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Cross-talk between carbon metabolism and the DNA damage response in *S. cerevisiae*

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Abstract

Yeast cells with DNA damage avoid respiration, presumably because products of oxidative metabolism can be harmful to DNA. We show that DNA damage inhibits the activity of the Snf1 (AMP-activated) protein kinase (AMPK), which activates expression of genes required for respiration. Glucose and DNA damage upregulate SUMOylation of Snf1, catalyzed by the SUMO E3-ligase Mms21, which inhibits SNF1 activity. The DNA damage checkpoint kinases Mec1/ATR and Tel1/ATM, as well as the nutrient sensing protein kinase A (PKA), regulate Mms21 activity towards Snf1. Mec1 and Tel1 are required for two SNF1-regulated processes—glucose sensing and *ADH2* gene expression—even without exogenous genotoxic stress. Our results imply that inhibition of Snf1 by SUMOylation is a mechanism by which cells lower their respiration in response to DNA damage. This raises the possibility that activation of DNA damage checkpoint mechanisms could contribute to aerobic fermentation (Warburg effect), a hallmark of cancer cells.

Introduction

Sugars have the potential to cause genotoxic stress for cells because their oxidative metabolism can generate reactive molecules that damage DNA. Indeed, yeast cells with DNA damage seem to avoid respiration: studies of the DNA damage response revealed decreased expression of genes involved in respiration and increased expression of genes involved in fermentation (Caba et al., 2005, Fry et al., 2003, Gasch et al., 2001, Jelinsky and Samson, 1999, Lee et al., 2000, Shalem et al., 2008), and one of the effects of DNA damage in yeast is the reduction of respiration (Kitanovic et al., 2009).

S. cerevisiae has three major glucose sensing pathways: the Gpa1/2-Ras2-PKA pathway that also regulates stress response, the Snf3/Rgt2/Rgt1 (Sensor/Receptor-Repressor, or SRR)

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pathway that regulates expression of genes encoding hexose transporters (Busti et al., 2010, Johnston and Kim, 2005) required for fermentation (Gamo et al., 1994), and SNF1, a central regulator of carbon metabolism in *S. cerevisiae* (Ghillebert et al., 2011, Hedbacker et al., 2004) that is the orthologue of the AMP-activated protein kinase (AMPK) of mammalian cells (Hardie et al., 2012, Jiang and Carlson, 1997). SNF1 protein kinase is a heterotrimer composed of the Snf1 catalytic subunit, the Snf4 regulatory subunit, and one of three localizing subunits (Sip1, Sip2 and Gal83). SNF1 stimulates expression of genes involved in respiration, the diauxic shift, ethanol and lactate catabolism, and gluconeogenesis by activating transcriptional activators such as Adr2, the activator of *ADH2* (which encodes alcohol dehydrogenase) (Bojunga and Entian, 1999, Hardie et al., 1998, Tachibana et al., 2005, Walther and Schuller, 2001, Young et al., 2003), and by inhibiting the Mig1 repressor of glucose-repressed genes (Vallier and Carlson, 1994). SNF1 also inhibits glucose sensing *via* the SRR pathway (Gadura et al., 2006, Pasula et al., 2007), which effects degradation of the Mth1 co-repressor of *HXT* genes encoding glucose transporters. Thus, active SNF1 increases Mth1 levels and thereby reduces *HXT* expression, and increases Adr1 function, thereby inducing *ADH2* expression.

SNF1 is active in glucose-starved yeast cells, in which the Snf1 catalytic subunit is phosphorylated on its activation loop threonine 210 (Elbing et al., 2006, Hedbacker et al., 2004, Hong et al., 2003). Addition of glucose to cells results in a reduction in ADP levels that leads to dephosphorylation of T210 by the Glc7-Reg1 protein phosphatase, thereby inactivating SNF1 (Ludin et al., 1998, Chandrashekarappa et al., 2011, Mayer et al., 2011). The Sit4 and Ptc1 phosphatases also have a role in dephosphorylation of this site (Ruiz et al., 2011, Ruiz et al., 2013). In addition, SNF1 is inhibited by its modification with the Small Ubiquitin-like Modifier protein SUMO, catalyzed by the SUMO E3-ligase Mms21 in response to glucose, which leads to ubiquitinylation and degradation of Snf1 (Simpson-Lavy and Johnston, 2013).

Methyl methanesulfonate (MMS), a DNA-alkylating agent that causes methylation of deoxyguanine and deoxyadenine, activates DNA damage repair pathways and causes cell cycle arrest (Evensen and Seeberg, 1982). The Mec1 protein kinase (orthologue of human ATR), and its homologue Tel1 (orthologue of human ATM), respond to DNA stress by catalyzing phosphorylation of Rad53 (Friedel et al., 2009, Paciotti et al., 2001, Pellicioli and Foiani, 2005) and other proteins involved in the response to genotoxic damage (Morrison et al., 2007, Mallory et al., 2003). Inhibition of SNF1 is involved in the response of cells to MMS, and is required for a concomitant switch in metabolism from respiration to fermentation (Kitanovic et al., 2009), but the basis of that inhibition is unknown. Here, we provide evidence that DNA damage inhibits SNF1 function by inducing its SUMOylation.

Although many targets of SUMO E3-ligases have been identified, the regulation of the ligases themselves remains little understood. To date, the only known phospho-regulation of a SUMO E3-ligase is the regulation of Siz1 stability by the Cdc28 protein kinase via an unidentified mechanism (Takahashi and Kikuchi, 2005). Here we present evidence that suggests that the SUMO (E3) ligase Mms21 is regulated by phosphorylation due to Mec1, Tel1 and PKA activity.

Results

Mms21 is a phosphorylated protein

One of the ways glucose inhibits Snf1 activity is by promoting its SUMOylation, catalyzed by the Mms21 SUMO (E3) ligase (Simpson-Lavy and Johnston, 2013). We observed an electrophoretic band-shift of Mms21-3HA that is reversed by λ-phosphatase treatment in cells exposed to either glucose or MMS (**Figure 1a**). Overexpression of the *PDE2* phosphodiesterase (Sass et al., 1986) reduced the amount of this glucose-induced phosphorylation of Mms21 (**Figure 1b**), suggesting a role for PKA in Mms21 phosphorylation. MMS treatment also results in phosphorylation of Mms21, but this is not inhibited by *PDE2* overexpression (**Figure 1c**). Expression of activated $Ras2^{G19V}$, which increases PKA activity (Tatchell, 1986) by stimulating adenyl cyclase (Cyr1) (Uno et al., 1985, Uno et al., 1987), increases basal phosphorylation of Mms21, and the level of phosphorylated Mms21 is increased further by MMS treatment (**Figure 1c**). Thus, both MMS and glucose (via PKA) treatments result in phosphorylation of Mms21.

To further characterize this phosphorylation we immunoprecipitated Mms21-3HA from cells treated with glucose or MMS, and probed with an antibody that recognizes phosphorylated serine in the motif RxxS (PKA's consensus site). Phosphorylation of this motif was increased about 2-fold in response to both glucose and MMS (**Figure 1d**). Expression of $Ras2^{G19V}$ increased basal phosphorylation of this sequence, whereas overexpression of *PDE2* attenuated its phosphorylation following addition of glucose (**Figure 1e**). Mms21 has four RxxS sequences, three in the N-terminal, Smc5-interacting domain, and one in the C-terminal SUMO ligase domain. Examination of the crystal structure of Mms21 bound to Smc5 (Duan et al., 2009) indicates that the former three are either buried or interacting with Smc5, but the site in the SUMO E3 ligase domain is exposed; it is also a more stringent PKA consensus, containing a double basic motif.

Phenotypes of Mms21 phospho-mutants

MMS activates the Mec1/Tel1 protein kinases (Flott et al., 2007, Gasch et al., 2001, Kato and Ogawa, 1994), which phosphorylate their substrates at SQ/TQ sequences (Mallory and Petes, 2000). A cluster of SQ sequence motifs in its C-terminal SUMO E3-ligase domain (residues 227-228, 253-254, 261-262) makes Mms21 a good candidate for a Mec1/Tel1 substrate (Cheung et al., 2012).

We examined the effect of single potential phospho-site mutations in *MMS21*. Mutation of a single SQ to AQ does not result in sensitivity to hydroxyurea or MMS (**Figure 2a**), nor in temperature sensitivity (**Figure 2b**), which are phenotypes of *mms21* mutants (Zhao and Blobel, 2005) (Santa Maria et al., 2007). However, $mms21^{S261A}$ cells grow considerably better than wild-type under strong respiratory conditions (**Figure 2c**), which suggests this serine may regulate Snf1 mediated processes. Mutation of one of these SQ motifs—S261—but not the other two, to AQ, reduces *HXT3* expression (**Figure 2d**) and increases *ADH2* expression (**Figure 2e**) to the same extent as the *mms21-11* mutation; mutation of S261 to the phosphomimicking aspartate has the opposite effect (**Figures 2d, e**). This is consistent with the idea that S261 mediates regulation of Snf1.

We changed to alanine residues in the vicinity of Serine 261 to better characterize the sequence required for its phosphorylation, using Mth1 degradation, and *HXT3* and *ADH2* expression as reporters of SNF1 function. Mutation of serine 261 to alanine results in an increase in *ADH2* expression (**Figures 3a, b**) and a decrease in *HXT3* expression (**Figures 3c, d**) to an extent similar to that caused by the *mms21*-11 mutation and the non-SUMOylatable $Snf1^{K549R}$ mutation, and impedes Mth1 degradation (**Figure 3e**). The phosphomimicking S261D mutation has opposite effects, reducing *ADH2* expression (**Figure 3a**) and increasing *HXT3* expression (**Figure 3c**). $Mms21^{S261D}$ cells exhibit a lower level of Mth1 in the absence of glucose, which is further reduced after exposure to glucose (**Figure 3g**). Mutation of S260A has no effect on *ADH2* or *HXT3* expression (**Figures 3a, c**) or on Mth1 degradation (**Figure 3e**), so the effect of Mms21 phosphorylation on Snf1 functions is specific to S261.

The S261 SQ motif overlaps a potential PKA phosphorylation site (R/K R/K X S*): $_{258}KRSSQ_{262}$. Changing to alanine R258 and K259 of the PKA motif, or Q262 of the Mec1/Tel1 motif, does not affect *ADH2* (**Figure 3a**) or *HXT3* (**Figure 3c**) expression or Mth1 degradation (**Figure 3e, f**). However, cells expressing $Mms21^{R258A,\ K259A,\ Q262A}$ show the same increase in *ADH2* (**Figure 3b**) and decrease in *HXT3* (**Figure 3d**) expression as do *mms21* E3-ligase domain mutants or non-phosphorylatable $Mms21^{S261A}$, and are defective for Mth1 degradation in response to glucose (**Figure 3g**). These results suggest that either PKA or Mec1/Tel1 are capable of phosphorylating S261.

Protein kinases that regulate Mms21

The $S_{261}Q_{262}$ motif of Mms21 is a possible target of the Mec1 and Tel1 protein kinases. Indeed, deletion of both *MEC1* and *TEL1* impairs glucose-induced Mth1 degradation (**Figure 4a**), reduces glucose-induced *HXT3* expression (**Figure 4b**), and enhances *ADH2* expression (**Figure 4c**), all phenotypes expected with loss of Mms21 SUMO E3-ligase activity towards Snf1. Mms21 does not become phosphorylated upon treatment with glucose in *mec1*Δ*tel1*Δ cells (**Figure 4d**). $MMS21^{S261D}$ introduced into *mec1*Δ*tel1*Δ cells restores wild-type Mth1 degradation (**Figure 4e**) and suppresses the enhanced *ADH2* expression of *mec1*Δ*tel1*Δ cells (**Figure 4c**). We conclude that promoting phosphorylation of S261 of Mms21 is the sole requirement of Mec1/Tel1 for Mth1 degradation and *ADH2* expression. [Note that the effects of $Mms21^{S261D}$ on the *mec1*Δ*tel1*Δ phenotypes examined are limited to Snf1 regulation: $Mms21^{S261D}$ does not suppress the sensitivity of *mec1*Δ*tel1*Δ cells to either hydroxyurea, MMS, or elevated temperature (37°C) (**Figure 2a**)].

Protein kinase A (PKA) affects glucose sensing at multiple levels [inhibition of Snf1 (Barrett et al., 2012), Rgt1 (Kim and Johnston, 2006, Roy et al., 2014) and Mth1 (Ma et al., 2014)], and is activated by Mec1 inhibition of the PKA regulatory subunit Bcy1 (Searle et al., 2011). We observed that phosphorylation of Mms21 in response to glucose (but not MMS) is reduced by overexpression of the *PDE2* phosphodiesterase (**Figures 1b, c**) and that phosphorylation of Mms21 on a PKA consensus motif (**RxxS, Figure 1e**) is affected by changes in PKA activity. It appears that increased stimulation of the PKA pathway compensates for lack of *MEC1* and *TEL1* because hyperactive $Ras2^{G19V}$ (McGrath et al.,

1984) restores Mms21 phosphorylation (**Figure 4d**), Mth1 degradation (**Figure 4f**) and *HXT3* expression (**Figure 4g**) in *mec1*Δ*tel1*Δ cells.

MMS treatment reduces *ADH2* expression via Snf1 SUMOylation

Because MMS treatment of *S. cerevisiae* cells reduces transcription of genes involved in respiration and increases transcription of genes involved in fermentation (Caba et al., 2005, Fry et al., 2003, Gasch et al., 2001, Jelinsky and Samson, 1999, Lee et al., 2000, Shalem et al., 2008), we monitored the effect of MMS on the expression of *ADH2*, which is usually induced under respiratory conditions. MMS reduces *ADH2* expression (**Figure 5a)** and increases SUMOylation of Snf1 (**Figure 5b**). The MMS-induced reduction of *ADH2* expression is curtailed to the same extent by deletion of *MEC1* and *TEL1*, or by mutation of *mms21*, or by preventing Snf1 SUMOylation (**Figure 5c**). These results suggest that MMS reduces *ADH2* expression *via* Mec1-Tel1 stimulated Mms21-catalyzed SUMOylation of Snf1. We previously showed that glucose-induced SUMOylation of Snf1 leads to its turnover (Simpson-Lavy and Johnston, 2013), and prolonged treatment of cells with a high dose of MMS likewise reduces Snf1 protein levels (**Figure 5d**).

SUMOylation and dephosphorylation regulate Snf1 function

Cells expressing Mms21^{S261D} have an increased amount of SUMOylated Snf1 in galactose-grown cells (**Figure 6a**). This is consistent with the idea that phosphorylation of S261 of Mms21 activates its SUMO ligase activity. Indeed, the *MMS21*^{S261D} mutation reduces Snf1 activity in galactose grown cells to levels comparable to those in glucose-grown cells, but not if Snf1 cannot be SUMOylated (Snf1^{K549R}) (**Figure 6b**).

What is the relationship of SUMOylation of Snf1 to dephosphorylation of threonine 210? Overexpression of *REG1* increases *HXT3* expression in wild-type cells [because it reduces SNF1 activity (Tu and Carlson, 1995, Ludin et al., 1998)], but it does not suppress the effects of either an *mms21* mutation or a deletion of the SUMO-directed Ubiquitin ligase Slx8 (which targets SUMOylated Snf1 for degradation) **(Figure 6c**). However, increasing Snf1 SUMOylation via the Mms21^{S261D} mutant increases *HXT3* expression, both in the presence and the absence of Reg1 (**Figure 6d**). This suggests that increasing Snf1 inhibition by SUMOylation can counteract Snf1 activation by T210-phosphorylation, but not vice-versa.

Deletion of both *reg1* and *sit4* is lethal to *S. cerevisiae*, even under fermentative conditions. This is due to overactive SNF1, because further deletion of *SNF1* restores viability (Ruiz et al., 2011). Introduction of plasmids bearing *MMS21*^{S261D} and *SNF1*^{WT} into *reg1*Δ*sit4*Δ*snf1* cells [also carrying *ubp8*Δ to reduce deubiquitinylation of Snf1 (Wilson et al., 2011)] resulted in viable cells on glucose media (**Figure 6e**). However, *MMS21*^{S261D} fails to restore viability when SUMOylation of Snf1 is blocked by the Snf1^{K549R} mutation (**Figure 6e**). Thus, inhibition of SNF1 by hyperSUMOylation of Snf1 (caused by the *MMS21*^{S261D} mutation) compensates for lack of Snf1 dephosphorylation. Loss of the *MMS21*^{S261D} plasmid resulted in their inviability, but viability is restored (to the same extent as in cells expressing kinase-dead Snf1^{K84R}) when selection for the Snf1-bearing plasmid is also relaxed (**Figure 6f**). These results are consistent with SUMOylation and dephosphorylation

of Snf1 occurring independently of each other in response to glucose. However, increasing SUMOylation of Snf1 using Mms21^{S261D} seems to be a more potent inhibitor of Snf1 than *REG1* overexpression.

Discussion

Little is known about how the activity of SUMO E3 ligases is regulated. Our results suggest that phosphorylation of serine 261 of Mms21 promoted by the Mec1 and Tel1 protein kinases in response to DNA damage, and also by PKA, induces SUMOylation of Snf1 and thereby inhibits SNF1 function, leading to reduced expression of genes for respiration and to the increased *HXT* gene expression necessary for fermentation (summarized in **Figure 7**). Recently, Carlborg et al published an investigation of Mms21 regulation under both ambient conditions and after DNA damage (Carlborg et al., 2015). In accordance with the results presented here, they report that Mms21 is phosphorylated on both S260 and S261. Indeed, we find no basal phosphorylation of Mms21 in *mec1*Δ*tel1*Δ cells (growing on galactose) (**Figure 4d**). Their report of increased phosphorylation of S261 following MMS treated is also in agreement with the results of our experiments. Our results, however, do not support a role for S261 phosphorylation in DNA repair, as *mms21-11* cells are clearly sensitive to MMS whereas all of the single SQ-AQ mutants exhibit WT growth (**Figure 2a**). Phosphorylation of Serine261 of Mms21 appears to be specific for SNF1 regulation because changing S261 to A does not affect the sensitivity of cells to the genotoxins hydroxyurea or MMS, or to elevated temperature, nor does Mms21^{S261D} suppress other *mec1*Δ*tel1*Δ phenotypes. Moreover, growth of cells expressing *mms21*^{S261A} on ethanol or glycerol is improved compared to wild-type cells; this correlates to increased SNF1 activity in these cells. Since sensitivity to hydroxyurea or MMS or to elevated temperature was not observed for Mms21^{S261A} cells, it is likely that these cells activate other metabolic solutions to genotoxicity, such as the pentose-phosphate pathway, as occurs in humans, where the ATM protein kinase (ortholog of Tel1) activates glucose-6-phosphate dehydrogenase (Zwf1), the rate-limiting enzyme of the pentose phosphate shunt that protects cells from oxidative damage by raising NADPH levels (Cosentino et al., 2011).

SNF1 is involved in regulating many cellular processes, including carbon metabolism, nitrogen availability (Orlova et al., 2006), response to stresses such as high pH (Casamayor et al., 2012), phosphate starvation (Thompson-Jaeger et al., 1991), and sodium ions (Hong and Carlson, 2007). These diverse roles for SNF1 may indicate the existence of different pools of SNF1, which may explain why only a small percentage of Snf1 needs to be SUMOylated to regulate carbon metabolism. Another explanation is that not all of the SNF1 in wild-type cells is active under respiratory conditions, as deletion of multiple phosphatases is lethal (Ruiz et al., 2011, Ruiz et al., 2013), suggesting unutilized Snf1 activity.

We compared the relationship of SUMOylation and dephosphorylation in the regulation of SNF1 activity. Lack of one of these modes of regulation of SNF1 does not prevent the other from occurring ((Simpson-Lavy and Johnston, 2013), **Figures 3e, 3f, 6a, 6e, 6f**). Hyperinduction of Snf1 SUMOylation with Mms21^{S261D} suppresses the effects of lack of dephosphorylation, as shown by the reduction in SNF1 activity when the Mms21^{S261D} mutant is grown in galactose (**Figure 6b**), a partial recovery of *HXT3* expression in *reg1*Δ

cells (**Figure 6d**), and viability of *reg1*Δ*sit4*Δ*ubp8*Δ *SNF1 Mms21*^{S261D} cells (**Figures 6e, f**).

The consequence of inhibition of SNF1 function by MMS is to throttle respiration, because SNF1 is a central component of the respiration/fermentation switch, inhibiting expression of genes encoding glucose transporters (Gadura et al., 2006, Pasula et al., 2007), which are required for fermentation (Gamo et al., 1994), and activating expression of genes required for respiration (Young et al., 2003). Thus, SUMOylation of Snf1 in response to MMS-induced DNA damage reduces respiration and increases fermentation. We have consistently observed that SUMOylation of Snf1 does not affect its phosphorylation at T210, thus SUMOylation of Snf1 during the DNA damage response downregulates Snf1 irrespective of the available carbon source.

Connections between DNA repair and carbohydrate metabolism are increasingly apparent. MMS treatment of *S. cerevisiae* decreases transcription of genes involved in respiration and increases transcription of genes involved in fermentation (Caba et al., 2005, Fry et al., 2003, Gasch et al., 2001, Jelinsky and Samson, 1999, Lee et al., 2000, Shalem et al., 2008) (though the effect upon respiration genes in these studies was limited since the experiments were conducted with glucose-grown cells). The requirement of either *MEC1 or TEL1* for glucose sensing by the SRR pathway suggests that increased Mec1/Tel1 activity may protect yeast cells from oxidative damage by driving the switch from respiration to fermentation. Thus, Mec1/Tel1 (and possibly ATM/ATR) play important roles in this metabolic switch, in addition to responding the DNA damage.

In mammalian cells p53 promotes respiration in a feed-forward mechanism with AMPK (the ortholog of SNF1), and the absence of p53 in tumor cells is a major contributor to the Warburg effect (the aerobic fermentation exhibited by tumor cells) (Lu et al., 2014, Matoba et al., 2006, Sinthupibulyakit et al., 2010, Wang et al., 2014, Contractor and Harris, 2012, Kim et al., 2013). Our results make us wonder if activation of DNA repair pathways, a hallmark of tumor cells, contributes to the Warburg effect. Our results suggest that the reduction of respiration in response to DNA damage is purposefully controlled, and offer a mechanism by which DNA damage can inhibit respiration by reducing SNF1 activity.

Experimental Procedures

Yeast strains, plasmids, mutagenesis and media

Strains used are listed in **Table S1;** plasmids used are listed in **Table S2**. Most experiments were conducted with strains related to W303a (Ma et al., 1987). Snf1 was immunoprecipitated from BY4741/2 (Brachmann et al., 1998) for determining its SUMOylation. Yeasts were transformed with DNA using the frozen lithium acetate method (Knop et al., 1999). To make strains with the *mms21*-CH mutations (C200A H202A) that abolish its SUMO E3 ligase function, plasmid pMMS21.47 (Santa Maria et al., 2007), which contains the 3' part of *MMS21* with the C200A H202A mutations, was linearized with *PvuII* to target its integration into the *MMS21* locus. Ura^{+} colonies were isolated and confirmed to carry the mutations by DNA sequencing of a PCR product. Mutations of *MMS21* in the vicinity of S261 were made using constructs produced from pYM3 [37] which introduced a

6HA-tag, *K.lactis TRP1* marker and mutations in the tail of Mms21 (the primer was codon optimised for codons corresponding to amino-acids 263-267. Primers for tagging *MMS21* are listed in **Table S3**). Mutagenesis was performed using Quickchange (Agilent); all mutations were confirmed by DNA sequencing. To construct the Mms21-3HA plasmid (used in **Figure 4d**), the 3HA-tag and the G418 resistance marker was cloned out of strain T74-17D (Zhao and Blobel, 2005) together with 244 bases upstream and 213 bases downstream of the STOP codon. This was inserted into plasmid Mms21-46 (Santa Maria et al., 2007) cut with BtrI & ClaI. The resultant plasmid contains Mms21-3HA expressed from its own promoter, and carries selection markers for both *URA3* and *KAN*.

With the exception of *snf1*Δ cells, which must be grown on 2% glucose, yeasts were grown for *HXT3* expression and Mth1 degradation assays in media containing 2% galactose with the appropriate amino acid and nucleotide base supplements. For *ADH2* expression assays, cells were grown in 4% glucose, washed 3x with water, and resuspended in media containing 3% glycerol. MMS (Sigma) was added directly to media to the indicated concentration. For serial dilutions, cells were grown overnight in galactose containing media (unless the cells required glucose) to saturation and diluted 10 fold per dilution. The first spotting is a 10-fold dilution from a saturated culture.

β-galactosidase assays

The β-galactosidase assay kit (Pierce, cat. no. 75768) was used in a 96-well plate format. Cell concentration was read at 600nm. Reactions typically proceeded for 5 minutes at room temperature. Additional β-galactosidase assays used a homemade kit, with β-galactosidase activity read at 415nm and cell concentration at 595nm. Rates of gene expression were determined by calculating the increase in LacZ activity per minute (or hour) over the course of the experiment (90 minutes for *HXT3* and 4 hours for *ADH2* expression) and normalized to that of wild-type for **each** experiment. All error bars are +− 1 standard deviation.

Immunopreciptations

Cells (40-50ml) were centrifuged for 2 minutes at 4400g in an Eppendorf 5702, resuspended in ice-cold B60 (Hanna et al., 2001), with 480mM KAc and 0.1% NP40 (for Snf1 immunoprecipitation) or 240mM KAc 0.1% NP40 (for Mms21 immunoprecipitation). Lysis buffer also contained 1mM N-ethyl maleimide (NEM), HALT protease and phosphatase inhibitors (Pierce #1861280), and pepstatin A (Sigma #P4265). Cells were vortexed at 4°C for 30 minutes with 1 minute rests every other minute and spun at 13000 rpm for 30 minutes at 4°C. The supernatant was transferred to a new tube and spun for an additional 5 minutes. Protein concentration was determined by BCA (Pierce #23228/1859078). With the exception of **Figure 1a**, extracts were incubated with Preconjugated EZ-View anti-Myc beads (Sigma #E6654) or anti-HA beads (Sigma #E6779) for 2 hours and washed before eluting by boiling into non-fluorescent sample buffer (Pierce #39001). For **Figure 1a**, extracts were precleared with a 50/50 mix of protein-A/protein-G beads, and incubated with 2μl anti-HA antibody overnight. A 50/50 mix of protein-A/protein-G beads was added for 3 hours. After the penultimate wash, samples were split and one-half treated with 400 units λ-phosphatase (NEB P0753S) for 40 minutes at 30°C. The beads were washed once more with B240, and proteins extracted by boiling in sample buffer.

Protein kinase assays

Snf1-8myc was immunoprecipitated in B60 with 0.1% Triton X-100 with 1mM NEM, and protease and phosphatase inhibitors as described above. The ADP-Glo protein kinase assay (Promega) kit was used with 1 mM SAMS peptide (Signalchem) as the phosphate acceptor substrate. All error bars are +−1SD.

Immunoblots

To prevent activation of SNF1 by processing of the cells (Wilson et al., 1996), cells were killed before centrifugation by addition of 1ml 100% TCA to 5ml of cells. Cells were vortexed with glass beads, pelleted, and resuspended in non-fluorescent sample buffer (Licor 928-40004). Protein concentration was determined by the Coomassie elution with SDS method (Marbach et al., 2001), except that destaining was done with water. Protein extracts were run on 10% (12% or 15% for Mms21-3HA) TGS gels (Biorad) and transferred to PVDF membranes. Phostag (Waco) was added at 0.15ml/10 mix when required. Membranes were probed with mouse anti-Myc (9E10, Santa Cruz), rabbit anti-S-tag (Abcam), rabbit anti-phospho-RxxS (Cell Signaling), mouse anti-HA (Roche or Santa Cruz), mouse anti-FLAG (M2, Sigma), with all antibodies diluted 1000-fold in blocking buffer (Rockland MB-070). Snf1 phosphorylated on T210 (P-Snf1) was detected using rabbit anti-phospho T172 AMPKα (Cell Signaling). Loading controls were detected with rabbit anti-actin (Epitomics, 500-fold diluted) or mouse anti-Pgk1 (Invitrogen, 10000-fold diluted) antibodies. Secondary antibodies were anti-mouse 680LT and anti-rabbit 800CW (LiCOR) or anti-mouse Dylight 488 and anti-rabbit Dylight 549 (Epitomics). Blots were visualized with a LiCOR odyssey or a Biorad imager. Quantifications were performed using NIH ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DNA damage and glucose both cause SUMOylation and inactivation of Snf1 by Mms21.
- Mec1 (ATR), Tel1 (ATM) and PKA phosphorylate Mms21at Serine 261.
- Inactivation of Snf1 by SUMOylation is independent from Snf1 dephosphorylation.
- This may contribute to the Warburg effect found in cancer cells

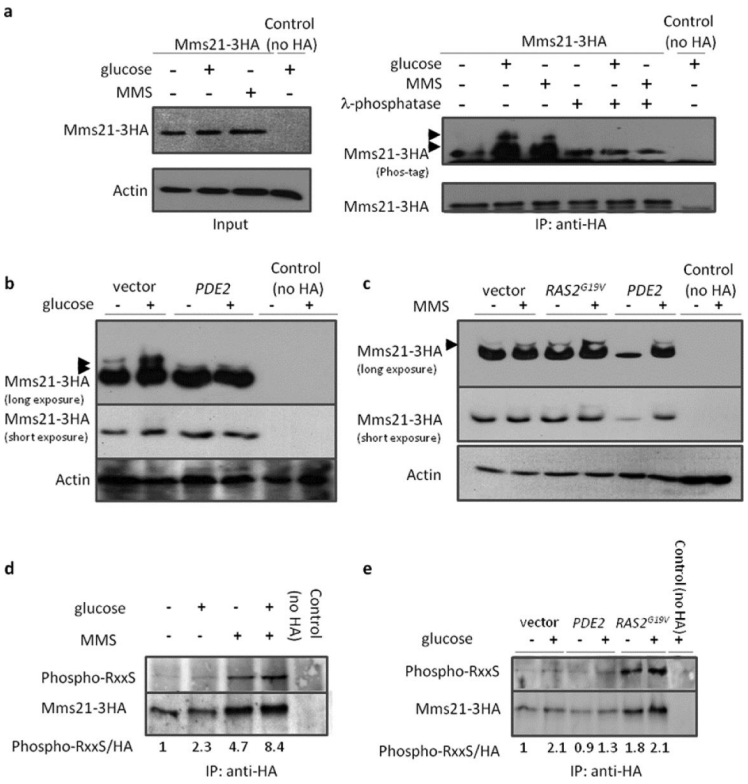

Figure 1. Mms21 is a phospho-protein

a. Glucose to 2% (15 minutes) or 0.03% MMS (1 hour) was added to media containing Mms21-3HA cells (or cells lacking the HA tag). Samples were taken for immunoprecipitations using anti-HA. Left: input. Right: Immunopreciptated Mms21-3HA was run on 15% phos-tag and regular gels, and probed with anti-HA. Phosphorylated Mms21 is indicated with arrows. **b, c**. Glucose to 2% (15 minutes) (**b**) or 0.03% MMS (1 hour) (**c**) was added to media containing Mms21-3HA cells (or cells lacking the HA tag) bearing indicated plasmids or empty vector. Cells were processed for immunoblots, and whole extracts loaded onto 15% gels containing phos-tag. A long and a short exposure for anti-HA are shown. After probing for HA, blots were stripped and reprobed for actin (**b**) or identical amounts of protein extract loaded onto a new gel and the membrane probed for actin (**c**). Phosphorylated Mms21 is indicated with arrows - only the upper phospho-Mms21 band is regulated by PKA. **d, e**. Mms21-3HA (or cells lacking the HA tag) were treated as with 4% glucose for 15 minutes and/or 0.3% MMS for 1 hour, and samples taken for immunoprecipitations using anti-HA beads. Immunoprecipated Mms21-3HA was run on 12% gels, and probed with mouse anti-HA and rabbit anti-RxxS* simultaneously using fluorescent secondary antibodies. Ratios of pRxxS/HA were calculated by quantification using ImageJ.

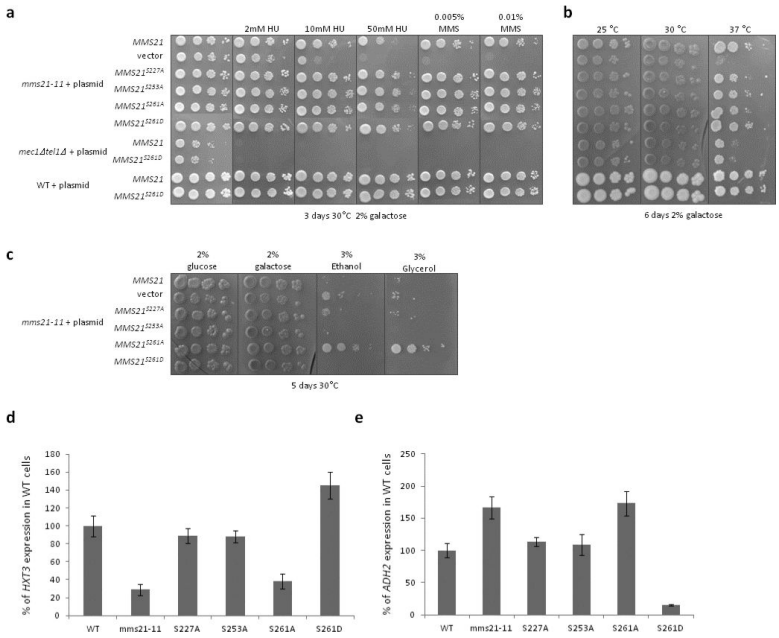

Figure 2. Phenotypes of Mms21 phospho-mutants

a, b, c. Cells bearing the indicated plasmids were serially diluted tenfold from saturated cultures onto SC-ura plates containing the indicated carbon sources and genotoxins, and grown at the indicated temperatures. Photographs were taken on the indicated days. For the genotoxin and temperature sensitivity assays, similar results were obtained for cells grown on glucose. For the carbon source growth assay, similar results were obtained at 25°C. None of the strains grew on either 3% ethanol or 3% glycerol at 37°C. *mec1*Δ*tel1*Δ cells are also *sml1*Δ. **d**. *mms21-11* cells bearing the indicated *MMS21* plasmids or empty vector and *prHXT3:LacZ* were grown overnight in 2% galactose, and glucose added to 2%. *HXT3* expression was measured by β-galactosidase activity every half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **e.** *mms21-11* cells bearing the indicated *MMS21* plasmids or empty vector and *prADH2:LacZ* were grown overnight in 4% glucose. Cells were washed three times with water and resuspended in media containing 3% glycerol. Samples were taken for β-galactosidase assays each hour for four hours. The rate of *ADH2* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation.

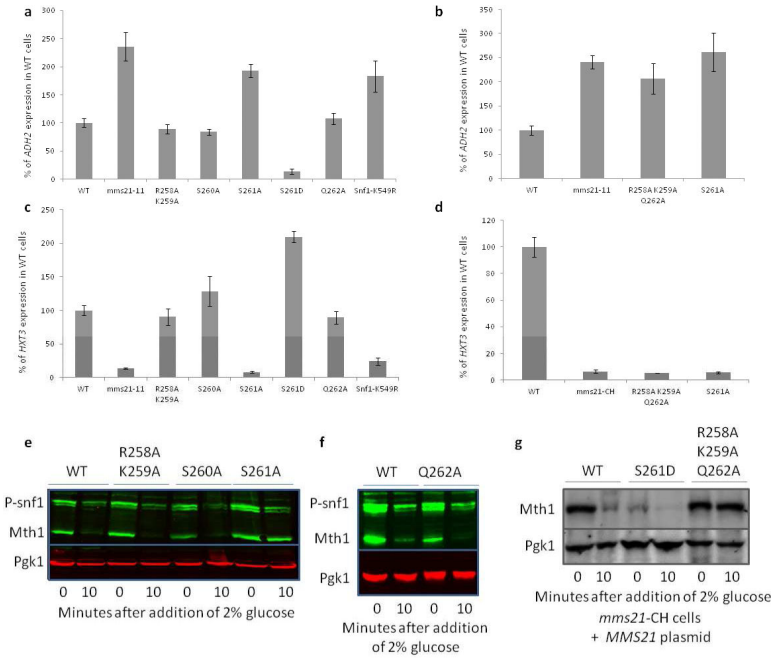

Figure 3. Alanine scan of the C-terminus of Mms21

a. WT, *mms21-11*, or cells with the indicated integrated mutations in the *MMS21* locus containing a *prADH2:LacZ* plasmid were grown overnight in 4% glucose. Cells were washed three times with water and resuspended in media containing 3% glycerol. Samples were taken for β-galactosidase assays each hour for four hours. The rate of *ADH2* expression is normalized to that of wild-type cells for each experiment. *ADH2* expression in *snf1*Δ cells bearing $Snf1^{K549R}$ is included for comparison. N=3. Error bars are +− 1 standard deviation. **b.** *mms21-11* cells bearing the indicated *MMS21* plasmids or empty vector and *prADH2:LacZ* were grown overnight in 4% glucose. Cells were washed three times with water and resuspended in media containing 3% glycerol. Samples were taken for β-galactosidase assays each hour for four hours. The rate of *ADH2* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **c.** WT, *mms21-11*, or cells with the indicated integrated mutations in the *MMS21* locus containing a *prHXT3:LacZ* plasmid were grown overnight in 2% galactose, and glucose added to 2%. *HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells for each experiment. *HXT3* expression in *snf1*Δ cells bearing $Snf1^{K549R}$ is included for comparison. N=3. Error bars are +− 1 standard deviation. **d.** *mms21-CH* cells bearing the indicated *MMS21* plasmids or empty vector and *prHXT3:LacZ* were grown overnight in 2% galactose, at 30°C. The temperature was raised to 34°C for one hour, and preheated glucose added to 2%. *HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **e, f.** Cells bearing the indicated integrated mutations in the *MMS21* locus were grown overnight in 2% galactose at 30°C. Glucose was added to 2%, and samples taken for immunoblotting at the indicated times. An extra copy of Snf1 on a *CEN* plasmid was present which did not affect the experiment. **g.** *mms21*-CH cells containing a plasmid with the indicated mutation in *MMS21* were grown overnight in 2% galactose at 30°C. The temperature was raised to 34°C

for one hour, and preheated glucose added to 2%. Samples were taken for immunoblots at indicated times.

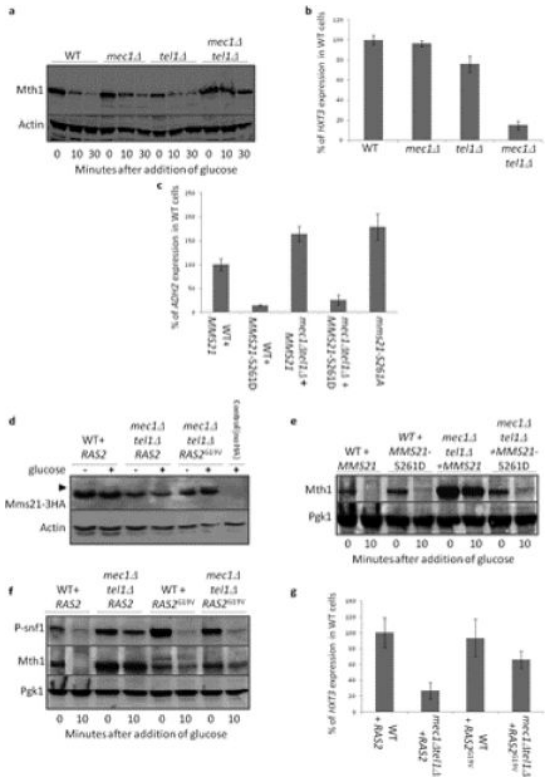

Figure 4. Kinases that regulate Mms21 activity towards Snf1 via phosphorylation of S261

*mec1*Δ and *mec1*Δ*tel1*Δ cells are also *sml1*Δ. **a.** Cells were grown overnight in 2% galactose at 30°C. Glucose was added to 2% and samples taken for immunoblots at the indicated times. **b**. Cells were grown overnight in 2% galactose at 30°C. Glucose was added to 2%. *HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **c.** Indicated cells containing a *prADH2:LacZ* plasmid and a plasmid with either $MMS21^{WT}$ or $MMS21^{S261D}$ were grown overnight in 4% glucose. Cells were washed three times with water and resuspended in media containing 3% glycerol. Samples were taken for β-galactosidase assays each hour for four hours. The rate of *ADH2* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **d.** Indicated cells with an extra copy of plasmid borne Mms21-3HA were grown overnight in 2% galactose at 30°C. Glucose was added to 2% and samples taken after 15 minutes. Cells were processed for immunoblots, and whole extracts loaded onto 15% gels containing phos-tag. After probing for HA, blots were stripped and reprobed for actin. Phosphorylated Mms21 is indicated with arrows. **e, f**. Indicated cells bearing indicated plasmids were grown overnight in 2% galactose at 30°C. Glucose was added to 2% and samples taken for immunoblots at the indicated times. **g.** Indicated cells bearing indicated plasmids were grown overnight in 2% galactose at 30°C. Glucose was added to 2%. *HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation.

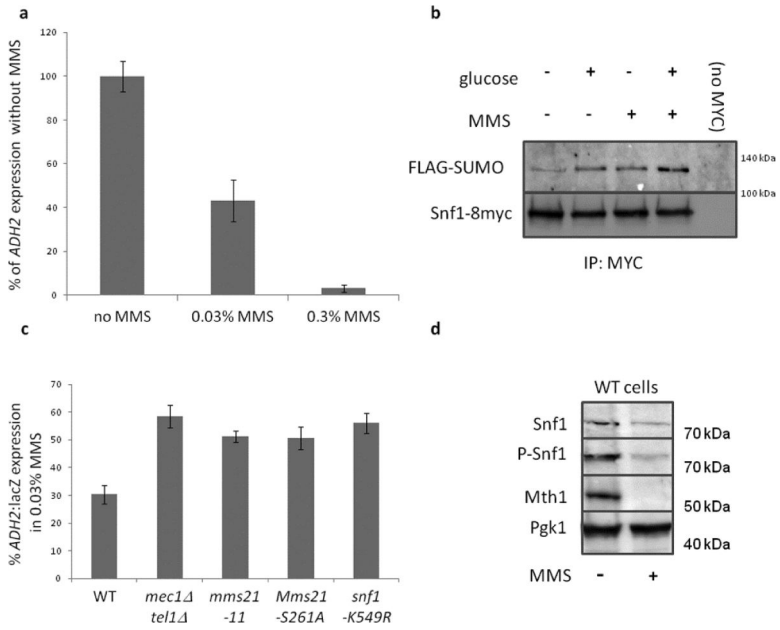

Figure 5. MMS downregulates *ADH2* expression

a. WT cells were grown overnight in 4% glucose at 30°C. Cells were washed three times with water and resuspended in media containing 3% glycerol and the indicated MMS concentrations. Samples were taken for β-galactosidase assays every hour for four hours. The rate of *HXT3* expression is normalized to that of untreated cells. N=3. Error bars are +− 1 standard deviation. *mec1*Δ*tel1*Δ cells are also *sml1*Δ. **b.** *ulp1*-ts cells (strain 1274 (Wykoff and O'Shea, 2005)) expressing Snf1-8myc (Liu et al., 2011), Snf^{1K549R}-8myc (Simpson-Lavy and Johnston, 2013) or Snf1 (with no tag) (Shirra et al., 2008), together with a plasmid containing GAL:His_6-FLAG-*SMT3* were grown overnight at 24°C in 2% galactose. The temperature was elevated to 37°C for 4 hours before addition of preheated glucose to 2% for 15 minutes or 0.3% MMS for 1 hour (or both conditions) as indicated. Samples were processed for immunoprecipitations with MYC. **c.** Cells bearing the indicated mutations were grown overnight in 4% glucose at 30°C. Cells were washed three times with water and resuspended in media containing 3% glycerol with and without 0.03% MMS. Samples were taken for β-galactosidase assays every hour for four hours and the rate of *ADH2* expression determined. The rate of *ADH2* expression in the presence of 0.03% MMS was normalized against the rate of *ADH2* expression *for that strain* in the absence of MMS (the mutant strains all have dramatically increased *ADH2* expression in the absence of MMS (**Figures, 2, 3, 4** and **(Simpson-Lavy and Johnston, 2013)**). Comparing the WT to any of the mutants gave a significant difference at $p<0.01$. In contrast, the differences between the mutants was not significant ($p>0.05$). N=3. Error bars are +− 1 standard deviation. **d**. Wild-type cells were grown overnight in 2% galactose at 30°C. 0.3% MMS was added for two hours. Samples with and without 0.3% MMS treatment for 2 hours were taken for immunoblots.

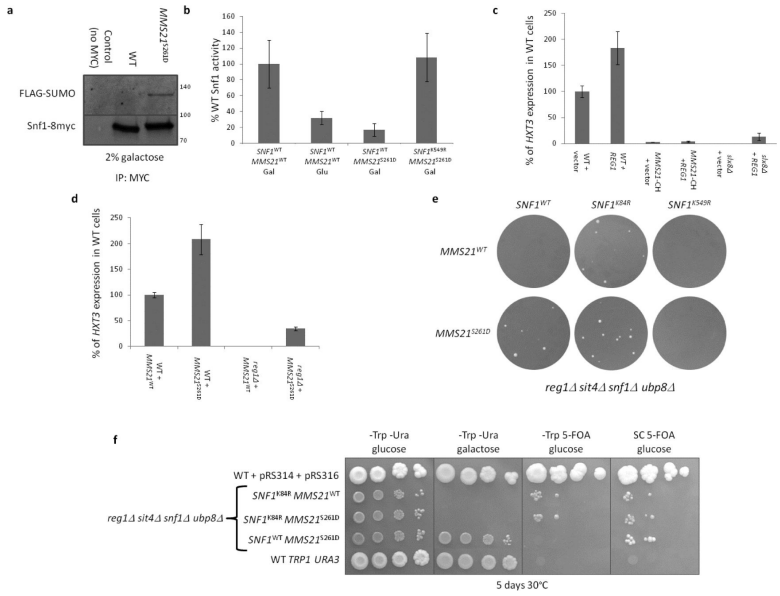

Figure 6. Snf1 SUMOylation and phosphorylation are independently regulated

a. *ulp1*-ts cells (strain 1274 (Wykoff and O'Shea, 2005)) expressing Snf1-8myc (Liu et al., 2011) or Snf1 (with no tag) (Shirra et al., 2008), a plasmid with either *MMS21* or *MMS21*^{S261D}, together with a plasmid containing GAL:His$_6$-FLAG-*SMT3* were grown overnight at 24°C in 2% galactose. The temperature was elevated to 37°C for 4 hours before harvesting for anti-myc immunoprecipitations with B480. Blots were probed with anti-myc (for Snf1-8myc) and anti-FLAG (for Smt3). **b.** Cells containing the indicated Snf1-8myc plasmid and *MMS21*^{S261D} plasmid where applicable were grown with either 2% galactose or 4% glucose as indicated overnight at 30°C. Cells were harvested and Snf1-8myc immunoprecipated. Cells with untagged Snf1 were used as a control. Protein kinase assays were performed using the ADP-glo kinase assay kit with SAMS peptide as a phosphate acceptor. N=3. **c.** Indicated cells containing a *prHXT3:LacZ* plasmid and either a 2μ plasmid overexpressing *REG1* or empty vector were grown overnight in 2% galactose at 30°C. The temperature was raised to 34°C for one hour, and preheated glucose added to 2%.*HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **d.** Indicated cells containing a *prHXT3:LacZ* plasmid and either a plasmid with *MMS21*^{WT} or *MMS21*^{S261D} were grown overnight in 2% galactose. *HXT3* expression was measured by β-galactosidase activity each half-hour for 90 minutes. The rate of *HXT3* expression is normalized to that of wild-type cells. N=3. Error bars are +− 1 standard deviation. **e**. *reg1*Δ*sit4*Δ*snf1*Δ*ubp8*Δ cells were transformed with prs314 (*TRP1*) bearing either *SNF1*^{WT}, *snf1*^{K84R} (kinase dead), or *snf1*^{K549R} (non-SUMOylatable) and with Ycplac33 (*URA3*) bearing either *MMS21*^{WT} or *MMS21*^{S261D} and plated onto –Trp -Ura 2% glucose plates, and photographed after 5 days incubation at 30°C. **f.** Wild-type *ADE2* cells containing plasmids prs314 (*TRP1*) and prs316 (*URA3*) (positive control for growth on 5-FOA containing media), wild-type *ADE2* cells with integrated *TRP1* and *URA3* (negative control for growth on 5-FOA containing media), and the three viable strains from **figure 6e** were serially tenfold diluted onto the indicated media. Plates were photographed after 5 days

incubation at 30°C. The 5-FOA promotes loss of the *MMS21*^{S261D} plasmid from *reg1*Δ*sit4*Δ*ubp8*Δ *SNF1 MMS21*^{S261D} cells, resulting in Snf1 toxicity on -Trp 5-FOA media.

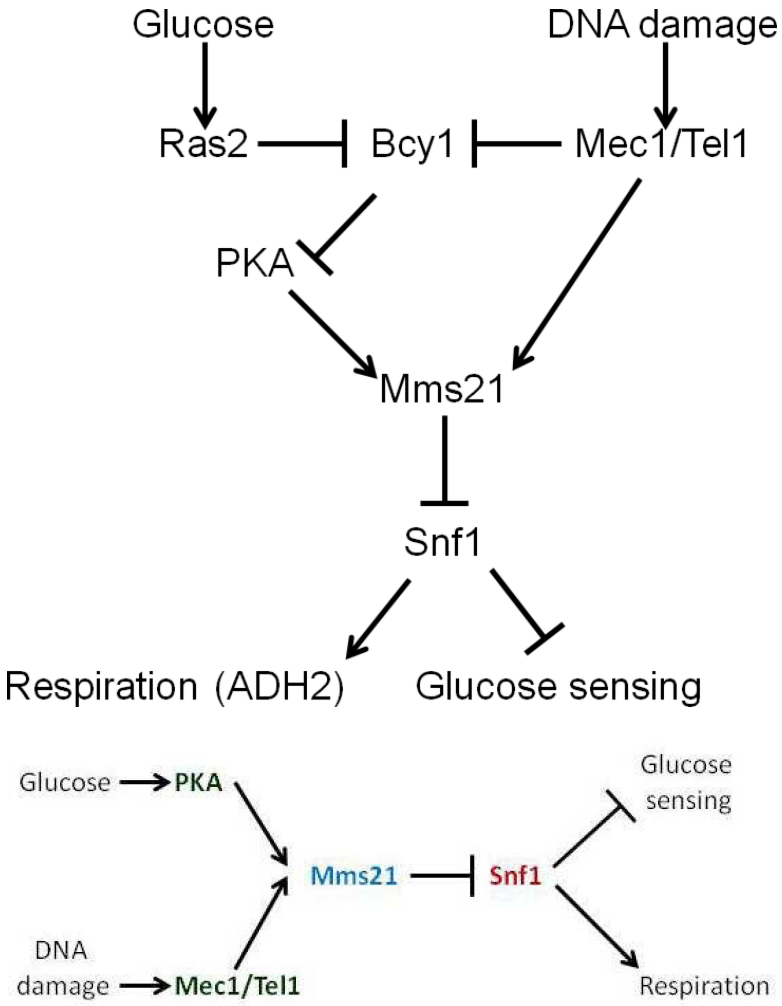

Figure 7. Model

Proposed signaling pathway based on data presented in this article.