

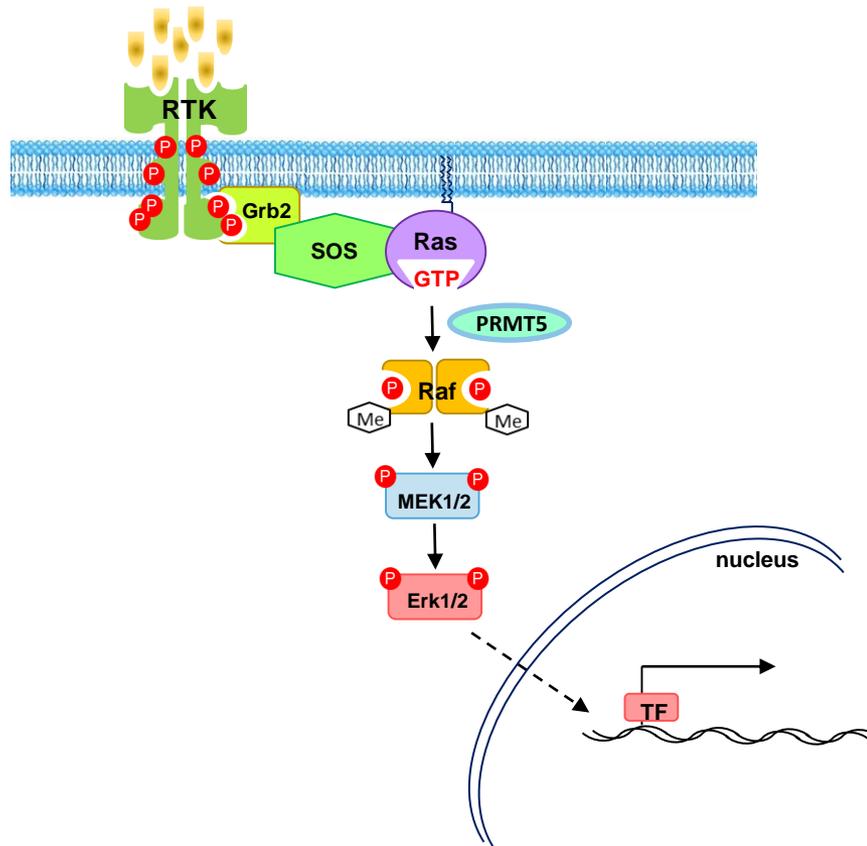
GTPase Biology: New Tools to the Rescue

Subhanjan Mondal, Ph.D.
Promega Corporation

welcome

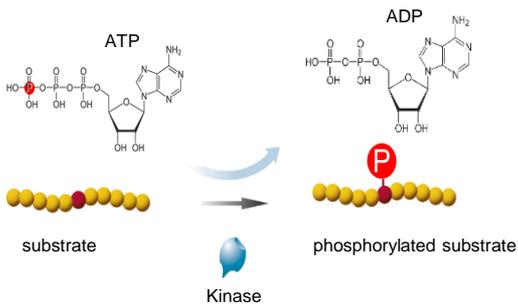
- Cell signaling overview: RTK-Ras-Erk pathway
- GTPases: families, mechanisms and human diseases
- GTPase-Glo™ assay principle
- Applications for the GTPase-Glo™ assay
 - Intrinsic GTPase activity
 - GAP-stimulated GTPase activity
 - GEF activity
- Assay optimization
- Conclusion and references

Cell signaling overview: RTK signaling



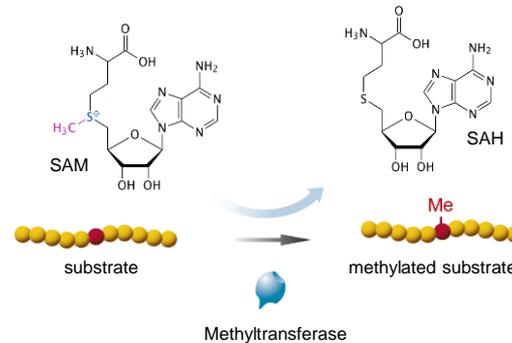
Cell signaling overview

Kinase



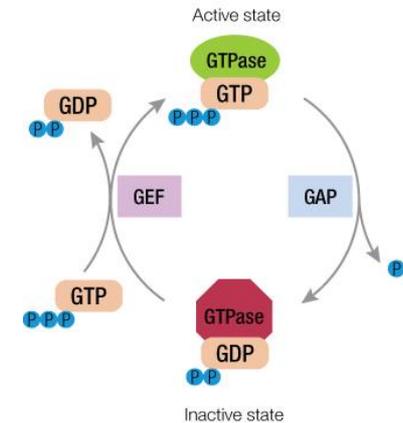
- Change in overall charge of the target protein.
- Change in protein conformation, enzymatic activity, protein-protein interaction or cellular localization of the target protein.

Methyltransferase



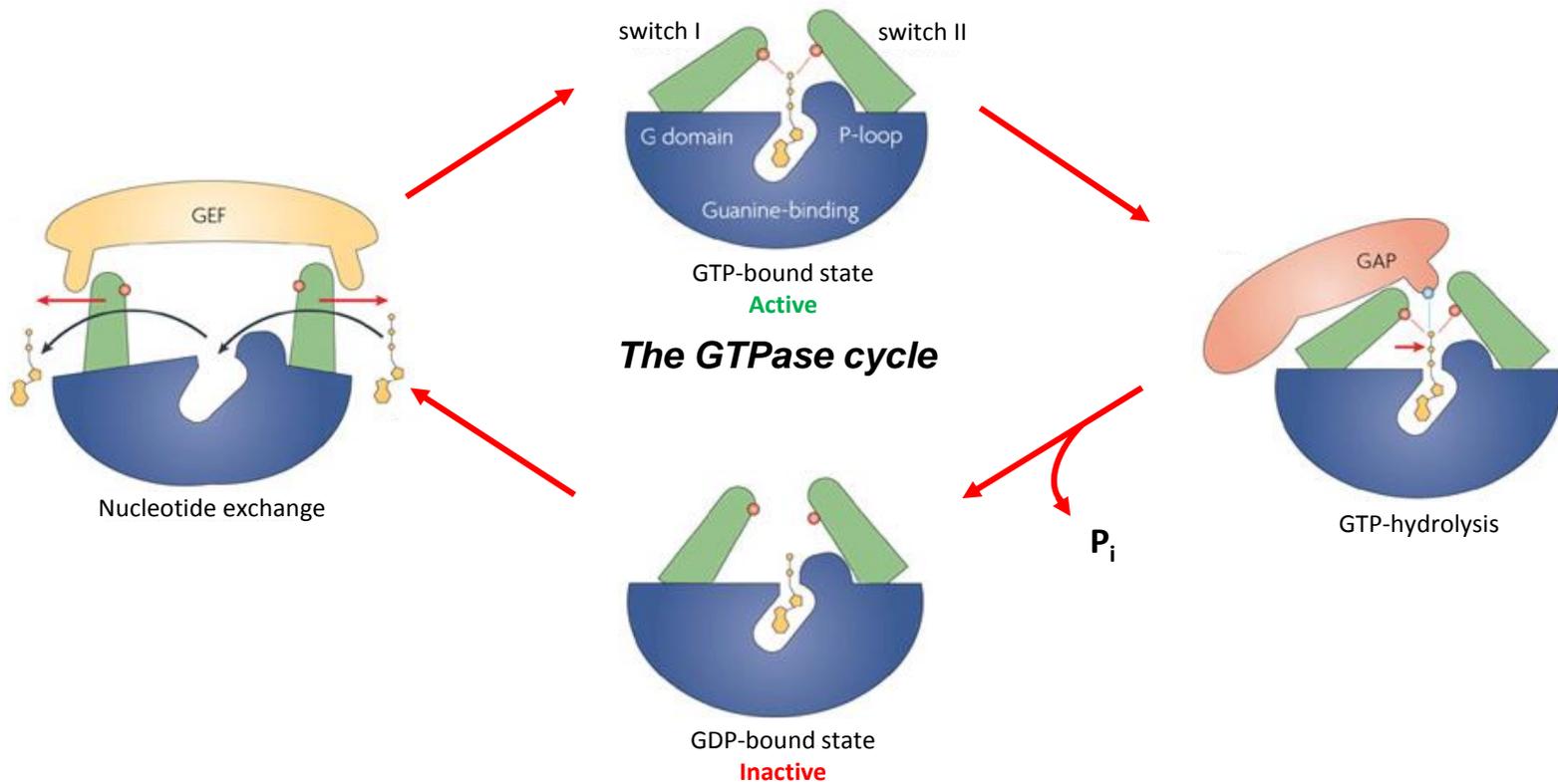
- Change in overall hydrophobicity of the target protein.
- Change in protein-protein interaction of the target protein.

GTPase



- Occupancy of GTP/GDP in the active site affects protein-protein interaction with effector proteins.
- Change in protein conformation or enzymatic activity of the effector protein.

The GTPase cycle

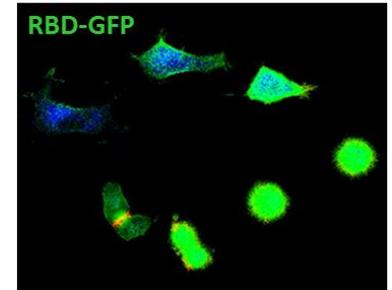
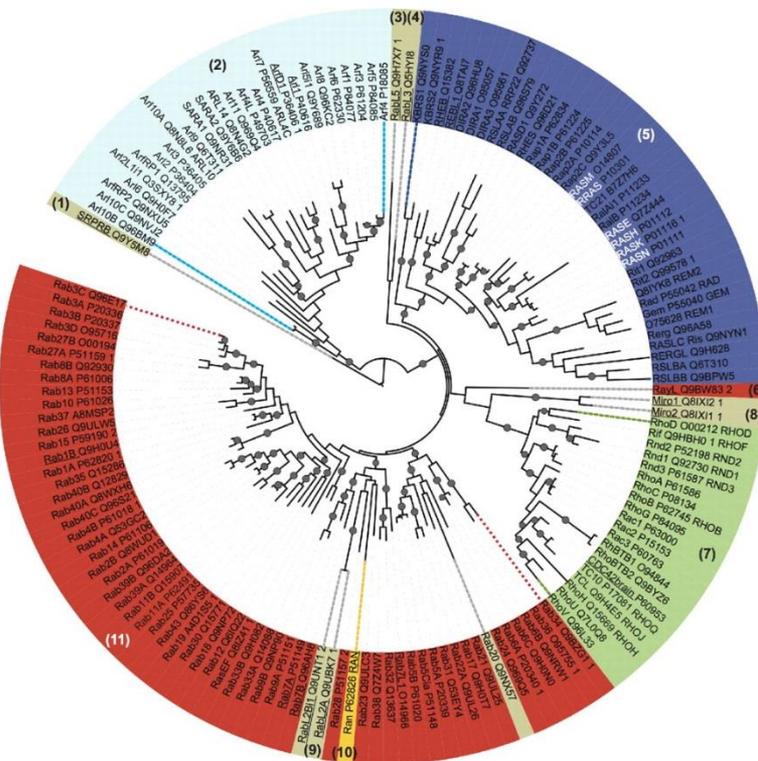


Weirich CS, et al. The septin family of GTPases: architecture and dynamics. *Nature Reviews Molecular Cell Biology* 2008 June; 9, 478-489.

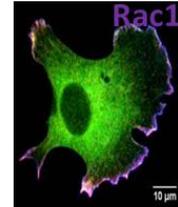
Human small GTPases (Ras superfamily)



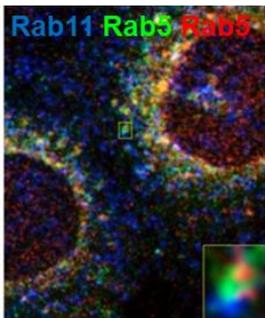
- Unclassified (9)
- ARF family (27)
- RHO family (20)
- RAB family (61)
- RAS family (36)
- RAN family (1)



Ras: cell growth and proliferation



Rho: actin cytoskeleton



Rab: vesicular transport

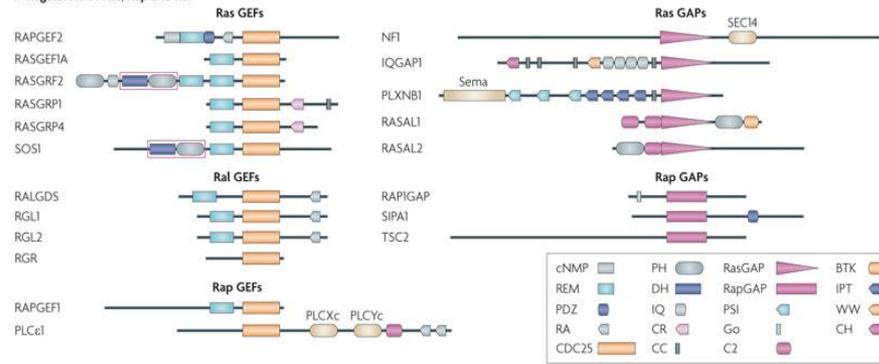
Rojas AM *et al. J Cell Biol* 2012;196:189-201.

Sönnichsen B *et al. J Cell Biol.* 2000 May 15;149(4):901-14.

Many GEFs and many GAPs for many GTPases



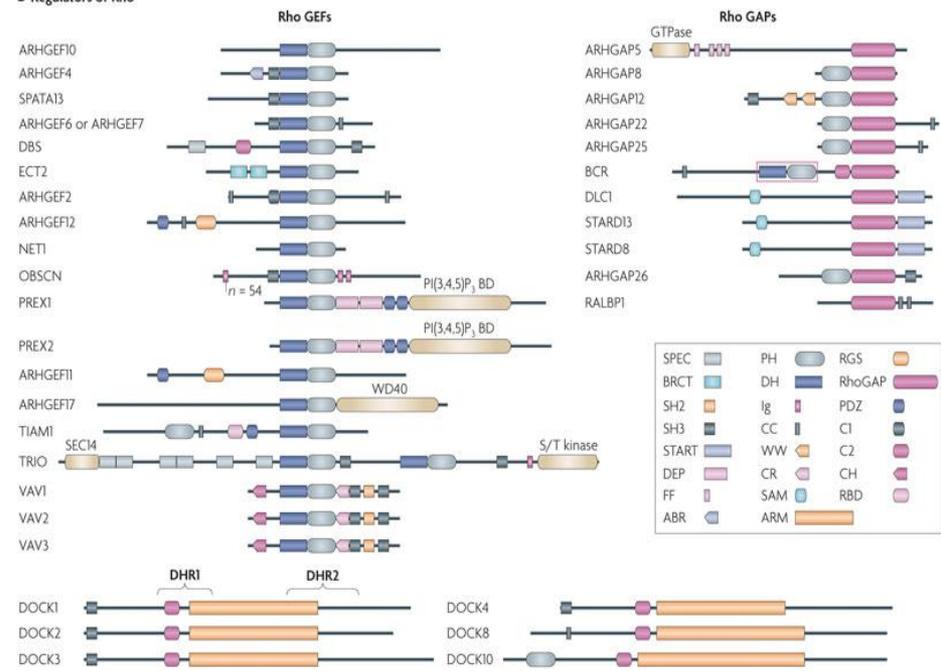
a Regulators of Ras, Rap and Ral



c Regulators of Arf

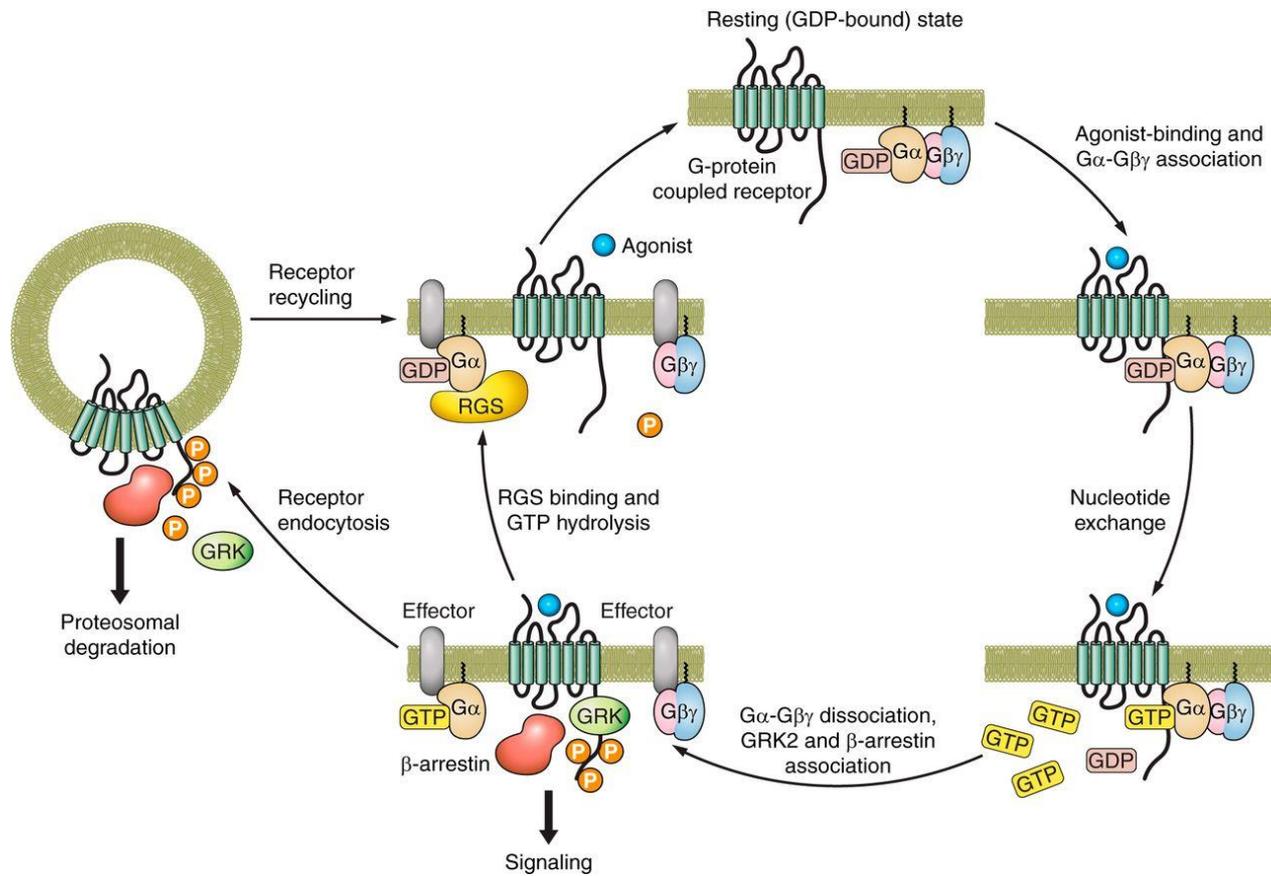


b Regulators of Rho



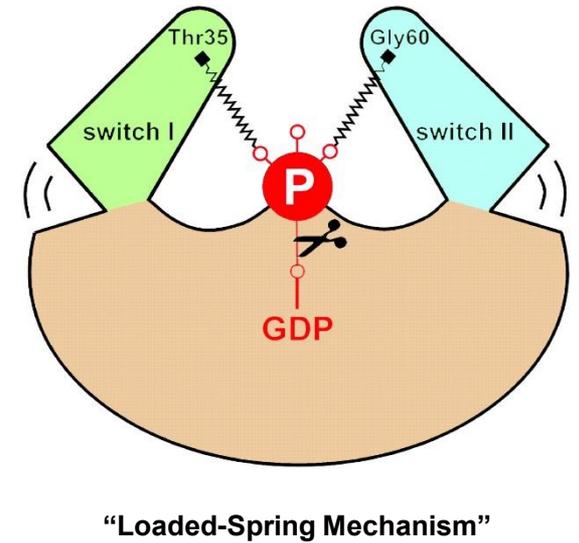
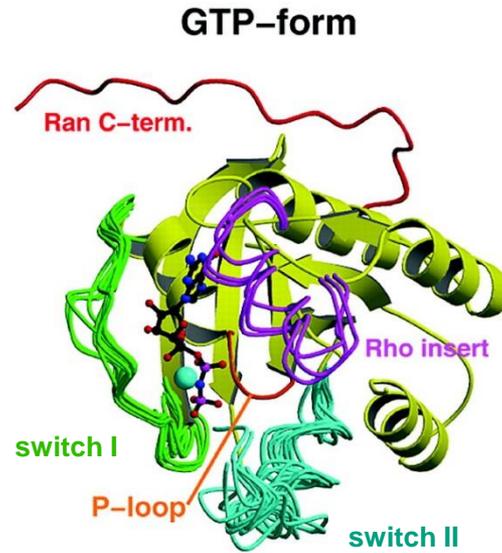
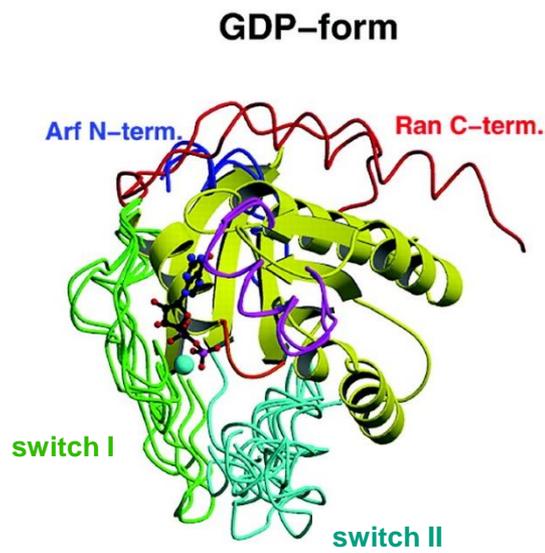
Vigil D, et al. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nature Reviews Cancer* 2010 Dec; 10, 842-857.

Heterotrimeric GTPases (G_{α})



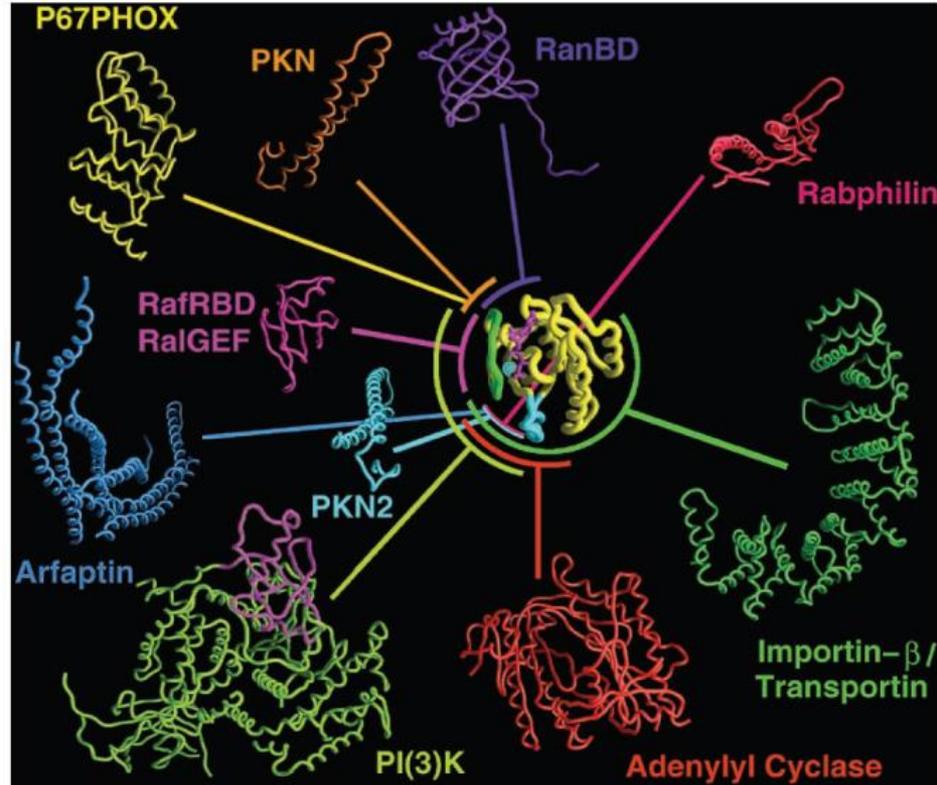
Sato PY et al. The evolving impact of G protein-coupled receptor kinases in cardiac health and disease. *Physiol Rev.* 2015 Apr;95(2):377-404.

How does the switch work?



Vetter IR, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. *Science*. 2001 Nov 9;294(5545):1299-304.

GTPase signaling: Protein-protein interactions



Vetter IR, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. *Science*. 2001 Nov 9;294(5545):1299-304.

GTPases in health and disease: Ras and cancer

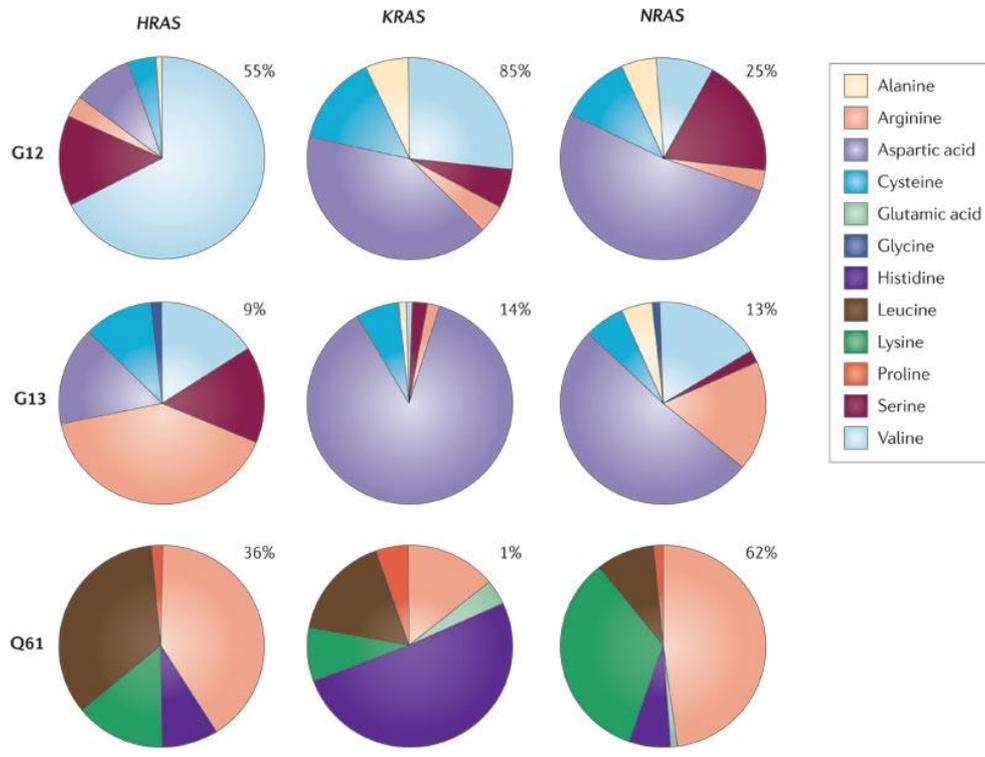


MORE THAN
30%
OF ALL HUMAN CANCERS
ARE DRIVEN BY MUTATIONS OF
RAS GENES

**RAS MUTATIONS
IN HUMAN CANCERS**

- PANCREAS — KRAS 95%
- COLORECTAL — KRAS 45%
- LUNG — KRAS 35%
- AML — NRAS 15%
- MELANOMA — NRAS 15%
- BLADDER CANCER — HRAS 10%

"RAS ONCOGENES ARE
THE **WORST** ONCOGENES."
— Dr. Frank McCormick,
RAS National Program Advisor

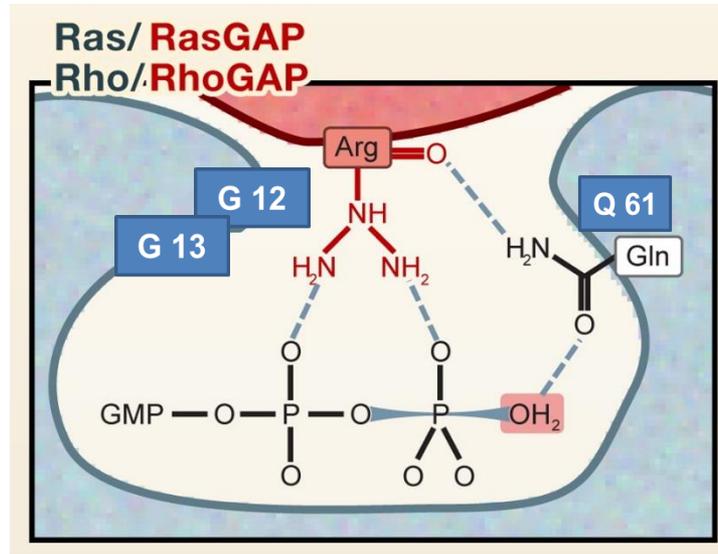
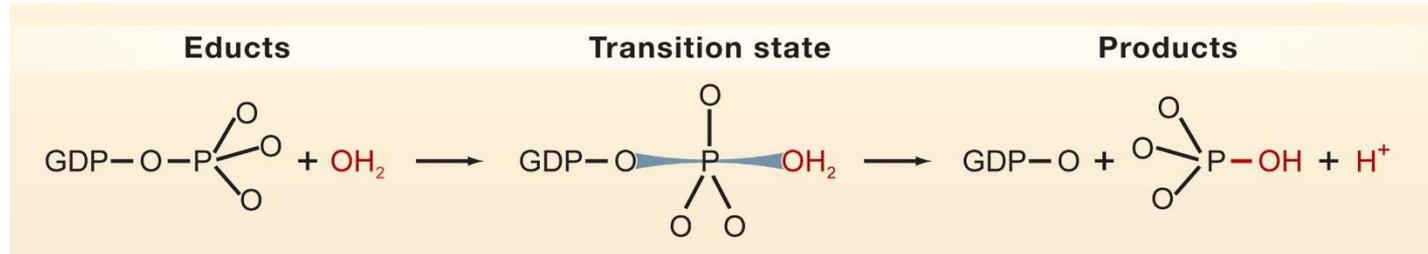


The unmet clinical need

- High incidence of Ras mutations in various cancers.
- No drugs that target Ras proteins directly or indirectly.
- No therapies effective for Ras driven cancers.
- Patients with Ras-driven cancers are excluded from treatment with targeted therapies.

Pylyayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer*. 2011 Oct 13;11(11):761-74.
<http://www.cancer.gov/research/key-initiatives/ras/the-problem>

GTPases in health and disease: Ras and cancer

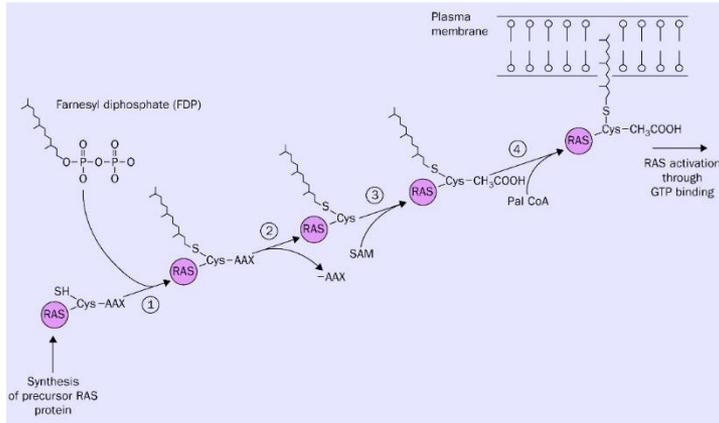


Bos JL, Rehmann H, Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. *Cell*. 2007 Jun 1;129(5):865-77.

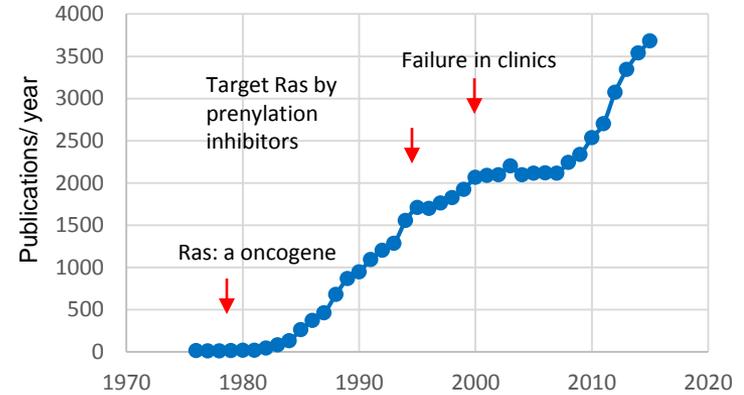
GTPases in health and disease: Ras and cancer



Targeting post-translational modifications of Ras



Johnston SR. Farnesyl transferase inhibitors: a novel targeted therapy for cancer. *Lancet Oncol.* 2001 Jan;2(1):18-26.



GTPases in health and disease: Ras and cancer

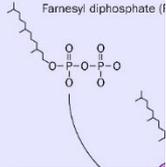


Targeting pAngewandte

Communications



Drug Discovery Hot Paper



Inhibition of Ras Signaling by Cyclic Peptides**

Punit Upadhyaya, Ziqing Qian, et al.

LETTER

International Edition: DOI: 10.1002/anie.201502763

clinics



doi:10.1038/nature12205

Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange at

Till Maurer^{a,1}, Lindsay S. Garrenton^{b,1}, Angela Oh^a, Benjamin P. Fauber^d, Borlan Pan^a, Shiva Malek^c, David Jiansheng Wu^a, Anthony M. Giannetti^f, Melissa A. Joachim Rudolph^d, Weiru Wang^{a,2}, and Guowei Fan^a

Small molecule inhibition of the KRAS–PDEδ axis disrupts oncogenic KRAS signalling

Shehab Ismail^{3*}, Nachiket Vartak², Anchal Chandra², Maike Hoffmann⁴.

A Small Molecule RAS-Mimetic Disrupts RAS Association with Effector Proteins to Block Signaling

Sai Krishna Athuluri-Divakar,^{1,2} Rodrigo Vasquez-Del Carpio,^{1,2} Kaushik Dutta,³ Stacey J. Baker,^{1,2} Stephen C. Cosenza,^{1,2} Indrani Basu,⁵ Yogesh K. Gupta,^{1,2} M.V. Ramana Reddy,^{1,2} Lynn Ueno,⁴ Jonathan R. Hart,⁴ Peter K. Vogt,⁴ David Mulholland,^{1,2} Chandan Guha,⁵ Aneel K. Aggarwal,^{1,2} and E. Premkumar Reddy^{1,2,*}

K-Ras(G12C) inhibits effector interactions

Jonathan M. Ostrem^{1*}, Ulf Peters^{1*}, Martin L. Sos¹, James A. Wells² & Kevan M. Shokat¹

GTPases in health and disease: Infectious diseases



Pathogen

Bacterial Toxin/ Host GTPase

Molecular Mechanism (post-translational modification)

Vibrio cholera

Ctx/*Ga_s*

Bordetella pertussis

Ptx/*Ga_i*

Clostridium botulinum

C3/*RhoA*

Pseudomonas

ExoS/*Ras*

Vibrio parahaemolytica

VopS/*Rho*

Histophilus somni

IbpA/*RhoA*

Legionella

DrrA/*Rab*

Clostridium difficile

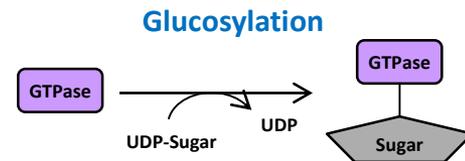
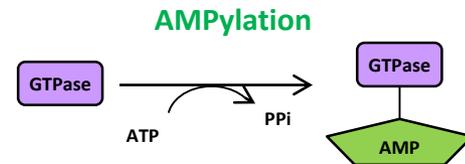
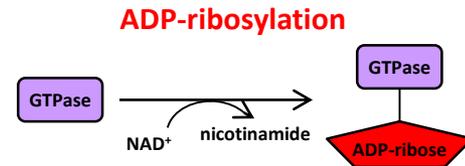
ToxinB/*RhoA*

Salmonella

SptP/*Rac1*
SopE/*RhoA*

Yersinia pestis

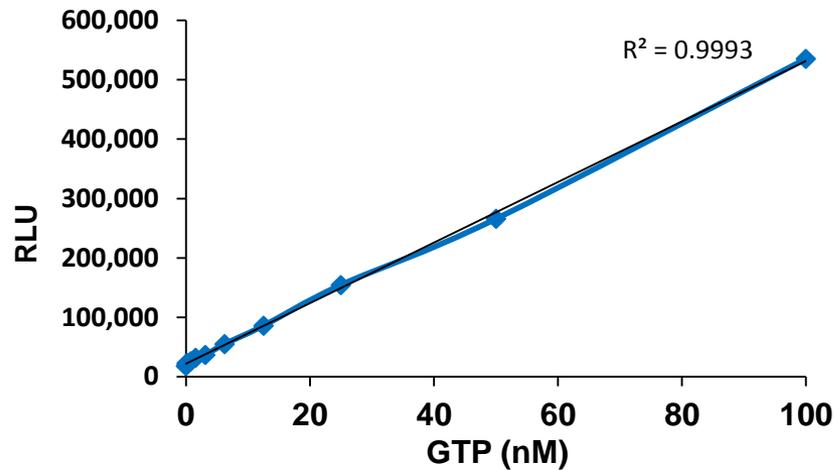
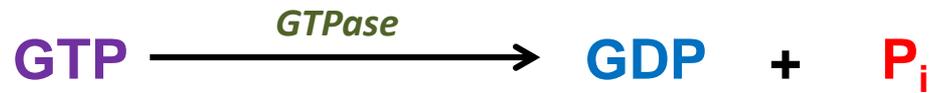
YopE/*Rho, Rac, Cdc42*



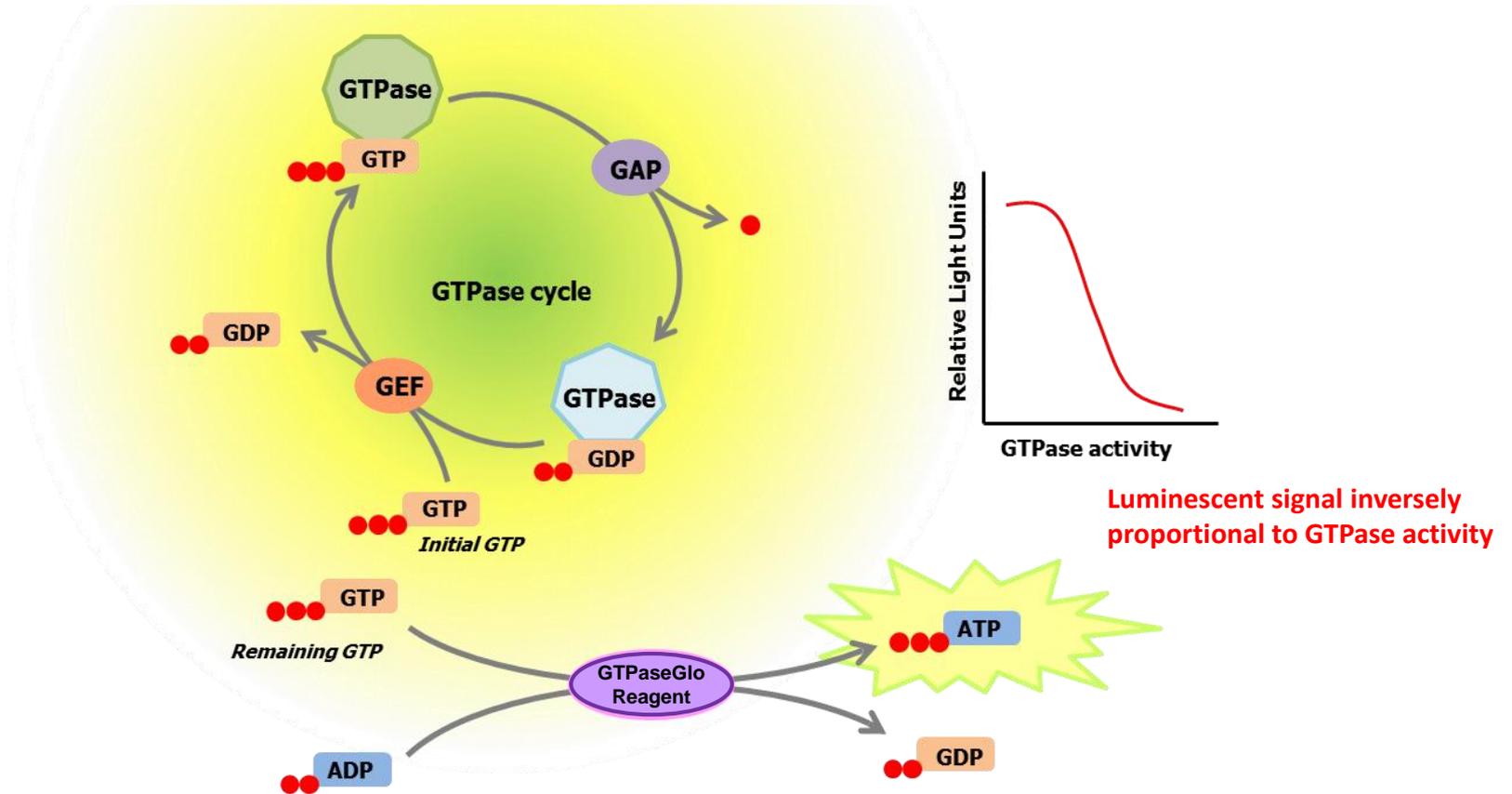
SptP acts as GAP for *Rac1*
SopE acts as GEF for *RhoA*

YopE acts as GAP for *Rho, Rac, Cdc42*

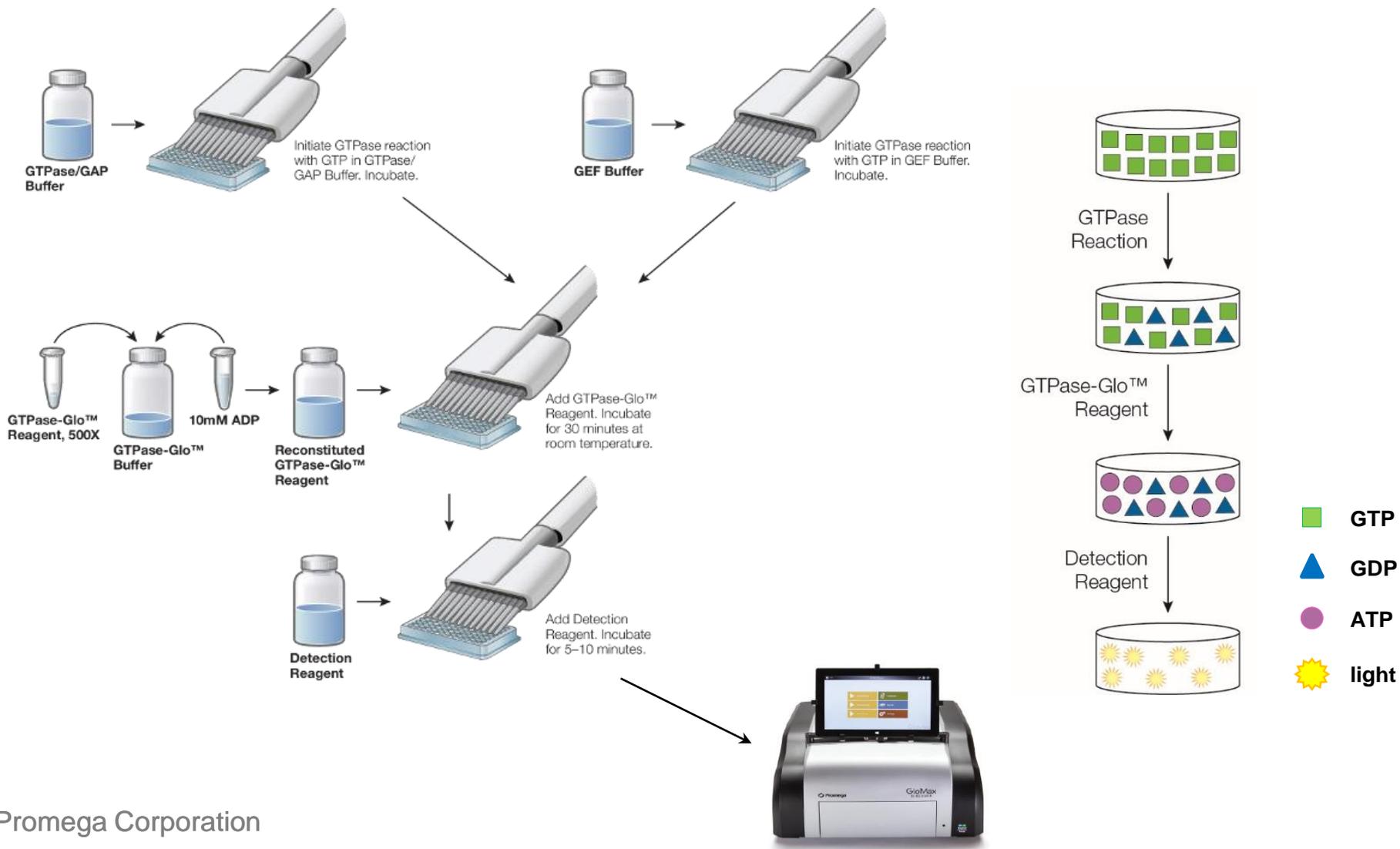
Assay development



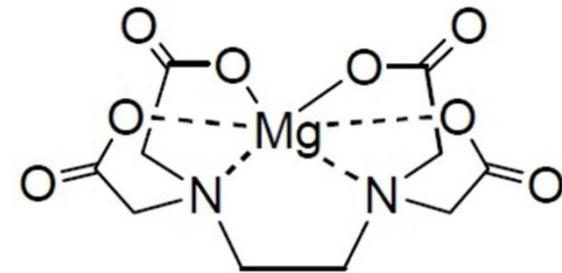
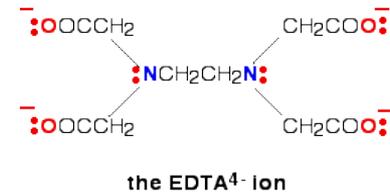
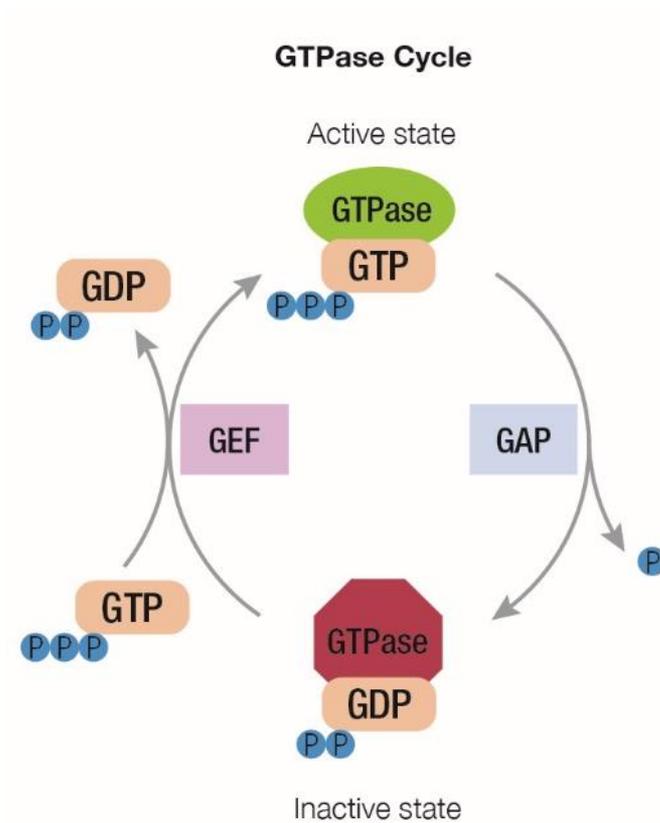
Assay principle



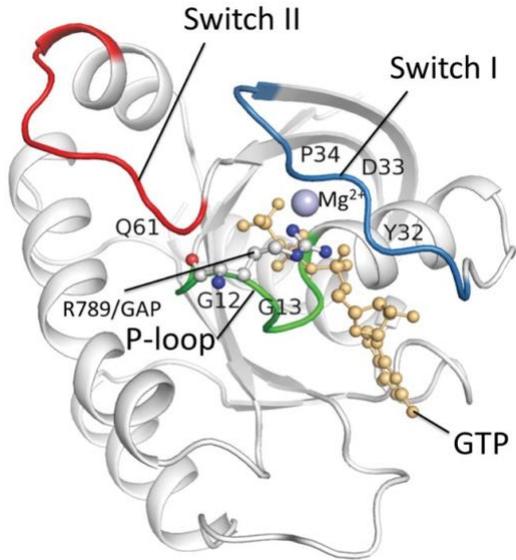
Assay workflow



Reaction buffers: GTPase/GAP Reaction Buffer



EDTA as a surrogate for GEF



GTPase/ GAP Reaction Buffer

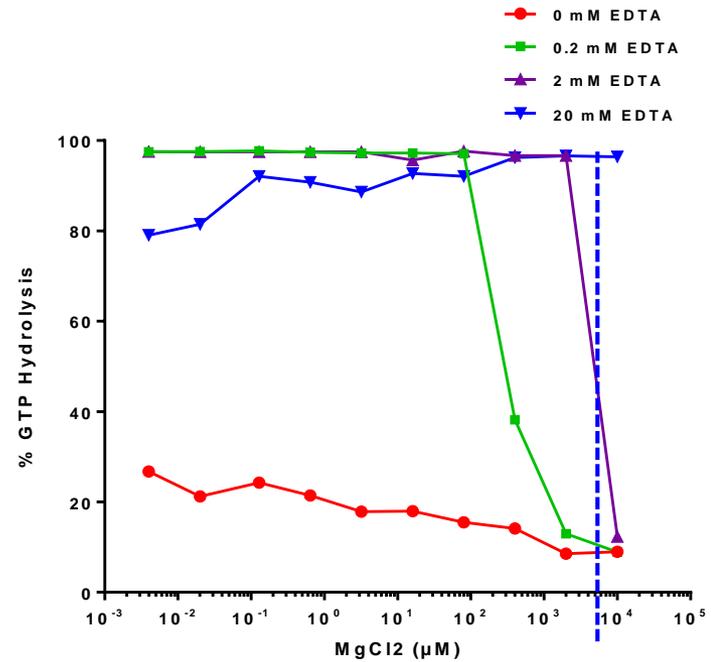
50 mM Tris-HCl, pH 7.5

50 mM NaCl

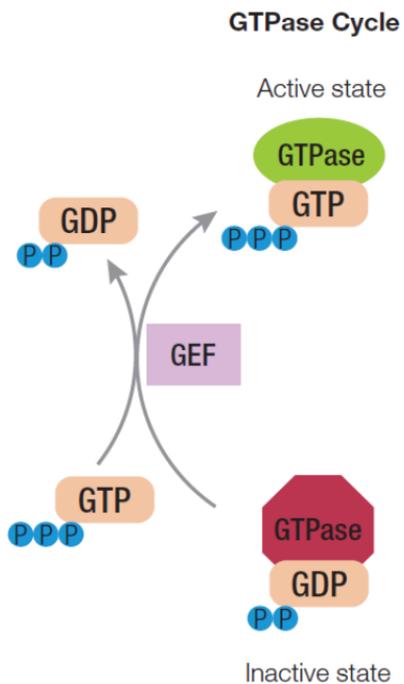
5 mM MgCl₂

20 mM EDTA

EDTA will chelate the Mg²⁺ in the active site and allow faster release of the bound GTP.



Reaction buffers: GEF Reaction Buffer



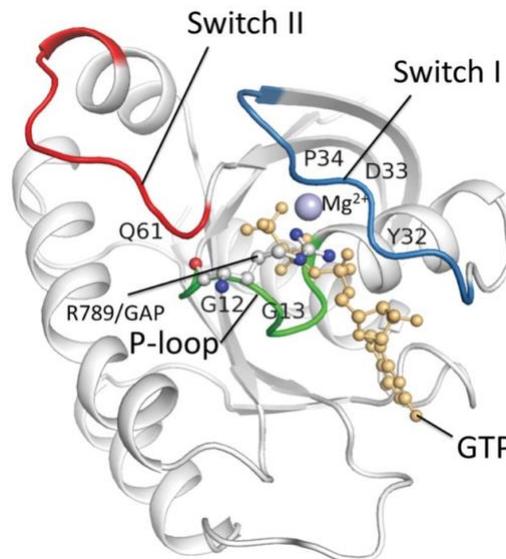
GEF Reaction Buffer

50 mM Tris-HCl, pH 7.5

50 mM NaCl

10 mM MgCl₂

1 mM EDTA



High Mg²⁺ in the buffer will allow nucleotide exchange in the presence of GEF proteins.

GTPase-Glo™ Buffer



GTPase-Glo™ Buffer

50 mM Tris-HCl, pH 7.5

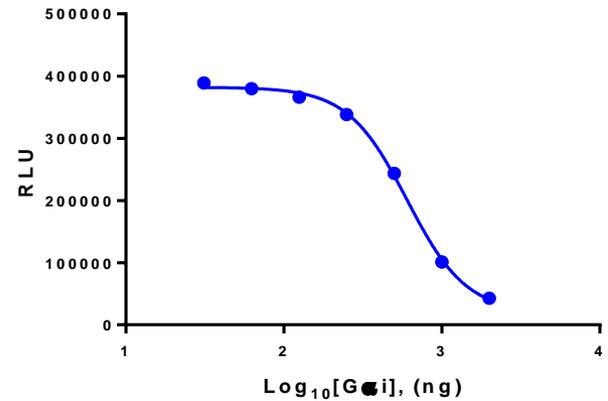
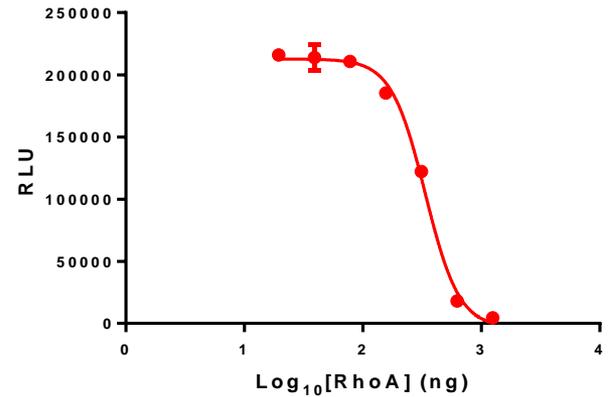
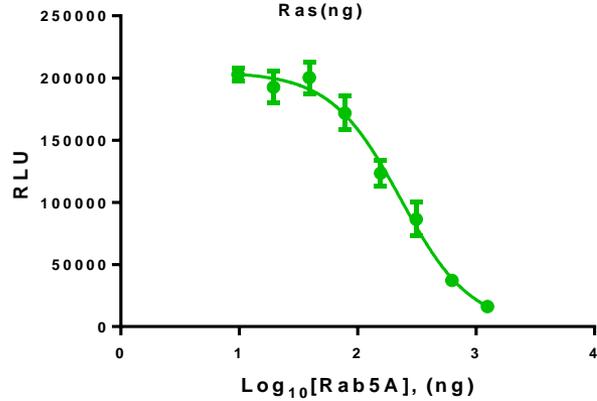
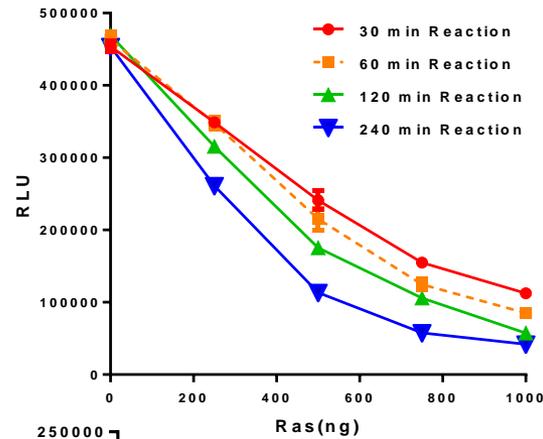
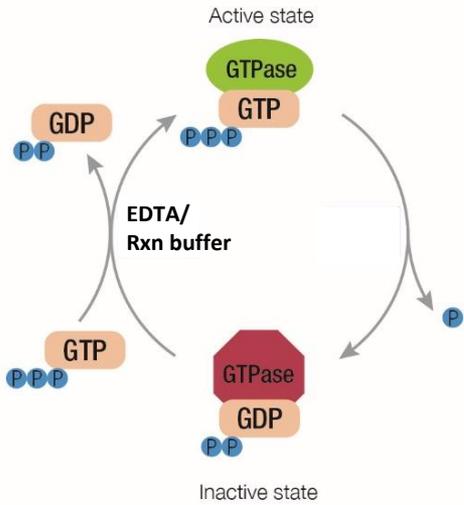
50 mM NaCl

40 mM MgCl₂

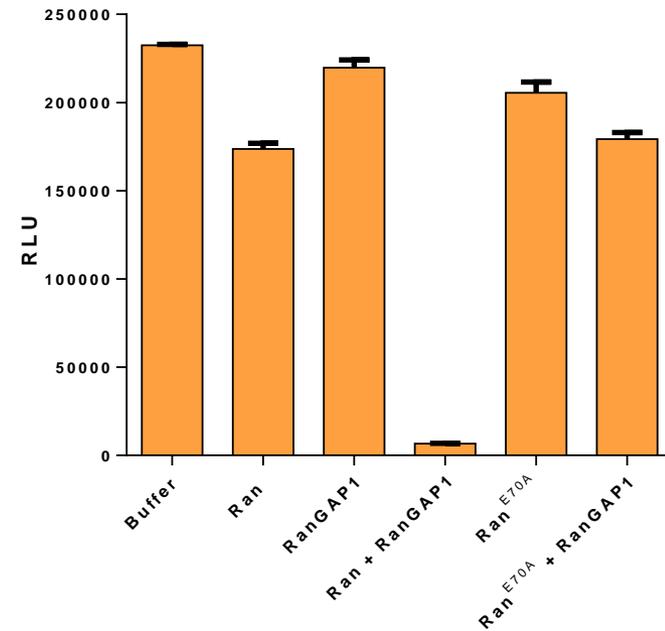
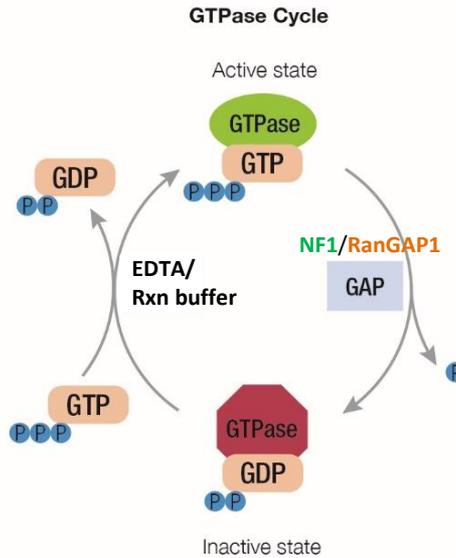
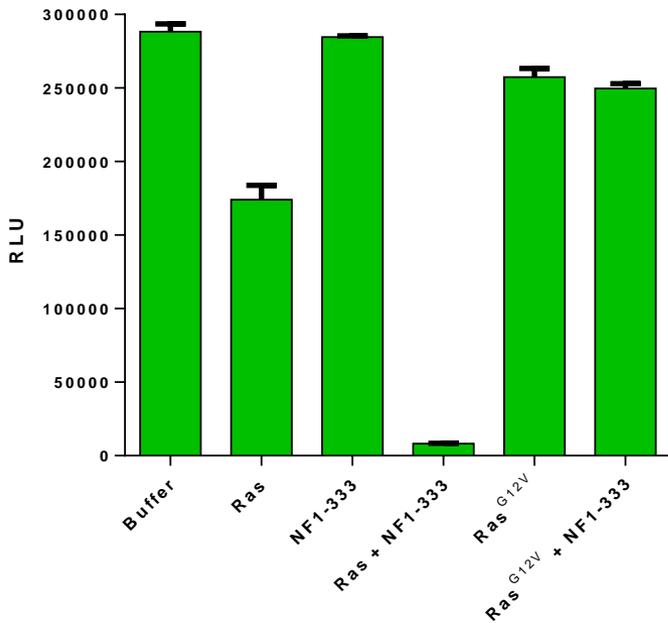
High Mg²⁺ in the GTPase-Glo™ Buffer converts a continuous reaction to a single turnover reaction, effectively stopping the GTPase cycle.



Intrinsic GTPase activity



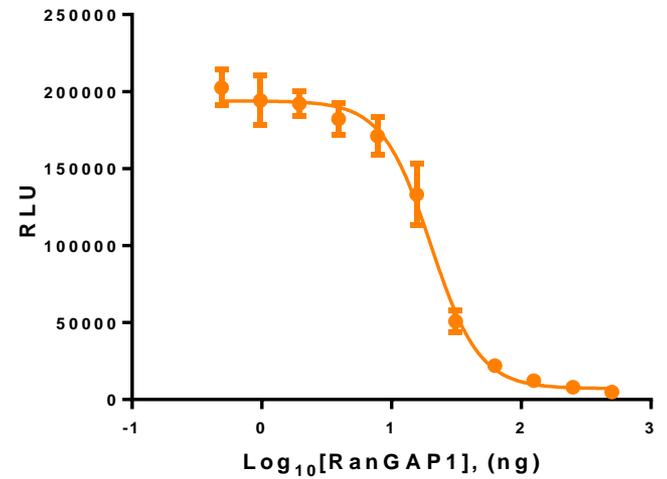
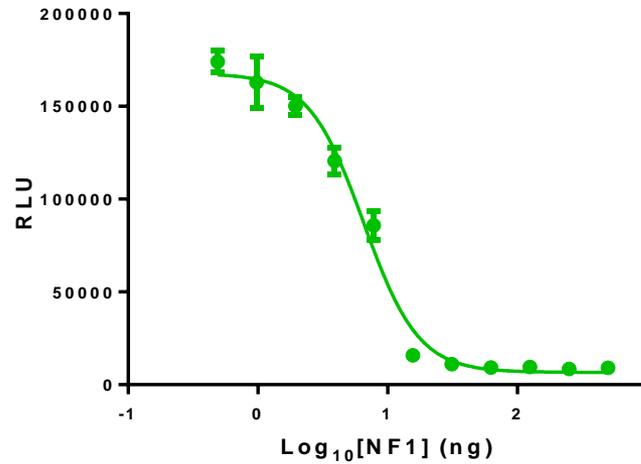
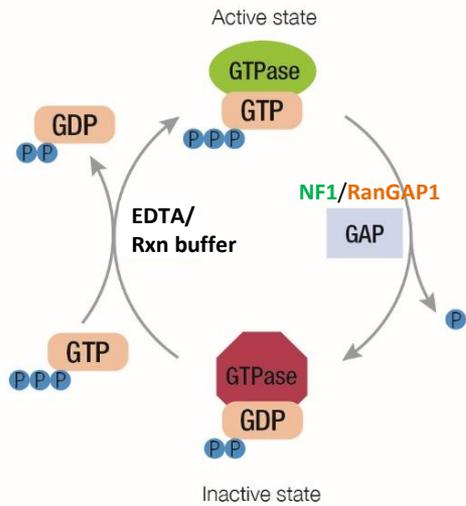
GAP-stimulated GTPase activity



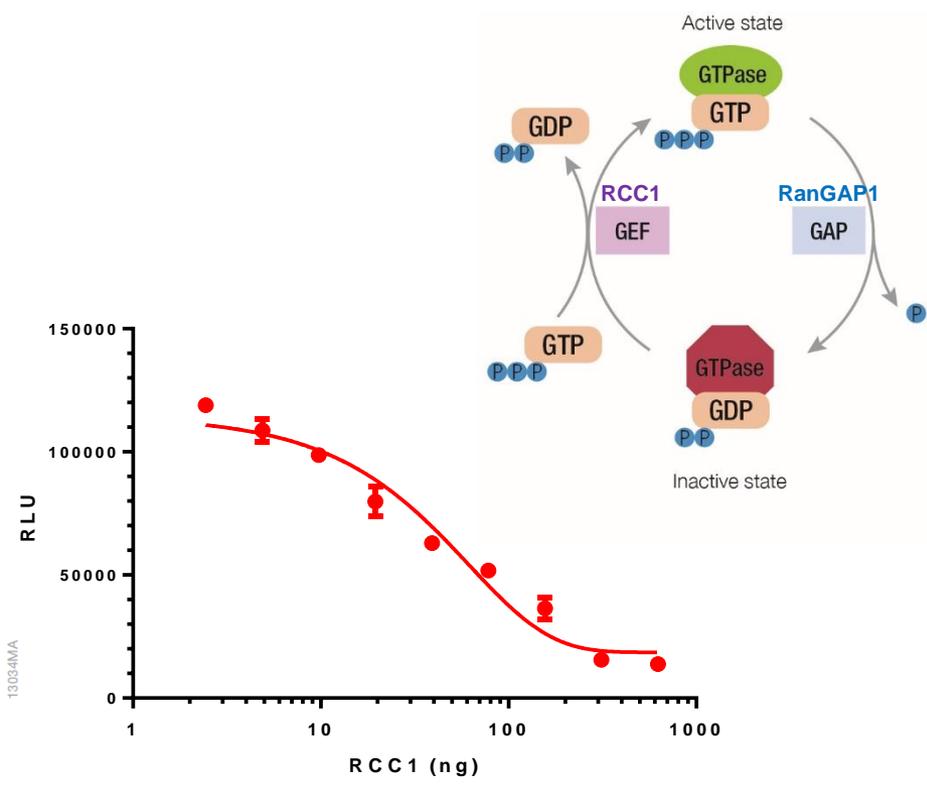
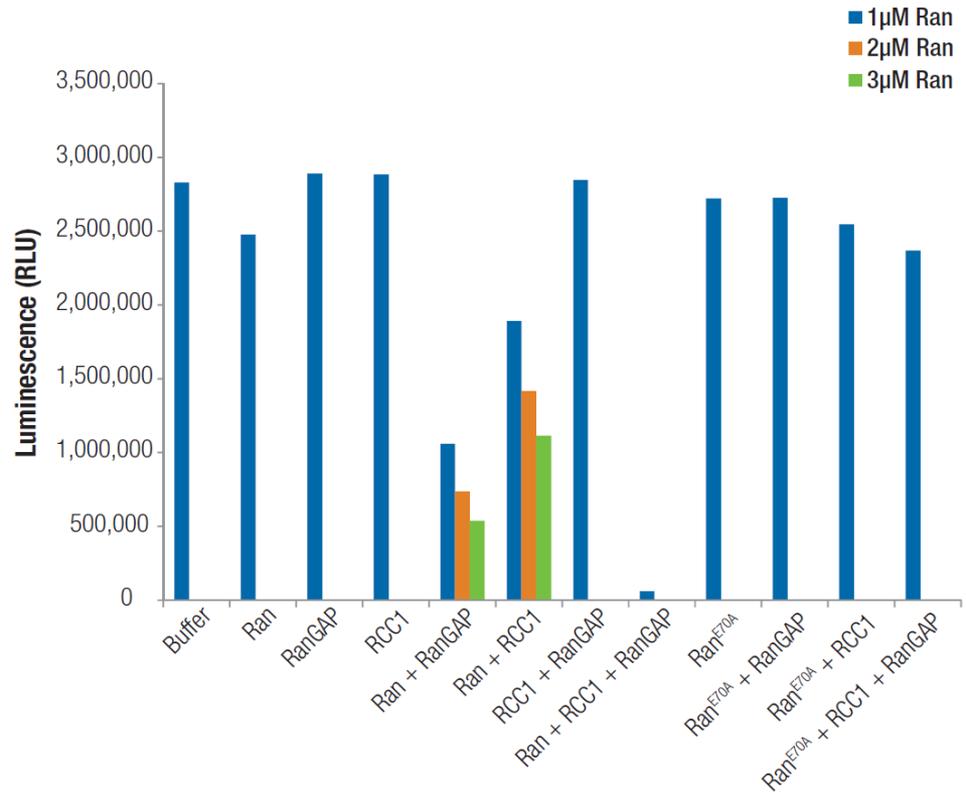
GAP-stimulated GTPase activity



GTPase Cycle



GEF activity



Optimizing the GTPase-Glo™ Assay

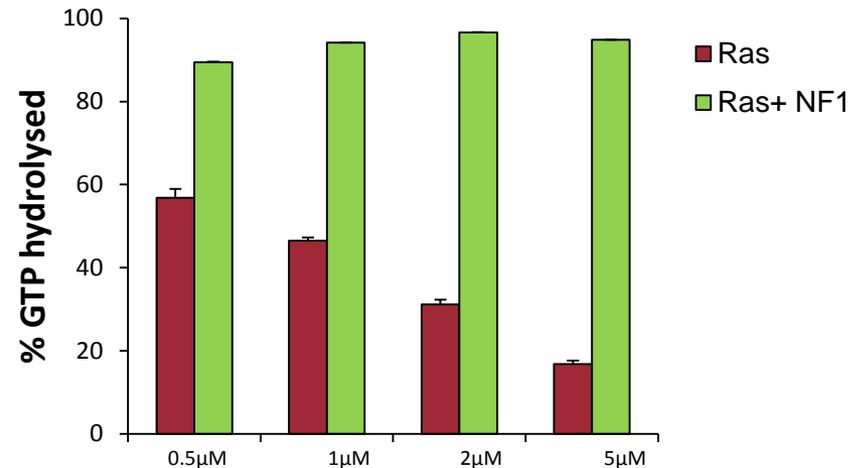


Problem: Low signal to background (S/B) ratio

GTP concentration in the reaction

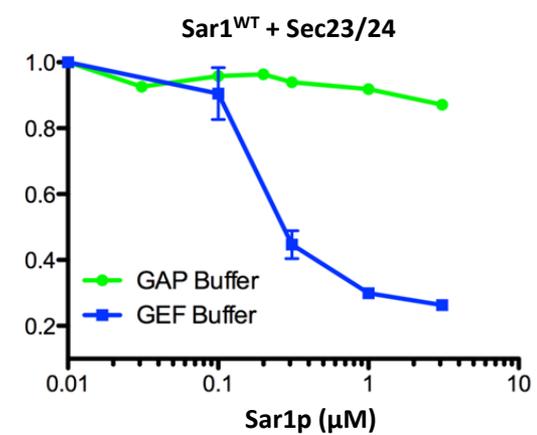
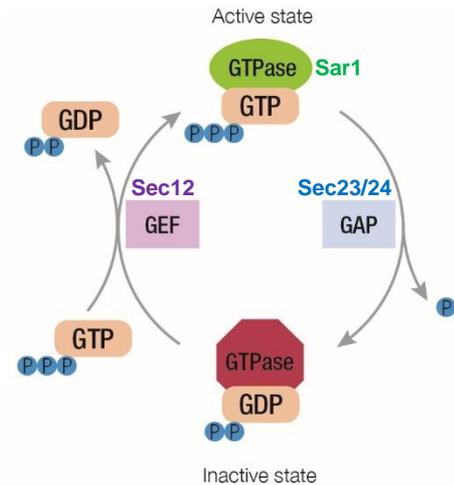
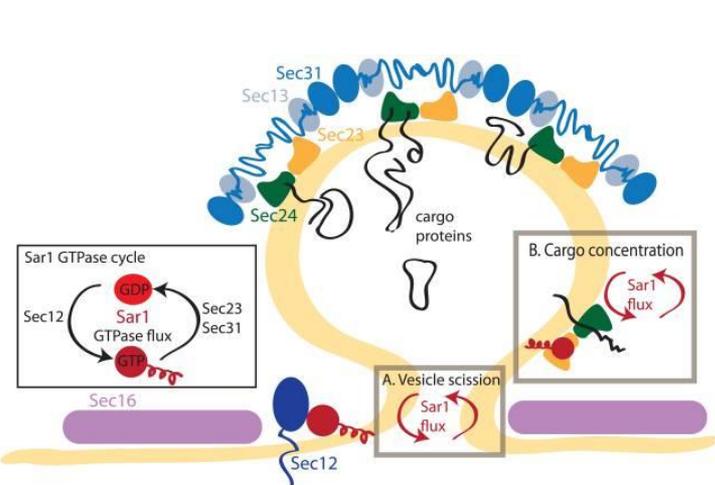
Intrinsic GTPase or GAP-mediated GTPase reactions were performed with different GTP concentrations.

- For GAP-mediated GTPase activity, changing GTP concentrations does not significantly improve percent of GTP hydrolysis.
- For intrinsic GTPase reaction, a lower amount of GTP in the reaction had a higher percent of GTP hydrolysis.



Optimizing GTP concentration in the reaction may yield better results (higher S/B ratios, larger dynamic range).

When buffers don't work as expected !!

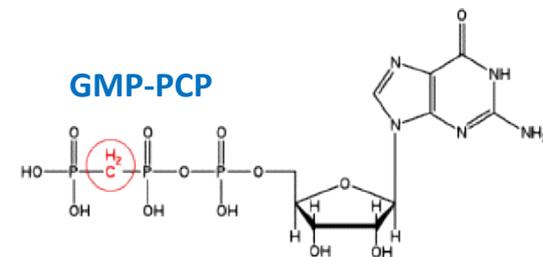
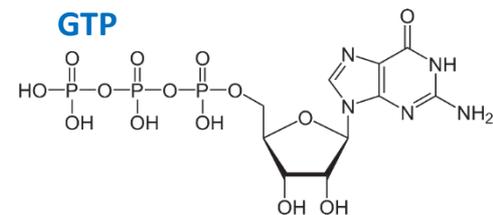
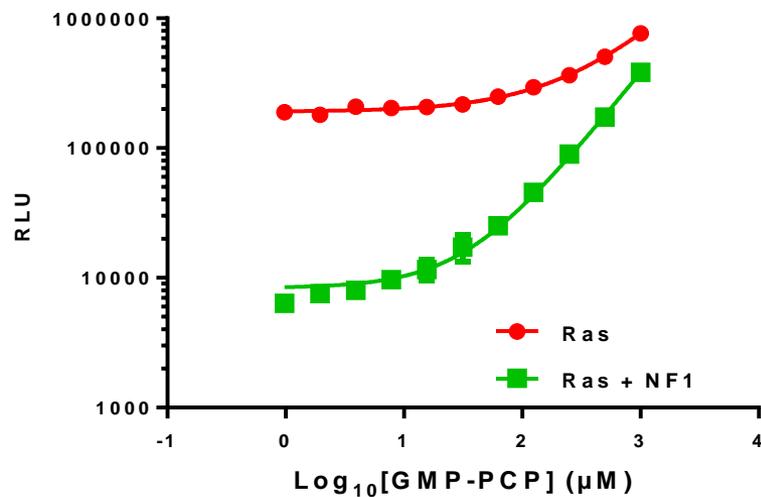


Arf and Sar family GTPases have different Mg²⁺ requirements for GTPase and GAP activities.

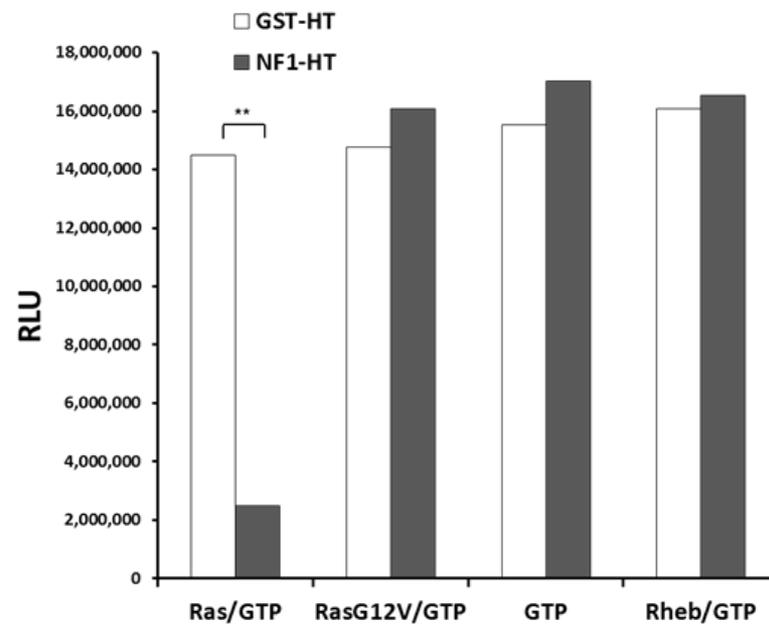
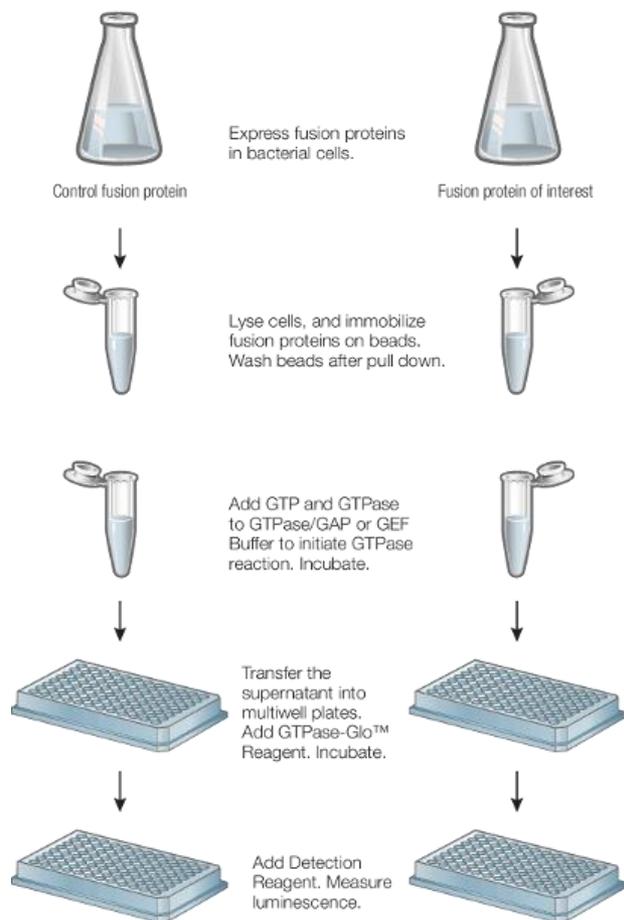
Sar GTPase reactions do not work in GAP Buffer (low Mg²⁺), the high Mg²⁺ containing GEF Buffer allows the reaction.

Miller EA, Barlowe C. Regulation of coat assembly--sorting things out at the ER. *Curr Opin Cell Biol.* 2010 Aug;22(4):447-53.
 Hanna MG 4th et al. Sar1 GTPase activity is regulated by membrane curvature. *J Biol Chem.* 2016 Jan 15;291(3):1014-27.

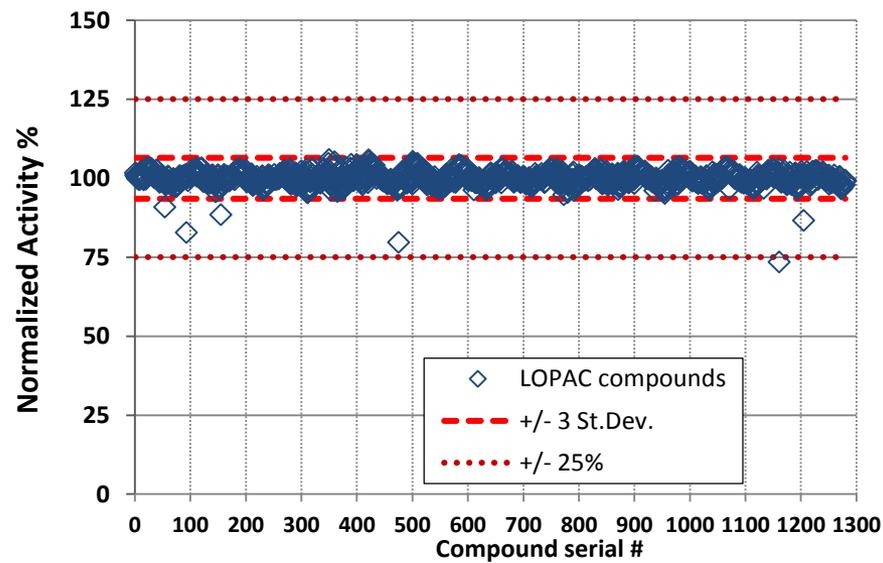
Inhibition of GTPase reaction by a competitive inhibitor



Biochemical GTPase assay using immobilized proteins



Robustness of the GTPase-Glo™ Assay



% CV = 2.2%

Z' = 0.931

GTPase Glo™ Assay is robust and is not affected by chemical inhibitors.

Key Features of the GTPase-Glo™ Assay

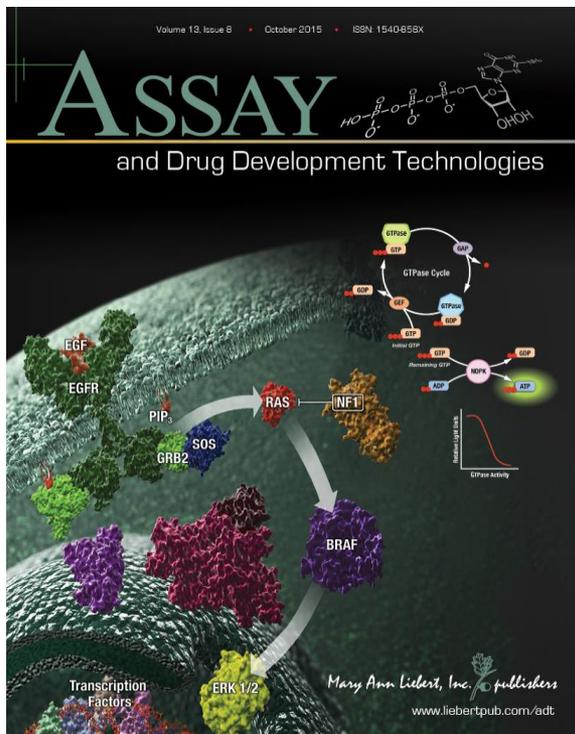


GTPase-Glo™ Assay:

- Convenient “add-mix-read” assay format.
- Analyzes **biochemical activities** of GTPase, GAPs and GEFs.
- Bioluminescent signal inversely proportional to GTPase activity.
- Large dynamic range.
- Requires low amounts of proteins.
- No pre-processing of purified proteins.
- Non-radioactive.

Disadvantages of current GTPase assays:

1. Fluorescent assays with labelled GTPases require pre-processing of GTPases.
2. Require large quantities of proteins.
3. Low dynamic range.
4. Large fluorescent GTP analogs have aberrant biochemical properties.



ORIGINAL ARTICLES

A Homogenous Bioluminescent System for Measuring GTPase, GTPase Activating Protein, and Guanine Nucleotide Exchange Factor Activities

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ABSTRACT

GTPases play a major role in various cellular functions such as cell signaling, cell proliferation, cell differentiation, cytoskeleton modulation, and cell motility. Deregulation or mutation of these proteins has considerable consequences resulting in multiple pathological conditions. Targeting GTPases and its regulators has

INTRODUCTION

Small GTPases are typically 20–25 kDa in size that shuttle between an active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations. The founding member of the small GTPase super family is the Ras GTPase, which is mutated in about 15% of all human tumors. The Ras superfamily GTPases comprise 154 members divided into five subfamilies: Ras, Rho, Rab, Arf, and Ran, and they control diverse cellular functions. The Ras family GTPases mediate signals emanating from cell surface receptors and culminating in transcription, cellular differentiation, and proliferation. The Rho family GTPases regulate cell shape and cytoskeletal processes like cell division and cell migration. Rab and Arf GTPases regulate ves-

Thank you!



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