MRAS: A Close but Understudied Member of the RAS family

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Abstract

MRAS is the closest relative to the classical RAS oncoproteins and shares most regulatory and effector interactions. However it also has unique functions including its ability to function as a phosphatase regulatory subunit when in complex with SHOC2 and PP1. This phosphatase complex regulates a crucial step in the activation cycle of RAF kinases and provides a key coordinate input required for efficient ERK pathway activation and transformation by RAS.

MRAS mutations rarely occur in cancer but deregulated expression may play a role in tumorigenesis in some settings. Activating mutations in MRAS (as well as SHOC2 and PP1) do occur in the RASopathy Noonan syndrome, underscoring a key role for MRAS within the RAS-ERK pathway. MRAS also has unique roles in cell migration and differentiation and has properties consistent with a key role in the regulation of cell polarity. Further investigations should shed light on what remains a relatively understudied RAS family member.

Introduction

The RRAS subgroup (RRAS, TC21/RRAS2 and MRAS/RRAS3) of the RAS family GTPases are the closest relatives to the classical RAS oncogenes (H/N/KRAS, hereafter referred to collectively as 'RAS'). These GTPases share many regulatory and effector proteins with RAS as well as transforming abilities (Chan et al. 1994; Saez et al. 1994; Kimmelman et al. 1997; Rodriguez-Viciana et al. 2006). Members of this group have distinct functions, and MRAS has been shown to play a number of roles in cellular processes such as differentiation, cytoskeletal remodelling and cell migration. Uniquely among RAS family GTPases, MRAS is part of a phosphatase complex that positively regulates RAF kinase activation and is required to cooperate with RAS proteins for efficient ERK pathway activation.

Sequence features of MRAS

The RRAS subgroup lies within a distinct branch of the tree of all small GTPases with classical RAS and ERAS (Figure 1A). MRAS is highly conserved between vertebrates and has considerable similarity to the *ras-2* gene in *C. elegans* (Figure 1B). Interestingly although classical

RAS orthologues exist in fly, fish and nematode, they are absent in ascidian, which does have orthologues to MRAS and RRAS (Figure 1B). Therefore MRAS evolved independently of RRAS in metazoans and has been suggested to compensate for the lack of classical RAS in ascidian (Keduka et al. 2009).

The G-domain of MRAS is similar in sequence to the classical RAS proteins (it shares 53% amino acid identity with KRAS), and like RRAS and TC21, it has a short N-terminal extension. The terminal residue of MRAS is lysine, which differs from the terminal residues of RAS (Figure 2A), and consequently rather than being farnesylated, MRAS is predicted to be post-translationally modified instead by geranylgeranylation (Zhang and Casey 1996). The hypervariable region (HVR) of MRAS has similarities to KRAS4B in that carboxy-terminal cysteines capable of palmytoylation are absent and instead a polybasic region is found (Figure 2A). Like KRAS4B, MRAS is found in disordered membrane domains rather than organised lipid rafts (Ehrhardt et al. 2002), which suggests these proteins may signal in similar pathways and/or be similarly regulated.

MRAS regulation and control of downstream pathways

MRAS shares many regulatory proteins with other RAS family GTPases (RFGs) – it can be activated by SOS1, RASGRF1, RASGRP1 and RASGRP3 GEFs and inactivated by p120 RASGAP, Neurofibromin and GAP1m GAPs (Mitin et al. 2005). Though some of these proteins also regulate RRAS/TC21, full analysis of this regulation links MRAS closely to classical RAS in terms of GEF/GAP specificity (Ohba et al. 2000). This implies that most physiological signals that activate RAS will also activate MRAS simultaneously and is consistent with MRAS functioning together with RAS to provide coordinate inputs for efficient RAF activation (see below).

Given its sequence similarity and identical effector domain (Figure 2B), it is unsurprising that MRAS can bind many of the same effectors as RAS such as A-, B-, and CRAF, AFDN/AF6, RASSF5, RalGEFs and PI3K (Quilliam et al. 2001; Ortiz-Vega et al. 2002; Rodriguez-Viciana et al. 2004) . Through binding RGL2/RLF, MRAS activates RAL and ELK1 in MCF-7 cells, in an ERK-independent manner (Ehrhardt et al. 2001; Castro et al. 2012). MRAS also controls activation of RAP activity through binding MR-GEF/RAPGEF5 (Rebhun et al. 2000) and RA-GEF2/RAPGEF6 (Gao et al. 2001), the latter being specifically linked to control of cell adhesion through TNFα-triggered integrin activation in haematopoietic cells (Yoshikawa et al. 2007).

MRAS is a weak activator of the ERK pathway compared to RAS (Kimmelman et al. 1997; Rodriguez-Viciana et al. 2004), which at least in part could be attributed to the lower affinity of MRAS for RAF compared to RAS. One reason for this may be the differences in sequence of MRAS, particularly in the Switch I region (Figure 2B). Both the GppNHp- and GDP-bound forms of MRAS have an open Switch I conformation, and mutation of these residues to those of RAS result in a higher proportion of MRAS in the closed state. In addition these mutations increase the affinity of MRAS for the RAS-binding domain (RBD) of RAF (Ye et al. 2005) which implies that there are regions unique to MRAS (with others yet to be identified) that govern its affinity, and specificity, for various effectors.

MRAS regulation of RAF activity through SHOC2/PP1

In addition to sharing interactions with many of RAS effectors, MRAS also has its own unique effector interactions with RAPGEFs (Gao et al. 2001) as well as SHOC2 and protein phosphatase 1 (PP1). (Rodriguez-Viciana et al. 2006). SHOC2 is a ubiquitously expressed protein comprised almost exclusively of leucine rich repeats that was originally identified in *C. elegans* as a positive modulator of the ERK pathway (Selfors et al. 1998; Sieburth et al. 1998). Active MRAS forms a ternary complex with SHOC2 and PP1 to form a phosphatase holoenzyme that specifically dephosphorylates a conserved inhibitory site in RAF kinases (S259 in CRAF, S365 in BRAF and S214 in ARAF, hereby referred to as 'S259') that functions as a 14-3-3 binding site and plays a key role in the RAF activation cycle (Rodriguez-Viciana et al. 2006).

The consensus model of RAF activation stipulates that RAF is maintained in an auto-inhibited inactive state in the cytosol by an intramolecular interaction between the N-terminal region and the catalytic domain, which is in part mediated by a 14-3-3 dimer bound to two phosphorylated residues (S259 and S621 in CRAF) (Figure 3A) (Tzivion et al. 1998; Matallanas et al. 2011; Lavoie and Therrien 2015). RAS-GTP binding to the RAS binding domain (RBD) of RAF results in RAF translocation to the plasma membrane where other activating steps then take place. Chief among this is the dephoshphorylation of the 'S259' site, which leads to 14-3-3 displacement from this site, destabilizes the closed conformation of RAF, allows the Cysteine-rich domain (CRD) to further anchor RAF to the membrane and facilitates RAF dimerization (Lavoie and Therrien 2015) (Figure 3B). The MRAS-SHOC2-PP1 complex functions as a key phosphatase promoting this dephosphorylation step, which occurs preferentially on the RAF that has been recruited to the plasma membrane by RAS proteins (Figure 3B) ((Rodriguez-Viciana et al. 2006).

PP1 is known to interact with hundreds of regulatory proteins that confer substrate specificity and unique properties to each resulting holoenzyme. By analogy with other PP1 holoenzymes as well as the heterotrimeric PP2A complex (Cho and Xu 2007; Shi 2009; Peti et al. 2013), MRAS and SHOC2 are predicted to 'remodel' the substrate recognition surface within the complex altering the physiochemical landscape to create a surface specific for recognition of P-S259 RAF but not other phosphorylation sites, even on the same target (Rodriguez-Viciana et al. 2006).

A role for MRAS in polarity

Active MRAS can also associate with the polarity protein SCRIB, although this interaction is indirect and mediated by SHOC2 (Young et al. 2013). SCRIB is also a PP1-interacting protein and MRAS has the potential to 'rearrange' the PP1 molecules within the SCRIB-SHOC2 complex. In the absence of MRAS, SHOC2 has very low affinity for PP1, and the PP1 in the complex is bound to SCRIB. (Figure 3A). In the presence of active MRAS, SHOC2 and PP1 form a ternary complex with high affinity and the PP1 interaction is now independent of SCRIB (Young et al. 2013) (Figure 3B).

Through its interaction with SHOC2, MRAS is expected to recruit SCRIB and its associated interactome to sites of activation (Richier et al. 2010; Anastas et al. 2012; Young et al. 2013). SCRIB antagonizes SHOC2-mediated RAF 'S259' dephosphorylation, at least partly, through a mechanism involving competition for PP1 molecules within the same macromolecular complex. SCRIB recruitment, through its interaction with PIX/GIT complex, SGEF and VANGL proteins, would also allow for the regulation of RAC/CDC42, ARF, RHOG, and the planar cell polarity pathways respectively (Ellerbroek et al. 2004; Frank and Hansen 2008; Tada and Kai 2012). Furthermore, MRAS impairs the association of NOS1AP and the exchange factor βPIX with the SHOC2-SCRIB complex and thus has the ability to regulate the SCRIB interactome. MRAS is also expected to recruit to the same signalling platforms other effectors such as RAPGEFs, RALGEFs and PI3K, which are also known to be involved in polarity adhesion and migration (Kimmelman et al. 2000; Ehrhardt et al. 2002; Rodriguez-Viciana et al. 2004). MRAS is therefore an excellent candidate to behave as a master regulator of polarity (Figure 4).

MRAS in human disease

Despite being the most closely related to RAS by sequence similarity, and given its shared interaction with many of the same effector proteins (as well as GAPs and GEFs) it is somewhat surprising that activating mutations in MRAS are rarely found in cancer, in clear contrast to RAS

genes ((Catalogue of Somatic Mutations in Cancer (COSMIC)). Considering the key role of the RAF-ERK pathway in mediating RAS oncogenic properties, MRAS lower affinity for RAF and its considerably weaker activation of the ERK pathway may at least partly account for this observation. Additionally, it is also possible that MRAS's unique role in polarity may make constitutive activation disfavoured in some contexts (Young et al. 2013). However, MRAS upregulation may be linked to cancer in other ways.

MRAS expression is upregulated in estrogen receptor (ER) negative breast carcinomas compared to ER-positive in three independent studies (van de Vijver et al. 2002; Chin et al. 2006; Hess et al. 2006), and overexpression of constitutively active MRAS enables MCF-7 breast carcinoma cells to proliferate in the absence of oestrogen (Castro et al. 2012). MRAS is part of the epithelial to mesenchymal signature (Huang et al. 2012) and expression of active mutants causes EMT and oncogenic transformation in mouse scp-2 cells. These cells can grow in the absence of serum, lose contact inhibition, gain the ability to grow in an anchorage-independent manner, and form tumours in mice that have elevated levels of P-ERK and P-AKT (Ward et al. 2004). Many of these MRAS-induced characteristics are dependent on an HGF autocrine mechanism (Zhang et al. 2004) which is interesting given that invasive growth and metastasis of mammary tumours correlates with HGF secretion (which itself occurs in the majority of tumours of that type) (Jeffers et al. 1996; Nagy et al. 1996).

The prominence of MRAS overexpression in cancer may also be context-specific. For example, *MRAS* (along with *MET*) is overexpressed in multiple tumour types in a cytokine-driven/STAT3 activity dependent manner (Yang et al. 2005). iTCGA studies show that MRAS is amplified in 17% Lung squamous cell carcinomas but very rarely in lung adenocarcinoma (Figure 5) which correlates inversely with the frequency of RAS mutations which is high in adenocarcionmas but low in squamous cell carcinomas (cBioportal) (Cerami et al. 2012; Gao et al. 2013). MRAS was also overexpressed and/or amplified in 11% Ovarian Serous Cystadenocarcinoma and 10% Head and Neck Squamous Cell Carcinoma (cBioportal). MRAS mutation frequency may be linked to specific subsets of cancers, for example Borrmann type IV gastric cancer, which carries particularly poor prognoses, has a higher frequency of nonsynonymous MRAS mutations (17%) compared to overall gastric cancers (0.7% MRAS mutations) (Yasumoto et al. 2017). However, functional assays are still required to show that these mutations are activating.

Two mutations in MRAS (p.Gly23Val and p.Thr68Ile) (Figure 6) have been identified in patients with Noonan syndrome (NS; MIM 163950), a developmental disorder which is part of the RASopathies family of related syndromes which are driven by mutations in components of the RAS-ERK pathway. NS typically features facial dysmorphisms, slow growth rates, skeletal anomalies, mental retardation, predisposition to malignancies and often cardiac defects. Of the two *MRAS* mutation positive patients so far described, both had developmental delays, facial dysmorphisms and cardiac hypertrophy. Similar clinical phenotypes are observed in patients with Noonan syndrome with loose anagen hair (NS-LAH) driven by mutations in MRAS binding partners – SHOC2 and PP1β (Cordeddu et al. 2009; Gripp et al. 2016). MRAS p.Gly23Val corresponds to Gly13 of H/N/KRAS – a known oncogenic mutation. G23V-MRAS is primarily GTP-bound and activates ERK in cells (Higgins et al. 2017), and although not functionally tested as yet, mutation of position T68 is predicted to be activating given that it is within the Switch II region and lies within the GTP/GDP binding pocket (Figure 5). Based on the observed phenotypes and what is known about its role in ERK pathway regulation, MRAS-driven NS is likely to be functioning through SHOC2/PP1 complex activity.

Gain-of-function *CRAF* mutations are also found in NS and cluster around S259 to disrupt 14-3-3 binding (Pandit et al. 2007; Razzaque et al. 2007; Kobayashi et al. 2010; Molzan et al. 2010), Of note, S259F as well as others in this region (S257L, 261S and V263A), have been identified in cancer as well as NS. These results underscore the important role of MRAS-SHOC2-PP1 and RAF S259 dephosphorylation on RAS-ERK pathway activation.

Large-scale genome-wide association studies have identified the MRAS locus as a risk factor in cardiovascular disease (Erdmann et al. 2009; Schunkert et al. 2011; Liu et al. 2013). Intriguingly, although MRAS is widely expressed, it has particularly high expression in the heart (biogps.org). Although a role for MAP kinase pathways in the heart is known (Rose et al. 2010), the precise role of MRAS remains to be elucidated.

Other functions of MRAS

MRAS signalling is linked to differentiation and development in a variety of mammalian cell types. It is activated by NGF and is required for ERK-dependent neuronal differentiation of rat pheochromocytoma PC12 cells (Kimmelman et al. 2002; Sun et al. 2006) and in developing mouse bone, BMP2 treatment not only activates MRAS, but also increases its expression levels.

Active MRAS stimulates transdifferentiation of C2C12 mouse myoblasts into osteoblasts, and causes osteoblast differentiation in a p38 and JNK-dependent manner (Watanabe-Takano et al. 2010). MRAS is up-regulated during dendrite development and contributes to dendrite growth via the ERK pathway. In these cells, Semaphorin4D (or Sema4D, a repulsive guidance molecule in the developing nervous system) binds to its receptor PLEXIN-B1, the cytoplasmic domain of which acts as a GAP on MRAS (and RRAS, but not TC21 or RAS). When MRAS activity is suppressed in this way, it results in growth cone collapse and remodelling of dendrite morphology (Saito et al. 2009). In the absence of Sema4D, active MRAS binds to an effector, LPD (lamellopodin), and recruits it to the membrane of growing dendrites where it participates in actin cytoskeleton remodelling via Ena/VASP proteins (Tasaka et al. 2012). However, other studies have suggested that sempahorins function as GAPs for RAPs not MRAS (Wang et al. 2012).

These ties to differentiation have also been studied in mouse embryonic stem cells (mESCs). Of all the *Ras* family members, *Mras* is the only gene whose expression is limited to undifferentiated mESCs. Prior to differentiation, expression is controlled by the cytokine LIF, and as such *Mras* is described as a marker of stemness (Mathieu et al. 2013). Persistent *Mras* down-regulation by removal of LIF affects the normal balance of expression of core pluripotency markers such as OCT4 and CAECAM1, and overexpression of MRAS leads to sustained expression of OCT4 and NANOG. In *Xenopus,* Mras is expressed throughout the embryo, maintaining pluripotency, until the blastula stage, and as in mice, it is required for neuronal differentiation, which indicates that this protein has conserved functions across vertebrates (Mathieu et al. 2013).

At the organismal level, *Mras* null mice were initially described as phenotypically normal (Nunez Rodriguez et al. 2006), but further studies since then link *Mras* to the control of normal urinary function (Ehrhardt et al. 2015b) and normal olfactory and/or social processes, in adult male mice since *Mras*$^{-/-}$ males are phenotypically more aggressive and exhibit increased sexual behaviour (Ehrhardt et al. 2015a).

Concluding remarks

Although *MRAS* mutations are rarely found in cancer but it is likely that dysregulation of *MRAS* at the expression level may be a contributing factor to tumourigenesis in some contexts. Considering this and the studies of MRAS in cell fate determination and stemness, understanding how MRAS expression is regulated in different cell types is of interest. The recent identification of activating

mutations in *MRAS* in Noonan syndrome (as well as *SHOC2* and *PPP1CB*), highlight a key role for the MRAS-SHOC2-PP1 complex in the regulation of the ERK pathway. MRAS has the ability to regulate multiple signalling pathways, including many that directly or indirectly regulate other GTPases of the RAS superfamily and that suggest a role for MRAS as a master regulator of polarity. More comprehensive biochemical and structural studies will deepen our understanding of how MRAS-specific signalling contributes to cell behaviour and will help to explain the phenotypes observed at the organismal level where MRAS function is modified.

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