Multiplexed Real-Time NMR GTPase Assay for Simultaneous Monitoring of Multiple Guanine Nucleotide Exchange Factor Activities from Human Cancer Cells and Organoids

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Ⓢ *Supporting Information*

ABSTRACT: Small GTPases (sGTPases) are critical switch-like regulators that mediate several important cellular functions and are often mutated in human cancers. They are activated by guanine nucleotide exchange factors (GEFs), which specifically catalyze the exchange of GTP for GDP. GEFs coordinate signaling networks in normal cells, and are frequently deregulated in cancers. sGTPase signaling pathways are complex and interconnected; however, most GEF assays do not reveal such complexity. In this Communication, we describe the development of a unique real-time NMR-based multiplexed GEF assay that employs distinct isotopic labeling schemes for each sGTPase protein to enable simultaneous observation of six proteins of interest. We monitor nucleotide exchange of KRas, Rheb, RalB, RhoA, Cdc42 and Rac1 in a single system, and assayed the activities of GEFs in lysates of cultured human cells and 3D organoids derived from pancreatic cancer patients. We observed potent activation of RhoA by lysates of HEK293a cells transfected with GEF-H1, along with weak stimulation of Rac1, which we showed is indirect. Our functional analyses of pancreatic cancer-derived organoids revealed higher GEF activity for RhoA than other sGTPases, in line with RNA-seq data indicating high expression of RhoA-specific GEFs.

Figure 1. Cartoons depicting the GTPase cycle (a) and signaling cascades downstream of Ras involving sGTPases comprising the assay (b). The signaling connections include activation of GEFs by direct interactions with Ras (black arrows), as well as transcriptional activation (brown arrows) (blue font: GEFs, Ras and Rho subfamily members are colored cyan and green, respectively).

The sGTPase proteins are molecular switches that interconvert between two different forms; a GDP-bound inactive and a GTP-bound activated form, which binds to downstream effector proteins to stimulate their respective signaling pathways. The intrinsic hydrolysis of GTP and nucleotide exchange (GDP to GTP) are slow, but can be catalyzed by regulatory proteins known as GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (Figure 1a), respectively. Deregulation of this "GTPase cycle" by alterations of sGTPases, GEFs or GAPs is associated with human ailments such as cancer and developmental disorders.[1a,b] The cellular signaling pathways mediated by sGTPases are highly interconnected;[2] for example, one sGTPase can promote the activation of another sGTPase by activating its GEF (e.g., Ras-GTP stimulates RalGEFs, Sos1 and Tiam1), or stimulating the transcription of its regulatory proteins (e.g., KRas

induces expression of GEF-H1).[2,3] The synchronized temporal and spatial activation of RhoA, Rac1 and Cdc42 during cell migration illustrate coordinated sGTPase signaling (Figure 1b).[4] Further, a single sGTPase can be activated by multiple GEFs and one GEF can act on multiple sGTPases.[5] Though the intricacies of sGTPase signaling are increasingly appreciated, few of the

commonly used in vitro sGTPase assays can reveal such complexity.

Fluorescence, colorimetric or radioactive methods are frequently used to measure GEF activity, but are limited to monitoring a single sGTPase at a time.[6] Pulling down a sGTPase with an effector protein can be used to assess its level of activation in vivo, but does not report specifically on GEF versus GAP activities. Previously we introduced a real-time NMR based GEF assay, which observes nucleotide-dependent structural changes in sGTPase proteins directly, and used this functional assay to characterize several sGTPases (Table S1). This assay is highly reliable because direct monitoring of protein provides an unambiguous readout and built-in control for folding. Importantly, the method does not require fluorescently tagged nucleotide analogs, which can alter the kinetics of exchange and hydrolysis.[7] Here we have taken advantage of the protein-specific readout to develop a multiplexed GEF assay to simultaneously monitor multiple sGTPases in real-time. We sought to develop a tool to explore and profile the GEF activities that regulate proliferative and metastatic properties of cancer cells, thus we selected sGTPase proteins that are relevant to these processes, however the approach should be generally applicable to any subset of sGTPases.

The Ras and Rho subfamilies of sGTPases regulate cell proliferation and migration and are strongly implicated in oncogenic processes.[1a,8] We therefore developed the assay to include KRas, Rheb and RalB from the Ras subfamily, as well as RhoA, Rac1 and Cdc42 from the Rho subfamily (Figure 1b). Thirty-percent of human cancers harbor oncogenic *RAS* mutations, which has led to extensive drug discovery efforts,[9] including targeting Ras interaction with the RASGEF SOS to inhibit activation.[10] Rheb drives cellular growth through mTOR and is upregulated in many types of cancer,[11] although the identity of its GEF remains unclear.[12] RalGEFs are Ras effector proteins, which activate Ral when activated by Ras-GTP,[13] and are important for the oncogenic effects of Ras mutants.[14] RhoA, Cdc42 and Rac1 are three well-studied members of the Rho subfamily of GTPases implicated in tumorigenesis.[15] Importantly, the human genome encodes numerous GEFs for Rho family GTPases, and increased expression of several RhoGEFs is associated with certain cancer types.[16] We have combined these six sGTPase proteins into a single system to build a tool to measure the activities of full-length GEFs from cancer cells and organoids, thus expanding the data obtained from these precious samples and maximizing NMR instrument time, while providing internal controls.

Our real-time NMR-based method measures nucleotide exchange of sGTPases based on intensities of protein cross-peaks that are sensitive to nucleotide binding. Briefly, an excess of nonhydrolyzable GTP analog is added to a purified ^{15}N-labeled GDP-loaded sGTPase protein, and $^{1}H-^{15}N$ HSQC NMR spectra are collected sequentially over the time course of the exchange reaction. The unique readout, which observes protein directly, provides an opportunity to develop this method into a multiplexed assay to observe several sGTPases at once. To overcome peak overlap (Figure S1), we used selective labeling strategies to simplify each spectrum, and further used two nuclei with distinct chemical shift ranges (^{13}C and ^{15}N). First, we identified backbone amide (NH) or side chain methyl (CH$_3$) peaks in each protein that undergo chemical shift changes upon nucleotide exchange, and do not overlap with peaks from the other proteins. By analyzing the overlaid spectra of the six proteins in both the GDP- and GTP-bound forms, we devised

the following labeling scheme; KRas and RhoA: ^{15}N-threonine, Rac1: ^{15}N-lysine, Rheb and Cdc42: ^{13}C-methyl groups on leucine and valine side-chains, and RalB: ^{13}C-methyl methionine (Figure S2).

To simultaneously acquire spectra of both ^{15}N- and ^{13}C-labeled sGTPases in the mixture of six proteins, we used a time-shared pulse sequence (Figure S3).[17] Time-shared NMR has been used to reduce acquisition time in protein structure determination;[18a−c] however, we have applied it to improve the temporal resolution of real-time NMR data to measure reaction kinetics of a mixture of several sGTPase proteins. The pulse sequence was optimized for the different magnitudes of the $1J_{NH}$ and $1J_{CH}$ heteronuclear coupling constants, reducing the t_1 increment for carbon compared to nitrogen to detect both chemical shift regions simultaneously, and applying gradients for coherence selection and water suppression (Figure S3).[17]

Each multiplex assay was prepared by mixing GDP-loaded sGTPases to a final concentration of 400 μM for each ^{15}N-labeled protein (KRas, RhoA, Rac1) and 200 μM for each ^{13}C methyl labeled protein (Rheb, Cdc42 and RalB). NMR data was collected from a 40 μL sample, thus each assay requires ~1.4 mg total labeled sGTPase protein. Despite this high protein concentration (35 mg/mL), no significant chemical shift perturbation or peak broadening was observed (Figure S4). After the addition of 6 mM GTPγS, the mixture was transferred to a 1.7 mm NMR tube and real-time time-shared (RT-TS) $^{1}H-^{15}N$ and $^{1}H-^{13}C$ HSQC spectra were acquired sequentially over the time course of the exchange process (Figure 2a,b). Within the mixture we were able to monitor well resolved GDP- and GTP-specific peaks for each of the six sGTPases. By combining selective labeling schemes with simultaneous acquisition of ^{13}C and ^{15}N, we could plot intrinsic exchange curves and calculate the rate of exchange for each sGTPase
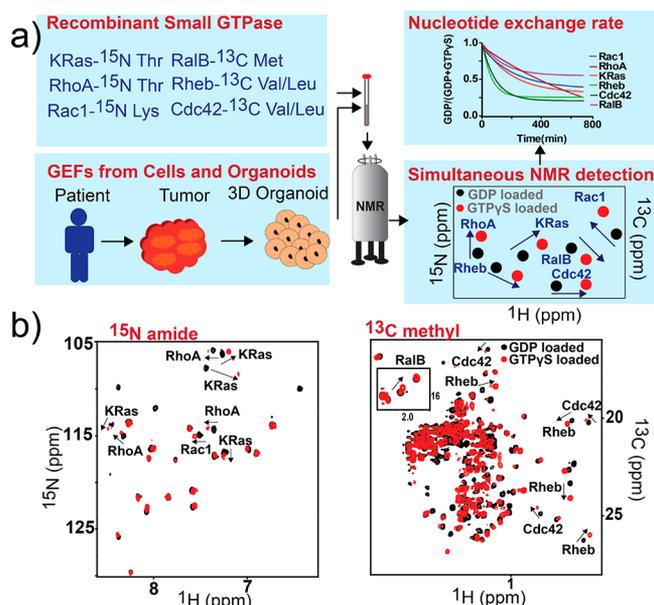
Figure 2. Schematic illustration representing the multiplex assay development. (a) sGTPase proteins are expressed in *E. coli* using selective isotope labeling strategies and the mixture is used to assay GEF activities present in lysates of cells or organoids. (b) $^{1}H-^{13}C$ and $^{1}H-^{15}N$ RT-TS HSQC spectra collected from a mixture of six sGTPases before and after nucleotide exchange. The arrows represent peaks used as probes for nucleotide exchange.

(Figure S5). The exchange rates and GTPγS loading at equilibrium of each sGTPase in this mixture were proportional to those in isolation.

Next, we used the assay to characterize GEF activities in cell lysates. GEF-H1 is a Rho-family GEF that can be inhibited by sequestration on microtubules and activated by multiple signaling events.[19a−c] GEF-H1 was initially reported to have GEF activity for both RhoA and Rac1;[20] however, subsequent reports focused mainly on RhoA.[21a,b] To resolve the substrate specificity of GEF-H1 and explore how GEF-H1 expression may indirectly perturb signaling of the other sGTPases, we performed an internally controlled multiplex assay to characterize GEF activities in lysates of cells overexpressing full-length GEF-H1.

Green fluorescent protein (GFP)-fused wild-type or catalytically impaired (E241K) GEF-H1 were transiently expressed in HEK293a cells and cell lysates were applied to the multiplex assay. As expected, lysate from cells expressing GEF-H1 increased the RhoA nucleotide exchange rate by more than 6-fold compared to the cells expressing GFP, whereas expression of the inactive GEF-H1 mutant did not affect exchange (Figures 3
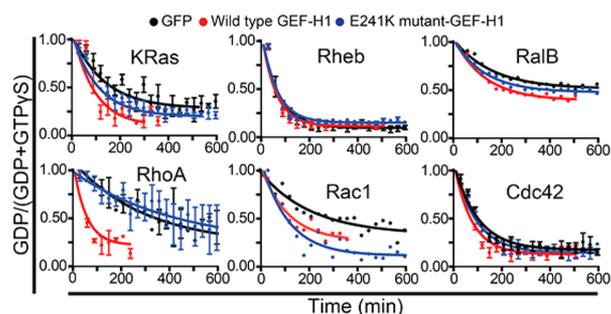
Figure 3. GEF-H1 activity in cell extracts. HEK293 cells were transfected with GFP, GFP-tagged GEF-H1, or a catalytically dead mutant (E241K) and lysates were analyzed by the multiplex assay. Exchange curves were fit to a one-phase exponential decay function.

and S6). The addition of GEF-H1 lysate doubled the exchange rate of Rac1; however, the GEF-H1 E241K lysate also stimulated Rac1 exchange to a similar extent (Figures 3 and S6). The other four sGTPases exhibited similar exchange rates in the presence of all three lysates. These data suggested the increase in Rac1 exchange activity may be an indirect effect of GEF-H1 overexpression. To determine definitively whether GEF-H1 possesses exchange activity for Rac1, we expressed the catalytic DH-PH domain (residues 201−601) in *E. coli*, and examined the GEF activity of the pure recombinant protein on [15]N RhoA, Rac1 and KRas. The DH-PH domain (1:5000 molar ratio) enhanced the nucleotide exchange rate of RhoA 17-fold, but had no detectable effect on Rac1 or KRas exchange, even at a higher molar ratio (1:1250) (Figure S7), indicating that Rac1 is not a direct substrate of GEF-H1. This demonstrates that the multiplex assay can detect GEF activities for multiple sGTPases in cell lysates. The increased Rac1 exchange activity may be the result of GEF-H1 perturbing cellular signaling, possibly stimulating the activity or expression of another GEF. The insensitivity of this effect to the E241K mutation suggests it may be mediated through a GEF-independent role of GEF-H1 (e.g., KSR-1 activation).[19a] These results demonstrate the remarkable sensitivity and selectivity of our assay for both direct and indirect effects of GEFs.

To further illustrate the utility of our assay, we used it to profile GEF activities of lysates from patient-derived organoids, self-organizing 3D cell cultures. Pancreatic cancer has a 5-year survival rate of only 7%, highlighting the need for better therapeutics.[22] These efforts require better preclinical models, which has driven the development of organoid models.[23] To test our assay with a patient-relevant model, two pancreatic ductal adenocarcinoma organoids were acquired from University Health Network Living Biobank.[24]

The organoids were lysed, and multiplexed GEF assays were performed using 70 μg of total protein from the lysate. In these experiments, we utilized a labeling scheme in which RhoA was uniformly ^{15}N labeled and Rac1 was ^{13}C-methyl labeled on isoleucines (Figure S8). Interestingly, both of the organoids, Org_2256 and Org_14553, showed high GEF activity for RhoA whereas this amount of lysate did not significantly alter the exchange rate for the other five sGTPases (relative to lysis buffer) (Figures 4a and S9). To investigate whether this functional
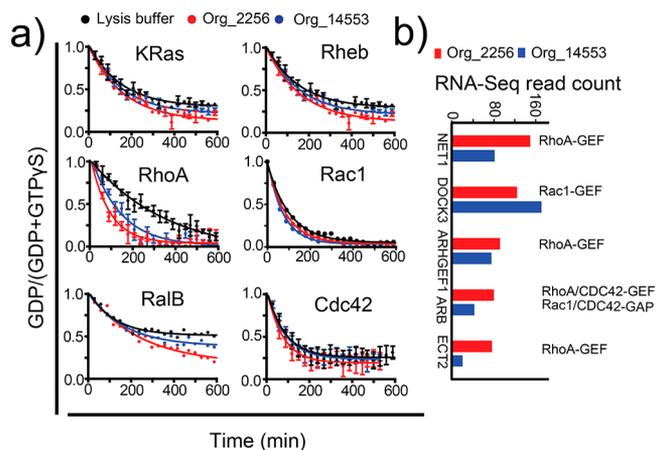
Figure 4. GEF activities in pancreatic cancer organoid lysates. (a) Lysates of pancreatic cancer organoids were assayed in the multiplexed system to generate exchange curves for six sGTPases. (b) RNA-seq read counts from 77 GEFs were compared for two organoids, and the data for the five most highly expressed genes are shown (full data in Figure S10).

observation correlates with gene expression, we compared the RNA-seq read counts of 77 GEF proteins of which 66 are RhoGEFs encoding a Dbl homology (DH) domain (Figure S10). Four of the five most abundant mRNAs encode GEFs with strong substrate specificity for RhoA (Figure 4b).[25a−d] Ras subfamily GEFs exhibited much lower RNA-seq counts, consistent with the lack of GEF activity observed for KRas and RalB. It is reassuring that our GEF protein activity data parallel the gene expression data on the same organoids. Our observation is also consistent with previous findings that pancreatic cancer invasion requires RhoA activation.[26]

In conclusion, we developed a multiplexed real-time NMR-based method to simultaneously measure the GEF activity for several sGTPases in lysates from cells and organoids. This functional assay based on simultaneous real-time acquisition of [1]H−^{13}C and [1]H−^{15}N HSQC spectra was applied to the detection of GEF activities of cells and organoids, and could serve as a functional read-out of GEF protein activities in cancers, complementing gene expression assays such as RNA-seq. This combination of selective isotopic labeling with time-shared and real-time NMR may enable multiplex observation of other biological processes (e.g., post-translational modifications).

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b13703.

Methods (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Fernandez-Medarde, A.; Santos, E. *Genes Cancer* **2011**, *2*, 344−58. (b) Vigil, D.; Cherfils, J.; Rossman, K. L.; Der, C. J. *Nat. Rev. Cancer* **2010**, *10*, 842−57.

(2) Rajalingam, K.; Schreck, R.; Rapp, U. R.; Albert, S. *Biochim. Biophys. Acta, Mol. Cell Res.* **2007**, *1773*, 1177−95.

(3) Kent, O. A.; Sandi, M. J.; Burston, H. E.; Brown, K. R.; Rottapel, R. *Oncotarget.* **2017**, *8*, 4484−4500.

(4) Martin, K.; Reimann, A.; Fritz, R. D.; Ryu, H.; Jeon, N. L.; Pertz, O. *Sci. Rep.* **2016**, *6*, 21901.

(5) Cherfils, J.; Zeghouf, M. *Physiol. Rev.* **2013**, *93*, 269−309.

(6) Marshall, C. B.; Meiri, D.; Smith, M. J.; Mazhab-Jafari, M. T.; Gasmi-Seabrook, G. M.; Rottapel, R.; Stambolic, V.; Ikura, M. *Methods* **2012**, *57*, 473−85.

(7) Mazhab-Jafari, M. T.; Marshall, C. B.; Smith, M.; Gasmi-Seabrook, G. M.; Stambolic, V.; Rottapel, R.; Neel, B. G.; Ikura, M. *J. Biol. Chem.* **2010**, *285*, 5132−6.

(8) Hanna, S.; El-Sibai, M. *Cell. Signalling* **2013**, *25*, 1955−61.

(9) Wilson, C. Y.; Tolias, P. *Drug Discovery Today* **2016**, *21*, 1915−1919.

(10) Lu, S.; Jang, H.; Zhang, J.; Nussinov, R. *ChemMedChem* **2016**, *11*, 814−21.

(11) Populo, H.; Lopes, J. M.; Soares, P. *Int. J. Mol. Sci.* **2012**, *13*, 1886−918.

(12) Parmar, N.; Tamanoi, F. *Enzymes* **2010**, *27*, 39−56.

(13) Tian, X.; Rusanescu, G.; Hou, W.; Schaffhausen, B.; Feig, L. A. *EMBO J.* **2002**, *21*, 1327−38.

(14) Ihle, N. T.; Byers, L. A.; Kim, E. S.; Saintigny, P.; Lee, J. J.; Blumenschein, G. R.; Tsao, A.; Liu, S.; Larsen, J. E.; Wang, J.; Diao, L.; Coombes, K. R.; Chen, L.; Zhang, S.; Abdelmelek, M. F.; Tang, X.; Papadimitrakopoulou, V.; Minna, J. D.; Lippman, S. M.; Hong, W. K.; Herbst, R. S.; Wistuba, II; Heymach, J. V.; Powis, G. *Journal of the National Cancer Institute.* **2012**, *104*, 228−39.

(15) Sahai, E.; Marshall, C. J. *Nat. Rev. Cancer* **2002**, *2*, 133−42.

(16) Barrio-Real, L.; Kazanietz, M. G. *Sci. Signaling* **2012**, *5*, pe43.

(17) Sattler, M.; Maurer, M.; Schleucher, J.; Griesinger, C. *J. Biomol. NMR* **1995**, *5*, 97−102.

(18) (a) Boelens, R.; Burgering, M.; Fogh, R. H.; Kaptein, R. *J. Biomol. NMR* **1994**, *4*, 201−13. (b) Wurtz, P.; Aitio, O.; Hellman, M.; Permi, P. *J. Biomol. NMR* **2007**, *39*, 97−105. (c) Nolis, P.; Perez, M.; Parella, T. *Magn. Reson. Chem.* **2006**, *44*, 1031−6.

(19) (a) Cullis, J.; Meiri, D.; Sandi, M. J.; Radulovich, N.; Kent, O. A.; Medrano, M.; Mokady, D.; Normand, J.; Larose, J.; Marcotte, R.; Marshall, C. B.; Ikura, M.; Ketela, T.; Moffat, J.; Neel, B. G.; Gingras, A. C.; Tsao, M. S.; Rottapel, R. *Cancer Cell* **2014**, *25*, 181−95. (b) Meiri, D.; Marshall, C. B.; Mokady, D.; LaRose, J.; Mullin, M.; Gingras, A. C.; Ikura, M.; Rottapel, R. *Nat. Commun.* **2014**, *5*, 4857. (c) Sandi, M. J.; Marshall, C. B.; Balan, M.; Coyaud, E.; Zhou, M.; Monson, D. M.; Ishiyama, N.; Chandrakumar, A. A.; La Rose, J.; Couzens, A. L.; Gingras, A. C.; Raught, B.; Xu, W.; Ikura, M.; Morrison, D. K.; Rottapel, R. *Sci. Signaling* **2017**, *10*, eaan3286.

(20) Ren, Y.; Li, R.; Zheng, Y.; Busch, H. *J. Biol. Chem.* **1998**, *273*, 34954−60.

(21) (a) Birkenfeld, J.; Nalbant, P.; Yoon, S. H.; Bokoch, G. M. *Trends Cell Biol.* **2008**, *18*, 210−9. (b) Krendel, M.; Zenke, F. T.; Bokoch, G. M. *Nat. Cell Biol.* **2002**, *4*, 294−301.

(22) Siegel, R. L.; Miller, K. D.; Jemal, A. *CA-Cancer J. Clin.* **2015**, *65*, 5−29.

(23) Baker, L. A.; Tiriac, H.; Clevers, H.; Tuveson, D. A. *Trends Cancer* **2016**, *2*, 176−190.

(24) Boj, S. F.; Hwang, C. I.; Baker, L. A.; Chio, II; Engle, D. D.; Corbo, V.; Jager, M.; Ponz-Sarvise, M.; Tiriac, H.; Spector, M. S.; Gracanin, A.; Oni, T.; Yu, K. H.; van Boxtel, R.; Huch, M.; Rivera, K. D.; Wilson, J. P.; Feigin, M. E.; Ohlund, D.; Handly-Santana, A.; Ardito-Abraham, C. M.; Ludwig, M.; Elyada, E.; Alagesan, B.; Biffi, G.; Yordanov, G. N.; Delcuze, B.; Creighton, B.; Wright, K.; Park, Y.; Morsink, F. H.; Molenaar, I. Q.; Borel Rinkes, I. H.; Cuppen, E.; Hao, Y.; Jin, Y.; Nijman, I. J.; Iacobuzio-Donahue, C.; Leach, S. D.; Pappin, D. J.; Hammell, M.; Klimstra, D. S.; Basturk, O.; Hruban, R. H.; Offerhaus, G. J.; Vries, R. G.; Clevers, H.; Tuveson, D. A. *Cell* **2015**, *160*, 324−38.

(25) (a) Dubash, A. D.; Guilluy, C.; Srougi, M. C.; Boulter, E.; Burridge, K.; Garcia-Mata, R. *PLoS One* **2011**, *6*, e17380. (b) Xiang, X.; Li, S.; Zhuang, X.; Shi, L. *FEBS Lett.* **2016**, *590*, 2940−55. (c) Yuce, O.; Piekny, A.; Glotzer, M. *J. Cell Biol.* **2005**, *170*, 571−82. (d) Chuang, T. H.; Xu, X.; Kaartinen, V.; Heisterkamp, N.; Groffen, J.; Bokoch, G. M. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 10282−6.

(26) Timpson, P.; McGhee, E. J.; Morton, J. P.; von Kriegsheim, A.; Schwarz, J. P.; Karim, S. A.; Doyle, B.; Quinn, J. A.; Carragher, N. O.; Edward, M.; Olson, M. F.; Frame, M. C.; Brunton, V. G.; Sansom, O. J.; Anderson, K. I. *Cancer Res.* **2011**, *71*, 747−57.