The spatial organization of Ras proteins into nanoclusters on the inner leaflet of the plasma membrane is essential for high fidelity signaling through the MAPK pathway. Here we identify two selective regulators of K-Ras nanoclustering from a proteomic screen for K-Ras interacting proteins. Nucleophosmin (NPM) and nucleolin are predominantly localized to the nucleolus but also have extranuclear functions. We show that a subset of NPM and nucleolin localizes to the inner leaflet of plasma membrane and forms specific complexes with K-Ras but not other Ras isoforms. Active GTP-loaded and inactive GDP-loaded K-Ras both interact with NPM, although NPM–K-Ras binding is increased by growth factor receptor activation. NPM and nucleolin both stabilize K-Ras levels on the plasma membrane, but NPM concurrently increases the clustered fraction of GTP–K-Ras. The increase in nanoclustered GTP–K-Ras in turn enhances signal gain in the MAPK pathway. In summary these results reveal novel extranuclear functions for NPM and nucleolin as regulators of K-Ras nanocluster formation and activation of the MAPK pathway. The study also identifies a new class of K-Ras nanocluster regulator that operates independently of the structural scaffold galectin-3.

Ras proteins are small GTPases that function as molecular switches on the inner leaflet of the plasma membrane, conveying extracellular signals to the cell interior. Ras proteins are critical regulators of signal transduction pathways controlling key cell fates such as cell growth, differentiation, and apoptosis. Deregulation of these pathways results in aberrant cell growth and tumor formation. Mutations that render Ras constitutively active are found in ~15% of human cancers, making Ras one of the most clinically significant proteins in human carcinogenesis. Oncogenic mutations are most prevalent in the K-Ras gene, accounting for a large proportion of solid tumors including 90% of pancreatic cancer, 50% of colon cancer, and 30% of non-small cell lung cancer (1, 2).

The three major Ras isoforms, H-, N-, and K-Ras generate distinct signal outputs in intact cells, signifying specific roles for each isoform. These functional differences stem from significant sequence divergence in the Ras C-terminal 25 amino acids of the hypervariable region (HVR) that directs post-translation attachment of different lipid anchors. The minimal membrane anchor of H-Ras comprises two palmitate groups and a farnesyl group, whereas K-Ras is tethered by a farnesyl group and a polybasic domain (3, 4). These minimal anchors, together with flanking protein sequences and the G-domain, interact with lipids and proteins of the plasma membrane, driving the Ras isoforms into spatially and structurally distinct nanodomains on the plasma membrane (5, 6). Ras lateral segregation is further modulated by the activation state of Ras; active GTP-loaded H-Ras is organized in cholesterol-independent nanoclusters, whereas inactive GDP-loaded H-Ras is arrayed in cholesterol-dependent nanoclusters (5, 7–9). Recent work has also shown that GTP–K-Ras clusters into nanodomains that are spatially distinct from GDP–K-Ras, although both types of nanocluster are cholesterol-independent and actin-dependent (7, 9). K-Ras-GTP nanoclustering, however, is regulated by galectin-3, which operates as a nanodomain scaffold (10, 11).

Ras–GTP nanoclusters are the sites of Raf/MEK and ERK recruitment to the plasma membrane. Scaffolding all components of the MAPK module within nanoclusters rewire the biochemistry to generate a digital ERKpp output. The operation of Ras–GTP nanoclusters as highly sensitive digital switches is critical to deliver high fidelity signal transmission across the plasma membrane (12–14). A key parameter in epidermal growth factor (EGF) receptor to MAPK signal transmission is the fraction of Ras–GTP that forms nanoclusters; this clustered fraction sets the gain for cellular MAPK signaling (15, 16).

NPM (also known as B23) and nucleolin are multifunctional phosphoproteins predominately localized to the nucleolus that play key roles in ribosome biogenesis (17–19). For example, NPM exhibits ribonuclease activity and preferentially cleaves...
pre-rRNA. NPM and nucleolin also have functions outside of the nucleolus. Both proteins shuttle between the nucleolus and the cytoplasm (20), and this shuttling may allow NPM to operate as a molecular chaperone (21). In addition cytosolic NPM is involved in centrosome duplication (22). Like Ras proteins, NPM and nucleolin regulate cell proliferation and transformation and are overexpressed in multiple cancers (23). However, the physiological role of NPM in carcinogenesis remains controversial because it has been described as both an oncogene and a tumor suppressor (23).

In this study we identify NPM and nucleolin as proteins that interact specifically with K-Ras but not H-Ras. Furthermore we definitively identify a subset of NPM and nucleolin on the inner leaflet of the plasma membrane where both proteins interact with K-Ras. Importantly, NPM and nucleolin stabilize K-Ras levels on the plasma membrane, leading to an increase in the K-Ras clustered fraction, which amplifies signal output from the MAPK pathway. Combined, our data indicate that NPM and nucleolin play a critical role in signal transduction via the MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—GST-CTH (166–189) and GST-CTK (166–188) were attained by subcloning CTH and CTK nucleotide sequence into pHASTBAC-DUAL transfer vector (Invitrogen). The recombinant pHASTBAC-DUAL-GST-CTH was transformed into MAX Efficiency DH10Bac-competent cells (Invitrogen) to generate recombinant bacmids. Recombinant bacmids were transfected into SF9 cells by the Protein Expression Facility of the University of Queensland. GFP-tH, GFP-CTH, GFP-tk, GFP-CTK, GFP-K-Ras, GFP-K-RasG12V, GFP-H-Ras, and GFP-H-RasG12V have been described previously (8, 24). GFP-H-Ras + KL and GFP-K-Ras + HL were generated by subcloning the linker region of K-Ras (amino acids 166–172) in GFP-H-Ras and the linker region of H-Ras (amino acids 166–172) in GFP-K-Ras. RFP-NPM and RFP-nucleolin was subcloned using cDNA (obtained from the Microarray Facility, Institute for Molecular Bioscience, University of Queensland) into an mRFP vector. NPM deletion mutants, the N-terminal region of NPM (Nt-NPM) (1–119), acidic domain of NPM (120–188), and the C-terminal region of NPM (189–294), were generated by PCR using full-length NPM as a template. PCR fragments were subcloned into pEGFP. All of the point mutations were introduced by PCR mutagenesis.

**Antibodies and Reagents**—Antibodies against NPM were purchased from Zymed Laboratories Inc., nucleolin was from Sigma, Raf-1 and Ras were from BD transduction Laboratories, ERKpp was from Cell Signaling Technology, and K-Ras and upstream binding factor (UBF) were from Santa Cruz. GFP and RFP antibodies were produced by immunizing rabbits with recombinant proteins and affinity-purified from serum. RFP antibodies were produced by immunizing rabbits with a recombinant protein and affinity-purified from serum. GFP-H-Ras and GFP-K-Ras antibodies were produced by immunizing rabbits with recombinant proteins and affinity-purified from serum.

**Cell Culture**—Baby hamster kidney (BHK) and 293T cells were grown and maintained in HEPES-buffered Dulbecco’s modified Eagle's medium containing 10% fetal bovine serum. The cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and harvested after 24 h. Where necessary, the cells were serum-starved for 3 h prior to harvest. Where indicated, the cells were stimulated with EGF (50 ng/ml) for the indicated time points.

**Affinity Chromatography**—GST fusion proteins were purified from SF9 cell lysates by binding to glutathione-agarose beads (Sigma); washed extensively in 1 M KCl, 1% Triton X-100, 1 mM dithiothreitol; and then equilibrated three times with 10 mM Tris-HCl (pH 7.5), 25 mM KCl, 2.5 mM CaCl2. Mouse brain cytosol was prepared by incubating minced tissue (14 mouse brains) in 5 volumes of a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 2.5 mM CaCl2) for 30 min on ice. The minced brain was homogenized with a Polytron homogenizer (Kinematica) at 15,000 rpm for 4–10 s bursts. The lysate was clarified by centrifuging at 23,000 × g for 30 min at 4 °C to remove cell debris. Clarified cytosol was incubated with the prepared GST beads for 2 h at 4 °C with rotation. The beads were then washed ten times for 5 min with buffer containing 100 mM KCl, 10 mM Tris-Cl, 2.5 mM CaCl2, and 1 mM dithiothreitol. The beads were transferred to a new tube, eluted in 30 μl of 1× Laemmli sample buffer, and analyzed by SDS-PAGE.

**Protein Identification by Mass Spectrometry**—Coomassie blue-stained gel bands were excised and digested with trypsin as described in Ref. 25. The peptides were spotted onto MALDI target using 5 mg/ml α-cyano-hydroxycinnamic acid in 50% acetonitrile, 5 mM ammonium phosphate, and 0.1% formic acid as matrix. A calibration mixture containing 1 pmol of des-Arg-bradykinin, 2 pmol of angiotensin I, 1.3 pmol of Glu-Fibrinopeptide B, 2 pmol of adrenocorticotropic hormone (ACTH) (1–17 clipping), 1.5 pmol of ACTH (18–39 clipping), and 3 pmol of ACTH (7–38 clipping) was diluted 10-fold in the matrix and spotted on the same MALDI plate. The samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF-TOF (Applied Biosystems). After plate calibration for MS and MS/MS, 1000 MS spectra were accumulated for all spots using a MS positive ion reflectron mode acquisition method at a laser energy of 3400. The TOF-MS spectra were analyzed using the Peak Picker software supplied with the instrument. The 10 most abundant spectral peaks that met the threshold (>20:1 signal/noise) criteria were included in the acquisition list for the TOF-TOF. MS/MS data from the TOF-TOF was acquired using the default positive ion, 1-kV collision energy, reflectron mode, at a laser energy of 4200. The threshold criteria were set as follows: mass range, 850–4000 Da; minimum cluster area, 500; minimum signal-to-noise, 20; maximum number of ms/ ms spectra per spot, 10. A mass filter excluding matrix cluster ions and trypsin autolysis peaks was applied. Data base searching of noninterpreted TOF-MS and TOF-TOF data were performed using the Mascot search engine (Matrix Science) and the MSDB data base. The following parameters were used: peptide mass tolerance, 0.4 Da; fragment mass tolerance, 0.2 Da; fixed cysteine carbamidomethylation, variable methionine oxidation, maximum one missed cleavage.

**Co-immunoprecipitation**—The cells were subjected to hypotonic lysis and the post-nuclear supernatant was isolated as previously described (3). Where required cytosolic (S100) and membrane (P100) fractions were further fractionated from the post-nuclear supernatant by a 30 min, 100,000 × g ultracentrifugation at 4 °C. The lysates were solubilized with Triton X-100 for 30 min, and then nonsoluble proteins were cleared by cen-
trifugation at 100,000 \times g. The appropriate antibody was coupled to protein G-conjugated magnetic D (Dynal) and then incubated with the lysate diluted in buffer A (10 mM Tris, pH 7.5, 25 mM NaF, 5 mM MgCl2, 1 mM EGTA, 0.33 ng/ml aprotinin, 0.1 mM Na3VO4, 3.3 ng/ml leupeptin, 1 mM dithiothreitol) for 90 min rotating at 4 °C. The beads were washed three times with washing buffer (10 mM Tris-Cl, 60 mM NaCl, 0.2% Triton X-100, 25 mM NaF, 5 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 100 \mu M NaVO4, 10 \mu g/ml aprotinin, and 10 \mu g/ml leupeptin). The beads were resuspended into sample buffer, resolved on SDS-PAGE gels, and transferred to polyvinylidene difluoride using semi-dry transfer. The membranes were probed with primary and secondary horseradish peroxidase antibodies and developed using enhanced chemiluminescence.

**Immunofluorescence**—Transfected cells grown on coverslips were fixed with 4% paraformaldehyde. The coverslips were quenched with 50 mM ammonium chloride and then permeabilized and blocked in 0.1% Triton, 3% bovine serum albumin in phosphate-buffered saline for 30 min. Primary and secondary antibodies were incubated for 1 h each, and then coverslips were mounted in Mowiol and visualized by confocal microscopy (Zeiss LSM 510). Fluorescent microscopy of plasma membrane sheets was performed based on Ref. 26. Briefly, the cells grown on coverslips were washed once in phosphate-buffered saline and then incubated in poly-l-lysine for 1 min. The coverslips were washed three times for 5 s in 1/3 Buffer A and then briefly in buffer A. The cells were blotted once or twice with 1 ml of buffer A, fixed in 2% formaldehyde, mounted in Mowiol, and imaged on a fluorescent microscope.

**Electron Microscopy**—Apical plasma membrane sheets were prepared from BHK cells, fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and labeled with anti-GFP or anti-RFP antibodies conjugated directly to 5-nm gold as described previously (7, 9). Digital images of the immunogold labeled plasma membrane sheets were taken at 100,000 \times magnification (Jeol 1011). Intact 1-\mu m2 areas of the sheet were identified using Image J, and the (x,y) coordinates of the gold particles were determined as described (7, 9). Bootstrap tests to examine the differences between replicated point patterns were constructed exactly as described previously (27–29), and the statistical significance was evaluated against 1000 bootstrap samples.

**RESULTS**

**K-Ras Interacts with Nucleolar Proteins NPM and Nucleolin**—To identify proteins that specifically interact with K-Ras, we used affinity chromatography with the farnesylated hyper-variable domain of K-Ras (Fig. 1A). The HVR of K-Ras (CTK) was expressed as a C-terminal GST fusion protein in SF9 cells. GST–CTH, constructed from the HVR of H-Ras, was similarly produced and used as a control to allow the identification of K-Ras-specific interacting proteins. GST–CTH and GST–CTK were separately purified onto glutathione-agarose beads and used affinity chromatography with the farnesylated hyper-variable domain of H-Ras or K-Ras (GST–CH or GST–CTK) purified from SF9 cells and used to isolate binding proteins. A representative Coomassie-stained SDS-PAGE gel from a typical experiment is shown. Bands of interest were excised, digested with trypsin, and analyzed by MALDI-TOF-TOF. Two CTK-specific bands (indicated) were identified as NPM and nucleolin (Nuc). The beads were resuspended into sample buffer, resolved on SDS-PAGE gels, and transferred to polyvinylidene difluoride using wet transfer. The membranes were probed with primary and secondary horseradish peroxidase antibodies and developed using enhanced chemiluminescence.

**Electron Microscopy**—Apical plasma membrane sheets were prepared from BHK cells, fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and labeled with anti-GFP or anti-RFP antibodies conjugated directly to 5-nm gold as described previously (7, 9). Digital images of the immunogold labeled plasma membrane sheets were taken at 100,000 \times magnification (Jeol 1011). Intact 1-\mu m2 areas of the sheet were identified using Image J, and the (x,y) coordinates of the gold particles were determined as described (7, 9). Bootstrap tests to examine the differences between replicated point patterns were constructed exactly as described previously (27–29), and the statistical significance was evaluated against 1000 bootstrap samples.
and used as bait with clarified mouse brain cytosol. After extensive washing of the beads, bound proteins were eluted, resolved by SDS-PAGE, and Coomassie-stained (Fig. 1A). Selected bands were excised, digested with trypsin, and analyzed by MALDI-TOF-TOF. Two proteins that bound specifically to CTK but not CTH were NPM and nucleolin (Table 1). To further explore these observations, we used GFP fusions of the HVR of K-Ras and H-Ras (GFP-CTK and GFP-CTH), and GFP fusions of the minimal membrane anchors of K-Ras and H-Ras (GFP-tk and GFP-th) (Fig. 1A). To confirm the identity of the CTK-binding proteins and the specificity of the interactions, we ectopically expressed GFP-CTK, GFP-tk, GFP-CTH, and GFP-th with RFP-NPM or RFP-nucleolin in BHK cells. We performed immunoprecipitations from post-nuclear supernatants using an anti-GFP antibody and immunoblotted using an anti-RFP antibody (Fig. 1C). These experiments demonstrated that RFP-NPM and RFP-nucleolin selectively bind to GFP-CTK but not GFP-CTH. RFP-NPM, but not RFP-nucleolin, also bound to the minimal membrane anchor of K-Ras (GFP-tk). Neither RFP-NPM nor RFP-nucleolin bound to the minimal membrane anchor of H-Ras (GFP-th). We next ectopically expressed full-length GFP-K-Ras and GFP-H-Ras in BHK cells and found that endogenous NPM and endogenous nucleolin both selectively bound to GFP-K-Ras but not GFP-H-Ras (Fig. 1D). These results indicate that although NPM and nucleolin are predominately nucleolar proteins, both have a non-nuclear fraction that selectively interacts with the C-terminal HVR of K-Ras. We chose to further explore the functional significance of the K-Ras-NPM interaction.

**NPM Binds to the Polybasic Region of K-Ras**—Fig. 1C shows that the hypervariable region (CTK) and the minimal membrane anchor (tk) of K-Ras both interact with NPM. To investigate whether the linker region of Ras (amino acids 166–172) contributes in any way to NPM binding, we replaced the linker region of full-length GFP-H-Ras (residues 166–172) with the corresponding linker residues of K-Ras (=GFP-H-Ras+KL). The complementary protein GFP-K-Ras+HL was engineered to comprise full-length K-Ras but with residues 166–172 replaced with the corresponding H-Ras linker residues. The results in Fig. 2A show that full-length K-Ras and K-Ras-HL both bound NPM, whereas H-Ras-KL did not. We conclude that the linker region of K-Ras does not contribute to the interaction with NPM and that the minimal membrane region is solely responsible for the specific interaction between K-Ras and NPM.

Two key motifs of the minimal membrane anchor of K-Ras are the polybasic domain and the prenylated cysteine at residue 185. To test whether these motifs are both necessary for the interaction with NPM, we replaced six consecutive lysines of the polybasic domain in constitutively active GFP-K-RasG12V with glutamines (GFP-K-RasG12V,6Q). We also replaced Cys185 with serine (GFP-K-RasG12V,C185S) to abolish farnesylation. When co-expressed with mRFP-NPM in HEK293T cells, GFP-K-RasG12V, but not GFP-H-RasG12V bound to NPM (Fig. 2B). GFP-K-RasG12V,6Q, however, did not co-immunoprecipitate mRFP-NPM, showing that the polybasic domain is essential for binding to NPM. Interestingly, blocking

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**TABLE 1**

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<tr>
<td>Nucleolin/C23 (P09405) #2</td>
<td>10</td>
<td>17</td>
</tr>
</tbody>
</table>

*The threshold score for p < 0.05 is 59.

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**FIGURE 2. NPM binds to the polybasic domain of K-Ras.** A. linker domain mutants of K-Ras and H-Ras. GFP-H-Ras+KL comprises full-length H-Ras with residues 166–172 of the linker region replaced by the corresponding residues of K-Ras. GFP-K-Ras+HL comprises full-length K-Ras with residues 166–172 replaced with the corresponding H-Ras linker residues (left panel). The GFP-Linker mutants as well as the positive control, GFP-K-Ras, and the negative control, vector only, were transiently expressed in BHK cells. Anti-GFP immunoprecipitates from post-nuclear supernatants were Western blotted with GFP antibody to verify expression and capture and with anti-NPM antibody to detect interaction with endogenous NPM (right panel). B. BHK cells transiently expressing GFP-K-RasG12V or GFP-K-RasG12V point mutants S181A, S181D, T183E, C185S, and 6Q were analyzed for NPM binding using anti-GFP immunoprecipitates. GFP-H-RasG12V expressing cells were used as a negative control. GFP-K-Ras point mutants were probed for with anti-GFP and anti-NPM antibody to detect bound endogenous NPM (left panel). The amount of endogenous NPM bound to mutants relative to K-RasG12V were quantified in Image J and graphed (right panel). NPM bound to K-RasG12V S181D and 6Q was significantly decreased (p = 0.02 and 0.04, respectively), whereas C185S was significantly increased (p < 0.0001) compared with K-RasG12V.
K-Ras farnesylation (GFP-K-RasG12V,C185S) actually increased the affinity of K-Ras for NPM, suggesting that prenylation negatively regulates the interaction. These experiments, in conjunction with those in Figs. 2A and 1D also show that the nucleotide, GTP or GDP, bound to K-Ras does not regulate NPM binding. Throughout the rest of this study the mutational analysis is confined to K-RasG12V, because this interaction between K-Ras and NPM is extranuclear. To further pinpoint the location of this interaction, we fractionated the post-nuclear supernatant of BHK cells into cytosolic (S100) and membrane (P100) fractions and assessed the localization of GFP-K-RasG12V and endogenous NPM (Fig. 4A). We observed NPM in both fractions, but it was more abundant in the membrane fraction. As expected, GFP-K-RasG12V, because this allows a functional output to be measured. Phosphorylation of Ser181 modulates the electrostatic interactions of K-Ras with the plasma membrane nanoclusters (10, 30). We therefore investigated whether the NPM-K-Ras interaction is also sensitive to Ser181 phosphorylation. Fig. 2B clearly shows that the phospho-mimetic mutation S181D reduced the affinity of GFP-K-RasG12V S181D for NPM. In contrast the phospho-mimetic mutation T183E had no significant effect on the affinity of GFP-K-RasG12V, T183E for NPM (Fig. 2B).

K-Ras Interacts with the N-terminal Region of NPM—To map the NPM binding site for K-Ras, we analyzed a set of GFP-tagged NPM deletion mutants (Fig. 3A) using co-immunoprecipitation assays and post-nuclear supernatants prepared from BHK cells. The results clearly show that Nt-NPM is responsible for the interaction with K-Ras (Fig. 3A). Of note, this N-terminal region of NPM has been shown previously to be important for NPM pentamer formation and chaperone activity (21, 31). The isolated acidic domain or C-terminal region of NPM showed no interaction with K-Ras.

By fluorescent microscopy, mRFP-NLS-NPM appears to be exclusively nucleolar, and we observed no convincing co-localization with GFP-K-RasG12V (Fig. 3B). However, because the extranuclear pool of NPM is of considerably lower abundance than the nucleolar fraction, it is difficult to detect by fluorescent microscopy. In contrast, the Nt-NPM fragment of NPM displays a much greater cytosolic fraction, and when co-expressed with K-RasG12V, it clearly co-localizes at the plasma membrane (Fig. 3B).

**FIGURE 3. K-Ras binds to the N-terminal region of NPM.** A, diagram of NPM deletion mutants and binding ability (+ or −). BHK cells were transiently transfected with RFP-K-RasG12V and GFP-NPM or the GFP-NPM deletion mutants. Immunoprecipitates were performed from post-nuclear supernatants. Anti-RFP immunoprecipitates were analyzed by Western blotting using anti-GFP antibody to detect NPM-deletion mutants and a pan-Ras antibody to probe for RFP-K-RasG12V (middle panel). Only the Nt region of NPM, or full-length NPM bound to RFP-K-RasG12V. Reciprocal anti-GFP immunoprecipitates (right panel) confirms this result. B, full-length RFP-NPM + GFP-K-RasG12V, GFP-Nt NPM alone, and GFP-Nt-NPM + RFP-K-RasG12V were analyzed by fluorescent microscopy. Clear co-localization of Nt NPM and K-RasG12V can be seen on the plasma membrane in the zoomed frame (bottom panel). Scale bar, 10 μm. WT, wild type; PNS, post-nuclear supernatant.

**FIGURE 4. NPM and nucleolin interact with K-Ras in the membrane fraction of cells.** A, BHK cells transiently transfected with GFP-K-RasG12V were fractionated into membrane and cytosolic fractions. The bottom panel shows the nuclear protein Histone H3 is only detected in the nuclear fraction. Anti-GFP immunoprecipitates (IP) from membrane and cytosolic fractions were analyzed by Western blotyping with antibodies against nucleolin, Raf-1, and NPM to detect respective endogenous proteins. Anti-Ras antibody was used to probe for ectopically expressed GFP-K-RasG12V. B, a P100 fraction isolated from nontransfected BHK cells was immunoprecipitated using anti-NP or anti-Flag (negative control). Western blot analysis was performed with anti-NP to detect endogenous NPM and anti-Ras to detect endogenous Ras. C, a post-nuclear supernatant isolated from non-transfected BHK cells was used for immunoprecipitations with control antibody, anti-NP, and anti-UBF. The immunoprecipitates were performed in Western antibody to specifically detect endogenous K-Ras, anti-NP, and anti-UBF (left panel). Expression of each protein in a crude nuclear and membrane fraction is also shown (left panel, last lane). D, BHK cells expressing GFP-K-RasG12V were separated into membrane and cytosolic fractions and analyzed by native PAGE. Western blots were probed with anti-GFP to detect GFP-K-RasG12V, and anti-NP and anti-nucleolin to detect endogenous proteins.
RasG12V was more abundant in the membrane fraction but also displayed a cytosolic pool. To exclude the possibility of contamination by nuclear material, we probed for histone H3 in the subcellular fractions. Histone H3 was detected exclusively in the nuclear fraction (Fig. 4A), demonstrating that the NPM detected in the P100 and S100 fractions was not due to nuclear contamination.

Next we immunoprecipitated GFP-K-RasG12V from S100 and P100 fractions and probed for NPM. Interestingly, we observed that NPM interacts with K-Ras exclusively in the membrane fraction (Fig. 4A). However, it is important to note that because the abundance of NPM and K-Ras G12V is considerably lower in the S100 fraction, we cannot exclude the possibility that an interaction in the cytosol is not detected because it is below the sensitivity of the Western blot. As a positive control, Ral-1, which also interacts with K-Ras only at the plasma membrane, was co-immunoprecipitated with K-Ras exclusively from the membrane fraction. We performed reciprocal immunoprecipitations with anti-NPM antibody on the P100 fraction but this time probed for endogenous Ras. Fig. 4B shows that endogenous Ras binds to endogenous NPM in the membrane fraction. To extend this demonstration of endogenous interactions, we immunoprecipitated NPM and the irrelevant nucleolar protein, UBF, from BHK cell post-nuclear supernatant and blotted for endogenous K-Ras. We could also specifically detect endogenous K-Ras interacting with endogenous NPM but not with UBF (Fig. 4C). Note that only a small amount of UBF was present in the post-nuclear supernatant, which was enriched by immunoprecipitation. However, the UBF antibody detected a band of the same expected size in combined crude nuclear and membrane extract (Fig. 4C, right lane). We further tested Ras binding specificity with a second nucleolar protein, fibrillarin, and again found no evidence of endogenous Ras in fibrillarin immunoprecipitates (Fig. 4C). The reverse experiment of identifying endogenous NPM in endogenous K-Ras immunoprecipitates is not technically possible because the isoform-specific K-Ras antibody recognizes and blocks the polybasic domain that binds NPM.

Interestingly, the immunoprecipitates in Fig. 4A show that endogenous nucleolin is also present in membrane immunoprecipitates with NPM and K-Ras (Fig. 4A). Therefore to determine whether K-Ras, NPM, and nucleolin might exist in the same complex, we used native PAGE analysis. Immunoblots of BHK cells expressing GFP-K-RasG12V show three bands corresponding to GFP-K-RasG12V, NPM, and nucleolin all migrating at the same approximate mass of 180 kDa specifically in the membrane fraction (Fig. 4A).
NPM Modulates K-Ras Signaling

membrane fraction and not in the cytosolic fraction (Fig. 4D).
Therefore, we conclude that K-Ras, NPM, and nucleolin are able to form a complex in the membrane fraction of BHK cells.

Given that the P100 fraction contains many cellular membranes, we wanted to determine whether NPM truly resides with Ras on the inner leaflet of the plasma membrane. To this end we prepared intact plasma membrane sheets attached to coverslips and imaged the sheets by fluorescent microscopy (26). GFP-K-Ras was expressed at high levels on the sheets. NPM displayed lower expression levels; yet we could still readily detect co-localization between NPM and K-Ras (Fig. 5A). We next used immunoelectron microscopy to image intact plasma membrane sheets prepared from BHK cells transiently expressing RFP-NPM and RFP-nucleolin. We observed immunogold labeling of 40–50 gold particles/μm² for both NPM and nucleolin, significantly higher than background labeling (Fig. 5B). This clearly demonstrates that there is a pool of NPM and nucleolin on the cytoplasmic face of the plasma membrane.

Given that a subset of NPM and nucleolin resides on the plasma membrane, we asked whether K-Ras recruits NPM and nucleolin to the membrane. To answer this question, we fractionated BHK cells expressing NPM or nucleolin, with or without K-RasG12V into membrane and cytosolic fractions. The Western blot revealed no difference in NPM and nucleolin localization to the membrane fraction between cells expressing GFP-K-RasG12V and those not (Fig. 5C). However, we observed a striking loss of NPM from the cytosolic fraction expressed in the presence of GFP-K-RasG12V.

Growth factor binding of cell surface receptors leads to Ras activation. Therefore, we considered whether the interaction between K-Ras and NPM/nucleolin is sensitive to EGF stimulation. Interestingly, a time course of cells stimulated with EGF showed the interaction between K-Ras, NPM, and nucleolin peaked between 2 and 5 min of stimulation and then progressively decreased (Fig. 5D). We have previously shown that GTP loading of K-Ras peaks at 2 min after EGF stimulation (14). Together these data indicate that although K-Ras does not appear to recruit NPM and nucleolin to the membrane, the association between these proteins is increased upon K-Ras GTP loading.

NPM and Nucleolin Increase K-Ras Clustering and MAPK Signal Output—We next examined whether NPM and nucleolin play a role in K-Ras signaling. We co-expressed NPM, nucleolin, or NPM and nucleolin together with K-RasG12V in BHK cells. We assessed ERK activation by measuring ERKpp levels by Western blot (Fig. 6). Co-expression of NPM and/or nucleolin significantly increased ERK activation compared with K-RasG12V alone. The expression level of K-RasG12V also concurrently increased when co-expressed with NPM and/or nucleolin. Thus the increase in ERK activation may in part result from an ability of NPM and nucleolin to stabilize the interaction of K-RasG12V with the plasma membrane. NPM and nucleolin co-expressed without K-RasG12V stimulated a small increase in ERK activation (Fig. 6), possibly a result of an effect on endogenous K-Ras. Similar experiments performed with H rasG12V showed no significant effect of NPM co-expression on ERKpp activation (not shown).

We next formally examined how plasma membrane-bound K-Ras was specifically affected by NPM and nucleolin. We prepared intact plasma membrane sheets from BHK cells expressing GFP-K-Ras or GFP-K-RasG12V alone or co-expressed with RFP-NPM, RFP-nucleolin, or both RFP-NPM and RFP-nucleolin. The plasma membrane sheets were labeled with anti-GFP 5-nm gold, and the spatial distribution was visualized by electron microscopy (Fig. 7A). Interestingly, we measured a significant increase in anti-GFP gold labeling on the plasma membrane when GFP-K-RasG12V was co-expressed with NPM and/or nucleolin. This result is consistent with the cell fractionation data shown in Fig. 6. A similar increase in anti-GFP gold labeling on the plasma membrane was observed when wild-type GFP-K-Ras was co-expressed with NPM and/or nucleolin. Taken together these data suggest that NPM and nucleolin can stabilize K-Ras binding to the plasma membrane and that this activity is independent of the nucleotide-bound state of K-Ras.

The spatial distribution of K-Ras on the plasma membrane is critical for functional activity (6, 7, 9, 32). Analysis of the K-RasG12V spatial distribution showed that NPM significantly increased the extent of K-RasG12V clustering but not the size of the nanoclusters, implying an increase in the K-RasG12V clustered fraction (Fig. 7B). Nucleolin expressed alone did not alter the extent of K-RasG12V clustering, nor...
did co-expression of nucleolin potentiate the enhanced clustering induced by NPM. (Fig. 7B). Essentially the same results were observed with wild-type K-Ras; clustering was increased by co-expression with NPM but not with nucleolin (Fig. 7C). Taken together our data demonstrate that a pool of NPM and nucleolin localizes on the inner leaflet of the plasma membrane and, by associating with K-Ras, stabilizes K-Ras retention on the membrane. NPM also increases the clustered fraction of K-Ras. Both of these effects amplify K-Ras-mediated signal transduction leading to increased ERK activation. An additional assessment of the physiological relevance of NPM and nucleolin loss of function to

MAPK signaling was not possible because of the toxicity associated with loss of expression of NPM and nucleolin (data not shown).

DISCUSSION

The plasma membrane imposes nonrandom distributions on lipids, proteins, and signaling complexes, concentrating or excluding them from specific nanodomains. For example, Ras proteins are laterally segregated into transient nanodomains, a distribution that is essential for high fidelity signal transduction via the MAPK pathway (13, 14). Here we identify NPM and nucleolin as novel regulators of K-Ras plasma membrane interactions that in turn influence signaling through the MAPK pathway.

We show definitively using electron microscopy that a fraction of NPM and nucleolin proteins are localized to the inner leaflet of the plasma membrane. Our imaging and biochemical data show that this pool of NPM and nucleolin, although low in abundance, specifically interacts with K-Ras and not H-Ras. The biochemical data attest to the physiological relevance of the interaction between K-Ras and NPM, because K-Ras specifically binds the N-terminal domain of NPM and not the acidic region, excluding the trivial explanation that K-Ras and NPM randomly interact because of electrostatic charge. Furthermore NPM and nucleolin both increase overall cellular K-Ras protein levels, which coincides with an increase in plasma membrane-bound K-Ras. A likely scenario is that NPM functions to stabilize the fraction of K-Ras associated with the plasma membrane. The precise mechanisms remain unclear and may be different for NPM, which interacts with the polybasic domain of the minimal K-Ras anchor, and nucleolin, which interacts with both the polybasic domain and protein sequences in the adjacent K-Ras hypervariable domain. In the case of NPM, binding to plasma membrane-associated K-Ras may be preferred over cytosolic K-Ras because the farnesyl group that inhibits the in vitro interaction will be partitioned into the lipid bilayer. Whatever the mechanisms, the consequence of NPM and nucleolin interaction is to increase the amount of K-Ras on the plasma membrane.
NPM Modulates K-Ras Signaling

In addition to stabilizing K-Ras on the plasma membrane, NPM also increases K-Ras nanoclustering; that is, it increases the fraction of K-Ras that exists in clusters. This property of NPM could well be related to its ability to form higher order oligomers (33). The formation of higher order oligomers is a biochemical property shared by galectin-3 (Gal-3), which we have shown recently also increases K-Ras nanoclustering and is a structural component of K-Ras-GTP nanoclusters (11). There are, however, some significant differences between the interactions of NPM and Gal-3 with K-Ras, which lead us to propose that NPM may be a new class of nanocluster regulator. Gal-3 binding is prenyl-dependent, is restricted to GTP-K-Ras, and occurs as a result of recruitment of Gal-3 from the cytosol to the plasma membrane by GTP-K-Ras (11). In contrast, a fraction of cellular NPM associates constitutively with the plasma membrane and interacts with GDP-K-Ras and GTP-K-Ras via binding that is prenyl-independent. These properties of NPM therefore will reset the basal clustered fractions of GDP- and GTP-K-Ras, depending solely on the availability or concentration of NPM on the plasma membrane. This is in contrast to the major role of Gal-3, which operates as a recruited, GTP-dependent scaffold. Given that GTP-K-Ras nanoclusters are the sites of Raf recruitment and MEK/ERK activation (10, 13, 14, 32, 34), increasing the clustered fraction of GTP-K-Ras will increase the gain in EGF receptor to MAPK signal transmission (15, 16); that is, for any given amount of K-Ras-GTP, an increased clustered fraction will increase the number of signaling nanoclusters and the consequent ERKpp output. The increasing total K-Ras levels on the membrane, the other biochemical effect of NPM and nucleolin, will also increase the maximum ERKpp output signal.

These effects of NPM and nucleolin could be especially significant during M phase of the cell cycle, when the cytosolic fraction of these largely nuclear components will increase (35, 36). We have previously observed that there is activation of Raf and MEK at this point in the cell cycle that does not correlate with an increase in actual Ras-GTP levels (37). The new data presented here could explain this result because elevated NPM and nucleolin levels would simply increase the number of K-Ras-GTP nanoclusters available to activate Raf and MEK without a requirement to stimulate Ras-GTP loading. Our results also suggest that the interaction between K-Ras and NPM is regulated by phosphorylation of Ser181 and therefore may be under the control of protein kinase C (30). We showed previously that Ser181 phosphorylation inhibited K-Ras nanoclustering by a mechanism that did not involve inhibiting Gal-3 binding (10). The new results here suggest that the inhibition of nanoclustering may in part be due to inhibiting K-Ras-NPM interactions as well as decreasing electrostatic interactions of the polybasic domain for acidic phospholipids.

In vitro studies have suggested that NPM may act as a molecular chaperone to prevent protein aggregation in the highly crowded environment of the nucleolus (21). The subset of NPM residing on the membrane may also function as a molecular chaperone in the sense that NPM may act to stabilize and assist assembly of K-Ras nanoclusters. In fact K-Ras specifically binds to the N-terminal region of NPM, which is required for pentamer formation as well as chaperone activity (21, 31). Intriguingly, nucleolin has also recently been shown to interact with the cytoplasmic domain of ERB4 receptors (38), which is consistent with our observation of nucleolin on the inner plasma membrane. In addition, a previous study demonstrates an interaction between K-Ras and nucleolin (39). However, in contrast to our data, this study concluded the K-Ras-nucleolin interaction was nucleolar.

Finally, it is worth noting that NPM and nucleolin are over-expressed in many cancers (23). Moreover, ~35% of adult acute myeloid leukemia cases have a frameshift mutation in the NPM gene that creates a functional nuclear export sequence and causes aberrant redistribution of NPM to the cytoplasm (40, 41). These NPM mutations frequently occur in association with mutations in K-Ras (42). It is therefore possible that mislocalized NPM augments K-Ras signaling by enhancing signal output from GTP-loaded K-Ras, contributing to leukemogenesis.

In summary, our data formally show that NPM and nucleolin localize to the cytoplasmic leaflet of the plasma membrane and play a critical role in signal transduction via the MAPK pathway. Nucleolin and NPM stabilize K-Ras association with the plasma membrane, and NPM in addition enhances K-Ras nanoclustering. This work sheds new light on the possible extranuclear roles of NPM and nucleolin that correlate with oncogenesis.

REFERENCES

Nucleophosmin and Nucleolin Regulate K-Ras Plasma Membrane Interactions and MAPK Signal Transduction
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