Stabilization of C-RAF:KSR1 Complex by DiRas3 Reduces Availability of C-RAF for Dimerization with B-RAF

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Abbreviations: KSR, kinase suppressor of Ras; RBD, Ras binding domain; M2PK, pyruvate kinase type M2; PARP, poly(ADP-ribose) polymerase; AMPK, AMP-activated protein kinase.

Abstract

RAF family kinases are central components of the Ras-RAF-MEK-ERK cascade. Dimerization is a key mechanism of RAF activation in response to physiological, pathological and pharmacological signals. It is mediated by a dimer interface region in the RAF kinase domain that is also conserved in KSR, a scaffolding protein that binds RAF, MEK and ERK. The regulation of RAF dimerization is incompletely understood. Especially little is known about the molecular mechanism involved in the selection of the dimerization partner. Previously, we reported that Ras-dependent binding of the tumour suppressor DiRas3 to C-RAF inhibits the C-RAF:B-RAF heterodimerization. Here we show that DiRas3 binds to KSR1 independently of its interaction with activated Ras and RAF. Our data also suggest that depending on the local stoichiometry between DiRas3 and oncogenic Ras, DiRas3 can either enhance homodimerization of KSR1 or recruit KSR1 to the Ras:C-RAF complex and thereby reduce the availability of C-RAF for binding to B-RAF. This mechanism, which is shared between A-RAF and C-RAF, may be involved in the regulation of Ras12V-induced cell transformation by DiRas3.

1. Introduction

The small GTPase DiRas3 (also referred as NOEY2 and ARHI) belongs to the Ras family of proteins and shares 55–62% homology with Ras and Rap [1-2]. However, it possesses entirely different functional properties compared with H-, N-, and K-Ras. In contrast to most Ras proteins, DiRas3 exists mainly in the active GTP-bound state in resting cells, and acts as a tumour suppressor. The DiRas3 gene encodes a 26-kDa protein that is monoallelically expressed and maternally imprinted [1]. It is lost or down-regulated in more than 60% of ovarian and breast cancers through several different mechanisms, including loss of heterozygosity, DNA hypermethylation, transcriptional regulation, and shortened mRNA half-life [2-3]. Loss of DiRas3 expression is associated with tumour progression and poor prognosis [4-5]. Re-expression of DiRas3 in cancer cells inhibits growth, decreases invasiveness, and induces apoptosis [1, 6]. Signalling alterations caused by introduction of the DiRas3 gene into cancer cells lacking DiRas3 expression can include activation of JNK, inhibition of the STAT3 transcriptional activity, down-regulation of cyclin D1 and inhibition of the Ras/ERK pathway [1, 7-8].

RAF is a central component of the Ras/ERK pathway, which uses a three-tiered kinase cascade, RAF-MEK-ERK, to mediate the effects of different extracellular stimuli on various cellular processes including proliferation, differentiation, transformation, motility and apoptosis [9-10]. While vertebrates express three RAF family members, C-RAF, A-RAF and B-RAF, lower eukaryotes and invertebrates have only one RAF gene, most closely related to B-RAF. The RAF activation/deactivation cycle is highly complex, and despite 30 years of research still incompletely understood. In quiescent cells RAF proteins exist in an inactive conformation maintained by autoinhibitory interactions between the N-terminal regulatory and the C-terminal catalytic domains. This inactive conformation is stabilized by binding of 14-3-3 dimers to conserved phosphoserine residues within the N- and C-terminus. Main RAF activators are Ras-GTPases, which are activated by many growth factor receptors. Their active Ras-GTP forms bind to the RBD (Ras binding domain) of RAF with high affinity, displacing 14-3-3 from the N-terminal binding site and presumably inducing conformational changes that initiate activating modifications such as the phosphorylation of activating sites in the N-region and activation loop [9, 11]. A host of recent papers has added dimerization as key mechanism of RAF activation in response to physiological, pathological and pharmacological signals [12-23]. The dimerization mechanism is unclear, but it was suggested that binding of Ras-GTP induces an open conformation in RAF that makes the dimerization surface available for interaction. The finding that Ras also forms dimers raises the intriguing possibility that Ras binding may directly promote RAF dimerization [24]. Structural studies revealed that RAF proteins dimerize side-to-side with contacts mainly made between the N-terminal lobes of the RAF kinase domain [12, 14, 20]. RAF dimerization leads to an allosteric activation of the binding partners provided that one partner has adopted an activated conformation [16]. Thus, a main role of dimerization seems to be the induction and stabilization of the activated conformation. This view is supported by findings

that RAF inhibitors have a paradoxical effect, inhibiting cells with mutated B-RAF but accelerating the growth of cells with mutated Ras [14-15, 19]. Recent studies suggest that, in Ras transformed cells, these drugs bind to and induce an activated allosteric conformation of wild-type B-RAF and C-RAF kinase domains that facilitates B-RAF:C-RAF dimer formation and results in the activation of downstream signalling pathways [14, 25]. Interestingly, some of the RAF inhibitors do not efficiently induce B-RAF:C-RAF dimers but can still activate MEK and ERK in Ras transformed cells [14, 19, 25]. These drugs induce dimerization between C-RAF and the structurally related KSR1 (kinase suppressor of Ras 1) [17]. Indeed, based on the symmetric packing of RAF molecules in the crystal structures, it has been suggested that the side-to-side dimer interface, conserved in KSR and in all RAF isoforms, mediates the ability of RAF to bind to itself or to KSR [20].

Upregulation of the ERK signal transduction pathway occurs in approximately 30% of all human cancers with mutations in Ras and B-RAF representing the majority of oncogenic mutations in most human cancers including malignant melanoma [26]. In light of the experimental evidence, this pathway represents an attractive target for the pharmacological intervention in proliferative diseases. Therefore, understanding how naturally occurring inhibitors, such as DiRas3, intercept signal transduction through the ERK pathway is crucial for the development and improvement of anti-cancer drugs and personalized treatment. We have previously found that DiRas3 inhibits C-RAF:B-RAF dimer formation, antagonizes Ras-GTP induced RAF activation and changes the cellular localization of RAF [27]. This discovery raised the question of the molecular mechanism underlying these DiRas3-induced effects. In the present study we show that DiRas3 binds to KSR1 independently of its interaction with activated Ras and RAF. Our data also suggest that depending on the local stoichiometry between DiRas3 and oncogenic Ras, DiRas3 can either enhance homodimerization of KSR1 or recruit KSR1 to the Ras:C-RAF complex and thereby reduce the availability of C-RAF for binding to B-RAF. This mechanism, which is shared between A-RAF and C-RAF, may be involved in the regulation of Ras12V-induced cell transformation by DiRas3.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: mouse anti-c-myc (9E10), rabbit anti-C-RAF (RAF-1, C-12), mouse anti-KDEL (10C3), mouse anti-pERK (E-4), rabbit anti-ERK2 (C-14), rabbit anti-B-RAF (C-19), mouse anti-vimentin (V9) and mouse anti-C-RAF-phospho-Ser621(6B4) from Santa Cruz; mouse anti-H-Ras (#R02120) from BD Transduction Laboratories; mouse anti-M2PK (DF4) from Schebo Biotech; mouse anti-PARP-1 (C-2-10) from Calbiochem; mouse anti-PARP (#556362) from BD Pharmingen; mouse anti-DiRas3 (HI-A8, ab45768) and rabbit anti-KSR1 (ab52196) from Abcam; anti-HA-peroxidase coupled (3F10) from Roche; mouse anti-V5 (#46-0705) from Life Technologies; anti-Flag peroxidase coupled (M2) from Sigma; rabbit anti-AKT-phospho-Ser473 (#9271) and rabbit anti-AKT (#9272) from Cell Signaling.

2.2. Plasmids

The following plasmids were used: human C-RAF-myc-His WT and R398A in pcDNA3.1; human HA-C-RAF WT, R398A and S621A in pcDNA3; human A-RAF-myc-His WT and R362A in pcDNA3.1; human A-RAF-HA WT and R362H in pcDNA3; human B-RAF WT in pCMV, human B-RAF-myc-His in pcDNA3.1; HA-BxB (human C-RAF-Δ1-303) in pcDNA3; human H-Ras12V in pcDNA3, mouse KSR1-Flag WT and R615H in pCMV, mouse HA-KSR1 WT, S392A and R615H in pcDNA3.

For construction of the pcDNA3-myc-DiRas3, pcDNA3-Flag-DiRas3 and pcDNA3-V5-DiRas3 expression vectors, PCR primers 5'–CCCAAGCTTGGCCACCATGGAGCAGAAG–3' (forward primer for the pcDNA3-myc-DiRas3 construct), 5'–CCCAAGCTTGGCCACCATGGAGCAGAAG–3' (forward primer for the pcDNA3-Flag-DiRas3 construct), 5'–CCCAAGCTTGCCGCCACCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTCTAG AGGTAACGCCAGCTTTGGCTC–3' (forward primer for the pcDNA3-V5-DiRas3 construct; contains sequence for the V5 tag) and 5'–ATAAGAATGCGGCCGCTTACATGATTATGCACTTGTCAAGCAG–3' (reverse primer for all three constructs) were used to amplify the human DiRas3 cDNA. The PCR fragments and the corresponding pcDNA3 vectors were digested with the restriction nucleases HindIII and NotI and, subsequently, ligated by use of T4 DNA ligase to give the expression plasmids for N-terminally tagged DiRas3.

The site-specific mutations were introduced using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The fidelity of the mutants was confirmed by DNA sequencing.

2.3. Immunoprecipitation

The required cDNA plasmids were transfected into COS7 cells under starvation conditions using jetPEI transfection reagent (Polyplus Transfection). Cells were lysed 24 h after transfection with buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 30 mM sodium pyrophosphate, 100 μM Na_3VO_4, 1% Triton X-100, and standard proteinase inhibitors for 45 min at 4 °C. The lysates were clarified by centrifugation at 27,000 × g for 15 min and incubated for 1 h at 4 °C with the appropriate antibody. After addition of protein G-agarose, the incubation was continued for 2 h at 4 °C. The agarose beads were washed 3 times with lysis buffer containing 0.1% Triton X-100. The immunoprecipitates were supplemented with Laemmli buffer, heated for 5 min at 95 °C, and applied to SDS-PAGE. After Western blotting the isolated proteins were visualized by appropriate antibodies.

2.4. In vitro kinase assay

The kinase assay was carried out directly with immunoprecipitated proteins in 25 mM Hepes, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4 and 500 μM ATP buffer (50 μl final volume). Recombinant MEK and ERK-2 were used as substrates. After incubation for 30 min at 30 °C, the kinase assay

mixtures were supplemented with Laemmli buffer, heated for 5 min at 95 °C and applied to SDS-PAGE. After Western blotting the extent of ERK phosphorylation was determined by an anti-phospho-ERK antibody.

2.5. Subcellular Fractionation

Cell fractions were isolated using the ProteoExtract subcellular proteome extraction kit (Calbiochem). COS7 cells were grown on 10-cm Petri dishes and transfected with appropriate cDNA constructs under starvation conditions. The cells were fractionated into four subproteomic fractions (cytosolic and nuclear fractions, fractions of whole membranes, and cytoskeleton) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The selectivity of subcellular extraction was documented by immunoblotting against marker proteins (M2PK (pyruvate kinase type 2) for cytosolic fraction, KDEL (ER retention signal) for membrane fraction, PARP (poly(ADP-ribose) polymerase) for nuclear fraction, and vimentin for cytoskeletal fraction).

2.6. Focus formation assay (transformation assay)

Low passage NIH3T3 cells were split into 10-cm dishes (20,000 – 30,000 cells per dish) and transfected with the expression vector for H-Ras12V (0.3 µg per 10 cm plate) using Lipofectamine® 2000 (Life Technologies). The transfected cells were cultured in DMEM supplemented with 10% donor bovine serum. 24 h and 48 h post transfection, one plate of cells was lysed for protein expression analysis and the rest were grown until the cell foci have appeared (14-21 days). For visualization of foci, cells were fixed with methanol and stained with Giemsa solution.

2.7. Quantification of cells

Cells were quantified from images randomly taken from plate with the light microscope. Image analysis was performed with CellProfiler 2.0 [28]. For identification of cells Otsu Global algorithm with two-class thresholding and weighted variance minimisation was used. All images were corrected for uneven background illumination.

3. Results

3.1. Heterodimerization of A-RAF and C-RAF with B-RAF is regulated by DiRas3

We previously have shown that DiRas3 associates with activated H-Ras and its effector protein C-RAF. The consequences of this complex formation is the DiRas3-induced suppression of C-RAF:B-RAF heterodimerization, inhibition of C-RAF kinase activation and recruitment of C-RAF to the cytoskeleton [27]. Here, we characterise the molecular mechanism for the DiRas3-induced inhibition of RAF heterodimerization and associated RAF functions. Due to the similarities in the regulation of C-RAF and A-RAF, we assumed that these isoforms might also share the same mechanism for the DiRas3-induced inhibition of their heterodimerization with B-RAF.

Therefore, we tested whether DiRas3 can interact with activated A-RAF and affect the heterodimerization of A-RAF with B-RAF. We expressed myc-DiRas3 and H-Ras12V together with HA-tagged A-RAF kinase in COS7 cells. DiRas3 was isolated by anti-myc immunoprecipitation and tested for the presence of associated A-RAF. The results revealed that A-RAF indeed binds to DiRas3, and that this interaction strongly depends on activated H-Ras (Fig. 1A). Moreover, cotransfection with DiRas3 reduced binding of B-RAF to both C-RAF and A-RAF (Fig. 1B and C), confirming that A-RAF and C-RAF association with B-RAF is regulated by DiRas3. In case of A-RAF, the negative effect of DiRas3 expression on binding of B-RAF to A-RAF was rescued by the R362A point mutation, which strongly increases the A-RAF:B-RAF heterodimerization (Fig. 1B) [29]. The importance of H-Ras as a mediator for the association of DiRas3 with RAF proteins is confirmed by the observation that DiRas3 preferentially binds to C-RAF followed by A-RAF and B-RAF (Fig. 1D). This is in line with the previously reported data demonstrating that due to the structural differences in the RBD of the RAF kinases, H-Ras associates with much higher affinity with C-RAF than with A-RAF [30]. In case of B-RAF, the binding of Ras to its RBD domain is known to be reduced in cells with prolonged and increased activation of ERK, due to the feedback phosphorylation of B-RAF at Ser151, which is in close proximity to the RBD (residues 155 to 227), by activated ERK [21]. Consistently, we observed that COS7 cells used in our study had strikingly high basal pERK level as compared to other cell lines, e. g. MCF7 (data not shown).

3.2. Kinase activity and intracellular localization of BxB is not affected by DiRas3

It has been previously reported that in contrast to H-Ras, which binds only to the N-terminal regulatory part of C-RAF, DiRas3 interacts with both the N-terminal part and the C-terminal kinase domain of C-RAF [31]. These data raise the question, whether DiRas3 may affect C-RAF functions potentially by direct binding to the kinase domain of RAF proteins. In this scenario, H-Ras12V would be required for the release of RAF auto-inhibition by its N-terminal domain to expose the kinase domain for interaction with DiRas3. In line with this assumption, we observed that binding of DiRas3 to C-RAF-BxB was Ras-independent (Fig. 2A). BxB is a C-RAF construct where amino acids 27-303 have been deleted, removing the N-terminal Ras binding domain but leaving the C-terminal kinase domain [32]. However, in contrast to full length C-RAF, DiRas3 did not affect the kinase activity and the intracellular localization of C-RAF-BxB (Fig. 2B-F). Our results and the previously published data suggest that the DiRas3-dependent regulation of RAF functions requires the presence of the N-terminal regulatory part in RAF.

3.3. DiRas3 regulates competition between B-RAF and KSR1 for binding to C-RAF or A-RAF

Previous studies have shown that RAF kinases dimerize also with the structurally related KSR proteins (KSR1 and KSR2) to form side-to-side RAF:KSR heterodimers [20]. Therefore, we assumed that DiRas3 may also inhibit the dimerization of RAF proteins with KSR. To test this hypothesis we examined the effect of DiRas3 expression on binding of the three RAF isoforms to Flag-tagged KSR1 in a co-immunoprecipitation assay. Surprisingly and contrary

to our expectation, the dimerization between all three RAF isoforms and KSR1 was not suppressed but even enhanced by DiRas3 co-transfection (Fig. 3A-C). Furthermore, DiRas3 did not have any effect on dimerization between KSR1 and BxB (Fig. 3D), showing that the N-terminal regulatory part is also crucial for DiRas3-dependent regulation of RAF dimerization with KSR. Interestingly, it has been shown that KSR1 can compete with C-RAF for binding to B-RAF in the presence of RAF inhibitors [18] and, therefore, can act as a suppressor of C-RAF:B-RAF heterodimerization. However, it is unknown how the selection of the dimerization partner is regulated under natural conditions. Based on our findings, we propose a hypothetical model, in which DiRas3 binds directly to KSR1. Heterodimerization of DiRas3 with activated H-Ras recruits KSR1 to the H-Ras:C-RAF or H-Ras:A-RAF complex and prevents the binding of B-RAF to C-RAF or A-RAF. This would ensure the time- and space-specific regulation of RAF heterodimerization (Fig. 3E).

In accordance with the proposed hypothesis we could show that DiRas3 can bind to overexpressed and endogenous KSR1 (Fig. 4A and B). In contrast to interaction with A-RAF and C-RAF, binding of DiRas3 to KSR1 is Ras-independent (Fig. 4B). Furthermore, results of sequential co-immunoprecipitation confirmed that all three proteins (C-RAF, KSR1 and DiRas3) are present in the same protein complex (Fig. 4C). The binding studies also revealed that overexpression of KSR1 enhances binding of DiRas3 to Ras-activated C-RAF and A-RAF, but not to B-RAF (Fig. 4D-F). This is consistent with the constitutive binding of KSR1 to B-RAF and the Ras-dependent binding of KSR1 to C-RAF [33-36].

Among others, the dimerization of RAF proteins is regulated by the structure of the side-to-side dimerization interface and by phosphorylation of the 14-3-3 binding sites [13-14, 20-21, 29, 37-38]. To further confirm our assumption that DiRas3 binds to KSR1 independently from its interaction with the Ras:C-RAF or Ras:A-RAF complex, we tested whether mutations of the side-to-side dimerization interface and the 14-3-3 binding sites in C-RAF, A-RAF and KSR1 would have any effect on the interaction with DiRas3. Previously, we have shown that substitution of C-RAF Arg398, which is part of the side-to-side dimerization interface (R^{398}KTR), by alanine inhibits the kinase activity and the homodimerization of C-RAF, but has little effect on the heterodimerization of C-RAF with B-RAF [29]. Here we show that this mutation also inhibits the interaction between C-RAF and KSR1 (Fig. 5A), and consequently impairs the binding of DiRas3 to C-RAF (Fig. 5B). In case of A-RAF, our previously published data [29] and the results of the present study revealed that substitution of Arg362 within the conserved side-to-side dimerization interface (RKTR^{362}) strongly increases the A-RAF:B-RAF heterodimerization (Fig. 1B), but does not have any effect on A-RAF:KSR1 dimerization or on binding of DiRas3 to A-RAF (Fig. 5C and D). In line with these results, the high affinity of A-RAF-R362A mutant to B-RAF gives the latter an advantage over KSR1 and shifts the equilibrium towards the A-RAF:B-RAF complex formation, thereby reducing the inhibitory effect of DiRas3 on A-RAF:B-RAF heterodimerization (Fig. 1B).

Previous studies have shown that growth factor-induced C-RAF:B-RAF heterodimerization is dependent on binding of 14-3-3 to a phosphorylation site in the C-

terminus of RAFs (C-RAF pSer621, B-RAF pSer729) [21, 37-38]. We were able to confirm that substitution of Ser621 reduces binding of C-RAF to B-RAF (Fig. 5E). In contrast, the binding affinity of C-RAF to KSR1 was not impaired by the S621A mutation (Fig. 5F). Consistently, the binding of DiRas3 to C-RAF appeared Ser621-independent (Fig. 5G). Also, phosphorylation of the 14-3-3 binding site Ser392 in KSR1, which has been shown to negatively affect the KSR1:B-RAF heterodimerization [18], did not have any effect on binding to DiRas3 and therefore is not involved in the regulation of KSR1:DiRas3 interaction (Fig. 5H). All together, these data strongly support the proposed model, in which binding of DiRas3 to KSR1 is independent of interaction with Ras-activated RAF and 14-3-3.

3.4. DiRas3 enhances the side-to-side homodimerization of KSR1

It is well established that numerous GTPases are regulated by dimerization [24, 39-40]. Also our previous experiments revealed that DiRas3 is able to heterodimerize with active H-Ras [27]. Therefore, it appeared very likely that DiRas3 is also capable of homodimerization. In fact, our co-immunoprecipitation data confirmed this assumption (Fig. 6B). Consequently, the next question we have asked was whether formation of DiRas3 homodimers may enhance the KSR1 homodimerization, as proposed in our model (Fig. 3E). To answer this question we expressed two differently tagged KSR1 constructs, i.e. wild type (WT) and a dimerization defective mutant (R615H), together with increasing amounts of DiRas3. DiRas3 strongly supported the formation of homodimers between KSR1-WT molecules, but not between the dimerization mutants (Fig. 6A), suggesting that DiRas3 dimers enhance the side-to-side homodimerization of KSR1. In support of our model, we also found that increasing the cellular DiRas3 concentration beyond a certain threshold led to an abrupt rise in binding of DiRas3 to KSR1 (Fig. 6C).

3.5. DiRas3 inhibits cell growth and H-Ras12V-induced transformation

To determine the possible biological effects of the proposed regulatory model we analysed the role of DiRas3 in H-Ras12V-induced cell transformation and cell growth. Low passage NIH3T3 cells were transfected with H-Ras12V and increasing amounts of DiRas3 expression vector. The transfected cells were used for three types of assays, i.e. (i) the analysis of biochemical pathways regulated by DiRas3 and H-RasV12; (ii) assessment of proliferation; and (iii) measurement of cell transformation.

It has been previously reported that expression of DiRas3 suppresses ERK and AKT activation [31, 41]. Therefore, we analysed the activating phosphorylation of these proteins 24 and 48 hours post transfection (Fig. 7A). 24 h post-transfection, we observed increased pERK and pAKT levels in samples expressing H-Ras12V, whereas co-transfection with DiRas3 did not affect activation of these kinases. Unexpectedly, 48 h post-transfection neither DiRas3 nor H-RasV12 expression had strong effects on ERK or AKT activation, although the expression of both transfected GTPases was easily detectable. Interestingly, both GTPases induced cleavage of the PARP protein indicative of apoptosis. Oncogenic Ras proteins can

induce apoptosis under certain conditions [42], and the data suggest that both DiRas3 and H-RasV12 overexpression can activate apoptosis.

These biochemical changes were reflected in a reduction in cell numbers observed in response to transfection with DiRas3 and H-RasV12 (Figs 7B and C). The strongest inhibitory effect was observed in samples transfected with H-Ras12V and DiRas3 together, suggesting that both GTPases cooperate to inhibit proliferation, presumably due to induction of apoptosis.

Interestingly, the effects of DiRas3 and H-RasV12 on cell transformation were different. To measure transformation, we used the classic focus assay, which scores the outgrowth of transformed foci from a layer of contact inhibited non-transformed cells. Thus, cells were grown to confluency and assessed for the appearance of transformed foci after two weeks. Despite its initial growth inhibitory effects H-RasV12 induced cell transformation, whereas co-transfection with DiRas3 reduced the foci number by approximately 40%, suggesting that DiRas3 inhibits H-Ras12V-iduced transformation (Fig. 7D and E). However, this effect was strongly concentration-dependent, as a further increase in the amount of DiRas3 transfected diminished the inhibition of transformation. These results are consistent with the model presented in Fig. 3E, where DiRas3 expression antagonises the formation of B-RAF:C-RAF heterodimers and thereby suppresses transformation. However, high DiRas3 levels preferentially induce KSR1 homodimerization leaving C-RAF and B-RAF available for dimerization and oncogenic signalling.

4. Discussion

The so-called paradoxical activation of RAF kinases poses a serious obstacle in cancer treatment with RAF inhibitors. The main reason for these undesirable properties is the induction of RAF heterodimers (for review see [43-44]). Heterodimerization is also part of the physiological activation mechanism of RAF kinases [13, 16, 45]. However, the regulation and the exact molecular mechanism of the RAF dimerization are incompletely understood. Especially little attention has been paid to the questions, how the selection of the dimerization partner is regulated under natural conditions, and how cells ensure the time- and space-specific regulation of RAF homo- and heterodimerization. In our previous study we have shown that DiRas3 binds to C-RAF in Ras-dependent manner. The consequences of this complex formation are inhibition of kinase activity and C-RAF:B-RAF heterodimerization and accumulation of C-RAF at the cytoskeleton [27]. Here, we show that DiRas3 also binds to activated A-RAF (though with lower affinity) and also inhibits A-RAF:B-RAF heterodimerization, suggesting that A-RAF and C-RAF share the same DiRas3-dependent regulatory mechanism. Knowing that DiRas3 does not bind to the RBD of RAF kinases [7, 31], and that the binding of C-RAF and A-RAF to DiRas3 is Ras-dependent, the difference between these two RAF isoforms in their binding affinity for DiRas3 might be a consequence of their difference in binding to Ras. The weak binding of A-RAF to the activated Ras has

been attributed to the presence of a lysine at position 22 of the RBD in A-RAF because exchanging the lysine to an arginine increases the affinity of A-RAF for H-Ras [30].

Previously, we have shown that DiRas3 binds to activated H-Ras independently of C-RAF, which led us to the suggestion that DiRas3 does not bind to C-RAF directly, but through dimerization with activated H-Ras [27]. Contrary to our hypothesis, we observed that DiRas3 binds to C-RAF-BxB, a C-RAF deletion mutant that encompasses the kinase domain but lacks the RBD and hence cannot bind H-Ras. Accordingly, binding of DiRas3 to C-RAF-BxB was Ras-independent. These results are in keeping with data showing that DiRas3 interacts with both, the N-terminal regulatory (aa 1-331) and the C-terminal catalytic (aa 323-648) fragments of C-RAF in the absence of activated H-Ras [31]. These data led us to reconsider our hypothesis and consider that DiRas3 might interfere with RAF dimerization by binding directly to the dimerization interface of C-RAF and A-RAF. However, our co-immunoprecipitation experiment with the A-RAF dimerization mutant did not support this hypothesis. Binding of DiRas3 to A-RAF was not impaired by substitution of Arg362 with histidine (Fig. 5D), which efficiently hinders the A-RAF:B-RAF heterodimerization [46]. Furthermore, although the side-to-side dimerization mode also applies to the heterodimerization between RAF kinases and the structurally related KSR proteins [20], we observed, to our surprise, that DiRas3 did not inhibit the RAF:KSR1 dimerization but rather enhanced it. These results disprove the hypothesis that DiRas3 hinders RAF heterodimerization by direct binding to the dimerization interface.

Since DiRas3 does not bind directly to the dimerization interface of RAF kinases, it is conceivable that by dimerizing with activated H-Ras it may recruit a protein (or several proteins), which would either change the phosphorylation status of C-RAF(A-RAF) or/and compete with B-RAF in binding to dimerization interface of C-RAF(A-RAF). The heterodimerization of RAF proteins is regulated by phosphorylation of the 14-3-3 binding sites and the N-region [13, 16, 38, 45]. However, expression of DiRas3 did not change phosphorylation of the C-RAF Ser621 14-3-3 binding site (data not shown) or Ser338 in the N-region [27]. Instead, our data suggest that DiRas3 recruits KSR1 to the H-Ras:C-RAF(A-RAF) complex, and in this way prevents binding of B-RAF. In support of this hypothesis we could show that (i) DiRas3 enhanced the dimerization between KSR1 and C-RAF(A-RAF), (ii) the H-Ras12V-dependent binding of DiRas3 to C-RAF(A-RAF) was increased, when KSR1 was overexpressed, (iii) all three proteins were present in the same complex, as confirmed by the sequential co-immunoprecipitation. Furthermore, our data suggest that binding of DiRas3 to KSR1 is independent of its interaction with RAF, as mutation of the Ser392 in KSR1, which has been previously shown to enhance RAF:KSR1 dimerization [18], did not affect binding of DiRas3 to KSR1. Moreover, mutation of C-RAF Arg398, which inhibits C-RAF:KSR1, but not C-RAF:B-RAF dimerization [29], decreased the levels of C-RAF in the anti-DiRas3 immunoprecipitates. Accordingly, mutation of C-RAF SerS621, which impairs C-RAF:B-RAF, but not C-RAF:KSR1 dimerization, did not have any effect on binding of DiRas3 to C-RAF.

The role of KSR1 as a RAF dimerization competitor has been proposed in several studies before. In one study it has been shown that KSR1 competes with C-RAF for inhibitor-bound B-RAF leading to attenuation of ERK activation [18], whereas in another study KSR1 was found to be required for the inhibitor-induced hyperactivation through promoting KSR1:C-RAF dimerization [17]. More recently, it has been shown that the affinity of the oncogenic KIAA1549-BRAF fusion protein found in astrocytomas to KSR1 appeared to be much higher than that of B-RAF wild type or B-RAF-V600E [23]. All these examples clearly show that the dimerization selectivity can be modulated by different conditions. Under natural conditions, AMP-activated protein kinase (AMPK) has been shown to modulate the B-RAF:KSR1 and B-RAF:C-RAF associations. Although the regulatory mechanism is not completely understood, the authors reported that activation of AMPK induced the phosphorylation of Ser729, the C-terminal 14-3-3 binding site in B-RAF, and disrupted the association of KSR1 with B-RAF, but not with C-RAF [22]. Based on the results of our study we suggest that DiRas3 may also act as a modulator of RAF and KSR1 dimerization. Thereby, the preferential formation of B-RAF:C-RAF(A-RAF) or KSR1:C-RAF(A-RAF) complexes may be regulated by the local ratio of activated H-Ras to DiRas3. The ability of DiRas3 to homo-oligomerize and to enhance the homo-oligomerization of KSR1 suggests that high concentrations of DiRas3 would favour the homo-oligomerization of DiRas3 and consequently the formation of KSR1 homo-oligomers, whereas a balanced ratio between activated H-Ras and DiRas3 would lead to increased formation of KSR1:C-RAF(A-RAF) complexes and in this way regulate the availability of C-RAF(A-RAF) for interaction with B-RAF (Fig. 3E).

By manipulating RAF dimerization one can alter the cellular response, such as cell transformation [13, 21]. Accordingly, our data revealed that expression of DiRas3 inhibited the transformation of NIH3T3 cells by oncogenic Ras. However, this effect depends on the expression level of DiRas3, since increasing the DiRas3 concentration beyond the certain threshold abolished the inhibitory effect. The DiRas3-induced impact on NIH3T3 transformation was not due to the delayed cell growth and increased cell death, since the transformation of the cells with the high expression levels of DiRas3 was not inhibited, although these cells experienced the strongest growth delay. The studies on the role of DiRas3 in tumourigenesis, that have been published so far, support its tumour suppressor function. However, there are several hints suggesting that DiRas3 does not always act as a tumour suppressor and that the anti-tumourigenic function of DiRas3 may depend on the cellular context. For example, the expression level of DiRas3 was decreased at the later stages but was markedly enhanced at the early stage during the transformation of immortalized human mesenchymal stem cells [47]. DiRas3 has been found overexpressed in malignant versus benign thyroid tumours [48]. The expression of DiRas3 appeared strongly increased in endometriosis epithelial cells as compared to normal ovarian epithelial cells [49]. Furthermore, the expression of the DiRas3 gene was up-regulated in the keratinocytes from the large congenital melanocytic nevi bearing the activating B-RAF-V600E mutation [50].

There is an increasing number of genes that originally have been assigned a tumour suppressor or an oncogenic role, but after further investigations appeared to have a dual function depending on cellular context. Also KSR1 has been assigned a dual function. It, facilitates ERK signal transduction in Ras-dependent cancers and is required for oncogenic Ras-induced transformation of MEFs [51-55]. However, recently an ERK-independent tumour suppressor function of KSR1 has been demonstrated in breast cancer, suggesting that the role of KSR1 in tumour progression is context-dependent [56]. Indeed, the controversial behaviour of KSR1 has been first observed by Kortum and Lewis [54] a decade ago. The authors reported that depletion of KSR1 blocked Ras12V-induced transformation of MEFs. Re-expression of KSR1 at low levels restored the transforming capacity of Ras12V, whereas the expression of KSR1 at higher levels completely inhibited transformation. At that time, when the KSR1 function was considered only in scaffolding of MAPK components, the loss of signalling at supra-physiologic KSR1 expression levels was proposed to be due to the titration of pathway members away from each other, a phenomenon termed as "combinatorial inhibition" [54]. However, emerging evidence suggests that KSR1 possesses dual activity as a scaffold protein and maybe also as active kinase phosphorylating C-RAF and MEK [17, 57-59]. Moreover, KSR1 can also form dimers with RAF and modulate its activity adding complexity to the simple view of KSR1 scaffolding function [17-18]. In light of these findings, our data suggest that the peculiar concentration-dependent effect of DiRas3 on Ras12V-iduced transformation may be due to involvement of KSR1, the protein whose function and regulation is barely understood. This means that in order to fully understand the role of DiRas3 in the Ras12V-induced signalling and cell transformation, we will need to gain a comprehensive insight into the molecular mechanisms underlying the regulation of cellular processes by KSR1.

5. Conclusions

Taken together, in this study we present data on the critical involvement of KSR1 in the DiRas3-dependent regulation of RAF dimerization. We show that DiRas3 binds to KSR1 independently of its interaction with activated Ras and RAF. Our data also suggest that depending on the local stoichiometry between DiRas3 and oncogenic Ras, DiRas3 can either enhance homodimerization of KSR1 or recruit KSR1 to the Ras:C-RAF complex and thereby reduce the availability of C-RAF for binding to B-RAF. This mechanism, which is shared between A-RAF and C-RAF, may be involved in the regulation of Ras12V-induced cell transformation by DiRas3.

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Figure legends

Fig. 1. Heterodimerization of A-RAF and C-RAF with B-RAF is regulated by DiRas3. COS7 cells were transfected with expression vectors as indicated. Dimerization of RAF proteins was induced by co-transfection with H-Ras12V. Protein complexes were isolated from cell lysates by anti-myc (A and B), anti-HA (C) or anti-V5 (D) immunoprecipitation (IP) and analysed by immunoblotting (IB). (*) B-RAF band; (**) C-RAF band; (***) A-RAF band. (A) Binding of DiRas3 to A-RAF is enhanced by H-Ras12V. (B and C) DiRas3 inhibits the C-RAF:B-RAF and A-RAF:B-RAF heterodimerization. (D) DiRas3 binds preferentially to C-RAF, followed by A-RAF and B-RAF. Antibodies used for IB: anti-HA, anti-DiRas3, anti-Ras, anti-B-RAF, and anti-myc.

Fig. 2. DiRas3 binds to BxB but does not affect its kinase activity and subcellular localisation. (A) HA-tagged BxB was transiently expressed either alone or together with H-Ras12V and myc-DiRas3 in COS7 cells. Protein complexes were isolated from cell lysates by anti-myc immunoprecipitation (IP) and analysed by immunoblotting (IB). Myc-His-tagged C-RAF (B) or HA-tagged BxB (C and D) were expressed either alone or together with H-Ras12V and myc-DiRas3 in COS7 cells. C-RAF and BxB were immunoprecipitated by anti-His or anti-HA antibodies, respectively. Their catalytic activity was measured by an *in vitro* kinase assay using purified MEK and ERK as substrates. (E and F) Western blot analysis of the subcellular distribution of C-RAF (E) and BxB (F). C-RAF-myc-His (E) or HA-BxB (F) was transfected either alone or together with DiRas3 and H-Ras12V into COS7 cells. 24 h after transfection, cytosolic (F1), membrane (F2), nuclear (F3), and cytoskeletal (F4) fractions were collected. Anti-M2PK, anti-KDEL, anti-PARP, and anti-vimentin immunodetection was used as fractionation controls for cytosolic, membrane, nuclear, and cytoskeletal fractions, respectively. Antibodies used for IB: anti-HA, anti-DiRas3, anti-Ras, anti-pERK, anti-ERK, anti-myc, anti-C-RAF, anti-M2PK, anti-KDEL, anti-PARP, and anti-Vimentin.

Fig. 3. DiRas3 regulates the choice between B-RAF and KSR1 for binding to C-RAF or A-RAF. COS7 cells were transfected with expression vectors as indicated. Protein complexes were isolated from cell lysates by anti-Flag immunoprecipitation (IP) and analysed by immunoblotting (IB). (A-C) DiRas3 enhances binding of RAF isoforms to KSR1. (D) DiRas3 has no effect on binding of BxB to KSR1. (E) A hypothetical model for the DiRas3-dependent regulation of RAF heterodimerization. DiRas3 recruits KSR1 to the H-Ras:C-RAF or H-Ras:A-RAF complexes and therefore prevents the binding of B-RAF to C-RAF or A-RAF. The increase of the intracellular concentration of DiRas3 relative to the concentration of active H-Ras

results in the DiRas3-induced homodimerization of KSR1. Antibodies used for IB: anti-HA, anti-DiRas3, anti-Flag, and anti-B-RAF.

Fig. 4. Binding of KSR1 to DiRas3 stabilizes the tetrameric complex consisting of H-Ras, C-RAF, KSR1 and DiRas3. NIH3T3 (A) or COS7 (B-F) cells were transfected with expression vectors as indicated. Protein complexes were isolated from cell lysates by anti-V5 (A) or anti-myc (B, D, E and F) immunoprecipitation (IP) and analysed by immunoblotting (IB). (A) Endogenous KSR1 binds to DiRas3. (B) Binding of DiRas3 to KSR1 is H-Ras12V-independent. (C) Sequential co-immunoprecipitation. COS7 cells were transfected with expression vectors as indicated. First, protein complexes were isolated from cell lysates by anti-V5 immunoprecipitation (1. V5-IP) and eluted from beads with 1mg/ml V5 peptide. The eluted protein complexes were then isolated from diluted eluate by anti-Flag immunoprecipitation (2. Flag-IP) and analysed by immunoblotting. (D, E and F) KSR1 enhances binding of C-RAF and A-RAF, but not B-RAF, to DiRas3. Antibodies used for IB: anti-KSR1, anti-V5, anti-Flag, anti-DiRas3, anti-Ras, anti-HA, and anti-B-RAF.

Fig. 5. Binding of DiRas3 to KSR1 is independent of interaction with Ras-activated C-RAF or A-RAF. COS7 (A-D) or NIH3T3 (E-H) cells were transfected with expression vectors as indicated. Protein complexes were isolated from cell lysates by anti-Flag (A), anti-V5 (B and H), anti-myc (C and D) and anti-HA (E-G) immunoprecipitation (IP) and analysed by immunoblotting (IB). (A and B) R398A mutation in C-RAF inhibits its binding to DiRas3 and KSR1. (C and D) Mutation of Arg362 in A-RAF does not have any effect on its interaction with KSR1 and DiRas3. (E-G) Mutation of the C-terminal 14-3-3 binding site in C-RAF inhibits B-RAF:C-RAF heterodimerization, but does not affect binding of C-RAF to KSR1 and DiRas3 (samples in (G) were run on the same blot). (H) Binding of KSR1 to DiRas3 is not regulated by phosphorylation on S392. Antibodies used for IB: anti-KSR1, anti-myc, anti-V5, anti-Flag, anti-DiRas3, anti-Ras, anti-HA, anti-B-RAF, anti-C-RAF, and anti-C-RAF-phospho-Ser621.

Fig. 6. DiRas3 enhances the side-to-side homodimerization of KSR1. COS7 cells were transfected with expression vectors as indicated. Protein complexes were isolated from cell lysates by anti-Flag (A and C) or anti-V5 (B) immunoprecipitation (IP) and analysed by immunoblotting (IB). (A) DiRas3-enhanced homodimerization of KSR1 requires intact side-to-side dimerization interface. (*) non-specific band detected by anti-HA antibody; (**) HA-KSR1 band. (B) Homodimerization of DiRas3. (C) Increasing the cellular DiRas3 concentration beyond a certain threshold leads to an abrupt rise in DiRas3 binding to KSR1. Antibodies used for IB: anti-HA, anti-V5, anti-Flag, and anti-DiRas3.

Fig. 7. Effect of DiRas3 expression on cell growth and transformation. (A) NIH3T3 cells were transfected with expression vectors as indicated and grown in DMEM supplemented with 10% donor bovine serum. 48 h post transfection, cells were lysed for protein expression analysis by Western blot. The amount of DiRas3 cDNA used in the experiments is indicated in the figure. (B) Eight random images of each plate were taken 3 days post transfection. (C) The images were used for quantification of cell numbers. (D and E) Cells were grown to

confluency. 14 days post transfection the cells were fixed and examined for focus formation by crystal violet staining. A representative plate of cells from each transfection combination is shown in (D). The quantification from 6 replicates for each transfection combination is shown in the bar graph (E). Antibodies used for IB: anti-pERK, anti-ERK, anti-Ras, anti-DiRas3, anti-PARP, anti-AKT, and anti-AKT-phospho-Ser473.

Figure 1
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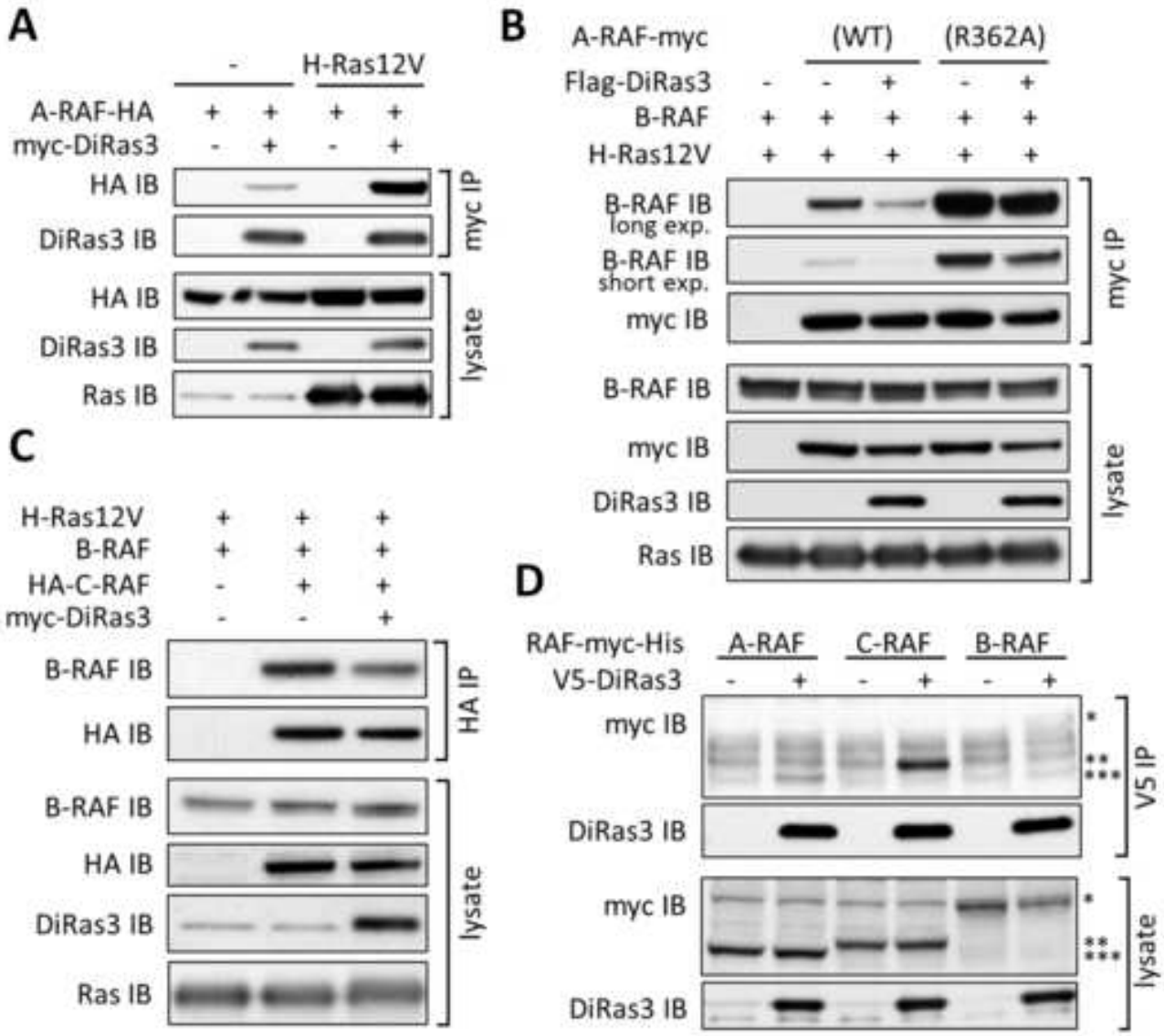
Figure 1



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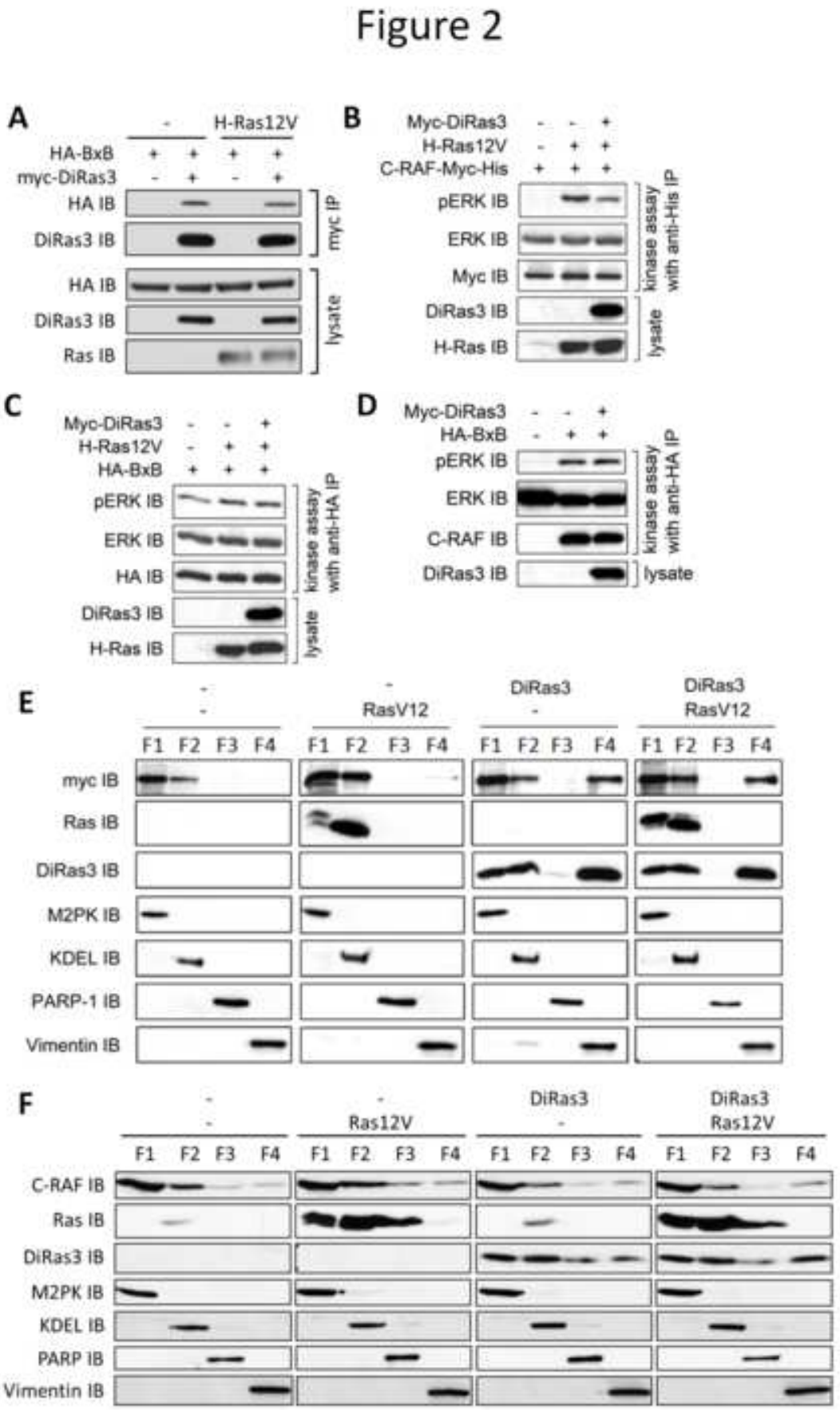

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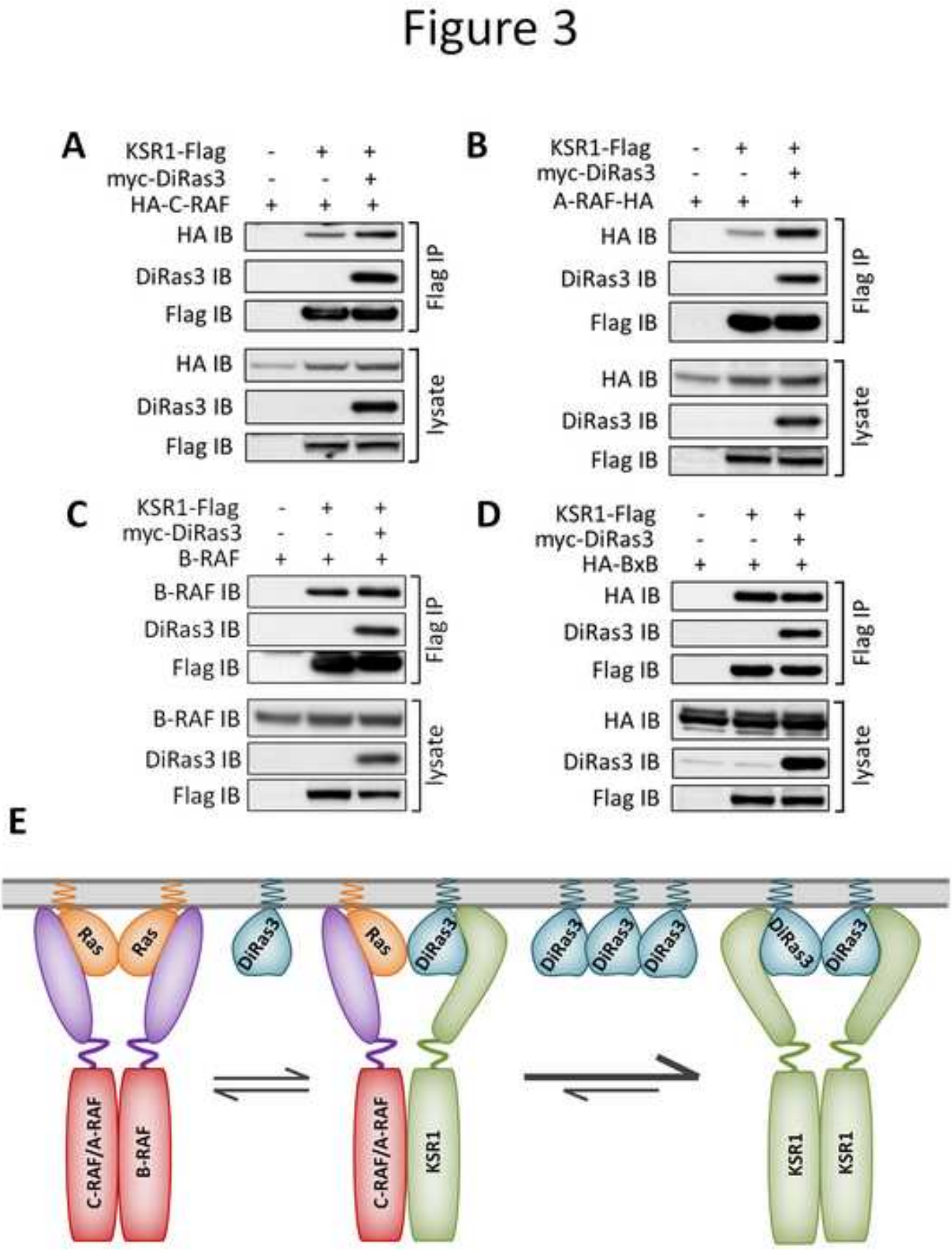

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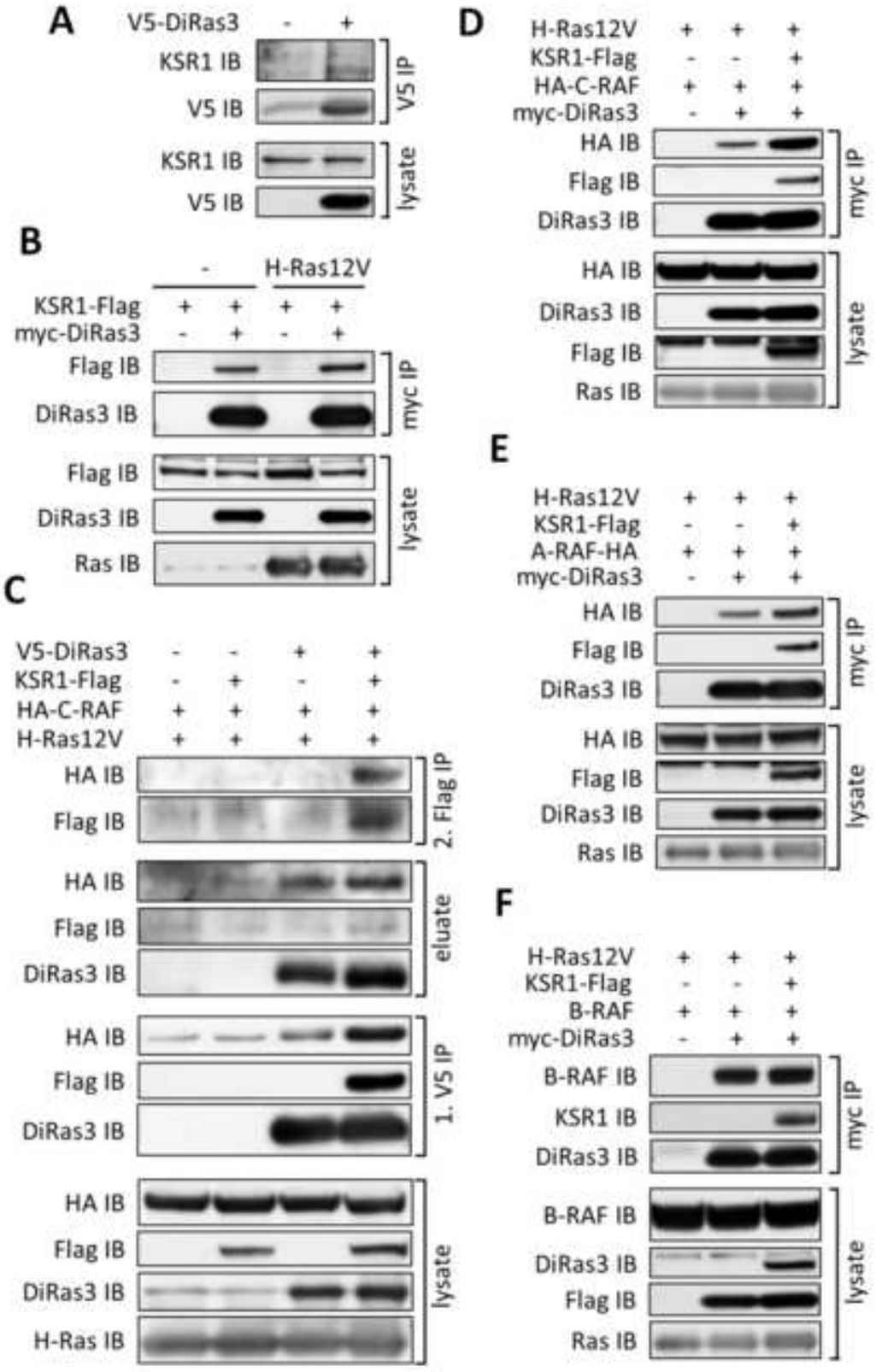

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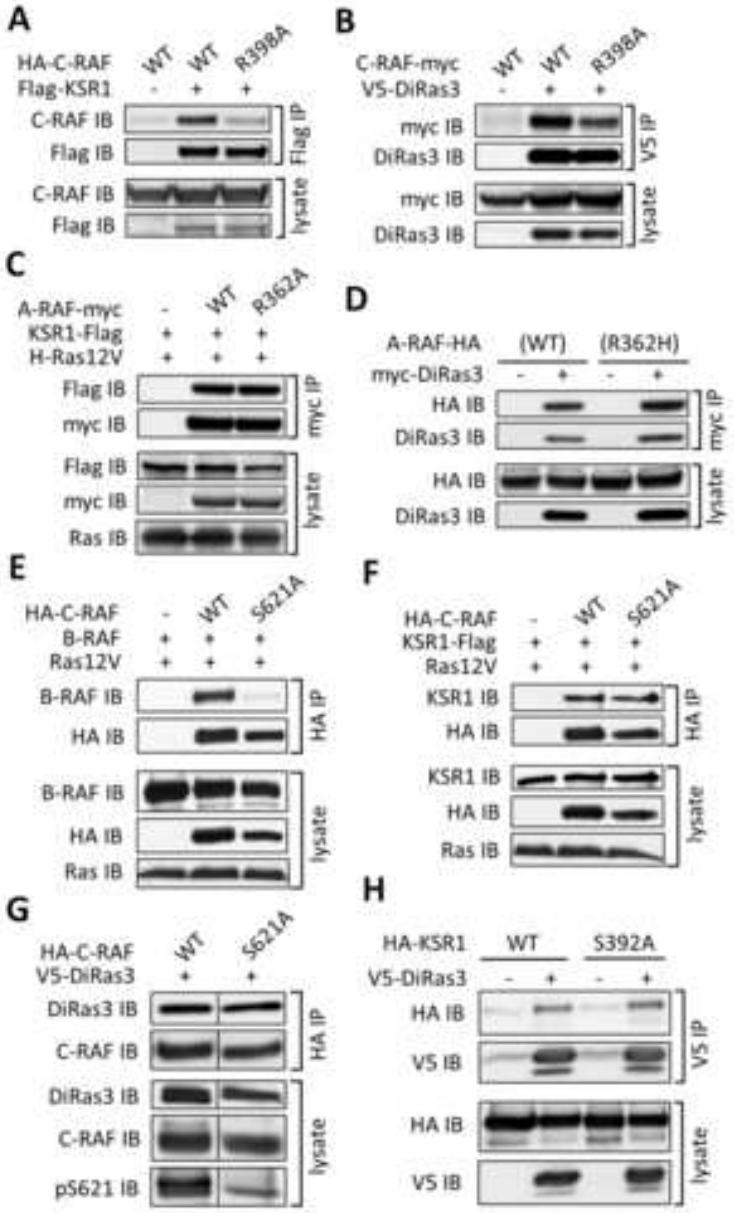

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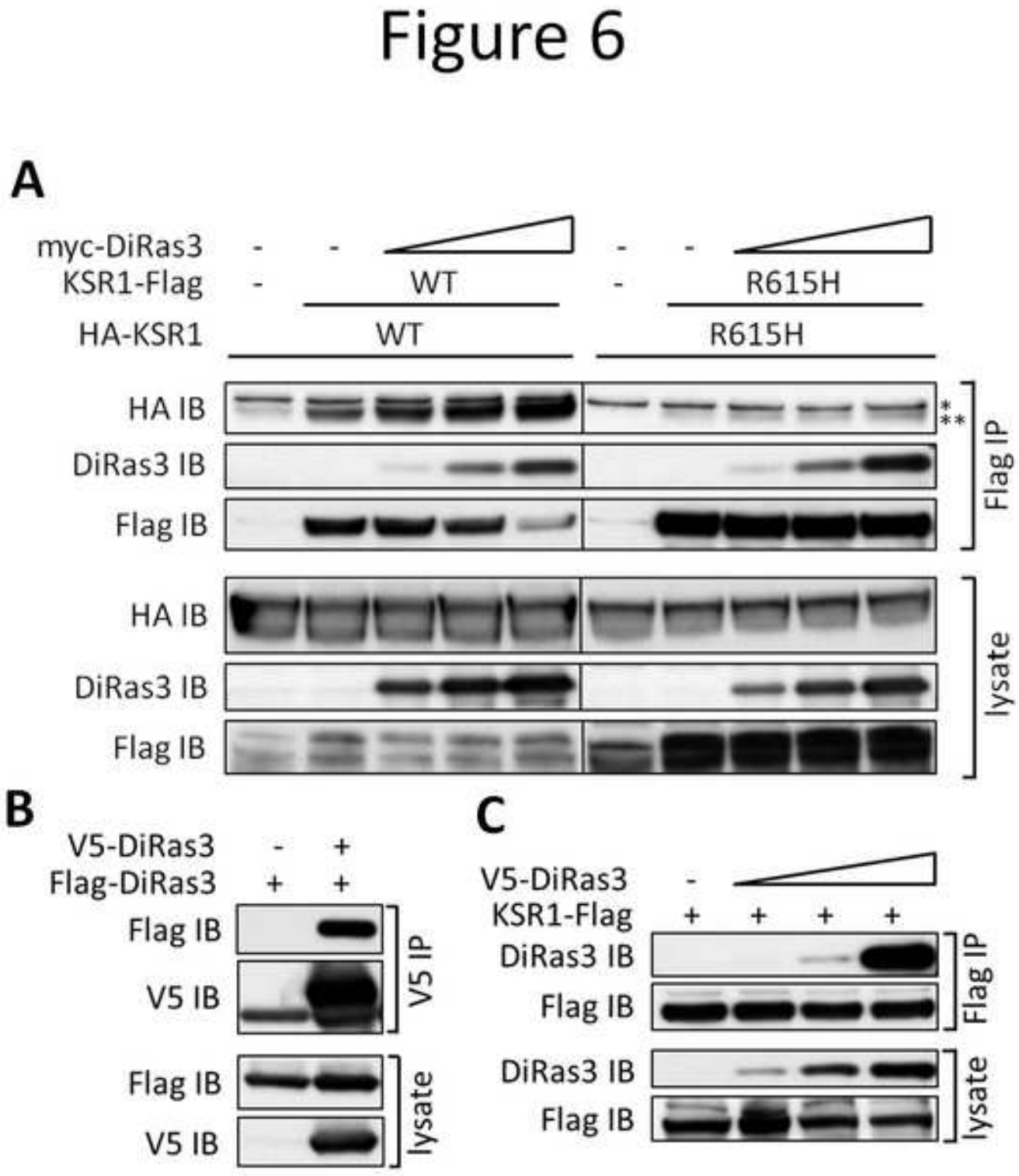

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