*5) I find it very interesting that a cycling between two forms of a switch is necessary rather than one or the other conformation. A similar effect has been observed in a cis-trans molecular switch in the circadian clock (Partch and coworkers, Mol. Cell, 2017, 66:447). Perhaps the authors can comment on a possible mechanism of how cycle dynamics, rather end populations can impact downstream biological processes?*

We agree with the reviewer that this is an interesting point. We believe that this point is beautifully presented in an essay we cite in the paper (Rush & D’Eustachio, BioEssays, 1996). The paper distinguishes “two distinct coupling mechanisms” of how a GTPase switch can regulate cellular processes (to which we, based on our systems level data, add a third one).

1.) A “Ras paradigm” where the cellular function is defined by a gradient of “active” GTP-bound GTPase. It is a system in which only the GTP-bound GTPase binds the “effectors”. In Ras signaling, where the GEF is localized to the membrane the levels of GTP-bound Ras are highest at the membrane and dilute towards the middle of the cell. The same mechanism of active state gradient, where Gsp1:GTP gradient radiates from chromatin (where GEF is localized), diluting towards the periphery of the cell/nucleus, guiding spindle assembly early in the cell cycle.

2.) For systems where a GTPase drives unidirectional transport across organelles, the difference in the GTP and GDP conformations between compartments is not gradual, as it involves regulated transport across a barrier (e.g. nuclear pore), which is mediated by effector that bind the GDP-bound and the GTP-bound GTPase, respectively. By this “Rab paradigm” of GTPase function, both the GDP and the GTP-bound conformations are recognized by effectors.

In brief, nuclear transport is driven by a following mechanism: After karyopherins have facilitated import of cargo protein into the nucleus, GTP-bound Gsp1 binds nuclear karyopherins and recycles them back to the cytoplasm where Gsp1:GTP is hydrolysed with the help of the GAP (Rna1), and the karyopherins are released, free to bind the next protein cargo bearing nuclear localization signal. The GDP-bound Gsp1 is then bound to Ntf2, which recycles Gsp1 back to the nucleus, where Gsp1 is converted to the GTP-bound conformation by the GEF (Srm1).

From this, it is clear that nuclear transport is coupled to the entire cycle of the GTPase, as it depends on the turnover between the two states.

Rather than “cycle dynamics”, we believe the word “turnover” describes this point better and we now use turnover in our Discussion section.

*6) While reading the manuscript, I felt that the work was not sufficiently placed in perspective of what is known about the mode of operation of other molecular switches. A short paragraph on this may help the manuscript.*

Ok add that. Not sure how to interpret this request? TO DO!!!!

Introduction text from the manuscript:

Proteins perform their cellular functions through interactions with many partners{Eisenberg, 2000, r04250;Mellis, 2015, r04971}, organized into functional signaling networks built of elementary cycles, such as GTPases or kinase/phosphatase pairs. The behavior of those switches is defined by their ultrasensitive response to regulation{Ferrell Jr, 2014, r05860; Ferrell, 2014, r05859; Ferrell, 2014, r05521}, however the fundamental question of the functional specificity of switches still remains: How can switches individually control different functions with the required precision and accuracy, when distinct cellular processes are interconnected and common regulators are often shared? Moreover, in highly interconnected networks even a small perturbation targeting individual interactions, introduced by posttranslational modifications, point mutations, or drug binding, could be magnified through the network and have widespread cellular consequences. Protein mutations in disease are enriched in protein-protein interfaces{Buljan, 2018, r04747;Schuster-Böckler, 2008, r01544}, but it is unclear whether the consequences of these mutations can be explained primarily by their effects on individual interactions. Similarly, drug compounds are typically designed against specific targets but could affect cellular functions more broadly. Determining the extent and mechanism by which molecular perturbations affect interconnected biological processes requires an approach that quantifies effects on both the cellular network and on the molecular functions of the targeted protein (**Fig. 1a**).

To develop such an approach, we targeted a central molecular switch, a GTPase. GTPases belong to a class of common biological motifs, where a two-state switch is controlled by regulators with opposing functions{Goldbeter, 1981, r05525;Pincus, 2008, r05733} (**Fig. 1a**). Add here other types of switches. For GTPases, the two states of the switch are defined by the conformation of the GTPase in either the GTP- or GDP-bound forms, and the interconversion between the two states is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (**Fig. 1b**). Switch motifs are often multi-specific, defined here as regulating several different processes{Dasso, 2002, r05390}. This multi-specificity raises the question of how a single switch motif differentially controls diverse processes at the cellular level.

In this study, we sought to uncover the mechanistic basis of functional multi-specificity in the small GTPase Gsp1 (the *S. cerevisiae* homolog of human Ran, which shares 83% amino acid identity with Gsp1), which is a single molecular switch with one main GEF and one main GAP{Bischoff, 2001, r05330}. Gsp1 regulates nucleocytoplasmic transport of proteins{Moore, 1993, r05366;Stewart, 2007, r02551} and RNA{Köhler, 2007, r05362;Delaleau, 2015, r05123}, cell cycle progression{Arnaoutov, 2003, r05389}, RNA processing{Ren, 1995, r05367} and nuclear envelope assembly{Hetzer, 2000, r05424}. Gsp1/Ran forms direct physical interactions with a large number of partners, and high-resolution crystal structures of Gsp1/Ran in complex with 16 different binding partners are known (**Extended Data Fig. 1**, **Supplementary File 1 Table 1**). We reasoned that by placing defined point mutations in Gsp1 interfaces with these partners, we could differentially perturb subsets of biological processes regulated by Gsp1. We then determined the functional consequences of these Gsp1 mutations on diverse biological processes in *S. cerevisiae* using quantitative genetic interaction mapping, measured changes to the physical interaction network using affinity purification mass spectrometry (AP-MS), and finally quantified molecular changes on the Gsp1 switch motif using biophysical studies *in vitro* (**Fig. 1 a, b**).

Discussion text from the manuscript:

Only five years after the discovery of the small GTPase RAN, Rush et al.{Rush, 1996, r05005} proposed that RAN must act by two different mechanisms: one in which the *cycling* of the GTPase is most important (‘Rab paradigm’), and the other in which the *amount* of “active” RAN:GTP is most important (‘Ras paradigm’). Our findings lead to a model where RAN/Gsp1 acts by *three* different paradigms of coupling the GTPase switch to cellular processes, defined by the ability of the switch to: (i) *turnover*, (ii) *turn off* by hydrolyzing GTP, and (iii) *activate* by producing Gsp1:GTP.

Other effects such as direct perturbations of interactions, binding partner competition, and changes in expression of Gsp1 or its partners also undoubtedly play a role in modulating the genetic interaction profile of our Gsp1 mutants. Nevertheless, our model explains to a remarkable degree how a single molecular switch motif can differentially control subsets of biological processes by using one of the three functional modes. Furthermore, our model is in agreement with previous studies of canonical GTPase mutants in RAN, in which mutants defective in hydrolysis, hyperactivated independently of GEF activity, or defective in nucleotide exchange were exogenously expressed or injected into cells and their effects on specific cellular processes were observed. For example, a mutant defective in GTP hydrolysis abrogated Mad1 turnover during spindle assembly checkpoint (Scott, 2009), and a mutant with impaired nucleotide exchange disrupted actin spindle assembly (Kalab, 1999). Due to the widespread allostery observed in Gsp1, precise design of novel mutations to perturb individual Gsp1 functions remains a significant challenge, but our work provides a set of viable mutants with a range of effects on the GTPase cycle that can be used to study further molecular details of the cellular functions of Gsp1.

The discovery of several allosteric sites (positions 34, 141, 147, and 157) in the model molecular switch Gsp1 both explains the widespread functional consequences we observe for single amino acid point mutations at interaction surfaces of Gsp1 and has important implications for revising our understanding of GTPase switch regulation. We show that mutations in distal interfaces allosterically modulate the switch cycle. This finding demonstrates thermodynamic coupling between interfaces and the classical switch region in the active site and thereby suggests that partners binding to distal sites also regulate the switch by affecting conformational equilibria at the active site. This hypothesis is supported by evidence that the Yrb1 homolog RanBP1 modulates GAP activity{Bischoff, 1995, r04759;Geyer, 1999, r04729;Seewald, 2002, r04903}. Our data provide a mechanistic explanation, where mutations at allosteric sites, including Thr34 in the Yrb1 binding interface, tune the population of Gsp1 in a hydrolytically-primed conformation. Since the overall switch mechanism is conserved across the small GTPase fold, we propose that thermodynamic coupling between distal interfaces and functional conformational changes may be a more general mechanism to regulate other GTPase switches, and may aid in the development of allosteric inhibitors.

Our observation of widespread functional effects induced by relatively small mutational perturbations is reminiscent of the ultrasensitivity achievable in biological motifs with opposing regulators{Goldbeter, 1981, r05525}. While switch-like ultrasensitivity is typically described for systems controlled by covalent modifications (such as phosphorylation), our results, as well as the observations that cellular levels of small GTPase regulators require tight control{Besray Unal, 2018, r04807;Görlich, 2003, r05565}, corroborate a model of ultrasensitivity for GTPase conformational switches{Barr, 2013, r05519}. While we investigated the changes to the GTPase cycle caused by mutations, similar effects on regulation could be exerted by partner binding or posttranslational modification.

Finally, deriving a model that explains the cellular multi-specificity of GTPases by differential sensitivity of biological processes to distinct parameters of the switch cycle was enabled by a quantitative analysis that integrated functional genomics, proteomics, and biophysics. Given the prevalence of biological two-state switch motifs controlled by opposing regulators (kinase/phosphatase, acetylase/deacetylase){Bashor, 2010, r05332}, we envision this approach to be fruitful for other studies of cellular regulation and to be extended to mammalian systems using CRISPR-based approaches to yield mechanistic insights into the drastic consequences of disease mutations targeting central molecular switches