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Paper

MST2-RASSF protein–protein interactions through SARAH domains

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Abstract

The detailed, atomistic-level understanding of molecular signaling along the tumor-suppressive Hippo signaling pathway that controls tissue homeostasis by balancing cell proliferation and death through apoptosis is a promising avenue for the discovery of novel anticancer drug targets. The activation of kinases such as Mammalian STE20-Like Protein Kinases 1 and 2 (MST1 and MST2)—modulated through both homo- and heterodimerization (e.g. interactions with Ras association domain family, RASSF, enzymes)—is a key upstream event in this pathway and remains poorly understood. On the other hand, RASSFs (such as RASSF1A or RASSF5) act as important apoptosis activators and tumor suppressors, although their exact regulatory roles are also unclear. We present recent molecular studies of signaling along the Ras-RASSF-MST pathway, which controls growth and apoptosis in eukaryotic cells, including a variety of modern molecular modeling and simulation techniques. Using recently available structural information, we discuss the complex regulatory scenario according to which RASSFs perform dual signaling functions, either preventing or promoting MST2 activation, and thus control cell apoptosis. Here, we focus on recent studies highlighting the special role being played by the specific interactions between the helical Salvador/RASSF/Hippo (SARAH) domains of MST2 and RASSF1a or RASSF5 enzymes. These studies are crucial for integrating atomistic-level mechanistic information about the structures and conformational dynamics of interacting proteins, with information available on their system-level functions in cellular signaling.

Key words: competing protein interactions; SARAH domains; signaling switches; apoptosis; cell fate decision; molecular dynamics

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Molecular modeling in cancer research

The complexity of cancer and the vast amount of experimental data available have made the use of modern computer-aided approaches crucial for investigating specific molecular interactions and their corresponding molecular mechanisms. For this reason, there is an increasing interest in these computational methods. The large variety of available computational tools ranges from sequence-based, bioinformatics-based approaches to advanced ab initio calculations. Some of the most used methods include docking proteins with associated drugs to estimate the interaction, coarse-grained models, elastic network models and atomistic molecular dynamics among others [1, 2]. To combat cancer and other diseases, several system-related approaches have become the focus of attention, a key element corresponding to the regulation of the Hippo pathway that controls cell death, in an attempt to induce apoptosis in cancer cells.

The Hippo pathway

The Hippo pathway was discovered in genetic screens in the fruit fly *Drosophila melanogaster*, where knocking out its name-giving component, the kinase Hippo, causes tissue overgrowth [3, 4]. This phenotype is owing to a reduction of cell death and stimulation of proliferation combined with a loss of proper organ size control. In *Drosophila* the core pathway consists of Hippo, which phosphorylates and activates Warts, another kinase, which phosphorylates the Yorkie protein. This phosphorylation sequesters Yorkie in the cytosol preventing it from transcribing antiapoptotic and growth stimulatory genes in the nucleus, such as cyclin E, Diap1 and other targets [5]. The peripheral stimuli that regulate the Hippo pathway in *D. melanogaster* include cytoskeletal cues and cell–cell contacts, but although several components are known, the biochemical mechanisms of this regulation remains to be discovered [6].

In mammalian cells, the core components are conserved: the Mammalian STE20-Like Protein Kinases 1 and 2 (MST1 and MST2) correspond to Hippo, the Large Tumor Suppressor Kinases 1 and 2 (LATS1 and LATS2) correspond to Warts, and the Yes-associated proteins 1 and 2 (YAP1 and YAP2) are homologues of Yorkie [4]. However, the functional wiring of the pathway is different in mammalian cells [7], where MST1/2 can directly regulate YAP [8] (Figure 1A), and where the regulation of MST1/2 includes inhibition by ARAF [9] and CRAF [10] kinases, which have no direct homologues in *D. melanogaster* (Figure 1B). Another example is RASSF proteins, which inhibit Hippo in *D. melanogaster*, but activate MST1/2 kinases in mammalian cells [11, 12]. The reasons for these discrepancies are unclear, but may be related to the fact that the RASSF–MST1/2 signaling pathway in mammals has evolved to become a major tumor suppressor pathway [13, 14]. By contrast, *D. melanogaster* has no naturally occurring tumors, and hence, the Hippo pathway plausibly may serve different functions in the fruit fly.

The mammalian Hippo pathway contains several tumor suppressors, such as RASSF1A, LATS1, p73 and p53 (Figure 1B). Thus, it is no surprise that in addition to the regulation of cell proliferation and organ size, the mammalian Hippo pathway is also an important mediator of apoptosis (Figure 1B). It contributes to apoptosis initiated by death receptors, such as Fas, by inducing the formation of a YAP/p73 transcription factor complex that activates the expression of the pro-apoptotic gene PUMA [12]. It also participates in apoptosis induced by oncogenic KRAS, which is the only RAS gene family member that when mutated can not only cause cell transformation and cancer, but also apoptosis [15]. In this scenario, mutated KRAS binds RASSF1A, leading to the activation of MST2 and LATS1, which inactivate the murine double minute protein MDM2 (an inhibitor of p53), thus promoting p53 accumulation and apoptosis [16]. These functions, where the same core pathway processes different upstream inputs into different effector mechanisms of apoptosis illustrate the versatility of RASSF and MST1/2 signaling and its potentially widespread roles in apoptosis regulation in mammalian cancer cells [17].

Thus, great efforts are being made to understand the Hippo pathway not only in terms of genetic interactions but also in

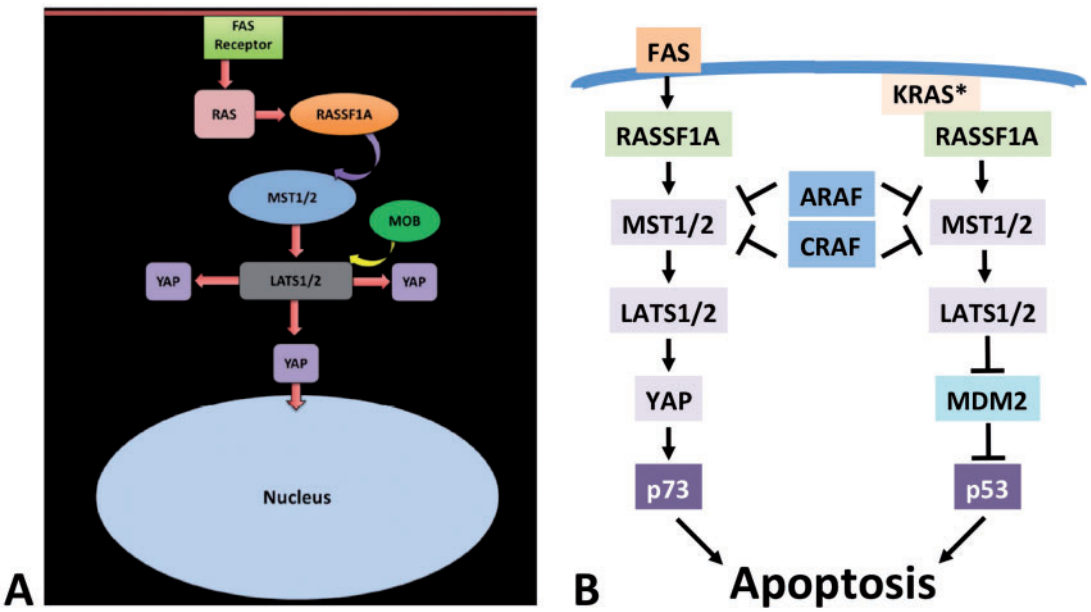

Figure 1. (**A**) Schematic representation of apoptosis regulation by the mammalian Hippo signaling pathway, along which (**B**) the regulation of MST1/2 involves the inhibition by ARAF and CRAF kinases, which do not contain a SARAH domain, leading to p73- and p53-mediated apoptosis (see text for details).

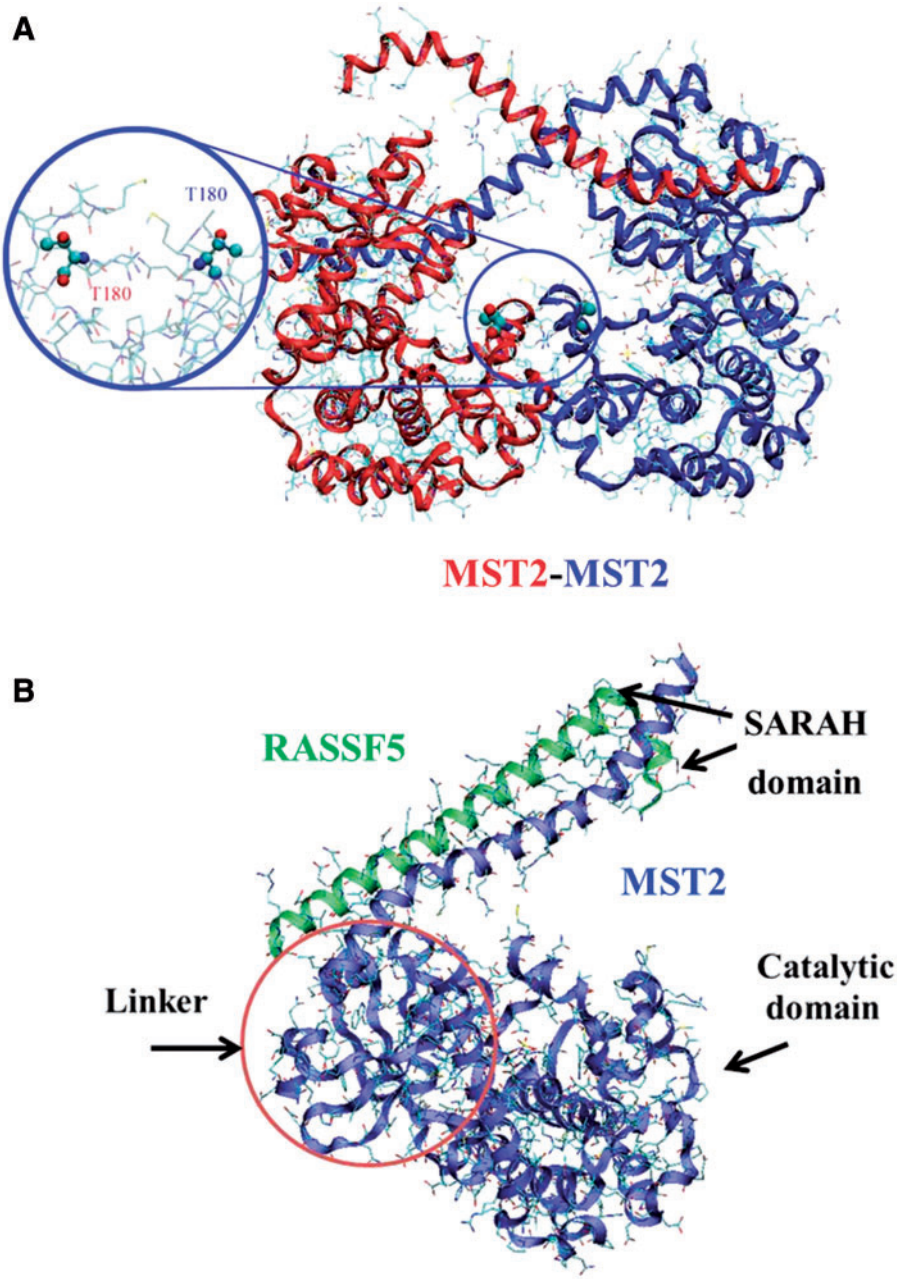

Figure 2. (**A**) MST2-MST2 dimer structure (PDB ID: 4LG4). Activation loop and T180 have been highlighted. (**B**) MTS2-RASSF5 complex from crystal structure (PDB ID: 4LGD) showing the direct interaction between the RASSF5 (green) and the MST2 (blue) SARAH domains. The MST2 kinase domain (blue) is also resolved in the 4LGD crystal structure. Representation of the possible linker between the MST2 kinase and SARAH domains is also shown though, due to its intrinsically disordered nature, it cannot be resolved experimentally. A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

terms of its biochemical and mechanistic regulation at the level of its protein components, in particular the core kinases MST1/2 and LATS1/2 [4, 18]. The regulation of protein–protein interactions seems to play an important role in the control and function of this pathway. Recent data show that RASSF1A can release MST2 from its inhibitory complex with CRAF. The competition between RASSF1A and CRAF for MST2 binding, combined with phosphorylation of key residues that modulate the binding affinities, causes switch-like transitions between MST2-RASSF1A and MST2-CRAF protein complexes, which coordinate the mutually exclusive decision between apoptosis and proliferation [19]. These interesting biochemical properties and their biological consequences are closely linked to structural features of the protein interactions that allow dynamic regulation and the adjustment of signal flux through the pathway appropriate for triggering highly specific biological responses. Here, we will focus our attention on the MST1/2 kinases, which once phosphorylated, transduce the cell signal toward the LATS1/2 kinases [20], and their interactions with RASSF scaffolds, which regulates their activity.

The activation of kinases such as MST1 and MST2 is modulated through both homo- and heterodimerization (Figure 2A and B respectively; e.g. interactions with RASSF scaffold proteins). While this is a key upstream event in this pathway, it remains poorly understood. On the other hand, RASSFs (such as RASSF1A or RASSF5) act as important apoptosis activators and

tumor suppressors, though their exact regulatory roles are also unclear.

SARAH domains

An important characteristic of MST proteins is a particular helical segment, known as the SARAH (Salvador/RASSF/Hippo) domain motif, which has been found to be essential in the activation process of those proteins, and therefore in the initiation of signal transduction [5].

The SARAH domain is located in the C terminus region in three types of eukaryotic proteins denoted in its name (i.e. Salvador, RASSF and Hippo), which are known to be tumor suppressors [5]. The SARAH domain mediates signal transduction from Hippo via the Sav (Salvador) scaffolding protein toward the protein Wts (Warts), downstream along the signaling pathway, by acting as a scaffold that facilitates the Wts phosphorylation by Hippo [3–5, 21]. The SARAH domain is also involved in the dimerization of the mammalian MST1/2 kinases, which form homodimers via their C-terminal SARAH domain. SARAH domains are also known to be associated with other protein domains [5, 21], such as in kinase domains, the WW/rsp5/WWP domain [22–24], the C1 domain [25], the LIM domain [26] or the Ras-associating (RA) domain [27, 28].

The process of signal transduction involves the formation of MST2 dimers, and the dimerization process is governed by the interaction through SARAH domains [12, 19, 29–32] (Figure 3A). In addition, RASSF scaffolds also interact with and regulate the activity of MST proteins through protein interactions mediated by their SARAH domain (Figure 3B). However, despite the fact that the SARAH domain is essential in the dimerization process, MST1/2 proteins may interact using their SARAH domain with another type of suppressors that do not contain a SARAH domain, such as ARAF [9] and CRAF (Figures 1B and 3C). One example is CRAF, which controls cell proliferation, oncogenic transformation, differentiation and apoptosis. CRAF binds to a small segment of the MST2 SARAH domain competing with RASSF1A [12, 19, 29] and RASSF5 scaffolds [33]. Other possibilities for the SARAH domain function have been considered and are still in progress of investigation by our group, for example, covering the activation loop in the MST itself (Figure 3D), and even interactions with three SARAH domain members. Trimers, have been postulated for Salvador [5] (Figure 3E) and between MST2, WW6 and RASSF1A (or RASSF6) [34, 35]. However, some studies suggest that Sav-RASSF5-MST1 trimers may not be likely to form stable complexes under experimental conditions [36].

We focus our attention on those SARAH domains that belong to proteins in the Hippo pathway, in particular to those from MST1/2 kinases and RASSF scaffolds. Despite intensive research in the past decade, the role of SARAH domains is still poorly understood. It is known that these long helixes play a crucial role in the MST dimerization, and through them, RASSF scaffolds control the activity of such kinases. In particular, members of the RASSF family (RASSF1-10) have been denoted as tumor suppressors scaffolds, which are frequently down-regulated by promoter hypermethylation in several types of cancer. These proteins present common types of RA and SARAH domains, which may potentially bind Ras oncoproteins and play an important role in protein-protein interactions with other proteins through their SARAH domains (e.g., with MST). However, among the entire RASSF family scaffold, only RASSF1-6 possess SARAH domains, while the SARAH domain is absent in the remainder (RASSF7-10) [37]. A multiple sequence alignment of the SARAH domains using Clustal Omega [38–40] is shown in Figure 4A. By comparing the primary structure of the SARAH domains from the RASSFn family, we observe a moderate to high level of sequence identity and sequence similarity among all of the SARAH domains belonging to the RASSF scaffolds family. In particular, RASSF5 (also known as Nore1 or RAPL) and RASSF1A are highly conserved and present a high percentage of sequence identity (54.1%) and similarity (89.4%) (Figure 4B) (G. Sánchez-Sanz *et al.*, unpublished data). In addition, MST and RASSF SARAH domains are also highly homologous, as both helical structures have fair sequence identity and similarity [41]. For example, MST2 and RASSF1 (Figure 4C) have 31.4% identity and 64.6% sequence similarity, which is reflected in their structural similarities (G. Sánchez-Sanz *et al.*, unpublished data). This is important because RASSF1A has been identified to mediate proapoptotic signals through binding of the mammalian sterile 20-like kinases 1 and 2 (MST1 and MST2) [42, 43].

It is also worth mentioning the significant pioneering efforts that have been made to crystallize SARAH domain structures. A thorough search on the UniProt [44] and RCSB PDB data banks reveals several crystal structures of SARAH domain monomers and homo- and heterodimers. The crystal structure of MST SARAH domains have been resolved by X-ray diffraction, for MST1, PDB ID: 2JO8 [36], 4OH8 [45], 4NR2 (A. Chaikuad *et al.*, unpublished data), 2YMY [41] and for MST2 PDBID: 4LGD [32]. In the case of RASSF family, there is a lack of crystal structures for any of the RASSF scaffolds, with the exception of RASSF5 (PDB ID: 4LGD [32] and 2YMY [41]). However, more important than studying the protomers alone is to look at dimeric structures, as the dimerization process between two SARAH domains is crucial in understanding the activation of MST kinases. As suggested by recent experiments, here we focus on understanding the specific dimeric interactions between the helical SARAH domains of MST2 and RASSF1A or RASSF5 scaffolds. Our discussion is aimed at the specific part of those proteins, SARAH domains and, in particular, at the structure of dimers formed between MSTn and RASSFn.

SARAH–SARAH domain interactions

As stated above, SARAH domains have been shown to form antiparallel, coiled coil dimers. In general, long helix–helix interactions, including dimer, tetramers and barrels of helices have been intensely studied [46] and the importance and nature of the intermolecular interactions between each have been highlighted. For example, the MST1-SARAH domain monomeric conformation has been shown to be thermodynamically unstable, and may unfold and later on dissociate without presenting any stable intermediate state [41]. More interactions of MST1 SARAH dimers and the influence of the intrinsically disordered inhibitory domain in the dimerization process have been previously studied [47].

The number of studies devoted to the SARAH–SARAH domains interactions is significant because they may govern the activity of MST kinases and therefore control cell signaling. Thus, SARAH domain interactions seem to act as a main driver for the activation of apoptosis. Here, we highlight some of those studies, which may contain not only experimental but also computational procedures related to crystal structures. Homodimers of MST1–MST1 have been studied and their crystal structures resolved (PDBID: 2JO8 [36], 4OH8 [46], 4NR2 (A. Chaikuad *et al.*, unpublished data)). Much attention has been attracted by MST2–MST2 homodimers, in which several crystal

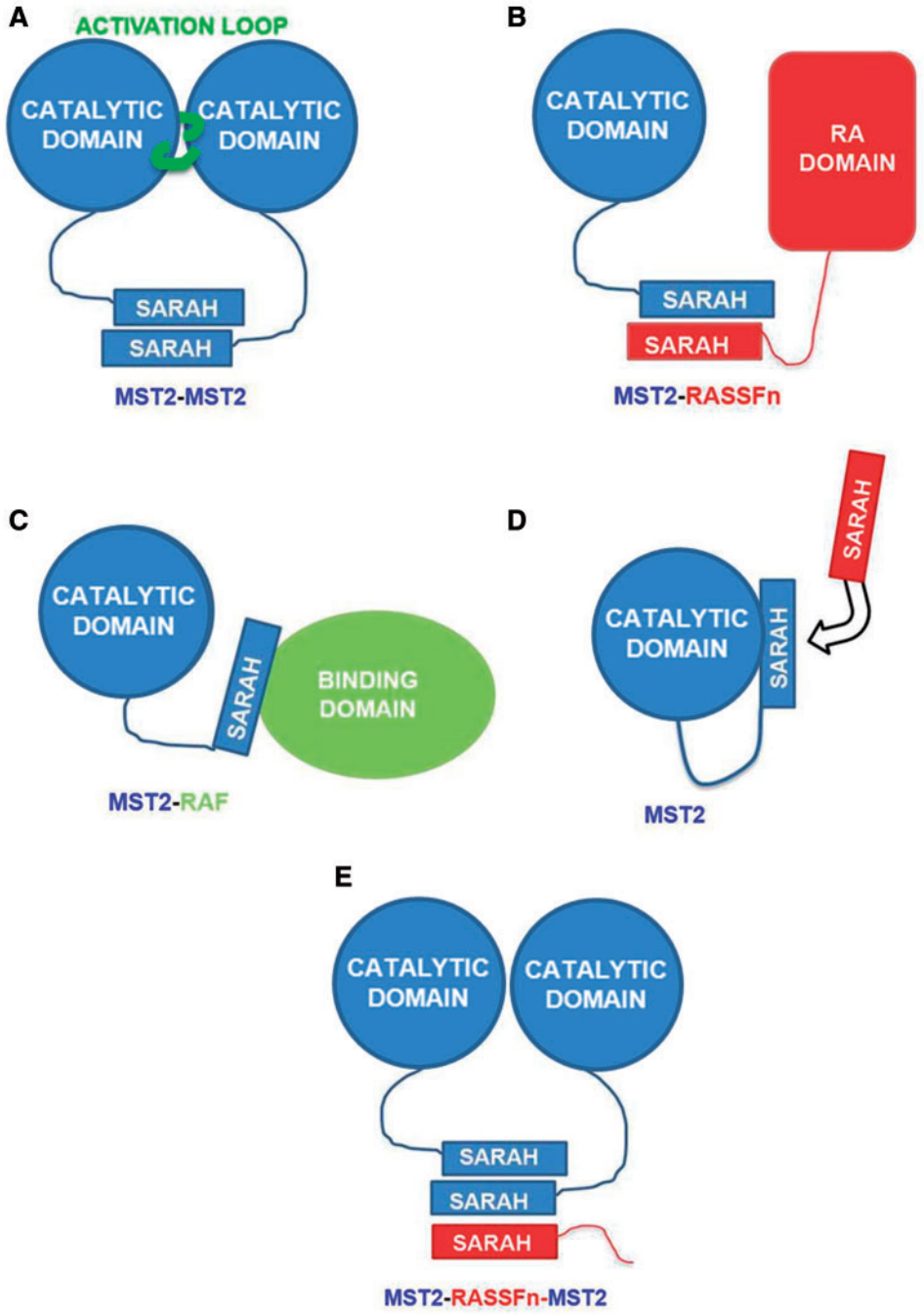

Figure 3. Schematic representation of possible SARAH-mediated intermolecular interactions. (**A**) MST2 (blue) homodimers can be subject to autophosphorylation mediated by an active loop (green). (**B**) RASSFn scaffolding proteins (red) can form competitively heterodimers with MST2 SARAH domains. (**C**) MST2 SARAH domains can interact with other binding domains from partners such as RAF. (**D**) Owing to its large and flexible linker, MST2 may present self-interactions of its SARAH and catalytic domains. (**E**) MST2 SARAH homodimers (blue) may be affected by tertiary (e.g. RASSFn) SARAH domains that could modulate the MST2 homo-interactions. A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

structures have been published (PDBID: 4OH9 [45], 4HKD (G.G. Liu *et al.*, unpublished data), 4L0N (A. Chaikuad *et al.*, unpublished data)). RASSF5-RASSF5 homodimers [41] have also been the subject of many studies. The structure has been successfully crystallized, and its structure in solution investigated including circular dichroism. Additionally, the dissociation energetics of RASSF5–RASFF5 dimers was also probed by isothermal titration calorimetry.

In the case of heterodimers, the majority of the experimental studies have been devoted to the MST–RASSF1 or MST–RASSF5 interactions [12, 19, 29, 30, 48]. For example, interactions between RASSF1A (and RASSF1C) and MST1 and MST2 have been identified and analyzed showing that this interaction depends on the C-terminal SARAH domain, i.e. dimerization of both SARAH domains of each protein [31]. Nevertheless, few crystal structures are available for

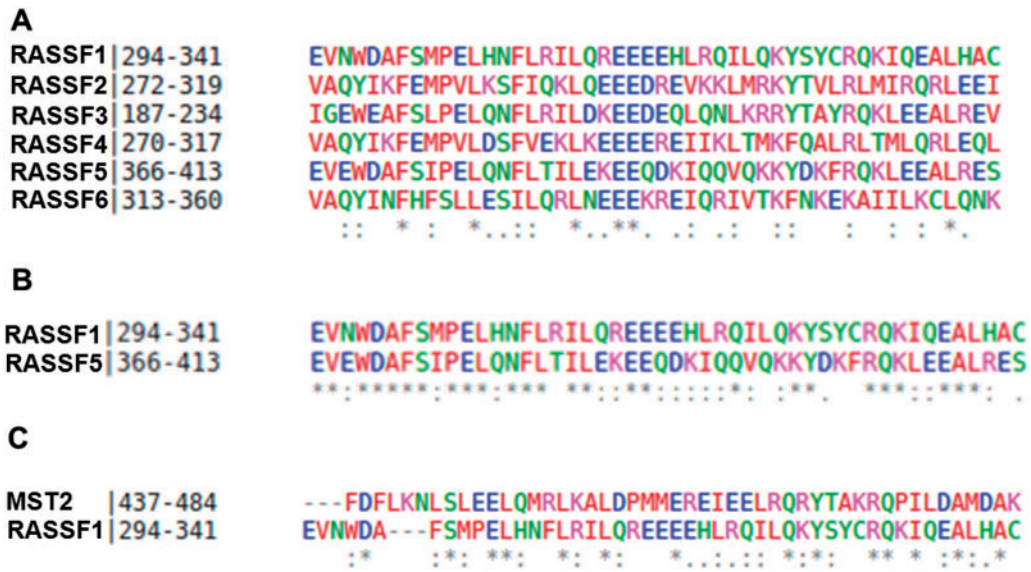

Figure 4. Multiple and pairwise alignments of SARAH domains using Clustal Omega software of (**A**) RASSFn, with n = 1–6, (**B**) RASSF1 and RASSF5 alone, and (**C**) MST2–RASSF1A. Color coding: red = small and amino acids (including aromatic - Y); blue = acidic; green = hydroxyl + sulfhydryl + amine + G; magenta = basic (without H). In the last row, the alignment results are represented as follows: an asterisk (*) = indicates positions that have a single, fully conserved residue, a colon (:) = indicates conservation between groups with strongly similar properties, a period (.) = indicates conservation between groups with weakly similar properties. In (C), the two domains have 31.4% sequence identity and 64.6% sequence similarity. A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

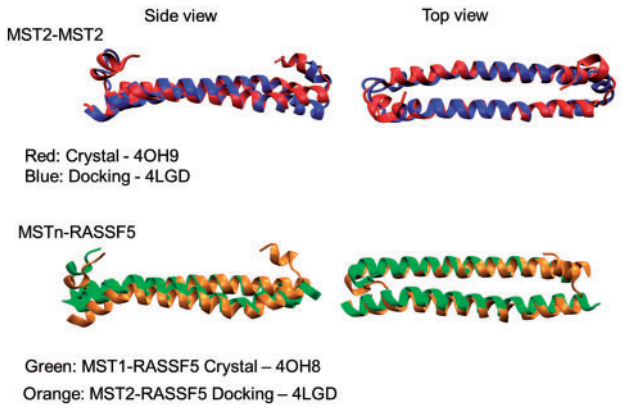

Figure 5. Structural alignment of crystal and docked structures of SARAH dimers. The top row shows MST2-MST2 SARAH homodimer structures in red: crystal structure from 4OH9, and in blue: based on the MST2 protomer structures from 4LGD. The bottom row illustrates in green: crystal structures of MST1-RASSF5 SARAH dimer (4OH9), and in orange: MST2-RASSF5 docked structures (based on the MST2 structures from 4LGD). A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

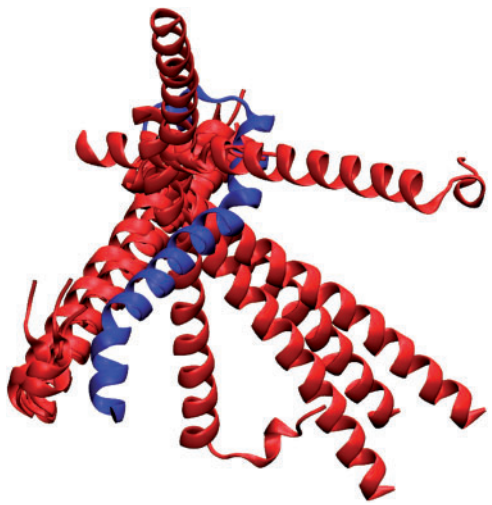

Figure 6. Top 10 highest scoring SARAH heterodimer structures from a docking study (using Zdock) of RASSF5 SARAH domains (red) docked on a target MST2 SARAH domain (blue, from the 4LGD structure). A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

heterodimers, i.e. MST1–RASSF5 dimers have been resolved (PDBID: 4OH8 [45], 2YMY [41]) and more recently MST2–RASSF5 SARAH dimers crystal structures have been published, 4LGD [32].

We have been actively researching the homo- and heterodimerization of MST2 and RASSF1 and 5 SARAH domains, paying special attention to the driving forces that govern the stability of such dimers (G. Sánchez Sanz *et al.*, unpublished data). We would like to highlight the importance of computational modeling for such tasks, in particular, those cases in which the crystal structure of a specific system is not available. Figure 5 shows a comparison between MST2-MST2 crystal structure (4OH9) and the one resulting from molecular docking simulation (Zdock [49–51]) using the MST2 monomer from 4LGD. As it is shown, the alignment of both structures match, revealing how the computational approach may reproduce the experimental structures. Furthermore, in Figure 5, two different heterodimers have been also compared; the MST1-RASSF5 SARAH crystal structure from 4OH8 and the MST2-RASSF5 modeled using 4LGD as template. This again shows that the computational approach predicts reliable structures directly comparable with experimental structures.

Recently, we have carried out an in-depth analysis not only of the possible poses arising from docking (Figure 6), but also using full atomistic molecular dynamic simulations to describe the behavior of those dimers in solvation and to study their intermolecular interactions (G. Sánchez Sanz *et al.*, unpublished data).

Figure 7 shows the different homo- and heterodimers investigated, MST2-MST2 (blue-blue), MST2-RASSF5 (blue-red) and MST2-RASSF1A (blue-green). It is worth noting that the MST2-MST2 and MST2-RASSF5 dimers were obtained from docking studies using 4LGD monomers as receptors and ligands. However, in the case of MST2-RASSF1A, there is no crystal structure available, so homology modeling was used using RASSF5 as a template, with RASSF1A sequence (Uniprot Q9NS23) using the SwissProt program [52]. Once again, computational modeling is shown to be a useful tool that gives insight into the biophysical properties, functions and biological mechanisms of proteins and their interactions within the cell (G. Sánchez Sanz *et al.*, unpublished data) [2, 53–55].

Interestingly, we also note that the SARAH–SARAH domain interactions are not restricted to dimers. In fact, the existence of SARAH domain trimeric complexes has been suggested by Scheel [5], though it has been subsequently questioned by Makbul *et al.* [41]. However, recent studies have successfully

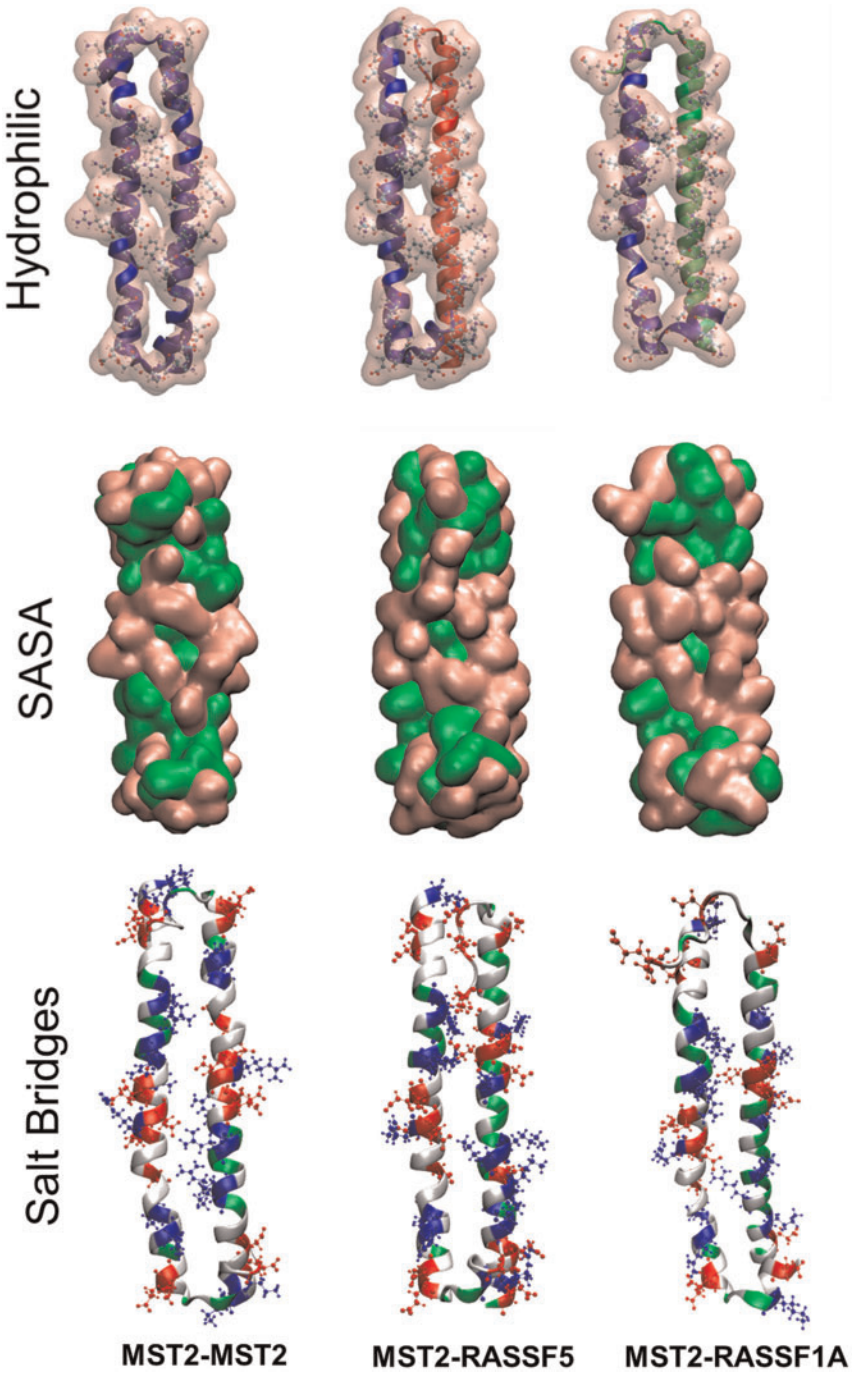

Figure 7. Molecular representations of MST2 homo- (left, blue backbone) and heterodimers with RASSF5 (center, red backbone) and RASSF1A (right, green backbone) SARAH domains. The first row illustrates the backbone and hydrophilic interactions. The middle row shows the solvent accessible surface area (SASA) for each dimer with the corresponding hydrophilic (pink) and hydrophobic (green) SASA fractions. The bottom row illustrates the same SARAH dimers with their characteristic electrostatic interactions (red: acidic, blue: basic, gray: hydrophobic, and green: hydrophilic amino acids). A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

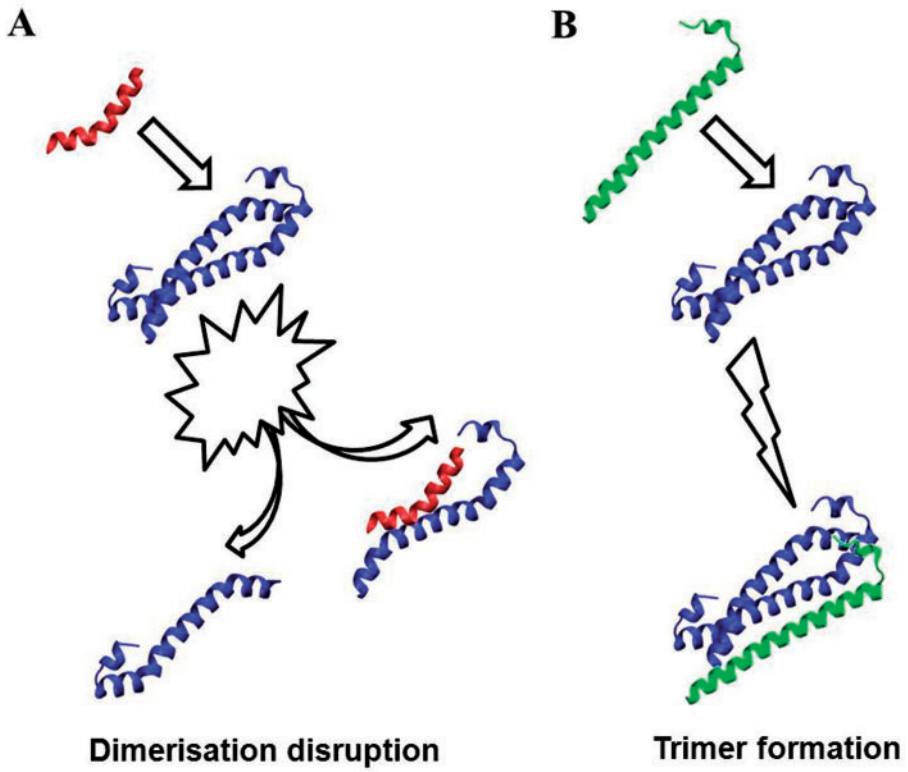

Figure 8. Schematic representation of possible molecular mechanisms involving MST2 homodimers (blue) and tertiary binding partners. (**A**) Small peptides can be designed to bind competitively and disrupt SARAH homodimers. (**B**) Sarah domains of other kinases (e.g. RASSFn, green) may bind noncompetitively to MST monomers or homomodimers and could facilitate and/or stabilize homodimeric interactions. A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

designed novel 'disruptor' peptides that can interfere efficiently with dimer formation, MST-MST, MST-RAF and MST2-RASSF1A [22] and have also probed the possibility of trimer formation, as illustrated in Figure 8.

Concluding remarks and outlook

The investigation of signaling pathways involved in cancer is one of the most important modern biomedical research avenues. Among all the possible complex signaling pathways that are under consideration in current cancer research, the Hippo pathway, which controls cell death, has been the objective of numerous investigations in the recent years. The MST protein family is particularly important for the activation of apoptosis and, therefore, for the possibility of controlling cancer cell death.

One specific domain of MST kinases has been highlighted to play a key role in the activation of the MST proteins and the subsequent signal transduction—the long terminal helix known as a SARAH domain. It has been also shown that the regulation of the MST kinases by its scaffolds, the RASSF protein family, is mediated by the interaction between MST and RASSF through their corresponding SARAH domains. Several crystal structures and publications have identified monomeric and dimeric (both homo- and heterodimers) structures involving MST and RASSF SARAH domains. Despite increasing efforts to characterize the structural interactions between SARAH domains, their interface and the implications of the dimerization on the activation process, the particular role of the SARAH domains remains poorly understood, both from an experimental and from a computational point of view.

Here, we have presented recent studies of SARAH-mediated interactions, describing the current state of the field in relation to the available crystal structures and modern computational molecular modeling approaches available. While there are several crystal structures for homo- and heterodimers, computational and mechanistic modeling is only recently catching up. We have focused our attention on describing structural properties of SARAH domain dimeric interactions both from the computational and experimental point of view. Recently, the molecular interactions between RAF1 and MST2 proteins has been studied both experimentally and computationally, showing that MST2 modulates the crosstalk between the mitogenic Raf and the pro-apoptotic MST2 pathway [19]. This study showed that a designed 17-mer peptide can disrupt effectively RAF1-MST2 dimerization [19]. Understanding at a molecular level how such 'disruptor' peptides affect the dimerization process can be crucial for future development of novel anti-cancer drugs that can activate MST2 by changing its inhibitory interaction with RAF1.

While experimental studies have provided a strong foundation to lead the cancer research investigation in this area, computational approaches are becoming both increasingly available and able to provide unique atomistic-level insights on the underlying molecular mechanisms, with reliable and experimentally testable results.

Key Points

- Hippo signaling pathway controls tissue homeostasis by balancing cell proliferation and death through apoptosis.
- MST1/2 kinase are key components of the mammalian Hippo signaling pathway.
- MST kinases are regulated by RASSF scaffolds by interacting through SARAH domains.
- Few crystal structures are available for MST-RASSF SARAH or MST-MST dimers, and exclusively for MST2, MST1 and RASSF5.
- Molecular modeling (e.g., homology-based methods, docking and molecular dynamics) provides essential tools to probe and understand protein structure and activity.

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