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Ras-GTP dimers activate the Mitogen-Activated Protein Kinase (MAPK) pathway

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Rat sarcoma (Ras) GTPases regulate cell proliferation and survival through effector pathways including Raf-MAPK, and are the most frequently mutated genes in human cancer. Although it is well established that Ras activity requires binding to both GTP and the membrane, details of how Ras operates on the cell membrane to activate its effectors remain elusive. Efforts to target mutant Ras in human cancers to therapeutic benefit have also been largely unsuccessful. Here we show that Ras-GTP forms dimers to activate MAPK. We used quantitative photoactivated localization microscopy (PALM) to analyze the nanoscale spatial organization of PAmCherry1-tagged KRas 4B (hereafter referred to KRas) on the cell membrane under various signaling conditions. We found that at endogenous expression levels KRas forms dimers, and $KRas^{G12D}$, a mutant that constitutively binds GTP, activates MAPK. Overexpression of KRas leads to formation of higher order Ras nanoclusters. Conversely, at lower expression levels, $KRas^{G12D}$ is monomeric and activates MAPK only when artificially dimerized. Moreover, dimerization and signaling of KRas are both dependent on an intact CAAX (C, cysteine; A, aliphatic; X, any amino acid) motif that is also known to mediate membrane localization. These results reveal a new, dimerization-dependent signaling mechanism of Ras, and suggest Ras dimers as a potential therapeutic target in mutant Ras-driven tumors.

Ras dimer | MAPK signaling | cancer | single molecule imaging | superresolution microscopy

The canonical rat sarcoma (Ras) GTPase family members *H-*, *N-*, and *K-ras* are frequently activated in human cancers (1–4) by recurrent point mutations at codons 12, 13, or 61. These mutations result in constitutive binding of Ras to GTP due to impaired GTP hydrolysis (5). Despite nearly identical G-domains, mammalian Ras isoforms serve nonredundant biological roles and exhibit different mutational spectra in human cancers (1, 4, 6). These functional differences are in part attributed to distinctions in the membrane-tethering motif at the C-terminal of Ras known as the hyper-variable region [HVR, which includes the "CAAX" (C, cysteine; A, aliphatic; X, any amino acid) motif] (6, 7). Although mechanisms regulating Ras-GTP levels in cells have been examined extensively, details of how Ras organizes and operates on the cell membrane have been elusive. Efforts on targeting mutant Ras to therapeutic benefits in human cancers by inhibiting membrane localization or GTP binding have not been successful, leaving mutant Ras an intractable drug target (8). Hence, identification of new mechanisms that regulate Ras oncogenesis is crucial to combating mutant Ras-driven cancers.

Recent studies using immuno electron microscopy (immuno-EM) have implicated a previously unappreciated spatial mechanism in regulating the biological functions of Ras. In particular, Ras proteins were found to form 5- to 8-membered nanoclusters that serve as signaling scaffolds for recruiting and activating downstream effectors such as Raf and PI3K on the cell membrane (9–11). This spatial regulation mechanism may offer a new venue for targeting mutant Ras in human cancers. However, the immuno-EM studies were limited by the need to separate membrane sheets from living cells to image Ras proteins on the inner leaflet of the membrane, and the need to overexpress Ras to compensate the low labeling efficiency with gold-conjugated antibodies (9, 10). These limitations leave the nature, molecular organization, and biological relevance of the observed Ras nanoclusters in question.

Superresolution light microscopy techniques such as photoactivated localization microscopy (PALM) circumvent these limitations and enable imaging of intact biological samples with 10- to 20-nm spatial resolution and single molecule sensitivity (12, 13). As demonstrated in several recent studies (14–16), PALM allows quantitative analysis of cellular proteins with nanometer spatial and single-copy stoichiometric resolutions, ideally suited for studying Ras nanoclusters. Here, we have combined PALM with biochemical analysis to measure the nanoscale organization of individual, PAmCherry1-tagged KRas molecules in intact cells and under physiological conditions in correlation with the biological outcome. Our studies implicate Ras-GTP dimers in Raf-MAPK activation, which contrasts some beliefs that Ras signals as a monomeric GTPase and others that Ras signals as a cluster with 5–8 monomers. Together with previous reports on Raf dimerization, our finding suggests a new, dimer model of Ras-Raf signaling and provides the molecular basis for alternative approaches to targeting mutant Ras in human cancers.

Results

Ras-GTP Dimer Formation Coincides with MAPK Activation. We constructed chimeric cDNAs encoding PAmCherry1 fused to wild-type

Significance

Rat sarcoma (Ras) proteins play central roles in both normal and oncogenic signaling. Mechanisms of how Ras interacts with its effectors on the cell membrane, however, are still poorly understood, significantly hampering efforts to target this molecule in human cancer. Here we have used quantitative superresolution fluorescence microscopy in combination with carefully engineered biological systems to show that Ras dimers drive oncogenic signaling through the Raf-MAPK pathway. Our study suggests a new, dimer model of Ras-Raf signaling and the potential value of Ras dimers as a therapeutic target.

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human KRas 4B (hereafter referred to as KRas) or a G12D mutant thereof (hereafter referred to as $KRas^{G12D}$) that stably binds to GTP. We verified the biological activities of the PAmCherry1-KRas fusion proteins using an NIH 3T3 cell transformation assay (Fig. S1). For tetracycline-regulated expression in mammalian cells, these constructs were incorporated into lentiviral plasmids with a CMV-TetOn promoter (17) in front of PAmCherry1-KRas, as illustrated in Fig. 1. The plasmids were then used to generate lentivirus for infecting BHK21 cells stably expressing the Tet-Repressor (tetR). In infected and isolated single BHK21 cell clones, PAmCherry1-$KRas^{G12D}$ expression was undetectable in the absence of doxycycline (Dox, a tetracycline analog) and increased in a Dox-concentration dependent manner (Fig. 1*B*). As expected, PAmCherry1-$KRas^{G12D}$ almost exclusively localized to the cell membrane in these cells (Fig. 1*C*).

Interestingly, MAPK activation by $KRas^{G12D}$ as measured by phospho-Erk (ppErk) levels did not increase linearly with the expression level of $KRas^{G12D}$. At a Dox concentration of 1 ng/mL, expression of PAmCherry1-$KRas^{G12D}$ was already evident, albeit at much a much lower level than that of endogenous KRas (Fig. 1*B*, lane 3); MAPK activation, however, was observed only at Dox concentrations of 2 ng/mL and above, at which point PAmCherry1-$KRas^{G12D}$ protein levels were comparable to endogenous KRas or slightly overexpressed (Fig. 1*B*, lanes 4–6). As a control, we studied the expression of PAmCherry1-KRas wild type by varying the Dox concentration and found that there was no significant MAPK activation, even at high expression levels when PAmCherry1-KRas molecules were clustered (Figs. S2 and S3). These data indicate that a functional transition occurred between 1 and 2 ng/mL Dox that enabled $KRas^{G12D}$ to activate MAPK.

PALM imaging revealed that $KRas^{G12D}$ underwent a monomer to dimer transition in these cells when Dox concentration increased from 1 to 2 ng/mL. Using a PALM imaging and spatial analysis procedure as described previously (14), we evaluated changes in the membrane density and spatial organization of PAmCherry1-$KRas^{G12D}$ in BHK21 cells as a function of Dox concentration. Consistent with Western blotting results, we detected 11 ± 5, 72 ± 14, and 116 ± 27 (mean ± SD) PAmCherry1-$KRas^{G12D}$ molecules per μm^2 in PALM images of cells exposed to 1, 2, and 5 ng/mL Dox, respectively. Both visual inspection (Fig. 1*D*, *Left*) and Ripley's K test (18) (Fig. 1*D*, *Right*) clearly showed that PAmCherry1-$KRas^{G12D}$ molecules predominated in monomeric form at 1 ng/mL Dox but became significantly aggregated at 2 ng/mL Dox. Cluster size analysis with simulation aided DBSCAN (SAD) (14) confirmed the dominance of monomeric $KRas^{G12D}$ at 1 ng/mL Dox. SAD analysis further revealed that at 2 ng/mL Dox, $KRas^{G12D}$ clusters were mostly dimers and that higher order structures including trimers are rare (Fig. 1 *E* and *F*). On average, the density of $KRas^{G12D}$ dimers increased from 0.2 ± 0.1 to 5.9 ± 1.0 per μm^2, and that of trimers showed only a slight increase from 0.1 ± 0.1 to 0.5 ± 0.3 per μm^2, when Dox concentration increased from 1 to 2 ng/mL.

In PALM images taken at 2 ng/mL Dox, the dimers appeared as pairs of closely spaced PAmCherry1 molecules (Fig. 1*D*, *Lower Left* and *Inset*) with an apparent size of ~30 nm based on Ripley's K test (Fig. 1*D*, *Lower Right*). We note that this apparent size of Ras-GTP dimers is mostly due to the limited precision (~18 nm) in localizing single PAmCherry1 molecules, and that the physical size of the dimers should be much smaller.

Dimers remained the dominant form of Ras clusters even at Dox concentration of 5–10 ng/mL (Fig. S3, *Top*), when $KRas^{G12D}$ was slightly overexpressed. Large (>4) Ras clusters were observed only at transient overexpression conditions comparable to those previously used in immuno-EM studies (Fig. S3, *Middle*) (9, 10). The same correlation between KRas dimer formation and MAPK activation was observed in TRex-293 cells expressing PAmCherry1-$KRas^{G12D}$ (Fig. S4).

Artificial Dimerization of Ras-GTP Monomers Activates MAPK. The coincidence between Ras-GTP dimer formation and MAPK activation suggests the possibility that Ras-GTP dimers activate MAPK; alternatively, MAPK activation could simply result from the elevated membrane density of Ras-GTP at Dox concentrations of 2 ng/mL and above. To distinguish the two possible mechanisms and better define the role of Ras dimer formation in MAPK activation, we used an artificial dimerization strategy

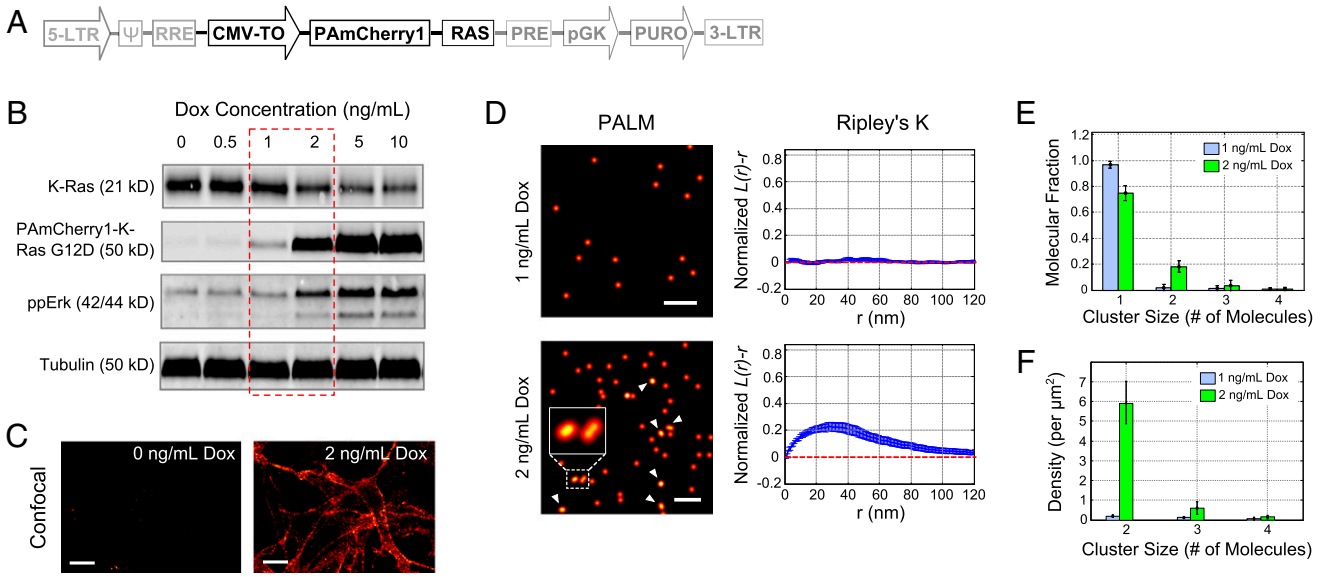

Fig. 1. $KRas^{G12D}$ forms dimers at expression levels that activate Raf-MAPK. We used a lentiviral vector construct with a CMV-TO (TetOn) promoter (*A*) to express PAmCherry1-KRas under tetracycline or doxycycline (Dox) regulation. (*B*) Western blotting demonstrated dose-dependent induction of PAmCherry1-$KRas^{G12D}$ expression, leading to varying degrees of activation of Raf/MAPK (as indicated by ppErk) in a single clone of BHK21 cells. Confocal fluorescence microscopy showed a clear membrane localization of the induced PAmCherry1-$KRas^{G12D}$ proteins (*C*). (*D–F*), PALM imaging and cluster analysis of PAmCherry1-$KRas^{G12D}$ molecules in cells from the same clone as in *B* induced at 1 and 2 ng/mL Dox. Each dot in the PALM images represents one PAmCherry1-tagged $KRas^{G12D}$ molecule. White arrows indicate dimers (see also the inset in the bottom PALM image). (Scale bars, 5 μm in *C* and 200 nm in *D*.)

to drive "monomeric" Ras-GTP into dimers without overexpression. Here, "monomeric" Ras-GTP is $KRas^{G12D}$ expressed at levels much lower than endogenous Ras (Fig. 1*D*). An FKBP-derived dimerization domain (DD, ~11 kDa) was genetically fused to the N terminus of PAmCherry1-$KRas^{G12D}$ (hereafter referred to as DD-PAmCherry1-$KRas^{G12D}$). A small molecule dimerizer, AP20187, can bind two and only two DDs (19) and in so doing forces DD-PAmCherry1-$KRas^{G12D}$ proteins into artificial dimers (Fig. 2*A*).

We established stable BHK21 cell lines using the same strategy described earlier and isolated single cell clones that expressed DD-PAmCherry1-$KRas^{G12D}$ with Dox induction (Fig. 2*B*). Similar to those shown in Fig. 1*B* for PAmCherry1-$KRas^{G12D}$, induction with 1 ng/mL Dox in isolated single BHK21 cell clones yielded detectable amounts of DD-PAmCherry1-$KRas^{G12D}$ but failed to activate MAPK (Fig. 2*B*, lane 3). However, addition of AP20187 (15 min at 100–500 nM) to these cells strongly activated MAPK (Fig. 2*B*, lane 4). Cells exposed to 2 ng/mL Dox also showed up-regulation in MAPK activation when AP20187 was added despite that MAPK was already activated; this is likely because at this Dox concentration, only a fraction of DD-PAmCherry1-$KRas^{G12D}$ was in dimer form before addition of AP20187, as evidenced in Fig. 1 *D–F*. As a control, AP20187 did not show any effects on MAPK activation in cells unexposed to Dox (Fig. 2*B*, lanes 1 and 2). PALM imaging confirmed that at 1 ng/mL Dox, DD-PAmCherry1-$KRas^{G12D}$ molecules were monomers but were converted into dimers by AP20187 without formation of higher order clusters or increase in membrane density of Ras proteins (Fig. 2*C*).

We observed the same MAPK activation by artificial dimerization of monomeric $KRas^{G12D}$ in other cell lines and using other expression systems. For example, in TRex-293 cells expressing low levels of DD-PAmCherry1-$KRas^{G12D}$ at 1 ng/mL Dox, treatment with AP20187 induced strong activation of MAPK within 15 min (Fig. S5). Additionally, low-level expression of DD-PAmCherry1-$KRas^{G12D}$ was also achieved by using a weak, phosphoglycerol kinase (PGK) promoter. In BHK21 cells stably expressing DD-PAmCherry1-$KRas^{G12D}$ under the PGK promoter, we measured only 15 ± 6 molecules per μm^2 on the membrane with PALM. This membrane density is similar to that achieved in the tetracycline-regulated expression system at 1 ng/mL Dox (Fig. 1*D*, *Top*); this expression level is ~1/5 that of endogenous, wild-type KRas, again in good agreement with Western blotting (Fig. 2*D*). Similar to that shown Fig. 2*B*, treatment of these BHK21 cells with AP20187 (100–500 nM, 15 min) also caused profound activation of MAPK (Fig. 2*D*). These data confirm that MAPK activation by Ras-GTP dimer formation is not limited to a single biological system, and that formation of Ras-GTP dimers, rather than increased membrane density alone, was responsible for the observed MAPK activation.

In agreement with Ras dimerization-induced MAPK activation, when TRex-293 cells expressing DD-PAmCherry1-$KRas^{G12D}$ under 1 ng/mL Dox were serum starved or treated with a MEK inhibitor (e.g., Trametinib; 50 nM) for a few days, we observed massive cell death in the absence of AP20187. By contrast, coincubation with AP20187 (100 nM) effectively rescued the cells, although the cells showed an apparently different morphology (Fig. S6).

Ras Dimerization and Signaling Depend on an Intact CAAX Motif. We next sought to explore the mechanisms that drive Ras dimer formation. First, we asked whether Ras dimerization is dependent on GTP-binding, a key event in Ras activation. Interestingly, PAmCherry1-KRas wild-type proteins (mostly GDP-bound) formed dimers to a similar extent to PAmCherry1-$KRas^{G12D}$ when expressed at similar expression levels (i.e., at a membrane density of ~70 molecules per μm^2) in serum-starved cells, as evidenced by comparing Fig. 3 *A* and *C* with Fig. 1*D*. Wild-type KRas and the G12D mutant also aggregated similarly when transiently overexpressed (Fig. S3, *Bottom*). Hence, KRas dimerization or higher-order cluster formation does not appear to require GTP-binding, and instead is a shared property between the GDP- and GTP-bound forms.

Next, we imaged and analyzed membrane clustering of PAmCherry1-CAAX, a chimera with PAmCherry1 fused to the HVR

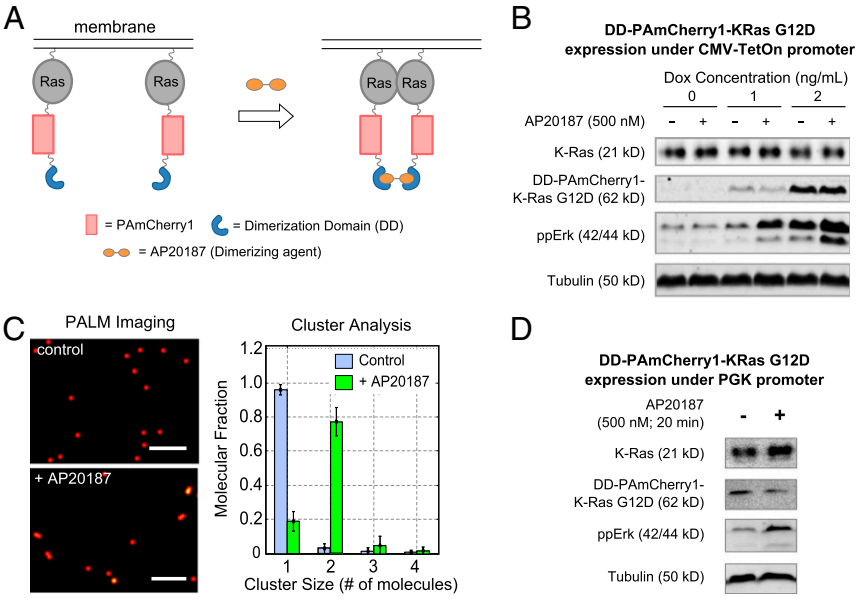

Fig. 2. Artificial dimerization of monomeric $KRas^{G12D}$ leads to Raf-MAPK activation. (*A*) Schematics of the artificial dimerization system, where a dimerization domain (DD) is genetically fused to the N terminus of PAmCherry1-$KRas^{G12D}$. A small molecule, AP20187, forces PAmCherry1-$KRas^{G12D}$ to dimerize by binding to two DD domains at once. (*B*) Western blot showing Dox-induced expression of DD-PAmCherry1-$KRas^{G12D}$ and effects of AP20187. Induction at 1 ng/mL Dox yielded detectable levels of DD-PAmCherry1-$KRas^{G12D}$ but no Raf/MAPK activation (lane 3) until AP20187 was added (lane 4). AP20187 also increased ppErk in cells treated with 2 ng/mL Dox but to a lesser extent (lanes 5–6). (*C*) PALM imaging and cluster size analysis of DD-PAmCherry1-$KRas^{G12D}$ expressed in cells induced at 1 ng/mL Dox, confirming a monomer to dimer conversion before and after AP20187 treatment. (*D*) Western blotting demonstrated Raf/MAPK activation by AP20187 in BHK21 cells expressing low levels of DD-PAmCherry1-$KRas^{G12D}$ under a weak, PGK promoter. (Scale bars, 200 nm.)

(last 21 residues at the C terminus) of KRas without a G-domain, expressed in BHK21 cells. Surprisingly, PAmCherry1-CAAX also aggregated similarly to both PAmCherry1-KRas and the G12D mutant at comparable expression levels (75 ± 23 molecules per μm^2, Figs. 3 *B* and *C*, and 1 *D*–*F*). This observation suggests that the KRas HVR alone can mediate protein dimerization in cells, in addition to its known role in membrane localization. Although the exact mechanisms are unclear, protein dimerization and clustering mediated by the Ras HVR has been previously reported, for examples on CRaf-CAAX with quantitative PALM (14) and on GFP-CAAX with immuno-EM (10).

Consistent with the critical role of the HVR in Ras dimerization and signaling, DD-PAmCherry1-KRas$^{G12D/C185S}$, an SAAX mutant with impaired posttranslational modifications and membrane localization (20), failed to activate MAPK even in the presence of AP20187 (Fig. 3*D*). In comparison, Y64A, a mutation in the G-domain of Ras, did not have an effect on either the spontaneous activation of MAPK by KRas^{G12D} or the induced activation by AP20187 (Fig. S7). The Y64A mutation was suggested to be at or coupled to the HRas–HRas dimer interface in a recent study (21), but the experiments used recombinant HRas tethered to an artificial lipid bilayer via a synthetic linker instead of the actual, posttranslationally modified HVR. Together, our data demonstrate that KRas dimerization and signaling depend on an intact CAAX motif.

We argue that it is unlikely that PAmCherry1 induces the dimer formation that activates MAPK signaling. The probe is a strictly monomeric protein even in crystallized form (22). More significantly, we compared the onset of MAPK signaling of DD-PAmCherry1-KRas with DD-KRas without PAmCherry1 in TRex-293 cells, and also with and without the addition of the dimerizing agent (Fig. S8). Note that the threshold for MAPK signaling in Fig. S8 in the absence of the dimerizing agent is now between 2 and 4 ng/mL. If the addition of PAmCherry1 to KRas were the cause of dimerization at the onset of MAPK signaling, we would expect that a lower concentration of DD-PAmCherry1-KRas^{G12D} would trigger MAPK signaling. Contrary to the prediction, we observed that the addition of PAmCherry1 to DD-KRas actually slightly reduces MAPK signaling.

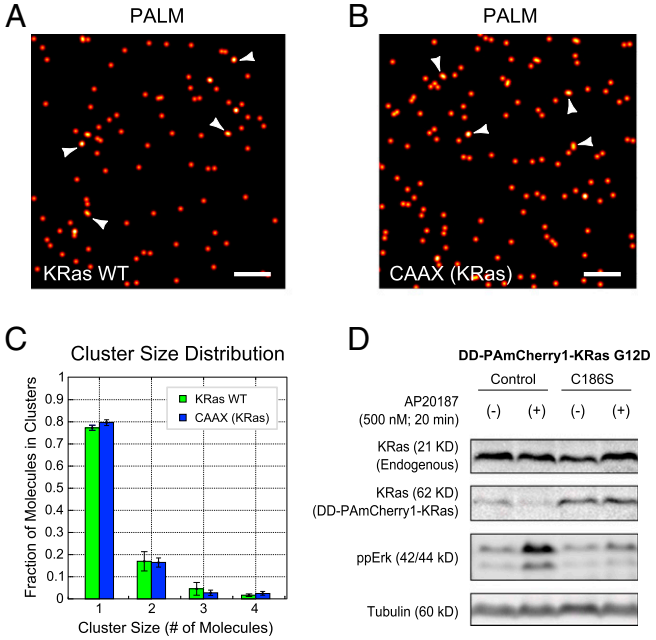

Fig. 3. Ras dimerization and signaling depend on an intact CAAX motif. PALM images of PAmCherry1-KRas wild type (*A*) and PAmCherry1-CAAX (CAAX = last 21 amino acids of KRas) (*B*) taken on cells expressing the proteins at membrane densities around 70 ± 19 molecules per μm^2. White arrows indicate dimers and occasional higher order clusters. (*C*) Cluster size analysis of both PAmCherry1-KRas (green) and PAmCherry1-CAAX (blue) indicated similar clustering properties between the two proteins as well as to PAmCherry1-KRas^{G12D} (Fig. 1*E*). (*D*) Western blot comparing the response of DD-PAmCherry1-KRas^{G12D} (lanes 1–2) and the DD-PAmCherry1-Kras$^{G12D/C185S}$ double mutant (lanes 3–4) to artificial dimerization by AP20187. Both proteins were expressed at low levels under a PGK promoter. (Scale bars, 200 nm.)

A New, Dimer Model for Ras-Raf Signaling. Taken together, our results demonstrate that the formation of Ras-GTP dimers at 6 dimers/μm^2 is sufficient to activate the Raf-MAPK pathway. Based on the results, we suggest the new, dimeric model for Ras/Raf/MAPK signaling shown in Fig. 4. In this model, Ras molecules undergo both dimerization and nucleotide exchange on the plasma membrane. When a Ras monomer is converted from its GDP- to GTP-bound form, it can recruit a Raf molecule but this event alone does not activate Raf because activation of wild-type Raf requires dimerization (23). Raf is activated when two Ras-GTP molecules, each bound to a Raf kinase, dimerize to bring two Raf molecules into a dimer. In an alternative scenario, Ras-GTP dimers may form before the recruitment and activation of two Raf molecules. Although largely based on data with wild-type Raf, our model may also apply to other Raf dimer configurations such as BRaf-CRaf (24) or even Raf^{WT}-Raf^{KD} (where KD = kinase dead) heterodimers (25). We note that certain Raf mutants (e.g., BRAF^{V600E}) have kinase activity independent of dimerization (26); in such cases, the functional relevance of Ras-GTP dimers remains unclear.

A prediction of the model is that an active Ras/Raf complex should comprise two Ras and two Raf molecules. A full proof of this active configuration would require quantitative PALM imaging in multiple colors or atomic scale structural models of Ras/Raf dimers; neither is yet available. Nevertheless, using a combined BiFC and PALM approach (BiFC-PALM), we have recently shown that the complex between KRas^{G12D} and the Ras binding domain of CRaf (Ras-GTP/CRaf-RBD) can further aggregate to form dimers and occasional higher order structures in cells (27). This observation suggests that tetrameric Ras/Raf complexes do exist under physiological conditions.

Discussions

Ras small GTPases regulate essential cellular functions and are highly desired drug targets. Despite intensive research, aspects of Ras biology and particularly how Ras operates on the cell membrane to activate its effectors are still poorly understood. In part for this reason, therapeutic targeting of mutant Ras remains a major challenge in cancer medicine. In the present work, we provide strong evidence to show that in addition to binding to GTP, Ras may also need to form dimers to activate the Raf-MAPK effector pathway. Along with previous reports on Raf dimerization (23, 28, 29), our study suggests a unified, dimer model of Ras-Raf signaling (Fig. 4) that sheds new light on previous observations of Ras-dependent formation of Raf dimers under physiologic and pharmacologic conditions. More importantly, given the pivotal role of the Ras-Raf-MAPK signaling axis in human cancer, our model also implies that it may be possible to target mutant Ras through disruption of Ras dimers.

Our data implicates Ras-GTP dimers in Ras-mediated Raf-MAPK activation, which contrasts existing views on Ras functioning as a monomeric GTPase as well as those on Ras forming 5–8 membered clusters (7, 9, 10) to activate effectors. The results presented here suggest that Ras proteins spontaneously form dimers and higher order clusters on the cell membrane; dimers, however, are the predominant form of clusters at physiological expression levels (~70 molecules per μm^2; Figs. 1 *D*–*F* and 3 *A*–*C*). Previous immuno-EM studies that reported 5–8 membered Ras clusters typically used a much higher expression level, at 250–1,000 gold particles per μm^2; in those experiments (9, 10), only a small fraction of

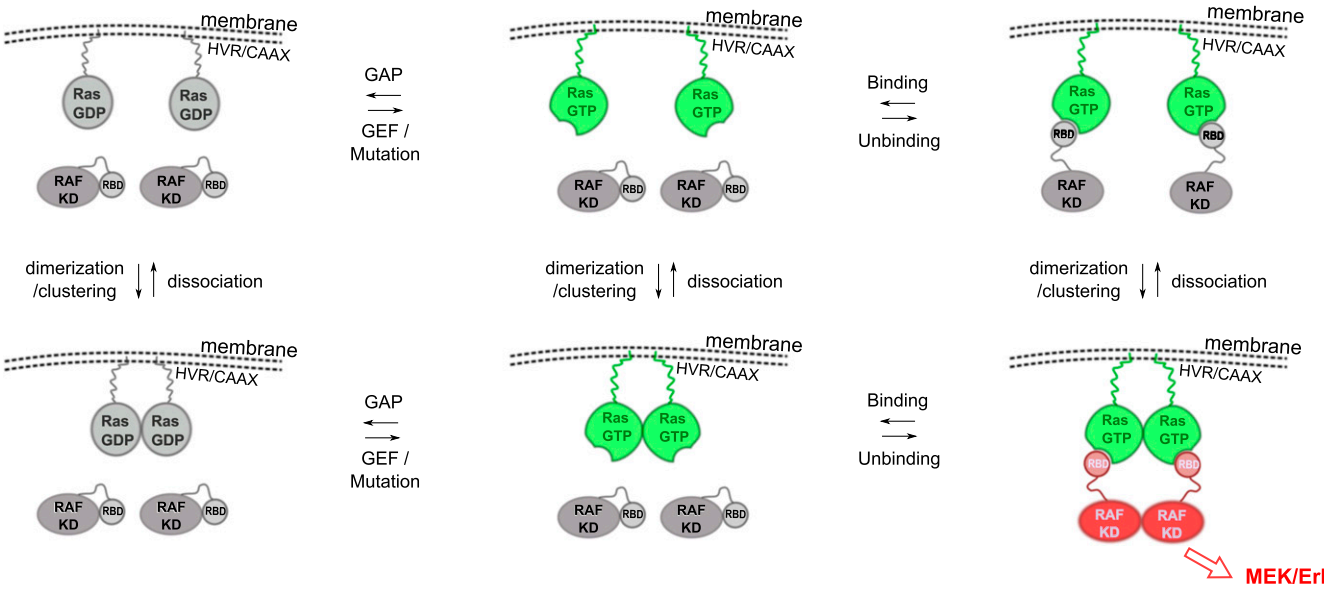

Fig. 4. Dimer model for Ras-mediated activation of Raf/MAPK. Ras attaches to the cell membrane via the C-terminal HVR including the CAAX motif. In the GDP-bound state, Ras is unable to bind Raf, leaving Raf auto-inhibited in the cytosol even when Ras-GDP forms dimers (*Left*). GTP-loaded Ras can bind to Raf (*Upper Right*) or form a Ras-GTP dimer (*Lower Center*); either event alone does not activate Raf. When two Ras-Raf complexes further dimerize or when a Ras-GTP dimer recruits two Raf molecules, the event results in a Raf-Raf dimer that in turn activates the Raf kinase and subsequently MEK/Erk (MAPK) (*Lower Right*). It is presently unclear how the Ras-Ras dimer formation and the Ras-Raf binding processes are ordered and coupled in Ras-mediated Raf activation. GAP, GTPase activating proteins; GEF, guanine-nucleotide exchange factor; KD, Kinase domain; RBD, Ras binding domain.

ectopically expressed Ras proteins were labeled by gold-conjugated antibodies, thus corresponding to an at least 3- to 14-fold higher expression level than what we used in our PALM imaging experiments. As such, it is likely that formation of high order Ras clusters in the immuno-EM studies was an effect of overexpression. Indeed, under transient overexpression conditions that yielded a membrane density of >300 PAmCherry1-KRas molecules per μm^2, we also observed large (>4) Ras clusters (Fig. S3), although the clustering parameters could not be accurately determined due to the high molecular density and finite spatial resolution of PALM.

The use of an artificial dimerization system has allowed us to further define the functional roles of Ras-GTP monomers and dimers in Ras-mediated MAPK activation. When expressed at very low levels (10–15 molecules per μm^2, ~5 times lower than that of endogenous KRas in BHK21 and TRex-293 cells), Ras-GTP molecules are predominantly monomers and unable to activate MAPK. Forced dimerization of monomeric Ras-GTP caused a strong up-regulation of MAPK without having to increase the expression level of Ras-GTP. In the present study, MAPK activation was achieved at a density of ~6 Ras-GTP dimers/μm^2 in three different experimental schemes: induced expression at 2 ng/mL Dox (Fig. 1 *D–F*), induced expression at 1 ng/mL with forced dimerization (Fig. 2 *B* and *C*), or constitutive expression with a PGK promoter with forced dimerization (Fig. 2*D*). We note that aside from the 6 Ras-GTP dimers/μm^2, at 2 ng/mL Dox there was a 10 times higher density (~60 per μm^2) of Ras-GTP monomers coexisting on the cell membrane. The overall MAPK activation in this case, however, was similar to that observed in the other two experimental schemes (Fig. 2*B*), where there were only ~6 Ras-GTP dimers and few Ras-GTP monomers per μm^2. This analysis suggests that the contribution to MAPK activation from Ras-GTP monomers is minimal, if any, compared with that from Ras-GTP dimers. Hence, Ras-GTP dimers instead of monomers are likely the functional unit of Ras in activating the Raf-MAPK pathway, although further investigations are necessary to address whether dimer formation is required for Ras-mediated MAPK signaling.

Our data also suggest that Ras dimerization is primarily mediated by the C-terminal HVR. As mentioned earlier, protein oligomerization by the Ras HVR has been reported in previous studies (9, 10, 14), although at present the exact mechanisms remain unclear. One possibility is that the HVR mediates protein-protein interactions through the lipid groups attached to the HVR by posttranslational modifications, as hinted by recent molecular dynamic simulations (30). This interaction may further be modulated through phosphorylation of Ras at the C terminus (31). Additionally, certain membrane proteins, such as galectin (32), nucleophosmin (33), and calmodulin (34) could serve as scaffolds to bring two or more Ras proteins into proximity. Lastly, albeit secondary in driving Ras dimer formation, direct G-domain contacts between the two Ras proteins should not be ruled out based on existing data. Indeed, two recent studies using cell-free systems have implicated the G-domain in dimer formation of HRas and NRas (21, 35). Intriguingly, whereas residue Y64, located in the conserved G-domain of H-, K-, and NRas, was suggested as a dimer interface in one study (21), the Y64A mutation does not affect $KRas^{G12D}$ signaling to MAPK in our experiments (Fig. S7). These discrepancies highlight the complexity of Ras dimerization and signaling, and urge further biochemical, microscopy, and structural studies to reveal the nature of Ras dimers.

Aside from the mechanisms of dimerization, many other questions regarding the functional roles of Ras dimers in cell signaling also need to be addressed. First, it is unclear whether Ras-GTP dimers are required for the activation of other Ras effector pathways including PI3K-Akt, another commonly activated pathway in human cancer. $KRas^{G12D}$ did not activate PI3K-Akt even at high expression levels in BHK21 or TRex-293 cells, the two cell lines used in this study. Second, it is unclear whether other Ras isoforms such as HRas and NRas, also need to form dimers to activate the effectors. Given that Raf is a common effector for all three Ras isoforms and that activation of wild-type Raf requires dimer formation, it is likely that other Ras isoforms also function as dimers. Third, because an active Ras dimer takes two Ras-GTP molecules, but Ras-GDP dimerizes similarly to Ras-GTP, it may be possible that Ras-GDP could act as an "inhibitor" of Ras-GTP activity by forming an inactive Ras-GDP:Ras-GTP heterodimer. This conjecture could explain a long standing observation that wild-type *Kras2* can suppress mutant KRas-driven lung carcinogens in mice (36).

Nevertheless, the interplays between wild-type and mutant Ras appear to be much more complex, as exemplified by a recent study (37).

As demonstrated by the present study, quantitative superresolution microscopy is an effective tool for addressing questions concerning the molecular mechanisms of cellular processes. In combination with carefully engineered biological systems, the use of quantitative PALM (14–16) has permitted simultaneous nanoscale localization and determination of the signaling stoichiometry of Ras in whole mammalian cells under physiological conditions. Although a few previous reports with alternative approaches have also proposed the formation of Ras dimers, they either did not provide direct evidence to distinguish Ras dimers from higher order clusters (38) or were performed on Ras proteins attached to artificial membranes via semisynthetic anchors (21, 35). To our knowledge, this paper reports the first direct proof that the formation of Ras-GTP dimers activates MAPK signaling.

In summary, we have shown that Ras dimer formation could be a new mechanism for regulating the biological activity of Ras, additionally to GTP-loading and membrane localization. Our finding has profound implications for both basic understanding of Ras biology and pharmaceutical targeting of Ras, the most frequently mutated oncogene in human cancers. Besides acting as a molecular driver for many types of cancer, activated Ras has also often been linked to tumor resistance to therapeutic agents targeting other oncogenic components such as Raf (28, 29, 39). Historically, it has been difficult to develop compounds that counter the oncogenic actions of mutant Ras (3, 4, 8) although new allosteric inhibitors that control GTP affinity and effector interactions are showing promise (40). We suggest the alternative approach of disrupting Ras dimerization to inhibit MAPK activation even in cells with activating Ras mutations. New therapeutic agents that accomplish this might prove effective in the control of Ras-driven tumor progression and drug resistance.

Materials and Methods

PAmCherry1-KRas wild-type and G12D mutants were generated by subcloning the respective KRas cDNA fragments into a Gateway entry vector, followed by LR reactions to make lentivirus expression vectors. Stable cell lines were established via lentiviral infection, manually picked and screened with both Western blotting and fluorescence microscopy. PALM microscopy was conducted on a custom built setup, and data analysis was performed using previously described scripts (14). A detailed description of materials and methods for cloning, cell culture, Western blotting, microscopy, and data analysis can be found in *SI Materials and Methods*.

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