UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA
Facoltà di Scienze MM. FF. NN.
Dipartimento di Biotecnologie e Bioscienze
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Protein kinase Snf1/AMPK: a new regulator of G1/S transition in *Saccharomyces cerevisiae*

Dott.ssa SARA BUSNELLI
Matr. 064203

Anno Accademico 2012-2013

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Riassunto

La proteina chinasi Snf1 di *Saccharomyces cerevisiae* appartiene alla famiglia delle serina-treonina chinasi SNF1/AMPK (*Sucrose Non-Fermenting 1/AMP-activated protein kinase*). Ortologhi di queste proteine sono stati individuati in tutti gli eucarioti, dove svolgono un ruolo fondamentale nel mantenimento dell'omeostasi energetica, garantendo la sopravvivenza cellulare (Hardie G., 2007; Ghillebert R. *et al.*, 2011).

In lievito, Snf1 è un complesso eterotrimerico formato da una subunità catalitica α (Snf1), da una subunità β (alternativamente Sip1, Sip2 o Gal83) e da una subunità γ (Snf4). Le subunità β determinano la localizzazione del complesso e concorrono all'interazione con i substrati (Vincent O. and Carlson M., 1999; Vincent O. *et al.*, 2001). La subunità Snf4, invece, è implicata nell'attivazione di Snf1. Essa, legando il dominio auto-inibitorio della subunità catalitica Snf1, permette la piena attivazione della chinasi (Rudolph M.J. *et al.*, 2005; Momcilovic M. *et al.*, 2008). Oltre al legame con Snf4, l'attivazione della proteina chinasi Snf1 richiede la fosforilazione del residuo di Treonina 210 posto nel dominio chinasico della subunità α. Tale fosforilazione è catalizzata da tre proteine chinasi (Sak1, Tos3 ed Elm1) che sono costitutivamente attive. La regolazione dell'attività di Snf1, quindi, è determinata dalla defosforilazione della Treonina 210 che è mediata dalla fosfatasi Glc7 associata alla subunità regolatoria Reg1 (Huang D. *et al.*,1996; Sanz P. *et al.*, 2000; Sutherland C. *et al.*, 2003; Hong S. *et al.*, 2003).

La funzione della chinasi Snf1 è di regolare l'espressione di numerosi geni implicati nel metabolismo cellulare, permettendo l'adattamento a basse concentrazioni di glucosio e la crescita su fonti di carbonio alternative al glucosio (saccarosio, glicerolo, etanolo). Snf1, inoltre, è implicata nella risposta a numerosi tipi di stress ambientali (stress osmotico o alcalino) e nella regolazione di diversi processi cellulari quali l'invecchiamento, la sporulazione, la crescita invasiva e filamentosa (Portillo F. *et al.*, 2005; Ashrafi K. *et al.*, 2000; Vyas V.K. *et al.*, 2003). Dati di letteratura dimostrano che Snf1 modula l'espressione genica agendo su più aspetti della trascrizione. Infatti questa chinasi regola diversi fattori trascrizionali, quali l'inibitore

Mig1 o i fattori trascrizionali Adr1, Cat8, Sit4, Gcn4 e Rgt1(Treitel M.A. *et al.*, 1998; Hedbacker K. and Carlson M., 2008; Kacherovsky N. *et al.*, 2008). Inoltre, Snf1 è implicata anche nel processo di rimodellamento della cromatina e nel reclutamento dei componenti del complesso di pre-inizio ai promotori. Infatti, Snf1 regola l'acetilazione dell'istone H3 sia fosforilandolo direttamente sul residuo di Serina10, sia modulando l'attività dell'acetil-transferasi Gcn5 (Lo W. *et al.*, 2005; van Oevelen C.J. *et al.*, 2006; Liu Y. *et al.*, 2010). Inoltre, Snf1 è coinvolta nel reclutamento a specifici promotori dei componenti del complesso del Mediatore (Young E.T. *et al.*, 2002), del complesso SAGA (van Oevelen C.J. *et al.*, 2006), della *TATA binding protein* (TBP) (Shirra M.K. *et al.*, 2005) e della RNA Polimerasi II (Tachibana C. *et al.*, 2007; Young E.T. *et al.*, 2012).

L'attività di ricerca del mio dottorato è stata principalmente volta a definire una nuova funzione di Snf1 nella regolazione della progressione del ciclo cellulare in *S. cerevisiae*.

Analisi preliminari svolte nel nostro laboratorio dimostravano che la delezione del gene *SNF1* causava un forte ritardo nella transizione G1/S, testimoniato dal ritardo nella formazione della gemma e nella replicazione del DNA. Le proteine regolatorie di tali eventi cellulari sono codificate da un gruppo di circa 200 geni la cui espressione coordinata avviene al termine della fase G1, abbiamo perciò voluto indagare se Snf1 fosse implicato nella regolazione di questi geni.

Una prima analisi svolta sul gene *CLB5*, uno dei principali geni di fase G1, ci ha permesso di dimostrare che la mancanza di Snf1 causa una forte diminuzione dell'espressione di tale gene. Questa minore espressione porta ad un decremento dei livelli della proteina Clb5 e quindi ad una minore fosforilazione dei substrati del complesso Clb5/Cdk1, quali da proteina Sld2 che è implicata nella replicazione del DNA. Inoltre, mediante saggi di co-immunoprecipitazione abbiamo dimostrato che Snf1 interagisce con il fattore trascrizionale Swi6 che, legando le proteine Swi4 o Mbp1 forma i complessi SBF (Swi6-Swi4) ed MBF (Swi6-Mbp1), i principali responsabili dell'espressione dei geni di fase G1 (Nasmyth, K. and Dirick, L., 1991; Koch C. *et al.*, 1993).

In particolare, le nostre analisi dimostravano che il ritardo nell'ingresso in fase S di un ceppo *snf1Δ* è osservabile solo in cellule cresciute in terreno contenente 2% glucosio. Concentrazioni più alte di questa fonte di carbonio (5% glucosio), invece, permettono la totale reversione del fenotipo, suggerendo che il ruolo svolto da Snf1 nella regolazione del ciclo cellulare dipenda dalla condizione nutrizionale delle cellule.

Questi dati, pubblicati nel 2010 (Pessina S. *et al.*, 2010), hanno evidenziato quindi per la prima volta un ruolo per la proteina chinasi Snf1 nella regolazione del ciclo cellulare.

Alla luce di questi dati abbiamo voluto ulteriormente indagare il meccanismo mediante il quale Snf1 regola la trascrizione dei geni legati alla progressione del ciclo cellulare ed abbiamo dimostrato che, oltre a *CLB5*, Snf1 è coinvolto nell'espressione sia dei geni regolati dal complesso SBF, sia dei geni dipendenti dal complesso MBF. Infatti, cellule delete del gene *SNF1*, sincronizzate in fase G1 mediante *α-factor* e rilasciate in terreno privo di feromone mostrano un profilo di espressione dei geni SBF- (*CLN2* e *PCL1*) ed MBF- (*CLB5* ed *RNR1*) dipendenti alterato rispetto a quello di un ceppo *wild type*.

Successivamente, nonostante analisi di *Chromatin ImmunoPrecipitation (ChIP)* indicassero che Snf1 non è rilevabile ai promotori dei geni di fase G1, abbiamo indagato se questa chinasi fosse coinvolta nella regolazione dei complessi SBF ed MBF.

Le nostre analisi hanno evidenziato che, come atteso, durante la fase G1 nelle cellule *wild type* i componenti di questi complessi (Swi4, Mbp1 e Swi6) si trovano nel nucleo, dove legano i promotori dei geni di fase G1. Diversamente, in un ceppo *snf1Δ* queste proteine risultano localizzate nel nucleo solo nel 60% delle cellule. Mediante esperimenti di *ChIP* abbiamo verificato che sia il legame di Swi4 e Mbp1 al DNA sia il successivo reclutamento di Swi6 risultano significativamente ridotti in un ceppo *snf1Δ*. Tale riduzione del reclutamento dei fattori trascrizionali ai promotori porta, come conseguenza, a difetti nel reclutamento di altri componenti del

complesso di pre-inizio della trascrizione, quali il complesso FACT, che regola il rimodellamento della cromatina, e l'RNA Polimerasi II.
I nostri dati, quindi, indicano che Snf1 svolge un ruolo fondamentale nella regolazione dell'espressione dei geni di fase G1 favorendo il reclutamento ai promotori dei maggiori regolatori della trascrizione.
Poiché è noto che Snf1 agisce da regolatore trascrizionale quando è attivata mediante la fosforilazione della Treonina 210, la fosforilazione di questo residuo è stata indagata durante la progressione del ciclo cellulare ed è stato rilevato che in cellule cresciute in 2% glucosio Snf1 risulta parzialmente fosforilata. Questo dato suggerisce che in questa condizione nutrizionale Snf1 sia parzialmente attiva e quindi che possa regolare la trascrizione dei geni di fase G1 fosforilando specifici substrati. Abbiamo, quindi, ipotizzato che Swi6 fosse un substrato di Snf1. L'analisi della sequenza di Swi6, infatti, aveva evidenziato la presenza di un putativo sito consenso per Snf1 centrato sulla Serina 760, posta nella regione carbossi-terminale di Swi6. Questo residuo risulta fosforilato *in vitro* da Snf1. Tuttavia, lo studio del fenotipo dei mutanti sito specifici *SWI6-S760A* e *SWI6-S760E* non ha mostrato alterazioni della transizione G1/S, suggerendo che Swi6 non sia il solo o principale substrato dell'attività chinasica di Snf1.
Per questo motivo abbiamo analizzato la transizione G1/S in cellule che esprimono mutanti di Snf1 caratterizzati da alterazioni dell'attività catalitica. I dati da noi ottenuti mostrano che cellule che esprimono un mutante di Snf1 non attivabile (*SNF1-T210A*) o una forma di Snf1 cataliticamente poco attiva (*SNF1-K84R*) presentano un ritardo nell'ingresso in fase S ed una diminuzione dei livelli di espressione dei geni SBF- ed MBF-dipendenti. In particolare, questi difetti trascrizionali non sono causati da alterazioni del reclutamento di componenti del complesso di pre-inizio della trascrizione. Infatti, nel mutante *SNF1-K84R*, Swi6, il complesso FACT e l'RNA Polimerasi II sono reclutati ai promotori come in cellule *wild type*. Tuttavia, in questo mutante, il legame del complesso FACT e dell'RNA polimerasi II alle regioni codificanti dei geni di fase G1 (quali *CLN2* ed *RNR1*) è ridotto, indicando che l'alterazione dell'attività chinasica

di Snf1 porti a difetti nella fase di allungamento della trascrizione di tali geni.
I nostri dati, quindi, dimostrano che la proteina chinasi Snf1 regolando a più livelli l'espressione dei geni SBF- ed MBF-dipendenti, promuove la transizione G1/S.

Abstract

The AMP-activated protein kinase (AMPK) family is a group of Serine/Threonine kinases highly conserved in eukaryotes, from yeast and insects to plants and mammals. Their primary role is the integration of signals regarding nutrient availability and environmental stresses, ensuring the adaptation to those conditions and cell survival (Hardie G., 2007; Ghillebert R. *et al.*, 2011). As its homologue AMPK, in *Saccharomyces cerevisiae* Snf1 exists as a heterotrimeric complex. Core of this enzyme is the catalytic α subunit (Snf1), made up of a canonical catalytic domain in its N-terminus and of an autoinhibitory C-terminal domain which mediates the interaction with the regulatory subunits of this kinase (Rudolph M.J. *et al.*, 2005). These subunits are: the β subunit (Sip1, Sip2 and Gal83, alternatively), which regulates Snf1 localization (Vincent O. *et al.*, 2000) and the γ subunit (Snf4) that, interacting with the autoinhibitory domain of Snf1, guarantees the complete activation of the kinase (Momcilovic M. *et al.*, 2008). Beyond the interaction with Snf4, the activation of the protein kinase Snf1 is determined by the phosphorylation of Thr210 residue in the α subunit (McCartney R.R. and Schmidt M.C., 2001). Three upstream kinases (Sak1, Tos3, Elm1) are responsible for such a phosphorylation. Those kinases are constitutively active, but metabolic signals, such as high glucose concentrations, promote the activity of the phosphatase complex Reg1/Glc7 which dephosphorylates and hence inactivates Snf1 (Huang D. *et al.*,1996; Sanz P. *et al.*, 2000; Sutherland C. *et al.*, 2003; Hong S. *et al.*, 2003).

In budding yeast, Snf1 is required for adaptation to glucose limitation and for growth on non-fermentable carbon sources. In those conditions Snf1 controls the expression of more than 400 genes. Apart from carbon metabolism, Snf1 affects several other processes; in fact, this kinase controls the expression of some important genes involved in the resistance to different environmental stresses (osmotic and alkaline stresses) or in the regulation of different cellular processes such as sporulation, aging, filamentous and invasive growth (Portillo F. *et al.*, 2005; Ashrafi K. *et al.*, 2000; Vyas V.K. *et al.*, 2003).

As a transcriptional regulator, Snf1 exerts its role modulating gene transcription at different levels. This kinase regulates different transcription factors, such as the transcription inhibitor Mig1 (Treitel M.A. *et al.*, 1998; Papamichos-Choronaris M. *et al.*, 2004) or some other transcription factors like Adr1, Sit4, Cat8 and Gcn4 which regulate the expression of genes involved in central metabolic functions, such as gluconeogenesis and respiration (Hedbacker K. and Carlson M., 2008; Smets B. *et al.*, 2010; Kacherovsky N. *et al.*, 2008). Moreover, protein kinase Snf1 is even able to positively regulate the transcription of some metabolic genes influencing the chromatin remodelling process and the recruitment of some Pre-Initiation Complex (PIC) components at promoters. In fact, Snf1 promotes acetylation of histone H3 by either the direct phosphorylation of Ser10 of histone H3 and the phosphorylation of acetyl-tranferase Gcn5 (Lo W. *et al.*, 2005; van Oevelen C.J. *et al.*, 2006; Liu Y. *et al.*, 2010). Moreover, Snf1 is involved in the recruitment to some promoters of Mediator complex (Young E.T. *et al.*, 2002), SAGA complex (van Oevelen C.J. *et al.*, 2006), TATA-binding protein (TBP) (Shirra M.K. *et al.*, 2005) and RNA Pol II (Tachibana C. *et al.*, 2007; Young R.T. *et al.*, 2012).

My PhD research activity was focused on the role of Snf1 in the regulation of the expression of G1-specific genes, and thus in its function as modulator of cell cycle progression.

Data obtained in our laboratory showed that, in cells grown in 2% glucose, deletion of *SNF1* gene caused a delayed G1/S phase transition, consistently with a decreased expression of *CLB5* gene. In keeping with that defective expression, the *snf1Δ* strain showed a severe reduction of Clb5 protein levels and a consequent decrease of phosphorylation of Clb5/Cdk1 complex targets, such as Sld2, which are responsible for the onset of DNA replication. Moreover, our co-immunoprecipitation assays highlighted that Snf1 interacts with Swi6, the common subunit of SBF (Swi4-Swi6) and MBF (Mbp1-Swi6) transcription complexes which regulate the expression of G1-specific genes (Nasmyth, K. and Dirick, L., 1991; Koch C. *et al.*, 1993).

Remarkably, the phenotype of the *snf1* null mutant was complemented by a glucose concentration higher than 2% (5%), suggesting that the role of Snf1 in the modulation of cell cycle progression could depend on the nutritional status of cells.

Those data, published in Pessina S. *et al.*, 2010, newly indicated that Snf1 was involved in the regulation of G1/S transition and pointed to a role for this kinase in the modulation of G1-specific gene expression.

To gain further insight into the function of Snf1, we then analyzed the expression profile of G1-specific genes in cells synchronized in G1 phase by α-factor treatment and released into fresh medium. Our analyses showed that loss of Snf1 (*snf1Δ* strain) severely affected the expression of *CLN2*, *PCL1* (SBF-dependent) and *CLB5*, *RNR1* (SBF-dependent) genes, suggesting that Snf1 regulates the expression of both SBF- and MBF-dependent genes.

Although protein Snf1 was not detectable at promoters of G1-specific genes, we investigated whether it could modulate the activity of SBF and MBF complexes and we found that in a *snf1Δ* strain the recruitment of Swi6 to G1-specific promoters was affected. Moreover, our Chromatin ImmunoPrecipitation (ChIP) assays also showed that in a *snf1Δ* strain the defective association of Swi6 to promoters led to a decreased recruitment of both the FACT complex, which is involved in the chromatin remodelling at G1-specific promoters, and of the RNA Pol II.

Since it is known that the subcellular localization of Swi6 influences its interaction with promoters, then we analyzed its localization in G1 synchronized cells. In keeping with literature data (Sidorova J.M. *et al.*, 1995; Taberner, F.J. and Igual, J.C., 2010), our analyses showed that in *wild type* cells synchronized in G1 phase by α-factor treatment Swi6 was essentially nuclear. Instead, in a *snf1Δ* strain Swi6 was localized in the nucleus only in the 60% of the G1-arrested cells, consistently with the reduced binding of Swi6 to G1-specific promoters.

It is well known that the Swi6 interaction to DNA is mediated by the DNA binding-proteins Swi4 and Mbp1 (Andrews B.J. and Moore L.A., 1992; Moll T. *et al.*, 1992). Therefore we extended our analyses

to those proteins and we found that also the nuclear localization and the subsequent binding to DNA of Swi4 and Mbp1 were affected in a *snf1Δ* strain.

Therefore, our data provide a representative snapshot of what occurs *in vivo* in a *snf1* null mutant, supporting the notion that Snf1 promotes the expression of G1-specific genes modulating the nuclear localization of SBF and MBF components and thus promoting the formation of a complete Pre-initiation Complex (PIC) at G1-specific promoters.

It is well known that phosphorylation of Snf1 at Thr210 leads to the full activation of the kinase (Hong S.P. *et al.*, 2003; Sutherland C.M. *et al.*, 2003). Then, in order to obtain insight into the Snf1 molecular mechanism in cell cycle regulation, we investigated its phosphorylation on Thr210 during cell cycle progression. Snf1 was slightly phosphorylated on the Thr210 residue during all the cell cycle, suggesting that this kinase was partially active. To determine whether the activation of Snf1 was involved in its function as regulator of G1 transcription, we analyzed the expression of SBF- and MBF-dependent genes in the *SNF1-T210A* mutant and we found that in this mutant the expression of those genes was reduced. In keeping with those data, the expression of G1-specific genes resulted affected also in a *SNF1-K84R* mutant, in which the ATP binding site has been destroyed causing a severe reduction of Snf1 kinase activity.

On the base of those findings, we investigated whether Snf1 could exert its role in G1 phase through the phosphorylation of specific substrates and we found that Snf1 phosphorylates *in vitro* Swi6 on Ser760. Nevertheless, analyses of site-specific mutants (*SWI6-S760A* or *SWI6-S760E*) did not show any alteration of G1/S transition, suggesting that this phosphorylation was not involved in the role of Snf1 as regulator of cell cycle.

The ChIP analyses of Swi6 binding to *CLN2* and *RNR1* promoters, then, showed that in the *SNF1-K84R* mutant the recruitment of Swi6 was slightly affected; nevertheless, that alteration was not severe as that of a *snf1Δ* strain. Consistently, neither the recruitment of FACT

complex nor the binding of RNA Pol II to G1-specific promoters was affected in the *SNF1-K84R* mutant.
Since this last finding seemed to disagree with the severe reduction of mRNA expression of SBF- and MBF-dependent genes observed in the *SNF1-K84R* mutant, we wondered whether defects in transcriptional elongation might occur in that strain. Thus, we analyzed the occupancy of FACT complex and of RNA Pol II at the internal regions of *CLN2* and *RNR1* genes by ChIP analyses; in the *SNF1-K84R* mutant the occupancy of both those complexes was decreased, suggesting that the kinase activity of Snf1 promotes the transcriptional elongation across G1-specific genes.
In conclusion, the sum of data here presented indicates that protein kinase Snf1 is involved at different levels in the modulation of the G1-specific gene expression, thus highlighting a new function for Snf1 in the regulation of G1/S transition.

Introduction

1. *Saccharomyces cerevisiae* cell cycle

Eukaryotic cell cycle is a complex process which is precisely regulated at many levels in order to coordinate cell growth and cell division. During the cell division cycle, the cell mass is doubled and all essential cellular components are duplicated, in order to generate two daughter cells (Herskovitz I., 1988).

The budding yeast *Saccharomyces cerevisiae* is a good model to study cell cycle. This organism is easier to manipulate genetically, permitting to study molecular processes in great details. Moreover, its genome is fully sequenced, and a complete list of all its genes and predicted protein sequences are available. Finally, many regulations present in *S. cerevisiae* cell cycle are conserved in higher eukaryotes, so information gained studying budding yeast can be used as the basis for understand multicellular organisms.

A particularity of budding yeast is the asymmetric cytokinesis. At division, mother and daughter cells have different size; thus daughter cell (the smaller cell) needs more time than the mother to reach the proper size to begin a new cell cycle, while the mother cell can enter the S phase almost immediately (Hartwell L.H., 1994).

Yeast cell cycle is divided into four different phases: G1, S, G2 and M During G1 (Gap1) phase the cell increases its mass and prepares itself for S (Synthesis) phase, in which DNA is duplicate. The G2 (Gap2) phase, then, is the temporal gap between the end of replication and the M (Mitosis) phase in which chromosome segregation, nuclear division and cytokinesis take place (Herskovitz I., 1988).

A regulatory step is present between G1 and S phase. During G1 phase, several metabolic, stress and environmental signals are integrated to determine whether the cell enter in a new mitotic cell cycle. Then the commitment to initiation of a new round of cell cycle occurs in late G1-phase, often referred to as START. Immediately after START, different cell-cycle specific processes take place, such as DNA replication, bud emergence and spindle pole body

duplication; all together those processes lead inevitably to cell division.

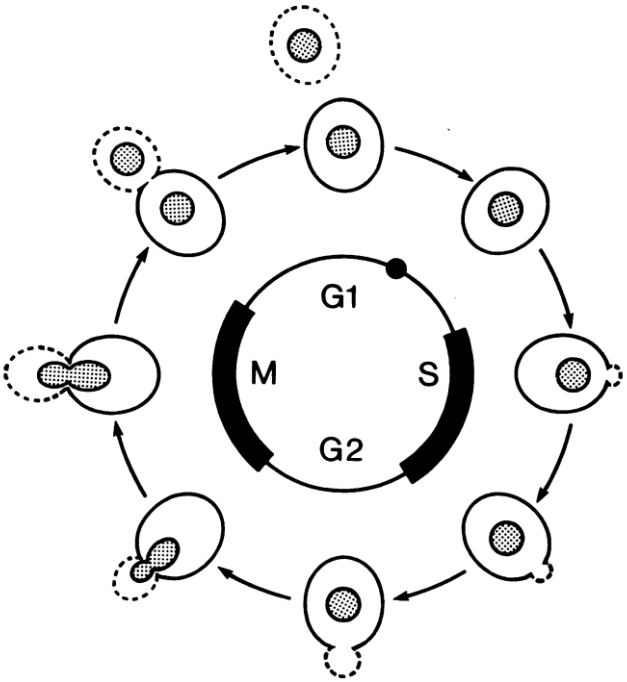


Fig.1- *Saccharomyces cerevisiae* cell cycle (Herskovitz I.-1988)

In yeast, cell cycle progression is regulated at different steps by the Cyclin-dependent protein kinase 1 (Cdk1), a Ser/Thr protein kinase which phosphorylates differents substrates at S/T-P consensus motif.
CDC28 gene, which encodes for Cdk1, is essential and constitutively expressed; thus, all regulations of this kinase take place at post-transcriptional level.
Cdk1 activity is regulated by 9 different proteins called cyclins, which bind Cdk1 and target it to specific substrates. Those proteins are divided into 3 classes on the basis of the cell cycle phase in which they are synthesized: Cln1,2,3 are expressed in G1 phase, Clb5,6 are S phase cyclins, while Clb1,2,3,4 regulate M phase (Mendenhall M.D. and Hodge A.E., 1998).

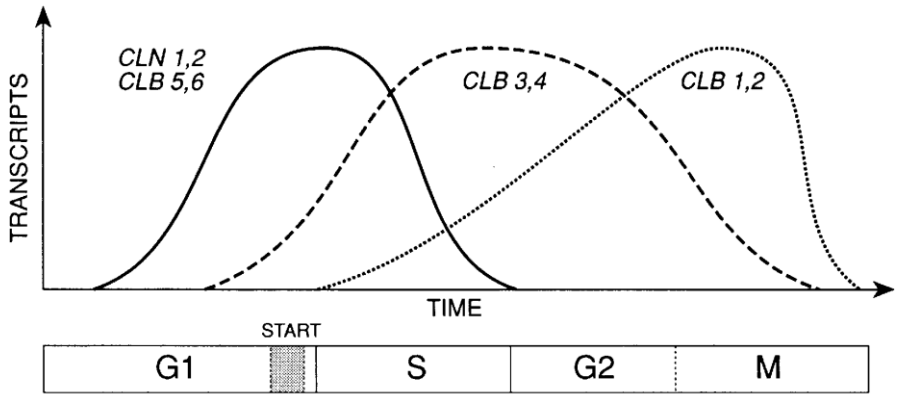


Fig.2- Phase-dependent expression of cyclins
(Schwob E. and Nasmyth K., 1993)

Full activation of Cdk1-cyclin complexes requires the phosphorylation of Thr169 residue of the Cdk1 T loop by the Cdk1-Activating Kinase (CAK) (Kaldis P. *et al.*, 1996; Espinoza F.H. *et al.*, 1998). In fact, a non phosphorylatable Cdk1 mutant (*CDC28-T169A*) is inactive *in vitro* and is unable to sustain cell division *in vivo* (Lim H.H. *et al.*, 1996). Besides those positive regulations, Cdk1 activity is also negatively regulated. Cdk1-cyclins complexes are inhibited by specific inhibitors such as Far1 which is involved in the response to pheromones (McKinney J.D. and Cross F.R.,1995) and Sic1, which specifically inhibits Clb5,6/Cdk1 and Clb2/Cdk1 complexes (Schwob *et al.*,1994). Cdk1, then, is also inhibited by phosphorylation. Protein kinase Swe1 recognize Cdk1-Clb2 complexes and phosphorylates Cdk1 on residues Thr18 and Lys19 (Booher R.N. *et al.*, 1993); on the contrary protein phosphatase Mih1 dephosphorylates those residues and activate Cdk1 (Sia R.A. *et al.*,1996).

1.1 Transcriptional circuit of cell cycle in budding yeast

As described above, the Cdk1 regulation of cell cycle is modulated by the interaction of this kinase with different cyclins. The expression of those proteins is finely cell cycle regulated and guarantees the correct progression of cell cycle. Beside cyclins, other genes are differently regulated during cell cycle.

Remarkably, in yeast between 10 and 20% of genes are expressed during a specific phase of cell cycle (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998). Those genes are typically involved in processes associated with cell cycle progression and show a peak in transcription when the process must take place. Thus, cell cycle progression is constitute of consecutive, interdependent waves of transcription. The transcriptional activation that function during one phase of the cell cycle contribute to the regulation of transcriptional activators that function during the following phase, forming a fully connected regulatory circuit (Simon I. *et al.*, 2001).

The coordination of those waves of transcription is guaranteed by different transcription factor (rewieved in Breeden L.L., 2003; Wittenberg C. and Reed S.I., 2005). In late-G1 phase the SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) complexes are active and lead the transcription of genes involved in G1 and S phase such as *CLN*1,2 and *CLB*5,6 genes (Koch C. and Nasmyth K., 1994). Moreover, they also regulate the expression of *YOX1*, a transcriptional inhibitor that recognizes ECB (Early cell-cycle box) binding sites in the promoter of Swi4 and turn off its expression. Besides this negative feedback loop, SBF and MBF complexes regulates also *NDD1* gene, one of the components of the transcription factor of M phase. In fact, in M phase Fkh protein, Mcm1 and Ndd1 form a complex and regulate the expression of M phase cyclins encoded by *CLB1,2* as well as *SWI5* and *ACE2* genes which encoded for two transcription factors. Swi5 and Ace2 and Mcm1 then regulate transcription in late M and early G1 phase (Dohrmann P.R. *et al.*, 1992). Mcm1 binds Swi4 promoter on ECB sequences and regulates its expression (Simon I. *et al.*, 2001). Differently, between genes regulated by Swi5 and Ace2 there are: the

Cdk1/Clb5,6 complexes inhibitor Sic1, the cyclin Cln3, the transcriptional activator Rme1 (which promotes the expression of the G1 cyclin Cln2) and Ash1 which, in daughter cells, is required to regulate the expression of *HO* gene (Schwob *et al.*,1994; Bobola N. *et al.*, 1996; Spellman P.T. *et al.*, 1998; Frenz L.M. *et al.*, 2001).
Threfore, transcriptional activation at each phase is the key element necessary to understand the regulation of the yeast cell cycle.

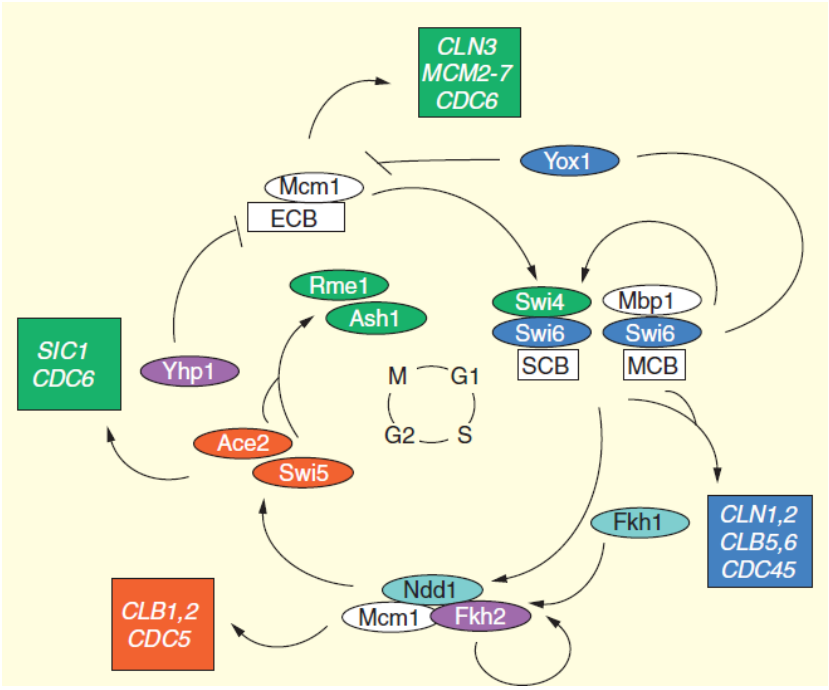


Fig. 3- Transcriptional circuit regulates yeast cell cycle
(Breeden L.L. 2003)

1.2 G1-regulon

In budding yeast commitment to initiation of a new round of cell cycle occurs in late G1-phase, a regulatory stage called START.
The earliest indicator of cell cycle initiation is the transcriptional activation of a large set of genes, more than 200, encoding the components of cellular machinery required for events associated with cell cycle initiation (Spellman P.T. *et al.*, 1998; Breeden L.L., 2003; Wittenberg C. and Reed S.I., 2005). Those genes are commonly divided into two classes on the base of the presence, in their promoters, of redundant specific sequences called Swi4-Cell cycle Box (SCB), CACGAAAA, or Mlu Cell cycle Box (MCB), ACGCGT (Breeden L.L., 1996; Lee T.I. *et al.*, 2002; Kato M. *et al.*, 2004). Those sequences are targeted respectively by SBF complex (SCB Binding Factor) and MBF complex (MCB Binding Factor), two heterodimeric transcription factors. Besides their regulation, SBF- and MBF-dependent genes are different also for their function. In fact, SBF targets are involved in bud emergence, spindle pole body duplication and other growth-related functions. On the other hand, MBF-regulated genes are implicated in DNA replication, repair and DNA processing in general.
Although each factor controls a specific set of genes, a considerable cross-talk between SBF and MBF occurs, since they can cross-recognize each other's DNA target elements and several genes presents both SCB and MCB elements in their promoters (Dirick L. *et al.*,1992; Patridge J.F. *et al.*, 1997; Taylor I.A. *et al.*, 2000). The overlapping activity of those transcription factors provide robustness to the G1-regulon expression, but make difficult to divide genes into specific classes. Currently, only for a few genes a specific regulation has been attributed: *CLN2*, *HO*, *PCL1*, *PCL2* are SBF-dependent, whereas *CLB5*, *CLB6*, *RNR1*, *POL1*, *CDC21* and *NRM1* are MBF-dependent (Spellman P.T. *et al.*, 1998; Iyer V.R. *et al.*, 2001; Simon I. *et al.*, 2001; Bean J.M. *et al.*, 2005; Beyer A. *et al.*, 2006; Holloway D.T. *et al.*, 2008; Ferrezuelo F. *et al.*, 2010).

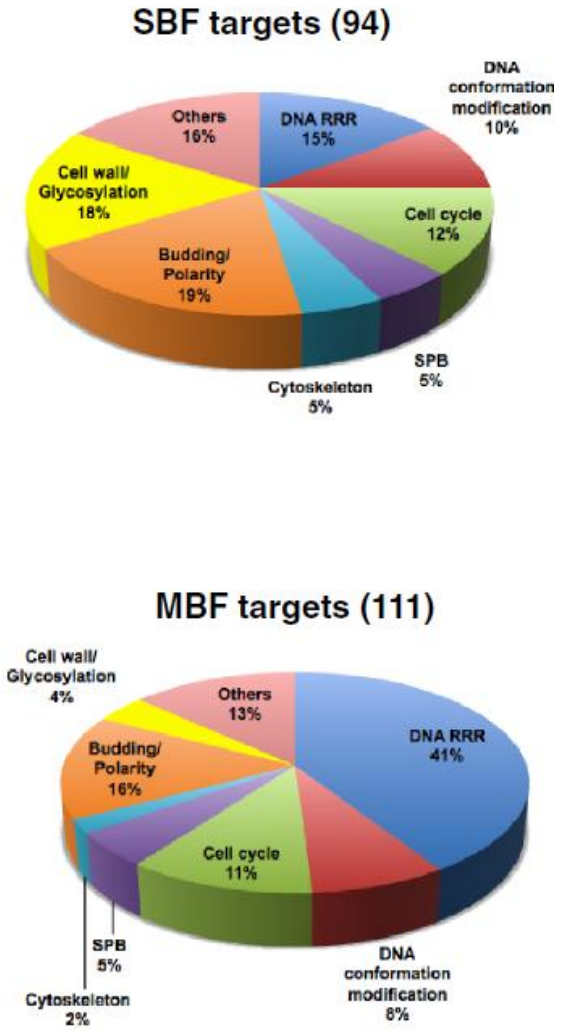


Fig.4- Functional classification of SBF- and MBF-dependent genes; in the figure DNA RRR is used for DNA replication, recombination and repair, while SPB indicates Spindle Pole Body regulation (Ferrezuelo F. *et al.*, 2010).

1.3 Different regulators of G1-specific gene expression

The burst in transcription of G1-regulon is finely regulated by complexes involved in chromatin remodeling and by specific transcription factors, whose interaction with G1-specific promoters is cell-cycle regulated.
Remarkably, the great part of data regarding the role of those factors in G1-specific transcription has been collected analyzing *HO* gene, an SBF-dependent gene which encodes for an endonuclease that initiates mating-type switching in haploid yeast cells.
Chomatin ImmunoPrecipitation (ChIP) analyses performed on the promoter of *HO* revealed interdependence of transcription regulatory proteins, showing that recruitment to promoters of a regulator leads to the proper recruitment of the next one. In keeping with the model proposed (Cosma M.P. *et al.,* 1999; Cosma M.P. *et al.,* 2001; Takahata S. *et al.,* 2009a and b), coactivator complexes such as the histone acetyl- transferase SAGA (Spt-Ada-Gcn5 Acetyl-transferase) complex and the Swi/Snf chromatin remodeling complex are recruited to DNA in late M phase. Those complexes then promotes the recruitment to G1-specific promoters of SBF or MBF transcription factors. In G1-phase, SBF and MBF recruit both the chromatin remodeling complex FACT and the Srb/Mediator complex, in a Cdk1-independent way. At the end, activation of Cdk1 allows recruitment of RNA Pol II. Thus, the association of all those factors with promoters ultimately leads to the formation of a complete pre-initiation complex (PIC) (Hahn S. and Young E.T., 2011).

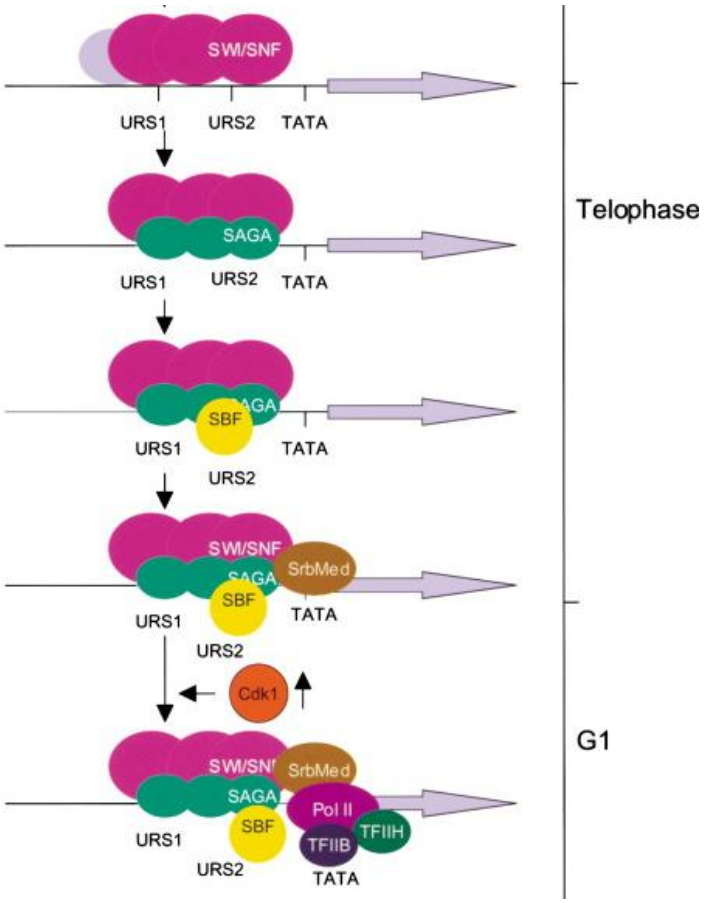


Fig.5- Ordered recruitment of transcription regulators to the SBF-dependent promoter of *HO* gene (Cosma M.P., 2002)

A fundamental aspect of transcription process is chromatin remodelling, since the presence of nucleosomes over the coding regions of genes constitutes a structural barrier that inhibits transcriptional initiation and elongation (Williams S.K. and Tyler J.K., 2007; Li B. *et al.*, 2007). For that reason, chromatin disassembly must occurs at G1-specific promoters in order to allow the binding of SBF or MBF complexes to DNA (Workmann J.L., 2006).
Swi/Snf and SAGA complex are the main transcriptional coactivators involved in the expression of G1-regulon

1.3.1 Swi/snf complex

Swi/Snf complex is an ATP-dependent remodelling factor; using ATP hydrolysis, it remodels chromatin structure and increases accessibility of DNA. Swi/Snf complex is highly conserved in all eukaryotes, in which it regulates the expression of highly inducible genes (Fry C.J. and Peterson C.L., 2001). In budding yeast, the members of this complex were originally identified by defects in mating-type switching (*SWI* genes) or growth in media containing sucrose (*SNF* genes) (Peterson C.L. and Tamkun J.W., 1995). The Swi/Snf complex is made by 11 different subunits. Six of them are present in single copy in the complex (Swi1, Swi2, Snf5, Swp73, Arp7 and Arp9); differently, subunits Swi3, Snf6, Snf11, and Swp82 are present in double copies, and Swp29 in three copies (Martens J.A. *et al.*, 2003). Between those subunits Swi2 is the catalytic subunit; alterations of that subunit cause defects in the expression of many genes and severe growth defects (Peterson C.L. and Tamkun J.W., 1995; Mitra D. *et al.*, 2006). Differently, the slight alteration of its activity (through the mutation *SWI2-E834K*) reduces the expression of *HO* gene, a defect complemented by the deletion of *SIN3*, one of the subunits of Histone Deacetylases Complex which is involved in the inhibition of G1-regulon expression (Mitra D. *et al.*, 2006).

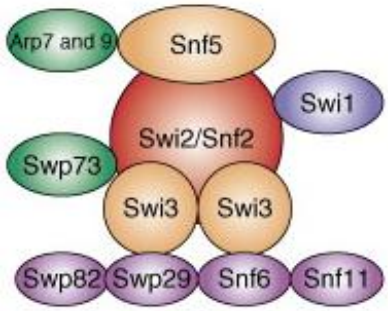


Fig.6- Swi/Snf complex of budding yeast
(Kwon C.S. and Wagner D., 2007)

The multisubunit complex Swi/Snf is recruited to promoters. In particular, the first analyses done on this complex showed that it is recruited to the SBF-dependent promoter of *HO* gene where it promotes the binding of another chromatin remodelling factor, SAGA complex (Cosma M.P. *et al.*, 1999; Cosma M.P. *et al.*, 2001; Krebs J.E. *et al.*, 1999). Nevertheless, the observation that deletion of *GCN5*, gene encoding for the active subunit of SAGA complex, leads to a defective recruitment of Swi/Snf complex to DNA seems to suggest that the binding of those two complexes is interdependent (Mitra D. *et al.*, 2006). Consistently, the double deletion of *SWI2* and *GCN5* is lethal (Biswas D. *et al.*, 2004). Recently also a negative regulation on Swi/Snf complex mediated by Gcn5 it has been proposed. In fact, Gcn5 is able to acetylate the Snf2 subunit of the Swi/Snf complex determining its dissociation from some promoters (such as the promoter of *SUC2*) and thus inhibiting gene expression (Kim J.H. *et al.*, 2010). Thus, the functional interaction between Swi/Snf complex and SAGA complex appears to be complicated and not completely understood.

In addition to its role in the recruitment of SAGA complex, it has been observed that Swi/Snf complex is also able to interact with TATAbox of *HO* gene, where it influences the recruitment of TATA Binding Protein (TBP) (Biswas D. *et al.*, 2004). Swi/Snf complex seems to be involved also in transcriptional elongation. In fact, yeast cells devoid of this complex are sensitive to drugs that inhibit RNA Pol II elongation. Moreover, components of Swi/Snf complex have been found associated with coding regions of different genes and deletion of *SWI2* causes a severe decrease of RNA Pol II occupancy at the coding regions of different genes, the indicator of defects in transcriptional elongation (Schwabish M.A. and Struhl K., 2007).

1.3.2 SAGA complex

SAGA complex (Spt-Ada-Gcn5 acetyltransferase) presents a modular structure with distinct functional units: a recruitment module (Tra1), an acetylation module (Gcn5, Ada2, Ada3), a TBP interaction unit (Spt3, Spt8), a Dub module (Ubp8, Sus1, Sgf11 and Sgf73) and a architecture unit (Spt7, Spt20, Ada1, TAF5, -6, -9 and -12) (Koutelou E. e*t al*., 2010). Deletion of genes encoding for Ada1, Spt7, or Spt20/Ada5 subunits causes disruption of SAGA complex and severe growth defect, suggesting that SAGA structural integrity is important for cells (Sterner D.E. and Berger S.L., 2000).
The active subunits of the complex are Gcn5 and Ubp8 which possess HAT (Histone AcetylTransferase) and histone deubiquitynase activities respectively. The core of the complex is composed of Gcn5, Ada2 and Ada3 which catalyzes the acetylation of N-terminal histone lysine residues, with a primary specificity for Lys9, Lys14, Lys18, Lys23 of histones H3 (Kuo M.H. *et al.*, 1996; Grant P.A. *et al.*, 1999).

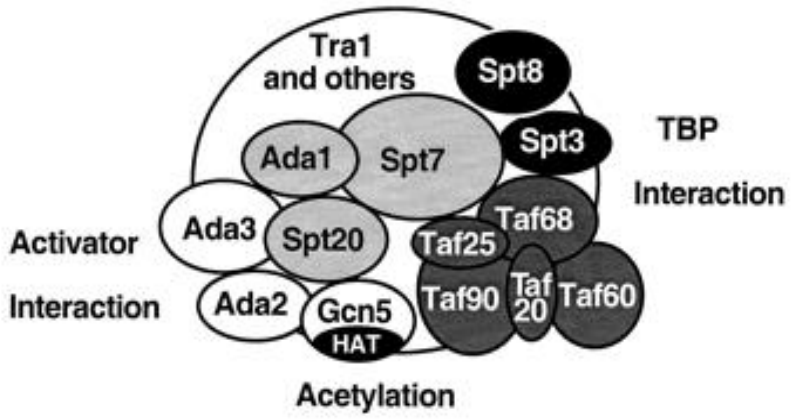


Fig.7- Yeast SAGA complex (Sterner D.E. and Berger S.L., 2000)

Histone acetylation neutralizes the positive charge generated by Lysin-rich domains present in the N-terminal tails of histones, thereby affecting nucleosome structure and enhancing DNA accessibility (Huang J.C. *et al.*, 2006). Therefore, histone acetyltransferases, as SAGA complex, bind transcription activators and promote transcription. On the contrary, histone deacetylases interact with transcription inhibitors, determining the inhibition of gene expression.
The binding of SAGA complex to histone H3 is promoted by others modification such as the di- and trimetylation of Lys4 residue (Pray-Grant M.G. *et al.*, 2005). Interestingly, different studies have also demonstrated that at some promoters Gcn5 preferentially acetylates H3 when it is phosphorylated on Ser10 residue, an event which is catalyzed by the protein kinase Snf1 (Lo W. *et al.*, 2001; Liu Y. e*t al.*, 2005).
The role of SAGA complex is to stimulate the recruitment of TBP (TATA Binding Protein) to promoters, through a direct interaction with TBP and the acetylation of histone H3 which makes DNA more accessible to TBP (Sterner D.E. *et al.*, 1999; Deckert J. and Struhl K., 2002). Moreover, SAGA complex is also able to directly recruit RNA Pol II, independently from TBP (Warfield L. e*t al.*, 2004). Besides this well defined function, it has also been observed that SAGA complex promotes transcription through the deubiquitylation of ubiquitinated-Lys123 of histone H2B at different SAGA-dependent promoters (Daniels J.A. *et al.*, 2004).
At *HO* promoter, SAGA complex is recruited during early G1 phase when it catalyze the acetylation of histones H3 in 1Kb upstream the ATG of *HO* which makes DNA more accessible to Swi4 (Krebs J.E. *et al.*, 1999). In particular, this event requires Swi/Snf complexes and is independent from transcription factor Swi4 (Krebs J.E. *et al.*, 1999).

Taken toghether, literature data about Swi/Snf and SAGA complexes suggest that those complexes regulate the recruitment of Swi4 and Mbp1 to DNA.

1.3.3 SBF and MBF complexes

The expression of G1-specific genes is regulated by two different, but related transcription factors: SBF and MBF complexes. In those heterodimeric complexes the binding to DNA depends on the two different proteins Swi4 (SWItching deficient 4) or Mbp1 (MluI-box Binding Protein), while the activation requires the common regulatory subunit Swi6 (SWItching deficient 4).
Swi4 and Mbp1 share the same structure: a DNA-binding domain (DBD) in the N-terminus, which leads the association of those proteins to specific elements called SCB and MCB respectively, a Nuclear Localization Signal (NLS), a series of ankyrin repeats and, in the C-terminal region, a domain which mediates the interaction with protein Swi6 (Siegmund R.F. and Nasmyth K.A., 1996).

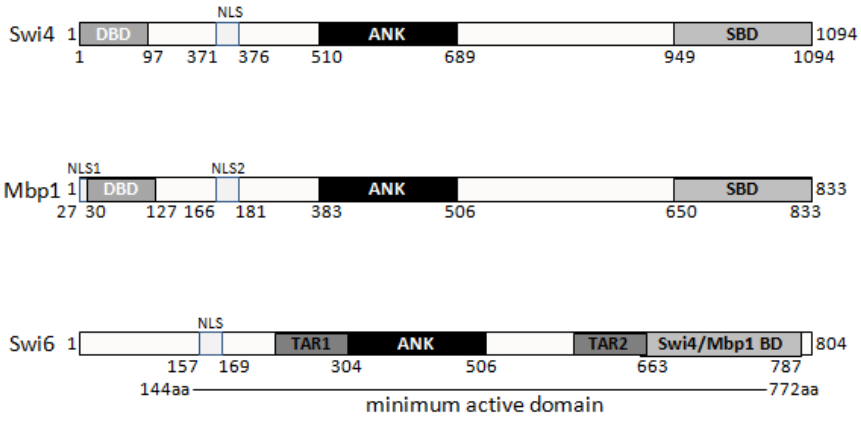


Fig.8 - Sequences of Swi4, Mbp1 and Swi6. In the figure DBD (DNA-binding domain), NLS (Nuclear localization signal), ANK (Ankyrin repeats), Transcriptional Activation Domains (TAR1 and TAR2) and SBD (Swi6-binginf domain) or Swi4/Mbp1 BD (Swi4/Mbp1 binding domain) are indicated

Despite their structural similarity, those two proteins present some differences both in regulation and in functions exerted.
The expression of protein Swi4 is cell cycle regulated with a peak of accumulation in G1 phase, immediately before the peak of *HO*. In the *SWI4* promoter there is an upstream activating sequence (UAS) between nucleotides +513 and +653 which presents a perfect MCB element (at +561) and two CGCG sequences (at +172 and +205); loss of those sequences leads to a 10-fold drop of *SWI4* expression, indicating that they promote the transcription of *SWI4* (Foster R. *et al.*,1993). Although *SWI4* promoter presents an MCB element this gene is not simply a MBF-dependent gene. In fact, deletion of Swi6 causes only the loss of periodicity of *SWI4* expression; in the *swi6Δ* strain, in fact, Swi4 remains at low levels during all the cell cycle, without neither the peak in transcription nor the loss of transcription, suggesting that Swi6 plays both a negative and a positive role in *SWI4* expression (Foster R. *et al.*, 1993). In addition, Swi4 promoter also show some ECB (Early cell-cycle box) elements which are recognized by the transcriptional inhibitor Yox1 which turns off Swi4 expression (MacKay V.L. *et al.*, 2001). In particular, since *YOX1* is a SBF-dependent genes, Swi4 seems to participate to its own inhibition (Bean J.M. *et al.*, 2005).
Remarkably, when *SWI4* is expressed constitutively through the cell cycle, the periodicity of *HO*, *CLN1* and *CLN2* transcription is lost, suggesting that the regulation of *SWI4* expression is fundamental for the proper expression of G1-specific genes (Foster R. *et al.*,1993; MacKay V.L. *et al.*, 2001).
Once synthesized in late M/early G1 phase, Swi4 enter into the nucleus thank to the classical import pathway which is constituted by the β-karyopherin Srp1 and Kap95. Those proteins, in fact, recognize the canonical NLS of Swi4 and determine its nuclear localization (Taberner F.J. and Igual J.C., 2010). In the nucleus, then, Swi4 binds DNA and leads the recruitment of Swi6 to SBF-dependent genes, the event which determines the activation of those genes. Intriguingly, SBF complex determines the expression also of genes which do not

present SCB elements necessary for the recruitment of Swi4, such as *YOX1* and *TOS4* (Bean J.M. *et al.*, 2005).
Since the expression of Swi4 is cell cycle regulated, the binding of Swi4 to SCB elements changes during cell cycle progression, reaching a peak of interaction during G1 phase (Harrington L.A. and Andrews B.J., 1996). Interestingly, it has been observed than protection of SCB elements *in vivo* is not detectable in a *swi6Δ* strain (Harrington L.A. and Andrews B.J., 1996; Koch C. *et al.*, 1996) because in that strain Swi4 seems to be unable to bind SCB elements (Baetz K. and Andrews B., 1999). Moreover, analyses of Swi4 sequence have revealed the presence of a C-terminal domain (last 144 amino acids) which, interacting with the DNA-binding domain of Swi4, prevents its binding to promoters. The interaction with Swi6 then relieves this inhibition promoting the association of the SBF complex to DNA (Baetz K. and Andrews B., 1999). Although those data suggest a cooperative role for Swi4 and Swi6, it is known that deletion of Swi6 causes a decreased expression of SCB-dependent genes, however only the concomitant deletion of both *SWI6* and *SWI4* genes determines a synthetic lethality (Nasmyth K. and Dirick L. 1991; Koch C. *et al.*, 1993). In addition, overexpression of *SWI4* eliminates Swi6 need for *HO* transcription, while in a *swi4Δ* strain the overexpression of *SWI6* does not rescue the low levels of *HO* expression (Sidorova J. and Breeden L., 1993), indicating that Swi4 is necessary for the regulation of SBF-dependent genes. Taken together those data suggest that although Swi6 is required for the proper interaction of Swi4 to DNA, Swi4 is slightly able to bind SCB elements and presents a low activity as transcriptional activator also in the absence of Swi6.
Another regulation on Swi4 is the phosphorylation of its Ser153 and Thr799 residues which is mediated by Cln3/Cdk1 complexes. Nevertheless, site-specific mutations of those residues do not determine any significant phenotype, suggesting that those phosphorylations are not required for the regulation of G1-specific gene expression (Wijnen H. *et al.*, 2002). At the end, it has been shown that Swi4, through its ankyrin repeats, interacts with Clb2, thus suggesting a possible regulation mediated by Clb2 on SBF complex.

Remarkably this interaction seems to be specific for Swi4, since Mbp1 does not interact with Clb2 and Swi6 interacts only slightly with this cyclin (Siegmund R.F. and Nasmyth K.A., 1996).
In the mechanism of G1-specific gene regulation Swi4 is a fundamental regulator. Deletion of Swi4 causes a slow growth phenotype, large cell size and altered morphology (Igual J.C. *et al.*, 1996). Consistently, in the *swi4Δ* strain the expression of SBF-dependent genes, such as *CLN1* and *CLN2*, is severely affected (Nasmyth K. and Dirick L. 1991; Koch C. *et al.*, 1993), indicating that Swi4 acts as a transcriptional activator.

Consistent with the functional overlap between SBF- and MBF-dependent regulation, a *swi4Δmbp1Δ* double mutant is lethal (Koch C. *et al.*, 1993); however, the single deletion of *MBP1* does not affect cell viability and causes only the increase of 20% of cell size (Bean J.M. *et al.*, 2005).
As Swi4 protein, also Mbp1 accumulates into the nucleus through the classical import pathway. In particular, Mbp1 has two NLS sequences recognized by Srp1 and Kap95 proteins; nevertheless, a slight nuclear localization of this protein is detectable also in a Kap95 mutant suggesting that others importins could regulate Mbp1 localization (Taberner F.J. and Igual J.C., 2010).
Despite that common aspect, differently from Swi4, Mbp1 is constitutively expressed during all the cell cycle, thus the binding of Mbp1 to MCB elements is constant during the cell cycle progression. The binding with Swi6, in G1 phase, then determines the activation of MBF-dependent genes (Dirick L. *et al.*, 1992).
In addition, deletion of *MBP1* causes only the loss of the periodicity of MBF-dependent genes expression, which in some case (such as for as *CLB2*, *SPT21*, *CLB5*, *POL1* and *TMP1* genes) exhibit higher levels of transcription. Therefore, differently from the transcription activator Swi4, Mbp1 seems to act as a transcription inhibitor, allowing the expression of MBF-dependent genes only in G1-phase and determining their inhibition in others phases (Koch C. *et al.*, 1993).

Although their differences, SBF and MBF complexes share the same regulatory subunit: Swi6.

Swi6 is a protein of 803 amino acids, which presents a minimum active domain from amino acid 144 to amino acid 772. This domain contains: a NLS, two Transcriptional Activation Domains (TAR1, 270-309 aa, and TAR2, 653-669 aa), four ankyrin repeats (ANK) and the C-terminal region necessary for the interaction with Swi4 and Mbp1 (from amino acid 663 to 787) (Sedgwick S.G. *et al.*, 1998).

As transcriptional activator, Swi6 is necessary for the proper expression of all G1-specific genes and for G1/S transition. In fact, a *swi6Δ* strain, synchronized by α-factor in G1 phase, after the release into fresh medium, shows an asynchronous bud emergence and DNA replication (Chiu J. *et al.*, 2011), in accordance with a severe reduction of G1 gene expression (Lowndes N.F. *et al.*, 1992; Dirick L. *et al.*, 1992).

Swi6 expression is not cell-cycle regulated, thus its activity is regulated at post-transcriptional levels.

The main regulation on Swi6 involves its subcellular localization. During G1 phase, Swi6 accumulates into the nucleus thanks to the β-importins Srp1 and Kap95 (Taberner F.J. and Igual J.C., 2010). Once entered into the nucleus, Swi6 forms SBF and MBF complexes interacting with Swi4 and Mbp1 respectively. That interaction, then, leads to the recruitment of FACT complex and other members of the PIC, promoting the proper expression of G1-specific genes (Takahata S. *et al.*,2009 a and b).

Between those genes, Swi6 permits the expression of the type B-cyclins Clb5 and Clb6; then Clb6, and not Clb5, associating with Cdk1, determines the phosphorylation of Ser160 residue of Swi6 which is the necessary signal for the nuclear export of Swi6 (Sidorova J.M. *et al.*, 1995; Geymonat, M. *et al.*, 2004). In fact, this residue is near the NLS of Swi6 and its phosphorylation causes the delocalization of Swi6 into the cytoplasm. Accordingly, a non phosphorylatable *SWI6-SER160ALA* mutant is always localized into the nucleus, while the *SWI6-SER160GLU* mutant remains into the

cytoplasm also during G1 phase (Sidorova J.M. *et al.*, 1995; Harreman M. T. *et al.*, 2004).

The exclusion from the nucleus of Swi6 is mediated by the karyopherin Msn5. Protein Swi6 and Msn5 interacts *in vivo* and loss of this exporter (*msn5Δ*) causes the accumulation into the nucleus of Swi6 which remains nuclear along all the cell cycle (Queralt E. and Igual J.C., 2003). Remarkably, the exit from the nucleus of Swi6 is a fundamental regulatory event for its activity in the next cell cycle. In fact, in *wild type* cells, in which Swi6 is localized into the cytoplasm from the end of G1 phase until mitosis, this transcription factor can be dephosphorylated by the phosphatase Cdc14 and, during the subsequent G1 phase, can bind Swi4 and Mbp1 and regulate G1-specific transcription (Geymonat, M. *et al.*, 2004; Queralt E. and Igual J.C., 2003). On the contrary, an alteration which prevents the nuclear exclusion of Swi6 (*msn5Δ*) and so its dephosphorylation leads to a Swi6 protein which is not able to bind *CLN2* promoter and to regulate the expression of SBF-dependent genes, suggesting that the nucleocytoplasmic shuttling of Swi6 is necessary for its functionality (Queralt E. and Igual J.C., 2003).

Besides the phosphorylation of Ser160 residue, Swi6 is regulated also by Rad53 in response to DNA damage. In fact, after the treatment with MMS (Methyl MathanSulfonate) Rad53 phosphorylates Swi6 on Ser547 in order to delay the expression of *CLN1/2* genes and the S-phase transition (Sidorova J.M. and Breeden L.L.,1993 and 2003). Moreover, Swi6 is also target of the protein kinase Mpk1 (mitogen-activated protein kinase 1). In response to cell wall stresses Mpk1 binds Swi4 and promotes the expression of *FKS2* gene, which codify for the catalytic subunit of 1,3-beta-glucan synthase, responsible for the synthesis of 1,3-beta-D-glucan, a polysaccharide that is the main structural component of the cell wall. Moreover this protein influences also the Swi6 localization through a non-catalytic mechanism (Kim *et al.*, 2008; Truman A.W. *et al.*, 2009). Then Mpk1 is also involved in Swi6-inhibition; in fact Mpk1 phosphorylates Swi6 on Ser238 preventing its interaction with the importin Kap120 and, so, the

nuclear localization of Swi6 (Madden K. *et al.*, 1997; Kim K.Y. *et al.*, 2010).

Recently it has been proposed a regulation of Swi6 which is independent from phosphorylation events. Swi6 seems to play a role as sensor of oxidative stress thank to its cysteine residue at position 404. In fact, oxidative stress causes the oxidation of Cys404 which leads to a severe decrease of G1-specific genes expression (such as *CLN1*, *CLN2*, *PCL1* and *PCL2*) and a consistent delay in the G1/S phase transition (Chiu J. *et al.*, 2011).

Therefore, literature data support the notion that Swi6 integrates different signals to determine the proper activation of G1-specific transcription.

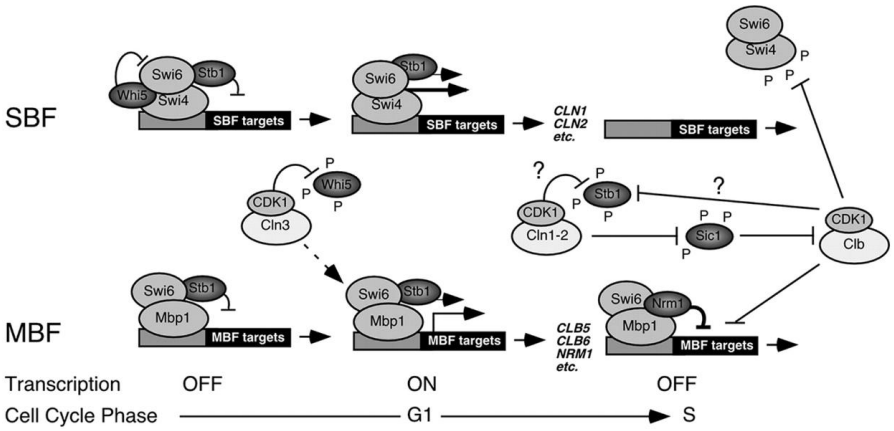


Fig.9- SBF- and MBF-dependent transcription
(de Bruin R. A. *et al.*, 2008)

The regulation of SBF and MBF activity is the central event of the G1-transcription mechanism, for that reason it is finely regulated. In particular, it is known that besides SBF and MBF components, also others transcription activators and inhibitors contribute to the

regulation of G1-regulon expression. Here the main regulators will be described.

Protein **Stb1** (Sin3 Binding Protein), through its interaction with Swi6, is recruited at both SBF- and MBF-dependent promoters (Costanzo M. *et al.*, 2003; de Bruin R. A. *et al.*, 2008).
Literature data show that deletion of *STB1* does not cause any growth defects (Ho Y. *et al.*, 1999). Differently, combined with the deletion of proteins responsible for G1-transcription activation such as Cln3 or Bck2 (*stb1Δcln3Δ*, *stb1Δbck2Δ*), loss of Stb1 leads to an increase of cell size and a delayed G1/S transition (Ho Y. *et al.*, 1999; Costanzo M. *et al.*, 2003) In particular, those defects depends on alterations of G1-gene expression; in those mutants, in fact, the levels of different G1-specific genes are decreased and the overexpression of either *CLN1* or *CLN2* can rescue this phenotype (Ho Y. *et al.*, 1999; Costanzo M. *et al.*, 2003), thus suggesting that Stb1 promotes the expression of G1-specific genes.
Stb1, however, seems not to be merely a transcriptional activator. During G1 phase, Stb1 is involved in the recruitment of Rpd3L histone deacetylase complex to determine the inhibition of G1-specific transcription; consistently, loss of Stb1 causes a de-repression of G1-genes at early G1 phase (de Bruin R. A. *et al.*, 2008). In late G1, then, Cdk1 phosphorylation on Stb1 relieve the inhibition mediated by Rpd3L and permits to Stb1 to function as transcriptional activator (Takahata S. *et al.*, 2009b).

Rme1 (Regulator of Meiosis 1) is a transcriptional activator expressed in M-G1 phase. Once synthesized, Rme1 promotes the expression of *CLN2* gene through a pathway independent from SBF complexes, since it interacts with *CLN2* promoter at sequences different from that recognized by Swi4 (SCB elements) (Toone W.M. *et al.*, 1995). Differently, the transcription activator **Msa1** binds both SBF- and MBF-dependent genes at late G1 phase and stimulates G1 transcription (Ashe M. *et al.*, 2008).

As previously said, it has been proposed that MBF complex could act as transcription inhibitor in order to restrict the expression of MBF-dependent genes to G1-phase (Koch C. *et al.*, 1993). In this model a negative feedback take place and depends on the transcriptional inhibitor **Nrm1** (Negative Regulator of MBF targets 1). *NRM1* is a MBF-dependent gene which is expressed in late G1 phase. Once synthesized, Nrm1 binds MBF complex and inhibit it, turning of the MBF-dependent transcription from S to M phase (de Bruin R.A. *et al.*, 2006). On the contrary, in early G1 phase the APC^{Cdh1} complex determines the degradation of Nrm1, an event which coincides with the expression of the MBF-dependent genes (Ostapenko D. and Solomon M.J., 2011).
Intriguingly, the phosphorylation of Nrm1 determined by Cdk1 seems to stabilize this inhibitor. In fact, inactivation of Cdk1 or mutations into Alanine of the four residues which present a Cdk1-consensus sequence (Thr163, Thr171, Thr207 and Thr231) lead to destabilization of Nrm1 (Ostapenko D. and Solomon M.J., 2011).

As MBF complex, also SBF activity is inhibited by a specific inhibitor: **Whi5**. Whi5, interacting with Swi4 and Swi6, is recruited to SBF-dependent promoters (Costanzo M. *et al.*, 2004; de Bruin R.A. *et al.*,2004), where it inhibits transcription. In fact, a *whi5Δ* strain shows a decrease of cell size of about 30% (Jorgensen P. *et al.*, 2002), and in synchronous cells loss of Whi5 causes a precocious bud emergence (de Bruin R.A. *et al.*, 2004). Protein Whi5 exerts its role as inhibitor promoting the recruitment of histone deacetylases Hos3 and Rpd3, and preventing the binding of FACT complex to SBF-dependent promoters (Huang D. *et al.*, 2009; Takahata S. *et al.*, 2009b). In particular, those deacetylases interacting with the subunit Sin3 form the Rpd3L complex; the interaction with both Whi5 and Stb1, then, determines the binding of the Rpd3L complex to G1-specific promoters and the inhibition of G1-specific gene expression (Takahata S. *et al.*, 2009b).

1.3.4 FACT complex

The binding of Swi6 to SBF- and MBF-dependent promoters allows the recruitment of FACT complex (Facilitating Chromatin Transcription) (Takahata S. *et al.*, 2009a and b). FACT complex is made by Spt16 (Cdc68) and Pob3, two essential proteins (Wittmeyer J and Formosa T., 1997) which interact with the accessory subunit Nhp6 (Singer R.A. and Johnston G.C., 2004).

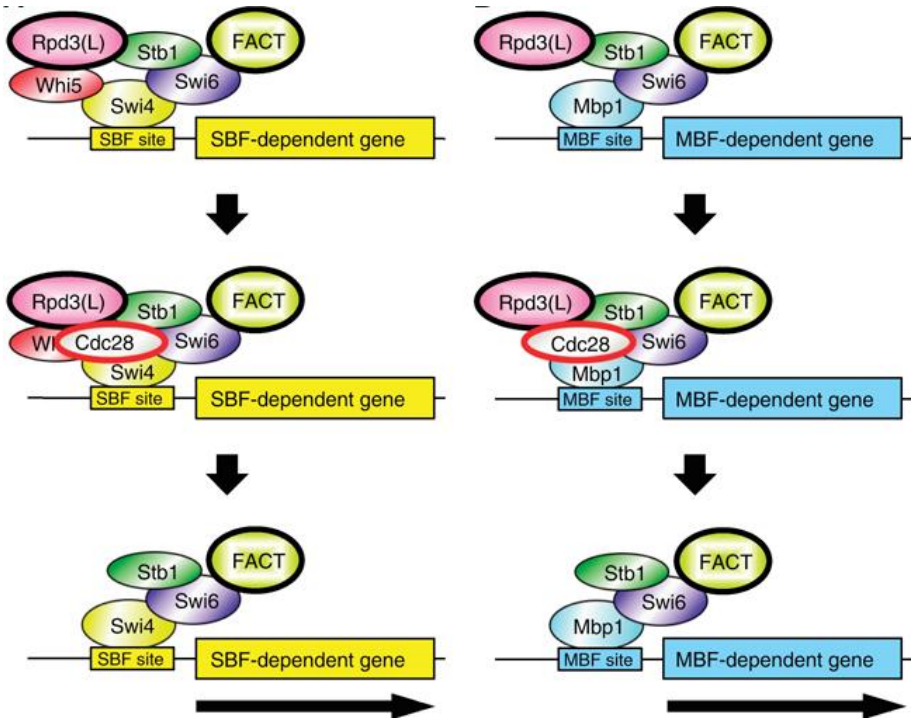


Fig.10 - Recruitment of FACT complex to G1-specific promoters (Takahata S. *et al.*, 2009)

FACT complex is the better-characterized transcription elongation complex. It binds transcribed regions of genes (Mason P.B. and Struhl K., 2003; Kim M. *et al.*, 2004) in order to facilitate the movement of RNA Pol II across transcribed genes (Reinberg D. and Sims R.J., 2006). It has been observed that FACT binds nucleosomes and alters the structure of chromatin destabilizing interaction between H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers *in vitro* (Belotserkovskaya R. *et al.*, 2003); this activity is independent on ATP (Formosa T. *et al.*, 2001). FACT complex, then, is also responsible for the reorganization of nucleosomes after the passage of RNA Pol II, a necessary regulation to maintain the proper structure of chromatin.

Besides this well transcribed role, FACT complex is also involved in transcriptional initiation. In fact, alterations of FACT components correlate with defects in the recruitment of TATA binding protein to promoters (Biswas D. *et al.*, 2005).

In particular, Spt16 and Pob3 are involved in the regulation of G1-specific gene expression. In fact, in *cdc68-1* mutant cells the levels of *CLN1* and *CLN2* transcripts are depleted (Rowley A. *et al.*, 1991) and alteration of FACT causes also a decreased expression of the SBF-dependent gene *HO* (Formosa T. *et al.*, 2001). At G1-specific promoters FACT is recruited thanks to protein Swi6 and also by a Swi6-independent mechanism, since deletion of *SWI6* causes only a delayed binding of FACT to promoters (Takahata S. *et al.*, 2009b). Therefore, FACT complex ensures the expression of G1-genes (Rowley A. *et al.*, 1991; Formosa T. *et al.*, 2001), allowing the formation of a complete Pre-Initiation Complex at promoters and probably influencing transcriptional elongation across the coding regions of those genes.

1.3.5 SRB/Mediator complex

SRB/Mediator complex binds *HO* promoter in late G1 phase. Mediator is a 21 subunits complex which includes Srb proteins, Med protein and several others polypeptides. This complex has a compact structure which changes upon interaction with RNA Pol II and presents three different regions. The "head" of the complex interacts with RNA Pol II, the middle module is the target of regulatory signals while the "tail" of the complex mediates the interaction with specific transcription factors (Casamassimi A. and Napoli C., 2007).
Mediator complex does not interact with highly active promoters; on the contrary, in response to stress signals it binds promoters (Fan X. *et al.*, 2006).
In particular, this complex is recruited to DNA independently from RNA Pol II (Fan X. *et al.*, 2006); differently, analyses performed on the promoter of *CYC* gene suggest that the recruitment of Mediator depends on SAGA complex (Lee S.K. *et al.*, 2010).
Although the regulation of Mediator binding to DNA has been deeply analyzed, the mechanism by which this complex regulates transcription has not been fully understood. However, Mediator complex is believed to play a role in the assembly of the Pre-Initiation complex (PIC), affecting the recruitment of RNA Pol II, and also to regulate the activation of poised RNA Pol II-dependent promoters (Johnson K.M. and Carey M., 2003; Lee S.K. *et al.*, 2010).
This complex is involved in the expression of G1-specific gene. In fact, different subunits of Mediator genetically interacts with the transcription factor Swi6, showing suppression of growth defect associated to *SWI6* deletion (Li L. *et al.*, 2005) Moreover, Mediator complex is recruited at *HO* promoter at late G1 phase in a Cdk1-independent way, suggesting that it could regulate G1-specific genes (Cosma M.P. *et al.*, 2001).

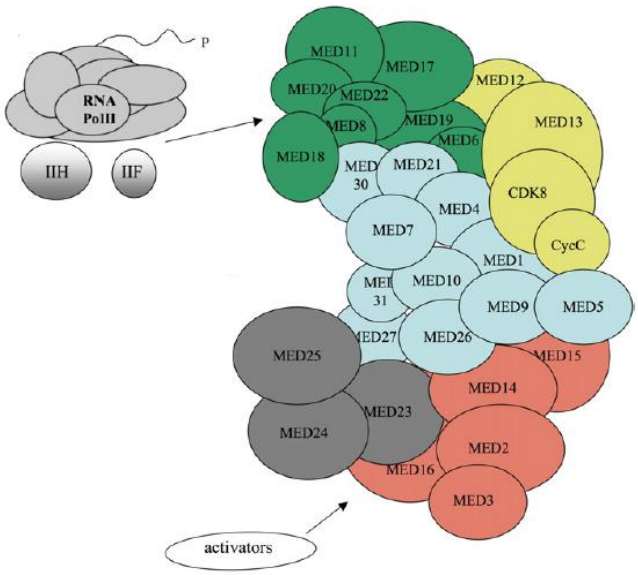


Fig.11- Structure and interactors of Mediator complex
(Casamassimi A. and Napoli C., 2007)

All those events of chromatin remodeling and formation of a complete transcriptional machinery at the promoters finally lead to the recruitment of RNA pol II and of general transcriptional factors, responsible for genes transcription (Cosma M. P. *et al.*, 1999 and 2001; Bhoite L.T. *et al.*, 2001). However, although the recruitment of those factors is independent from Cdk1-activity, the binding of RNA Pol II to promoters requires the activation of Cdk1/Cyclins complexes (Cosma M.P. *et al.*, 2001). Remarkably, this requirement of Cdk1 activity is a characteristic of G1-specific promoters (such as those of *HO*, *CLN1*, *CLN2* and *PCL1*), but not of others genes (such as *GAL10*) in which the recruitment of RNA Pol II depends only on Mediator complex (Cosma M.P. *et al.*, 2001).

1.3.6 RNA Pol II complex

In budding yeast the core of RNA Pol II is made of 12 subunits (Rpb1-Rpb12), which range in size between 6 and 200 kDa.
At promoters the core of RNA Pol II interacts with the so-called general transcription factors (GTFs), which together with RNA Pol II for a complete PIC and stimulate transcriptional initiation. GTFs are: TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. In particular, TFIID is composed of TBP (TATA binding protein) and 12 TAFs (TBP-associated-factors) and it is responsible for the binding of the PIC complex to TATA box sequences.

The main subunit of the RNA Pol II core, Rpb1, presents a highly conserved C-terminal domain (CTD). This region presents 25 to 52 tandem copies of the consensus repeat heptad $Y_1S_2P_3T_4S_5P_6S_7$ (Corden J.L., 1990), a substrate of different kinases which, through the phosphorylation of Rpb1 tail, regulate RNA Pol II activity (Myer V.E. and Young R.A., 1998). In particular, phosphorylations of CTD regulate the interaction of RNA Pol II with transcriptional elongation factors (such as Paf1 complex) which are responsible for nucleotides disassembly, a necessary event for RNA Pol II passage across actively transcribed genes (Qiu H. *et al.*, 2006).
The most phosphorylated residues are Ser2 and Ser5. In particular, the phosphorylation of Ser5 residue is detectable in RNA Pol II complexes present near the beginning of genes, whereas polymerases near the ends of genes are extensively phosphorylated on Ser2 residues (Komarnitsky P. *et al.*, 2000; Morris D.P. *et al.*, 2005).
Ser2 residues are phosphorylated by CTDK-I which is involved in the stimulation of transcriptional elongation (Lee J.M. and Greenleaf A.L., 1997). On the contrary, dephosphorylation of Ser2 by the phosphatase Fcp1 turns off transcriptional elongation (Cho E.J. *et al.*, 2001).
Differently, two kinases phosphorylate Ser5 residue. Srb10 and Srb11 form a complex and phosphorylate Ser5 of the Rpb1 CTD before the assembly of PIC complex, supporting the inactivation of transcription.

Differently, the Kin28(TFIIH)/Ccl1 complex phosphorylates Ser5 residue during transcription, in order to stimulate transcriptional elongation (Hengartner C.J. *et al.*, 1998).

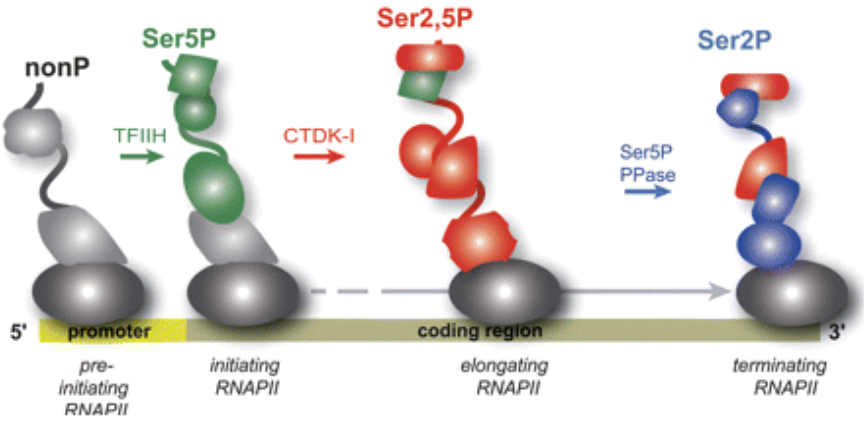


Fig.12- Different phosphorylations of RNA Pol II CTD during transcription initiation and elongation process
(Phatnani H.P. and Greenleaf A.L. 2006)

1.4 START execution

Although the binding of co-activators and of SBF/MBF complexes to G1-specific promoters is necessary, it is not sufficient for transcriptional activation (Koch C. *et al.*, 1993; Cosma M.P. *et al.*, 2001). In fact, transcriptional activation occurs only in late G1 phase as a consequence of Cdk1 activation (Tyers M. *et al.*, 1992; Stuart D. and Wittenberg C. 1994; Dirick L. *et al.*, 1995;Cosma M.P. *et al.*, 2001).

During G1 phase cells increase their size until they reach a critical cell size (called Ps) which allows the execution of START. This size depends on nutritional conditions, since cells grown in rich medium (glucose) show a bigger cell size than cells grown in poor medium, such as the non-fermentable carbon source ethanol (Alberghina L. *et al.*, 2004). Remarkably, due to the asymmetric cell division of budding yeast, newborn cells (the small ones) have to grow more than mother cells before being able to overcome the cell size checkpoint, differently, larger mother cells can reach the critical cell size earlier. Since the duration of the budded phase is constant for all generations, what differs from mothers and daughters is the duration of the G1 phase, as represented in Fig. 13.

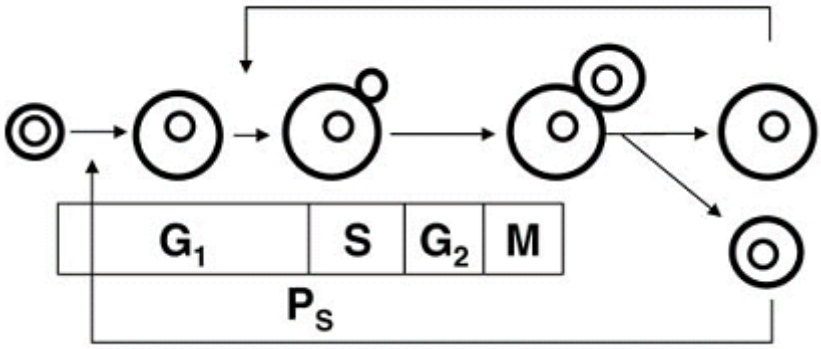


Fig.13- The asymmetrical division of the budding yeast *Saccharomyces cerevisiae* (Barberis M. *et al.*, 2007).

The G1 cyclin Cln3, in combination with Cdk1, is a key regulator of START, and is critical for the size-dependent activation of SBF and MBF (Tyers M. *et al.*, 1992). Consistently, when Cln3 level is increased, or when Cln3 is expressed in a stable form cells divide at a size smaller than *wild-type* cells and G1 phase become shorter. On the contrary, deletion of *CLN3* gene delays START and leads to an increased cell division size (Cross F.R., 1988; Tyers *et al.*, 1992; Futcher B. *et al.*, 1996).

Cln3 levels depend on nutritional conditions; they are high when fermentable carbon sources are present in the medium (glucose) and low in media containing non-fermentable carbon sources such as glycerol or ethanol (Hall D.D. *et al.*,1998; Parviz F. *et al.*, 1998). In particular, the induction of Cln3 expression mediated by glucose is stimulated by glycolisis, in fact, the 2-deoxyglucose, an analog of glucose transported into the cell and phosphorylated in the initial step of glycolysis as glucose but unable to be further metabolized, prevents *CLN3* induction (Parviz F. *et al.*, 1998).

Interestingly, the amount of Cln3 is regulated by different molecular patways. In response to cAMP levels, PKA pathway regulates Cln3 translation (Hall D.D. *et al.*, 1998). On the contrary, the Snf1 pathway seems not to affect Cln3 level; in fact, *mig1Δ*, *snf1Δ* or *reg1Δ* have the same amount of Cln3 as a *wild type* strain (Parviz F. *et al.*, 1998).

Since the level of Cln3 is a crucial indicator which integrates signals regarding nutritional conditions and cell cycle progression, thus Cln3 is finely regulated both at transcriptional and at translational level.

Cln3 transcription is only mildly cell cycle regulated, with a peak in late M or early G1 (McInerny C.J. *et al.*, 1997). *CLN3* promoter shows a set of repeated AAGAAAAA elements recognized by the transcription factor Azf1. Mutations of those sequences in the *CLN3* promoter reduce its transcription and prevent glucose induction of *CLN3* expression (Parviz F. *et al.*, 1998; Newcomb L.L. *et al.*,2002). *CLN3* promoter presents also some ECB (Early cell-cycle box) elements which are recognized by the transcription factor Mcm1 (McInerny C.J. *et al.*,1997; Mai B. *et al.*, 2002).

In addition, *CLN3* mRNA shows an upstream open reading frame (uORF) encoding the tripeptide Met-Asp-Phe at position -315 in the 5' mRNA leader. This sequence has a complex function; it slightly stimulates Cln3 synthesis and cell division in rich media, but it inhibits those events in poor nutritional conditions (Polymenis M. and Schmidt E.V., 1997).

The integration of Cln3 level and START execution is still not perfectly defined.
In G1 phase the level of Cln3 increases proportionally to cell mass and Cln3 accumulates into the nucleus (Cross F.R. and Blake C.M., 1993). Since the size of the nucleus is constant during G1 phase, it has been proposed that cells pass START upon reaching a critical Cln3 concentration in the nucleus (Futcher B., 1996).
However, several evidences indicated that Cln3 cannot be the only determinant of the cell sizer mechanism, and it was suggested that a threshold, involving an activator (Cln3) and an inhibitor, could control entry into S phase (Alberghina L. *et al.*, 2004). This inhibitor was proposed to be Far1, a Cdk1/cyclin inhibitor involved in the cell cycle arrest mediated by pheromone (Alberghina *et al.*, 2004). During G1 phase the level of Cln3 increases, on the contrary the levels of Far1 remain constat. When the level of Cln3, localized into the nucleus, overcomes the amount of Far1 the threshold of cell cycle is passed and Cln3/Cdk1 complexes can activate SBF- and MBF-dependent transcription (Alberghina *et al.*, 2004).
Besides Far1, others molecules were proposed as Cln3 inhibitors. In fact, it has been shown that in early G1 phase Cln3 is retained into the endoplasmic reticulum (ER), bound to the Ssa1,2 chaperones. In late G1, then, Cln3 is released form the ER by Ydj1 ATPase activation, leading to the nuclear accumulation of Cln3. Since chaperone levels increase as cells grow in G1 phase, it has been proposed that Ydj1 could be the critical regulator of START execution (Vergés E. *et al.*, 2007).
At the end, more recently another regulation mechanism has been indicated by Futcher and co-workers. Considering that Cln3 was

found at *CLN2* promoter they hypothesized that the binding of Cln3 to SBF- and MBF-dependent promoters might be the critical size requirement for START. In particular, they proposed that in early G1 phase Cln3 levels are low and this cyclin is not able to interact with the great number of SBF- and MBF- binding sites present in the yeast genome (around 400). Yet the number of SBF- and MBF- binding sites is fixed, whereas the number of Cln3 molecules increases during G1 phase; thus, at a certain point Cln3 could reach a proper level in order to interact with all SBF- and MBF-binding sites determining the passage of START (Wang H. *et al.*, 2009).

As transcriptional activator the role of Cln3 is to target Cdk1 to phosphorylate Whi5, the transcriptional inhibitor which interacts with SBF complexes and represses G1-specific transcription by recruiting histone deacetylases (HDACs) Rpd3 and Hos3 which work synergistically to inhibit G1-gene expression (Stephan O. and Koch C., 2009; Huang D. *et al.*, 2009). In late G1 phase, Cdk1/Cln3 complexes phosphorylate Whi5 and promote the dissociation of Whi5 from the transcription factor SBF and MBF (Costanzo M. *et al.*, 2004; de Bruin R.A. *et al.*, 2004). As for Swi6, also Whi5 after the phosphorylation exits from the nucleus; the exclusion from the nucleus requires the karyopherin Msn5, which recognizes specific phosphorylated residues (Taberner F.J. *et al.*, 2009). On the other hand, the nuclear accumulation of Whi5 depends on its dephosphorylation which is mediated by the phosphatase Cdc14 (Taberner F.J. *et al.*, 2009).
Remarkably, activation of SBF complexes leads to the accumulation of Cln1/Cdk1 and Cln2/Cdk1 complexes which then act on Whi5 to establish a positive feedback loop that gives coherence to the G1/S transition (Skotheim J.M. *et al.*, 2008). Later in the cell cycle, the inactivation of this transcriptional wave take place when Clb2/Cdk1 complexes phosphorylate and inactivate SBF (Koch C. *et al.*, 1996) and the inhibitor Nrm1 binds MBF complexes determining turning them off (de Bruin R.A. *et al.*, 2006).

Besides this well known function of Cln3, some evidences show that Cln3 can affect size also in a *Δwhi5* strain (Costanzo M. *et al.*, 2004), suggesting that, apart from Whi5, other Cln3 substrates could be involved in G1-specific transcription. Analyses aimed at found others possible inhibitor of SBF complexes identified Pho23 (a component of the histone deacetylase Rpd3L complex), Cdh1 (component of the SAGA complex) and Stb1 (Wang H. *et al.*, 2009)
In particular, Rpd3 and Sin3 (the main components of the Rpd3L complex) were recruited at *CLN2* promoter in a Swi6-dependent manner and deletion of *RPD3* or *SIN3* was able to rescue the lethal phenotype of a *Δcln3Δbck2*. Moreover, Sin3 and Rpd3 were lost after *CLN3* induction, suggesting that besides counteracting the inhibitory function of Whi5, Cln3 is also involved in the rescue of the deacetylase-dependent inhibition of SBF complexes (Wang H. *et al.*, 2009).
In addition, the observation that a *whi5Δstb1Δ* strain is not responsive to alterations of Cln3 levels suggests that Stb1 constitutes another regulatory pathway by which Cln3/Cdk1 complexes modulate the G1-specific transcription (Wang H. *et al.*, 2009).

Despite its delayed G1/S transition, *cln3Δ* strain is viable because alternative proteins guarantee the transcription of *CLN1* and *CLN2* genes. Apart from Cln3, the most important protein that can activate G1-specific transcription is Bck2. A *cln3Δbck2Δ* strain is barely viable because of severe defects in the expression of G1-specific genes; in fact, overexpression of *CLN2* in this background rescues the defective phenotype (di Como C.J. *et al.*, 1995). In particular, the mechanism by which Bck2 might activate *CLN1* and *CLN2* expression is still largely unknown. Nevertheless, it has been observed that Bck2 is involved in the regulation of cell-cycle genes at different phases and that it regulates G1-specific transcription independently from protein Swi6, thus suggesting that the function played by Bck2 is fundamentally different from that of Cln3 protein (Ferrezuelo F. *et al.*, 2009).

Another regulator of G1-gene expression is the Cyclin-dependent kinase Pho85. In fact, *cln3Δpho85Δ* cells arrest in G1 phase with an unreplicated DNA; deletion of *WHI5*, as well as deletion of the HDAC *HOS3* or *RPD3*, rescues the growth defect of this background (Huang 2009). In addition, it has been observed that, through its regulatory cyclin Pcl9, Pho85 is recruited to SBF-dependent promoters (such as *CLN2*) where it binds Whi5. All together those data support the notion that Pho85/Pcl9 complexes could activate G1-specific expression counteracting the inhibitory activity of Whi5 and Rpd3L complex (Huang D. *et al.*, 2009).

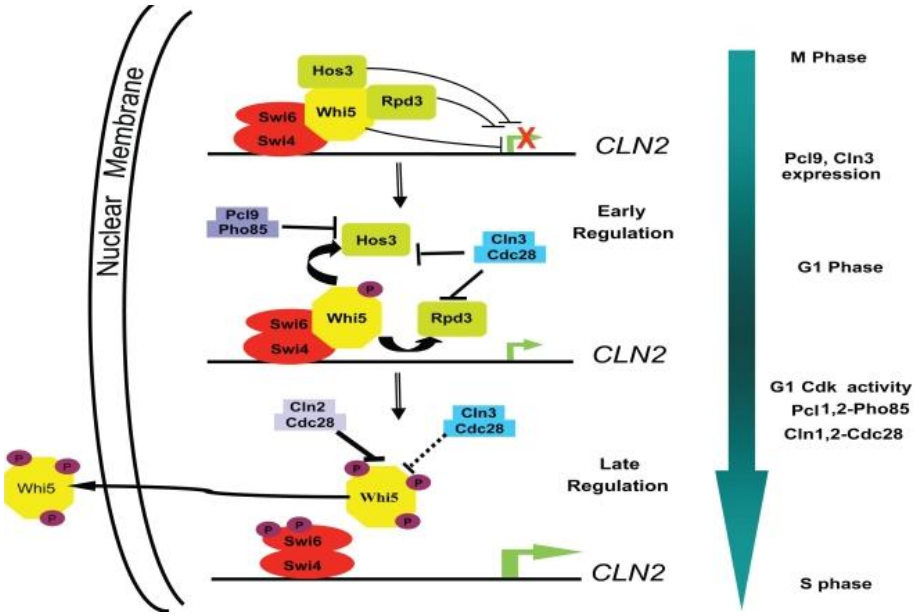


Fig.14- Model for CDK-dependent regulation of Whi5 activity and G1/S-specific transcription (Huang D. *et al.*, 2009)

1.5 Principal proteins expressed in G1-phase

At START, the expression of more than 200 genes takes place. In particular, some of them codifies for fundamental regulators of cell cycle progression.

1.5.1 G1 cyclins: Cln1/Cln2 and Pcl1/Pcl2

Late in G1 phase, SBF complex drives the expression of *CLN1* and *CLN2* genes, which encode Cdc28 cyclins, and *PCL1* and *PCL2*, that encode Pho85 cyclins (Ferrezuelo F. *et al.*, 2010). Those cyclins promote post-START processes such as bud formation, Spindle Pole Body duplication and degradation of Clb5,6/Cdk1 complexes inhibitor Sic1 (Dirick L. *et al.*, 1995; Moffat J. and Andrews B., 2004). On the contrary, they seem not involved in others S phase events such as DNA replication (Moffat J. and Andrews B., 2004). Consistently with their critical role in cell cycle progression, a strain lacking the four G1-cyclins (*cln1Δcln2Δpcl1Δpcl2Δ*) is inviable (Measday V. *et al.*, 1994). This strain can be kept alive by the conditional expression of Pcl2 from a *GAL1* promoter and when the expression of this cyclin is inhibited cells arrest with severe defects in cell morphology , while DNA is correctly replicated (Moffat J. and Andrews B., 2004), indicating that the role in bud emergence is the central function of G1-cyclins.

Although those proteins seem to play redundant roles, the main G1-cyclins are Cln1 and Cln2, in fact *cln1Δcln2Δ* strain shows growth defects and aberrant morphology (Hadwiger J.A. *et al.*, 1989), consistent with a delayed bud emergence (Stuart D. And Wittenberg C., 1995). Cln1 and Cln2 are very similar proteins, with an identity of 75% at sequence level, and are functionally redundant. Nevertheless, neither *CLN1* deletion nor *CLN1* overexpression cause any significant phenotype. On the contrary, loss of Cln2 leads to an increased size and a delayed bud formation and overexpression of *CLN2* causes a

reduction of cellular size (Queralt E. and Igual J.C., 2004). Thus Cln2 seems to play the primary role in the control of budding, whereas Cln1 become relevant only whenever Cln2 is absent.
In the absence of both Cln1 and Cln2, Pcl1 and Pcl2 become essential (Measday V. *et al.*, 1994).

It is known that Cln1,2/Cdc28 complexes regulate bud formation promoting the polarization of cortical actin cytoskeleton to form the pre-bud site; an event, that take place 10 minutes after START and depends on Cln2 levels (Lew D.J. and Reed S.I., 1993). However, the role of Cln1,2/Cdc28 and Pcl1,2/Pho85 complexes in the regulation of bud emergence is still not completely defined.
Literature data suggest a role for those complexes in the regulation of Cdc42, a central regulator of bud emergence, whose activation is essential for polarization of growth. In fact, both Pcl1 and Pcl2 localize to the bud neck, where Cdc42 is present (Moffat J. and Andrews B., 2004) and *CLN2* (but not *CLN1*) become essential for viability when *CDC42* is deleted (Queralt E. and Igual J.C., 2004).
Cdc42 is a Rho-type GTPase which is regulated by the antagonistic activity of the activatory GEFs (Guanine-nucleotide exchange factors) and the GAPs (GTPase-activating proteins), which determine its inactivation. The GEF of Cdc42 is Cdc24; whereas, the GAPs are Rga1, Rga2, Bem1 and Bem2 (Pruyne D. *et al.*, 2004).
Cln1,2/Cdc28 and Pcl1,2/Pho85 complexes influence the activation of Cdc42. In fact, in early G1 phase Cdc24 is sequestered into the nucleus via the physical interaction with Far1 (Shimada Y. *et al.*, 2000). Then, Cln1,2/Cdc28 complexes phosphorylate and trigger the degradation of Far1, allowing the exit from the nucleus of Cdc24 which can localize to the bud neck where Cdc42 is present (Nern A. and Arkowitz R.A.., 2000). In addition, also the inhibition of Cdc42 is regulated by G1-cyclins associated to CDKs. In fact, it has been recently demonstrated that the GAP Rga2 is substrate of Cln1,2/Cdc28 and Pcl1,2/Pho85 complexes, which phosphorylate and inhibit it, promoting Cdc42 activation (Sopko R. *et al.*, 2007).

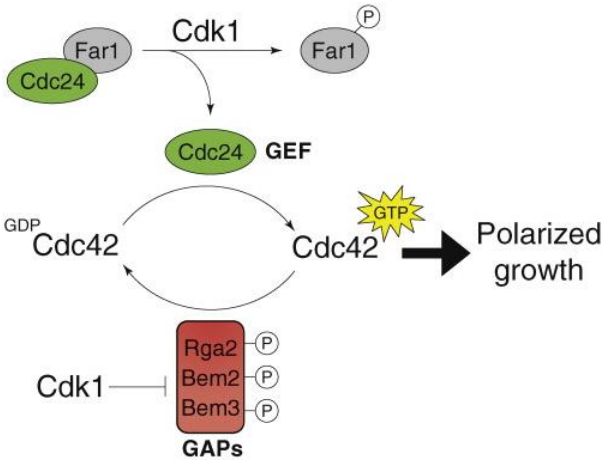


Fig.15 - Cdk1 promotes polarized growth by increasing Cdc42 GEF activity and inhibiting GAPs (Moseley J.B. and Nurse P., 2009)

1.5.2 B-type cyclins: Clb5 and Clb6

CLB5 and *CLB6* are MBF-dependent genes (Ferrezuelo F. *et al.*, 2010) and thus are expressed at START. Those genes codify for Clb5 and Clb6, which are defined as B-type cyclins since they present a conserved sequence of 118 amino acids (Kühne C. and Linder P., 1993; Schwob E. and Nasmyth K., 1993).

Once expressed, those proteins associate with Cdk1, but they are inhibited by Sic1; only after Sic1 degradation the Cdk1/Clb5,6 complexes are active and could regulate S phase events (Alberghina L. *et al.*, 2004). Clb5 and Clb6 are involved in the regulation of DNA replication and the deletion of both of them (*clb5Δclb6Δ*) causes defects in S phase entrance and duration (Schwob E. and Nasmyth K., 1993). Remarkably, those proteins plays different but correlated functions. Either Clb5 and Clb6 promotes the onset of DNA replication and S phase entrance; whereas, only Clb5 is required to regulate the progression through S phase (Schwob E. and Nasmyth K., 1993; Kühne C. and Linder P., 1993). In fact, in a *clb5Δ* strain there is

a defective firing of the late origins, a defect not detectable in *clb6Δ* cells (Donaldson A.D. *et al.*, 2000). Consistently, Clb6 degradation is determined by SCF^{Cdc4} (Skp1-Cdc53/Cullin-F box protein) complex and takes place just after the G1/S transition, whereas degradation of Clb5 is mediated by the APC^{Cdc20} (Anaphase Promoting Complex) and happens only during mitosis (Jackson L.P. *et al.*, 2006).
As regulators of DNA replication, Clb5 and Clb6 promote the recruitment of DNA Polymerase to the ARS (Autonomously Replicating Sequences). DNA Polymerase, associating with protein Sld2, forms the pre-Loading Complex (pre-LD) which, then, binds protein Dpb11. Dpb11 is associates also with Sld3, a protein posed at ARS; thus, Dpb11 mediates the recruitment of DNA polymerase to ARS. The formation of Sld2-Dpb11-Sld3 complex marks the beginning of S phase (Zagerman P. and Diffley J., 2007). All those events are regulated by Clb5,6/Cdk1 complexes. In fact, those complexes phosphorylate Sld2 on 6 residues and, in particular, on Thr84 promoting its interaction with Dpb11 (Masumoto H. *et al.*, 2002; Tak Y. *et al.*, 2006). Moreover Clb5,6/Cdk1 complexes phosphorylate Thr600 and Ser622 residues of Sld3, positively regulating its association with Dpb11 (Tanaka S. *et al.*, 2007; Zagerman P. and Diffley J., 2007).

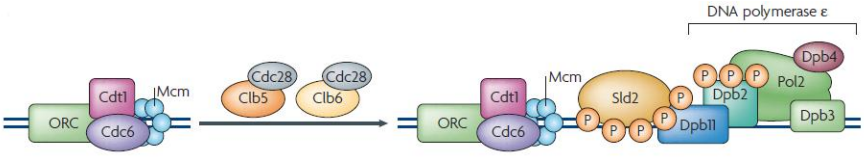


Fig.16- Cyclin control of DNA replication
(Bloom J. and Cross F.R 2007)

Clb5,6/Cdk1 complexes also regulate disassembly of the Pre-replication Complex (pre-RC). The pre-RC, which binds ARS, is composed by: the ORC complex (ORC1-6), some members of the MCM (MiniChromosome Manteinance proteins) family such as Mcm2-7, Cdc6 and Cdt1. Clb5/Cdk1 complexes phosphorylate Cdc6

and Mcm2-7 leading to their degradation and, thus, preventing a second round of DNA replication in the same mitotic cycle (Nguyen V.Q. *et al.*, 2000; Drury L.S. *et al.*, 2000).

1.5.3 RNR genes

Between genes of the G1-regulon, different regulators of DNA replication are present. In fact, MCB elements (recognized by Mbp1) are found in the promoters of many cell cycle-regulated genes involved in DNA replication such as *CDC9* (DNA ligase), *POL1* (DNA Polymerase 1), and *RNR* genes (Toyn J. H. *et al.*, 1995). In addition to being cell cycle regulated, the expression of some MBF-controlled genes (such as *CDC9*, *POL1* and *RNR1*) is also induced by DNA damage (Ho Y. *et al.*, 1997).

Both DNA replication and DNA repair after damage require deoxyrebonucleotides (dNTPs) as building blocks to maintain genomic integrity. The rate-limiting enzyme in the production of dNTPs is Ribonucleotide reductase (RNR), which catalyzes the conversion of precursor ribonucleotide diphosphates into its deoxy-form (Elledge S.J. *et al.*, 1993). In budding yeast, RNR is a tetrameric complex composed by two large subunits of Rnr1 and two small subunits, Rnr2 and Rnr4 respectively. The Rnr1/Rnr1 homodimer contains the regulatory and catalytic sites, and the Rnr2/Rnr4 heterodimer presents the essential diferric-tyrosyl radical cofactor (Xu H. *et al.*, 2006).
Consistently with the cell cycle modulated expression of *RNR* genes, ribonucleotide reductase activity is periodic during the cell cycle, rising from an initial low level to a maximum early in S phase, then declining at the end of S phase (Lowdon M. and Vitols E., 1973). In S phase, this enzyme is essential; in fact the inhibition of ribonucleotide reductase activity by hydroxyurea treatment causes the arrest of cells in S phase as large-budded, uninucleate cells (Wang P.J. et al., 1997). Thus, this enzyme plays a critical role in the regulation of DNA synthesis and so of cell cycle progression.

2. Protein kinase Snf1

Cell growth and proliferation require a high amount of energy for biosynthetic pathways. Cells take energy from nutrient intake and both unicellular and multicellular organisms have evolved systems that allow dynamic sensing of energy sources, mainly sugars. The class of Snf1/AMPK (Sucrose non-fermenting/AMP-activated protein kinase) is a central element playing a key role as a guardian of cellular energy (Hardie D.G., 2007). Proteins of this family are Serine/Threonine kinases highly conserved in eukaryotes. The primary role of those proteins is the integration of signals regarding nutrient availability and environmental stresses, ensuring the adaptation to those conditions and cell survival (Hardie D.G., 2007; Ghillebert R. *et al.*, 2011).
The *Saccharomyces cerevisiae* protein kinase Snf1 is a member of the Snf1/AMPK family and regulates the expression of several genes involved in metabolic pathways, which permit adaptation to glucose limitation or growth on carbon sources different from glucose, such as the alternative fermentable carbon sources (sucrose, maltose, galactose) or the non-fermentable ethanol and glycerol. In the presence of those nutrients, Snf1 is activated and regulates the expression of genes involved in central metabolic functions, such as gluconeogenesis and respiration. Moreover, Snf1 results also involved in the response to numerous cellular stresses and in the regulation of cellular processes (reviewed in Hedbacker K. and Carlson M., 2008; Smets B. *et al.*, 2010).

2.1 Snf1 structure

Protein kinase Snf1 in yeast exists as a heterotrimeric complex made by the catalytic α subunit (Snf1) and the regulatory β (Gal83, Sip1 and Sip2) and γ (Snf4) subunits (Hedbacker K. and Carlson M., 2008).

2.1.1 The α subunit: Snf1

The catalytic α subunit (encoded by *SNF1* gene) was firstly identified in a screening of mutant cells unable to growth in presence of sucrose, or on non-fermentable carbon sources such as glycerol and ethanol (Carlson M. *et al.*, 1981). The Snf1 subunit is a constitutively expressed 633 amino acid protein which comprise a kinase domain near the N-terminus and a C-terminal regulatory region.

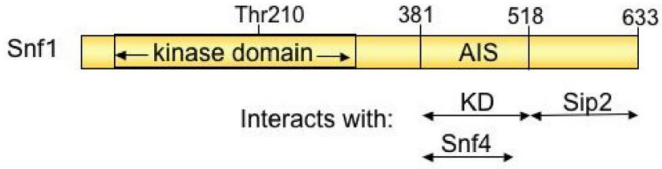


Fig.17- Structure of the α subunit Snf1; in the figure the autoinhibitory sequence (AIS) is shown. Arrows indicate regions mapped by deletion analysis as sufficient for interaction with kinase domain (KD), Snf4 or Sip2, as indicated (Hedbacker K. and Carlson M., 2008)

The first N-terminal amino acids (18-30 aa) show an histidine rich domain (13 histidine residues), that allows the purification of Snf1 (Celenza J.L. and Carlson M.,1989). The N-terminal domain of Snf1 (30-138 aa) then presents the canonical structure of kinase domains: a small lobe made by β-strands which interacts with a big lobe made by α-helixs. In this domain the ATP binding site is present; in particular, it is centered on Lys84, a residue whose mutation into Arginine determines a severe decrease of the Snf1 catalytic activity (Celenza J.L. and Carlson M.,1989). This kinase domain also includes a

disordered activation loop (200-215 aa), in which the activatory Thr210 residue is present (Rudolph M.J. *et al.*, 2005). Differently, in the C-terminal domain of Snf1 there is a short autoinhibitory sequence (AIS) (380-415 aa) and the domain which mediates the interactions with the β subunits of the complex, such as Sip1, Sip2 and Gal83. The autoinhibitory domain presents 3 α-helix and interacts with both the regulatory subunit Snf4 and the kinase domain of Snf1. The interaction with Snf4 relieves the inhibition of the AIS allowing the phosphorylation of Thr210 residue of Snf1 that determines its activation (Rudolph M.J. *et al.*, 2005; Chen L. *et al.*, 2009).

2.1.2 The β subunits: Sip1, Sip2 and Gal83

The *S. cerevisiae* genome encodes three β subunits, Sip1, Sip2, and Gal83 (Erickson J.R. and Jhonston M., 1993). The β subunits are fundamental for the activity of Snf1 complex. In fact, a *sip1Δsip2Δgal83Δ* strain presents growth defects in presence of glycerol or ethanol and is unable to phosphorylate Snf1 targets such as Mig1 (Schmidt M.C. and McCartney R.R., 2000).
Those subunits contain a conserved C-terminal sequence in which two domains are present: the KIS domain (Kinase Interacting Sequence) that mediates the interaction with the α-subunit Snf1 (Yang X. *et al.*, 1994) and the ASC domain (Association with SNF1 kinase complex) that allows the interaction with Snf4 (Jiang R. and Carlson M., 1997). Differently, the N-terminal sequence is specific for each β subunit and confers a different subcellular localization patterns to each protein.
All three proteins are cytoplasmic in presence of high glucose concentrations. Upon glucose depletion, Sip1 relocalizes to the vacuolar membrane, Gal83 relocalizes to the nucleus, and Sip2 remains cytoplasmic (Vincent O. *et al.*, 2001). Thus, the role of the β subunits is to interact with Snf1 and to modulate its subcellular localization (Vincent O. *et al.*, 2001; Hedbacker K. *et al.*, 2004).

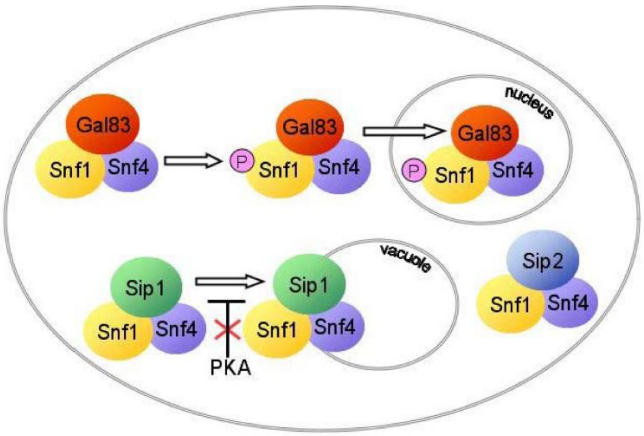


Fig.18 - Snf1 β-subunits determine the subcellular localization of the Snf1 complex (Hedbacker K. and Carlson M., 2008)

Remarkably, the β subunits exhibit various differences. Protein Sip1 alone (*gal83Δsip2Δ* strain) is not able to sustain growth on glycerol or ethanol and determines a very low kinase activity for the Snf1 complex (Nath N. *et al.*, 2002). However, it is known that this protein plays a fundamental role; in high glucose it is retained into the cytoplasm by PKA, differently, in low glucose concentrations Sip1 localizes to the vacuolar membrane thank to a myristilation sequence present in its N-terminal domain (Hedbacker K. *et al.*, 2004).
The β-subunit Sip2 remains in the cytoplasm and its function seems to be involved in the mechanism of cellular ageing (Ashrafi K. *et al.*, 2000).
Gal83 seems to play the main role in the regulation of Snf1 function. In fact, when Gal83 is the only β-subunit (*sip1Δsip2Δ* strain) Snf1 shows a high kinase activity (75% respect to the *wild type* strain). In low glucose then Gal83 determines the nuclear localization of the Snf1 complex thank to its NLS (Nuclear localization sequences); on the contrary, NES (Nuclear Export Signals) present in the sequence of Gal83 allows the exit from the nucleus when high glucose concentrations are present (Hedbacker K. and Carlson M., 2006). In addition, Gal83 mediates the interaction of Snf1 with some substrates,

such as the transcription activator Sip4 (Vincent O. and Carlson M., 1999), and possibly with the transcriptional apparatus (Kuchin S. *et al.*, 2000). More recently, it has also been shown that deletion of the glycogen binding domain (GBD) of Gal83 leads to a constitutive activation of Snf1 which results able to activate the expression of some Snf1-regulated genes (such as *SUC2*, *HXT1* and *PCK1*) also in high glucose concentrations. In addition, GBD interact with the Reg1/Glc7 phosphatase complex (Momcilovic M. *et al.*, 2008) Taken together those data suggest that Gal83 plays a dual role regulating nuclear localization of Snf1 in low glucose and supporting the inactivation of Snf1 in high glucose.

2.1.3 The γ subunit Snf4

The gene encoding the γ subunit, *SNF4*, was identified by isolation of a sucrose-nonfermenting mutant (Neigeborn L. and Carlson M., 1984). Snf4 is a constitutively expressed protein of 322 residues that binds the Snf1 and β subunits of the Snf1 complex (Celenza J.L. and Carlson M., 1989; Jiang R. and Carlson M., 1997). The role of Snf4 is to relieve the inhibition of Snf1 interacting with its AIS domain, ensuring the complete activation of Snf1 (Leech A. *et al.*, 2003). In fact, *SNF4* deletion causes a decreased kinase activity of Snf1, whereas deletion of the AIS domain of Snf1 fully complement the phenotype of a *snf4Δ* strain (Celenza J.L. and Carlson M., 1989; Leech A. *et al.*, 2003). Remarkably, the activating phosphorylation of Thr210 residue of Snf1 is still detectable in a *snf4Δ* strain (McCartney R.R. and Schmidt M.C., 2001) and in high glucose Snf4 seems to be required for the proper inactivation of Snf1 mediated by the phosphatase complex Reg1/Glc7 (Momcilovic M. e*t al.*, 2008). Thus, these findings indicate that Snf4 plays a complex role in the regulation of Snf1, a role that is not completely understood.
In addition, the observation that Snf4 localizes into the nucleus also in presence of high glucose concentrations, suggests a possible role for Snf4 independently from Snf1 (Vincent O. *et al.*, 2001)

2.2 Snf1 activation

Snf1 complex is activated through the following two steps: the association of the α-subunit Snf1 with the γ subunit Snf4, necessary to counteract the autoinhibition of Snf1, and the phosphorylation of the Thr210 by the three upstream kinases Sak1, Tos3 and Elm1 (Hong S.P. *et al.*, 2003; Sutherland C.M. *et al.*, 2003). This phosphorylation is essential for Snf1 activity; in fact the *sak1Δtos3Δelm1Δ* strain shows the same phenotype of a *snf1Δ* strain, such as growth defects in presence of limiting glucose or carbon sources like glycerol or ethanol (Hong S.P. *et al.*, 2003). Thus, Snf1 phosphorylation on Thr210 is commonly considered a marker for Snf1 activation. On the contrary, in response to high glucose concentrations, Snf1 is inactivated through the action of the Glc7 protein phosphatase (also called protein phosphatase 1, PP1), which is targeted to Snf1 by the regulatory subunit Reg1 and dephosphorylates Thr210 (Ludin K. *et al.*, 1998; Sanz P. *et al.*, 2000). Protein Reg1 interacts both with Glc7 and Snf1 when glucose is largely available in the culture medium and loss of Reg1 (*reg1Δ*) leads to the constitutive activation of Snf1 (Frederick D.L. and Tatchell K., 1996; Huang D. *et al.*, 1996; Rubenstein E.M. *et al.*, 2008).

Moreover, in high glucose concentrations, Hxk2 (Hexokinase 2) regulates the activity of PPI an thus the activation of Snf1 kinase (Sanz P. *et al.*, 2000).

Therefore, on the base of those data it has been proposed a model in which the three upstream kinases Sak1, Tos3 and Elm1 are active independently from the glucose concentration in the culture medium; whereas the nutrient-dependent activation of Snf1 is mainly regulated through the Glc7/Reg1-mediated dephosphorylation of Thr210 residue.

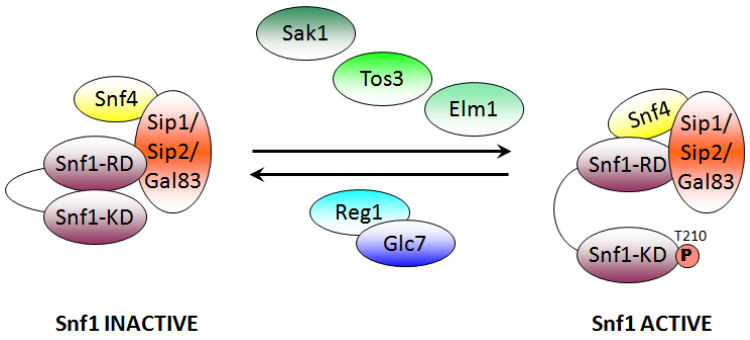


Fig.19- Snf1 activation requires both the phosphorylation of Thr210 residue in the Kinase Domain (Snf1-KD) by Sak1, Tos3 and Elm1 kinases, and the interaction of the Regulatory Domain (RD) with Snf4

Although Snf1 phosphorylation is a key step of its activation, several data support the notion that a non-phosphorylatable Snf1 mutant (*SNF1-T210A*) still keeps a low catalytic activity, giving a partial rescue of different *snf1Δ* phenotypes such as sensitivity to *o*-dinitrobenzene, Higromycin B or UV irradiation. Only the mutation of the invariant Lysine which constitutes the ATP binding site in the kinase domain (*SNF1-K84R*) mimics loss of Snf1 protein causing sensitivity to those compounds (Portillo F. *et al.*, 2005; Shinoda J. and Kikuchi Y. 2007; Wade S.L. *et al.*, 2009).

Differently from its mammalian homolog AMPK, protein kinase Snf1 is not allosterically activated by AMP (Adenosine MonoPhosphate) nucleotides (Wilson W.A. *et al.*, 1996). However, recently it has been demonstrated that ADP (Adenosine DiPhosphate) molecules are able to bind the subunit Snf4 preventing Snf1 dephosphorylation mediated by Glc7 (Mayer F.V. *et al.*, 2011; Chandrashekarappa D.G. *et al.*, 2011). Moreover, another regulation of Snf1 activity has been recently proposed. In fact, Dent's group recently demonstrated that Ubp8, one of the subunits of the SAGA complex, interacts with Snf1 and deubiutinate it, positively influencing its activity (Wilson M.A. *et al.*, 2011).

2.3 Snf1 functions

As previously mentioned, in yeast Snf1 is involved in the regulation of metabolism; in fact, it is required for the expression of genes that permit cell growth in presence of fermentable carbon sources different from glucose (such as sucrose, galactose, maltose) or non-fermentable carbon sources (ethanol, glycerol).
Once activated Snf1 phosphorylates the transcriptional inhibitor Mig1 promoting the expression of genes such as those involved in the metabolism of maltose (*MAL* genes), galactose (*GAL* genes) or sucrose (*SUC2* gene which codify for the invertase, the enzyme which catalyze the hydrolysis of sucrose into a molecule of glucose and a molecule of fructose) (Carlson M., 1999; Schüller H., 2003). In addition, through the regulation of the transcription factors Cat8 and Sip4, Snf1 modulates the expression of gluconeogenic genes (Vincent O. and Carlson M., 1998); whereas in response to amino acid starvation Snf1 regulates the transcription activator Gcn4 and promotes the expression of amino acid biosynthetic genes (Shirra M.K. *et al.*, 2008).
As part of its function in controlling cellular energy status, protein Snf1 phosphorylates and inactivates acetyl-CoA carboxylase enzyme (Acc1) inhibiting fatty acid biosynthesis during glucose-limiting conditions (Woods A. *et al.*, 1994).

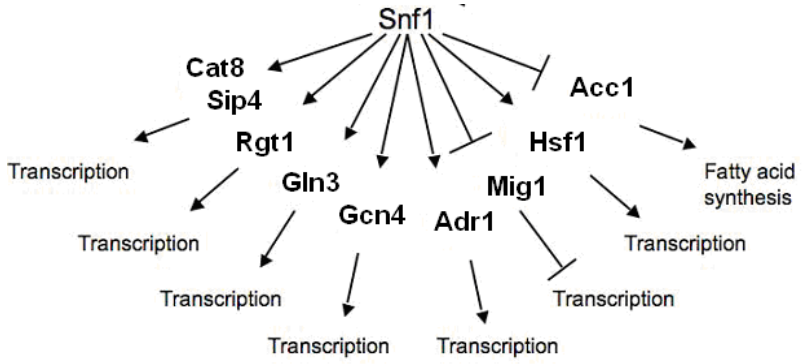


Fig.20- Snf1 substrates

Besides nutritional stresses, Snf1 is also involved in the response to different cellular stresses. In fact, *snf1Δ* cells are sensitive to genotoxic stresses caused by cadmium (Thorsen M. *et al.*, 2009), hygromycin B (Portillo F. *et al.*, 2005) or Hydroxyurea (Dubacq C. *et al.*, 2004). However, the mechanism used by Snf1 to regulate the response to those stresses is still unknown. On the contrary, for the resistance to osmotic or alkaline stresses (pH6-8) it has been determined that Snf1, once activated, counteracts the activity of the transcriptional inhibitor Nrg1, promoting the expression of *ENA1*, which codify for a Na+-ATPase responsible for Na+ ions detoxification (Platara M. *et al.*, 2006; Ye T. *et al.*, 2008). Snf1 also regulates HSF (Heat Shock transcription Factor), ensuring the cellular resistance to high temperatures (Hahn J. and Thiene D.J., 2004; Hong S. and Carlson M., 2007).

In addition, protein kinase Snf1 is also a regulator of various nutrient-responsive cellular processes. Snf1 is involved in the connections between energy metabolism and aging; overexpression of Snf1 and the absence of the β subunit Sip2 promote aging, while deletion of *SNF4* extends cell life span (Ashrafi K. *et al.*, 2000; Lin S.S. *et al.*, 2002). Snf1 is a positive regulator of autophagy, a process for recycling organelles and macromolecules (Wang Z. *et al.*, 2001). Moreover it modulates both diploid pseudohyphal growth and haploid filamentous invasive growth in response to glucose limitation (Cullen P.J. and Sprague G.F., 2000; Palacek S.P. *et al.*, 2000; Kuchin S. *et al.*, 2002).
Protein kinase Snf1, then, modulates meiosis. In fact, literature data shows that in response to nutrient depletion, Snf1 promotes the expression of *IME1* and *IME2* which encodes for master regulators of early and late stages of meiosis respectively (Honingberg S.M. *et al.*,1998).
Thus, several literature data support the notion that protein kinase Snf1, besides its critical role in the regulation of cellular metabolism, exerts also important functions in the regulation of different cellular processes.

2.4 Snf1-mediated transcriptional regulation

Snf1 regulates transcription of several genes through numerous way, such as activation of transcription factors or inhibition of transcription inhibitors, regulation of chromatin remodelling and perhaps direct modulation of the transcriptional apparatus. Having multiple roles in the activation of a single gene Snf1 provides cells with a finer control of gene expression in response to nutrient conditions and cellular stresses.

2.4.1 Regulation of transcription factors

Snf1 regulates several transcription factors. One of them, Mig1, is the most studied and constitute a good case of Snf1 regulation of transcription factors.
Mig1 is a transcriptional repressor which belongs to the GC box-binding proteins, a subfamily of evolutionary related zinc finger proteins. In fact, Mig1 binds the sequence [C/T]GG[G/A]G present in numerous promoters and represses the expression of various genes in presence of high glucose concentrations. Its action is inhibited by Snf1 which phosphorylates Mig1 both *in vitro* and *in vivo* (Ostling J. *et al.*,1996; Treitel M.A. *et al.*, 1998; Smith F.C. *et al.*,1999). In particular, it has been demonstrated that Mig1 is phosphorylated by Snf1 on 4 sites: Ser222, Ser278, Ser311, Ser381. Analyses of Mig1 proteins mutated in those sites (S-A) showed that *in vitro* those were the only sites phosphorylated by Snf1 (Smith F.C. *et al.*,1999) while the study of the phosphorylation status of Mig1 by immunoblot analysis showed that Mig1-4SA mutant was different than Mig1 *wild type* observed in a *snf1Δ* strain, suggesting that Mig1 was phosphorylated also on others sites (Treitel M.A. *et al.*, 1998; Ostling J. *et al.*,1998). Regulation of Mig1 function is mediated by Snf1, but different effects have been proposed. Since subcellular localization is regulated by glucose (Mig1 is present into the nucleus in high glucose and then it translocates to the cytoplasm in low glucose) and Mig1

interacts with the nuclear exportin Msn5 it has been supposed that regulation of Mig1 is mediated by its localization. In particular, Msn5 seems to recognize a long and unusual NES (nuclear exportation signal) after Mig1 phosphorylation mediated by Snf1 and to exclude Mig1 from the nucleus allowing glucose-repressed genes expression (DeVit M.J. and Johnston M., 1999). *MSN5* deletion abolishes its nuclear export but it does not affect neither Mig1 phosphorylation nor Mig1inhobition mediated by Snf1 (DeVit M.J. and Johnston M., 1999), suggesting that localization is not the only level of Mig1 regulation. Remarkably, loss of Snf1 causes a decreased nuclear association of the exporters of the importin-β family, among which Msn5, suggesting that it could regulate Mig1 localization also through an indirect way (Quan X. *et al.*, 2007).

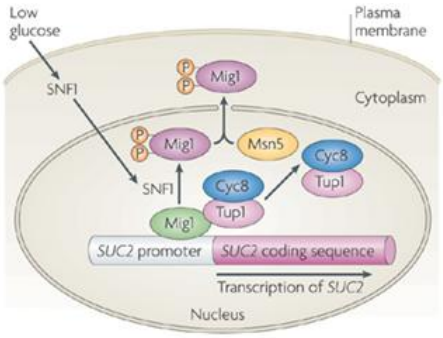


Fig.21 - Snf1-mediated inhibition of Mig1 and consequent expression of the Mig1-regulated gene *SUC2* (Hardie D.G., 2007)

Mig1 function requires the presence of two co-repressors: Cyc8(SSN6) and Tup1. Those repressors were found as inhibitors of *SUC2* gene expression, since loss of those proteins causes invertase derepression. In particular it has also been shown that Cyc8 and Tup1 work together to inhibit invertase expression (Williams F.E. and Trumbly R.J., 1990). Analyses of interaction between Cyc8/Tup1 and Mig1 showed that Cyc8/Tup1 are at *GAL1* promoter independently on

the presence of Mig1 and that also in low glucose Mig1 is localized at *GAL1* promoter suggesting that the Snf1-mediated phosphorylation on Mig1 causes the loss of Mig1 interaction with Cyc8, in addition to its exclusion from the nucleus (Papamichos-Choronaris M. *et al.*, 2004).
Finally, also Hxk2 is involved in this mechanism. Hxk2 is the protein that initiates the intracellular metabolism of glucose by phosphorylation at C-6, but it plays also a role in glucose repression; deletion of *HXK2* causes loss of glucose-repression on different genes. Hxk2 interacts both *in vitro* and *in vivo* with Mig1 which seems to sequestered Hxk2 into the nucleous; the Hxk2 decapeptide, previously identified as essential for Hxk2 nuclear localization, is required for such an interaction (Ahuatzi D. *et al.*, 2004). Moreover this interaction requires Mig1 phosphorylation on Ser311 and is strong in high glucose (Ahuatzi D. *et al.*, 2007).
So, Hxk2 interacts with Snf1 both in high and low glucose and regulates phosphorylation of Mig1 in fact in a *hxk2Δ* mutant Mig1 is phosphorylated also in high glucose, probably because in this mutant strain Snf1 is more active. Thus data suggest that Hxk2 protein functions as bridge between Snf1 and Mig1 and is required to inhibit Mig1 phosphorylation on Ser311 in high glucose growing cells (Ahuatzi D. *et al.*, 2007).

Besides Mig1, Snf1 regulates also others transcription factors.
Snf1 sustains dephosphorylation of Ser230 residue of the transcription factor Adr1, promoting the expression of genes involved in ethanol metabolism and in the fatty acids oxidation (Kacherovsky N. e*t al.*, 2008). In response to glucose limitations, Snf1 also activates the transcription factors Cat8 and Sip4 which regulate the expression of gluconeogenic enzymes (Vincent O. and Carlson M., 1998). In particular, Snf1 promotes the expression of Cat8-dependent genes promoting the expression of *CAT8* through the inactivation of Mig1 transcriptional inhibitor, as well as activating directly Cat8 through phosphorylation (Charbon G. *et al.*, 2004). Moreover, Snf1 inhibits the transcription factor Rgt1, determining the inactivation of *HXT1*

gene expression, a gene which codifies for a low affinity glucose transporter (Tomas-Cobos L. and Sanz P., 2002).
Snf1 seems to be also involved in the regulation of nitrogen metabolism. In the presence of low glucose concentrations Snf1 promotes the nuclear localization of Gln3, a transcriptional factor which regulates the expression of genes necessary for the metabolism of nitrogen sources different than glutamine (such as the less preferred glutamate) (Bertram P.G. *et al.*, 2002).
At the end, Snf1 regulates also the synthesis of Gcn4, the transcription factor which regulates genes involved in amino acids biosynthesis. The presence of a great amount of amino acids, protein kinase Gcn2 is activated and phosphorylates Ser51 of eIF2 (a specific translational inhibitor), promoting the inhibition of Gcn4 synthesis. Besides, glucose starvation leads to the activation of Snf1 which seems to inhibits the protein phosphatases Glc7 and Sit4 that physically interacts with eIF2, sustaining the inhibition of Gcn4 translation (Shirra M.K. *et al.*, 2008).

2.4.2 Regulation of chromatin remodelling

The covalent modification of histone tails is a crucial step in controlling gene expression. In particular, phosphorylation of Ser10 residue of Histone H3 is known to be a fundamental signal for gene expression, a signal highly conserved in all eukaryotes (Nowak S.J. and Corces V.G., 2004).
In budding yeast, protein kinase Snf1 phosphorylates Ser10 residue of histone H3, promoting its the acetylation on Lys14 residue by Gcn5, a component of SAGA complex (Lo W. e*t al.*, 2000 and 2001).

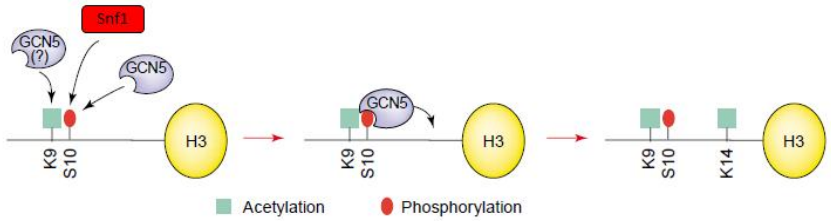


Fig. 22 - A simplified representation of the modifications of histone H3 N-terminal domains during gene activation.

Interestingly, this phosphorylation is detectable at different promoters, such as those of *INO1, HIS3* and *GAL1* genes. However, only at *INO1* promoter loss of Snf1 causes a severe decrease of H3 acetylation and SAGA complex recruitment, suggesting that Snf1-mediated regulation of histones involves only specific genes (Lo W.S. *et al.*, 2005). Recently, it has been demonstrated that the Snf1-mediated regulation of histone H3 is involved also in the expression of *ADY2* gene. In fact, Snf1 stimulates the binding of Gcn5 and the acetylation of histone H3 at *ADY2* promoter, promoting the transcription of this gene (Abate G. *et al*., 2012).

Besides histone H3, it has been shown that Snf1 also directly regulates Gcn5. Snf1 physically interacts and phosphorylates Gcn5 (Liu Y. e*t al.*, 2005). Analyzing the catalytic domain of Gcn5 three consensus sequences for AMPK/Snf1 (Φ-x-R-x-x-S/T-x-x-x-Φ, Φ = hydrophobic residue) were found: Thr203, Ser204, Thr211. Alteration of those residues caused a severe reduction of Gcn5 phosphorylation. Then, *in vitro* analyses showed that also Tyr212 was somehow phosphorylated in a Snf1-dependent way (Liu Y. *et al*., 2010). The mutation of those four residues into Alanine did not affect the recruitment of Gcn5, histone H3 or Snf1 to the promoter of *HIS3* gene; however, the *GCN5-TSTY/4A* mutant showed a severe decrease of H3 acetylation, consistent with a decreased expression of *HIS3* gene and a consequent hypersensitivity to 3-aminotriazole (3-AT), an inhibitor of histidine biosynthetic enzyme. Intriguingly, both the hypoacetylation of histone H3 and the transcription of *HIS3* gene were complemented by Snf1-overexpression (Liu Y. *et al*., 2010). Thus, on the base of those observations, it has been proposed that Gcn5 is not the sole component of PIC complexes regulated by Snf1, and that probably this protein kinase uses Gcn5 as docking site to reach promoters where it could phosphorylate other members of the PIC

2.4.3 Regulation of transcriptional apparatus

Snf1 is known to regulate the formation of a complete Pre-Initiation Complex at promoters. Literature data show that Snf1 physically interacts with three components of the Srb/Mediator complex (Srb10, Srb11 and Sin4) (Kuchin S. *et al.*, 2000) and that Snf1 deletion causes defects in the recruitment of some Mediator components (Srb2,4 and Med6) to promoters (Young E.T. *et al.*, 2002). Moreover also the recruitment to promoters of SAGA complex (van Oevelen C. *et al.*, 2006), TATA-binding protein (TBP) (Shirra M.K. *et al.*, 2005) and RNA Pol II (Tachibana C. *et al.*, 2007; Young E.T. *et al.*, 2012) are affected in a *snf1Δ* strain.
Besides, the regulation of PIC formation, it has been proposed that Snf1 could exert a direct role in the regulation of RNA Pol II activity. In fact, a Snf1 mutant characterized by an high kinase activity (*SNF1-G53R*) stimulates transcription through RNA Pol II holoenzyme (Kuchin S. *et al.*, 2000).

Recently, Young and co-workers (Young E.T. *et al.*, 2012) have revealed a new Snf1 function.
To analyze the function of the kinase activity of Snf1 they used a Snf1 mutant, called Snf1^{as} (*SNF1-I132G*) sensitive to 2-naphthylmethyl pyrazolopyrimidine 1 (2NM-PPI), which causes the inactivation of Snf1. Analyzing the expression of different Snf1-dependent genes, they showed that the inhibition of Snf1 activity causes a slight alteration of PIC formation; in fact, the binding of the transcription factor Adr1 and of the RNA Pol II as well as the hyperacetylation of histone H3 were still detectable at promoters of genes such as *ADH2* and *POX1*. Nevertheless, inhibition of Snf1 caused the inhibition of transcriptional process and enhanced the average rate of decay of several mRNA (such as those of *FDH*, *PCK1* and *FBP1*) 2-4 fold, suggesting that protein kinase Snf1 could act also at a post-transcriptional level to stabilize transcripts.

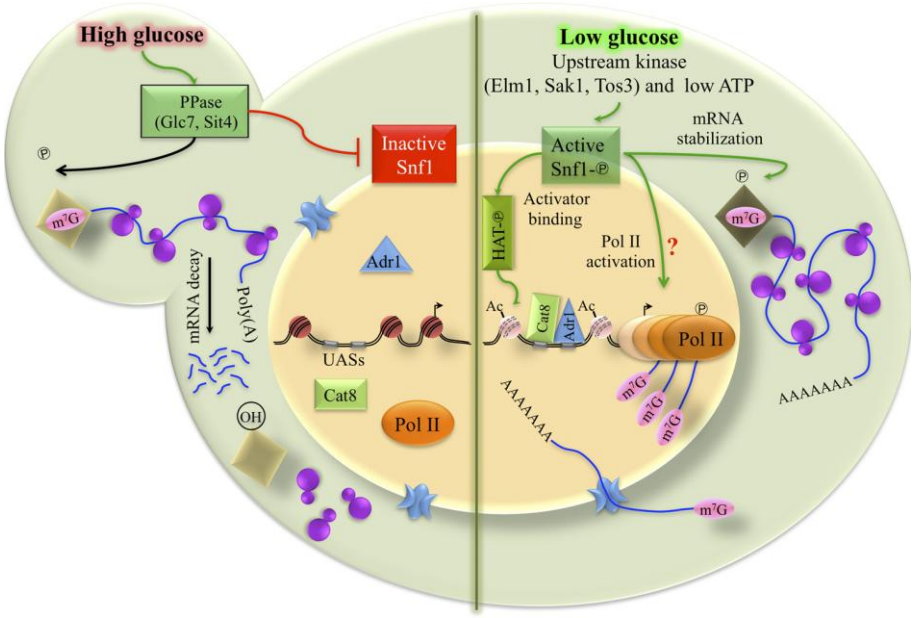


Fig. 23- Model of Snf1 regulation of Adr1-dependent gene expression (Young E.T. *et al.*, 2012)

3. Snf1/AMPK family

The Snf1/AMPK (AMP-activated protein kinase) protein kinases are a family of highly conserved heterotrimeric Serine/Threonine kinases with orthologs found in all eukaryotes, from fungi (yeast Snf1), roundworms (AMP-activated kinase, AAK), and insects (AMPK), to plants (SnRK1) and animals (AMPK).
Consistent with the ancestral role in the response to nutrient starvation (Snf1 functions), all AMPK orthologues are involved in the integration of signals regarding nutrient availability and environmental stresses in order to induce the required adaptation to maintain energy homeostasis and cell survival (Hardie D.G., 2011). Besides their function, those proteins share also a similar structure and a comparable activation.

3.1 Structure

As in yeast, also in higher eukaryotes Snf1/AMPK proteins function as heterotrimeric complexes that require a catalytic α-subunit and two regulatory subunits (β and γ). However, each organism presents different isoforms for each subunit.

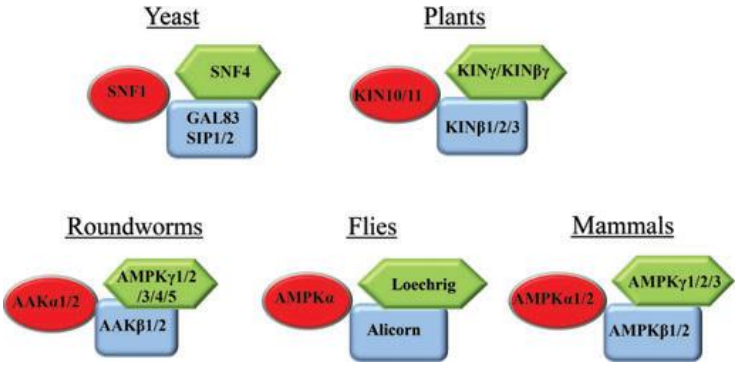


Fig.24- composition of Snf1/AMPK complexes among different organisms (Ghillebert R. *et al.*, 2011)

As shown in figure 24, in *Caenorhabditis elegans* 5 genes encode for the γ-subunit and two different genes encode for the α and β subunits, allowing the potential formation of 20 heterotrimeric complexes. Differently, in *Drosophila melanogaster* each subunit is encoded by single genes: AMPKα, alicorn (β subunit) and loechrig (γ subunit) (Ghillebert R. *et al.*, 2011).

In the plans *Arabidopsis thaliana*, the proteins that display most similarity to Snf1 are KIN10 and KIN11; those proteins, together with KINβ1/ KINβ2 (similar to yeast β subunits) and KINγ (the γ subunit), form the SnRK1 and SnRK2 (Snf1-Related protein Kinases 1 and 2) complexes.

In mammalian cells, then, AMPK presents different isoforms for the three subunits of the complex: α1, α2, β1, β2, γ1, γ2, γ3. Those proteins can generate 12 different complexes which seem to be similar, however some differences can be detected. In fact, α1 and α2 have an identity of 46% at sequence level, and are functionally redundant. Nevertheless, α1 is present almost in all tissues and it is responsible for the 90% of the kinase activity of AMPK complexes, measured *in vitro* (Carling D. *et al.*, 1994; Salt I. *et al.*, 1998). Differently, α2 is less active than α1 isoform and it is expressed only in muscle tissues and in the liver (Salt I. *et al.*, 1998).

As the catalytic subunit, also the β subunit isoforms are different. They both present the ASC (Association with SNF1 kinase Complex) and the KIS (Kinase-Interacting Sequence) domains; on the contrary they have different N-terminal and their expression is tissue-specific with β1 that is expressed in the liver, while β2 is present almost only in muscle tissues (Thornton C. *et al.*, 1998).

3.2 Activation

Proteins of the Snf1/AMPK family are activated through the phosphorylation of a Threonine residue placed in the catalytic domain of the α subunit (Thr210 for Snf1 in yeast, Thr175 of KIN10 and Thr176 for KIN11 in plants and Thr172 for AMPK in mammals). As previously described, three kinases (Sak1, Tos3 and Elm1) phosphorylate Thr210 in yeast. In plants, sequence comparison has implicated GRIK1/2 as possible activating kinases of KIN10 and KIN11 (Shen W. *et al.*, 2009).
AMPK in mammals is activated by two kinases: LKB1 and CaMKKβ. In particular, LKB1 is an heterotrimeric kinase which exerts its role with the regulatory subunits MO25 and STE-20 (Hawley S.A. *et al.*, 2003); this kinase is constitutively active and it seems to phosphorylate both α1, and α2 isoforms (Carling D. *et al.*, 2008). Differently, CaMKKβ is an isoform of the kinase CaMKK (CalModulin-dependent protein Kinase Kinase) which is activated by signals that use Ca^{2+} ions as second messanger such as muscle movement (Tamas P. *et al.*, 2006). In addition, also the mammalian TAK1 kinase is able to complement the phenotype of the *sak1Δtos3Δelm1Δ* yeast strain and hence it could be involved in the phosphorylation of AMPK (Momcilovic M. e*t al.*, 2006).
The inactivation of AMPK is still under investigation, however PP2A and PP2C phosphatase complexes were reported to dephosphorylate and inactivate mammalian AMPK and plant SnRK1, suggesting that those phosphatases could be involved in AMPK regulation (Ruiz A. *et al.*, 2011).

In addition to the regulation described above, mammalian AMPK is subject to allosteric regulation by the AMP/ATP ratio, a sensitive indicator of cellular energy status. In fact, binding of AMP to the γ subunit of the AMPK complex promotes the phosphorylation of Thr172 residue by uprstream kinases and protect it from dephosphorylation (Davies S.P. *et al.*, 1995). Moreover AMP is known to directly interact and activate LKB1 kinase (Hawley S.A. *et*

al., 2003). Thus, AMP and ATP, competing for the same binding sites, exert a fundamental role in the regulation of AMPK.
Differently, neither Snf1 in yeast nor SnRK1/2 in plants seem to be allosterically regulated by AMP/ATP; nevertheless, the activation of both those kinases correlates with an increase of the AMP/ATP ratio (Wilson W.A. *et al.*, 1996; Sudgen C. *et al.*, 1999).

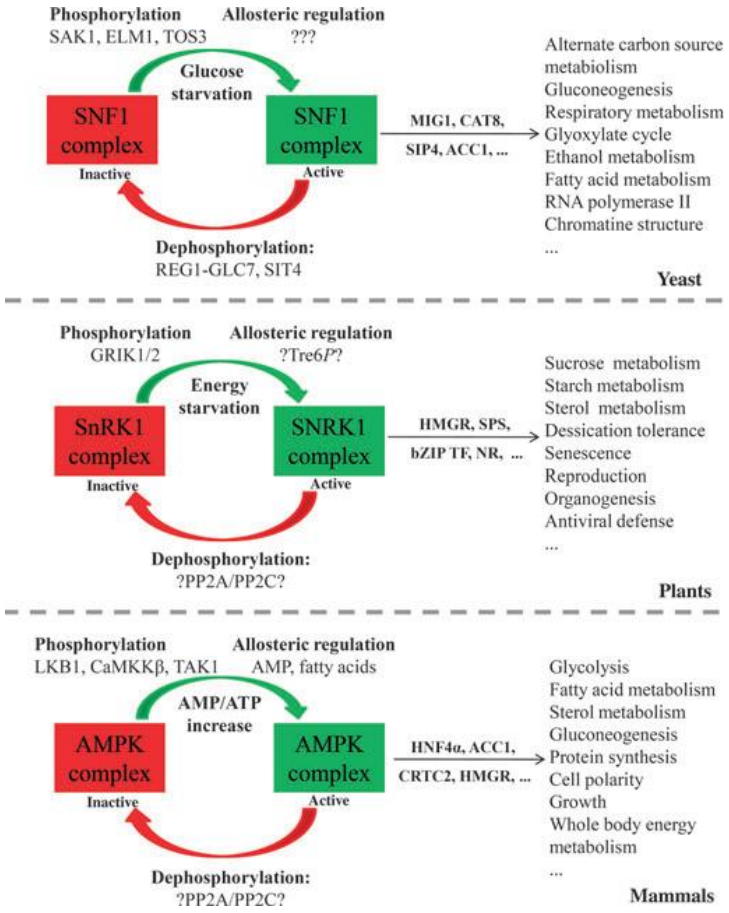


Fig.25- activation and functions of Snf1/AMPK complexes among different organisms (Ghillebert R. *et al.*, 2011)

3.3 Function

The Snf1/AMPK orthologs exert very similar functions.
In *Caenorhabditis elegans*, AAK functions as a metabolic sensor that couples lifespan to information about energy levels. Larvae that lack AAK rapidly consume their stored energy, are unable to develop vital organs and die prematurely (Narbonne P. and Roy R., 2009).

In *Drosophila melanogaster*, AMPK has a critical function in the regulation of epithelial integrity and cell division throughout development and in response to energetic stress. In fact, abnormal mitotic phenotypes were observed for both AMPKa and LKB1 null mutant in *Drosophila melanogaster* (Lee J.H. *et al.*, 2007).
In addition, AMPK is also involved in the regulation of cell cycle in response to alterations of mitochondrial functions. It has been observed that deletion of the gene encoding cytochrome c oxidase subunit Va (CoVa) leads to the accumulation of ROS (Reactive Oxigen Species), the increase of AMP molecules and a severe alteration of the G1/S transition. In that condition, in fact, AMPK positively regulates p53 which promotes the expression of p21, the inhibitor of CyclinE/Cdc2 complexes, responsible for the G1/S transition (Mandal S. *et al.*, 2005; Owusu-Ansah E. *et al.*, 2007).

Therefore, literature data support the notion that in *Drosophila melanogaster* AMPK could coordinate the energy status of cells with fundamental processes, including cell division and cell polarization.
Remarkably, also in plants and in mammalian cells AMPK seems to exert a similar function.

In plants, darkness is equivalent to a period of nutrient starvation in an animal. In *Physcomitrella patens*, plants lacking the two genes encoding catalytic subunit orthologs of AMPK (PpSNF1a and PpSNF1b) are viable if grown under constant illumination, but fail to grow in alternate light:dark cycles (Thelander M. *et al.*, 2004).

In the higher plant *Arabidopsis thaliana*, overexpression of the catalytic subunit KIN10 causes resistance to the effects of carbohydrate starvation and promotes lifespan extension. On the contrary, the concomitant loss of KIN10 and KIN11 prevents the normal switch in gene expression which allows the expression of genes necessary for the adaptation to a dark period, a nutritional stress for plants (Baena-Gonzalez E. *et al.*, 2007). Those data, thus, indicate that also in plants the AMPK orthologs are central regulators of gene transcription in response to nutritional stresses. In particular, that hypothesis has been recently confirmed, since it has been observed that also OsSnRK1 and OsSnRK3, the rice orthologs of SnRK1/2, regulate the expression of a large set of genes in response to nutritional stress (Cho Y. *et al.*, 2012).
Intriguingly, Baena-Gonzalez and co-workers (Baena-Gonzalez E. *et al.*, 2007) had also observed that, differently from what happened in *P. patens*, neither continuous light irradiance nor addition of 1% sucrose could complement the phenotype of the *kin10kin11* mutant of *A. thaliana*. Those data, thus, suggest that this defect is not merely due to impaired catabolism and support the notion that KIN10/KIN11 could be involved in the regulation of normal vegetative and reproductive growth.

The mammalian, AMPK is a key regulator of energy sensing; in response to nutritional stresses this kinase stimulates catabolic processes to generate ATP and inhibits ATP-consuming anabolic processes. In particular, AMPK exerts its role through both the direct regulation of metabolic enzymes and the modulation of gene expression (Cantó C. and Auwerx J., 2010).
Interestingly, several AMPK substrates have been found using AICAR (5-AminoImidazolo-4-Carboxamide Riboside) an adenosine analog which in cells is converted into ZMP (5-aminoImidazolo-4-carboxamide-1-D-ribofuranosil-5'-monofosfato). ZMP, similar to AMP, activates AMPK; remarkably this compound is specific for AMPK, in fact it does not influence the activation of others enzymes regulated by AMP (Corton J.M. *et al.*, 1995).

As its ortholog Snf1, in mammalian cells AMPK phosphorylates acetyl-coA carboxylase 1 and 2 (ACC1 and ACC2), promoting the fatty acid oxidation (Hardie D.G. and Pan D.A., 2002). Moreover, in response to nutritional stresses, AMPK phosphorylates Ser7 residue of glycogen synthase enzyme in order to inhibit its activity and to decrease the glycogen synthesis rate (Carling D. and Hardie D.G., 1989). At the same time, AMPK also promotes the glycolytic flux through the phosphorylation of Ser466 residue of 6-phosphofructo-2-kinase (PFK-2) enzyme which catalyzes the synthesis of fructose 2,6-bisphosphate, a stimulator of glycolysis (Marsin A.S. *et al.*, 2000).
In addition, AMPK activation allows the localization of the glucose transporter GLUT4 to the plasma membrane, causing the increase of glucose uptake by skeletal muscle (Hardie D.G., 2003).
At the end, activation of AMPK either by glucose-deprivation or by the AMP-mimetic drug AICAR triggers phosphorylation of Thr2446 residue of mTOR, which leads to the inhibition of protein synthesis (Cheng S.W. et al., 2004). Moreover, AMPK prevents protein synthesis also regulating TIF-IA, the transcriptional factor which regulates the synthesis of rRNA (Hoppe S. *et al.*, 2009).
As previously mentioned, AMPK is also a fundamental regulator of gene expression. In muscle cells, AMPK complexes which present α2 as catalytic subunit localize into the nucleus in response to muscle contraction and causes a specific change in gene-expression pattern (McGee S.L. *et al.*, 2003). In fact, in the nucleus AMPK regulates transcription regulators of the FOXO family and the coactivator PGC-1α which is involved in the modulation of PPARα, PPARβ and CREB transcription regulators (Cantó C. and Auwerx J., 2010). In addition, AMPK also counteracts the inhibitory activity of the Histone Deacetylase 5 (HDAC). AMPK phosphorylates Ser259 and Ser498 of HDAC causing its dissociation from MEF2/GEF transcription factors and thus promoting the expression of MEF2/GEF-dependent genes (McGee S.L. *et al.*, 2008).
In other tissues the localization of AMPK has not been determined, however it is known that also in liver AMPK plays a critical role in the modulation of transcription factors. In fact, through the

modulation of transcription factors such as CREB and HNF4α AMPK promotes the expression of genes involved in gluconeogenesys, glycolisis and fatty acids oxidation (Cantó C. and Auwerx J., 2010).
Besides its well known role as modulator of cellular metabolism, AMPK is also involved in the direct regulation of cellular processes. The activation of AMPK determined by glucose-depletion or AICAR treatment leads to the arrest of cells in G1 phase. In particular, AMPK phosphorylates p53, promoting its activity and the consequent expression of p21, the inhibitor of CyclinE/CDK complexes. Therefore, activation of AMPK in response to nutrient stresses prevents the G1/S transition (Jones R.G. *et al.*, 2005; Igata M. *et al.*, 2005).
Recently, a role for AMPK as positive regulator of cell cycle it has been proposed. In fact, it has been observed that, once activated, AMPK directly binds the mitotic apparatus and ensures the correct cell division and chromosomal segregation during mitosis (Vazquez-Martin A. *et al.*, 2012).

Results

Snf1/AMPK promotes S-phase entrance by controlling *CLB5* transcription in budding yeast

In budding yeast Snf1 is required primarily for adaptation to glucose limitation and for growth on non-fermentable carbon sources. However, this kinase plays also roles in various nutrient-responsive cellular processes such as meiosis and sporulation, haploid invasive growth and diploid pseudohyphal growth (for a review see Hedbacker K. and Carlson M., 2009)

In the present work we present a previously unrecognized role for Snf1 in the regulation of cell cycle progression.

In cells grown in a medium supplemented with 2% glucose, lack of Snf1 α-catalytic subunit down-regulates the growth rate and the expression of *CLB5*, delaying Sld2 phosphorylation and G1/S transition. Moreover, we show that a non-phosphorylatable Snf1 mutant (*SNF1-T210A*) rescues the slow growth phenotype of a *snf1Δ* strain, but still presents a slightly delayed G1/S transition. On the other hand, a phosphomimetic Snf1 mutant (*SNF1-T210E*) complements the G1/S delay of the *snf1Δ* strain, indicating that the activation of Snf1 is involved in its role as cell cycle regulator.

Our data also reveal the existence of a specific interaction of Snf1 with Swi6, the regulatory subunit of the SBF and MBF transcription complexes, which modulate the expression of G1-specific genes.

Remarkably, both the altered phenotype of the *SNF1-T210A* mutant and the severe defect in cell cycle progression of *snf1Δ* cells are complemented by a glucose concentration higher than 2% (5%), suggesting that the role of Snf1 depends on the nutritional condition of yeast cells.

Data here described were published in Pessina S, Tsiarentsyeva V, Busnelli S, Vanoni M, Alberghina L and Coccetti P - Snf1/AMPK promotes S-phase entrance by controlling *CLB5* transcription in budding yeast (2010) Cell Cycle. 9(11):2189-200.

RESULTS

Transcription of *CLB5* and activity of Clb5/Cdk1 complexes are down-regulated in *snf1Δ* strain

We tested the role of Snf1 in yeast cell cycle progression by evaluating several cell cycle parameters of a *snf1Δ* strain during exponential growth in YP supplemented with 2% glucose. *snf1Δ* cells had a slightly longer mass duplication time (MDT) than the *wild type*, as reported (von Plehwe U. *et al.*, 2009), and no significant alteration in DNA and protein distributions and in the protein content at the onset of S-phase (Ps value) were observed (Fig.1 A). The level of G1-cyclins Cln1 and Cln2, Cdk1 and Sic1, were not affected in the *snf1Δ* background. Interestingly, the level of Clb5 protein was strongly reduced in the *snf1Δ* strain (Fig.1 B). The down-regulation of Clb5 did not depend on a reduced stability of the protein, since doxycycline treatment on *clb5Δ* and *clb5Δsnf1Δ* strains, expressing Clb5-9myc under the control of a tetracycline-repressible promoter, depleted cellular Clb5 within 100 min in the *snf1Δ* strain as well as in the control (Fig.1 C).

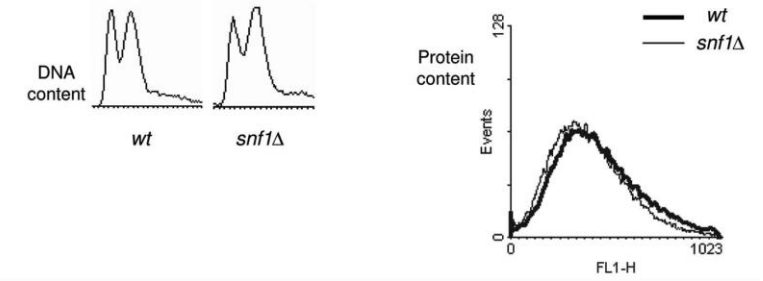
On the contrary, the down-regulation of Clb5 protein is correlated to a reduced expression of *CLB5* mRNA in the *snf1Δ* strain, as judged by quantitative relative Real-time PCR (Fig.1 D).

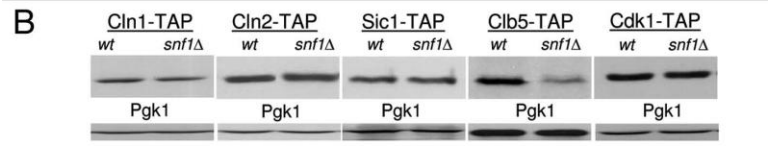
To examine the role of Snf1 on *CLB5* transcription, a physical interaction of Snf1 with MBF, the transcription factor of *CLB5*, was investigated. We showed that Snf1 interacts with the regulator subunit Swi6 by specific detection of Swi6 in endogenous Snf1-myc immunocomplexes and that reversely Snf1-myc is detectable in Swi6 immunocomplexes (Fig.1 E). We did not find the similar recovery of Mbp1 in the Snf1-myc immunoprecipitates (data not shown), suggesting that Snf1 may bind MBF *via* its interaction with Swi6.

Taken together our results show that the absence of Snf1 protein reduces the transcription of *CLB5* and, as a consequence, decreases the level of Clb5 protein.

A

	MDT (min.)	Budding index, %	P (a)	Ps (b)
wt	78 ± 4	69 ± 7	436 ± 6	355 ± 8
snf1Δ	91 ± 1	60 ± 6	400 ± 8	363 ± 5





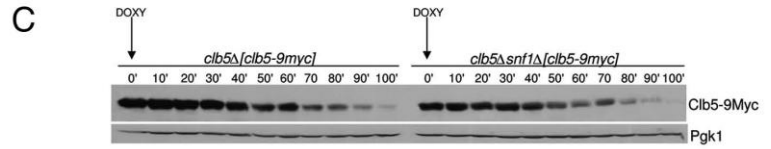


FIGURE 1 (A-C). Effects of *SNF1* deletion on growth rate, cell cycle parameters, G1/S proteins level, *CLB5* transcription and degradation, of exponentially growing cells.

(A) Mass duplication time (MDT), budding index and FACS analysis of protein and DNA content of *snf1Δ* and *wild type* strains exponentially growing in YP media, 2% glucose; (a), (b) determined from 2D CFF data as reported in materials and methods: average protein content (P), protein content at the onset of DNA replication (Ps). (B) Western blot using anti-TAP antibody. (C) Stability of Clb5 protein in *clb5Δ[pCM189-CLB5-9MYC]* and *clb5Δsnf1Δ[pCM189-CLB5-9MYC]* strains was detected by western blot using anti-myc antibody.

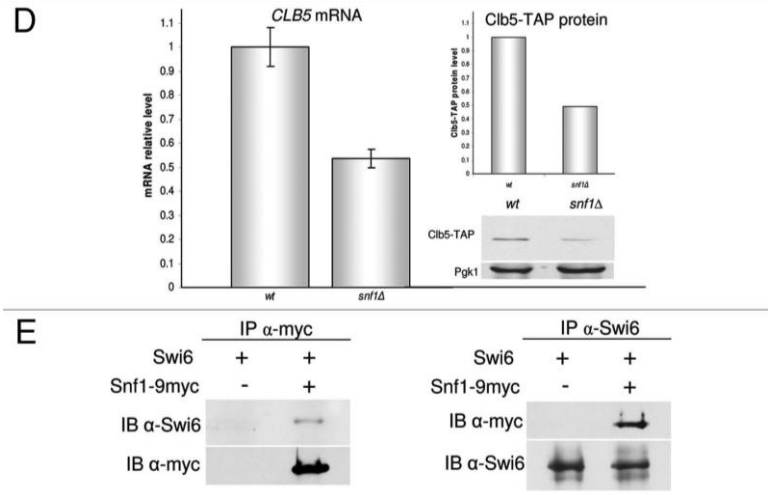
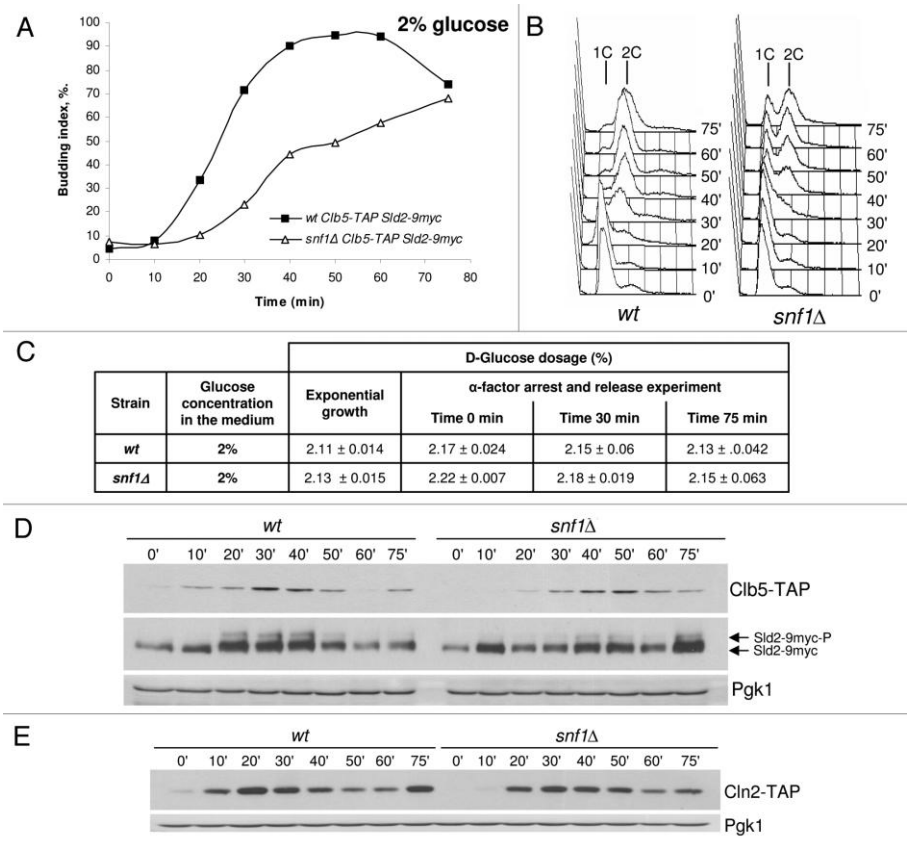


FIGURE 1 (D-E).
(D) Quantitative relative Real-time PCR on *CLB5* mRNA. The histogram shows mean values of three biological replicates, error bars indicate standard deviations. Clb5 protein by western blot using anti-TAP antibody of exponentially growing cells used for the Real-time PCR. The signals have been quantified, normalized on loading control and reported in the histogram. (E) The immunocomplexes (IP) were analyzed by western blot (IB) with anti-myc antibody and anti-Swi6 antibody.

Clb5 availability influences S-phase entrance (Schwob E. and Nasmyth K., 1993). Hence, to focus on G1/S transition, exponentially growing *snf1Δ* cells were synchronized in G1 phase by α-factor treatment and released. The entrance into S-phase of *snf1Δ* strain was severely delayed. In fact, while control cells started to enter into S-phase within 20-30 min after release, in keeping with the synthesis of Clb5 protein (Fig.2 A, B and D), the increase of budding index in the *snf1Δ* strain was substantially delayed and did not even reach the same values as the *wild type*, as confirmed by the analysis of post-replicative DNA content (Fig.2 A and B). In the *snf1Δ* strain, DNA replication was less synchronous than in *wild type* (Fig.2 B). The dosage of the concentration of D-glucose in the medium in exponential phase and in α-factor release experiments showed no difference in glucose usage in *snf1Δ* cells *vs wild type* strain (Fig.2 C).
Consistently with the analysis of DNA profiles, Clb5 accumulation started with a 10-20 min delay in the *snf1Δ* strain (Fig.2 D). We therefore analyzed the phosphorylation of the more relevant substrate of Clb5/Cdk1 complex, Sld2, a protein required to trigger, in its multi-phosphorylated state, the onset of DNA replication (Tanaka S. *et al.*, 2007). In the *wild type* the slower migrating forms of Sld2 were detectable at early S-phase (20 min) disappearing when essentially all cells completed DNA replication (Fig.2 B and D); on the other hand in *snf1Δ* cells Sld2 phosphorylation was severely delayed and reduced, and started to be barely detectable 40 min after the release (Fig.2 D).
In the *snf1Δ* mutant there was a 10 min delay in the synthesis of Cln2 (Fig.2 E), which, in complex with Cdk1, is responsible for bud emergence (Cvrcková F. and Nasmyth K., 1993), while, as noted above the levels and pattern of expression of Clb5 (as well as the phosphorylation of Sld2) were different throughout the experiment. Taken together these results indicate that Clb5 is more severely affected than Cln2 by *SNF1* deletion. We do not completely exclude a role of Snf1 in regulating *CLN2* expression considering the interaction between Snf1 and Swi6 reported above (Fig.1 E).
Interestingly, a progressive reduced S-phase entrance delay and a rescued synchronism of DNA replication was observed in the *snf1Δ*

strain grown in media containing increased glucose concentrations (from 2% to 5% glucose, Fig.2 F and G). Specifically, in the presence of 5% glucose, no difference was scored between *wild type* and *snf1Δ* mutant regarding Cln2, Clb5 protein expression and Sld2 phosphorylation (Fig.2 H and I), suggesting that the Snf1 catalytic subunit is required to promote *CLB5* expression only during growth in 2% glucose, being dispensable at higher glucose concentrations. *snf1*Δ strain showed a G1/S delay when grown in 2% glucose + 3% sorbitol media, excluding also the hypothesis that 2% glucose should be a condition of osmotic stress for *snf1Δ* cells (Supplementary Fig.S2).



Strain	Glucose concentration in the medium	D-Glucose dosage (%)			
		Exponential growth	α-factor arrest and release experiment		
			Time 0 min	Time 30 min	Time 75 min
wt	2%	2.11 ± 0.014	2.17 ± 0.024	2.15 ± 0.06	2.13 ± .0.042
snf1Δ	2%	2.13 ± 0.015	2.22 ± 0.007	2.18 ± 0.019	2.15 ± 0.063

FIGURE 2 (A-E). Clb5 level and Sld2 phosphorylation are affected in *snf1Δ* mutant grown in YP media containing 2% glucose.

(A-E) *snf1Δ* and *wild type* strains were grown in 2% glucose and G1-arrested by α-factor. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analysis, (C) D-glucose concentration dosage in exponential phase and in α-factor release experiments, (D-E) Clb5, Sld2 and Cln2 proteins by western blot using anti-TAP and anti-myc antibody.

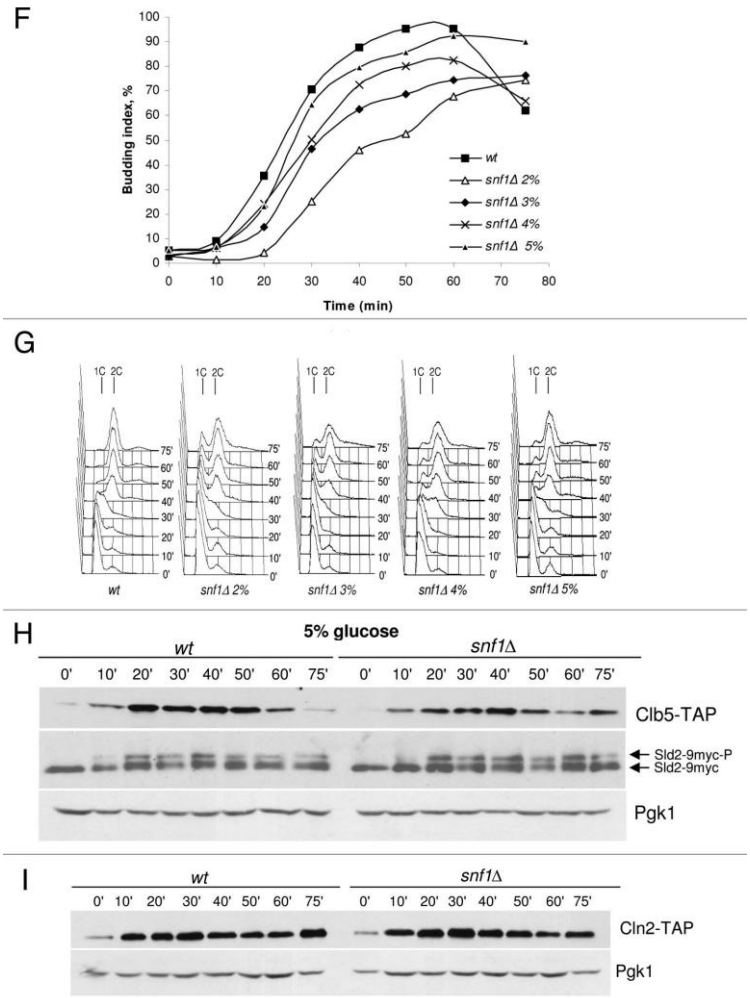


FIGURE 2 (F-I).
(F-G) *snf1Δ* and *wild type* strains were grown in 2%, 3%, 4%, 5% glucose, G1-arrested by α-factor and released in the same glucose concentrations. Samples were taken at the indicated time points to assay (F) budding index, (G) DNA content by FACS analysis. (H-I) Clb5, Cln2 level and Sld2 phosphorylation are not affected in *snf1Δ* mutant grown in YP media containing 5% glucose. *snf1Δ* and *wild type* strains were grown, G1-arrested by α-factor and released. Samples were taken at the indicated time points to assay Clb5, Sld2 and Cln2 proteins by western blot using anti-TAP and anti-myc antibody.

Deletions of other players of the Snf1 pathway do not affect entrance into S phase.

In order to delve more into the relationship between activated Snf1 and the promotion of *CLB5* expression, a *reg1Δ* strain, in which the Snf1 kinase is constitutively active due to the deletion of the regulatory subunit of the Glc7/Reg1 phosphatase (Sanz P. *et al.*, 2000), was analysed. The MDT of the *reg1Δ* was slower than the control culture (Fig.3 A), as reported (Schwob E. and Nasmyth K., 1993). No significant alterations in DNA, protein distributions and Ps value were observed (Fig.3 A). The constitutive activation of Snf1 in the *reg1Δ* strain did not alter the level of any protein of the G1/S transition (Fig.3 B) and no difference was observed between the *wild type* and *reg1Δ* strain after α-factor release (Fig.3 C-E). In conclusion, constitutive Snf1 activation yields a correct G1 to S transition in budding yeast growing in 2% glucose.
Considering the well known regulatory role of Snf4 on Snf1 complex activity (Momcilovic M. *et al.*, 2008), the phenotype of *snf4Δ* strain was also investigated by monitoring entrance into S phase after α-factor treatment. Deletion of the *SNF4* gene did not alter the kinetic of entrance into S-phase (Fig.4), showing that the γ-subunit does not influence the Snf1 function in S-phase entrance.

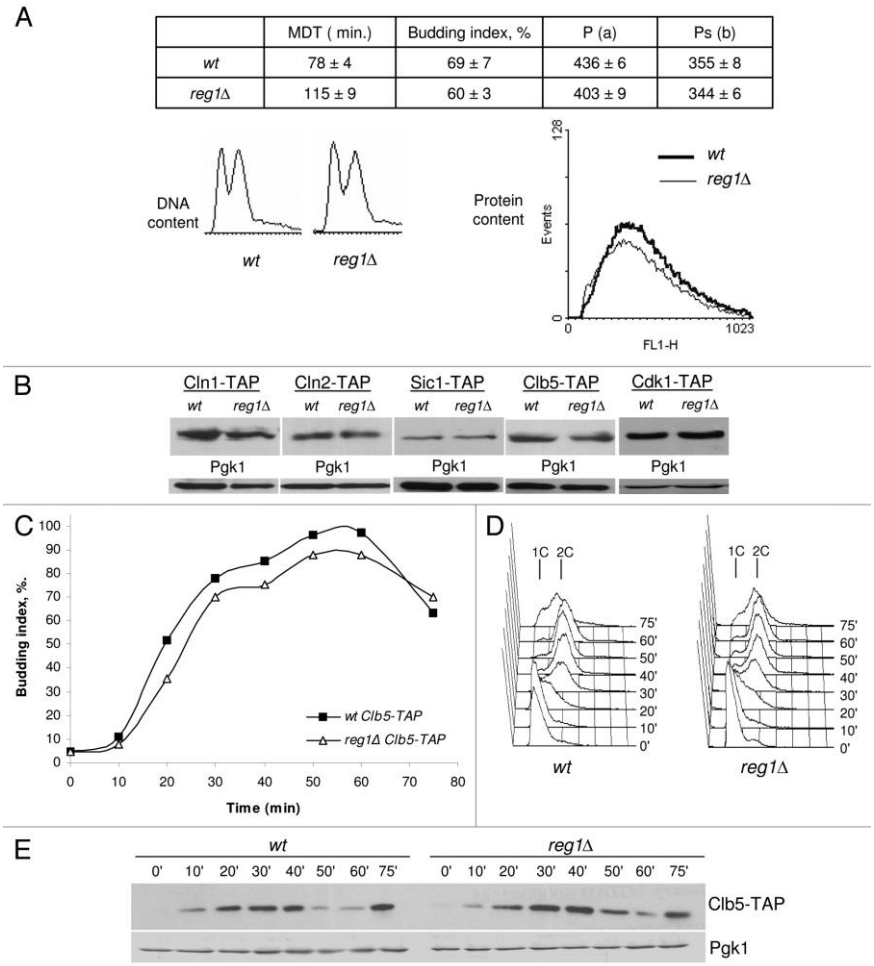


FIGURE 3. Clb5 level is not affected in *reg1Δ* mutant.
reg1Δ and *wild type* strains were grown in YP media containing 2% glucose. (A) Mass duplication time (MDT), budding index and FACS analysis of protein and DNA content; (a), (b) determined from 2D CFF data as reported in materials and methods: average protein content (P), protein content at the onset of DNA replication (Ps). (B) Western blot using anti-TAP antibody of samples from exponentially growing cells. (C-E) *reg1Δ* and *wild type* strains were G1-arrested by α-factor and released. Samples were taken at the indicated time points to assay (C) budding index, (D) DNA content by FACS analysis, (E) Clb5 protein by western blot using anti-TAP antibody.

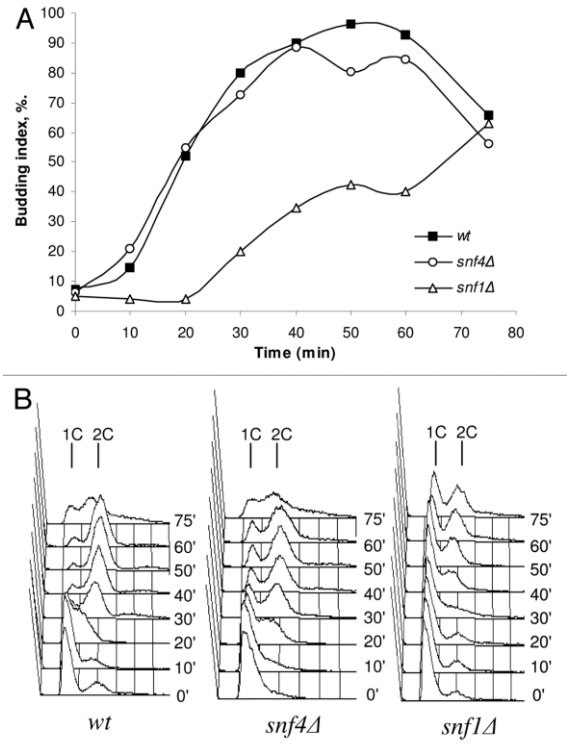


FIGURE 4. The absence of the γ subunit of Snf1 complex does not affect the G1/S transition.

snf4Δ, *snf1Δ* and the *wild type* strains were grown in YP media with 2% glucose until exponential phase and synchronised in G1 by α-factor treatment. They were released in the same fresh medium at time 0. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analysis.

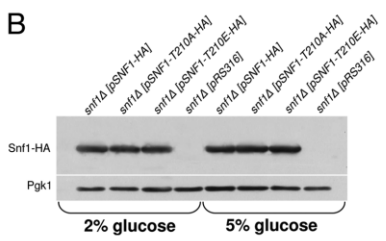
The *SNF1-T210E* mutant fully rescues the phenotype of the *snf1*Δ strain

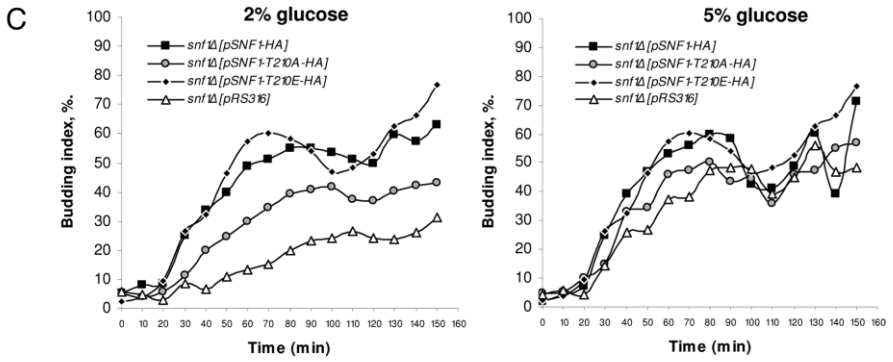
To gain further insight into the relationship between the entrance into S-phase and Snf1-T210 phosphorylation, the strains *snf1Δ[pSNF1-HA]*, *snf1Δ[pSNF1-T210A-HA]* and *snf1Δ[pSNF1-T210E-HA]* were analysed both in exponential phase and in G1 synchronous populations entering into S phase. To maintain the plasmids, cells were grown in synthetic media, as reported (Estruch F. *et al.*, 1992), with 2% or 5% glucose. First of all, the growth parameters of the *snf1Δ[pSNF1-HA]* strain were tested and found to be the same as the *wild type*, indicating a full complementation of the *SNF1* deletion (data not shown). The growth rate of the *snf1Δ[pSNF1-HA]* was the same both in 2% and 5% glucose (Fig.5 A), while the *snf1*Δ strain showed a slower growth rate, especially in 2% glucose. The expression of *SNF1-T210A* gave partial complementation of the growth rate of the *snf1*Δ strain in 2% glucose and full complementation in 5% glucose (Fig.5 A). The inability of *SNF1-T210A* to fully complement the *snf1*Δ phenotype in 2% glucose was not merely due to an instability of the mutated protein, as shown (Fig.5 B). Interestingly, growth kinetic of the *snf1*Δ mutant was fully restored by the *Snf1-T210E* protein in both glucose concentrations (Fig.5 A). Although the *Snf1-T210D* mutant was reported to be unable to grow on raffinose (Estruch F. *et al.*, 1992), the *Snf1-T210E* mutant partially complemented the nonfermenting phenotype of a *snf1Δ* strain on sucrose and fully complemented Snf1 function under carbon limitation (data not shown). In G1-synchronous cells entering S-phase in 2% glucose, the mutant *snf1Δ[pSNF1-T210E-HA]* showed the same kinetic of budding and DNA synthesis as the *wild type* (*snf1Δ[pSNF1-HA]*) (Fig.5 C and D). Interestingly, the mutant unable to be phosphorylated on the T210 residue (*snf1Δ[pSNF1-T210A-HA])* had a phenotype intermediate between the *wild type* and the *snf1*Δ strain (Fig.5 C and D). Moreover in this mutant, in 2% glucose, the synthesis of Clb5 protein was sensibly reduced (Fig.5 E). While the

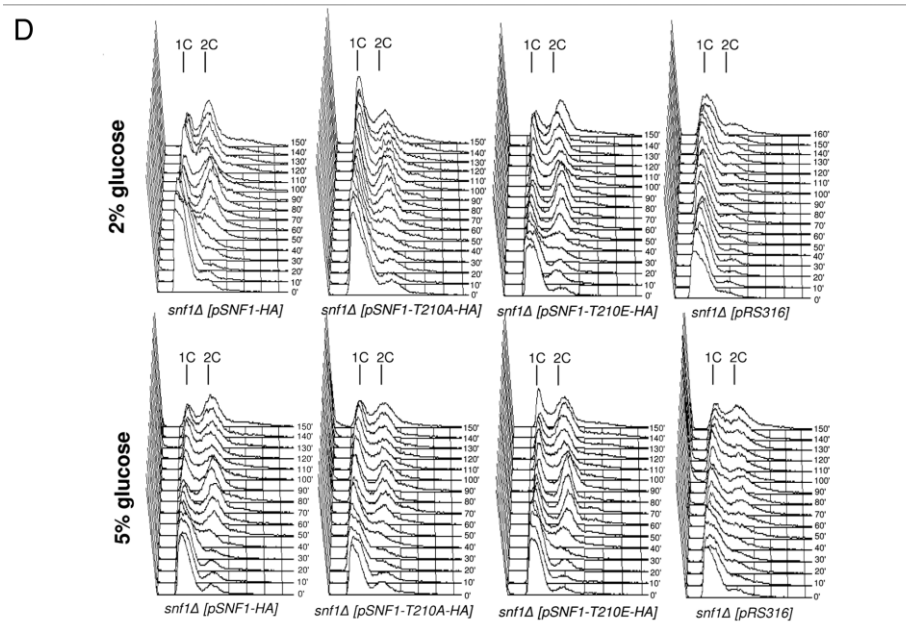
snf1Δ[pSNF1-T210E-HA] strain had the same Clb5 expression as the *wild type* (Fig.5 E).
No major difference was observed among all the mutant strains and the *wild type* in 5% glucose in terms of budding index, post-replicative DNA content and Clb5 protein level (Fig.5 C-E).

A

Strain	MDT (min), 2% glucose	MDT (min), 5% glucose
snf1Δ [pSNF1-HA]	140 ± 7.4	139 ± 8
snf1Δ [pSNF1-T210A-HA]	179 ± 16	142 ± 9.8
snf1Δ [pSNF1-T210E-HA]	150 ± 6.5	127 ± 1.4
snf1Δ [pRS316]	250 ± 15	183 ± 14







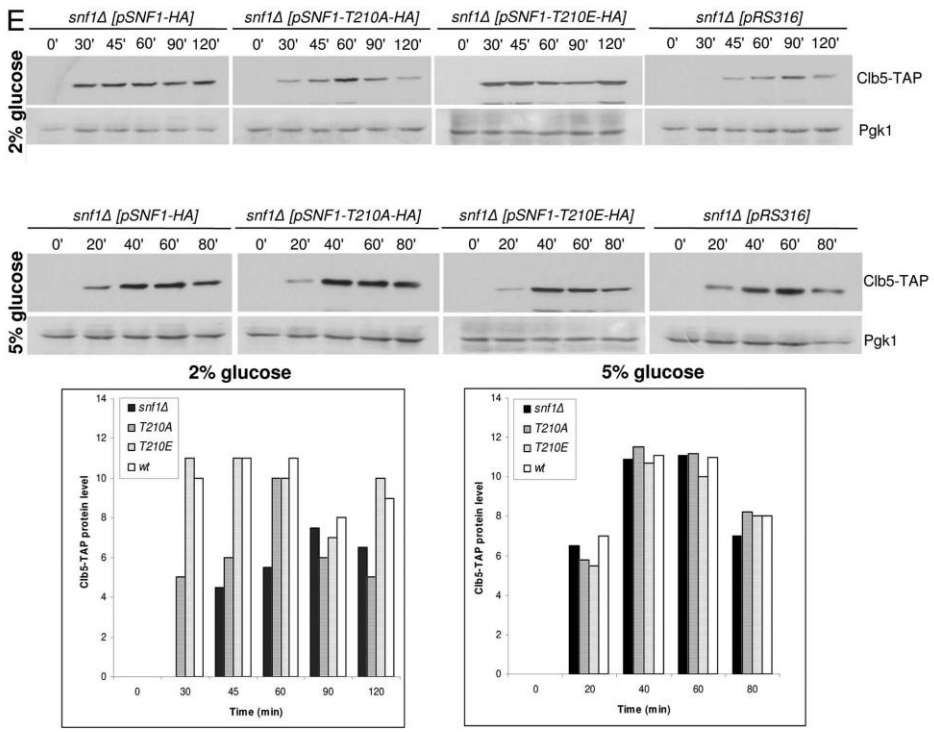


Figure 5. The *SNF1-T210E* mutant fully rescues the phenotype of the *snf1*Δ strain.

The strains *snf1Δ[pSNF1-HA]*, *snf1Δ[pSNF1-T210A-HA]*, *snf1Δ[pSNF1-T210E-HA]* and *snf1*Δ*[pRS316]* were grown in synthetic media containing 2% or 5% glucose. (A) MDT in 2% and 5% glucose of exponentially growing cultures; (B) western blot using anti-HA antibody. (C-E) Cells were grown until exponential phase, G1-arrested by α-factor and released. Samples were taken at the indicated time points to assay (C) budding index, (D) DNA content by FACS analysis and (E) western blot using anti-TAP antibody. The signals have been quantified, normalized on loading control and reported in the histograms.

SUPPLEMENTARY MATERIALS

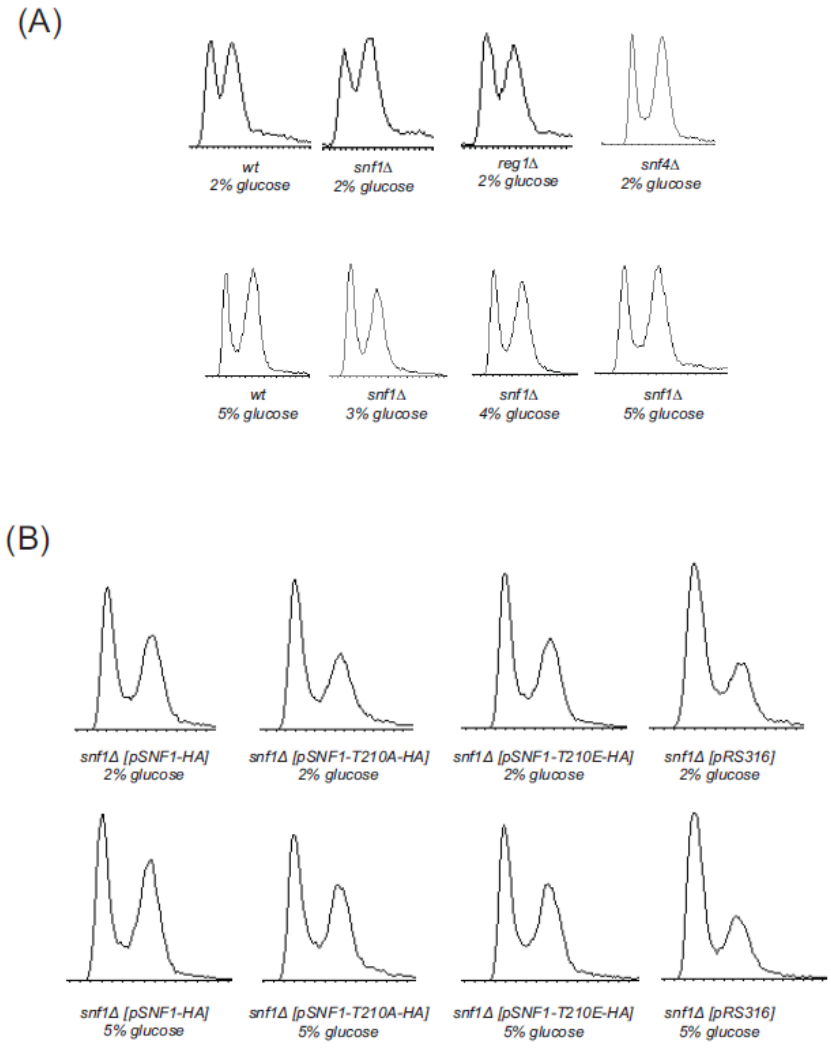


Figure S1. DNA distributions of exponential phase growing cells. Cells were grown in YP media containing 2%-3%-4%-5% glucose (A) and in synthetic media containing 2% or 5% glucose (B). Cells were harvested in exponential phase and DNA content was determined by FACS analysis (A, B).

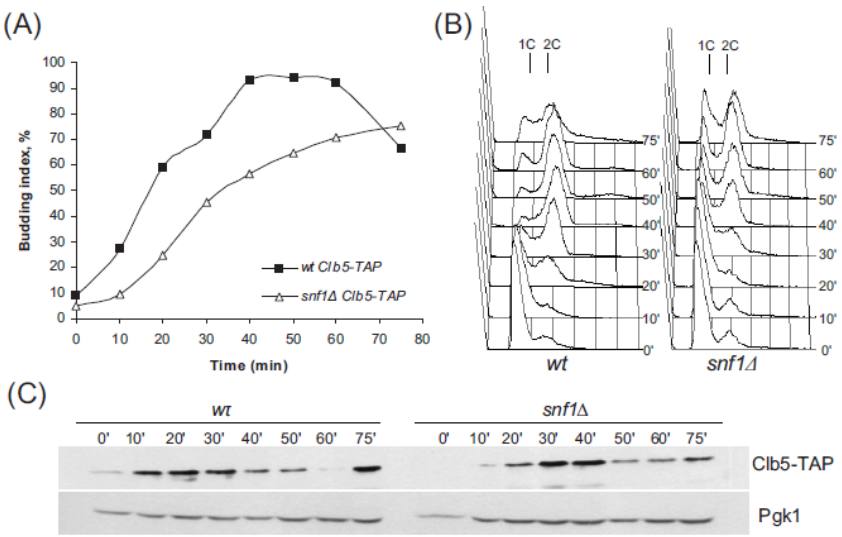


Figure S2. G1/S transition of a *snf1Δ* strain grown in 2% glucose + 3% sorbitol. *snf1Δ* and *wild type* strains were grown in YP media containing 2% glucose + 3% sorbitol. (A-C) *snf1Δ* and *wild type* strains were G1-arrested by α-factor and released. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analysis, (C) Clb5 protein by western blot using anti-TAP antibody.

MATERIALS AND METHODS

Yeast strains, growth conditions and plasmids. *S. cerevisiae* strains used in this study are listed in Table 1. YP media contained 1% yeast extract, 2% peptone and 2% - 3% - 4% - 5% glucose; synthetic media contained 6.7 g/L of Yeast Nitrogen Base (Difco), 100-50 mg/L of required nutrients and 2% or 5% glucose. Glucose concentrations were measured by the D-Glucose HK (R) assay Kit K-GLUHKR (Megazyme International Ireland). Cell density of liquid cultures grown at 30°C was determined with a Coulter counter on mildly sonicated and diluted samples. Percentage of budded cells was determined by direct microscopic count of at least 300 cells after mild sonication. For G1 synchronization, cells were grown to exponential phase and α-factor (GenScript) was added to a final concentration of 3 µM. Silencing of *CLB5* expression was obtained by adding 0.5 µg/ml doxycycline to exponentially growing cells carrying *CLB5* under the control of the tetracycline repressible promoter on *pCM189-CLB5-9myc* plasmid.

Recombinant DNA techniques and genetic manipulations. DNA manipulations and yeast transformations were carried out according to standard techniques. Oligonucleotides used in this study are listed in Table 2. *SNF1* deletion was obtained by Polymerase Chain Reaction (PCR) with primers *SNF1-KAN-F* and *SNF1-KAN-R* and controlled by PCR with the oligos *SNF1-47-F* and *SNF1-KAN-R*, by kanamycin resistance and by the inability to grow in the presence of non-fermenting carbon source. To obtain *REG1* deletion the primers *REG1 F* and *REG1 R* were used. The Sld2-9myc tagged protein was obtained by *in-locus* 3' in-frame insertion of *SLD2* gene with a *9myc-URA3* epitope sequence. The *9myc-URA3* sequence was amplified from *pST-9myc-URA3* plasmid. This plasmid was obtained by inserting the PCR amplified *9-myc* sequence and the *URA3* marker in *pSTBlue-1* plasmid digested with *EcoRI* and *BamHI*. To obtain *CLB5* overexpression, *CLB5-9myc* was amplified by PCR using *pSTBlue1-CLB5-9myc* (Coccetti P. *et al.*, 2004) as template and cloned in *pCM189* after a

Pst1 digestion. Then, the strains *clb5*Δ and *clb5*Δ*snf1*Δ were transformed with the obtained plasmid *pCM189-CLB5-9myc* carrying *CLB5* gene under the control of the tetracycline repressible promoter. The strains *snf1Δ[pSNF1-HA], snf1Δ[pSNF1-T210A-HA], snf1Δ[pSNF1-T210E-HA]* and *snf1Δ[pRS316]* were obtained by transforming the Clb5-TAP *snf1*Δ cells with the plasmids *pSNF1-HA, pSNF1-T210A-HA, pSNF1-T210E-HA* and the empty vector *pRS316* (Ye T. *et al.*, 2008). Site directed mutagenesis was performed by PCR using *pSNF1-HA* as a template and primers *SNF1-T210E R* and *SNF1-T210E F* (Table 2) resulting in the plasmid *pSNF1-T210E-HA* which was sequenced.

Protein extractions, immunoprecipitation assays and immunoblottings. Samples were collected by filtration and immediately freezed at -80°C. Protein extractions, immunoprecipitations and western blot analysis were performed as described in Coccetti P. *et al.*, 2006. For coimmunoprecipation experiments endogenous Swi6 and Snf1-myc were immunopurified from 5 mg of total protein extract. Anti-TAP rabbit polyclonal antibody (Open Biosystems), anti-c-myc mouse 9E10 monoclonal antibody (Santa Cruz Biotechnology), anti-Swi6 rabbit polyclonal antibody (kindly provided by Breeden L.L.), anti-HA mouse monoclonal antibody (Roche), anti-Pgk1 mouse monoclonal antibody (Molecular Probes) (1:1000 dilution for each antibody; 1:500 dilution for anti-c-myc) were used. Pgk1 was always used as loading control. Scion Image (Scion Corporation) was used for densitometric analysis.

RNA extraction and qReal-Time PCR. $1.2x10^9$ cells/sample were collected by filtration and rapidly freezed at -80°C. They were resuspended in LETS buffer (200 mM LiCl, 20 mM EDTA, 20 mM Tris, SDS 20%) and lysed with the glass beads. Two steps of phenol:chloroform:isoamyl alcohol (PCI) extraction were performed. RNA was selectively precipitated with LiCl (0.5 M) at -80°C. 40μg of RNA were treated with 9 units of DNase I (RNase-free, Qiagen) for 1h at 37°C, followed by a PCI extraction and by an ethanol

precipitation at -80°C. Reverse transcription of 0.5 µg of mRNAs was carried out with iScript cDNA Synthesis Kit (BIO-RAD). Quantitative Real-time PCR for *CLB5* gene expression used three biological replicates. The reactions were performed using primers reported in Table 2 and Sso Fast EvaGreen Supermix (BIO-RAD). The obtained data were normalized on *ACT1*, *PGK1* and *CDC34* reference genes and organized with Genex (BIO-RAD).

Flow cytofluorimetric analysis (FACS analysis). Flow cytofluorimetric analysis were performed using a BD FACStarPlus as described in Tripodi F. *et al.*, 2007. DNA/Protein biparametric staining was performed as described in Coccetti P. *et al.*, 2004. Cells were collected during exponential phase and sequentially stained with FITC for protein content and PI for DNA content, and analysed simultaneously for both parameters. Protein and DNA contents are expressed in fluorescence channel number and can be compared to each other within the same analysis.

TABLES

Table1. Strains used in this study.

Strain	Genotype	Source
wt	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open
Cln1-TAP	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN1-TAP:HIS3*	Open
Cln2-TAP	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN2-TAP:HIS3*	Open
Clb5-TAP	*BY4741; MATa his3Δ1 leu20 met15Δ0 ura3Δ0 CLB5-TAP:HIS3*	L.Alberghina
Sic1-TAP	*BY4741; MATa his3Δ1 leu20 met15Δ0 ura3Δ0 SIC1-TAP:HIS3*	Open
CdK1-TAP	*BY4741; MATa his3Δ1 leu20 met15Δ0 ura3Δ0 CDK1-TAP:HIS3*	Open
Cln1-TAP snf1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN1-TAP:HIS3*	this study
Cln2-TAP snf1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN2-TAP:HIS3*	this study
Clb5-TAP snf1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3*	this study
Sic1-TAP snf1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIC1-TAP:HIS3 snf1::KAN*	this study
Cdk1 -TAP snf1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CDK1-TAP:HIS*	this study
Cln1-TAP reg1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN1-TAP:HIS3*	this study
Cln2-TAP reg1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLN2-TAP:HIS3*	this study
Clb5-TAP reg1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3*	this study
Sic1-TAP reg1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SIC1-TAP:HIS3*	this study
Cdk1 -TAP reg1Δ	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CDK1-TAP:HIS3*	this study
Snf1-9myc	*BY4741: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SNF1-9MYC:URA3*	this study
snf4Δ	*BY4741; MATa his3Δ1 leu20 met15Δ0 ura3Δ0 snf4::KAN*	Euroscarf
clb5Δ [pCM189-CLB5-9Mvc]	*BF264-15D; MATa ade1, leu2-3,112, ura3, his2, KanS, clb5::ARG4 [pCM189-CLB5-9mvc]*	this study
clb5Δ snf1Δ [pCM189-CLB5-	*BF264-15D; MATa ade1, leu2-3,112, ura3, his2, KanS, clb5::ARG4 snf1::KAN [pCM189-CLB5-9mvc]*	this study
Clb5-TAP Sld2-9Myc	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 SLD2-9MYC:URA3*	this study
Clb5-TAP snf1Δ Sld2-9Myc	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 snf1::KAN SLD2-9MYC:URA3*	this study
Clb5-TAP snf1Δ[pSNF1-HA]	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 snf1::KAN [pSNF1-HA]*	this study
Clb5-TAP snf1Δ[pSNF1-	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 snf1::KAN [pSNF1-T210A-HA]*	this study
Clb5-TAP snf1Δ[pSNF1-	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 snf1::KAN [pSNF1-T210E-HA]*	this study
Clb5-TAP snf1Δ[pRS316]	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CLB5-TAP:HIS3 snf1::KAN [pRS316]*	this study

Table 2. Primers used in this work.

Name:	Sequence (5' → 3'):	*Used for:*
SNF1-KAN-F	ACTGTATGATGTTATCAAATCCAAAGATGAAATCATTATGGTTATAG AGTACGCCGGGAACGAATTGTTTGATCTGTTTAGCTTGCCTCG	*snf1*Δ
SNF1-KAN-R	ATATTTCCACCTTAATTTGATAGTCCATAAATCCTCTTCAGATGGCTT GGCCCATTCGGCACCCAAATTCATAGGCCACTAGTGGATCTG	*snf1*Δ
SNF-47-F	TAGCCACCACCACCACCATC	*Confirmation of SNF1 deletion*
REG1 F	TGATGATTATGATGACGGCTATCAGGAACACTCAACCTCCGTTTCT CCACCGCCGGCGGATAATGATAGCTAACTGTGGGAATACTC	*reg1*Δ
REG1 R	GGCTGGTGAAGGAGTATTATTTACAATTCTAAAGCTTTGTGAACTAT CGGACGCTTTTGGCTGCTTTGGACCTACCCTATGAACATATTCC	*reg1*Δ
CLB5-9myc F	CGATTCTGCAGATGGGAGAGAACCACGAC	*[pCM189-CLB5-9myc]*
CLB5-9myc R	CGACCTGCAGTTACGGTATCGGATCCAG	*[pCM189-CLB5-9myc]*
Sld2-9Myc F	TTCAGAAGACTAAAACTGCCAAAGAAAAACCGATTCTCTAATGGAC GATGGGGAAGAAGGAGATCTCTTAGCGGCCGCTCT	*Sld2-9Myc*
Sld2-9Myc R	ACTGAATACTTAATAGGTTTCTATAAATTACAAATGTTTGTATTATTT ACGCCATCACGCGCATGCTGCAGACGCGTTACG	*Sld2-9Myc*
SNF1-F	TTCAGCCTACCCATTTTTACATTTAACAACAAAACTAATTATGGAATT AGCCGTTAACAGTCAAAGCAATAGATCTCTTAGCGGCCGCTCT	*Snf1-9Myc*
SNF1-R	AAGATGTTGCAAATACGTTACGATACATAAAAAAAAGGGAACTTCCA TATCATTCTTTTACGTTCCACCAGCATGCTGCAGACGCGTTACG	*Snf1-9Myc*
SNF1-T210E F	ATGGTAATTTCTTAAAGGAGTCTTGTGGATCCCCCAATTATGCGGC TCCTGAAGTTAT	*[pSNF1-T210E-HA]*
SNF1-T210E R	ATAACTTCAGGAGCCGCATAATTGGGGGATCCACAAGACTCCTTTA AGAAATTACCAT	*[pSNF1-T210E-HA]*
ACT1 F	ACGTTCCAGCCTTCTACGTTTCCA	*qReal Time PCR*
ACT1 R	ACGTGAGTAACACCATCACCGGAA	*qReal Time PCR*
CLB5 F	AACGGCAGCAGAGCAAGAAGAAGA	*qReal Time PCR*
CLB5 R	AGGTCCTGCCAGCCTACTATTTCA	*qReal Time PCR*
PGK1 F	GGCTGGTGCTGAAATCGTTCCAAA	*qReal Time PCR*
PGK1 R	GTGTTGGCATCAGCAGAGAAAGCA	*qReal Time PCR*
CDC34 F	CCCACTTCTGAATCGGCGTACATA	*qReal Time PCR*
CDC34 R	ACCCGTTCTCATCGTCGTCCAAAT	*qReal Time PCR*

Snf1/AMPK promotes Swi6, FACT complex and RNA Polymerase II recruitment to G1-specific genes

Data obtained in our group and described in the first part of this thesis indicate that Snf1 regulates S-phase entrance and interacts with Swi6, the regulatory subunit of SBF (Swi4-Swi6) and MBF (Mbp1-Swi6) transcription complexes.

Here we show that, although Snf1 is not detectable at the promoters of G1-specific genes, it promotes the nuclear localization and the recruitment to G1-promoters of the transcription factor Swi6 and the DNA binding proteins Swi4 and Mbp1. Snf1, ensuring the recruitment of Swi6 allows the proper binding of FACT complex and RNA Polymerase II at *CLN2*, *PCL1* (SBF-dependent), *CLB5* and *RNR1* (MBF-dependent) promoters. Thus, protein Snf1, modulating the formation of Pre-Initiation Complex (PIC), regulates the expression of G1-genes and the execution of G1/S transition.

Moreover, our findings indicate that the kinase activity of Snf1 supports transcriptional elongation across G1-genes such as *CLN2* and *RNR1*, leading to a proper expression of those genes.

Busnelli S, Cirulli C, Tripodi F, Tedeschi G, Alberghina L and Coccetti P - Snf1/AMPK promotes Swi6, FACT complex and RNA Polymerase II recruitment to G1-specific genes-2013 submitted

RESULTS

Snf1 promotes transcription of G1-specific genes

Previous work from our group has revealed a connection between protein kinase Snf1 and cell cycle control at the G1/S phase transition. In particular it was found that Snf1 is required for the proper expression of *CLB5* gene (Pessina S. *et al.*, 2010). To further investigate if Snf1 was involved in the regulation of others genes of the G1-regulon, we extended our analyses to both SBF- (*CLN2*, *PCL1*) and MBF-dependent (*CLB5*, *RNR1)* genes. A *wild type* and a *snf1Δ* strain were synchronized by α-factor treatment in G1-phase and then released into fresh medium. In *wild type* cells the expression of G1-specific genes increased after the release and reached a peak at 20 minutes for both SBF- and MBF-dependent genes (Fig.6 C), in accordance with the earlier onset of bud emergence and DNA replication (Fig.6 A and B). As expected the *snf1Δ* strain showed a delayed G1/S transition (Fig.6 A and B). Consistently, the expression of G1-specific genes was severely reduced (Fig.6 C) at all time points considered, suggesting that protein kinase Snf1 is involved in the regulation of both SBF- and MBF-dependent transcription.

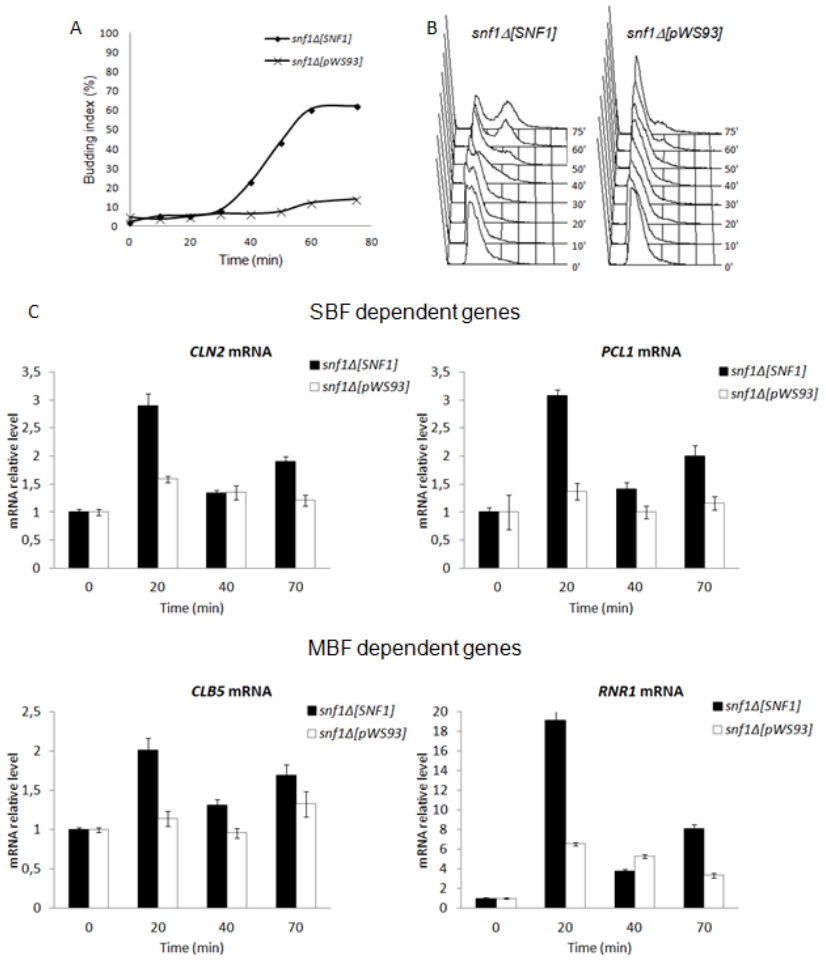


FIGURE 6 (A-C). Snf1 is required to ensure the correct transcription of G1-genes

(A) *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were grown in synthetic medium containing 2% glucose until exponential phase, G1-arrested by α-factor treatment and released into fresh medium. Samples were taken at the indicated time points to assay (A) budding index, (B) DNA content by FACS analyses, (C) the level of *CLN2*, *PCL1*, *CLB5* and *RNR1* mRNA by quantitative relative Real-time PCR. Reported values are the mean ± standard errors of three independent experiments.

The expression of G1-specific genes is negatively regulated by Whi5. This transcription inhibitor, in its unphosphorylated state, binds the SBF complexes to repress transcription. In late G1 phase, then, the Cdk1-dependent phosphorylation of Whi5 causes the nuclear export of this inhibitor, leading to activation of SBF- and MBF-dependent genes transcription (Costanzo M. *et al.*, 2004; de Bruin R.A.M. *et al.*, 2004). One of the better understood functions of Snf1 is to counteract the activity of transcription inhibitors such as Mig1 and its co-inhibitors Cyc8 and Tup1 to activate the expression of numerous genes (Treitel M.A. *et al.*, 1998; Papamichos-Chronakis M. *et al.*, 2004). Thus, we hypothesized that Snf1 could exert its role in the modulation of G1-specific transcription modulating the inhibitory activity of Whi5. Nevertheless, the deletion of *WHI5* did not rescue the *snf1Δ* phenotype (Fig.6, D and E), indicating that this inhibitor is not involved in the Snf1-mediated regulation of G1-gene expression.

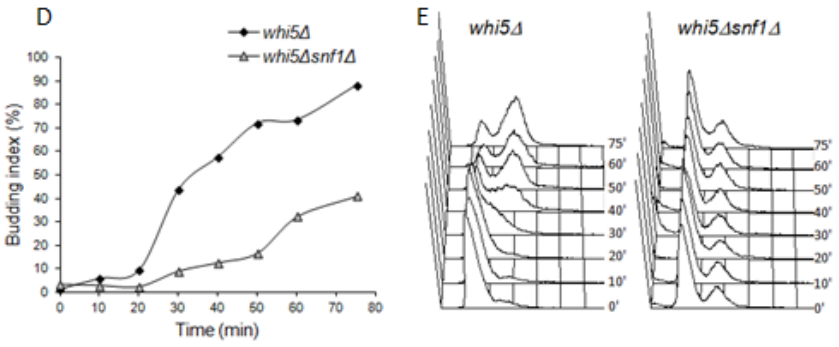


FIGURE 6 (D-E)

whi5Δ and *whi5Δsnf1Δ* strains were grown in synthetic medium containing 2% glucose, synchronized by α-factor treatment and then released into fresh medium. Samples were taken at the indicated time points to assay (B) budding index, (C) DNA content by FACS analysis.

Snf1-Swi6 interaction is not mediated by DNA

The induction of G1-specific transcription requires the transcription factor Swi6 which is tethered to promoters through its binding with the DNA-binding proteins Swi4 and Mbp1 (Lowndes N.F. *et al.*, 1992; Dirick L. *et al.*, 1992).
Since we had previously shown that Snf1 interacts with Swi6 (Pessina S. *et al.*, 2010), we investigated by Chromatin ImmunoPrecipitation (ChIP) experiments whether Snf1 was recruited to G1-specific promoters. *Wild type* strains expressing respectively myc-tagged Snf1 or Swi6 proteins were synchronized in G1 phase by α-factor treatment and released into fresh medium. Samples were collected at 0 and 20 minutes after the release and a ChIP assay was performed using an anti-myc antibody. Immunopurified DNA was analyzed by Real-time PCR using primers which amplify the SCB or MCB elements present in the promoters of *CLN2* or *CLB5* genes respectively. Besides, also TATA box sequences and an internal region of those genes were amplified (position of sequences shown in Fig.7 A). Snf1-myc was not detectable at promoters or coding regions of *CLN2* and *CLB5*, while Swi6-myc, here used as a positive control, was found at both SCB and MCB elements, as expected (Fig.7 B).
Consistently, the association between Snf1 and Swi6 was still detectable after DNAse treatment, indicating that this interaction was not mediated by DNA (Fig.7 C). In keeping with that finding, Snf1 interaction with Swi6 was not affected in cells lacking the DNA-binding proteins Swi4 or Mbp1 (Fig.7 D). The same held true in cells lacking Gal83 (data not shown) or Snf4 (Fig.7 D), the two subunits of Snf1 complex which are known to present a nuclear localization (Vincent O. *et al.*, 2001).
Collectively our data indicate that Snf1 modulates G1-specific transcription without a direct interaction with SBF- and MBF-regulated promoters.

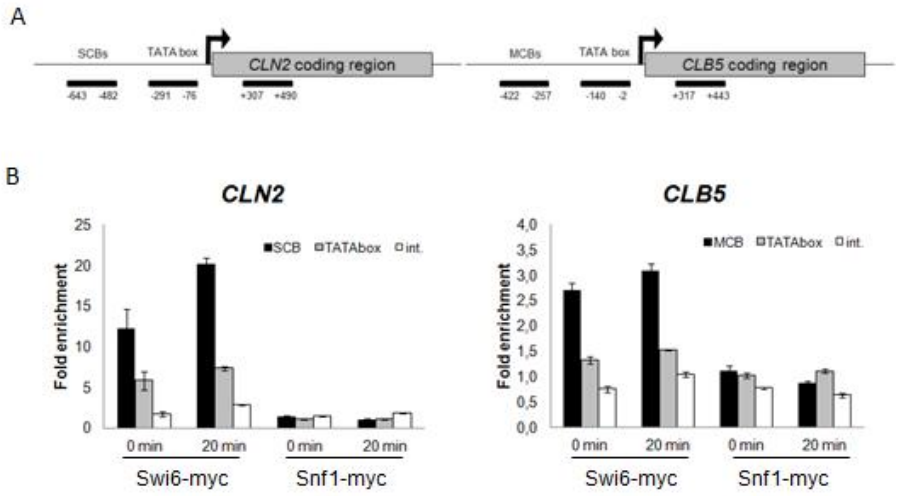


FIGURE 7 (A-B). Snf1 does not interact with G1-specific genes
(A) Diagrams of *CLN2* and *CLB5* genes with PCR amplicones corresponding to SCB or MCB sequences, TATA box and a mRNA-coding region at the indicated positions upstream or downstream from the ATG codon. (B) Snf1-myc and Swi6-myc cells were grown in synthetic medium containing 2% glucose, G1-arrested with α-factor treatment and released into fresh medium; samples were collected at the indicated time points after the release. Chromatin ImmunoPrecipitation analyses were performed with anti-myc antibody. Specific regions of *CLN2* and *CLB5* genes promoter containing SCB or MCB elements were amplified, as controls the TATA box and a sequence of the coding region of those genes was included, as shown in the diagram in (A). The amount of immunoprecipitated DNA was normalized on a non-transcribed region of Chromosome I sequence present in the IP DNA and considering the input DNA. Relative quantification was performed with respect to a *no tag* strain control, which was set as 1.

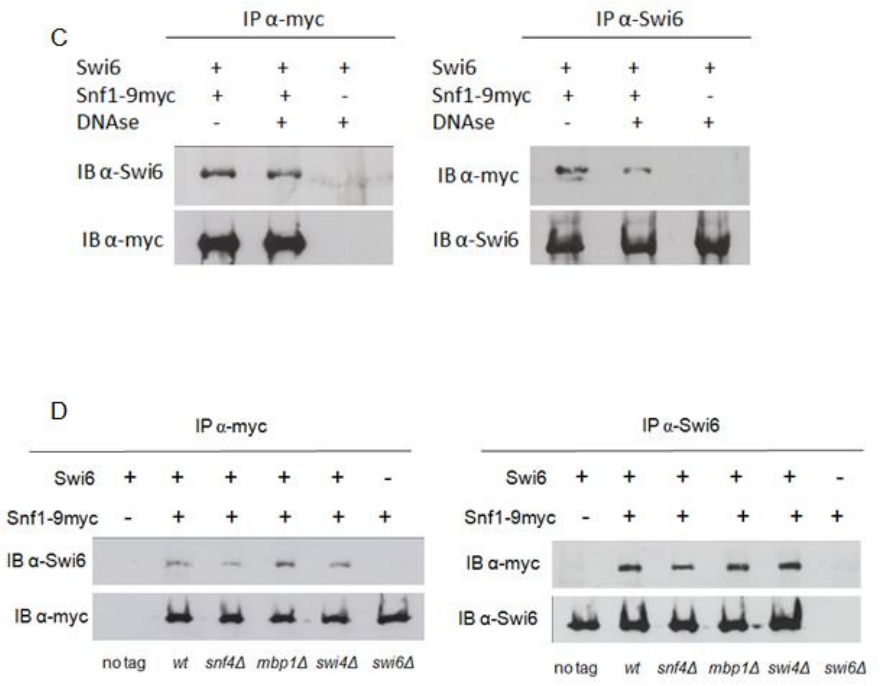


FIGURE 7 (C-D).

(C) Co-immunoprecipitation experiment was performed in a Snf1-myc strain. Part of the lysate was incubated with DNAse, as reported in Materials and Methods. (D) *Wild type*, *swi4Δ*, *mbp1Δ*, *swi6Δ* and *snf4Δ* strains expressing Snf1-myc were grown in synthetic medium supplemented with 2% glucose. In those experiments the immunocomplexes (IP) were precipitated with anti-myc antibody or anti-Swi6 antibody and analyzed by immune-blot (IB) with anti-Swi6 antibody and anti-myc antibody.

SNF1 deletion hampers the binding of Swi6 to SBF- and MBF-dependent promoters

Although Snf1 was not found anchored to G1-specific genes, we wondered if it could exert its function regulating Swi6 interaction to promoters. The binding of Swi6 to *CLN2* or *CLB5* promoters was then analyzed by ChIP experiments. *Wild type* and *snf1Δ* strains expressing Swi6-myc were G1 synchronized by α-factor treatment. *Wild type* cells showed an increase of Swi6 binding to promoters after 10-20 minutes from the release (Fig.8 A), coherently with the maximum expression of the corresponding mRNAs (Fig.6 C). On the contrary, in *snf1Δ* cells the binding of Swi6 was severely impaired from the beginning of the release, remaining low and without any significant increase at all the time points analyzed (Fig.8 A).

It is known that the subcellular localization of Swi6 influences its binding to promoters. In G1 phase, Swi6 accumulates into the nucleus by an import mechanism mediated by a NLS (Nuclear Localization Signal) present in its sequence (Sidorova J.M. *et al.*, 1995). Then, the phosphorylation of Swi6 on Ser160 residue by Clb6/Cdk1 complexes determines its delocalization into the cytoplasm, where Swi6 remains until the end of mitosis (Sidorova J.M. *et al.*, 1995; Geymonat M. *et al.*, 2004). In order to investigate whether the decreased binding of Swi6 to promoters could depend on an altered nuclear accumulation of this protein, we analyzed Swi6-myc localization in a snf1 null mutant by immunofluorescence experiments.

As expected, in *wild type* cells synchronized in G1 phase by α-factor treatment (data collected at 0 minutes after the release into fresh medium) Swi6 was essentially nuclear (Fig.8 B and C). Instead, in G1-synchronized *snf1Δ* cells Swi6 was localized in the nucleus only in the 60% of cells (Fig.8 B and C), consistently with the reduced binding of Swi6 to G1-specific promoters (Fig.8 A).

Therefore, the findings here reported indicate that protein Snf1 promotes the nuclear localization of Swi6 and its binding to SBF- and MBF-dependent genes.

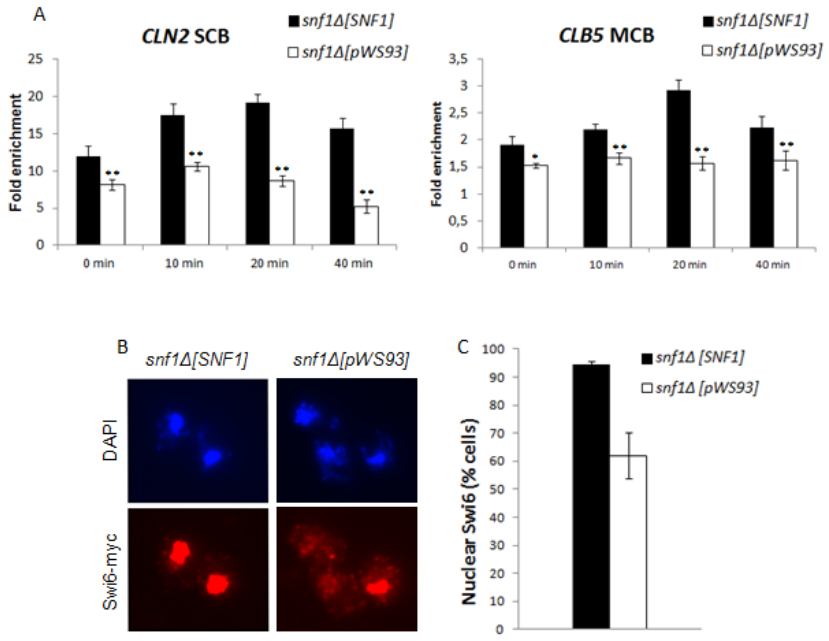


FIGURE 8. Snf1 is required for the binding of Swi6 to G1-specific promoters

(A) *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were G1-arrested by α-factor treatment and released in 2% glucose. At the indicated time points samples were taken and a ChIP essay was performed with anti-Swi6 antibody to test the binding of Swi6 to specific regions of *CLN2* and *CLB5* genes promoter which contains SCB or MCB elements. The amount of immunoprecipitated DNA was normalized on a non-transcribed region of Chromosome I sequence present in the IP DNA and considering the input DNA. Relative quantification was performed with respect to a *no antibody* control, which was set as 1. *p < 0.05, **p < 0.005. (B-C) Detection of Swi6-myc by indirect immunofluorescence on *snf1Δ[SNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains synchronized in G1 phase by α-factor treatment and released (sample taken at 0 minutes after the release). The cells were mounted with DAPI-containing solution for nuclei visualization. (B) Images of the cells are shown, (C) quantification of cells with Swi6 localized only in the nucleus. Reported values are the mean ± standard errors of two independent experiments.

Loss of Snf1 affects the binding of Swi4 and Mbp1 to DNA

It is well known that the Swi6 interaction to DNA is mediated by the DNA binding-proteins Swi4 and Mbp1 (Andrews B.J. and Moore L.A., 1992; Moll T. *et al.*, 1992). In fact, those proteins present a DNA-binding region which allow the interaction with specific elements in the promoters of G1-specific genes (Tailor I.A. *et al.*, 2000).

Since our data showed a defective Swi6 recruitment to promoters in a *snf1Δ* strain, we wondered whether that alteration could reflect a defective interaction of Swi4 and Mbp1 to DNA. So, we performed ChIP analyses in G1 synchronized cells in order to quantify the binding of Swi4 to the SCB sequences in the promoter of *CLN2* and of Mbp1 to the MCB elements present in *CLB5* promoter. Our analyses showed that in a *wild type* strain those proteins were recruited to promoters and their binding increased and reached the maximum after 10 minutes from the release into fresh medium (Fig.9 A and D). On the other hand, in a snf1 null mutant the interaction of both Swi4 and Mbp1 to promoters tested was significantly impaired (Fig.9 A and D).

On the base of those data we then analyzed the subcellular localization of Swi4 and Mbp1. Differently from Swi6, Swi4 and Mbp1 localize into the nucleus during all the cell cycle (Taberner F.J. and Igual J.C., 2010); therefore, we analyzed their localization in exponentially growing cells. In *wild type* cells, as expected, both Swi4 and Mbp1 accumulated into the nucleus, whereas in about 40% of *snf1Δ* cells there was a partial delocalization of those proteins into the cytoplasm (Fig.9 B-C and E-F).

Taken together, our findings support the hypothesis that protein Snf1 ensures the nuclear localization and the binding to G1-specific promoters of the SBF and MBF complexes components.

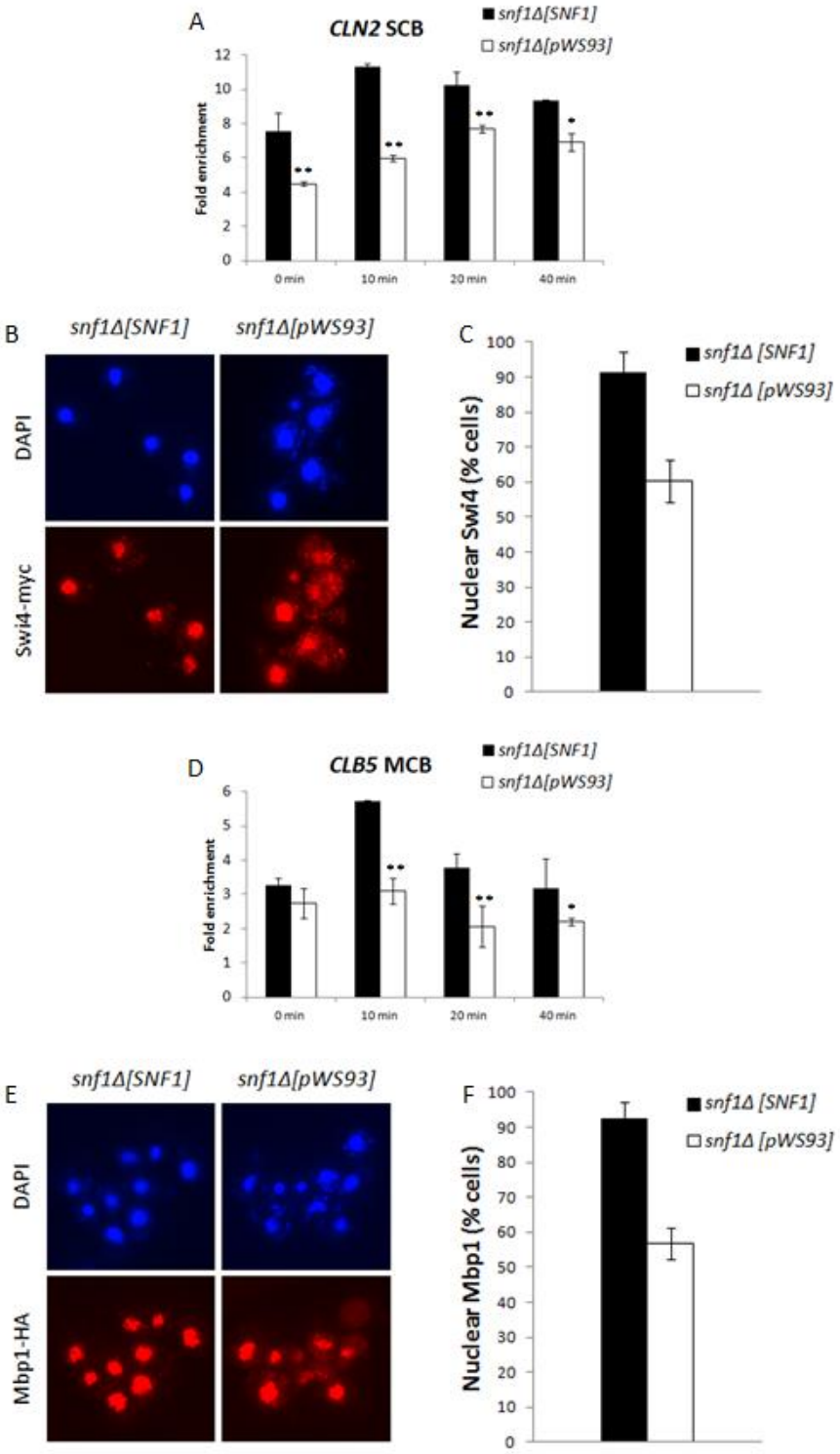

A
CLN2 SCB
snf1Δ[SNF1]
snf1Δ[pWS93]
Fold enrichment
0 min
10 min
20 min
40 min
B
snf1Δ[SNF1]
snf1Δ[pWS93]
DAPI
Swi4-myc
C
Nuclear Swi4 (% cells)
snf1Δ [SNF1]
snf1Δ [pWS93]
D
CLB5 MCB
snf1Δ[SNF1]
snf1Δ[pWS93]
Fold enrichment
0 min
10 min
20 min
40 min
E
snf1Δ[SNF1]
snf1Δ[pWS93]
DAPI
Mbp1-HA
F
Nuclear Mbp1 (% cells)
snf1Δ [SNF1]
snf1Δ [pWS93]

FIGURE 9. The interaction of Swi4 and of Mbp1 with DNA is positively regulated by protein Snf1

(A) *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains expressing either a Swi4-myc or a Mbp1-HA protein were grown in 2% glucose and arrested in G1 phase using α-factor. At the indicated time points samples were taken to test the binding of Swi4-myc to the SCB elements present in the promoter of *CLN2* and of Mbp1-HA to the MCB sequences present in the promoter of *CLB5*. The amount of immunoprecipitated DNA was normalized on a non-transcribed region of Chromosome I sequence present in the IP DNA and considering the input DNA. Relative quantification was performed with respect to a *no tag* strain control, which was set as 1. *$p < 0.05$, **$p < 0.005$. (B-C, E-D) Detection of Swi4-myc and of Mbp1-HA by indirect immunofluorescence on *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains in exponential phase. The cells were mounted with DAPI-containing solution for nuclei visualization. Images of (B) Swi4-myc cells and (E) Mbp1-HA cells are shown; (C-D) quantification of cells with Swi4-myc or Mbp1-HA localized only in the nucleus. Reported values are the mean ± standard errors of two independent experiments.

Snf1 is necessary for FACT complex and RNA PoI II recruitment at promoters of G1-genes

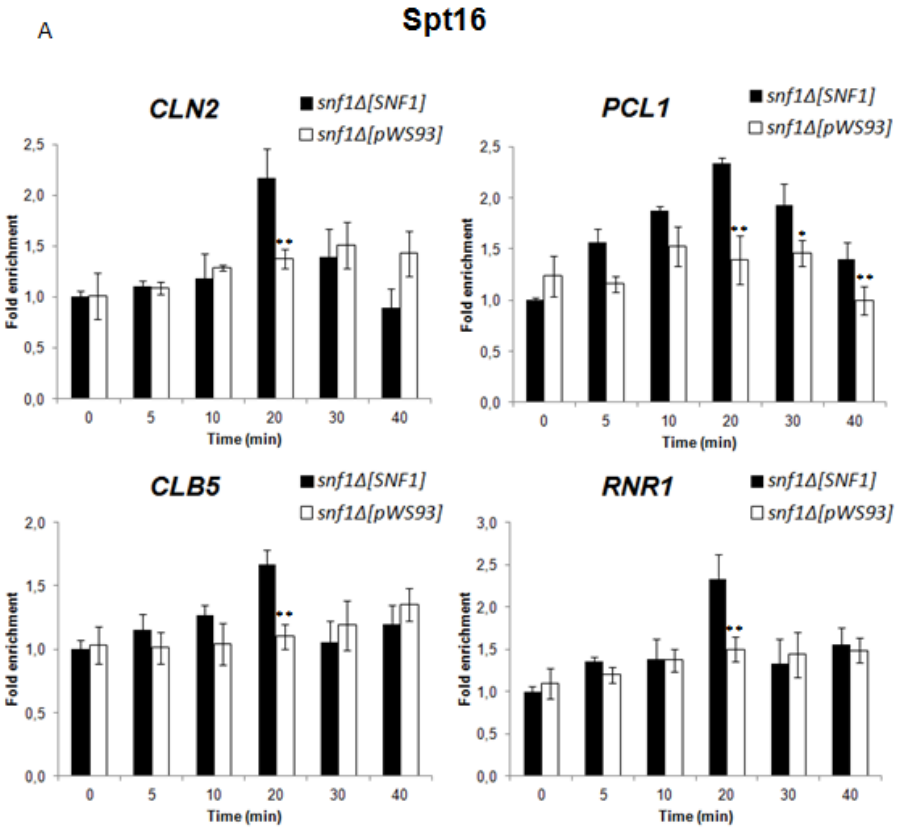
The binding of Swi4 and Mbp1 proteins to DNA and the recruitment of Swi6 set the stage for the binding of co-activators, such as the FACT complex which regulates chromatin remodelling to guarantee transcriptional initiation (Takahata S. *et al.*, 2009 a and b). The co-activators, then, promote the binding of RNA Pol II to promoters, the event which determines the formation of a Pre-Initiation Complex (PIC) necessary for transcription initiation (Cosma M.P. *et al.*, 1999; Cosma M.P. *et al.*, 2001; Bhoite L.T. *et al.*, 2001; Takahata S. *et al.*, 2009 a).

Since our data indicate that *SNF1* deletion determines the reduction of SBF and MBF binding to promoters of G1 genes, we explored whether this defect could alter the formation of the Pre-Initiation Complex. For that reason, we analyzed the binding of FACT complex and of RNA Pol II to the promoters of G1-specific genes by a ChIP assay. *Wild type* and *snf1Δ* strains were synchronized in G1 phase by α-factor treatment and released into fresh medium. Subsequently, formaldehyde-cross-linked chromatin was isolated and a ChIP assay was performed using an antibody specific for Spt16 (the main subunit of FACT complex) or for the CTD repeats of Rpb1 (the large subunit of RNA Pol II) at different time points after the release. Immunopurified DNA was analyzed by Real-time PCR using primers which amplify TATA box sequences of *CLN2*, *PCL1*, *CLB5* and *RNR1* promoters.

The recruitment of FACT complex to G1-specific promoters depends on Swi6 and shows a sharp peak of binding consistently with nucleosome loss and transcription initiation (Takahata S. *et al.*, 2009 a and b).

Our ChIP analyses performed in a wild type strain showed, as expected, a peak of FACT binding at promoters 20 minutes after the release from α-factor arrest (Fig.10, A). This peak was sharp for *CLN2*, *CLB5* and *RNR1* promoters, whereas at *PCL1* promoter the

binding of FACT increased gradually reaching the maximum level at 20 minutes (Fig.10 A).
On the contrary, in a *snf1Δ* strain no peak of Spt16 recruitment was shown at any promoter analyzed (Fig.10 A).
Consistently, the association of RNA Pol II with SBF- and MBF-dependent promoters was strongly impaired in a *snf1Δ* mutant (Fig.10 B), although the level of RNA Pol II of the snf1 null mutant was comparable with that of a *wild type* strain (Supplementary figure S1).
Therefore, our findings indicate that protein kinase Snf1, ensuring the nuclear localization and the binding of SBF and MBF components to DNA, promotes the formation of the Pre-Initiation Complex and the expression of G1-specific genes.



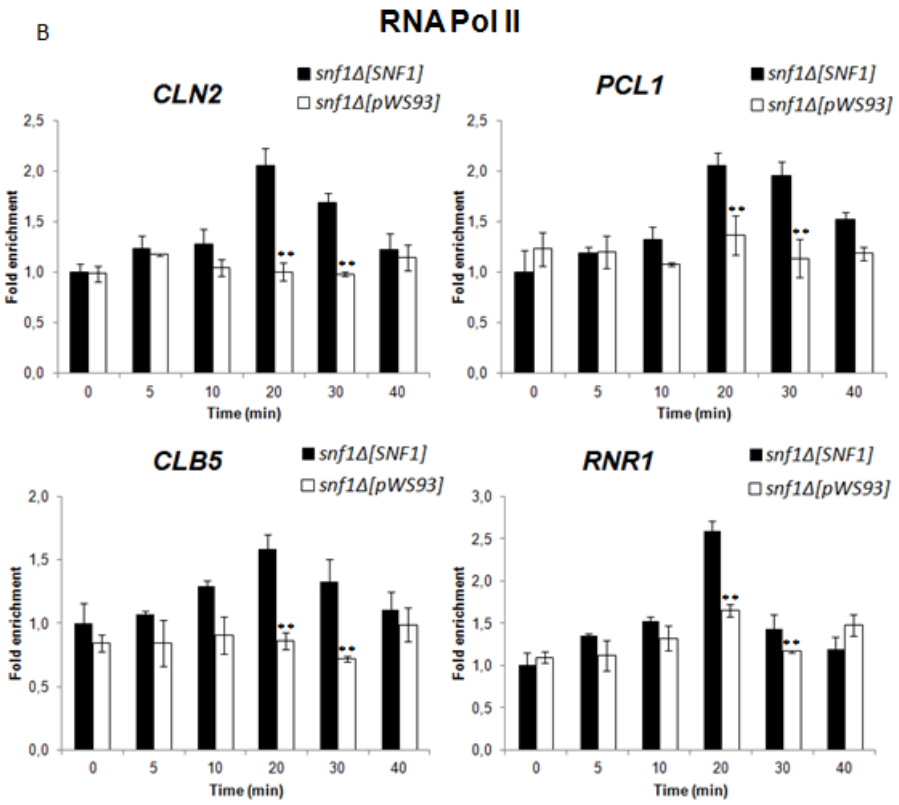


FIGURE 10. FACT and RNA Pol II recruitment to SBF- and MBF- dependent promoters depends on Snf1

(A-B) *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were grown in exponential phase, G1-arrested by α-factor treatment and released into fresh medium. Samples were taken at the indicated time points to perform Chromatin Immunoprecipitation assay using (A) anti-Spt16 antibody or (B) anti-RNA Pol II antibody (8WG16). Relative FACT and RNA Pol II occupancy at the TATA box of *CLN2* and *PCL1* and *CLB5* and *RNR1* genes was set with respect to occupancy of *wild type* strain at 0 minutes. Reported values are the mean ± standard errors of two independent experiments, all data were normalized on a non-transcribed region of Chromosome I. *$p < 0.05$, **$p < 0.005$.

The kinase activity of Snf1 in required for the proper expression of G1-specific genes

It is well known that phosphorylation of Snf1 at Thr210 leads to the full activation of the kinase (Hong S.P. *et al.*, 2003; Sutherland C.M. *et al.*, 2003).

Then, in order to obtain insight into the Snf1 molecular mechanism in cell cycle regulation, we investigated its phosphorylation on Thr210 during cell cycle progression. A strain expressing Snf1-myc was grown in minimal medium supplemented with 2% glucose, synchronized in G1 phase by α-factor treatment and released into fresh medium. The level of Snf1 α catalytic subunit was not cell cycle-regulated and its phosphorylation on Thr210 was only slightly detectable during the G1/S phase transition (Fig.11 A), suggesting that Snf1 might be partially active. Snf1-Thr210 was slightly phosphorylated also in cells growing in exponential phase (Supplementary figure S2), thus indicating that the phosphorylation of Thr210 did not depend on α-factor treatment.

To determine whether the activation of Snf1 was involved in its function as regulator of G1 transcription, we compared the expression of SBF- and MBF-dependent genes in the *SNF1-T210A* mutant with that detectable in *wild type* and *snf1Δ* strains. *Wild type*, *SNF1-T210A* and *snf1Δ* strains were grown until exponential phase and synchronized in G1 phase. In *wild type* cells a peak in transcription of G1-genes took place at 20 minutes after the release into fresh medium (Fig.11 D and E), consistent with bud emergence and the onset of DNA replication (Fig.11 B and C). Differently, the non-phosphorylatable Snf1 mutant (*SNF1-T210A*) and the *snf1Δ* strain, as reported (Pessina S. *et al.*, 2010), showed a delay of the G1/S phase transition (Fig.11, B and C) and exhibited a consistent reduction of transcription of both SBF- and MBF-dependent genes (Fig.11, D and E).

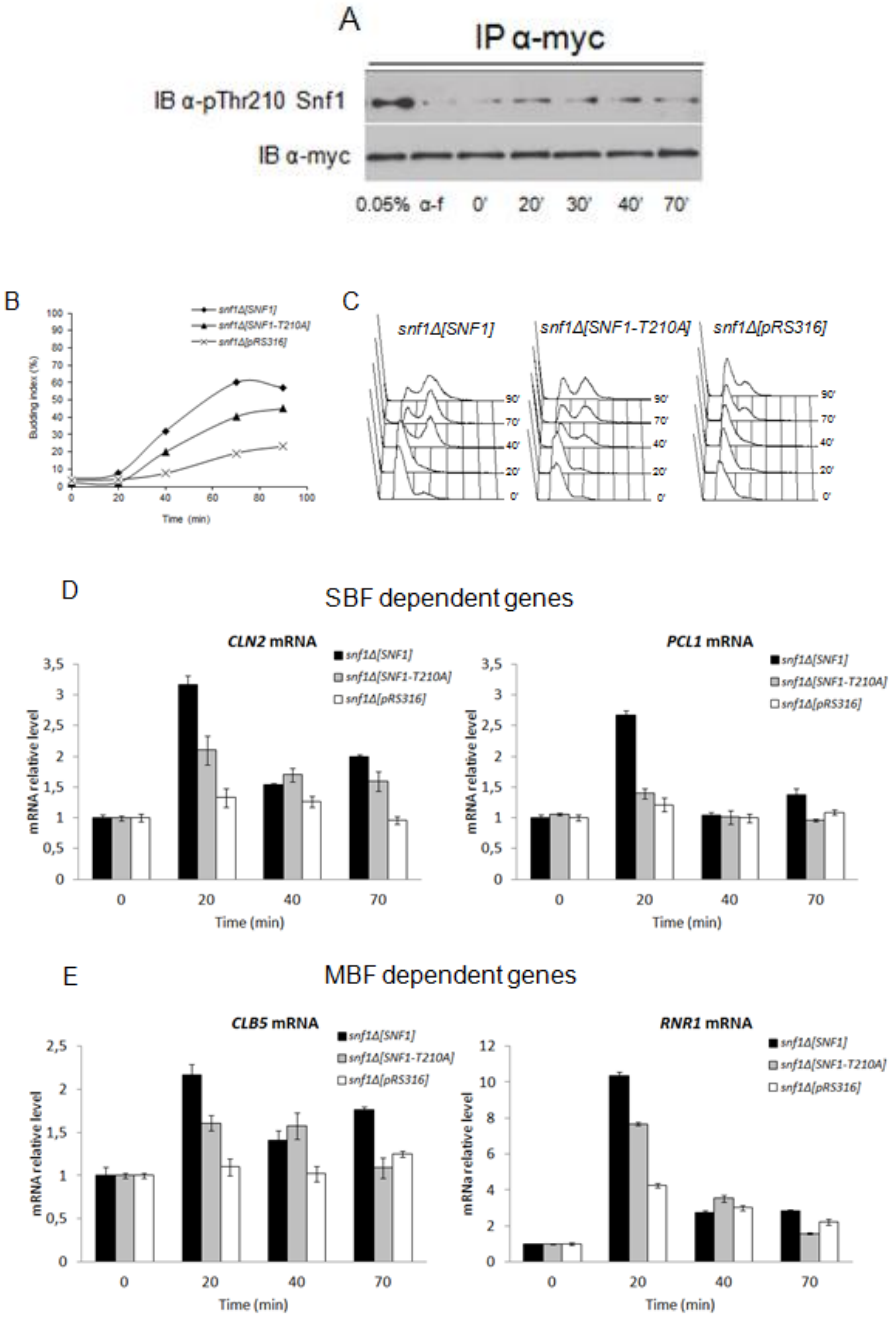

A
IP α-myc
IB α-pThr210 Snf1
IB α-myc
0.05% α-f 0' 20' 30' 40' 70'
B
snf1Δ[SNF1]
snf1Δ[SNF1-T210A]
snf1Δ[pRS316]
Budding index (%)
Time (min)
C
snf1Δ[SNF1]
snf1Δ[SNF1-T210A]
snf1Δ[pRS316]
90'
70'
40'
20'
0'
D
SBF dependent genes
CLN2 mRNA
PCL1 mRNA
mRNA relative level
Time (min)
E
MBF dependent genes
CLB5 mRNA
RNR1 mRNA

FIGURE 11. Snf1 is phosphorylated on Thr210 in 2%glucose growing cells

(A) A Snf1-myc expressing strain was grown in synthetic medium containing 2% glucose until exponential phase (exp), then a part of the culture was filtered, washed and shifted into synthetic medium containing 0.05% glucose for 10 minutes (0.05%). The rest of the culture was synchronized in G1 phase by α-factor treatment (α-f); cells were released into fresh medium and samples were taken at different time points (0, 20, 30, 40, 70 minutes). Snf1-myc was immunoprecipitated (IP) with anti-myc antibody and analyzed by immune blot (IB) with anti-pT210 Snf1 antibody or anti-myc antibody. (B-E) *snf1Δ[SNF1]* (*wild type*), *snf1Δ[SNF1-T210A]* (*SNF1-T210A*) and *snf1Δ[pRS316]* (*snf1Δ*) strains were grown in synthetic medium containing 2% glucose, G1-arrested by α-factor treatment and released into fresh medium. Samples were taken at the indicated time points to assay (B) budding index, (C) DNA content by FACS analysis and the level of (D) *CLN2*, *PCL1* and (E) *CLB5*, *RNR1* mRNA by quantitative relative Real-time PCR. Reported values are the mean ± standard errors of three independent experiments.

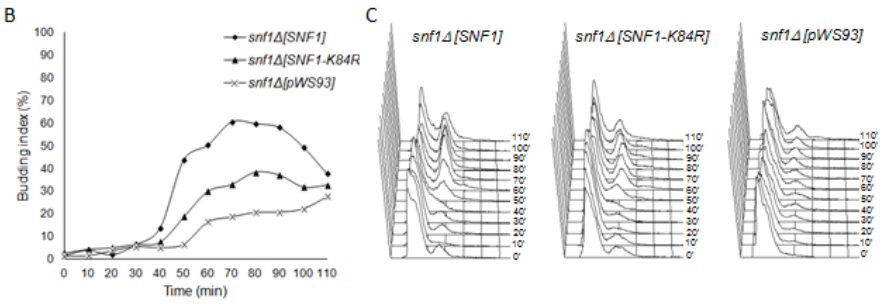
It has been reported that a mutation which prevents Snf1 activation (*SNF1-T210A*) still retains a residual kinase activity, giving a partial rescue of *snf1Δ* phenotype in response to different cellular stresses (Dubacq C. *et al.*, 2004; Portillo F. *et al.*, 2005, Shinoda, J. and Kikuchi Y., 2007; Wade S.L. *et al.*, 2009). To further investigate the role of the Snf1 kinase activity on G1-genes expression, we analyzed the phenotype of the *SNF1-K84R* strain in which the mutation of Lysine 84 residue determines the destruction of the ATP binding site. This mutant was largely used in *in vitro* assays as negative control by Carlson M. and co-workers, which defined it as a kinase-dead mutant (Celenza J.L. and Carlson M., 1989; Estruch F. *et al.*, 1992; Treitel M.A. *et al.*, 1998). Nevertheless, in further experiments a weak kinase activity was shown by this mutant (Nath N. *et al.*, 2002; Shinoda, J. and Kikuchi Y., 2007), suggesting that it is not completely kinase-dead. However, the *SNF1-K84R* mutant is unable to complement different phenotypes of a *snf1Δ* strain such as defective growth on sucrose, sensitivity to UV irradiation or response to stresses such as treatment with Hygromycin B or *o*-dinitrobenzene (*o*-DNB) (Portillo F. *et al.*, 2005, Shinoda, J. and Kikuchi Y., 2007; Wade S.L. *et al.*, 2009); thus, the weak activity of this mutant seems not to be sufficient to sustain Snf1-functions *in vivo*.

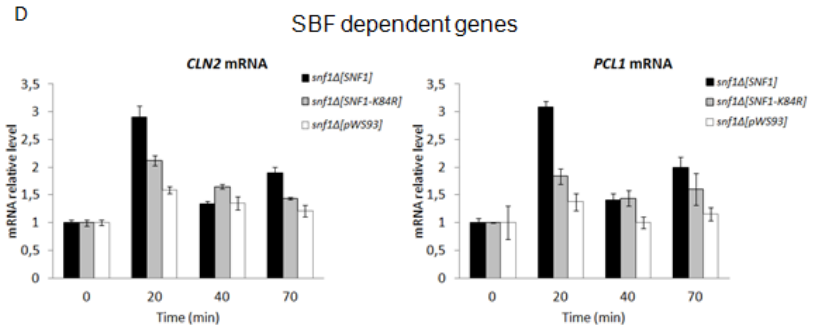
In order to ascertain the role of the Snf1 kinase activity for its function as cell cycle regulator we thus analyzed the G1/S transition in the *SNF-K84R*. The expression of *SNF1-K84R* partially complemented the slow growth phenotype of the *snf1Δ* strain (Fig.12 A). G1-synchronous cells expressing the *SNF1-K84R* mutant showed a delayed kinetic of bud formation and DNA synthesis (Fig.12 B and C). Accordingly, the expression of both SBF-dependent (*CLN2* and *PCL1*) and MBF-regulated genes (*CLB5* and *RNR1*) was significantly affected (Fig.12 D and E).

Taken together our findings suggest that in cells grown in 2% glucose Snf1 is partially active and that its kinase activity plays a significant role in the regulation of G1 phase transcription.

A

Strain	MDT (min)
snf1Δ[SNF1]	132.97 ± 7.56
snf1Δ[SNF1-K84R]	153.24 ± 8.37
snf1Δ[pWS93]	198.02 ± 1.44





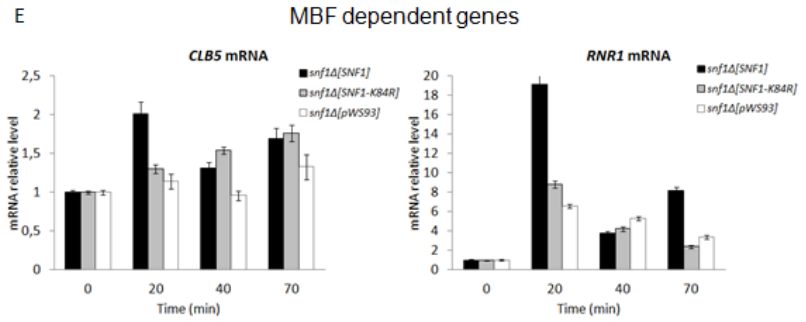


FIGURE 12. Snf1 kinase activity promotes the transcription of G1-genes

snf1Δ[pSNF1] (*wild type*), *snf1Δ[pSNF1-K84R]* (*SNF1-K84R*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were grown in synthetic medium containing 2% glucose. (A) MDT (mass duplication time) of exponentially growing cultures in 2% glucose; (B-E) cells were G1-arrested by α-factor treatment and released into fresh medium. Samples were taken at the indicated time points to assay (B) budding index, (C) DNA content by FACS analysis and the level of (D) *CLN2*, *PCL1*, (E) *CLB5* and *RNR1* mRNA by quantitative relative Real-time PCR. Reported values are the mean ± standard errors of three independent experiments.

The formation of a complete Pre-Initiation Complex at G1 promoters does not involve Snf1 catalytic activity

It is well known that Swi6 is a substrate of different protein kinases, such as Cdk1 or Rad53, which regulate its nuclear localization and its activity as transcriptional factor (Sidorova J. *et al.*, 1995; Sidorova J. and Breeden L., 1997; Queralt E. and Igual J.C., 2003). Moreover, we had shown that Snf1 and Swi6 interact, and that lack of Snf1 protein causes an altered localization of Swi6 (Fig.8 B and C). Thus, we hypothesized that Swi6 could be a substrate of the protein kinase Snf1. The analysis of Swi6 sequence showed that Ser760 resembled the AMPK consensus (Φ-x-R-x-x-S/T-x-x-x-Φ, Φ=hydrophobic residue) (Dale S. *et al.*, 1995). By using mass spectrometry analysis we found that Ser760 was actually phosphorylated by Snf1 *in vitro* (Fig.13 A). However, site-specific mutants (*SWI6-S760A* or *SWI6-S760E*) showed neither a remarkable alteration of growth rate (data not shown) nor relevant defects in S-phase entrance (Fig.13 B and C). In addition, the interaction of Snf1 with Swi6 was still detectable in a *SNF1-K84R* strain (Fig.13 D).

The ChIP analyses of Swi6 binding to *CLN2*, *PCL1* and *CLB5*, *RNR1* promoters showed that in the *SNF1-K84R* mutant the recruitment of Swi6 was slightly affected (Fig.14 A and Supplementary figure S3); nevertheless, that alteration was not severe as that of a *snf1Δ* strain (Fig.8 A).

Consistently, the recruitment of FACT complex to G1-specific promoters, an event dependent on protein Swi6 (Takahata S. *et al.*, 2009 a), was not affected neither at SBF- nor at MBF-dependent promoters (Fig.14 B and Supplementary figure S3). Moreover, accordingly with the proper recruitment of FACT complex, also the RNA Pol II binding to G1-promoters was not affected in the *SNF1-K84R* mutant (Fig.14 C and Supplementary figure S3), indicating that the kinase activity of Snf1 is not required for the proper formation of the PIC at G1-specific promoters.

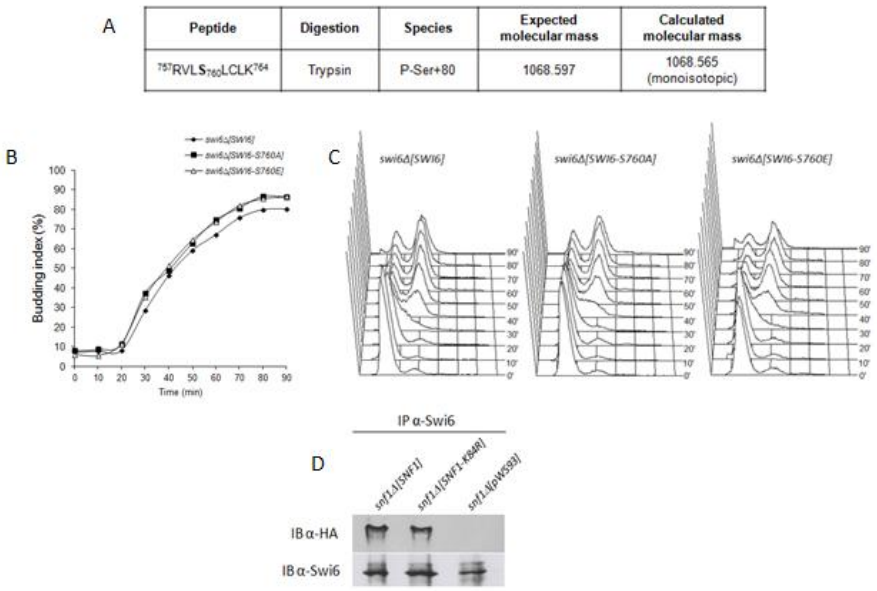


FIGURE 13. Snf1 phosphorylation on Swi6 is not involved in the Snf1-mediated regulation of cell cycle progression

(A) 10 µg of His_6-Swi6 were phosphorylated *in vitro* by Snf1-HA immunopurified from yeast cells, reaction mixtures were incubated at 30°C for 30 min. Samples preparation and mass spectrometry analysis are described in Materials and Methods. (B-C) *swi6Δ[pSWI6]* (*wild type*), *swi6Δ[pSWI6-S760A]* (*SWI6-S760A*), and *swi6Δ[pSWI6-S760E]* (*SWI6-S760E*) strains were G1-arrested by α-factor treatment and released into fresh medium. Samples were taken at the indicated time points to assay (B) budding index and (C) DNA content by FACS analysis. (D) *snf1Δ[pSNF1]* (*wild type*), *snf1Δ[pSNF1-K84R]* (*SNF1-K84R*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were grown in synthetic medium containing 2% glucose until exponential phase. Swi6 was immunoprecipitated with anti-Swi6 antibody and the immunocomplexes were analyzed by immune-blot (IB) with anti-HA antibody and anti-Swi6 antibody.

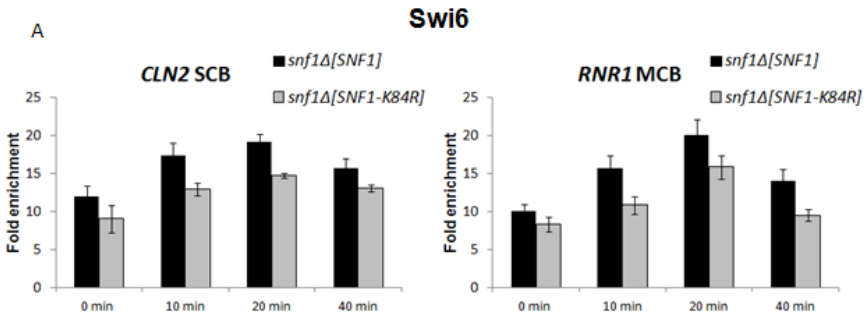


FIGURE 14 (A). Snf1 catalytic activity is not required for the recruitment of Swi6, FACT complex or RNA Pol II to promoters
snf1Δ[pSNF1] (*wild type*) and *snf1Δ[SNF1-K84R]* (*SNF1-K84R*) strains were grown in synthetic medium containing 2% glucose until exponential phase and G1-arrested by α-factor treatment. At the indicated time points samples were taken to test the binding of Swi6 to SCB or MCB elements of *CLN2* and *RNR1* promoters respectively by a ChIP analysis performed using an anti-Swi6 antibody. The amount of immunoprecipitated DNA was normalized on a non-transcribed region of Chromosome I sequence present in the IP DNA and considering the input DNA. Relative quantification was performed with respect to a *no antibody* control, which was set as 1.

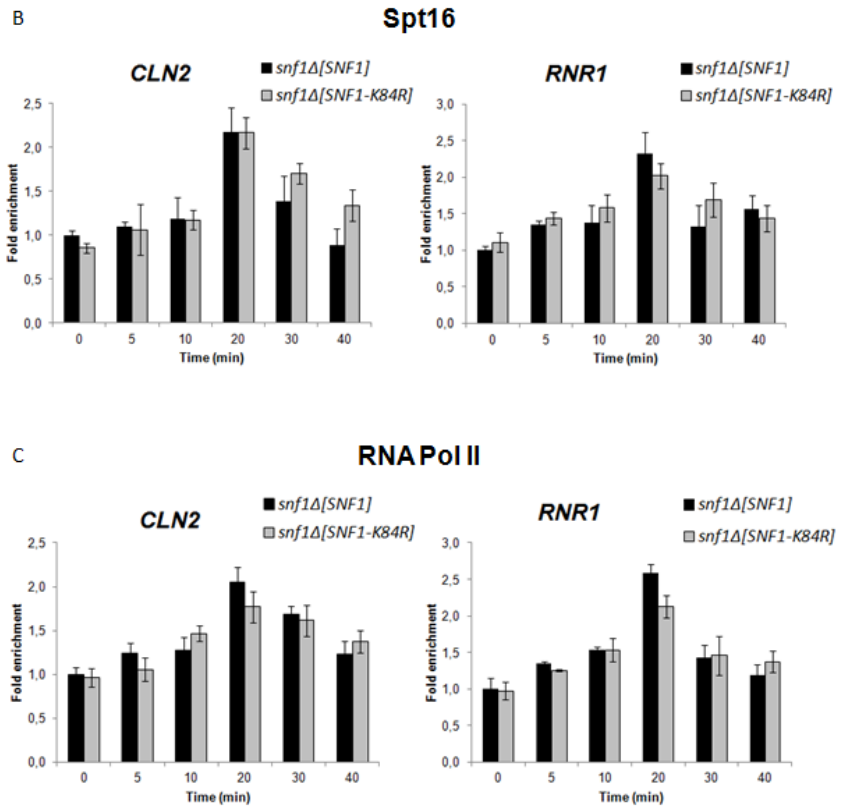
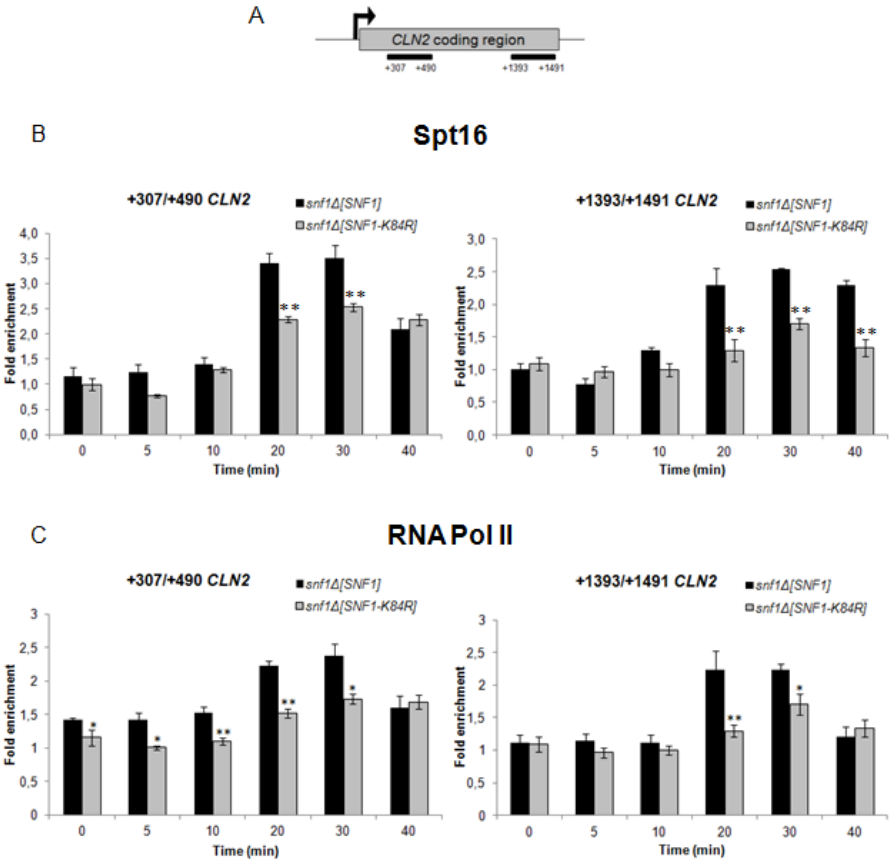


FIGURE 14 (B-C).
In *snf1Δ[pSNF1]* (*wild type*) and *snf1Δ[SNF1-K84R]* (*SNF1-K84R*) arrested in G1-phase and released; a ChIP analysis was performed using (B) anti-Spt16 antibody or (C) anti-RNA Pol II antibody and TATA box sequences present in the promoter of *CLN2* or *RNR1* were amplified. The amount of immunoprecipitated DNA was normalized on a non-transcribed region of Chromosome I sequence present in the IP DNA and considering the input DNA. Relative quantification was performed with respect to a *no antibody* control, which was set as 1.

Since this last finding seemed to disagree with the severe reduction of mRNA expression of SBF- and MBF-dependent genes observed in the *SNF1-K84R* mutant (Fig.11 D and E), we wondered whether defects in transcriptional elongation might occur in the *SNF1-K84R* mutant.
Trascriptional elongation process requires the remodelling of nucleosomes at promoters and across active genes (Sims J.R. *et al.*, 2004; Workman J.L., 2006). The transcription elongation complex FACT binds the coding regions of genes and alters the structure of chromatin in order to facilitate the movement of RNA Pol II across active transcribed genes (Mason P.B. and Struhl K. 2003; Reinberg D. and Sims R.J. 2006). To investigate defects in transcriptional elongation we then compared the FACT complex occupancy at G1-specific genes detectable in a *SNF1-K84R* mutant, with that of a *wild type* strain. Using anti-Spt16 antibodies we performed ChIP experiments and the immunopurified DNA was then analyzed by Real-time PCR to quantify the binding of FACT complex to the coding regions of *CLN2* and *RNR1* (at +307/+490 and +1393/+1491 from the ATG codon of *CLN2* gene and at +951/+1059 and +2201/+2340 of *RNR1* gene) as described in figure 15, A and D.
Although in the *SNF1-K84R* mutant the localization of Spt16 to promoters of both SBF- or MBF-dependent genes was not affected (Fig.14 B and Supplementary figure S3), its occupancy at the coding regions of the same genes was significantly altered (Fig.15 B and E). In fact, in the *SNF1-K84R* mutant the binding of FACT to *CLN2* coding regions was severely decreased at all time points considered. Differently, in the *SNF1-K84R* mutant the FACT occupancy at the +307/+490 region of *RNR1* reached its maximum at 30 minutes and became higher than that of the *wild type* strain at the same time point. However this level of FACT occupancy was lower than that detectable in the *wild type* strain at 20 minutes after the release, indicating that in the *SNF1-K84R* mutant FACT recruitment to coding region of *RNR1* gene was both delayed and decreased.
To further analyze that defect in transcriptional elongation, the level of RNA Pol II occupancy at the same regions of the Open Reading Frame of *CLN2* and *RNR1* was investigated by ChIP experiments.

In *wild type* cells the density of RNA Pol II molecules at internal regions of *CLN2* and *RNR1* genes reached the maximum 20-30 minutes after the release from α-factor arrest (Fig.15 C and F). In contrast, in the *SNF1-K84R* strain there was a statistically significant decrease in RNA Pol II occupancy at coding regions, especially at 20 minutes (Fig.15, C and F). Remarkably, as for FACT complex binding, our analyses indicate that in the *SNF1-K84R* mutant also the recruitment of RNA Pol II at the *RNR1* coding regions was both delayed and decrease (Fig.15, F).

Taken together our findings support the notion that the kinase activity of Snf1 stimulates FACT and RNA Pol II occupancy to the coding regions of G1-specific genes, promoting the expression of those genes.


A
CLN2 coding region
+307 +490
+1393 +1491
B
Spt16
+307/+490 CLN2
snf1Δ[SNF1]
snf1Δ[SNF1-K84R]
Fold enrichment
Time (min)
+1393/+1491 CLN2
C
RNA Pol II

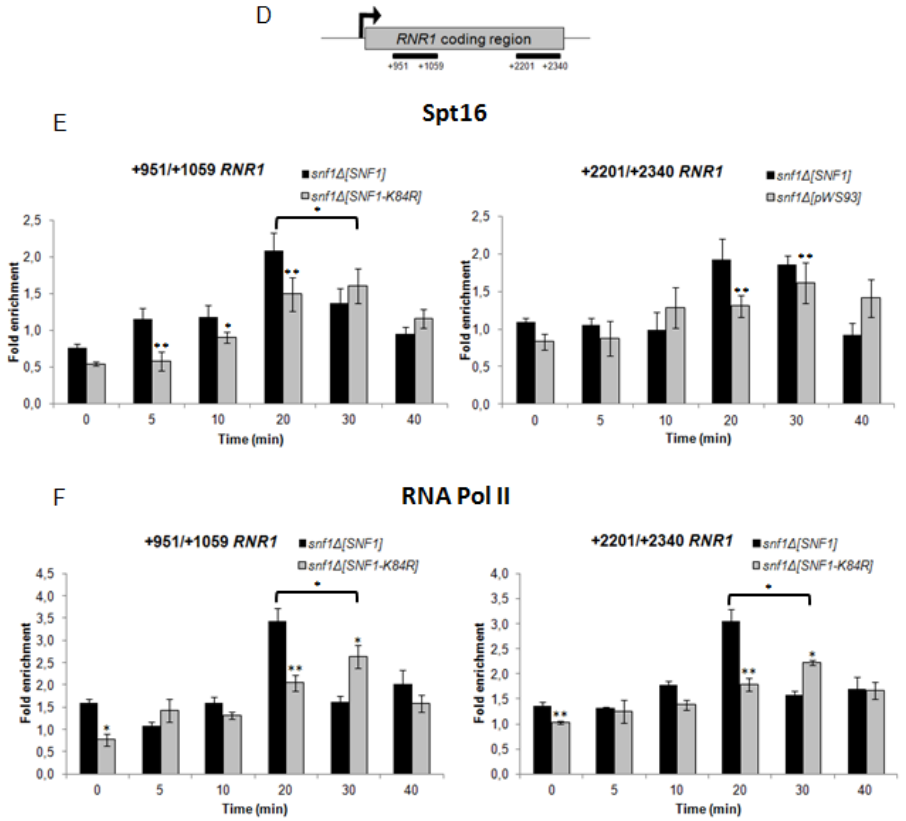
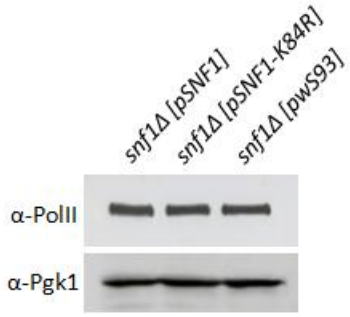


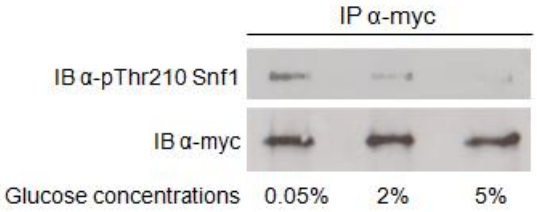
FIGURE 15. The kinase activity of Snf1 is important for normal levels of FACT complex and RNA Pol II occupancy at the coding regions of G1-specific genes.

(A-D) Diagrams of *CLN2* and *RNR1* genes with PCR amplicones corresponding to the mRNA-coding regions at the indicated positions downstream from the ATG codon. (B-E) Spt16 and (C-F) RNA Pol II occupancy at *CLN2* and *RNR1* coding regions at different time points after α-factor release. Relative quantification was performed with respect to a *no antibody* control which was set as 1. Reported values are the mean ± standard errors of two independent experiments, all data were normalized on a non-transcribed region of Chromosome I. *$p < 0.05$, **$p<0.005$.

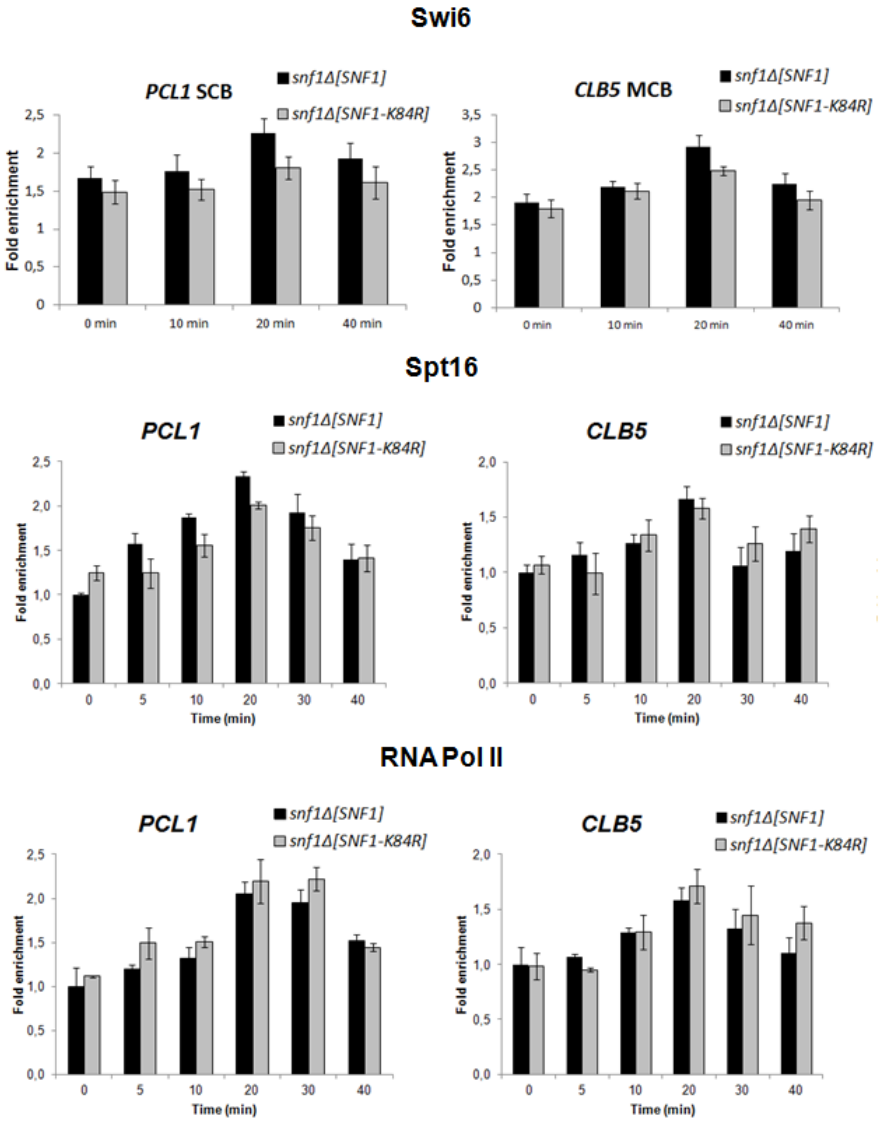
SUPPLEMENTARY MATERIALS



Supplementary Figure S1.
snf1Δ[SNF1] (*wild type*), *snf1Δ[SNF1-K84R]* (*SNF1-K84R*) and *snf1Δ[pWS93]* (*snf1Δ*) strains were grown in synthetic medium containing 2% glucose. Samples were taken in exponential phase to detect the level of RNA Pol II by western blot analysis using anti-RNA Pol II antibody (8WG16) (anti-Pgk1 antibody was used as loading control).



Supplementary Figure S2.
A Snf1-myc expressing strain was grown in synthetic medium containing 0.05%, 2% or 5% glucose until exponential phase. Snf1-myc was immunoprecipitated (IP) with anti-myc antibody and analyzed by immune-blot (IB) with anti-pT210 Snf1 antibody or anti-myc antibody.



Supplementary Figure S3.
Analyses of Swi6, Spt16 or RNA Pol II binding to the promoters of *PCL1* or *CLB5* genes; ChIP experiments were performed as described in Fig.14

MATERIALS AND METHODS

Yeast strains and growth conditions

S. cerevisiae strains used in this study are listed in Table 1. Standard genetic methods were used for strain construction. Synthetic medium contained 2% glucose, 6.7 g/L of Yeast Nitrogen Base (Difco), 100-50 mg/L of required nutrients, at standard pH (5.5). Cell density of liquid cultures grown at 30°C was determined with a Coulter counter on mildly sonicated and diluted samples. For G1 synchronization, cells were grown to exponential phase and α-factor (GenScript) was added to a final concentration of 3 μM. Percentage of budded cells was determined by direct microscopic counting of at least 300 cells after mild sonication.

Protein extraction, immunoprecipitation assays and immunoblotting

Cells samples were collected by filtration and immediately frozen at -80°C. Protein extractions, immunoprecipitations and western blot analysis were performed essentially as previously described in Pessina S. *et al.*, 2010. For coimmunoprecipation experiments endogenous Swi6 and Snf1-myc were immunopurified from 1-5 mg of total protein extract. DNAse treatment was performed as in Wilson M.A. *et al.*, 2011. Anti-HA mouse monoclonal antibody (12CA5, Roche), anti-c-myc mouse monoclonal antibody (9E10, Santa Cruz Biotechnology), anti-Swi6 rabbit polyclonal antibody (kindly provided by L.L. Breeden), anti-RNA Pol II (8WG16, Abcam), Anti-Spt16 (kindly provided by T. Formosa), anti-Pgk1 mouse monoclonal antibody (Molecular Probes) and anti-Snf1 pThr210 (kindly provided by S. Hohmann) (1:500 dilution for anti-c-myc; 1:1000 dilution for all other antibodies) were used for western blot analysis.

Flow cytofluorimetric analysis

Cells were collected, fixed in 70% ethanol, stained with SYTOX Green nucleic acid stain (Molecular Probes) and analyzed for DNA content as previously described in Pessina S. *et al.*, 2010. Flow cytofluorimetric analysis were performed using a BD FACScan (Beckton-Dickinson).

RNA isolation and qReal-time PCR

Total RNA was isolated using a phenol-chloroform protocol, essentially as previously described Pessina S. *et al.*, 2010. Reverse transcription of 0.5 µg of mRNAs was carried out with iScript cDNA Synthesis Kit (BIO-RAD). Quantitative Real-time PCR for *CLN2*, *PCL1*, *CLB5* and *RNR1* genes expression were performed using SsoFast EvaGreen Supermix (BIO-RAD), oligos available upon request. The obtained data were normalized on *PGK1* and *CDC34* reference genes and organized with CFX manager software (BIO-RAD). Data are presented as the mean value ± standard deviation from three independent experiments.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was performed essentially as previously described by Baetz K. *et al.*, 2001. In a few instances the protocol was changed as described afterward. Cells were grown and treated with 1% formaldehyde for 15 min. Glycine was added to a final concentration of 125 mM and the cultures were further incubated for 5 min. The cells were then washed once with cold HBS buffer (50 mM Hepes pH 7.5, 140 mM NaCl); after filtration cell pellets were resuspended in 400 µl ChIP lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 0,1% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, proteases inhibitor mix Complete EDTA free Protease Inhibitor Cocktails Tablets, Roche). The cell suspension was then mixed with an equal volume of glass-beads and the cells were broken at 4°C on vortex. Cell lysates were then sonicated to yield an average DNA fragment size of 500 bp and clarified by centrifugation at 10000 x g for 15 min. Protein concentration for each sample was measured by the Bradford method using a BIO-RAD protein assay kit. An equal amount of proteins (1 mg) were incubated overnight at 4°C with 5 µl of anti-c-myc mouse monoclonal antibody (9E10, Santa Cruz Biotechnology) or anti-RNA Pol II antibody (8WG16, Abcam) and 30 µl of protein A magnetic beads (Millipore) previously equilibrated in ChIP lysis buffer. The beads-bound immunocomplexes were washed three times with ChIP lysis buffer, twice with TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and once with TE (10 mM Tris

pH 8.0, 1 mM EDTA). Each wash was performed for 10 min at 4°C. The immunoprecipitated material was eluted from magnetic beads by incubation at 65°C for 10 min with 150 µl of TE containing 1% SDS. The eluted material was incubated at 65°C for 8-10 h to reverse the cross-link and the immunoprecipitated DNA was purified using a PCR purification kit (Qiagen) following the instructions of the manufacturer. Control immunoprecipitations without anti-RNA Pol II or anti-Spt16 or anti-Swi6 antibody (no Ab) or from a strain without myc-tag (no tag) were also performed to subtract the background. Real-time PCRs were performed to quantify the relative enrichment of target DNA fragments after Immunoprecipitation. Reactions were performed in triplicate using the Sso Fast EvaGreen Supermix (BIO-RAD) and carried out in a MiniOpticon (BIO-RAD). The enrichment of target regions were calculated using an intergenic region of Chromosome I as a reference for nonspecific DNA as reported in Takahata S. *et al.*, 2011. For each experiment shown, ChIP analysis was performed at least twice using independently prepared batches of chromatin. Results were compared by using a two-sided Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Immunolocalization

Immunolocalization experiments were performed essentially as in Rossi *et al.*, 2005, using primary anti-HA or anti-myc antibody (9E10, Santa Cruz Biotechnology, 1:200) and secondary anti-mouse antibody conjugated with AlexaFluor555 (Invitrogen, 1:400). Images were taken with a Nikon Eclipse E600 microscope equipped with a Leica DC 350F ccd camera.

Purification of recombinant His_6-Swi6

E. coli strain *BL21(DE3)[pLysE]* was used for expression of recombinant His_6-Swi6. *SWI6* was cloned into the *pIVEX2.4a* plasmid using customly designed primers as a *SalI-PstI* fragment. His_6-Swi6 protein was purified on Ni^{2+}/nitrilotriacetate beads (Qiagen) following the manufacturer's instructions and eluted with 250 mM imidazole. Protein concentration was measured by the Bradford method using a BIO-RAD protein assay kit. The purified protein was dialyzed against

kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100) and stored at -80°C.

***In vitro* phosphorylation assay**

Active protein kinase Snf1 was immunopurified from protein extracts of a *snf1Δ[SNF1-HA]* strain grown in YPD medium containing 0.05% glucose. 50 mg of total protein extract were immunoprecipitated using 50 µl of immobilized anti-HA antibody agarose beads (Roche) according to the manufacturer's instructions. The beads were washed three times and resuspended in 40 µl of kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100) as previously described in Treitel *et al.*, 1998. The kinase reaction was started by adding 10 µg of recombinant His_6-Swi6 and 1 mM ATP, incubated at 30°C for 30 min and stopped by adding 50 µl of SDS-sample buffer. Proteins were resolved by SDS-PAGE and the gel was stained using colloidal Coomassie (Gel Code, Pierce).

Sample preparation and mass spectrometry analysis

Coomassie blue-stained protein bands were excised from SDS-PAGE and washed once with MilliQ grade water (5 min). Protein gel pieces were then destained first with acetonitrile and then with 0.1 M NH_4HCO_3 pH 8 (three times, 15 min). Protein samples were reduced by incubation in 10 mM DTT, 0.1 M NH_4HCO_3 buffer for 45 min at 56°C; then free cysteines were alkylated by incubation in 55 mM iodoacetamide, 0.1 M NH_4HCO_3 buffer for 30 min at room temperature in the dark. The supernatant of the alkylating solution was discarded and the reaction stopped by washing gel pieces with acetonitrile and digestion buffer (10 mM NH_4HCO_3 pH 8). Enzymatic digestion was performed incubating gel pieces in sequencing grade trypsin (Promega) (1 ng/µl) in 10 mM NH_4HCO_3 pH 8, for 2 h at 4°C. Trypsin solution was removed and gel pieces were incubated in 40 µl of 10 mM NH_4HCO_3 pH 8 for 18 h at 37°C. Peptides were then extracted by washing the gel pieces with 1% formic acid in 50% acetonitrile. The extracted peptides were subject to a desalting/concentration step on a µZipTipC18 (Millipore) using 40% CH_3CN in 0.1% TFA as eluent before LC-ESI-MS/MS analysis, using a Dionex UltiMate 3000 HPLC System (Dionex S.p.A) with a

Hypersil Gold column (150 mm, internal diameter of 180 µm) filled with 3 µm Reprosil-Pur C18-AQ resin (Dr. Maisch GmbH). The gradient consisted of 5-15% acetonitrile in 0.1% formic acid for 10 min, 15-40% acetonitrile in 0.1% formic acid for 52 min and 40-95% acetonitrile in 0.1% formic for 68 min at a flow rate of 1.2 µl/min. The eluate was electrosprayed into an LTQ Orbitrap Velos (Thermo Fisher Scientific) through a Proxeon nanoelectrospray ion source. The LTQ Orbitrap Velos was operated in a CID top 5 mode. The resolution was 60,000 for the Orbitrap whereas fragment spectra were read out at low resolution in the LTQ. Ion trap and Orbitrap maximal injection times were set to 50 ms and 1000 ms, respectively. The ion target values were 5000 for the ion trap and 1000000 for the Orbitrap. Raw files were processed using version 1.1 of Protein Discoverer (Thermo Scientific). For protein identification the Sequest program was used to search the NCBI protein Data Bank setting carbamidomethylation as fixed modification and oxidation (M), phosphorylation (Ser, Thr and Tyr) and deamidation (Asn and Gln) as variable modifications. Initial peptide mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.8 Da. Two missed cleavages were allowed. Peptide quality scores were derived by processing against decoy shuffled databases.

TABLE 1 Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
snf1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KAN*	Pessina *et al.*, 2010
whi5Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, whi5::kanMX4*	Open Biosystems
whi5Δsnf1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, whi5::kanMX4 snf1::HPH*	This study
Snf1-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SNF1-9MYC:URA3*	Pessina *et al.*, 2010
mbp1Δ Snf1-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, mbp1::kanMX4 SNF1-9MYC:URA3*	This study
swi4Δ Snf1-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, swi4::kanMX4 SNF1-9MYC:URA3*	This study
swi6Δ Snf1-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, swi6::kanMX4 SNF1-9MYC:URA3*	This study
snf4Δ Snf1-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, snf4::kanMX4 SNF1-9MYC:URA3*	This study
Swi6-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SWI6-9MYC:URA3*	This study
snf1ΔSwi6-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SWI6-9MYC:URA3 snf1::HPH*	This study
Swi4-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SWI4-9MYC:HPH*	This study
snf1ΔSwi4-9myc	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SWI4-9MYC:HPH snf1::KAN*	This study
Mbp1-HA	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MBP1-HA:URA3*	This study
snf1ΔMbp1-HA	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MBP1-HA:URA3 snf1::HPH*	This study
swi6Δ[pSWI6]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, swi6::kanMX4 [pSWI6(URA)]*	This study
swi6Δ[pSWI6-S760A]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, swi6::kanMX4 [pSWI6-S760A(URA)]*	This study
swi6Δ[pSWI6-S760E]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, swi6::kanMX4 [pSWI6-S760E(URA)]*	This study
snf1Δ[pSNF1]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KAN [pSNF1-HA(URA)]*	Pessina *et al.*, 2010
snf1Δ[pSNF1-T210A]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KAN [pSNF1-T210A-HA(URA)]*	Pessina *et al.*, 2010
snf1Δ[pRS316]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KAN [pRS316 (URA)]*	Pessina *et al.*, 2010
snf1Δ[pSNF1]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::HPH [pSNF1-HA(URA)]*	This study
snf1Δ[pSNF1-K84R]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::HPH [pSNF1-K84R-HA(URA)]*	This study
snf1Δ[pWS93]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::HPH [pWS93 (URA)]*	This study

All strains are isogenic to BY4741

Discussion

In *Saccharomyces cerevisiae* the main control of cell cycle progression occurs in late G1 phase at START, when cells decide whether or not to initiate a new cell cycle depending on external (nutrient availability, presence of pheromone) and internal (protein synthesis/cell size, DNA integrity) signals (Mendenhall M.D. and Hodge A.E.,1998; Alberghina L. *et al.*, 2012).

The passage through START consists in the activation of a transcriptional program that implies the coordinated expression of about 200 genes (G1-regulon) and determines the S phase entrance. In fact, those genes codify for proteins which regulate the essential processes of cell duplication: bud formation, initiation of DNA replication and Spinde Pole Body duplication (Spellman P.T. *et al.*, 1998; Breeden L.L., 2003; Wittenberg, C. and La Valle, R., 2003;).

Data presented in this thesis newly highlight that protein kinase Snf1, a master regulator of cellular energy balance (Usaite R. *et al.*, 2009), plays a role in the regulation of G1-specific gene expression and thus in the modulation of cell cycle progression.

Our first analyses, collected in Pessina S. *et al.* 2010, showed that deletion of *SNF1* gene affected the growth rate of yeast cells causing a delayed G1/S transition (Fig.2). Moreover, we found that in a *snf1Δ* strain the expression of *CLB5* gene was decreased, consistently with a low level of Clb5 protein. This reduced protein level, then, led to a lower phosphorylation of Sld2 by the Clb5/Cdk1 complex, the fundamental event that determines the onset of DNA replication (Zagerman P. *et al.*, 2007; Tanaka S. *et al.*,2007).

Therefore, our preliminary data showed that the function played by Snf1 in the coordination between cell growth and cell cycle could not be accounted simply by its role in the regulation of cellular metabolism. In particular, by the demonstration that Snf1 was physically associated with Swi6, our findings provided a mechanistic link between Snf1 and *CLB5* transcription and pointed to a new role for Snf1 as a modulator of G1-specific transcription.

That notion was supported by further analyses which demonstrated that deletion of *SNF1*, besides *CLB5*, affected also *CLN2*, *PCL1* and

RNR1 gene transcription, extending the role of Snf1 to the regulation of the large set of SBF- and MBF-dependent genes (Fig.6).
On the base of those data, we then wondered whether Snf1 could regulate G1-specific transcription influencing the formation of a complete Pre-Initiation Complex (PIC), a necessary requirement for transcriptional initiation. Literature data supported that hypothesis demonstrating that Snf1 promotes transcription regulating the recruitment of Mediator components (Srb2,4 and Med6) (Young E.T. *et al.*, 2002), SAGA complex (van Oevelen C. *et al.*, 2006), TATA binding protein (Shirra M.K. *et al*., 2005) and RNA Pol II (Tachibana C. *et al.*, 2007; Young E.T. *et al.*, 2012) to different promoters.
It is well known that in early G1 phase coactivator complexes such as the histone acetylase SAGA (Spt-Ada-Gcn5 Acetyl-transferase) complex and the Swi/Snf chromatin remodeling complex bind promoters. Those complexes, then, recruit transcription factors SBF and MBF (Cosma M.P. *et al.*, 2001). SBF and MBF are heterodimeric complexes composed by a DNA-binding protein, Swi4 or Mbp1 respectively, and a common regulatory subunit Swi6 (Sidorova J.M. *et al.*, 1995). The association of those complexes to G1-specific promoters set the stage which is necessary to recruit both the chromatin remodeling complex FACT and the Srb/Mediator complex (Bhoite L.T. *et al.*, 2001; Cosma M.P. *et al.*, 1999; Takahata S. *et al.*, 2009 A and B). While those interactions occur in a Cdk1-independent way, Cdk1 activation allows the recruitment of RNA Pol II which interacts with the Mediator complex. Thus, the binding of SBF and MBF complexes to promoters is required for the proper formation of a complete pre-initiation complex (PIC), which leads to the synchronous and coherent expression of the G1-regulon (Cosma M.P. *et al.*, 2001; Hahn S. and Young E.T., 2011).
On the base of those data we investigated the binding of Swi4 and Mbp1 to DNA as well as the recruitment of Swi6 and we found that in a snf1 null mutant the interaction of all those proteins with promoters was severely affected. In keeping with the well known role of SBF and MBF complexes, also the recruitment of FACT complex and RNA Pol II to promoters was decreased in a *snf1Δ* strain, suggesting

that the defect in G1-gene expression depends on a defective PIC formation at G1-specific promoters.
Our further analyses on Swi4, Mbp1 and Swi6 localization showed that in a *snf1Δ* strain synchronized in G1-phase by α-factor treatment, only the 60% of cells showed a correct nuclear localization of those proteins, consistently with their reduced binding to G1-promoters.
Taken together our findings suggest that Snf1, promoting the nuclear localization of SBF and MBF components, ensures the formation of PIC complexes at G1-specific promoters and thus the transcription of G1-specific genes.
The mechanism by which Snf1 may influence the localization of Swi4, Mbp1 and Swi6 remains to be investigated; however, some findings support the notion that Snf1 may regulate the nuclear trafficking. In fact, literature data show that *SNF1* deletion causes a decreased nuclear association of the exporters of the importin-β family, among which Msn5 (Quan X. *et al.*, 2007). Remarkably, this defect is detectable also in a *snf1Δ* strain grown in medium supplemented with glucose as carbon source and in a *reg1Δ* strain, suggesting that the role of Snf1 in the modulation of importin nuclear association is independent from the activation status of Snf1 and that it is important also in nutrient conditions in which the Snf1-pathway is inactive. Moreover, the analysis of the sequence of Kap95, the importin responsible for Swi4, Mbp1 and Swi6 nuclear localization, shows a putative Snf1 consensus-site centered on Ser836, a residue which in a global phosphorylation analysis has been found phosphorylated *in vivo* (Albuquerque C.P. *et al.*, 2008). Therefore, in keeping with literature data, it would be of great interest to further analyze the trafficking mechanism in order to define whether Snf1 could regulate it.
It is known that one of the major Snf1 substrates, Mig1, once phosphorylated by Snf1 is exported from the nucleus to the cytoplasm by Msn5 protein (DeVit M.J. *et al.*, 1999). Besides, another protein involved in the repression of glucose-repressed genes, Hxk2, localizes into the nucleus thank to Kap95 and Kap60, where it binds Mig1 ensuring the inhibition of Mig1-dependent genes (Fernández-García P.

et al., 2012). Thus, the study of a possible Snf1-dependent regulation of importers and/or exporters could be also interesting to reveal a new role for Snf1 in the modulation of metabolic gene expression.

In order to obtain insight into the function of Snf1 in G1 phase, we then investigated the relevance of the kinase activity of Snf1 for its role as a regulator of cell cycle progression.
In fact, analyzing the activation state of Snf1 we found that in 2% glucose growing cells, Snf1 was slightly phosphorylated on the Thr210 residue (Fig.11 A and Supplementary Figure S2). Consistently, the phenotype of a *snf1Δ* strain was fully rescued by the expression of a phosphomimetic Snf1 mutant (*SNF1-T210E*). On the contrary, Snf1 mutants with a low kinase activity gave only a partial complementation of the *snf1Δ* phenotype. In fact, both a non phosphorylatable Snf1 mutant (*SNF1-T210A*) or a Snf1 mutant in which the ATP binding site is destroyed (*SNF1-K84R*) showed a delayed G1/S transition and a defective expression of G1-specific genes, although those phenotypes were not so compromised as the phenotype of *snf1Δ* strain.
Thus, our results suggest that in 2% glucose Snf1 might be partially active and that its kinase activity could be involved in its function as cell cycle regulator.
On the base of those data, we investigated whether the transcription factor Swi6 could be a substrate of Snf1 and we found that Swi6 was phosphorylated by Snf1 on Ser760. Nevertheless, analyses of site-specific mutants for that residue (*SWI6-S760A* or *SWI6-S760E*) showed no alterations of the G1/S transition, supporting the notion that Snf1 regulates G1-gene expression independently from its phosphorylation on Swi6.
In keeping with that last finding, we also demonstrated that alteration of Snf1 kinase activity does not affect the recruitment of Swi6 to G1-specific promoters. In fact, in the *SNF1-K84R* mutant Swi6 binds G1-specific promoters and is able to lead the recruitment of FACT complex and RNA Pol II as in a *wild type* strain.

Despite the correct recruitment of PIC members at SBF- and MBF-dependent promoters, in the *SNF1-K84R* mutant the expression of G1-specific genes was severely reduced (Fig.12). Further analyses showed that in the *SNF1-K84R* mutant the decreased expression of G1-genes was due to a defective transcription elongation. In fact, our ChIP analyses revealed that in this mutant the RNA Pol II occupancy along the coding regions of G1-specific genes such as *CLN2* and *RNR1* (Fig.15) was affected, accordingly with the reduced transcription of SBF- and MBF-dependent genes and the delayed S-phase entrance (Fig.12). Intriguingly, also the binding of the FACT complex across G1-specific genes was reduced in the *SNF1-K84R* mutant, suggesting that Snf1 could influence transcriptional elongation through the direct regulation of the RNA Pol II and/or modulating the process of chromatin remodelling, which is necessary for the passage of RNA Pol II across the transcribed regions of genes.

Literature data suggest that the kinase activity of Snf1 could directly stimulate RNA Pol II-dependent transcription (Kuchin S. *et al.*, 2000). In addition, Snf1 interacts with Ctk1 (a component of the CTD kinase complex I) which phosphorylates the largest subunit of RNA Pol II (Rpb1) during elongation (Cho E.J. *et al.*, 2001; van Driessche B. *et al.*, 2005). Moreover, it has been observed a direct interaction between Snf1 and components of the Mediator complex (such as Srb10, Srb11, and Sin4) (Kuchin S. *et al.*, 2000), a complex which it has been recently demonstrated to regulate transcriptional elongation across different genes (Kremer S. *et al.*, 2012).

Thus, our findings reinforce the role of Snf1 as a modulator of the general transcriptional machinery and highlight a new function in the regulation of transcription of cell cycle genes.

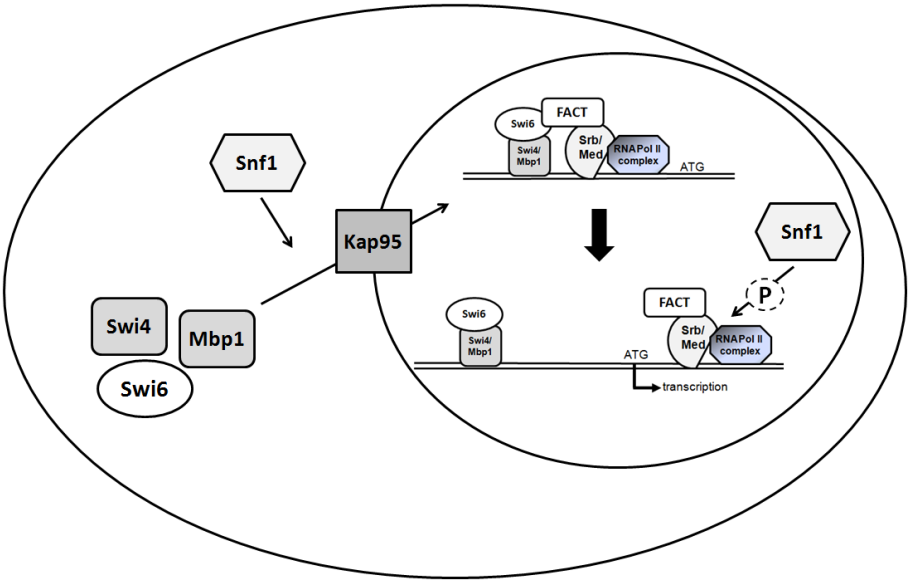


FIGURE 16 – Proposed model for Snf1-dependent regulation of G1-gene expression

Taken together, results reported in this thesis newly indicate that Snf1 plays a dual role in the activation of G1-phase transcription, since: (*i*) Snf1 protein, promoting the nuclear localization of Swi4, Mbp1 and Swi6, ensures the proper recruitment of SBF/MBF complexes, FACT complex and RNA Pol II at G1-specific promoters; (*ii*) Snf1 kinase activity is required for the transcriptional elongation process (Fig.16).

Novel findings here presented indicate a function for protein kinase Snf1 in the regulation of cell cycle progression. Remarkably, that role of Snf1 depends on the nutritional status of cells. The standard laboratory growth medium for budding yeast contains 2% glucose as carbon source, a high glucose concentration in which yeast cells proliferate rapidly using fermentation (glycolisis producing ethanol) to make ATP. In that nutritional condition Snf1 is considered inactive. In fact, Snf1 does not localize into the nucleus (Vincent O. *et al.*, 2001; Hedbacher K. *et al.*, 2004) and does not phosphorylate its substrates,

such as Mig1 and Sip4 (Ahuatzi D. *et al.*, 2004; Ye T. *et al.*, 2008; Lesage P. *et al.*, 1996). When glucose present in the medium decreases, growth slows (a phenomenon called diauxic shift) and cells begin to use oxidative phosphorylation to product ATP. A functional Snf1 kinase is necessary for such a shift (Galdieri L. *et al.*, 2010**;** Rao A.R. and Pellegrini M., 2011).

Nevertheless, in accordance with previous findings (McCartney R.R. *et al.*, 2005; Momcilovic M. *et al.*, 2008), we found that in 2% glucose growing cells Snf1 is slightly phosphorylated on Thr210, suggesting that this kinase could be partially active. Moreover, our analyses showed that the delayed S-phase entrance of a *snf1Δ* strain and of Snf1 mutants with a decreased kinase activity (*SNF1-T210A* and *SNF1-K84R*) was rescued by glucose concentrations higher than 2% (such as 5%). Thus our findings indicate that, although 2% is commonly considered a not-limiting glucose concentration, in that condition Snf1 is necessary to guarantee the proper progression of cell cycle. Differently, when glucose is largely available in the medium (5%), Snf1 is dispensable for the G1/S transition, probably because of the activation of other pathways involved in the regulation of cell cycle progression.

On the base of those data, it will be interesting to compare the expression of genes in cells growing in 2% and 5% glucose using a genome-wide approach. For that reason, in our laboratory a gene chip analysis is going to be performed to determine which genes are differently expressed in those conditions, and thus which pathways are differently regulated.

Until now, the importance of Snf1/AMPK has been limited to its role as a regulator of cellular metabolism. However, a link between metabolic regulation and cell cycle control is required in order to ensure that cell division occurs only if the energy status of the cell can support it. Several evidences prove the existence of such a link in higher eukaryotes, but this regulation appears to be complex.

Literature data indicate that in mammalian cells the activation of AMPK correlates with the arrest of cell cycle in G1 phase. In fact, a

low glucose concentration (1mM) or the treatment with AICAR (a compound which activates AMPK) increase the phosphorylation of Thr172 residue of AMPK. In those conditions, AMPK promotes the expression of p53 and consequently of p21, an inhibitor of the CyclinE/CDK complexes, which promotes the arrest of cell cycle in G1 phase (Jones R.G. *et al.*,2005; Igata M.*et al.*, 2005).

On the other hand, data from *Arabidopsis thaliana* indicate a function for Snf1-related protein kinases KIN10/KIN11 in normal vegetative and reproductive growth and development in flowering plants (Baena-González *et al.*, 2007). In addition, studies in *Drosophila* and mammalian cells have demonstrated that the role of AMPK in energy sensing is coupled with fundamental cell biology functions, such as cell polarity and cell division (Williams T. and Brenman J.E., 2008). AMPK also regulates mitotic progression in mammalian cells, independently from a low cellular energy status (Banko M.R., *et al.*, 2011; Vazquez-Martin A. *et al.*, 2012). Similarly, findings here presented show a role for Snf1 in the regulation of cell cycle in not-limiting glucose conditions.

Thus, the sum of literature data and our novelties clearly indicate that, besides the regulation of cellular processes in response to energy stresses, the conserved protein kinases of the AMPK family can coordinate essential and basic cellular functions under physiological conditions as well.

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