Change 1 - the biological effects of known Ran mutations and how the effects are consistent with our model (rev 2 on designing more mutants, point 2 by DL)

*1) The main conclusion from this work is a model for Gsp1 function, where different biological processes are sensitive to different aspects of the switching cycle, either cycling, GTP hydrolysis or nucleotide exchange (GDP to GTP). The authors ended up with this model starting with mutations to perturb various Gsp1-protein interaction interfaces. The power of this model is that it can now predict which processes will be affected if various parts of the cycle are tickled. However, this model has not been validated. The authors must now go the reverse direction, rationally design mutations to perturb the three aspects of the switch (cycling, activation and nucleotide exchange) and demonstrate that the model can successfully predict the effects of these mutations on downstream biological processes. Without this validation step, the predictive power of the model remains unclear and the manuscript remains one-sided.*

We agree with the reviewer that it would be desirable to support our model with additional experiments and mutations, ideally even probing all residues of Gsp1. However, there are several considerations that ultimately lead us to conclude that new insights might be limited by simply adding more attempted mutations, and that tests of the model would best be done using additional orthogonal approaches (which, to do rigorously, would require a new study of similar scope to our current manuscript). Below we outline our reasoning which we hope is agreeable:

First, because of the considerable and unexpected allostery we uncovered in Gsp1, it is beyond current structural modeling methods to predict mutations that will differentially perturb the different aspects of the GTPase switching cycle with notable success. It would thus be difficult to *rationally* design mutations that would affect specific ratios of the kinetics of switching and in turn specific functional consequences. We thus do not propose that we can predict the effects of point mutations of Gsp1 on cellular phenotype. We apologize if this was misleading and we have now explicitly stated that in the manuscript (see modified manuscript text below).

There are of course known mutations in GTPases with drastic effects on the switching cycle, such as the Q to L mutation in switch II or G to V mutations in the P-loop that impair intrinsic and GAP-catalyzed hydrolysis; the fast cycling F to A mutation in switch I that decreases nucleotide affinity, increases the intrinsic rate of nucleotide release and bypasses the requirement for activation by GEFs; and the T to N mutation at position 24 in RAN that causes increased binding to RanGEF, inhibiting nucleotide exchange. We have not tested such drastic mutations in our system because they are likely to not be viable when made in the *S. cerevisiae* genomic copy of Gsp1 (we attempted to make some mutations in the P loop, switch I or switch II (Supplementary File 1 Table 3) but none of them were viable). However, effects of these mutations in overexpression / protein injection assays are broadly consistent with our conclusions. For example:

a) The G to V mutant with impaired GTP hydrolysis abrogates the turnover of Mad1 during spindle assembly checkpoint (Scott, 2009), agreeing with our observation that OFF switch mutants correlate with spindle assembly regulation genes (Fig. 4).

b) The T24N mutant which inhibits nucleotide exchange, disrupts spindle assembly in Xenopus oocyte extracts (Kalab et al, 1999), agreeing with our observation that the ON switch mutants correlate with actin and polarity related genes (Extended Data Fig. 10)

In response to the above points, we modified the Discussion (Page XXX):

Our findings lead to a model where Ran/Gsp1 acts by *three* different paradigms that are defined by the sensitivity of different biological processes to perturbations of different characteristics of the Gsp1 GTPase cycle, i.e. the ability to (i) *cycle*, (ii) *turn off* by hydrolyzing to Gsp1:GDP, 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 phenotype of our Gsp1 mutations. 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, precisely designing 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.

References:

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Change 2 - what is known about conformations in Ras when describing the Ran allostery (point 3 by DL)

(ii) Data interpretation. We agree that the 31P data alone do not provide structural detail on the differences between the two states across the entire protein. However, regardless of the extent of conformational change that leads to the observed chemical shift difference of the γ phosphate in the active site, this change in the active site is caused by several mutations that are distal (at least 18 Å away) from the site of the probe, as well as distributed across the protein surface (Fig. 3e,f and Extended Data Fig. 7d). Our data therefore support a widespread allosteric mechanism, as opposed to local changes in the vicinity of the active site.

Although 31P NMR reports only on the chemical environment around the γ phosphate, previous work has structurally characterized the differences between the two γ conformations in more detail. In RANjust as inAS, (Geyer et al, 1999; Seewald, 2002)

Furthermore, in addition to shifting RAN into the γ2 conformation, binding of the RanBP1 effector (homolog of Yrb1) to RAN increases the *Kd* and *kon* of GAP binding to Ran-GPPNP (a close analog of GTP) but does not affect the rate-limiting step of GAP-mediated hydrolysis (the hydrolysis and subsequent release of inorganic phosphate).

Taken together, these studies suggest that RanBP1 accomplishes its activation of GAP-mediated hydrolysis by contributing binding energy towards distorting RAN to γ2 state, and that this distortion is achieved through binding at a site distal from the active site loops and the GAP interface. Given that the T34 position is in the Gsp1 interface with Yrb1 (the yeast RanBP1 homolog), we propose that the T34 mutants that primarily populate γ state 1 (T34E/Q/A/L) increase the energetic cost that GAP binding must overcome to distort Gsp1-GTP to γ state 2. This is a similar but opposite effect to RanBP1 binding, which decreases the cost that GAP binding must overcome, and is associated with decreased (tightened) *Kd* and *Km* values (Seewald et al 2003).

We have updated the text in a way we hope more clearly puts our data in the context of prior data on shifts in active site conformations.

*CHANGES TO THE MAIN TEXT:*

To probe the mechanism of these allosteric effects, we examined the impact of Gsp1 point mutations on the conformational distribution in the active site of GTP-bound Gsp1 using 1D 31P nuclear magnetic resonance (NMR) spectroscopy. Prior 31P NMR data on human RAN{Geyer, 1999 #139} and RAS{Geyer, 1996 #118} showed two distinct peaks for the γ-phosphate of bound GTP arising from differences in the local chemical environment of the γ-phosphate in each of two distinct conformations (termed γ1 and γ2) (Extended Data Fig. 7a). This work also showed that the ratio of γ1 and γ2 active state conformations can be tuned by mutations close to the active site, as well as by effector binding. Our 31P NMR spectra of *S. cerevisiae* wild-type Gsp1:GTP showed two distinct peaks for the γ-phosphate of bound GTP with 87% of wild-type Gsp1:GTP in the γ2 state conformation. Strikingly, the populations of the γ2 state in Gsp1 interface mutants ranged from close to 0% for T34E and T34Q, to close to 100% for H141R, Y157A, and K132H (Fig. 3c).

Furthermore, we observed a striking linear relationship between the relative log-transformed ratio of the two γ conformations and the log-transformed relative catalytic efficiency of GAP-mediated GTP hydrolysis (Fig. 3d) and intrinsic GTP hydrolysis (Supplementary File 1 Table 8, Extended Data Fig. 7b, c). From this relationship we can infer that the γ2 state represents the active site conformation of Gsp1:GTP that is more compatible with GAP-mediated GTP hydrolysis, compared to the γ1 state. Remarkably, the mutated residues that tune the population of the γ2 state (T34, H141, Q147, and Y157) are all distal, affecting the chemical environment of the Gsp1-bound GTP γ phosphate from at least 18 Å away (Fig. 3e, f). Furthermore, neither of these sites overlap with the allosteric inhibitor pockets successfully targeted by small molecule inhibitors in RAS{Canon, 2019, r05670;Kessler, 2019, r05463;Ostrem, 2013, r05039} (Extended Data Fig. 7d). Exceptions to the linear relationship are the K132H mutation, which is in the core of the GAP interface and is hence expected to directly affect the interaction with the GAP, and the D79S and R78K mutations, which are on the edge of the GTPase switch II region (from residues 69 to 77) and could lead to different perturbations of the nucleotide binding site geometry.

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