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LINKAGES BETWEEN *CCR4* AND G1-PHASE CYCLINS

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Genetic analysis has revealed that G1-phase cyclins are involved in the relationship in terms of cell size and commitment to cell division. When *CLNs* were expressed at more than normal physiological level they dramatically reduce the critical cell size and advance the progression past Start point. In contrast, reduction or delay of the expression of *CLNs* will produce abnormally large cells and delay the Start. To identify the genetic pathways that may link G1-phase cyclins to cell size, we conducted a systematic genomic-wide genetic screen in yeast. In this screen we identified several genes affecting cell size. Some of those gene products interact physically in highly conserved Ccr4-Not complex. Loss of function of these genes dramatically increases cell size at which cells are being divided. To investigate the role of cell size mutants in the coordination of cell growth with proliferation we choose Ccr4, one of the core components of the Ccr4-Not complex, for further study. We found that the strain with deleted *CCR4* gene has delayed *CLN1* and *CLN2* transcription which results to formation of large cell phenotype.

G1-phase cyclin – Ccr4-Not complex – cell size – Start

Գենետիկական վերլուծությունը ցույց է տվել, որ G1 փուլի ցիկլինները ներգրավված են բջջի չափերի կարգավորման գործում: Երբ ցիկլինների քանակությունը բջջում գերազանցում է նորմալ ֆիզիոլոգիական մակարդակը, բջջի բաժանման ժամանակ տեղի է ունենում ,Ստարտե սահմանային կետի վաղաժամ անցում և բջջի կրիտիկական չափսերի փոքրացում և ընդհակառակը: G1 փուլի ցիկլինների և բջջի չափսերի փոխկապվածության հիմքում ընկած գենետիկական մեխանիզմի բացահայման համար իրականցրել ենք գեների հաջորդական հետազոտություն խմորասնկերում: Այս հետազոտության ընթացքում բացահայտվել են բազմաթիվ գեներ, որոնցից կոդավորվող սպիտակուցները ուղղակիորեն փոխազդում են CCR4-Not համալիտի հետ: Այս գեների ֆունկցիայի բացակայությունը առաջ է բերում բջջի բաժանման խթանման համար անհրաժեշտ չափսերի աճ: Հետագա հետազոտությունների համար ընտրվել է CCR4 գենը, որը հանդիսանում է Ccr4-Not համալիրի հիմնական բաղադրիչներից մեկը: Մենք հայտնաբերել ենք, որ Ccr4 գենի դելեցիա պարունակող մուտանտի մոտ CLN1 և CLN2 գեների տրանսկրիպցիան հետաձգված է, որն էլ հանդիսանում է բջջի չափսերի աճի հիմնական պատճառը:

G1 փուլի ցիկլիններ - Ccr4-Not համալիր - բջջի չափսեր - ,Ստարտե

Генетический анализ показал, что в процесс регулирования размеров клетки вовлечены циклины фазы G1. Но взаимоотношения между циклинамы (*CLN1* и *CLN2*), размером клетки и готовностью клетки к делению весьма сложны. Экспрессия *CLN*-ов выше нормы, резко снижает критический размер клетки, необходимый для старта начала клеточного цикла и наоборот. С целью выявления генетических путей связывающих циклини фазы G1 и размер клетки, мы провели систематический всегеномный скрининг у дрожжей.

В ходе анализа мы выявили несколько генов, генетические продукты которых физически взаимодействовали в высоко консервативном комплексе Ccr4-Not. Потеря функции этих генов критически увеличивала размер клетки, который необходим для начала процесса деления. Для дальнейшего изучения роли размера клетки в координации процесса деления, мы выбрали *CCR4*, один из основных составляющих комплекса Ccr4-Not. Мы обнаружили, что у штамма с делеций *ccr4* гена, транскрипция *CLN1* и *CLN2* циклинов задержана которое приводит к образованию фенотипа клеток, отличающихся большим размером.

Циклины фазы-G1 – Ccr4-Not комплекс – размер клетки – «Старт»

In most eukaryotes, cells become committed to round of cell division at an event in G1 phase. This event is called Start in the yeast and restriction point in mammalian cells [1, 2, 6, 8].

In *Saccharomyces Cerevisiae*, it is well known that Start is dependent upon the G1-phase cyclins Cln1, Cln2, Cln3 and its associated cycline dependent kinase (Cdk) Cdc28 [1, 2, 6, 10, 11 and 14]. Just prior to Start, as cells approach the required critical size, Cln3-Cdc28 kinase phosphorylates Whi5, promoting its dissociation from transcription factors SBF and MBF, whose basal activity is kept low by Whi5. SBF and MBF induce the transcription of about 200 genes [7, 13] which are involved in DNA synthesis and repair, but the key transcripts are the G1 cyclins *CLN1* and *CLN2* and the B-type cyclins *CLB5* and *CLB6* [7, 13]. When no Cln3 is present, other cyclins, such as Bck2, may substitute for it, although quite inefficiently; therefore, the entrance into S phase takes place after a longer G1-phase than in wild-type cells [9].

As G1-phase cells grow in size toward Start, the abundance of the Cln1 and Cln2 mRNAs and proteins increases [4, 15]. In both yeast and mammalian cells it is widely believed that a critical amount of G1-phase cyclins must accumulate to induce cell cycle progression [1, 2, 6, 10, 11, and 14]. However, the relationship between G1-phase cyclins, cell size and commitment to cell division has not been well understood.

To identify the genetic pathways that may link G1-phase cyclins to cell size, we conducted a systematic genomic-wide genetic screen in yeast. Several hundred genes were identified that dramatically altered cell size and are involved in cell size homeostasis. Most of the currently known cell size control genes directly or indirectly affect the expression or activity of G1-phase cyclins, demonstrating the integral role of cell cycle regulation and G1-phase cyclins in cell size control. Interestingly, the gene products of five large mutants, Ccr4, Hpr1, Paf1, Pop2, and Rlr1, physically interact with or are components of Ccr4-Not complex. This complex plays an essential role in the control of gene expression. Characterization of several cell size mutants revealed that these genes function by regulating the timing of expression of G1-phase cyclins. By elucidating the role of *CCR4* in the coordination of cell growth with proliferation we found that *CLN1* and *CLN2* transcription were delayed in *ccr4*Δ strain which lead large cell phenotype.

Materials and Methods. *Strains and media*

The strains used in this work were derived from S288c S. Cerevisiae. Yeast cultures were grown in YEP-based medium (20.0 g of Difco Bacto Peptone and 10.0 g of Difco Bacto Yeast Extract dissolved in 900 ml of water, 100 ml of 20% glucose, sucrose, or raffinose).

Quantification of cell size, persentage of budded cells, and cell cycle distribution.

Cell cycle synchronizations were performed by centrifugal elutriation as previously described (3). The percentage of budded cells was determined by coding samples and then counting the cells with visible buds in a minimum of 200 cells. Analysis of the cell size distribution of yeast strains was done with a Coulter Counter Channelyzer ZM or Z2.

Recombinant DNA technique

Escherichia coli transformations, plasmid extractions, restriction digestions, and molecular cloning techniques were conducted using standard protocols as previously described (Ausubel 1987). To make a *GAL-CCR4* construct, oligonucleotides were designed for the amplification of the complete open reading frame. A typical 100-ml PCR reaction contained 10 ng of DNA template, 2–5 units of Pfu polymerase, 100 pmol of each primer, 1 ml of 25 mm dNTPs, and 10 ml 10X Pfu buffer. Typical reactions had 30 cycles consisting of denaturation for 1 min at 95°C, annealing at 50°C –58°C for 1 min, and a 1- to 3-min extension at 72°C (1 min for a 1-kb fragment) performed with an Eppendorf Mastercycler.

Preparation of RNA and Northern analysis

RNA preparation and Northern analysis were conducted using standard protocols as previously described (12). Quantification of Northern data was conducted with the FluorChem 2.0 spot densitometry analysis program (Alpha Innotech). Images captured on film were digitized and analyzed. To ensure linearity of the signal from film exposures, three to seven exposures were analyzed in each case. To control for loading, *CLN* mRNA signals were normalized to the *ACT1* mRNA.

Results and Discussion.

G1-phase cyclin CLN1 and CLN2 expression linked to cell size threshold

G1-phase cycline *CLN1* and *CLN2* are cell cycle regulators that promote transition from G1 to S phase. Deletion of these genes cost abnormally large cell phenotype and delay G1/S transition (Start). (Fig. 1A).

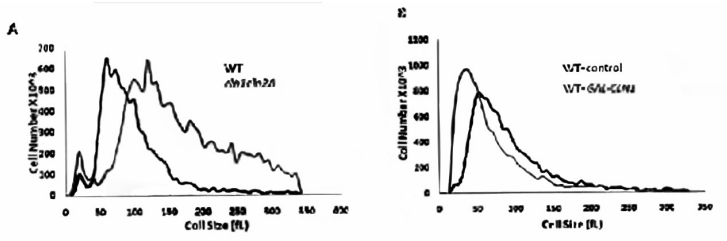

Fig. 1. Loss of Cln1 and Cln2 functions results in abnormally large cells. Average mean cell sizes were 92±1 fl (WT), 148±5 fl fl (cln1cln2Δ) (P=0.0005) (A) Ectopic overexpression of *CLN1* reduces the size of wild-type cells. The mean cell sizes of diploid wild-type cells transformed with control plasmid (e.g., an empty GAL-promoter construct), *GAL-CLN1*, grown in YEPRG as described were 84±5 fl, 53±4 fl, respectively (P=0,005) (B) Similar data (56±3 fl) were obtained with overexpression of *CLN2* (not shown).

In log phase the average size of wild-type diploid cells from the S288c background is 92±1 fl, when they grow in rich media. Under the same condition the size of *cln1cln2*Δ double deletion strain significantly increased (148±5 fl; P=0.0005). This data suggests that cell size requirements may in part reflect the need for a critical amount of the Cln1 and Cln2 proteins. Since the absence of G1-phase cycline *CLN1* and *CLN2* expression produce large cells, next we examined whether overexperssion of *CLN1* or *CLN2* reduce cell size of wiled-type cells. Wild type strains containing integrated *GAL-CLN* constructs give high, nonphysiological levels of *CLN* expression when induced with 1% galactose. We found that overexpression of *CLN1* (or *CLN2*) significantly decrease the size of wild type cells (Fig. 1B). Overall this data suggest that there is a threshold requirement for G1-phase *CLNs* which is a major determinant of critical cell size required for G1-S transition.

Cell cycle control and Ccr4-Not complex genes are involved in cell size homeostasis

To identify the genetic pathways that link G1-phase cyclin expression and proliferation to cell size, we conducted a systematic genomic-wide genetic screen in yeast. In this light, mutations were identified that either dramatically increased or decreased average cell size. Interestingly a group of mutants that dramatically altered cell size are member of cell cycle control genes.

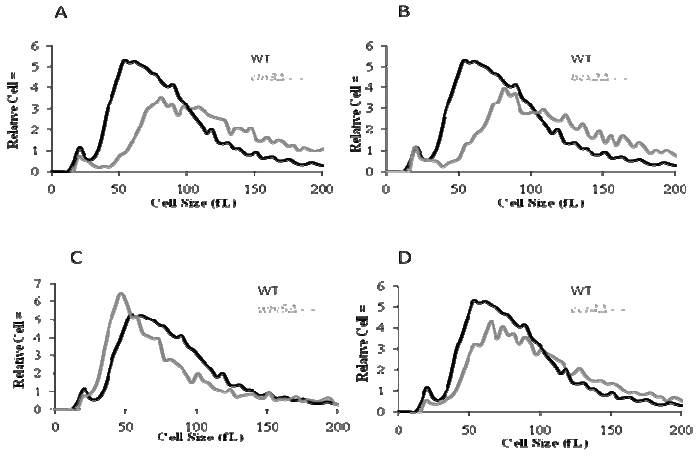

Fig. 2 Loss of Cln3, Bck2 and Ccr4 function result in abnormally large cells. In contrast loss of Whi5 function results abnormally small cells. The mean cell sizes of diploid wild-type, *cln3*Δ (A), *bck2*Δ (B), *whi5*Δ (C) and *ccr4*Δ (D) cells grown in YPD as described were plotted as a function of cell number. Average mean cell sizes were 92±1 fl (WT), 128±10 fl (*cln3*Δ), 110±5 fl (*bck2*Δ), 76±5 fl (*whi5*Δ) and 106±4 fl (*ccr4*Δ) (P=0.0005-0.02).

In our screen we found that deletion of either *bck2*Δ or *cln3*Δ increases cell size (Fig. 2A and 2B; P=0.02-0.05). Since it has been shown that Cln3 and Bck2 activate the transcription of the downstream G1-phase cyclins *CLN1* and *CLN2*, we suggest that large cell phenotype of *bck2*Δ or *cln3*Δ is reason of down-regulation of *CLN1* and *CLN2* expression. In contrast we found that in whi5Δ deletion strain average cell size was decreased (Fig. 2C). Whi5 protein, functionally equivalent with pRB tumor suppressor gene in mammalian cells, suppresses G1-phase cyclins *CLN1* and *CLN2* expression. Therefore loss of Whi5 function results in premature *CLN1* and *CLN2* expression.

We also found a group of cell size mutants that are members of the highly conserved Ccr4-Not complex or associated with this complex. We elucidated the role of Ccr4 in the coordination of cell growth with proliferation. We found that deletion of *ccr4*Δ produce abnormally large cells (Fig. 2D). Since the strain with *CCR4* gene deletion (*ccr4*Δ strain) has similar phenotype with *bck2*Δ and *cln3*Δ strain we suggest that Ccr4 may also regulate *CLN1* and *CLN2* expression.

Overexpression of CCR4 dramatically decreases the size of wild-type strain

Since deletion of *ccr4*Δ increased the average cell size we examined whether overexpression of *CCR4* could reduce cell size. We created *GAL-CCR4* construct, which gave high, nonphysiological levels of *CCR4* expression when induced with 1% galactose. By overexpressing this construct in wild type strains we found that average cell size was decreased (Fig. 3).

During our study we also examined the role of Ccr4 in G1/S transition point Start. For that reason we synchronized wild type and *ccr4*Δ strains using centrifugal elutriation. Similar –sized unbedded early G1 cells were collected (≈35 to 45 fl), resuspended in fresh YEPD media and incubated at 30°C. Samples were taken at regular intervals, and cell size and percent budding were measured. When the budding data are plotted as a function of time (Fig. 4A) and the same budding data are plotted as a function

of cell size (Fig. 4B) we found that budding was delayed around 30 min in *ccr4*Δ cells compare with wild type strain. Moreover, data indicated that *ccr4*Δ cells were budded 8-10 fl larger size than wild type strain.

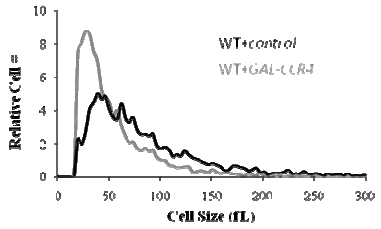

Fig. 3. Ectopic overexpression of *CCR4* reduces cell size. The mean cell sizes of diploid wild-type cells transformed with control plasmid (e.g., an empty GAL-promoter construct), *GAL-CCR4*, grown in YEPRG as described were 84±5 fl, 58±4 fl, respectively.

During our study we also examined the role of Ccr4 in G1/S transition point Start. For that reason we synchronized wild type and *ccr4*Δ strains using centrifugal elutriation. Similar –sized unbedded early G1 cells were collected (≈35 to 45 fl), resuspended in fresh YEPD media and incubated at 30°C. Samples were taken at regular intervals, and cell size and percent budding were measured. When the budding data are plotted as a function of time (Fig. 4A) and the same budding data are plotted as a function of cell size (Fig. 4B) we found that budding was delayed around 30 min in *ccr4*Δ cells compare with wild type strain. Moreover, data indicated that *ccr4*Δ cells were budded 8-10 fl larger size than wild type strain.

*Overexpression of CLN1 and CLN2 dramatically decrease the size of ccr4*Δ *strain*

It has been shown that *CLN3* and *BCK2* are major regulators of *CLN1* and *CLN2* expression. So deletions of other *CLN3* or *BCK2* will down-regulate *CLN1* and *CLN2* expression and produces abnormally large cells.

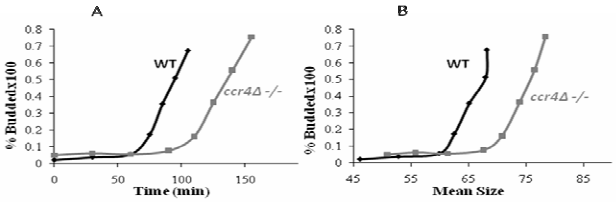

Fig. 4. Progression past Start is delayed in cells lacking *CCR4*. Centrifugal elutriation was used to isolate small, unbudded G1-phase cells. Following elutriation, cells were resuspended in fresh medium at 25°C and time points were taken every 15 min. The percentage of budded cells is plotted as a function of time (A) and as a function of cell size (B).

Since overexpression of *CLN1* or *CLN2* will reduce the cell size (Fig. 1B) we examined whether overexpession of *CLN1* or *CLN2* will rescue large cell phenotype of *ccr4*Δ strain. By overexpressing *CLN1* gene under the control of GAL promoter in *ccr4*Δ cells we found that the average size *ccr4*Δ strain was decreased (Fig. 5). This data indicated that the large phenotype of *ccr4*Δ is the reason of *Cln1* and *Cln2* deficiency.

*CLN transcription is delayed in ccr4*Δ *deletion strain*

To address the question whether *ccr4*Δ cells are abnormally large due to *CLN1* and *CLN2* deficiency we compared the abundance of *CLN1* and *CLN2* mRNA during mid-log phase in mutant and wild type strain. Northern analysis revealed that the levels

of *CLN1* and *CLN2* mRNAs were only modestly changed in mutants (Fig. 6A), suggesting that *CLN1* and *CLN2* mRNA expression levels were unlikely to be the reason that cells are unusually large.

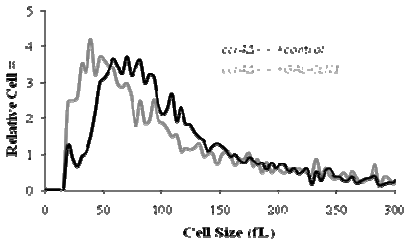

Fig. 5. Ectopic overexpression of *CLN1* reduces cell size. The mean cell sizes of diploid ccr4Δ cells transformed with control plasmid (e.g., an empty GAL-promoter construct), *GAL-CLN1*, grown in YEPRG as described were 94±6 fl, 85±6 fl, respectively.

The large critical cell size and the delayed Start of *ccr4*Δ cells strongly suggested that *CLN* expression might also be delayed. To examine this possibility, RNA was isolated from the same time points from for budding and cell size. The expression of *CLN1* and *CLN2* mRNA were then measured by Northern. Consistent with the timing of Start, in the wild type cells, the expression of *CLN1* and *CLN2* mRNA was undetectable until the 60 min time point and peaked between 75-90 min. In contrast, in the cells *CLN1* and *CLN2* mRNA was undetectable until the 90 min time point and peaked between 110~125min (Fig. 6B).

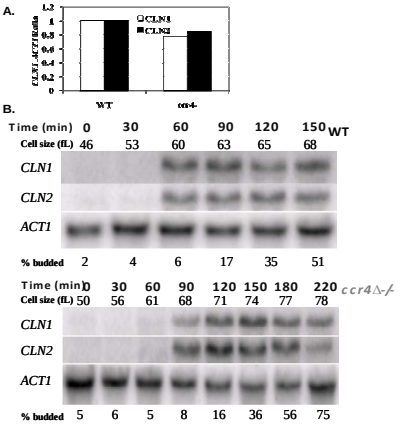

Fig. 6 *CLN1* and *CLN2* mRNA expression is advanced or delayed in cells cell size mutants. Total RNA isolated from mid-log-phase wild-type and cell size mutants YPD cultures were analyzed by Northern analysis. Blots were hybridized with *CLN1* and *CLN2* probes. *ACT1* probes were used as loading controls. Quantitation revealed that CLN1 and CLN2 levels were reduced only 10 and 15%, respectively, in *ccr4*Δ mutant (A). Total RNA was isolated from the elutriation fractions. Blots from wild-type cells and ccr4Δ cells were hybridized with *CLN1* and *CLN2* probes. ACT1 probes were used as loading controls. Analysis of data reveals that, in ccr4Δ cells, ≈50% of cells have progressed past G1-phase when cells are 75–79 fl, while in wild-type cells this happens at ≈73 fl.

The result indicated that *CLNs* expression also delayed in *ccr4*Δ strain. From the above data, we concluded that Ccr4 positively regulated *CLN1* and *CLN2* mRNA expression and determines the timing of their expression.

Finally, the present study is the first investigation concerning the role of Ccr4 in the cell cycle control, such as regulation of the expression of G1-phase cycline *CLN1* and *CLN2*.

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