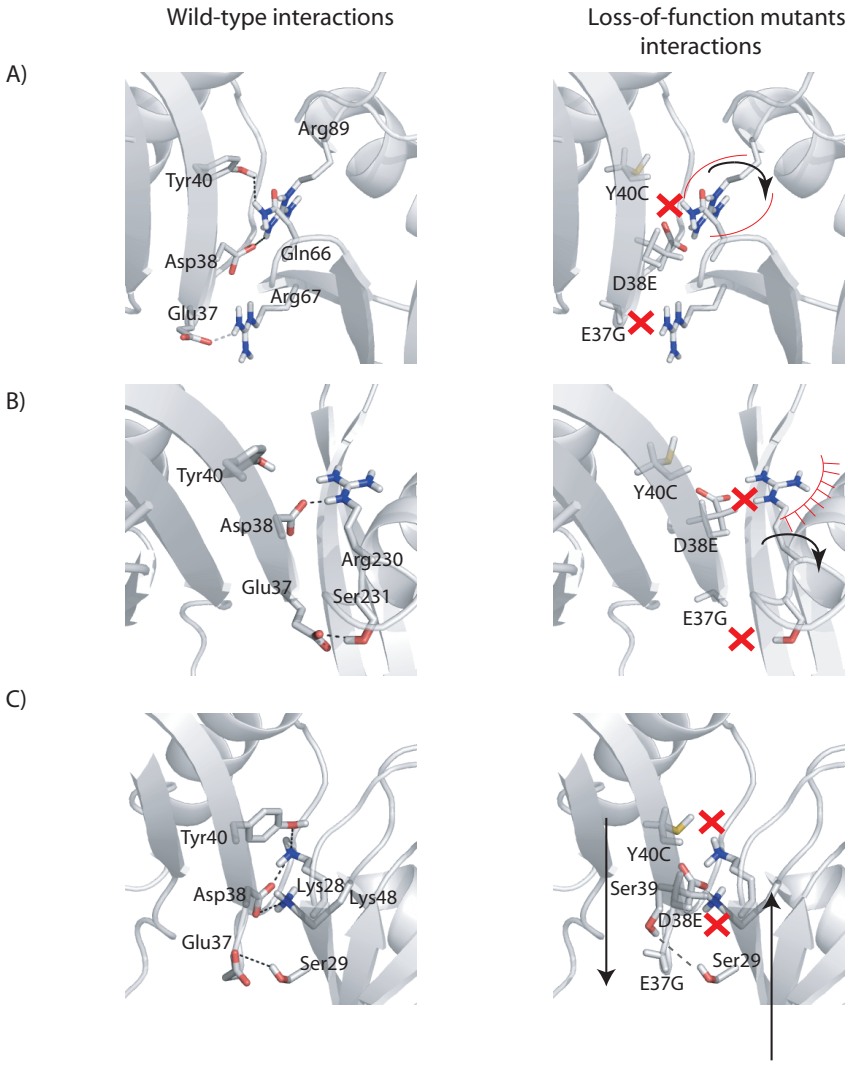

Figure 4. Interactions at residue level between Ras and its effectors for the wild type (left column) and the loss-of-function mutants (right column). This analysis points at different mechanisms, such as rotations of side chains (A), steric restrictions (B) and sliding of interfaces (C), by which the interaction with these diverse effectors is selectively affected by a point mutation.