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Compartmentalized Ras signaling differentially contributes to phenotypic outputs

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ABSTRACT

Ras isoforms are membrane bound proteins that differentially localize to the plasma membrane and subcellular compartments within the cell. Whilst the cell surface is the main site for Ras activity the extent to which intracellular pools contribute to Ras function is debated. We have generated Ras chimeras targeting Ras to the ER, Golgi, mitochondria and endosomes to compare the capacity of each of these locations to support activity equivalent to normal Ras function. We find that all locations are capable of regulating the MAP kinase and Akt pathways. Furthermore, whilst endomembranous Ras pools show location-specific competence to support proliferation and transformation, Golgi-Ras is as potent as N-Ras.

1. Introduction

Ras operates as a molecular switch near the top of many signaling cascades and regulates diverse cellular functions including proliferation, differentiation, migration, apoptosis and senescence. Despite a high degree of sequence homology and ubiquitous expression profiles the Ras isoforms H-Ras, N-Ras, and K-Ras generate different signal outputs. These include distinct contributions to embryonic development, cancer development, cellular homeostasis and differential coupling to canonical effector pathways [1–3]. Differences in relative compartmentalization of Ras isoforms both within and between organelles are suggested to be responsible for these signaling differences [4].

The 25/26 amino acid C-terminal hypervariable domain (HVR) of Ras is post-translationally modified to facilitate membrane binding and correct trafficking [5]. Each isoform has a distinct set of modifications and targeting motifs that result in overlapping but distinctive localizations. Whilst the plasma membrane is the main site of action for Ras proteins, association with the plasma membrane is highly dynamic and regulated by the activation state of the Ras protein [4]. Consequently, endomembranous pools of each isoform have been characterized that include the endosome, mitochondria and the ER/Golgi complex [6–13]. Endocytic and cytosolic transfer of activated Ras and *in situ* activation of Ras on endomembranous platforms have all been observed [14–17]. The presence of activated Ras on intracellular organelles suggests a functional role that is supported by data from T cells where opposing effects on immunological selection are produced by Ras activation on the cell surface versus the Golgi [18].

Despite these results there is still relatively little insight into the ability of endomembranous Ras to mediate canonical Ras functions. Dissecting the role of specific compartments in regulating Ras function is complicated by the pools of endogenous Ras still present on other organelles. One strategy to investigate this has involved ectopic expression of a constitutively active Ras chimera where mutations or motifs have been introduced into the N- or C-terminus to direct the Ras protein to the required subcellular compartment. This allows the potential of subcellular platforms to sustain particular signaling pathways to be characterized. A recent study using this approach found that a plasma membrane restricted Ras protein was unable to efficiently induce NIH3T3 cell transformation in comparison with Ras that could also access endomembranes [19]. Crespo and colleagues have conducted the most systematic attempt to characterize organellar Ras signaling and found that Golgi-Ras is unable to support Ras-induced NIH3T3 cells transformation or proliferation [20]. This was associated with an inability of Golgi-Ras to activate the ERK and Akt pathways. In contrast, endoplasmic reticulum (ER)-restricted Ras and Ras occupying distinct plasma membrane microdomains could equivalently sustain normal Ras signaling and function.

In this study we have extended these previous observations by using an isogenic NIH3T3 cell line approach to compare the signaling and function of Ras proteins targeted to all organelles where Ras has been detected. We find that all locations are able to modulate signaling via the Raf and PtdIns-3 kinase (PI3K) pathways. All subcellular locations are also able to promote proliferation and transformation to varying degrees. In contrast to previous data we find that Golgi-Ras performs

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at least as well as N-Ras in these functions. Our data reveal a broad capacity of subcellular organelles to support basic Ras functions albeit with important location-specific differences.

2. Results and discussion

Early work to compare location-specific Ras signaling focused on the role of the ER/Golgi and plasma membrane signaling domains in modulating Ras outputs [21,22,7,20]. Manipulating the C-terminal HVR and adding specific organelle targeting motifs enabled redirection or restriction of constitutively active Ras proteins to specific areas of the cell and measurement of cell signaling outputs. Since these influential studies, it has become increasingly clear that activated Ras isoforms are able to access the endocytic network and mitochondria from where they are proposed to modulate proliferative and pro-apoptotic signaling respectively [23,24,9,10,13,25]. What is not clear is the relative potency of these locations in supporting Ras function.

To address this, we constructed a series of targeted Ras chimeras encoding GFP at the N-terminus and an organelle-targeting motif at the C-terminus to replace the conventional targeting encoded within the Ras HVR (Fig. 1A). Whilst N-Ras was used as the donor for the N-terminal Ras G-domain, these constructs can be described as generic Ras molecules since Ras isoform-specificity is encoded within the HVR that has been replaced. Previous work to target Ras to distinct subcellular locations has typically involved fusing a membrane targeting signal to the N-terminus of H-Ras. For ER/Golgi-Ras this incorporated residues 1–66 of M1 avian infectious bronchitis virus and for Golgi-Ras a mutant (N193D) KDEL receptor with impaired ability to recycle to the ER was used [20]. With higher levels of expression of KDELr-Ras, clear ER labeling can be observed (data not shown). Therefore to reduce this and to standardize the topology of the fluorescent reporter and membrane targeting motifs amongst all of our constructs, we generated a new Golgi-Ras using the Golgi-targeting domain of GM130 [26]. Almost complete co-localisation with the Golgi resident protein GRASP55 is observed (Fig. 1B). Previous work from our lab had also generated an alternative ER/Golgi-Ras by modifying the HVR of N-Ras [27]. Specific localization to the ER/Golgi can be seen including peripheral reticular, nuclear envelope and peri-nuclear Golgi cisternal stack labeling (Fig. 1B).

In addition to these constructs that reproduce targeting to locations compared in previous studies we also generated mitochondrial and endomembrane-specific Ras. Addition of the C-terminal 36 amino acid transmembrane and basic domain from mitochondrial outer membrane protein 25 (OMP25) [28] resulted in exclusive mitochondrial Ras localization (Fig. 1B). For endosomal/endomembrane targeting we used tandem FYVE domains from FENS1 to promote enrichment on the early endocytic network via PtdIns-3P interactions [29]. Whilst this is the least specifically localized of our organellar Ras proteins, clear co-localisation with the early endosomal marker EEA1 is observed (Fig. 1B). N-Ras is used as a positive control for normal Ras signaling. This was chosen due to it being the source of the G domain in our organelle Ras constructs and because it displays the most endomembranous distribution of the three Ras isoforms [2], with peri-nuclear ER/Golgi labeling being clearly evident (Fig. 1B).

NIH3T3 cells lines stably expressing wild type and constitutively active version of each compartmental-Ras were generated using FLP recombinase technology [30]. A single integration site is present within the Flp-In NIH3T3 cell genome for receipt of the organellar Ras DNA. This reduces the opportunity for non-specific effects due to random integration of DNA associated with conventional stable cell line generation. Expression amongst the wild type and active organellar Ras constructs

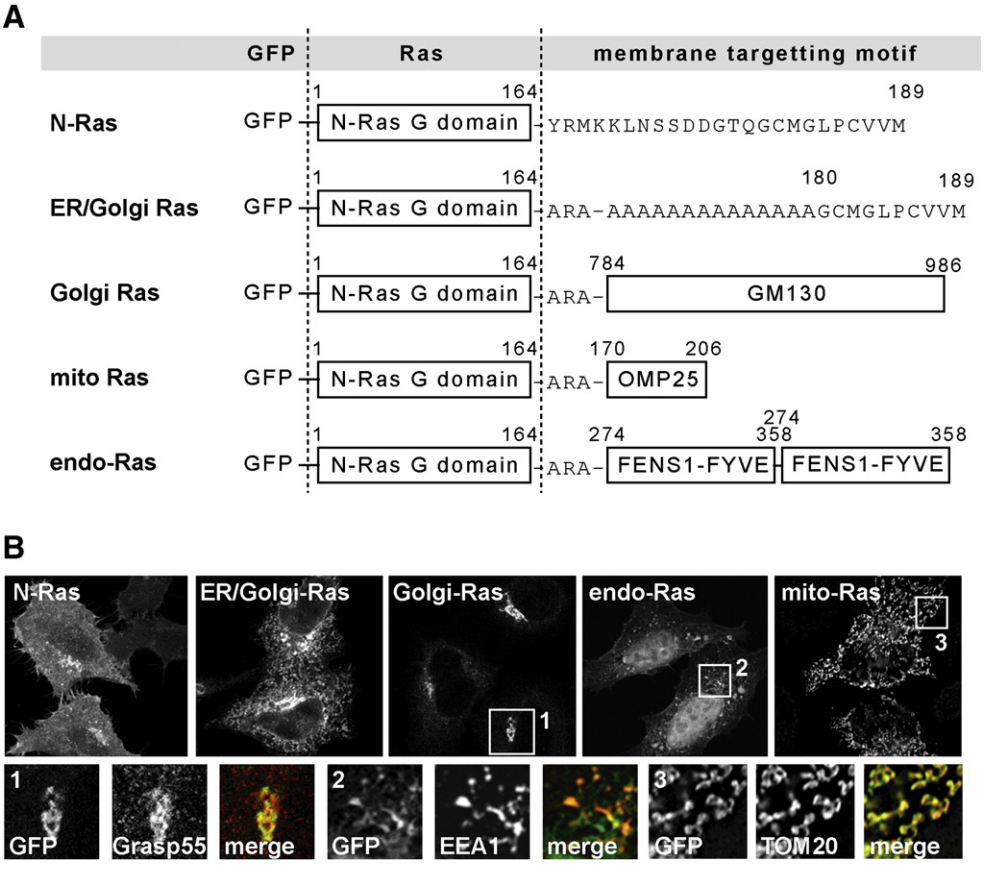

Fig. 1. Tethering Ras to different subcellular locations. (A) Schematic profile of all constructs generated. (B) Confocal images showing subcellular locations of the transient expressed constructs in HeLa cells. Ras proteins were visualized by anti-GFP (green) and co-localized with anti-Grasp55 (red) to confirm Golgi tethering, anti-EEA1 (red) to confirm endosomal location and anti-TOM20 (red) to confirm mitochondrial location.

was broadly equivalent although none of them achieved the same level of expression as GFP-N-Ras (Fig. 2A). Analysis of signaling via canonical Ras effector pathways revealed that in the absence of serum, N-Ras, Golgi-Ras and ER/Golgi-Ras each exhibited potent MEK activation in the presence of activated versus wild type Ras (Fig. 2B). In contrast, mito-Ras and endo-Ras showed little to no difference in MEK activation

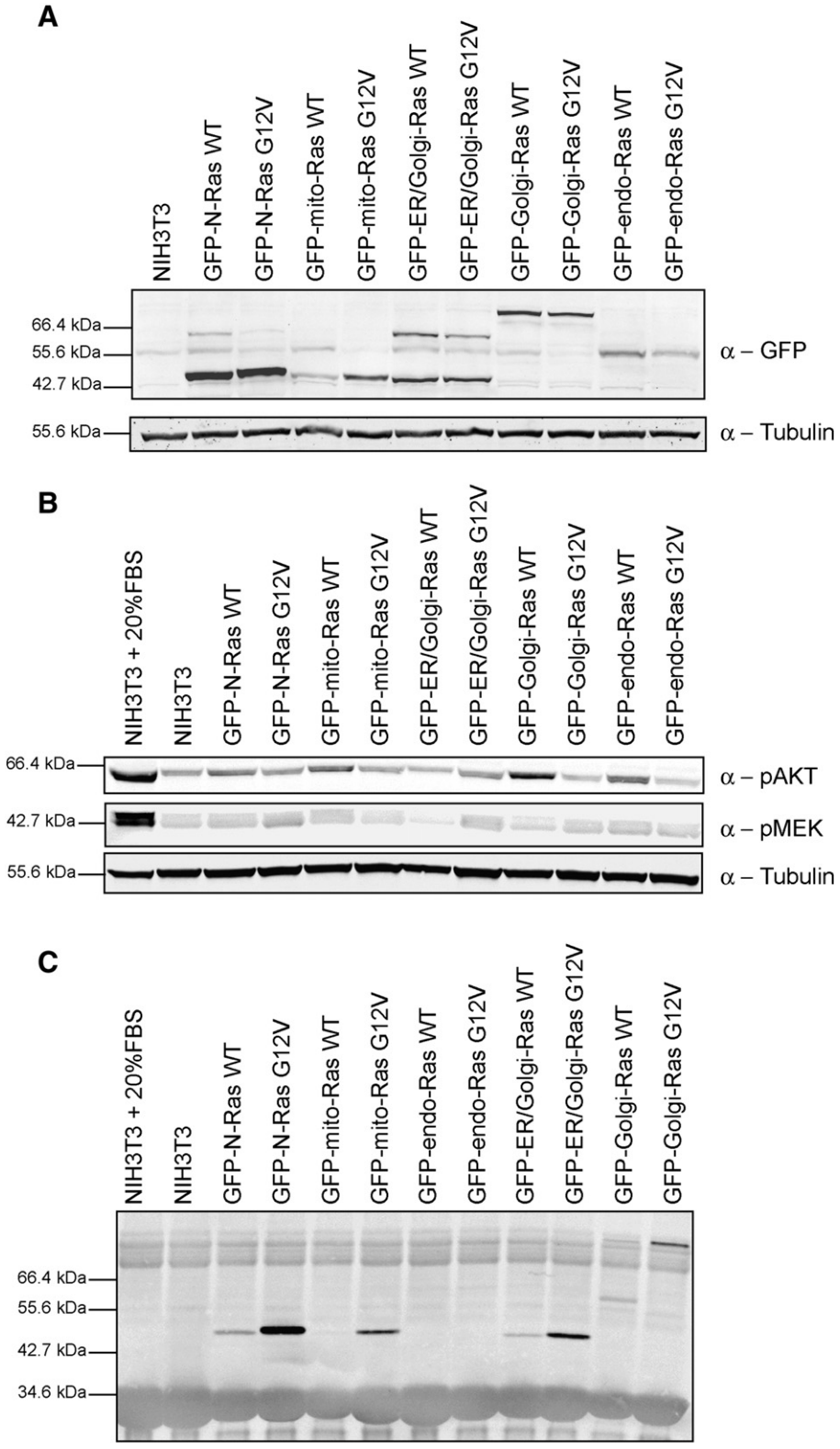

Fig. 2. Expression levels of the targeted Ras proteins, their ability to engage effectors and bind GST-RBD. (A) Ras protein expression was determined by anti-GFP-immunoblotting using 20 μg lysates from the NIH3T3 FLP-IN clones. Anti-tubulin was used as loading control. (B) Ras effector activation at different compartments was analyzed. Following 6 h starvation, 20 μg lysates from NIH3T3 FLP-IN cells stably expressing the indicated constructs were immunoblotted to determine pAKT, pMEK and the loading control tubulin levels using anti-pMEK, anti-pAKT and anti-tubulin respectively. (C) Ras activation was assayed *via* pull-down GST–RBD K85A fusion protein linked to glutathione–Sepharose 4B. Bound protein was detected by immunoblot using anti-GFP antibody. This figure shows representative blots from three biological repeats.

compared to their wild type versions and unstimulated control NIH3T3 cells. An inability of endo-Ras to activate the MAPK pathway is consistent with previous data that showed that ubiquitination of Ras resulting in enhanced Ras endocytosis attenuated Ras-dependent ERK activation [10]. We confirmed that the G12V mutant Ras proteins were active in each location albeit to different extents; endo-Ras activation was below the limit of detection (Fig. 2C). Similarly to N-Ras, Golgi-Ras and ER/Golgi-Ras show residual activation associated with the wild type constructs when grown in standard cell culture media that includes FBS.

Our results with Golgi-Ras superficially appear to be in contrast to the study of Matallanas et al. (2006) that showed an impaired ability of Golgi-Ras to activate ERK and Akt. However in this previous study only activated (G12V) mutants were compared whereas we also include the wild type versions too. Therefore whilst Golgi-Ras shows a clear enhancement of MEK activation when constitutively active, the increased activation attained with this condition was not much greater than those seen with the non-stimulated control NIH3T3 lane. ER/Golgi-Ras showed more potent MEK activation but was similarly characterized by a profound inhibitory activity of the wild type variant on MEK phosphorylation. Whilst wild type Ras is not constitutively active we still found evidence of activation in our pull-downs (Fig. 2C). One interpretation of our data is that with low levels of activation more typical of normal Ras signaling, Golgi-Ras is able to engage pathways that negatively regulate MEK activity. An example could be RKTG that sequesters Raf on to the Golgi and attenuates ERK activation [31]. With chronic Golgi-Ras stimulation this negative regulation is then lost and/or there is specific recruitment and activation of the Raf-MAP kinase cascade to the Golgi. Whilst we cannot discriminate between these possibilities it is clear that Golgi-Ras plays an active role in modulating MEK function.

A complex relationship is evident between Ras location, activation state and Akt activation. In the presence of activated G12V N-Ras Akt phosphorylation is reduced (Fig. 2B). This pattern is observed for Ras targeted to all locations except ER/Golgi-Ras where the reverse is true. Attenuated signaling in the presence of mutant Ras is seen in cells with long-term expression due induction of negative feedback mechanisms [32,33]. Therefore the observation of reduced Akt activation for constitutively active N-Ras and most of the other compartmental Ras proteins compared to wild type variants is not necessarily unexpected. Importantly, Golgi-Ras follows a similar pattern to N-Ras. Therefore analogous to the MEK data, whilst the G12V version appears non-responsive compared to unstimulated control there is a clear influence of Golgi-restricted Ras on Akt activation state when the wild type version is considered. Taken together, we see that whilst MEK modulation appears to be restricted to ER, Golgi and plasma membrane Ras, all locations are able to influence Akt activation.

The ability of compartmental Ras to sustain a range of phenotypic responses associated with normal Ras signaling was assayed. Measurement of cell proliferation was inferred using an MTS cell viability assay. All locations were able to sustain equivalent Ras dependent levels of proliferation to that seen with activated N-Ras (Fig. 3A). Furthermore, in our focus formation assays N-Ras, endo-Ras and Golgi-Ras generated significantly more foci than the GFP transfected control (Fig. 3B). Previous analysis of compartmental Ras signaling found that KDELr(N193D)-Ras localized to the Golgi displayed reduced proliferation and transformation compared to Ras targeted to the ER and plasma membrane [20]. One explanation for the discrepancy in our results could be that the Golgi-Ras used in the Matallanas study is impaired in its ability to cycle back to the ER. A similar argument was posited for the lack of transforming activity of Golgi-Ras that contrasted with previous studies using non-mutant KDELr as the Golgi targeting motif [7,14,20]. Although we cannot exclude the possibility that the tiny fraction of Golgi-Ras that is resident on the ER at steady state is solely responsible for our phenotypic observations we note that similarly low levels of peripheral ER labeling were also observed with KDELr(N193D)-Ras [20]. Furthermore, our Golgi-Ras construct exhibited more foci that the ER/Golgi-Ras arguing against a predominant role for the ER in our phenotypic observations.

Finally, oncogenic Ras is able to provide resistance to pro-apoptotic cisplatin-based chemotherapy in part via modulating MEK-ERK signaling [34,35]. Whilst activated N-Ras, mito-Ras and endo-Ras exhibit reduced apoptotic signaling as expected of normal Ras signaling, we intriguingly observed the opposite pattern for the Golgi and ER/Golgi-Ras proteins (Fig. 3C). The protective effect of wt Golgi-Ras and ER/Golgi-Ras correlates well with the minimal MEK activation observed with these constructs and is consistent with the known requirement for MEK-ERK signaling to mediate cisplatin-induced apoptosis in many cell types [36,34,37].

3. Conclusions

Our study represents the first comparative analysis across all locations where Ras isoforms are known to reside. Since Ras signaling involves membrane resident components in addition to recruitment of cytosolic factors we might expect that some locations would not be competent for sustaining targeted Ras function. In fact we found that Ras targeted to all locations was able to modulate signaling via the canonical Ras pathways. This was accompanied by regulation of phenotypic outputs including the ability for Golgi-Ras to promote proliferation and transformation equivalent to that seen with N-Ras. Whilst we cannot confirm the extent to which these effects are directly or indirectly mediated from each location our data highlight important capacity for endomembranous Ras to modulate Ras outputs.

4. Materials and methods

4.1. Antibodies

Rabbit anti-phospho-Akt (Ser473), rabbit anti-phospho-MEK1/2 (Ser217/221), and mouse anti-cleaved PARP (Asp214) were all from Cell Signaling Technology; mouse anti-tubulin (Sigma); mouse anti-Tom20 (BD Biosciences); rabbit anti-GFP [38]; rabbit anti-EEA1 [39]. Sheep anti-Grasp55 antibody was a kind gift of Francis Barr.

4.2. Targeted Ras constructs

Wild type and activated (G12V) GFP-N-Ras, GFP-ER/Golgi-Ras (GFP-N-HVR-Ala) and GFP-N-Ras acceptor constructs lacking the C-terminal HVR have been previously described (Laude and Prior, 2008). The GFP-N-Ras acceptor constructs allow subcloning of membrane targeting motifs C-terminal to the Ras G domain separated by a three amino acid ARA-linker sequence. The following primers were used to amplify the FENS1 FYVE domain (aa. 274–358), the GM130 Golgi targeting domain (aa 784–986) and the OMP25 mitochondrial targeting domain (aa 170–206) from plasmids encoding the relevant proteins: FENS_FYVE_F: 5′-ACTCGAGGTGAGCTCAGCAGAGAAGAGGC TCCT-3′ and FENS_FYVE_R: 5′-CTCGAGGCCCCCACCGCCACGAGAAGT CCGATCTTCATCTTTGATGGAGTC-3′. GM130_F: 5′-CTCGAGCTGCTCAC TTGGCAGCCCCA-3′ and GM130_R: 5′-GAATTCTTATATAACCATGATTT TCACCTCGTCGTTCTC-3′; OMP25_F: 5′-CTCGAGGACATCGAGGCGACG GAGAGGCC-3′ and OMP25_R: 5′-GAATTCCCTCAGAGCTGCTTTCGGTA TCTCACGAAGGC-3′. The sequenced targeting motifs were subcloned into the N-Ras acceptor construct and correct targeting confirmed in HeLa cells by immunofluorescence microscopy. HeLa cells were grown at 37 °C in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS. Cells were transfected with GeneJuice (Novagen) according to the manufacturer's instructions.

4.3. Stable cell line generation

NIH3T3 FLP-IN cells (Invitrogen) were grown in the presence of 100 μg/ml Zeocin. For the stable expression of compartmentalized

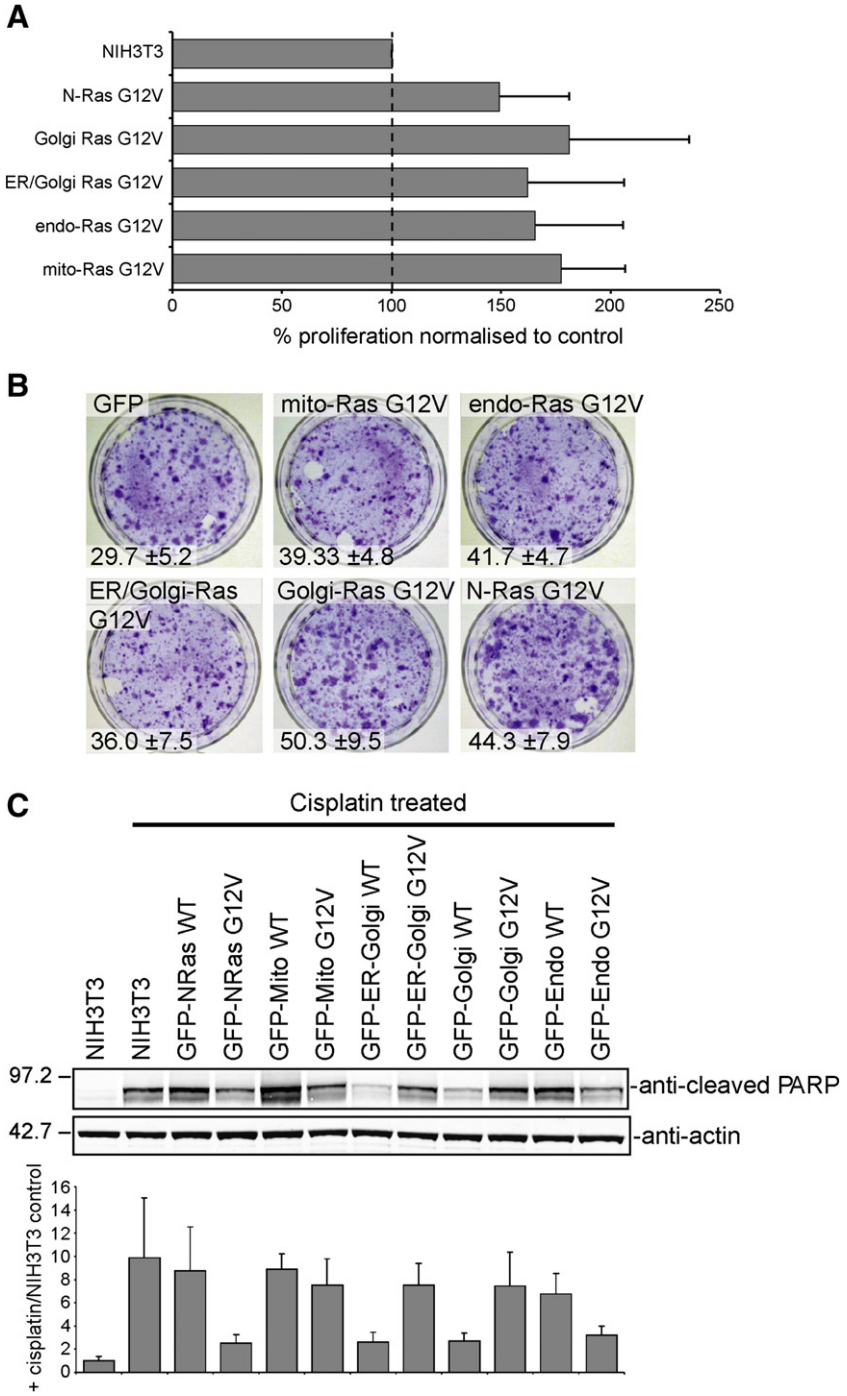

Fig. 3. Phenotypic responses of compartmentalized Ras signaling. (A) Levels of cell proliferation normalized to NIH3T3 control. Mean values for percentage of proliferation are shown. The described clones were seeded in triplicates on day 1 and cell numbers were determined 48 h post seeding by the MTS assay measuring absorbance at 490 nm (data obtained from 6 independent experiments). (B) Effects of compartmentalized Ras on cellular transformation. NIH3T3 cells were transfected with 1 μg of each of activated organellar Ras and cultured for 14 days in the presence of DMEM, 10% FBS containing 750 μg/ml G418. Foci were stained and the numbers of foci ≥2 mm diameter were counted; data represent means ± SEM of foci per well from three biological repeats. (C) Levels of cleaved PARP induced by cisplatin. NIH3T3 stably expressing the Ras targeted constructs were treated with 30 μM cisplatin for 24 h. PARP cleavage was monitored in 20 μg lysates by anti-cleaved PARP immunoblotting. This corresponds to a representative blot of three independent experiments. Graph shows levels of cleaved PARP normalized to control.

Ras, NIH3T3 FLP-IN cells were transfected with pEF5/FRT/V5 TOPO-GFP-Organellar Ras constructs together with pOG44 as indicated by the manufacturer's instructions. Transfections were performed using TransIT-3T3 transfection kit (MirrusBio). After 48 h, exchange into selection media (DMEM, 10% FBS, 200 μg/ml Hygromycin B) initiated death of non-transfected cells. Clones derived from single cells were picked and expanded and those positive for organellar Ras expression were identified and verified using fluorescence imaging and western blotting.

4.4. Viability and cisplatin resistance assays

To measure proliferation, cells were cultured at a density of 2000 cells per well in 96-well plates. After 48 h, CellTiter 96 Aqueous One Solution Reagent (Promega) was added to each well according to the manufacturer's instructions. After 4 h in culture the cell viability was determined by measuring the absorbance at 490 nm using a Multiskan Spectrum plate reader (Thermo Labsystems). Values were

converted from absorbance to cell number using a standard curve prepared for each experiment. For cisplatin resistance measurements, subconfluent cell cultures were incubated with 30 μM cisplatin (cDDP; Sigma) for 24 h. Cells were lysed on ice using RIPA buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Triton, 0.1% SDS, 1% sodium deoxycholate) lysates were separated by SDS–PAGE followed by immunoblotting, and finally analyzed with a LI-COR Odyssey 2.1 system.

4.5. Focus formation assay

NIH3T3 cells were plated in DMEM containing 10% FBS at a density of 5×10^5 cells/60-mm plate. Sixteen hours later the cells were transfected with 1 μg of each of activated organellar Ras and the following day media was exchanged for DMEM, 10% FBS containing 750 μg/ml G418. After 14 days the cells were fixed with 0.2% glutaraldehyde, 0.5% formaldehyde in PBS for 10 min on ice and stained with 0.2% crystal violet for 10 min at room temperature. The number of foci $\geq$2 mm diameter was counted; data represent means $\pm$ SEM of foci per well from three biological repeats.

4.6. Ras activation assay

Confluent organellar Ras NIH3T3 cells were washed with ice-cold PBS and lysed on ice in 50 mM Tris-Cl (pH 7.2), 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate and 1:250 protease-inhibitor cocktail (Sigma). In order to affinity-purify activated Ras from 200 μg of lysates, 11 μg of GST-RBD K85A fusion protein linked to glutathione–Sepharose 4B was added to each lysate together with at least a five-fold volume excess of binding buffer (50 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin (BSA), 0.5 mM dithiothreitol and 150 mM NaCl) and incubated for 30 min at 4 °C. Beads were washed twice in binding buffer (without BSA) before boiling in SDS sample buffer for 10 min. Activated Ras in the pull-downs was detected by western blotting with anti-GFP. GST-RBD K85A plasmid was a gift from Dr Tony Burgess, Ludwig Institute, Melbourne, Australia [40].

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