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TORC1: a Central Controller of Entry into and Exit from G_0 in the Model Organism *Saccharomyces cerevisiae.*

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" TORC1 : a Central Controller of Entry into and Exit from G_0 in the Model Organism *Saccharomyces cerevisieae* "

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Résumé

I) Contrôle de l'entrée en G_0 chez la levure *Saccharomyces cerevisiae*

I.1) Introduction

I.1.1) Généralités

La compréhension des mécanismes qui déterminent la prolifération ou la non-prolifération des cellules fait partie aujourd'hui d'un enjeu biomédical déterminant en ce qui concerne notamment le développement de médicaments anticancéreux ou de thérapies immunosuppressives (8). En effet, dans un organisme adulte, la plupart des cellules sont dans un état de non-division dû à une différenciation terminale ou à leur entrée dans un état de quiescence appelé aussi G_0 qui est réversible (9). C'est le cas des cellules précurseurs des cellules musculaire, les myoblastes, qui restent dans un état latent à moins que, suite à une blessure, le muscle ne doive être régénéré. Une réactivation inappropriée de ces cellules dormantes ou un défaut d'entrée en quiescence peut ainsi être à l'origine du développement d'un cancer. Lors de greffes, l'activation de cellules précurseur des cellules T ou B conduit à une réponse immunitaire responsable du rejet d'allogreffe. Cette réponse peut être réduite au moyen de drogues qui maintiennent artificiellement ces précurseurs en G_0. Finalement, une meilleure connaissance des mécanismes régissant l'entrée et la sortie des cellules de G_0 pourrait être utile pour élaborer des stratégies dans le but d'éliminer les microorganismes pathogènes qui, lorsqu'ils se trouvent dans le corps humain sous forme quiescente, échappent aux traitements pharmacologiques et au système immunitaire. Ces microoganismes n'étant qu'endormi ils peuvent à tout moment se réveiller et être à l'origine d'une infection secondaire. Ces pathogènes quiescents pourraient notamment être éliminés en trouvant un moyen de prévenir leur entré en ou leur sortie de G_0.

I.1.2) Croissance et division de la levure *Saccharomyces cerevisiae*

La levure de bière *Saccharomyces cerevisiae* est couramment utilisée en biologie moléculaire comme organisme modèle notamment parce qu'il s'agit d'une cellule eucaryote qui partage avec les cellules de mammifères un certain nombre de caractéristiques y compris les mécanismes fondamentaux gouvernant la croissance et la division cellulaire (10, 11). L'ajustement de ces deux paramètres a lieu en G_1, une phase du cycle cellulaire où la cellule fille issue de la mitose augmente sa masse et son volume. C'est à la fin de ce stade que la cellule prend la décision soit d'initier un nouveau cycle de division et d'entrer dans la phase S (synthèse de l'ADN), soit de s'arrêter et d'entrer dans un état de quiescence appelé

G_0 (12, 13). Les informations reçues par la cellule en provenance du milieu extracellulaire influencent cette décision. Ainsi, l'entrée en G_0 des cellules d'un organisme multicellulaire tel que le corps humain est principalement régulée par la présence ou non de facteurs de croissance ou d'hormones, tandis que chez la levure, qui est un organisme unicellulaire et donc en contact direct avec l'environnement externe, l'entrée des cellules en G_0 est régulée par la quantité d'éléments nutritifs essentiels présents dans le milieu de culture (*i.e.*, phosphate, azote ou hydrates de carbone) (8). L'entrée des cellules de levure en G_0 est une forme de différentiation qui leur permet de survivre à de longues périodes de carence en élément nutritifs.

I.2) Régulation de la prolifération cellulaire par les éléments nutritifs

Alors qu'une carence en éléments nutritifs essentiels provoque l'entrée des cellules en G_0, une quantité limitée d'un certain élément essentiel provoque la reprogrammation de la cellule pour l'utilisation d'éléments nutritifs de remplacement. Cette section de l'introduction va se focaliser sur la façon dont les cellules changent leur métabolisme en réponse à la qualité et à la quantité des éléments nutritifs présents dans le milieu.

I.2.1) Les différentes phases de croissance d'une culture de levures en milieu discontinu

En général, dans des conditions de culture classiques en laboratoire, lorsque les cellules croissent dans un milieu riche tel que le YPD, l'entrée en G_0 est causée par une carence en carbone. Avant d'atteindre cet état de non-prolifération, la culture présente différentes phases. Durant la **phase exponentielle**, qui est une phase de croissance rapide, les levures obtiennent leur énergie de la fermentation du glucose. Lors de la **transition diauxique**, qui a lieu lorsque le glucose présent dans le milieu est épuisé, les cellules cessent de façon transitoire leur croissance, et reprogramment leur métabolisme de façon à utiliser comme source de carbone et d'énergie l'éthanol produit lors de la phase de croissance exponentielle. La **phase** de croissance **diauxique** est une phase de croissance lente sur l'éthanol durant laquelle les cellules obtiennent leur énergie de la respiration. Enfin, lorsque toutes les sources de carbone ont été consommées, les cellules cessent totalement de se diviser et entrent en G_0 (2, 8, 14). Les cellules peuvent aussi entrer en G_0 suite à une carence en azote ou en phosphate (8).

I.2.2) Régulation par les principaux éléments nutritifs de la croissance des cellules de levures

Les cellules de levures sont capables de faire une distinction qualitative entre les différents éléments nutritifs présents dans le milieu. Ainsi, les sources de carbone sont séparées en deux groupes distincts, les sources de carbone fermentescibles qui comprennent les sucres (p.ex. le glucose, le fructose, le saccharose, le galactose et le raffinose) et les sources de carbone non-fermentescibles comprenant notamment l'éthanol et le glycérol. Les sources de carbone non-fermentescibles soutiennent un taux de croissance plus lent (8). La présence dans le milieu de culture des deux sources de carbone dites "préférées", le glucose et le fructose, réprime l'utilisation des autre sucres (tels que le mannose le galactose ou le saccharose) suivant un processus nommé répression catabolique (15). Ces deux sources de carbone répriment un certain nombre de gènes codant pour des protéines impliquées notamment dans la respiration, la gluconéogenèse et le métabolisme des autres sources de carbone (16, 17).

Les sources d'azotes sont également réparties en deux groupes, les sources d'azote dites "bonnes" comme la glutamine et l'asparagine soutiennent un fort taux de croissance et répriment la transcription des gènes de la répression catabolique de l'azote (gènes NCR codant pour des transporteurs de composant azoté et pour des enzymes impliquées dans la dégradation et l'utilisation de sources d'azote plus pauvres) (4). Les sources d'azotes dites "mauvaises", comprenant notamment la proline, l'arginine, l'allantoïne, l'ornithine l'urée et dans certains cas l'ammonium, déclenchent la mise en activité des gènes NCR (4, 18). L'utilisation de ces sources non-préférées d'azote passe par leur dégradation en glutamate et ammoniaque et leur conversion en glutamate et en glutamine (4). La régulation rétrograde (RTG) est un autre système impliqué dans la régulation du métabolisme de l'azote au niveau transcriptionnel (19, 20). Ce système, fortement réprimé par le glutamate et la glutamine, est apparemment activé par des source d'azote riches en ammoniaque, telles que l'ammoniaque elle-même ou l'urée (21). L'activation du système RTG conduit à l'induction de l'expression de gènes codants pour des enzymes mitochondriales et perroxysomales impliquées dans la synthèse d'un intermermédiaire du cycle de Krebs, l'α-cétoglutarate, composant le squelette de carbone qui, suite à la fixation de l'ammoniaque, forme le glutamate et la glutamine (20-22). Ainsi, ce système couple le métabolisme de l'azote à celui du carbone (22).

Finalement, de façon similaire au glucose, le phosphate inorganique (Pi) prévient la transcription des gènes impliqués dans l'utilisation des sources alternatives de phosphate en

activant le complexe Pho80-Pho85 qui phosphoryle et inhibe le facteur de transcription Pho4 (23).

I.2.3) Caractéristiques des cellules au moment de leur entrée en G_0

Les cellules arrêtées en G_0 présentent un certain nombre de caractéristiques correspondant à des changements morphologiques, physiologiques et biochimiques. Ces cellules n'ont pas de bourgeons, leur chromosomes sont condensés, leur paroi cellulaire s'est épaissie et elles deviennent résistantes à divers stress y compris aux chocs thermiques. Les cellules en G_0 présentent aussi une diminution importante de la synthèse protéique et de la transcription. Plus précisément, la transcription dépendante des ARN polymérases Pol(I) et Pol(III) est réduite tandis que la transcription dépendante de Pol(II) change pour promouvoir l'expression des gènes impliqués dans la réponse au stress ou dans la transition diauxique (gènes contenant dans leurs promoteurs l'élément STRE ou PDS). Enfin, ces cellules accumulent des hydrates de carbone de réserve tels que le glycogène et le tréhalose. Ces changements permettent à la cellule de survivre à de longues périodes de carence nutritives (8, 12).

I.3) Les principales voies de signalisation régulant l'entrée des cellules en G_0

Différentes voies de signalisation intègrent les informations nutritionnelles du milieu pour réguler l'entrée ou non des cellules en G_0. Conceptuellement, la voie de signalisation Snf1 (**s**ucrose **n**on-**f**ermenting) est un régulateur positif de l'entrée des cellules en G_0 tandis que les voies de signalisations PKA (**p**rotéine **k**inase **A**), du complexe TORC1 (**t**arget **o**f **r**apamycin **c**omplex **1**) ainsi que la protéine kinase Pho85 (**pho**sphate metabolism) sont, au contraire, des régulateurs négatifs de l'entrée des cellules en quiescence (8).

I.3.1) La voie de signalisation Snf1

Snf1 est une sérine/thréonine (Ser/Thr) kinase qui est inactive en présence de glucose dans le milieu. Lorsque le niveau de glucose chute, Snf1 est activée ce qui conduit notamment à une dérépression des gènes codant pour des protéines impliquées dans l'utilisation de sources de carbone autres que le glucose et dans l'activation des voies métaboliques générant de l'ATP (17). En l'absence de Snf1, les cellules ne peuvent pas utiliser l'éthanol et meurent rapidement après la transition diauxique (24).

I.3.2) La voie de signalisation PKA

La voie de signalisation PKA est conservée dans la plupart des cellules eucaryotes. Elle est activée par la présence de sources de carbone fermentescibles (p. ex. glucose, saccharose, et fructose) qui, lorsqu'elles sont ajoutées à des cellules carencées en carbone, induisent une augmentation transitoire de la synthèse d'AMP cyclique (AMPc) qui, à son tour, active la protéine kinase A (PKA) (25, 26). Cette réponse implique probablement l'activation des petites protéines G Ras1 et Ras2 (27, 28). Ces protéines sont régulées positivement par Cdc25 et Sdc25, deux facteurs d'échange GDP-GTP (GEF) qui stimulent la liaison de Ras au GTP, et négativement par Ira1 et Ira2 qui stimulent l'hydrolyse du GTP en GDP (29, 30). La transmission du signal des sucres aux protéines Ras nécessite que ceux-ci soient phosphorylés à l'intérieur de la cellule par les hexokinases. Cependant la nature exacte du signal en provenance des hexokinases doit encore être élucidée. Le second système de détection des sucres comporte un récepteur transmembranaire (Gpr1) couplé à une protéine G (Gpa2) (31, 32). Ras1 et Ras2 ainsi que Gpa2 activent l'adénylyl cyclase (Cyr1/Cdc35) et induisent la synthèse d'AMPc (27). Tandis que Ras1 et Ras2 sont aussi impliqués dans la production d'un niveau basal d'AMPc nécessaire à la survie des cellules, le système Gpr1 et Gpa2 est spécifiquement impliqué dans l'augmentation transitoire de l' AMPc qui se produit lors de l'addition de glucose et de saccharose dans un milieu carencé en carbone (32). Enfin, la PKA est un tétramère composé de deux sous-unités régulatrices (codée par un seul gène *BCY1*) et de deux sous-unités catalytiques (codées par trois gènes *TPK1,2,3*). La liaison de l'AMPc aux deux sous-unités régulatrices libère les sous-unités catalytiques qui phosphorylent alors les protéines cibles de la PKA (33).

Les effecteurs de la PKA

La PKA inhibe l'entrée des cellules en G_0 et est donc active lors de la phase de croissance exponentielle (34). Elle promeut la croissance et la division cellulaire notamment: (i) en soutenant la synthèse de protéines via l'activation de la transcription des gènes codants pour les protéines ribosomiques (35-38), (ii) en régulant probablement l'expression et la stabilisation de la cycline Cln3, impliquée dans le passage du point de contrôle "START" en G_1 (39) et (iii) en inhibant l'acquisition des caractéristiques nécessaires à l'entrée des cellules en G_0 (39). Par exemple, l'activité de la voie PKA prévient l'accumulation de glycogène et de tréhalose en inhibant les enzymes nécessaires à leur synthèse (Tps1, Tps2 et Gsy2) et en activant les enzymes impliquées dans leur dégradation (Nth1 et Gph1) (40-45). En plus d'une régulation post-traductionnelle, la PKA inhibe également la transcription des gènes

codant pour ces protéines. En fait, elle réprime d'une façon générale la transcription des gènes contenant dans leur promoteurs des éléments de réponse au stress (STRE) et à la transition diauxique (PDS) en empêchant entre autres l'importation nucléaire des facteurs de transcription Msn2/4 et en inhibant la protéine kinase Rim15 (46-48).

Rim15 est une (Ser/Thr) kinase qui est phosphorylée et inhibée par la PKA (48). Rim15 est nécessaire pour que, suite à une carence en éléments nutritifs, les cellules entrent correctement en G_0. En son absence, les cellules, lors de la transition diauxique, sont incapables d'accumuler du tréhalose et du glycogène, de transcrire les gènes *HSP12*, *HSP26* et *SSA3* (gènes STRE et PDS) et d'acquérir la thermotolérance. De plus comme les mutants *rim15Δ* n'entrent pas correctement en G_0, leur taux de survie est réduit dans cet état (48). Rim15 est un membre distant de la famille des kinases NDR (**n**uclear **D**bf2-related kinases). Ces kinases sont caractérisée par la présence d'un insert qui scinde le domaine catalytique (domaine kinase) en deux (49). Bien que la fonction de ce domaine ne soit pas encore établie, il a été démontré, dans le cas d'un autre membre de cette famille, que cet insert contenait un domaine de localisation nucléaire. Rim15 régule positivement l'expression d'un set de gènes (dont ceux mentionnés ci-dessus) connus pour être sous contrôle direct des facteurs de transcription Msn2/4 et/ou Gis1 et qui contiennent dans leur promoteur des éléments PDS et/ou STRE (50). Des études génétiques ont d'ailleurs démontré que Gis1 était situé en aval de Rim15 (51).

La voie de signalisation induite par un milieu soutenant la fermentation (FGM)

L'addition de glucose ou de saccharose dans une culture de cellules carencées en glucose provoque une augmentation transitoire de l'AMPc qui est nécessaire pour que des cellules post-diauxiques ou quiescente adaptent rapidement leur métabolisme à une croissance fermentative (31). L'addition d'azote, de phoshate ou de soufre à des cellules carencées en azote, phosphate ou soufre, respectivement, conduit également à l'activation des cibles de la PKA (52). Cependant, dans ces conditions, cette activation n'est pas associée à une augmentation de l'AMPc. Sur la base de ces observations, Thevelein *et al.* (1994) ont proposé qu'une autre voie de signalisation, la voie FGM (pour **f**ermentable **g**rowth **m**edium-induced pathway), était impliquée dans l'activation de la PKA ou des éléments en aval de la PKA indépendamment de l'AMPc (53). Cette voie de signalisation FGM ne possède à présent qu'un seul membre, la Ser/Thr protéine kinase Sch9 qui appartient comme la PKA à la famille des kinases AGC (c**A**MP-, c**G**MP-dependent protein kinases and protein kinase **C**) (54-56).

I.3.3) La voie de signalisation TOR (target of rapamycin)

La voie de signalisation TOR a été identifiée grâce à l'action d'une drogue, la rapamycine, qui induit artificiellement l'entrée des cellules en G_0. La voie de signalisation TOR est, comme la voie PKA, conservée de la levure à l'homme. Elle contrôle positivement la croissance cellulaire en réponse à des informations provenant des éléments nutritifs présents dans le milieu (57).

Les complexes TOR

La rapamycine est un antibiotique de la classe des lactones macrocycliques connue pour inhiber la croissance et la prolifération des cellules de levures. En fait, les cibles de la rapamycine sont, chez la levure deux Ser/Thr kinases Tor1 et Tor2, et chez les mammifères une seule protéine homologue mTor. Plus précisément, la rapamycine entre dans les cellules par diffusion et lie la protéine Fpr1 (FKBP12). Le complexe rapamycine-Fpr1 lie à son tour les deux protéines Tor1 et Tor2 et inhibe les fonctions de ces dernières (7, 58). Les protéines Tor font parties de deux complexes protéiques différents dont seulement un de ces complexes, TORC1, est sensible à la rapamycine. Le second complexe nommé TORC2 est impliqué dans la régulation du cytosquelette d'actine et donc dans la polarisation de la croissance (59). TORC1 est formé de Tor1 ou Tor2, en association avec les protéines Kog1, Lst8 et Tco89 tandis que TORC2 est formé de Tor2, Lst8, avo1, avo2, avo3 et Bit61 (60).

Régulation de TORC1 par les éléments nutritifs

Grâce aux effets de la rapamycine, le complexe TORC1 a été identifié comme étant un élément central dans le contrôle de la croissance et de la prolifération cellulaire (58). Cependant, Les mécanismes moléculaires conduisant à l'activation de TORC1 ne sont pas encore entièrement élucidés. Chez les cellules de mammifères, la voie de signalisation TORC1 est activée en réponse aux hormones de croissance et aux acides aminés (57). Chez la levure, en revanche, ces mécanismes sont moins bien compris. Puisque l'inactivation la voie de signalisation TORC1 promeut l'acquisition de caractéristiques similaires à celles observées suite à une carence éléments nutritifs, il est probable que TORC1 signale la présence de nutriment à ses éléments situés en aval. De plus, les analyses transcriptionnelles indiquent que le set de gènes induit par un traitement à la rapamycine est similaire à celui induit lorsque les cellules sont transférées d'un milieu riche en azote (contenant p. ex. de la glutamine) à un milieu contenant une source d'azote plus pauvre (p. ex. proline ou urée) ou lorsque les cellules sont confrontées à une carence en carbone (61-63). Ces résultats suggèrent que TORC1 répond à la quantité et à la qualité des

éléments nutritifs présents dans le milieu, et plus particulièrement à la présence de carbone et/ou d'azote dans le milieu.

TORC1 et les phosphatases

Les régulateurs directs de TORC1 n'ont pas encore été identifiés chez la levure. En revanche, de nombreux effecteurs situés en aval du complexe sont connus. Parmi eux, la protéine Tap42 est potentiellement une cible directe du complexe TORC1 puisqu'elle est phosphorylée *in vitro* par Tor2 (64). Cette phosphorylation est corrélée à l'association de Tap42 avec les phosphatases, Pph21, Pph22 et Sit4 (64, 65). L'association de Tap42 avec phosphatases, qui pourrait selon le modèle le plus courant inhiber l'activité de ces dernières, est dissoute suite au traitement des cellules avec de la rapamycine ou à une carence en éléments nutritifs et (65, 66). Ainsi, les phosphatases libérées de Tap42 interviendraient pour déphosphoryler les cibles du complexe TORC1 (66-68). Tap42 constitue la branche principale de la voie de signalisation TORC1. Cette branche est notamment impliquée dans: (i) l'initiation de la traduction (69, 70), (ii) la régulation de la distribution des transporteurs d'acides aminés à la surface de la cellule (67), (iii) la régulation de la transcription des gènes de la répression catabolique par l'azote (gènes NCR) (62, 68, 71, 72), probablement des gènes de la réponse au stress (gènes STRE) (72), et peut-être des gènes de la réponse rétrograde (gènes RTG) (72).

TORC1 et la régulation de la transcription

TORC1 de manière générale réprime la transcription des gènes induits en réponse à une carence en éléments nutritifs tels que les gènes NCR, STRE et RTG en retenant dans le cytoplasme leurs facteurs de transcription respectifs soit Gln3, Msn2/4 et le module Rtg1-3 (22, 68). Gln3 est impliqué dans la transcription des gènes de la répression catabolique de l'azote (4). Lorsque Torc1 est actif, Gln3 est phosphorylé et maintenu dans le cytoplasme par grâce à son association avec la protéine Ure2 (73). A l'inverse, le traitement des cellules avec de la rapamycine conduit à la déphosphorylation de Gln3 qui s' accumule dans le noyau et l'induit la transcription des gènes NCR (61, 68). Le module comprenant Rtg1-Rtg3 régule quant à lui l'expression des gènes de la réponse rétrograde (20). Lors de la phase de croissance exponentielle, l'activité de TORC1 favorise la phosphorylation de Mks1 qui, en association, avec Bmh1/2 empêche l'entrée de Rtg1-3 dans le noyau (74, 75). Suite au traitement des cellules avec de la rapamycine, Mks1 est déphosphorylé et s'associe avec Rtg2 qui l'inhibe permettant l'entrée du complexe Rtg1-Rtg3 dans le noyau et l'activation consécutive des gènes de la réponse rétrograde (22, 76-78). TORC1 inhibe la transcription des gènes de réponse au stress en promouvant: (i) l'exportation de leurs facteurs de

transcription (Msn2/4) hors du noyau et (ii) en favorisant la rétention cytoplasmique de Msn2/4 en régulant positivement sa liaison aux protéines d'ancrage Bmh1 et/ou Bmh2 (46, 68).

TORC1 et la régulation de la traduction

L'activité de TORC1 a un impacte positif la traduction. En fait, TORC1 favorise la traduction: (i) en inhibant GCN2, une protéine kinase qui phosphoryle et inhibe le facteur d'initiation de la traduction eIF2 (ii) en inhibant Eap1, une protéine qui empêcherait la formation du complexe d'initiation de la traduction (complexe eIF4E-eIF4G-eIF4A) en se liant de manière compétitive à eIF4E (79), (iii) en stabilisant la liaison eIF4E-eIF4G (iv) en favorisant la biogenèse des ribosomes notamment en activant la synthèse des gènes codant pour les protéines ribosomales et pour les ARN ribosomiques (35, 61, 80, 81).

TORC1 et la régulation de l'autophagie

TORC1 inhibe la macroautophagie en empêchant la formation du complex (Atg1-Atg13-Atg17) (82, 83). La macroautophagie est un processus qui implique la formation dans le cytoplasme de vésicules cytosoliques à double membrane, les autophagosomes, qui séquestrent des portions du cytoplasme. La fusion des autophagosome avec la vacuole (un compartiment de dégradation aussi impliqué dans le stockage des acides aminés) permet la dégradation de leur chargement et éventuellement le recyclage des acides aminés et des autres composantes générées (84). D'autre part TORC1 pourrait réguler positivement la microautophagie, un processus qui implique la dégradation de morceaux de la membrane vacuolaire par invagination de cette membrane à l'intérieur du lumen de la vacuole (85, 86).

I.3.4) Interractions croisées entre les différentes voies de signalisationd régulées par les éléments nutritifs

Il existe de nombreuses interactions croisées entre les différentes voies de signalisations régulées par les éléments nutritifs. Ces interactions permettent aux cellules d'assurer une croissance optimale en réponse aux changements environnementaux. Ainsi, les voies de signalisation TORC1 et PKA inhibent la formation du complexe Atg13-Atg1, nécessaire aux premières étapes de la macroautophagie, (87) ainsi que la protéine kinase Yak1, un régulateur négatif de l'expression des gènes codant pour les protéines ribosomiques (35, 88). Enfin, ces deux voies de signalisation empêchent l'accumulation nucléaire du facteur de transcription Msn2/4, soit en inhibant son importation dans le noyau (PKA) soit en favorisant

sa rétention dans le cytoplasme (TORC1) (47, 68). Différentes études indiquent que les voies de signalisation TORC1 et PKA pourraient agir sur leur cibles communes en parallèle ou que TORC1 pourrait contrôler l'activité de la PKA (88, 89). TORC1 inhibe la protéine kinase Gcn2. En parallèle cette protéine kinase reçoit aussi des informations concernant la quantité d'acides aminés présents dans le milieu. En fait Gcn2 est activée par les ARN de transfert (ARNt) non chargés qui s'accumulent lors d'une carence en acides aminés. Lorsque les cellules sont traitées avec de la rapamycine, en revanche, la quantité d'acides aminés présents dans le milieu ne varie pas. Dans ce cas l'inactivation de TORC1 augmente l'affinité de Gcn2 pour les ARNt non-chargés (90). Finalement, le facteur de transcription Gln3 est régulé par au moins trois signaux différents soit: Snf1 qui promeut l'entrée de Gln3 dans le noyau, TORC1 qui retient Gln3 dans le cytoplasme et enfin les source d'azote présentes dans le milieu (91-93). Ces interactions croisées placent les voies de signalisation auparavant décrites comme linéaires dans un vaste réseau de communications.

II) Présentation du travail de recherche et résultats

II.1) Présentation générale

Ce travail de recherche a été entrepris dans le but d'acquérir de nouvelles connaissances concernant certains aspects importants du contrôle de la croissance et de la prolifération cellulaire chez l'organisme modèle *Saccharomyces cerevisiae,* en étudiant les mécanismes gouvernant l'entrée et la sortie des cellules de l'état de quiescence (G_0). En effet, la compréhension de ces mécanismes apparaît comme étant un important enjeu biomédical puisque leur dérégulation, causant soit une incapacité pour les cellules d'entrer en G_0 soit une sortie inappropriée des cellules de leur état quiescent, sont susceptibles, chez les organismes multicellulaires, de mener au développement d'un cancer.

Ce travail de thèse est divisé en deux parties. Dans un premier chapitre, nous nous sommes intéressés aux mécanismes impliqués dans l'entrée des cellules en G_0 en étudiant la régulation de la protéine kinase Rim15 qui est la protéine phare de ce laboratoire. Ces mécanismes bien que largement étudiés sont encore mal connus. Dans un second chapitre, nous nous sommes intéressés à l'étude des mécanismes qui régulent la sortie des cellules de G_0. Au contraire des signaux régulant l'entrée des cellules en G_0, les signaux régulant la sortie des cellules de cet état de non-prolifération sont peu connus. Ils comprennent notamment l'addition de carbone à des cellules carencées en carbone ou, pour des cellules dont la croissance a été arrêtée suite à un traitement à la rapamycine, le retrait de cette drogue du milieu de culture.

II.2) Résumé du premier article publié

(Pedruzzi, I*., Dubouloz, F*., Cameroni, E., Wanke, V., Roosen, J., Winderickx, J., and De Virgilio, C. (2003) TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. *Mol Cell* 12, 1607-1613)

Les recherches effectuées précédemment dans le laboratoire du professeur C. De Virgilio ont établit que la protéine kinase Rim15 est directement phosphorylée et inhibée par la PKA et que cette protéine est nécessaire à l'entrée des cellules en G_0 (48). Cependant, la façon dont cette protéine est régulée n'a pas encore été totalement élucidée. Sachant que l'activation de Rim15 (suite à l'inhibition de la PKA) met en place les changements physiologiques nécessaires à l'entrée des cellules en G_0 et que ces changements sont

similaires à ceux observés suite à l'inactivation de la voie TORC1, nous nous sommes demandé si TORC1 était aussi un régulateur de Rim15.

En premier lieu, nous avons déterminé le rôle de Rim15 dans l'acquisition des phénotypes résultant de l'inactivation de TORC1. Pour ce faire, nous avons étudié un certain nombre de réponses induites par l'addition de concentrations inhibitrices de rapamycine sur des cellules sauvages ou des cellules dans lesquelles le gène *RIM15* a été supprimé. Cette première approche a établit que certaines de ces réponses sont dépendantes de la présence de Rim15. Nous avons ensuite évalué le rôle de la voie de signalisation PKA et du principal effecteur en aval TORC1, Tap42, dans la réponse transcriptionnelle induite par la rapamycine et dépendante de Rim15. L'analyse transcriptionnelle, par hybridation Northern de divers mutants de la voie PKA a démontré qu'une diminution (*ras2Δ*) ou une hyperactivation (*bcy1Δ* ou RAS^{Val19}) de la voie PKA affectait sensiblement l'expression de *HSP12*, *HSP26* et *SSA3* en réponse à la rapamycine mais qu'en l'absence totale de PKA, ces gènes étaient encore inductibles par la rapamycine via Rim15. De même, ni l'absence des phosphatases associées à Tap42 (*pph21Δ pph22Δ* ou *sit4Δ*), ni la présence dans des cellules sauvages d'un allèle de *TAP42* (*tap42-11*) qui rend les cellules partiellement résistante aux effets de la rapamycine n'a abolit l'induction des gènes régulés par Rim15 suite à l'inhibition de TORC1, indiquant que TORC1 régule cette protéine via un mécanisme indépendant de Tap42.

L'étude de la localisation de Rim15 *in vivo*, en utilisant une construction *GFP-RIM15*, a révélé que l'inhibition de TORC1 conduisait à un changement de localisation de Rim15 du cytoplasme au noyau. En accord avec les analyses génétiques précédentes, ni l'inhibition de la voie PKA (-AMPc), ni son hyperactivation ($RAS2^{Val19}$) n'affecte ce processus. De même l'absence de Sit4 n'influence pas la façon dont la localisation de Rim15 est régulée par la rapamycine. Sachant que Rim15 est une phosphoprotéine, nous avons aussi analysé son état de phosphorylation par retard de migration de la protéine sur gel d'acrylamide. Cette analyse a révélé que Rim15 était hyperphosphorylé dans des conditions où le complexe TORC1 est inactif et Rim15 nucléaire. A la recherche de nouveaux régulateurs de Rim15 nous nous sommes aussi intéressés à Sch9, une Ser/Thr kinase connue pour être impliquée dans la régulation des cibles de la PKA. Nous avons ainsi découvert que GFP-Rim15 localisait de façon constitutive dans le noyau des mutants *sch9Δ*. Cependant, au contraire de l'inactivation de TORC1, l'absence de Sch9 n'est pas associée à une hyperphosphorylation de Rim15. Finalement Rim15 étant une kinase régulée par les éléments nutritifs, nous nous sommes demandé si ceux-ci pouvaient aussi réguler la localisation de Rim15. Nous avons

réalisé une analyse dynamique de la localisation de Rim15 et de son état de phosphorylation au cours du temps à partir d'une culture de cellules en milieu liquide contenant au départ 2% de glucose. Cette analyse a révélé que, dans ces conditions, l'entrée de Rim15 dans le noyau a lieu quand la moitié du glucose présent dans le milieu a été consommé. L'entrée de Rim15 dans le noyau est aussi corrélée avec l'induction transcriptionnelle du gène *SSA3* et l'apparition d'une forme hyperphopshorylée de la protéine.

L'ensemble des résultats obtenus nous ont permis d'établir que TORC1, comme la PKA, régule négativement Rim15 via une branche effectrice indépendante de Tap42. Selon notre modèle lorsque les éléments nutritifs sont abondants la PKA et la voie TORC1 interviennent indépendamment l'une de l'autre pour inactiver Rim15. La première inhibe l'activité kinase de Rim15 tandis que la seconde prévient la translocation de la protéine dans le noyau par un mécanisme encore inconnu mais qui pourrait impliquer la phosphorylation de la protéine. Sch9 inhibe aussi probablement la localisation nucléaire de Rim15 et semble agir en parallèle à TORC1. Selon ces données, Rim15 est donc régulé par trois kinases différentes. Au contraire, lorsque les éléments nutritifs viennent à manquer ou lorsque les cellules sont traitées avec de la rapamycine, Rim15 est transporté dans le noyau (un environnement où l'activité de la PKA est supposée être faible) où il est actif et participe à l'acquisition des changements nécessaires à l'entrée des cellules en G_0.

II.3) Résumé du second article publié

(Dubouloz, F., Deloche, O., Wanke, V., Cameroni, E., and De Virgilio, C. (2005) The TOR and EGO protein complexes orchestrate microautophagy in yeast. *Mol Cell* 19, 15-26)

Cette seconde publication est le résultat d'une toute nouvelle approche mise au point dans le but d'élargir notre caractérisation de l'état G_0 chez la levure. Nous avons donc entrepris d'analyser une collection de mutants (chacun comportant une altération dans un parmi 4857 gènes non-essentiels) afin d'identifier ceux qui, après avoir subit un arrêt en G_0 artificiellement induit par un traitement à la rapamycine, ne peuvent pas reprendre leur croissance une fois la drogue enlevée. Cette méthode a pour but de révéler les gènes dont les produits sont impliqués dans la sortie des cellules d'un état G_0 induit par la rapamycine. De cette manière nous avons identifié sept mutants dont trois (*ykr007wΔ*, *gtr2Δ* et *ybr077cΔ*) ont été sélectionnés pour notre étude. Nous avons renommé *YKR007W* et *YBR077C*, *EGO1* et *EGO3* respectivement (pour **e**xit from a rapamycin-induced **gro**wth arrest). Par coloration au bleu de trypan nous nous sommes assuré que ces cellules restaient viables 48h suivant

le retrait de la rapamycine dans le milieu. L'étude d'un certain nombre de caractéristiques des cellules quiescentes a démontré que les mutants *ego* entrent correctement en G_0. Par des analyses génétiques, biochimiques et microscopiques nous avons ensuite établi que les protéines correspondant aux trois gènes mutants identifiés forment un complexe (EGO) localisé à la membrane de la vacuole.

L'analyse morphologique que des mutants *ego* a révélé que ceux-ci présentent un phénotype vacuolaire anormal durant la phase qui suit le retrait de la rapamycine du milieu (ou phase de réveil). Cette observation nous a permis d'élucider le mécanisme par lequel le complexe pourrait favoriser le retour à la croissance des cellules arrêtées en G_0. En effet, durant cette phase, les mutants *ego*, au contraire des cellules sauvages, présentent une vacuole anormalement grande qui occupe presque tout le volume de la cellule indiquant que le complexe EGO pourrait jouer un rôle dans le processus de réduction de la taille de la vacuole qui a lieu lors du retrait de la rapamycine. Conformément à cette prédiction, nous avons observé in *vivo* par microscopie à fluorescence que l'expression conditionnelle des gènes codant pour Ego1, Gtr2 et Ego3 durant la phase de réveil, induisait la formation de vésicules microautophagiques. L'induction de la microautophagie précédait la réduction de la taille des vacuoles et la reprise de la croissance cellulaire. Etant donné que ce processus implique l'internalisation et la dégradation de fragments de la membrane vacuolaire, il est idéalement situé pour réduire la taille de la vacuole.

Nous avons également démontré que les mutants *ego* étaient hypersensibles à la rapamycine tandis que la surexpression des gènes *EGO* sauvages conférait aux cellules une croissance partiellement résistante aux effets inhibiteurs de la rapamycine. Ces données indiquent que ce complexe pourrait faire partie de la voie TORC1. Puisque nos résultats montraient aussi qu'à la suite du retrait de la rapamycine divers traits régulés par TORC1 restaient bloqués dans les mutants *ego*, nous avons suggéré que le complexe EGO pourrait agir en amont de TORC1. Afin de définir un peu plus la façon dont le complexe EGO fonctionne, nous avons initié, en utilisant la collection de mutants précédemment décrite, un crible génétique pour la létalité synthétique dans le mutant *gtr2Δ* (dont le produit code pour une petite GTPase potentielle). Cette analyse a confirmé le lien existant entre le complexe EGO et TORC1 puisqu'en l'absence de Gtr2, une réduction de la voie TORC1 (*tor1Δ* ou *tif3Δ*) affectait fortement la croissance des cellules. De plus, elle a mis en évidence le fait que le glutamate et/ou la glutamine pourraient jouer un rôle important dans la croissance des mutants *ego* (*rtg3* et *rtg2* sont létaux avec la délétion de *gtr2*) ainsi que dans la suppression

du phénotype ego (*i.e.,* l'incapacité qu'ont les mutants *ego* de reprendre la croissance cellulaire suite à une exposition transitoire à la rapamycine).

Sur la base de ces résultats nous avons construit le modèle suivant. Les complexes EGO et TORC1 régulent la microautophagie en utilisant deux voies parallèles. Lors de la sortie des cellules de G_0, ce processus qui assure la redistribution des membranes cellulaires pourrait être impliqué dans la réactivation de TORC1. En effet, TORC1 qui est un complexe associé aux membranes pourrait sentir le flux membranaire initié par la microautophagie (60, 94). La vacuole est aussi un réservoir d'acides aminés. Ainsi, la réduction de la taille de la vacuole pourrait concentrer les acides aminés contenus dans ce compartiment (en particulier la glutamine) et envoyer un signal positif à TORC1(95).

II.4) Résumé du Chapitre IV

Ce Chapitre expose brièvement les résultats les plus récents de notre étude du complexe EGO. Je présente sous forme de tableau es résultats préliminaires d'un criblage double hybride systématique à l'échelle génomique entrepris dans le but d'identifier les régulateurs et/ou effecteurs du complexe EGO. Pour ce criblage, nous avons utilisé une collection de souches de levure contenant chacune l'un des 6000 cadres de lecture ouvert (ORF) présent chez la levure fusionné en N-terminal avec le domaine d'activation de Gal4. Cette collection a été croisée avec chacun des clones exprimant nos appâts (*EGO1*, *GTR2* et *EGO3)* fusionnés au domaine de liaison à l'ADN de Gal4. Avec ce système, lorsque deux protéines interagissent, elles reconstituent le facteur de transcription Gal4 qui peut ensuite activer les gènes rapporteurs *HIS3* et/ou *ADE2*, permettant aux cellules de croître en l'absence d'histidine et/ou d'adénine dans le milieu (96). Les résultats des premiers criblages nous ont permis d'identifier un nouveau membre du complexe EGO, Gtr1 qui est une petite GTPase potentielle appartenant à la même famille que Gtr2. Nous avons donc par la suite étendu les criblages doubles hybrides en utilisant comme appât cette petite GTPase ainsi que les variants des petites GTPases supposés être soit constitutivement liés au GTP ($Gtr2^{Q66L}$, $Gtr1^{Q65L}$) ou constitutivement liés au GDP ($Gtr2^{S23L}$, $Gtr1^{S20L}$). Nous avons aussi exploré plus en avant les relations entre TORC1 et le complexe EGO et nous présentons par l'analyse double hybride la première évidence d'une interaction directe entre les deux complexes (interaction entre la protéine Ego1 et Tco89).

III) Discussion Générale et Perspectives

Dans l'ensemble ces deux études complémentaires mettent en évidence le fait que le complexe TORC1 joue un rôle important non-seulement dans la régulation de l'entrée des cellules en G_0 mais également dans la sortie de cet état.

III.1) L'entrée en G_0

Notre étude de la protéine kinase Rim15, un régulateur clé de l'entrée des cellules en G_0, a démontré que les signaux transmis par les éléments nutritifs présents dans le milieu de culture régulaient non-seulement l'activité mais également la localisation intracellulaire de cette protéine. De plus, nous avons trouvé, qu'en plus de la PKA, cette kinase intégrait des signaux en provenance de deux autres protéines soit Sch9 et TORC1. Tandis que la PKA inhibe l'activité de Rim15, TORC1 et Sch9 agissent parallèlement à la PKA pour contrôler la localisation nucléocytoplasmique de Rim15. Ces deux kinases régulent aussi probablement l'activité de Rim15 puisqu'elles l'empêchent d'atteindre le compartiment nucléaire qui, selon la littérature, constituerait un environnement à faible activité PKA (97). Ces résultats démontrent que la protéine kinase Rim15 peut intégrer des signaux en provenance des différentes voies de signalisation régulées par les éléments nutritifs. Cependant, de nombreuses questions restent encore à élucider, notamment la façon dont est régulé le transport de Rim15 dans et hors du noyau ainsi que les mécanismes par lesquels TORC1 et Sch9 régulent la localisation et/ou l'activité de Rim15.

Les mécanismes régulant l'entrée et la sortie de Rim15 du noyau ont pu être en partie décryptés grâce à par l'étude de Wanke *et al.* (2005) qui a découvert un autre régulateur de Rim15, le complexe Pho80-Pho85 (98). Ce papier démontre que la protéine Msn5 est responsable de l'exportation de Rim15 hors du noyau. Il démontre également que le complexe Pho80-85 prévient la translocation de Rim15 dans le noyau en phosphorylant le résidu Thr1075 situé entre les deux domaines kinase de Rim15. Cette phosphorylation favorise l'interaction de Rim15 avec les protéines Bmh1/2 qui ancrent Rim15 dans le cytoplasme. Un tel mécanisme pourrait également être utilisé par Sch9 ou TORC1 pour retenir Rim15 dans le cytoplasme. En effet, le domaine de Rim15 contenant ce résidu Thr1075 possède aussi des sites consensus de phosphorylation pour Sch9 (RXRXXS/T) imbriqués dans des sites consensus de liaison à Bmh1/2. De plus, des résultats préliminaires ont établi que l'activité de TORC1 était nécessaire au maintient du résidu Thr1075 dans un état phosphorylé (98).

Je profite de cette partie pour commenter le résultat de l'hybridation Northern faite avec le mutant *sch9Δ* et exposé dans la première publication. Celui-ci démontrait qu'en l'absence de Sch9, qui est supposé réguler négativement Rim15, les gènes *HSP12*, *HSP26* et *SSA3* étaient constitutivement réprimés même à la suite d'un traitement des cellules avec de la rapamycine. En fait, il se pourrait que ces résultats soient dus à l'acquisition d'une mutation supplémentaire qui pourrait masquer le phénotype des mutants *sch9Δ*. En effet, d'après nos données les plus récentes, il semblerait que les mutants *sch9Δ* acquièrent facilement des mutations activatrices de la voie PKA. Cette activation constitutive de la voie PKA empêcherait toute transcription des gènes *HSP12*, *HSP26* et *SSA3*.

Dans le futur, nous projetons d'étudier plus en détail les mécanismes utilisés par TORC1 et Sch9 pour réguler la localisation de Rim15 et/ou son activité. La découverte des mécanismes moléculaires utilisés par Sch9 et TORC1 pour réguler Rim15 pourrait confirmer que ces deux voies de signalisation agissent en parallèle. Notamment, il nous pourrons tester *in vitro* (par essai kinase) la phosphorylation de Rim15 par TORC1 et/ou Sch9. Par spectrométrie de masse nous pourrons aussi déterminer si la phosphorylation de résidus spécifiques a lieu *in vivo*. Si cette phosphorylation a lieu, nous pourrons aussi établir si elle influence l'activité ou la localisation de Rim15. Il sera également intéressant de mieux définir la façon dont la localisation de Rim15 est régulée en trouvant, par exemple la protéine responsable de son importation dans le noyau. Pour cela, nous disposons d'une collection de mutants d'importines (responsables de l'importation des protéines dans le noyau). L'analyse par hybidation northern de ces mutants devrait révéler un défaut dans l'induction de la transcription des gènes dépendants de Rim15 à la suite d'un traitement des cellules à la rapamycine dans le mutant responsable de l'importation de la protéine.

III.2) La sortie de G_0

Dans la seconde partie de cette thèse, nous avons découvert l'existence d'un nouveau mécanisme potentiel de contrôle de la croissance cellulaire dont l'origine est à la membrane de la vacuole. Par le biais d'analyses génétiques, biochimiques et morphologiques, nous avons mis en évidence un nouveau complexe protéique nommé EGO, associé à la membrane vacuolaire, et dont la présence est nécessaire pour que les cellules arrêtée artificiellement en G_0 suite à un traitement avec de la rapamycine puissent reprendre leur croissance une fois la drogue enlevée. Le complexe EGO semble jouer un rôle important dans la réactivation de TORC1 à la suite d'un traitement transitoire des cellules à la

rapamycine et agit en parallèle à ou dans la voie TORC1 pour promouvoir la redistribution des membranes. Les analyses complémentaires décrites dans le Chapitre IV indiquent par ailleurs que le complexe EGO pourrait, par sa position à la membrane de la vacuole et son interaction potentielle avec des transporteurs vacuolaire, agir comme un senseur capable de mesurer de la disponibilité intracellulaire des éléments nutritifs (notamment le contenu vacuolaire en acides aminés). De plus, nos résultats les plus récents indiquent que le complexe EGO pourrait agit directement dans la voie de signalisation TORC1.

Cependant beaucoup de questions restent encore en suspend. En effet, les résultats présentés dans le Chapitre IV sont préliminaires et devront être confirmés. Notamment, les interactions protéine-protéine les plus prometteuses seront répétées dans un second test double hybride et confirmées par Co-précipitation. Il faudra aussi confirmer génétiquement que le complexe EGO agit bien en amont de TORC1 notamment en démontrant que d'autres phénotypes contrôlés par TORC1, comme l'induction de la transcription des protéines ribosomales restent bloqués dans les mutants *ego* suivant le retrait de la rapamycine. Matteo Binda, qui a repris le sujet s'attèle à cette tâche. De plus, la façon dont le complexe EGO pourrait réguler TORC1 ainsi que le rôle joué par la glutamine dans le processus de sortie des cellules de G_0 reste aussi à établir. Dans cette optique, il sera intéressant de mesurer la concentration de cet acide aminé dans les mutants *ego* en comparaison des cellules sauvages. Enfin, nous envisageons aussi de rechercher un homologue du complexe EGO dans les cellules de mammifères. En effet deux homologues de Gtr1 (RragA et RragB) ainsi que deux homologues de Gtr2 (RragC et RragD) ont déjà été identifiés (99). Cela pourra être réalisé par le crible en double hybride d'une banque d'ADNc de mammifère ou humaine en .utilisant comme proie les différentes protéines Rrag. Parmi les interactions protéine-protéine nous espérons trouver des protéines homologues à Ego1 et Ego3.

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Table of contents

List of abbreviations

3-AT	3-amino-1, 2, 4-triazole
acetyl-CoA	acetyl-coenzyme A
AGC	protein kinase A, G and C family
ALP	alkaline phosphatase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AD	activation domain
BD	binding domain
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent kinase
Co-IP	Co-immunoprecipitation
DIC	differential interference contrast
DAPI	4,6-diaminido-2-phenylindole
DNA	deoxyribonucleic acid
EGO	exit from a rapamycin-induced growth arrest
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
EtOH	ethanol
FAD	flavin adenine dinucleotide
FGM	fermentable growth medium
5-FOA	5-fluoroorotic acid
FGM	fermentable growth medium-induced
FRB	FKBP12/Frp1-rapamycin-binding domain
G_0	quiescence
Gal	galactose
GAP	guanine activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GSE	GTPase-containing complex required for Gap1 sorting in the endosome
GTP	guanosine triphosphate
kDa	kilo Dalton
OAA	oxaloacetate
OD	optical density
ORF	open reading frame
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
M	molar
MSX	L-methionine sulfoximine; glutamine synthase inhibitor
NAD	nicotinamide adenine dinucleotide
NCR	nitrogen catabolite repression system
NDP	nitrogen discrimination pathway
NES	nuclear export signal
NLS	nuclear localisation signal
ORF	open reading frame
PDS	post-diauxic shift
Pi	inorganic phosphate
PI3K	phosphatidyl inositol 3-kinase
PKA	protein kinase A

PKB	protein kinase B
PP2A	protein phosphatase type 2A
Pol(I)	RNA polymerase (I)
Pol(II)	RNA polymerase (II)
Pol(III)	RNA polymerase (III)
PP2A	protein phosphatase 2A
rDNA	ribosomal DNA
rap	rapamycin
Ras	Ras1 and Ras2 proteins
Ribi	ribosome biogenesis
RIM	regulator of *IME2*
RNA	ribonucleic acid
RP	ribosomal protein
RPGs	ribosomal proteins genes
RS-ALP	retrieval sequence-alkaline phosphatase
RTG	retrograde system
rRNA	ribosomal RNA
S6K1	S 6 kinase 1
SAP	Sit4 associated protein
SMIR	small-molecule inhibitor of rapamycin
STRE	stress response element
SPS	Ssy1, Ptr3, Ssy5 sensor of extracellular amino acids
TCA	tricarboxylic acid cycle
TOR	target of rapamycin pathway
Tor	Tor1 and Tor2 proteins
TORC1	TOR complex one, rapamycin-sensitive
TORC2	TOR complex two, rapamycin-insensitive
TORCs	TORC1 and TORC2
ts	temperature sensitive; thermosensitive
tRNA	transfer RNA
wt	wild-type

Gene and protein nomenclature in *S. cerevisiae*

Tor1	wild-type protein
$Tor1^{Ser1972Arg}$, $Tor1^{Arg1972}$	mutant protein (with an arginine instead of a serine at position 1972)
TOR1	wild-type allele
tor1	recessive mutant allele
$tor2^{ts}$	temperature sensitive mutant allele
tor1Δ	deletion mutant
$TOR1^{Ser1972Arg}$	dominant active mutant allele encoding an arginine instead of a serine at codon 1972

List of frequently-mentioned genes and their gene products

ARG3	ornithine carbamoyl transferase; enzyme involved in the conversion of glutamine into citrulline, an arginine precursor
ATG13/APG13	protein required for autophagy
ATG1/APG1	protein kinase required for autophagy
BCY1	cAMP-dependent protein kinase A: regulatory subunit
BMH1, 2	14-3-3 proteins; bind phosphorylated proteins
CDC25	Ras guanyl-nucleotide exchange factor
CDC55	regulatory subunit of PP2A with Rts1
CRF1	co-repressor of Fhl1
CPA2	carbamoyl-phosphate synthase; enzyme involved in the conversion of glutamine into citrulline, an arginine precursor
CYR1/CDC35	adenylyl cyclase
EGO1/MEH1/GSE2	component of the EGO complex, which is involved in the regulation of microautophagy; localises to the vacuolar membrane, myristoylated protein
EGO3/SLM4/GSE1	component of the EGO complex, which is involved in the regulation of microautophagy; localises to the vacuolar membrane; putative transmembrane protein
ERS1	protein with similarities with human cystinosin, involved in the transport of cystine from the lysosome
ESA1	histone acetyltransferase; acetylates histone H4 N-terminal tail
FHL1	transcriptional regulator of RPGs
FPR1	peptidyl-prolyl cis-trans isomerase (PPIase), binds to the drug rapamycin
GAP1	general amino acid permease
GAT1	transcriptional activator of NCR-responsive/-sensitive gene transcription
GCN2	protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2
GCN4	transcriptional activator of amino acid biosynthetic genes
GIS1	C_2H_2 zinc finger transcription factor required for transcription of genes containing a PDS-element
GLC7	catalytic subunit of the type1 Ser/Thre phosphatase; involved in complex with Reg1 in the inhibition of Snf1
GLN3	GATA-type zinc finger transcription factor; activates NCR-responsive/-sensitive transcription; localisation and activity regulated by quality and quantity of nitrogen source
GPA2	heterotrimeric G-protein alpha subunit
GPR1	G-protein-coupled receptor; low affinity glucose sensor
GTR1	small GTP binding protein; potential component of the TORC1 complex
GTR2	small GTP binding protein; component of the EGO complex; belongs to the Ras like small GTPase superfamily
HAP2, 3, 4, 5	subunits of the heme-activated, glucose-repressed HAP complex; regulator of respiratory gene expression
HSP12	12 kDa heat shock protein
HSP26	26 kDa heat shock protein
HXK1, 2	hexokinases one and two; involved in intracellular glucose sensing
IFH1	co-activator of Fhl1
IRA1, 2	Ras GTPase activating proteins
KOG1	component of the TORC1 complex

LST8	component of the TORC1 complex; involved in regulating Gap1 activity and RTG-target genes
MEP2	ammonium permease
MON1	protein required for fusion of vesicles and autophagosomes with the vacuole
MKS1	negative regulator or RTG-target gene transcription when bound to Bmh1 and Bmh2
MSN2, 4	C_2H_2 zinc finger transcription factor involved in STRE-controlled gene activation
MSN5	importin-beta family member required for nuclear export of Msn2 and Rtg1-Rtg3
NPR1	protein kinase that stabilises several plasma membrane amino acid transporters by antagonising their ubiquitin-mediated degradation
NVJ1/VAB36	nuclear envelope protein; interacts with the vacuolar membrane protein Vac8 to promote formation of nucleus-vacuole junctions during piecemeal microautophagy of the nucleus
PDE1, 2	low and high affinities 3', 5', cyclic nucleotide phosphodiesterases
PHO80	cyclin which associates with Pho85
PHO81	cyclin-dependent kinase inhibitor; inhibits the Pho80-Pho85 complex
PHO85	cyclin-dependent kinase, can associate with ten cyclin partners; in phosphate-rich conditions, Pho85p-Pho80p complex phosphorylates Pho4
PPH21, 22	redundant catalytic subunits of PP2A; involved in regulating some branches of the TORC1 pathway
PKB	mammalian protein kinase B
RAP1	DNA-binding protein involved in either activation or repression of transcription
RRAGA, B	mammalian Ras-related GTP-binding proteins; homologous to Gtr1
RRAGC, D	mammalian Ras-related GTP-binding proteins; homologous to Gtr2
RAS1, 2	small G-proteins; positive regulators of adenylyl cyclase
REG1	regulatory subunit of the phosphatase Glc7; forms with Glc7 a complex involved in the inhibition of Snf1
RIM15	Ser/Thr nutrient-regulated protein kinase; repressed by glucose and PKA; required for proper entry into G_0
RTG1, 3	basic helix-loop-helix transcription factors, form together a complex to activate RTG-target genes
RTG2	activates Rtg1p and Rtg3p; the transcriptional activators of the retrograde (RTG) pathway
RTS1	regulatory subunit of PP2A with Cdc55
RRN3	recruits RNA polymerase (I) to rDNA
SSA3	70 kDa heat shock protein
SCH9	AGC Ser/Thr kinase that regulates cell size; homologous to PKB/Akt
SFP1	transcription factor that controls expression of ribosome biogenesis genes
SIT4	Ser/Thr protein phosphatase involved in regulating some branches of the TORC1 pathway
SNF1	sucrose non-fermenting; Ser/Thr kinase required for glucose repression
TAP42	essential protein involved in the TOR signalling pathway; physically associates with the protein phosphatase 2A and the Sit4 protein phosphatase catalytic subunits
TAT2	tryptophan permease

TIF3	non-essential translation initiation factor eIF4B, promotes ATP-dependent RNA helicase activity of eIF4A; its loss confers a phenotype synthetic sic with loss of Gtr2
TIP41	Tap42 interacting protein
TCO89	component of the TORC1 complex
TOR1, 2	target of rapamycin proteins
TPD3	scaffolding subunit of PP2A
TPK1, 2, 3	cAMP-dependent protein kinase A, catalytic subunits
URE2	regulator of nitrogen catabolite repression
VPS27	endosomal protein required for recycling Golgi proteins and sorting ubiquitylated proteins to the vacuole.
YAK1	Ser/Thr protein kinase that is part of a glucose-sensing system involved in growth control in response to glucose availability
YOL092W	putative vacuolar amino acid permease homologous to Ers1

Summary

The mammalian protein kinase A (PKA), protein kinase B (PKB/Akt) and mTORC1 function in complex signalling pathways to positively control cell metabolism and growth in response to hormone and growth factors. Aberrant function of these pathways contributes to a number of pathologies ranging from metabolic disease to hyperproliferation disorders and cancer. Thus, understanding of their function is of major importance in order to build strategies to fight against these diseases. PKA, Sch9 and TORC1 are the yeast *Saccharomyces cerevisiae* counterparts of PKA, PKB/Akt and mTORC1. They exert in yeast similar physiological roles in response to nutrient availability. The yeast Rim15 protein kinase is under direct negative control of PKA and its activity is important for cells to adapt their growth rate and metabolism to nutrient depletion (48, 51). Rim15 activity is notably required for cells to arrest growth properly upon unfavourable nutrient conditions and its study is particularly interesting to elucidate in greater details how cells establish the program leading cells to enter into the non-proliferating and low metabolic state called quiescence (G_0). Although extensively studied in our laboratory, regulation of this protein is still not completely understood. Notably, some evidence indicated that a signalling pathway independent from the PKA pathway could negatively regulate Rim15. These negative regulators of Rim15 could be TORC1 and/or Sch9, since both nutrient-regulated kinases control phenotypes globally similar to those controlled by PKA.

In this thesis, we studied the possibility that the two nutrient-regulated kinases TORC1 and Sch9 converge on Rim15 to negatively control entry into quiescence (G_0) via a mechanism that is independent from PKA. One important finding of this work is that, upon TORC1 inactivation, full induction of the Rim15-dependent phenotype requires two distinct processes, *i.e.,* nuclear accumulation of Rim15 and release from the PKA-mediated inhibition of the protein kinase activity of Rim15. This study also reveals that three nutrient signalling kinases, *i.e.*, TORC1, Sch9 and PKA, directly or indirectly regulate Rim15. Our findings are in line with the new picture obtained by genomics and proteomic approaches, where these individual kinases are part of an integrative and converging network, which assures a dynamical and balanced transmission of extracellular signals

Eukaryotic cell proliferation is tightly controlled by specific growth factors and the availability of essential nutrients. If either one of these signals is lacking, cells may enter into the specialised non-dividing resting state called G_0 or quiescence. Most cells in adult human body are in G_0. While mechanisms that regulate the entry into G_0 have been extensively

studied in several organisms, including yeast and mammalian cells, the way cells get back into proliferation remains largely unknown (100).The exit from the cell division cycle can be temporary or permanent, as is the case with terminally differentiated cells like neurons. Understanding how cells exit from G_0 can be of biomedical importance. Notably, quiescent cells inappropriately resume proliferation during development of cancer. The highly conserved TORC1 protein complex may have come in for that role in positively regulating cell growth in this context.

To elucidate the mechanisms implicated in G_0 exit, we first established a functional profile of the yeast *Saccharomyces cerevisiae* genome with respect to the rapamycin-induced G_0-like growth arrest. In this screen, we were able to identify a vacuolar complex (EGO), consisting of the conserved putative small GTPase Gtr2 and two new proteins, Ego1 and Ego3, which are required for recovery from a rapamycin treatment. This EGO complex acts in parallel or upstream of TORC1 to positively regulate microautophagy (the formation and release of vesicles in the vacuolar lumen). This process is thought to counteract macroautophagy (the fusion of vesicles with the vacuole) and could affect several metabolic processes (*i.e.*, intracellular distribution of membranes and amino acid levels). Interestingly, our recent findings point towards a role of the complex upstream of the TORC1 pathway. Results of these latest studies are presented in Chapter III and Chapter IV.

Chapter I.

Nutrient-Regulated Control of G_0 Entry in the Yeast *Saccharomyces cerevisiae*.

A literature overview

I.1) Introduction

Most cells on earth exist in a non-proliferating and non-growing state known as quiescence or G_0, characterised by an overall decreased metabolic activity (2, 89). This holds true for many human cells like neuronal or liver cells and for nutrient-starved microorganisms. Failure of higher eukaryotic cells to enter or to stay into G_0 may lead to cancer whereas in microorganisms it may cause premature cell death (101). Thus, understanding of how cells enter into or exit from G_0 has important implications for the fight against cancer on one hand and against microbial pathogens on the other hand, since some pathogenic microorganisms can survive for years in a quiescent and hence, drug resistant state in human tissues (89). The basic cellular processes governing entry into and exit from quiescence appear to be conserved among eukaryotic cells. Accordingly our researches on the study of G_0 entry and exit were accomplished in the yeast *Saccharomyces cerevisiae,* introduced as an experimental organism in the mid-thirties of the 20th century (102, 103), and which is now widely accepted as a model organism for the study of eukaryotic cells.

I.1.1) The yeast *Saccharomyces cerevisiae* as a model organism

The budding or brewer's yeast *Saccharomyces cerevisiae* is a unicellular fungus commonly used as model organism in the biomedical research because it offers several advantages:

- It grows and divides quickly. Normal laboratory strains have a doubling time of about 90 min when grown on nutrient-rich medium, which is very short when compared to the generation time of a mammalian cell (*i.e.,* 24h). Additionally, a fairly large number of yeast cells can be grown in culture in a relatively limited amount of space (10).
- It can be efficiently transformed, which facilitates genetic studies and manipulations (104).
- It can live either as haploid or as diploid organism. Diploid yeast can, like mammalian cells, undergo meiosis and form four recombinant haploid cells. This process, called sporulation, provides an important tool to understand recombination and the transmission of genetic material. Importantly recessive mutations can be conveniently isolated and their phenotype can be studied in haploid cells.
- As eukaryotic cell, it shares with plants and animals, most of the complex internal cell structures (including mitochondria and a nucleus) as well as the control mechanisms over fundamental cell processes (*e.g.*, cell growth and cell division) (10, 11). Many signalling pathways and proteins are conserved from yeast to human (8, 105), and

many yeast genes have orthologs in the human genome (*S. cerevisiae* shares about 31% of its genome with the human genome), including many human genes whose defects cause diseases (105, 106). Consequently, yeast has been used for many years as a model for the study of certain human diseases and mammalian pathways (107, 108).

- *S. cerevisiae* has a relatively small genome that was the first eukaryotic genome to be completely sequenced (109). Information gathered on the function of its 6466 open reading frames (ORFs) and protein products was collected in several, usually public, databases including SGD (SGD; www.yeastgenome.org). These databases contain notably new knowledge on: (i) the transcriptional output of wild-type or mutant cells in response to different growth conditions, obtained via genome-scale DNA microarrays (61, 110); (ii) synthetic lethal interactions (111, 112); (iii) suppressors interactions, in which a mutant is lethal, except when combined with a second mutation or overexpression of a protein (113); and (iv) protein-protein interactions (114). Thus, in the post-genomic era, yeast, which has been in the vanguard of genomics (*i.e.*, DNA analysis), is becoming a useful tool for large-throughput analyses (108, 115-118).

I.1.2) The life cycle of *S. cerevisiae*

In yeast, as in other sexual microorganisms, the complete sexual cycle is present but is much simpler than in higher plants and animals. Permanent differentiation between germ line and somatic cells does not occur. Haploid cells have either one of two mating types named a and α. Cells of the α type secrete the α-pheromone or α-factor (119), a small peptide molecule, which is sensed by a cells via the α-receptor (Ste2) and, conversely, cells of the a type produce the a-pheromone, which is sensed by the Ste3 receptor of α cells (120). When haploid yeast cells sense the pheromone of the opposite mating type, they become

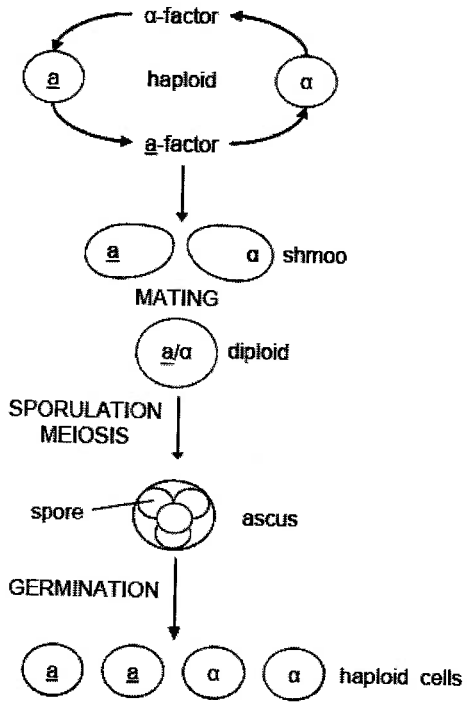


Fig. I.1 Yeast life cycle

Mating of a and α cells leads to diploid/zygote formation. The diploid can undergo meiosis to form an ascus containing four haploid spores. Adapted from (1).

elongated and form a projection called "shmoo", polarised toward their mating partner (121). Cells of opposite mating types that are in contact or close proximity, eventually join at the surface of the shmoo, fuse together and fuse their nuclei to form a diploid cell, the zygote. This process is called mating (for a review see (1)). The resulting diploid cells can proliferate by mitosis or undergo meiosis. During meiosis, the nucleus divides twice following a single replication of the chromosomes yielding four haploid spores contained in a sac-like structure named ascus. The process of meiosis and spore formation is called sporulation (1). It should be noted that in nature yeasts are almost exclusively found in the diploid state and that spore formation is a mean for yeast to survive during adverse conditions (Fig. I.1).

I.1.3) The yeast cell cycle

During vegetative growth, diploid and haploid yeast cells proliferate by mitosis. However, under certain environmental conditions yeast cells can abandon proliferation. The cell cycle in simple yeast is very similar to the cell cycle in humans and is commonly broken down into the four standard phases: G_1, S, G_2, and M (122) (Fig. I.2).

G_1: When the daughter cell breaks away from the mother cell, it is typically smaller than the mother cell. During the G_1 phase, it will grow until it reaches the critical size for entering the cell cycle. The G_1 phase of the cell cycle is important for determining the fate of the cell. Under unfavourable environmental conditions, the cell may enter G_0 (quiescent state) and re-enter the cell cycle when the environmental conditions become more favourable. Under some specific conditions of nutrient starvation G_1 diploid can undergo meiosis. It is again during the G_1 phase that cells of opposite mating type can fuse to form diploids that can then enter the mitotic or meiotic cell cycle (123, 124).

S: Once the cell attains a critical size, it passes the START point and enters the S phase, where it duplicates its DNA. The initiation of DNA replication is closely correlated with bud emergence. However, these two events are not dependent on each other (*i.e.*, if DNA synthesis is blocked, the cell can still bud, and if budding is blocked the cell can still replicate its DNA).

G_2: This relatively short phase defines the time after DNA replication but before mitosis.

M: The cell undergoes mitosis, partitioning DNA between the mother cell and the daughter bud. One difference between budding yeast and most other eukaryotic cells is that the nuclear membrane in budding yeast remains intact. After the DNA has been partitioned, the cell undergoes cytokinesis separating the mother cell from the daughter cell.

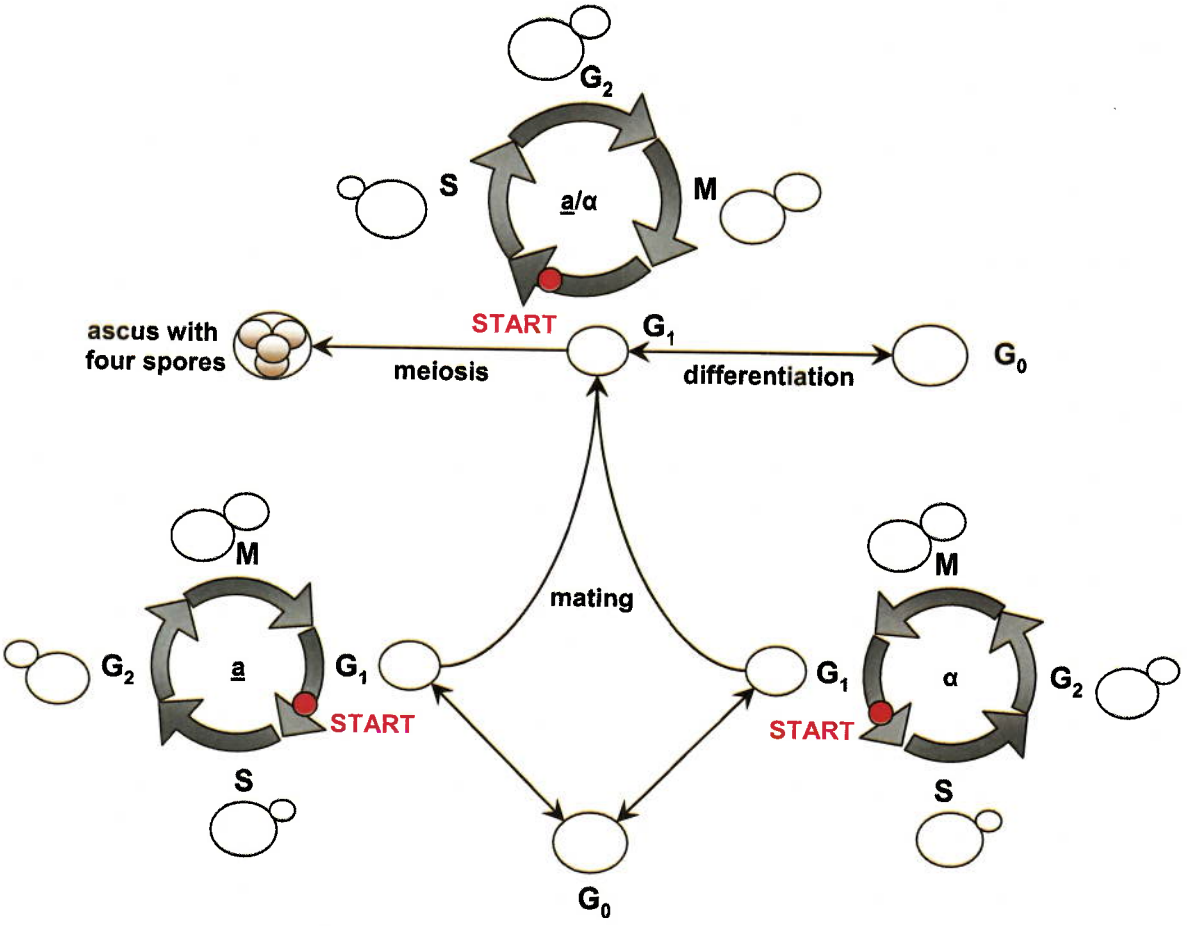


Fig. I.2 The yeast cell cycle

Passage through START initiates another round of the cell cycle. In nutrient-rich conditions and in the presence of cells of the opposite mating type, haploid cells, that are in G_1, can undergo mating and form diploid cells that can proliferate by budding. Starvation for any essential nutrient causes G_1 haploid and diploid cells to enter a non-proliferating state called G_0. Under some specific nutrient conditions, (nitrogen starvation in presence of non-fermentable carbon sources) diploid cells undergo sporulation (see section I.2).
G_1: gap1; S: DNA synthesis; G_2: gap2; M: mitosis; START: G_1/S checkpoint.

The cell cycle in yeast has two checkpoints where the cell commits itself to proceed to the next stage in the cycle. The first point called START occurs near the end of G_1. Passage through START has several requirements including: (i) growth to a critical cell size, (125) (ii) nutrient sufficiency (126), and (iii) attainment of a critical translation rate, three conditions that are likely to be interrelated (127, 128). The critical size requirement and minimum translation rate explain why slowing growth rate increases the length of the G_1 phase, whereas the time required to transit the rest of the cell cycle is relatively constant (127). The second important checkpoint is at the beginning of the M phase when the cell commits itself to chromosomal condensation and the subsequent mitotic steps (11, 13, 129).

I.2) Regulation of cell proliferation by nutrients

Cells of all living organisms are able to sense environmental stimuli and to respond appropriately. Human cells, in the context of the human body, mostly sense growth factors and hormones, while yeast cells sense nutrients present in their environment (8). In *S. cerevisiae*, the availability of nutrients is a major factor that regulates cell proliferation. As unicellular organisms, yeast cells may be exposed to abrupt changes and fluctuations of the external conditions including changes in nutrient availability. Consequently, yeast cells must rapidly adapt by adjusting their internal milieu and their growth rate to the changing environmental conditions. They are able to sense the extracellular information through cell surface receptor and transmit it via signalling pathways to effectors triggering the appropriate cellular differentiation processes. Limiting amounts of certain nutrients causes the cells to reprogram their metabolism for utilisation of any alternative nutrient, while starvation for any of the essential nutrients, including carbon, nitrogen, sulphur and phosphate, causes arrest in G_1 following completion of the ongoing cell cycle (2). Starved cells arrest before the START point and enter G_0 (130). G_0 cells are single, unbudded cells with unreplicated nuclear DNA and exhibit a variety of characteristic traits (see section I.2.5). Proper entry into G_0 is very important for yeast to survive prolonged periods of nutrient starvation. Notably, quiescent cells retain the ability to resume growth when nutrients become available (8, 131).

The following sections will focus on how nutrients (*i.e.,* carbon, nitrogen and phosphate) regulate cell proliferation and developmental programs, and more particularly, on how cells sense and change their metabolism in response to the quantity and the quality of nutrients. The term starvation, used in the next sections will always refer to the complete lack of any essential nutrient, while the term limitation will refer to a nutrient that has become scarce.

I.2.1) Growth phases of a yeast culture in nutrient-rich medium

Under laboratory conditions G_0 arrest is usually achieved by growing haploid or diploid cells to saturation in a glucose-based rich liquid medium (*i.e.,* YPD), under aerobic conditions and at the optimal temperature of 30°C. Within such a culture, cells attain G_0 after 4-7days of growth. Before reaching G_0, cells go through distinct growth phases: the exponential, the diauxic and the post diauxic phases (8).

- During the **exponential phase** of growth, yeast cells grow by fermentation, a process by which cells obtain energy anaerobically through the breakdown of glucose into

CO_2 and ethanol. In yeast, alcoholic fermentation occurs even under aerobic conditions. Typically, cells grow rapidly and divide every 90 min in this phase (132).

- When glucose is depleted, cells transiently stop growth and adapt their metabolism for the following respiratory phase of growth. This period of transition is called the **diauxic phase** (2).

- During the **post-diauxic phase** of growth, yeast cells metabolise the ethanol produced during the exponential phase of growth for use as a carbon and energy source. Cells shift their metabolism from glycolysis and fermentation to gluconeogenesis and respiration. This is a period of slow growth, cells substantially increase the time they spend in G_1 and undergo one or two cell divisions over a period of days (2).

- When ethanol is exhausted, the cell culture enters **stationary-phase**. At that point, most cells are in G_0 (quiescent) and there is no further increase in cell number (2, 8) (Fig. I.3).

Thus, in a YPD batch culture, yeast cells are believed to enter G_0 as a result of carbon starvation. However, yeast cells growing exponentially in a glucose-containing medium can also enter into G_0-like states when rapidly starved for other key nutrients like nitrogen, phosphate or sulphur (8, 133).

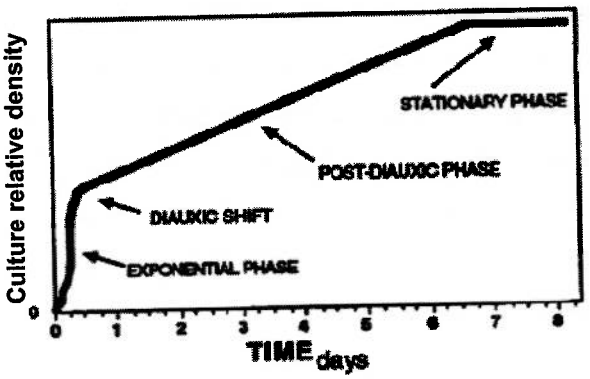


Fig. I.3 Typical growth curve of the yeast *S. cerevisiae* when grown on nutrient-rich medium
Adapted from (2).

I.2.2) Key nutrients in yeast: Carbon metabolism

Yeast can distinguish between the qualities of different carbon sources. Glucose is the most important energy and carbon source for yeast. It is also the preferred one, probably because it can be rapidly converted to ethanol, which inhibits the growth of competing microorganisms (134-136). Growth on glucose is faster than growth on other carbon sources, but a wide variety of other carbon sources can sustain growth of yeast cells (137, 138). These carbon sources are separated in two groups: the fermentable and the non-fermentable carbon sources.

I.2.2.1) Metabolism of the different carbon sources

All fermentable carbon sources are sugars. Glucose and fructose are the two preferred carbon sources (15, 134, 139). These two simple sugars enter directly the glycolytic pathway after phosphorylation by hexokinases[1] (Hxk1, 2 and Glk1), which results in glucose-6-phosphate and fructose-6-phosphate, respectively. However, yeast can consume a wide variety of additional sugars such as galactose, mannose, maltose, raffinose and sucrose. Galactose and mannose are converted into glucose-6-phosphate and fructose-6-phosphate, respectively, before entering the glycolytic pathway, while disaccharides (*e.g.*, maltose and sucrose) and trisaccharides (*e.g.*, raffinose) are first cleaved by specific glycosidases into the monosaccharides, glucose, fructose and/or galactose before entering the glycolytic pathway (Fig. I.4). These sugars sustain different growth rates. Fermentable sugars are processed through the glycolytic pathway into pyruvate and energy in the form of ATP. During fermentative growth, pyruvate is then converted into ethanol and CO_2 (138).

Non-fermentable carbon sources include glycerol, ethanol, acetate and lactate (140). They are also referred to as poor carbon sources as they sustain only a slow growth rate. These carbon sources are exclusively metabolised by respiration under aerobic conditions. Pyruvate is converted into acetyl-coenzyme A (acetyl-CoA), which enters the mitochondrial tricarboxylic acid cycle (TCA) for final conversion into two molecules of CO_2. This cycle releases four energy-rich molecules (3 NADH and 1 $FADH_2$) which are converted into ATP, by a process called oxidative phosphorylation (138). Respiratory cells also undergo gluconeogenesis (more or less the reverse process of glycolysis) to synthesise glucose-6-

[1] Kinase: protein that catalyses the transfer of phosphate from a donor, usually ATP (or other nucleotides), to the hydroxyl group (-OH) of particular serine, threonine or tyrosine residue in another protein or set of proteins called substrate(s).

phosphate, a key component for biosynthesis of macromolecules. Thus, growth on non-fermentable carbon sources requires fully functional mitochondria, for respiration, as well as a fully functional gluconeogenic pathway (17, 141). Most of the non-fermentable carbon sources enter the TCA cycle at the level of pyruvate and acetyl-CoA and hence bypass the glycolytic pathway (142) (Fig. I.4).

I.2.2.3) Regulation of carbon metabolism

Glucose and fructose being the two preferred, high quality carbon sources, their presence prevents the utilisation of other carbon sources (15, 25). This regulation takes place mainly at the level of gene expression and is named catabolite repression (17, 137, 142, 143). Genes under catabolite repression (*i.e.*, that are repressed during growth on glucose and fructose) code for:

- proteins involved in glucose biosynthesis (gluconeogenesis) (*e.g.*, Fbp1, Pck1) (16, 144).
- mitochondrial enzymes involved in the TCA cycle and respiration (16).
- proteins involved in the uptake and metabolism of alternative carbon sources such as galactose and maltose (16, 144).

Catabolite repression affects mainly transcription, but in a few case it also affects the stability of some corresponding mRNAs (17). The degree of catabolite repression depends on the sugar present in the medium: it is maximal in glucose and fructose-grown cells, while cells are completely derepressed when grown on a non-fermentable carbon source (*e.g.*, after the diauxic phase) (15, 138). Accordingly, cells grown on galactose, maltose and sucrose, present an intermediate phenotype with respect to catabolite repression (145). For assimilation of these sugars, cells have to derepress, and in some cases activate, the genes encoding the enzymes required for their corresponding metabolism (17). For example the *GAL* genes (*GAL1, 2, 7, 10* and *MEL1*) and the *MAL* genes (*MAL1, 2, 3, 4, 6*), encode enzymes required to metabolise galactose and maltose, respectively. These genes are relieved from glucose repression when cells are grown on galactose (*GAL* genes) or maltose (*MAL* genes) containing-medium. Derepressed *GAL* or *MAL* genes are additionally induced by Gal4 (146, 147) or Mal63 (148), respectively, two transcription factors activated by galactose (for Gal4) or maltose (for Mal63). However, glucose repression, which is mediated by the repressor Mig1, is stronger than Gal4- or Mal63-mediated transcriptional activation. Consequently, in the presence of glucose in the growth medium, galactose or maltose fail to induce the *GAL* or *MAL* genes, respectively (15, 17, 148). *SUC2*, the gene required for

metabolising sucrose, is also derepressed when glucose is removed from the medium, but, in contrary to the *GAL* and *MAL* genes, there is no evidence for its transcriptional activation by sucrose (148). The intermediate phenotype conferred by these sugars is reflected by the fact that cells grown on either galactose, maltose or sucrose keep genes encoding the mitochondrial and gluconeogenic enzymes at least in part repressed, in order to sustain fermentative growth and to avoid futile cycling between catabolic and anabolic reactions (17, 145, 149-152) (Fig. I.4).

Another phenomenon called carbon catabolite inactivation is responsible for inactivation and degradation of many enzymes (*e.g.*, Fbp1, Pck1) and sugar transporters (*e.g.*, Gal2) following glucose addition to cells growing in a non-fermentable or in a non-preferred carbon source (5). This involves notably phosphorylation of these protein, and their targeting to the proteasome (Fbp1) or the vacuole (Gal2) for degradation (153) (Fig. I.4).

I.2.2.4) Glucose sensing systems

Besides being a carbon source, glucose also serves as an important signalling molecule (5, 16). Yeast cells possess different systems to sense the glucose present in their environment. Glucose levels can be monitored through membrane proteins able to bind extracellular glucose and to generate an intracellular signal and/or through glucose internalisation by glucose transporters and phosphorylation by the hexokinases (154).

- The two glucose transporters homologues, Snf3 and Rtg2, act as glucose receptors and activation of these proteins, by glucose binding, results in the induction of glucose transporter gene expression (*HXT* genes) (155, 156).

- Intracellularly, glucose can be sensed by the hexokinases (Hxk1, 2 and Glk1). Hxk2 is particularly important for signalling glucose repression and is required for the adaptation of cells to fermentative growth (134, 156). For most of the regulatory effects of glucose on yeast cells, both, transport and phosphorylation of the sugar are required.

- The **G-p**rotein **c**oupled **r**eceptor (GPCR) Gpr1 is required to sense extracellular glucose and is involved in the glucose-induced increase in intracellular cAMP, required for the adaptation to fermentative growth. This GPCR system is composed of Gpr1 and its Gα protein Gpa2, which transduces the signal, and of the **r**egulator of **G**-protein **s**ignalling, Rgs2. (31, 134).

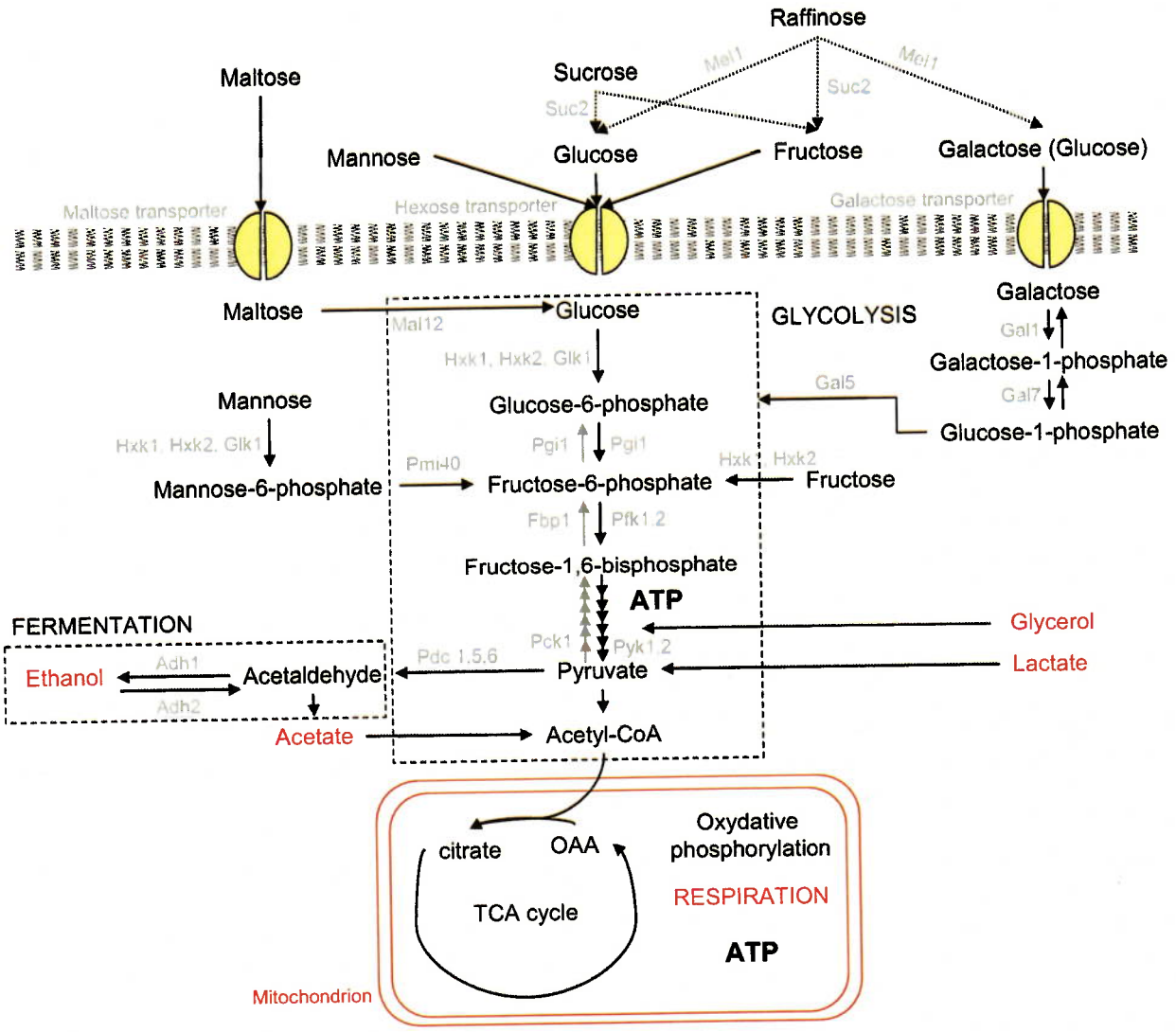


Fig. I.4 Fermentable (black) and non-fermentable (red) sugars enter into the glycolytic pathway

Mannose, glucose and fructose enter the cells through hexose transporters (Hxt), galactose through the galactose transporter (Gal2) and maltose through the maltose transporter (Mal11). Glucose can also enter cells through Gal2. The disaccharide sucrose is hydrolysed into fructose and glucose by the secreted enzyme invertase (Suc2) and the trisaccharide raffinose is decomposed into galactose and fructose through reactions that involve invertase (Suc2) and galactosidase (Mel1). The disaccharide maltose first enters yeast cells and is then converted into two glucose molecules by maltase (Mal12). These fermentable sugars then enter the glycolytic pathway at the level of glucose 6-phosphate and fructose 6-phosphate.
GLYCOLYSIS (dashed box) leads to energy production in the form of ATP and to degradation of pyruvate into acetaldehyde and finally ethanol (a non-fermentable carbon source). This process is called FERMENTATION, as it does not require oxygen. During RESPIRATION (red) the non-fermentable carbon sources ethanol, acetate, lactate and glycerol are converted to acetyl-CoA, which enters the mitochondrial ticarboxylic acid cycle for respiration and energy production (ATP).
Adh1, 2: alcohol dehydrogenase 1 and 2; Fbp1: fructose 1,6-bisphosphatase (gluconeogenesis); Gal1: galactokinase; Gal5: phosphoglucomutase; Gal7: galactose1-phosphate uridyl transferase; Glk1: glucokinase1; Hxk1, 2: hexokinase 1 and 2; OAA: oxaloacetate; Pck1:phosphoenol pyruvate carboxykinase (gluconeogenesis); Pdc1, 5, 6: pyruvate decarboxylase; Pfk1, 2: phosphofructokinase (glycolysis); Pgi1: phosphoglucose isomerase; Pmi40: phosphomannose isomerise; Pyk1, 2: pyruvate kinase 1 and 2; TCA cycle: tricarboxylic acid cycle; OAA: oxaloacetate.

I.2.3) Key nutrients in yeast: Nitrogen metabolism

Nitrogen starvation or nitrogen limitation is able to trigger different developmental responses according to the cell type and to the environmental cues. Yeast cells can also discriminate between the qualities of various nitrogen sources, which in turn trigger different metabolic responses. This latest distinction is tightly linked to nitrogen metabolism. In diploid cells, nitrogen starvation in the presence of a non-fermentable carbon source induces sporulation (1). Interestingly, spores share some common features with G_0 cells in that they can survive long period of nutrient starvation (14). Nitrogen limitation in the presence of a good carbon source, such as glucose, may stimulate a pseudohyphal growth program. In haploid cells, the opposite, namely glucose starvation in the presence of abundant nitrogen, activates a similar differentiation program called invasive growth (157-160). During pseudohyphal growth, yeasts undergo a dimorphic switch and form chains of elongated cells, the pseudohyphae, which grow away from the colony and have the ability to invade agar (161, 162). This form of growth is thought to be an adaptation that allows *S. cerevisiae* to forage for additional nitrogen compounds. This phenomenon, however, occurs only in the laboratory Σ strain background (163). In all other situations, nitrogen starvation (in presence of glucose) causes cells to arrest and to enter into G_0 (125, 164).

I.2.3.1) Nitrogen assimilation

Nitrogen is a major component of complex macromolecules and the yeast *S. cerevisiae* can obtain it from a wide variety of nitrogen sources including glutamine, asparagine, ammonia, glutamate, proline, allantoin, ornithine, arginine or urea (18). All the nitrogenous compounds are degraded to yield ultimately ammonia and glutamate (Fig. I.6). Ammonia is assimilated via the formation of glutamate and glutamine which are important nitrogen donors in the flow of nitrogen into organic compounds like amino acids and nucleotides (18, 165). Glutamate can be synthesised from α-ketoglutarate, which provides the carbon skeleton, by the glutamate dehydrogenase (Gdh1) or from glutamine by the glutamate synthase (Glt1). Finally, glutamine is synthesised by the glutamine synthase (Gln1) (Fig. I.5). Activities of these different enzymes and transcription of their encoding genes vary depending on what nitrogen and carbon source are present in the medium (165, 166). For example, during growth in an ammonia- and glucose-rich medium Gdh1 is preferentially used for glutamate production, while in a glucose-limited ammonia-rich medium, Glt1 is preferentially used to produce glutamate (166) (Fig. I.5). The four enzymes (Gdh1, Gdh2, Gln1 and Glt1) involved in these biosynthetic reactions are regulated at the transcriptional level by multiple signals (18). For example, *GLN1* transcription is controlled by at least three regulatory systems: (i)

the nitrogen catabolite repression (NCR also called NDP for nitrogen discrimination pathway) system, which responds to nitrogen quantity and quality; (ii) the general amino acid control system, which responds to amino acid availability and notably controls Gcn4-dependent transcription; and (iii) the HAP system, which responds to quality of the carbon sources (167). This complex and multifactorial mode of regulation ensures appropriate responses to diverse nutrient conditions.

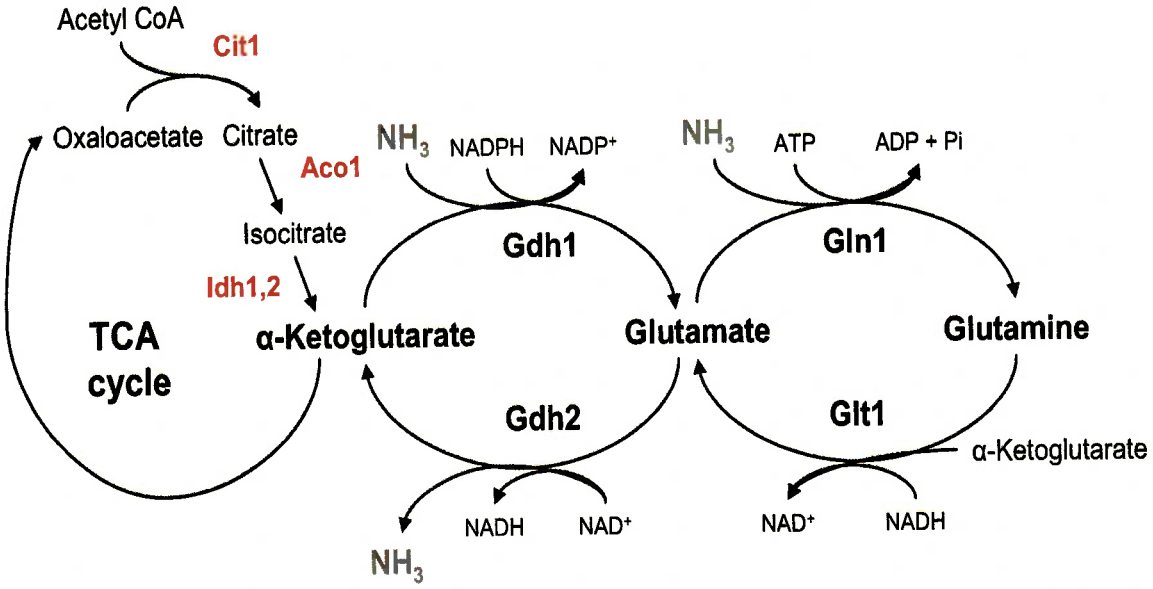


Fig. I.5 Central pathway for nitrogen metabolism

Central nitrogenous compounds, such as amino acids and nucleotides, are synthesised from either glutamate or glutamine. Ammonia (NH_3) is assimilated via its incorporation into glutamate (Gdh1) and glutamine (Gln1). Glutamate is synthesised from glutamine by Glt1 (this reaction results in the production of two glutamate) or from α-ketoglutarate by Gdh2 (this reaction results in the production of one glutamate). Glutamine is synthesised from glutamate by Gln1 and ammonia is released from glutamate by Gdh2.
Aco1: aconitase; Cit1: citrate synthase; Gdh1: $NADP^+$ glutamate dehydrogenase; Gdh2: NAD^+ glutamate dehydrogenase; Gln1: glutamine synthase; Glt1: glutamate synthase; TCA tricarboxylic acid cycle. Cit1, Aco1, Idh1, 2 are genes regulated by Rtg1 and Rtg3 and required for α-ketoglutarate biosynthesis. Figure adapted from (4).

I.2.3.2) Regulation of nitrogen metabolism

Two systems regulate nitrogen metabolism at the transcriptional level: the NCR system and the retrograde (RTG) gene expression system. The nitrogen catabolite inactivation (NCI) system post-transcriptionally regulates some components of the NCR system such as permeases (4, 18, 20).

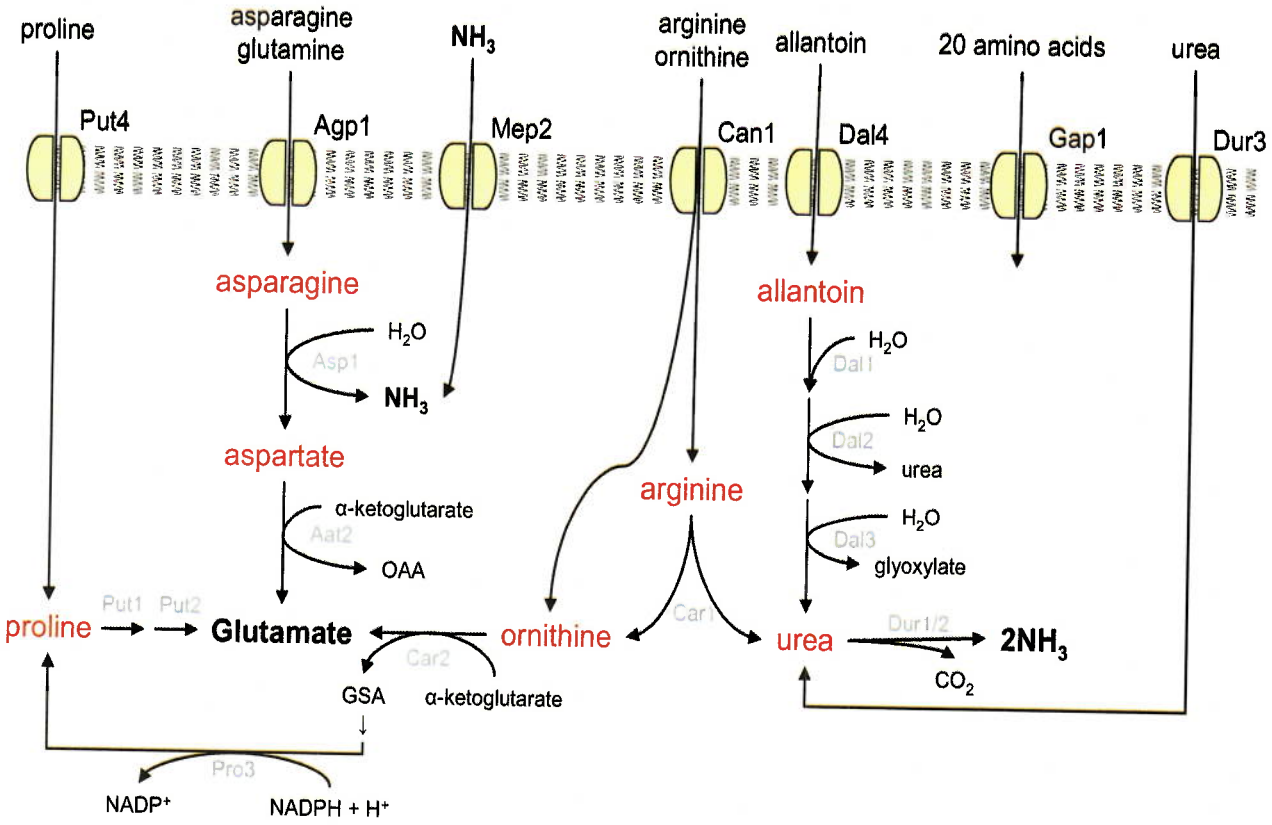


Fig. I.6 Conversion of nitrogen sources into ammonia and glutamate.

The nitrogen sources (red) enter into the cell via different membrane transporters (yellow boxes) to provide ammonia (NH_3) and/or glutamate. These two compounds can be used to synthesise glutamine.
Aat2: aspartate aminotransferase; Agp1: asparagine, glutamine permease; Asp1: cytosolic asparaginase; Can1: arginine permease; Car1: arginase; Car2: ornithine aminotransferase; Dal1: allantoinase; Dal2: allantoicase; Dal3: ureidoglycolate hydrolase; Dal4: allantoin permease; Dur1/2: urea amydolase; Dur3: urea permease; Gap1: general amino acid permease; GSA : glutamate semialdehyde; Mep2: high affinity ammonia permease; NH_3: ammonia; OAA: oxaloacetate; Pro3: pyrroline 5-carboxylate reductase; Put1: proline oxydase; Put2: delta-1-pyrroline-5-carboxylate dehydrogenase; Put4: proline permease; Red: nitrogen sources; Yellow: nitrogen-regulated permeases.

I.2.3.2.1) Transcriptional control: Nitrogen catabolite repression (NCR)

Not all nitrogen sources support growth equally well and certain nitrogenous compounds like glutamine, asparagine and ammonia, are referred to as good nitrogen sources, since they are preferentially used by *S. cerevisiae* and yield higher growth rates than poor/low quality nitrogen sources such as glutamate, proline, allantoin, ornithine, arginine or urea (4, 18). In the presence of good nitrogen sources, yeast cells repress the transcription of genes encoding proteins necessary for the uptake and metabolism of poor nitrogen sources. This response is called nitrogen catabolite repression (NCR), and genes that are repressed under

these conditions are called NCR-responsive genes (168). When cells are shifted from high to low quality nitrogen sources or when good nitrogen sources become limiting, NCR is relieved and NCR-responsive genes are induced by the two transcription factors Gln3 and Gat1 (169-171). This leads to an increased expression of genes encoding the enzymes used for degradation and conversion of the poor nitrogen sources into glutamate and glutamine, such as Put1 and Put2, and of genes encoding a class of amino acids transporters named nitrogen-regulated permeases, which provide the cells with amino acids to be used as a source of nitrogen (4) (Fig. I.6). The nitrogen-regulated permeases comprise among others Gap1, which transports all naturally occurring amino acids, Mep2, the high affinity ammonium permease, and Put4, which transports proline (18, 172) (Fig. I.6). Mep2 might also be required to retain ammonia inside the cells during growth on poor nitrogen sources, which explains its positive regulation by poor nitrogen sources (173).

I.2.3.2.2) Post-transcriptional control: Nitrogen catabolite induction

Once properly expressed, the nitrogen-regulated permeases also have to be activated by nitrogen source-dependent posttranslational modifications (18). On poor nitrogen sources, Npr1, a Ser/Thr kinase, is required for plasma membrane targeting of Gap1 and Put4 and may act either through Gap1 phosphorylation, or inhibition of Gap1 ubiquitylation (4, 174). In contrary, addition of a good nitrogen source to cells grown in proline or urea triggers Gap1 inactivation through its ubiquitylation, which promotes its targeting to the vacuole where it is degraded (175). Interestingly, in a glutamate-containing medium, NCR-responsive genes, including Gap1, are derepressed but Gap1 transport activity is repressed (4).

I.2.3.2.1) Transcriptional control: Retrograde gene expression

The RTG gene expression system couples carbon and nitrogen metabolism and is involved in providing the α-ketoglutarate carbon backbone for glutamate and glutamine synthesis when the TCA cycle is inoperative, which occurs during growth on glucose or following mitochondrial dysfunction (19, 20, 176). This system does not distinguish between good and poor nitrogen sources but, instead, it seems to distinguish between the capacity of a nitrogen source to generate glutamate or ammonia when degraded (21) (Fig. I.6). Indeed, growth on ammonia generally activates RTG-target gene transcription while growth on glutamate and glutamine generally represses RTG-target gene transcription. However, the exact nature of the metabolic signal(s) that regulate(s) RTG-target gene expression is still a matter of debate (21, 95). RTG-target genes encode key enzymes of the TCA cycle necessary for α-ketoglutarate production, including Cit1, Aco1 and Idh1/2, and enzymes of the glyoxylate

cycle such as Cit2. Their transcription is positively regulated by the complex formed by the two transcription factors Rtg1 and Rtg3 (19, 20) (Fig. I.5).

I.2.3.3) Nitrogen sensing systems

S. cerevisiae possesses three sensors of extracellular nitrogenous compounds namely the amino acid SPS sensor system, Mep2 which senses ammonia, and Gap1, which senses amino acids (177).

The trimeric SPS sensor system, composed of **S**sy1, **P**tr3 and **S**sy5, is located at the plasma membrane where it senses the presence of amino acids, including possibly glutamate (177-179). Its activity is required for: (i) proper localisation of basic amino acids in the vacuole (178, 180); (ii) transcriptional induction of genes encoding a subset of permeases including the tryptophan (Tat2) and the glutamine (Gnp1) permeases (177, 181-183); and (iii) full repression of *GAP1* transcription in cells grown in ammonia-based rich media (178, 182).

Mep2, the high affinity ammonium permease, may act, in addition to its role in ammonium ion uptake, as an ammonium sensor that detects ammonium limitation and generates a signal for the induction of pseudohyphal growth (177, 184, 185). Mep2 is also probably implicated in signalling the presence of ammonium in ammonium-starved cells (159, 184). Similarly, Gap1 has recently been suggested to act as an amino acid sensor to signal the presence of nitrogenous amino acids in amino acid starved cells (184, 186)

I.2.4) Key nutrients in yeast: Phosphate metabolism

Inorganic phosphate (Pi) is an essential nutrient for all organisms. It is used in the biosynthesis of many cellular components such as nucleic acids, proteins, lipids, and sugars and it is also required for energy metabolism and signal transduction. *S. cerevisiae* preferentially utilise inorganic phosphate (Pi) as the phosphate source. When Pi becomes limiting, yeast cells first derepress the PHO (phosphate) pathway, a genetic regulatory circuit, which allows adaptation to the low Pi condition notably by favouring transcription of genes encoding proteins used to scavenge phosphate from the environment. However, if starvation persists, cells arrest growth and cell division and enter G_0 (187).

I.2.4.1) The PHO pathway: Control of phosphate metabolism and transport

Similarly to glucose repression, high levels of inorganic phosphate repress transcription of the *PHO* genes. These genes encode proteins involved notably in the utilisation of the poorer phosphate sources (organic phosphate), and include the secreted acid phosphatases (Pho5, Pho11 and Pho12) and the vacuolar alkaline phosphatase (Pho8). *PHO* genes encode also proteins involved in the uptake of Pi from outside the cells, such as the high affinity Pi permeases Pho84 (23, 188). In accordance with the fact that they are secreted, acid phosphatases are probably responsible for retrieving Pi from extracellular sources of organic phosphate, while Pho8 retrieves phosphate from intracellular products (23). The expression of the *PHO* genes is negatively regulated by the cyclin-dependent kinase complex (CDK) Pho80-Pho85. Activated Pho80-Pho85 phosphorylates the transcription factor Pho4, reducing, both, Pho4 nuclear localisation and its association with the co-activator Pho2, and consequently inhibits the transcriptional activity of Pho4 (187, 189). When Pi becomes limiting, the Pho80-Pho85 complex is inhibited by Pho81 leading to a hypophosphorylated form of Pho4 which preferentially localises in the nucleus. Once in the nucleus, Pho4 activates together with Pho2 transcription of *PHO* genes (189, 190). The way Pho81 is activated has not been determined yet.
Pi availability not only controls *PHO84* transcription but it also controls plasma membrane activity of the Pho84, which is highest upon Pi-limitation (189). Under conditions of high Pi, or following complete Pi starvation, Pho84 is targeted to the vacuole for degradation (188).

I.2.4.2) Phosphate sensing systems

Little is known about how cells sense the environmental Pi level. It has been proposed that Pho84, similarly to the mechanism described previously for Mep2, acts as a Pi sensor to trigger the rapid metabolic changes that occur following addition of Pi to phosphate-starved cells, including rapid targeting of Pho84 to the vacuole (184, 191). It has also been suggested that an intracellular sensing system may also occur to initiate the PHO pathway. This system may be driven by Pho81, as it plays a central role in the regulation of this pathway (188, 192).

I.2.5) Quiescent cells and their characteristics

As described above, key nutrients have regulatory effects on nutrient transport, nutrient metabolism, cell development and cell growth. Starvation of any of these nutrients affects cell

growth and developmental programs by triggering entry into G_0, which is an out-of-cell-cycle phase distinct from G_1. The distinction between G_0 and a prolonged G_1 is based notably on the discovery of a conditional yeast mutant, *gcs1*, which is defective in restarting proliferation from quiescence at restrictive temperature, but which does not otherwise exhibit defects in the mitotic cell cycle (2, 130). Yeast cells entering G_0 display the following characteristics that differentiate them from proliferating cells:

- The cell population is predominantly unbudded.
- Cells display a high level of storage carbohydrates such as glycogen and trehalose. Trehalose, in addition to its role as a reserve carbon source, acts also as a stress protectant in yeast. It displays potent protective effects against denaturation of proteins and stabilises the membrane (193). In contrary to trehalose synthesis that starts at the diauxic transition, glycogen synthesis starts earlier during exponential phase, when about half of the glucose has been consumed (193, 194).
- Cells have a dramatically reduced transcription rate. More precisely, polymerase(I) (Pol(I)) and polymerase(III) (Pol(III)) transcription of the rRNAs and tRNAs genes is repressed while the transcriptional pattern of Pol(II), which transcribes mRNAs, changes (195, 196). Indeed, under starvation conditions, Pol(II) mediates transcription of a subset of genes containing in their promoter a **s**tress **r**esponse **e**lement (STRE) or a **p**ost-**d**iauxic **s**hift element (PDS), while at the same time Pol(II)-dependent transcription of ribosomal protein genes is repressed (8, 14, 144, 197, 198).
- Cells have a decreased protein synthesis rate which is less than 0.3% of the rate found in exponentially growing cells (199). Starvation conditions cause a decrease in cap-dependent translation initiation, notably through phosphorylation and inhibition of the eukaryotic translation initiation factor two, eIF2, on its α subunit (90, 200). In return, the reduced translation rate favours translation of some G_0 specific proteins such as Gcn4 (14, 90).
- Cells have thickened cell walls resistant to cell wall degrading enzymes such as zymolyase (14).
- Cells can retain viability without added nutrients.
- Cells are resistant to stress in general and to heat in particular. They can survive potentially lethal temperature (50 to 57°C) for quite some time (2).
- Cells have electron-dense vacuole due to macroautophagy induction. Macroautophagy denotes the formation of double membrane vesicles

(autophagosomes) around constituents of cytoplasm or organelles and their delivery to the vacuole for degradation and recycling (82, 201, 202).

- Cells have chromosomes in the condensed state, which reflects the low transcription rate (2).

Acquisition of many of these characteristics is not necessarily unique to G_0 cells. These characteristics may also be observed in cells that have experienced certain stresses different from nutrient starvation such as, for instance, an abrupt elevation of the growth temperature (heat shock) (130). Consequently, only the induction of the entire set of the above listed characteristics is diagnostic of a G_0 arrested cell. It has to be noted also that post-diauxic cells have already acquired many characteristics of the G_0 cells (138). For example, a first decrease in the protein synthesis rate occurs at the diauxic shift before stationary phase (14) and transcriptional induction of STRE-controlled genes such as *HSP12* and *HSP26* also starts during the diauxic phase and persists for at least the early stages of quiescence (144). However, in contrary to G_0 cells, the post-diauxic cell population continues slowly to proliferate following its arrest at the diauxic transition, indicating that acquisition of the G_0 metabolic changes are not simply the result of the growth arrest but are ultimately the result of changes in nutrient availability. Finally, entry into G_0 can be characterised by induction of quiescence-specific transcriptional pattern (89, 203).

I.3) Nutrient-regulated pathways controlling entry into G_0

At least three major signalling pathways regulate the differentiation program, which leads to the acquisition of G_0 properties: the Ras-regulated cAMP-dependent **p**rotein **k**inase **A** (PKA), the **s**ucrose **n**on-**f**ermenting (Snf1) and the **t**arget **o**f **r**apamycin (TOR) pathways. While conceptually, the TOR and PKA pathways act to repress entry into G_0, Snf1 acts to promote acquisition of G_0 characteristics (8). Accordingly, TOR and PKA pathways are downregulated and the Snf1 pathway is upregulated at the diauxic transition (4). These three pathways control G_0 entry, notably by modulating cell growth and cell metabolism in response to nutrient availability (5).

The following sections will give an overview of these three signal transduction pathways, focussing particularly on the PKA and TOR pathways. Their upstream and downstream components will be described and the way the two pathways may crosstalk to allow proper acquisition of G_0 traits will be discussed.

I.3.1) The SNF1 pathway

The Snf1 pathway has been implicated in the phenomenon of catabolite derepression that occurs following glucose limitation and/or depletion (204). Activation of this pathway is required for aerobic growth on non-fermentable carbon sources (*i.e.*, the postdiauxic growth), is also essential for growth on non-preferred sugars (140, 205) and is required for proper entry into G_0 (8). Accordingly, *snf1Δ* mutants, which cannot utilise alternative carbon sources such as ethanol and glycerol, die soon after the diauxic transition (24).

I.3.1.1) Components of the Snf1 pathway

Snf1 is a serine/threonine (Ser/Thr) protein kinase that is structurally and functionally related to mammalian AMP-activated protein kinase (206). Snf1 is found in three different heterotrimeric complexes containing, in addition to Snf1, the activating γ subunit Snf4 and one of the three scaffolding β subunits Sip1, Sip2, Gal83. When glucose levels become limiting, Snf1 is phosphorylated at residue Thr210 of its activation loop probably by the Pak1, Elm1 and Tos3 kinases, and activated (207-209). Snf4 also participates in activation of the Snf1 kinase through direct interaction with the Snf1 regulatory domain, but this mechanism is not sufficient to activate Snf1 by itself (208). Upon glucose addition, Snf1 is dephosphorylated and inactivated by the Reg1-Glc7 protein phosphatase complex (210). The nature of the glucose signal that activates Snf1 complexes is still unknown (134). It has

been suggested that increased AMP/ATP ratio, resulting from glucose depletion, might activate the Snf1 kinase (204). Alternatively, the glucose kinase Hxk2, which is required to maintain glucose repression during growth on glucose, may negatively regulate Snf1 by stimulating the ability of the Reg1-Glc7 module to promote the return of the Snf1 complex to an inhibited state (134, 211, 212).

I.3.1.2) Snf1-regulated cellular processes

Snf1 regulates the expression of genes that are repressed in glucose-containing media, notably by inhibiting the transcriptional repressor Mig1 and by stimulating the transcriptional activators of gluconeogenic genes Cat8 and Sip4 (143, 152, 213, 214). Snf1-mediated Mig1 inhibition derepresses: (i) genes regulated by the Hap2, 3, 4, 5 complex (required for mitochondrial function); (ii) the *ADH2* gene (required for ethanol consumption); and (iii) genes encoding transcriptional activators such as Gal4, Mal63 and Cat8 (required for transcriptional induction of *GAL*, *MAL* and gluconeogenic genes, respectively) (17, 143, 214-216). Snf1 notably stimulates *CAT8* transcription (via inhibition of Mig1), and may also activate Cat8 transcriptional activity probably trough direct phosphorylation of the protein (140). Finally, Snf1 may also negatively regulate transcription of some glucose-induced genes, including the hexose transporter-encoding *HXT1* (16, 217).

Snf1 promotes acquisition of the characteristics of quiescent cells. It is notably required for proper induction of autophagy, glycogen accumulation and Gln3-dependent transcription following nutrient deprivation (91, 193, 218). Snf1 appears to control glycogen accumulation via posttranslational mechanisms, since strains defective in Snf1 have been shown to have their glycogen synthase (Gsy2) blocked in an inactive dephosphorylated state, while transcription of *GSY2* is only modestly affected in these mutants (219). It should be noted that, besides glucose depletion, the Snf1 pathway can also be activated by other stresses such as heat shock or osmotic stress (210).

I.3.2) The PKA pathway

The PKA pathway is conserved in all eukaryotic cells and is involved in regulating cell growth and/or cell cycle progression, in particular in response to changes in the nutritional status, for yeast cells, or in response to hormonal signal, for mammalian cells (184, 220). This pathway is notably involved in the process of carbon catabolite inactivation previously described (17) (section I.2.2.3). Yeast cells deficient in PKA activity show physiological changes normally

associated with post diauxic or G_0 cells including trehalose and glycogen accumulation and enhanced expression of genes involved in the stress response (8, 221). These observations have led to the concept that the PKA pathway signals the availability of an optimal growth medium.

I.3.2.1) Components of the PKA pathway

The PKA signalling pathway is also called Ras/cyclic AMP (cAMP) pathway. It is notably activated by the fermentable sugar glucose and sucrose, and ultimately positively controls via cAMP the activity of the protein kinase A (PKA) (25, 26, 133, 222). Upstream components of this pathway include two G-protein systems: Ras1 and Ras2, and a G-protein coupled receptor system (GPCR).

Ras1 and Ras1, which are homologous to the human proto-oncogene[2] ras, are two proteins essential for growth (223). As they are redundant, loss of either Ras1 or Ras2 has no effects on growth on glucose (223). Nevertheless, *RAS1* and *RAS2* differ in their transcription rate, *RAS1* being more weakly expressed than *RAS2*. Consequently, most studies focused on the Ras2 protein (16, 224, 225). Ras1 and Ras2 are monomeric guanine nucleotide binding proteins, which are active in their GTP-bound form and inactive in their GDP bound form (226). The Ras-GDP inactive configuration is favoured by the two **G**TPase **a**ctivating **p**roteins (GAPs) Ira1 and Ira2 (30), while the Ras-GTP configuration is favoured by the two **g**uanine nucleotide **e**xchange **f**actors (GEFs) Cdc25 and Sdc25 (29, 227-230). Ira1 and Ira2 enhance the intrinsic GTPase activity of the Ras proteins, which results in hydrolysis of GTP into GDP, and Cdc25 and Sdc25 stimulate the exchange of GDP for GTP. It should be noted that, in contrary to Sdc25, Cdc25 is essential for cell survival and constitutes the major Ras GEF (230). The component(s) upstream of Cdc25 which possibly activate(s) this GEF in response to nutrient have not been identified yet (184) (Fig I.7).

The second G-protein system (GPCR system) involves the seven transmembrane receptor Gpr1, its cytoplasmic associated Gα-like protein, Gpa2, and the Gpa2 GAP, Rgs2 (31, 231-233). This system is specifically involved in glucose and sucrose sensing via Gpr1 (184, 234). Recent studies identified two proteins, Gpb1 and Gpb2, which associate preferentially with Gpa2-GDP and inhibit Gpa2 by preventing its coupling with Gpr1 (235). These two proteins have also been found to associate with and to stabilise Ira1 and Ira2, the two

[2] A gene that, when mutated or otherwise affected, causes a cell to develop into a cancerous tumour cell.

components of the first G-protein system. This stabilisation probably leads to increased Ras-GTP hydrolysis (236). Harashima *et al.* (2006) propose that the glucose signal stimulates GDP-GTP exchange on Gpa2 liberating Gpb1 and Gpb2, which can then interact with Ira1 and Ira2, and attenuate the Ras-GTP-mediated signal on adenylyl cyclase (236) (Fig. I.7).

Both, the GTP-bound Ras proteins and Gpa2 activate adenylyl cyclase (Cyr1/Cdc35), the enzyme which catalyses the synthesis of cAMP from ATP (25, 27, 32, 226, 237). cAMP is an important second messenger that functions exclusively in yeast to activate PKA (238). The PKA, in its inactive conformation, is a heterotetramer consisting of two negative regulatory subunits, encoded by *BCY1*, and two of three possible catalytic subunits, encoded by *TPK1, TPK2* or *TPK3* (33, 239, 240). The catalytic subunits of the PKA have a largely overlapping function in cell viability (239). During growth on glucose, which correlates with an active PKA, the tetramer is primarily located in the nucleus, while it is also cytoplasmic in post-diauxic and G_0 cells (97, 241). cAMP activates PKA by binding to Bcy1 (242): this binding induces a conformational change that causes dissociation of the tetramer into one dimeric regulatory subunit (Bcy1-Bcy1), which remains into the nucleus, and two catalytic subunits, which move to the cytoplasm (97, 243). Once in the cytoplasm, Tpk1, Tpk2 or Tpk3 can phosphorylate target proteins, thereby promoting, ultimately cell growth and cell proliferation (16, 161). The intracellular cAMP level is tightly regulated by the low (Pde1) and high (Pde2) affinity phosphodiesterases, which are responsible for hydrolysis of cAMP into AMP (244-246) (Fig. I.7).

I.3.2.2) Phenotypic properties controlled by the PKA pathway

It is generally accepted that PKA activity is high during exponential growth on glucose, while it is low during post-diauxic growth and in G_0 (34, 54, 133, 184). Accordingly, activation of the PKA pathway stimulates glycolysis, triggers trehalose and glycogen mobilisation, ensures rapid progression through the cell cycle, represses transcription of stress-related and gluconeogenic genes, increases stress sensitivity, and stimulates transcription of growth-related genes such as **r**ibosomal **p**rotein **g**enes (RPGs) (5, 53, 247-249). These traits are characteristics of fast growing cells. In addition, activated PKA pathway affects cell development, inasmuch as it negatively regulates sporulation (250).

Loss of Bcy1 or Ira2 leads to constitutively high activity of the PKA pathway. Cells lacking Bcy1 or Ira2 are constitutively sensitive to heat shock, unable to accumulate glycogen and trehalose, and die rapidly upon glucose depletion at the diauxic transition (8, 248). Thus,

proper downregulation of the PKA pathway is necessary for successful transit to the post-diauxic phase and ultimately entry into G_0. Introduction of a constitutively active *RAS* allele (*RAS2*^{Val19}), which increases Ras2-GTP association, also causes constitutive activation of PKA pathway (16, 27, 251).

Cells with reduced PKA activity behave like nutrient limited cells, even on nutrient-rich medium (8). Accordingly, these mutants, when grown on a nutrient-rich medium, generally exhibit a slow growth phenotype, accumulate storage carbohydrates and acquire heat shock resistance (8). Among mutations that cause low PKA activity, there are notably: (i) the *bcy1*^{Ala145} allele encoding a regulatory subunit with increased affinity for the Tpks (252); (ii) the *ras2Δ* (253); and (iii) the thermosensitive (ts) alleles of *cdc25* and *cdc35* (2, 254). The *bcy*^{Ala145} mutant exhibits also a lower growth rate than wild-type (wt) cells on non-fermentable carbon sources, and enters much earlier into G_0 (8), while *ras2Δ* mutants are completely unable to grow on non-fermentable carbon sources (224, 255). This indicates that a basal level of the PKA pathway is required for growth on non-fermentable carbon sources. Interestingly, the *tpk*^{w} *bcy1Δ* strain, which has a constitutively low level of PKA activity, whatever the cAMP level may be, responds appropriately to nutrient conditions and executes a normal transition to G_0, indicating that cells do not absolutely require signalling through Ras and adenylyl cyclase to enter G_0 (256). It should be also noted that decreased PKA activity (*e.g.*, in *ras2Δ* mutant) increases chronological life span, which denotes the time G_0 cells remain viable in culture (257). Thus, downregulation of PKA activity is important for G_0 survival.

Finally, loss of Ras1 and Ras2 (27), Cdc25 (via gene deletion or inactivation of a thermolabile protein) (254, 258), Cdc35 (via gene deletion or inactivation of a thermolabile protein) (254, 259), or of Tpk1, Tpk2 and Tpk3 together (239) disrupts any activity of the PKA pathway, and is lethal for cells (27, 239, 259, 260). In other words, PKA activity is essential in yeast.

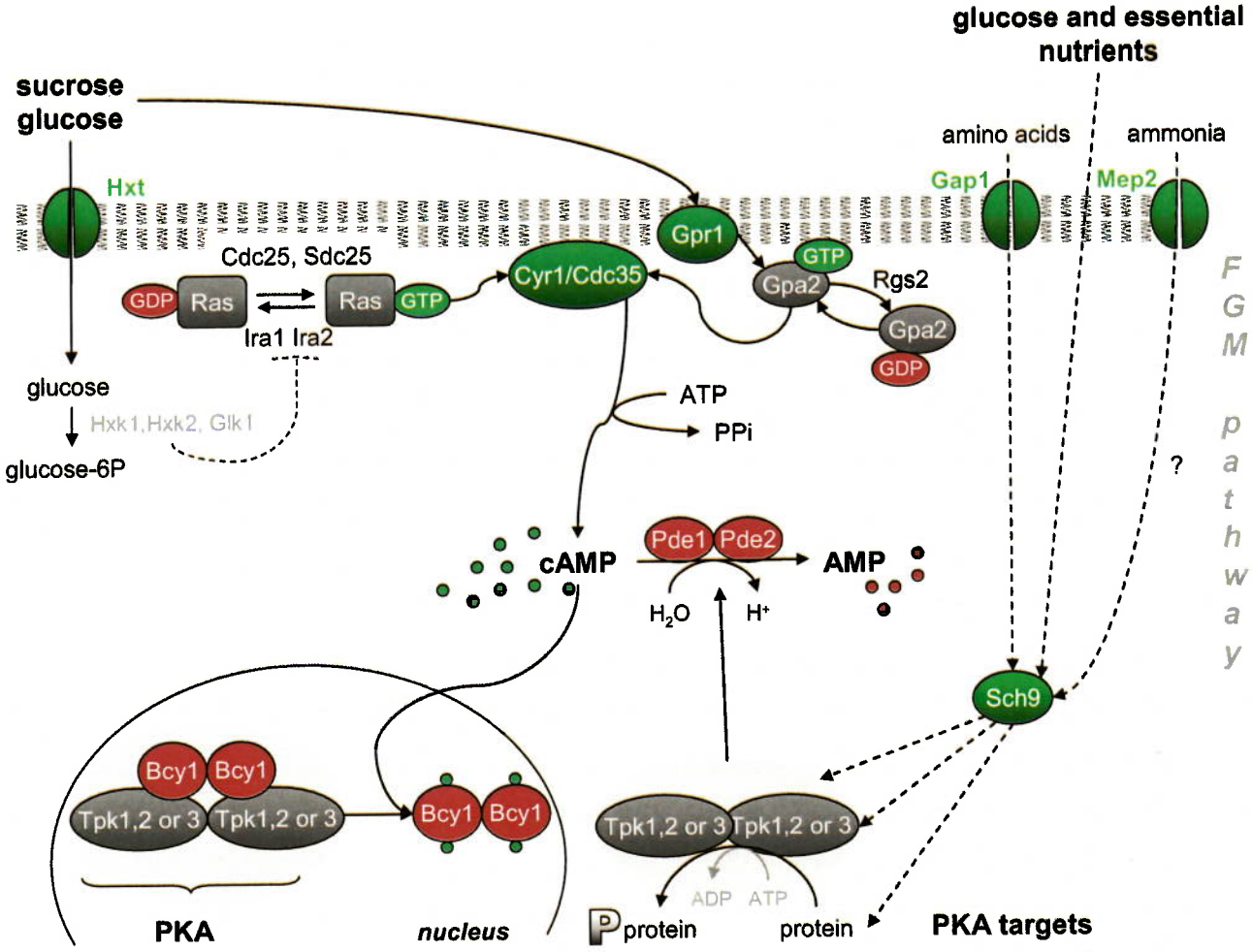


Fig. I.7 Elements of the cAMP- PKA pathway

The small GTPase proteins, Ras1 and Ras2 are responsible for basal activation of the pathway. Ras-GTP bound form activates adenylyl cyclase (Cdc35/Cyr1), which synthesises cyclic AMP (cAMP: green dots). GTP binding to Ras1 and Ras2 is favoured by Cdc25 and Sdc25, the two GTP exchange factors (GEFs). Hydrolysis of GTP into GDP is favoured by Ira1 and Ira2, the two GTPase activating proteins (GAPs). cAMP controls PKA activity by binding to the regulatory subunits, Bcy1, allowing their dissociation from the catalytic subunit (Tpk1, 2 or 3). During growth on glucose, the inactive tetramer and the Bcy1 dimer exclusively localise in the nucleus while the dissociated monomeric Tpks are cytoplasmic. Intracellular cAMP is quickly degraded by the low (Pde1) and high (Pde2) affinity phosphodiesterase. Pde1 is part of the feedback regulation loop involved in quick degradation of the cAMP following glucose activation. Glucose signalling requires: the G-protein-coupled receptor system (GPCR), composed of Gpr1 (receptor), Gpa2 (Gα-protein) and the negative regulator Rgs2, which enhances Gpa2 GTPase activity, and the three sugar kinases Hxk1, Hxk2 and Glk1. The Sch9 kinase is the single component the FGM pathway and is required for nitrogen-induced activation of PKA. The FGM pathway integrates the availability of different nutrients, to maintain high PKA activity during fermentative growth, independently from cAMP. Gap1 and Mep2 are involved in amino acid and ammonia detection, respectively. Green: PKA activators; Red: PKA inhibitor; Dashed arrow: presumable, indirect activation; Dashed bar: presumable, indirect inhibition; P: phosphorylation.

I.3.2.3) Regulation of the PKA pathway by glucose

The most potent activator of cAMP synthesis is glucose (or sucrose). Addition of high concentrations of glucose or sucrose to glucose-deprived cells induces an acute and transient increase in intracellular cAMP levels detectable within 30 seconds after glucose addition (31). cAMP concentration then rapidly declines (in a matter of 2 min.) to a level somewhat higher than in the prestimulated cells (248). This rapid synthesis of cAMP requires the GPCR system (*i.e.,* Gpr1 and Gpa2) for extracellular glucose and sucrose detection, as well as an intracellular glucose sensing mechanism that is dependent on glucose phosphorylation, in a way that is not fully understood (5, 16, 25, 28). Finally, the transient burst in cAMP beyond the basal level results in activation or inhibition of PKA targets, and is required for the rapid adaptation of post-diauxic or G_0 cells to glucose-rich growth conditions (184).

I.3.2.3.1) The extracellular glucose sensing system

The GPCR system is activated by elevated concentration of extracellular glucose (20mM) (25). Addition of glucose or sucrose activates the transmembrane sensor Gpr1, which stimulates in the cytoplasm Gpa2. Gpa2 activates in turn adenylyl cyclase and cAMP synthesis (31). Loss of Gpr1 and/or of Gpr2 renders cells specifically defective in the transient glucose-induced cAMP spike but not in the basal Ras-mediated cAMP synthesis (which is essential for cell viability). Thus, this defect delays, but does not prevent, adaptation of these mutant cells to exponential growth conditions (31, 248).

I.3.2.3.2) The intracellular glucose sensing system

The intracellular glucose sensing system requires glucose transport inside the cell, via Hxt1-7 and/or Gal2, and glucose phosphorylation by the thee hexokinases Hxk1, Hxk2 or Glk1 (16) (Fig. I.4). No further metabolisation of the sugar is required to induce the increase in cAMP level (5, 261) (Fig. I.7). Actually, intracellular glucose sensing may occur through the activity of all three hexokinases, since their catalytic activity seems to correlate with the level of the glucose-induced cAMP burst (5, 16, 25, 26, 262). Accordingly, fructose and maltose, which are both metabolised by Hxk1 and Hxk2, are also activators of this intracellular sensing system (26) (Fig. I.4). However, the exact nature of how hexose kinases, or a corresponding metabolite, impinge on glucose-induced cAMP synthesis remains to be elucidated. Activation of this intracellular sensing system, even by low glucose concentrations, is a prerequisite for the burst in cAMP synthesis, triggered by activation of the glucose/sucrose-induced GPCR system, to occur. This suggests that glucose

phosphorylation is required for rendering adenylyl cyclase somehow responsive to activation by the GPCR system (5, 26, 28, 184). Interestingly, both systems can be experimentally uncoupled (26).

I.3.2.3.3) Connexion between the two glucose-sensing systems

The GPCR system and the intracellular glucose sensing system have been proposed to be connected at the level adenylyl cyclase through Gpa2 and Ras, respectively, which both stimulates this enzyme (5). Accordingly, Ras1 and Ras2 are required to mediate the glucose-induced cAMP signal (222, 263). Moreover, a recent publication demonstrated that glucose-induced cAMP signalling increased the GTP loading of Ras2, in a way that is dependent on glucose phosphorylation but not on the GPCR system, indicating that intracellular glucose signal most probably impinges on Ras (16, 28). This increase has been proposed to involve indirect inhibition of Ira1 and Ira2 by Hxks or Cdc25 activation (28). What is the role of Ras in glucose-mediated cAMP signalling? Ras may be required to increase the responsiveness of adenylyl cyclase to the GPCR system. Interestingly, cells with a $Ras2^{Ser318}$ mutant as the sole Ras protein, display a wild-type basal level of cAMP, but are unable to trigger the glucose-induced cAMP spike (16, 248). This mutation prevents the association of Ras2 with the plasma membrane, suggesting that Ras2 may be required for adenylyl cyclase membrane targeting. Finding adenylyl cyclase at the plasma membrane may, indeed, facilitate its activation by the membranous GPCR system (264). Glucose-induced signalling remains, up to now, the single nutrient-induced signal known to activate cAMP synthesis (5, 248).

I.3.2.3.4) Downregulation of cAMP synthesis

Activation of the PKA pathway by glucose or sucrose is a transient phenomenon required for the rapid adaptation of respiratory or G_0 cells to fermentative growth conditions. Consequently, following glucose or sucrose stimulation, there is a quick inhibition of cAMP production. This feedback mechanism involves inactivation of Cdc25 and possibly Gpb1 and Gpb2-mediated stabilisation of Ira1 and Ira2 (236, 265). PKA itself exerts a strong inhibition on the glucose-induced cAMP level through phosphorylation and activation of the low affinity phosphodiesterase Pde1, which degrades cAMP (54, 133, 266-268) (Fig. I.8). It should be noted that Pde1 is one of the few known *bona fide* PKA substrates and its activity is not required for regulation of basal cAMP. In contrast, Pde2, the high affinity phosphodiesterase, seems to be involved in the control of the basal cAMP level (269). In line with this notion, exponential and nutrient-deprived *pde2Δ* cells exhibit increased basal cAMP levels (269). Finally, PKA has been proposed to exert feedback inhibition on other components of the PKA pathway such as Cdc25 (263), Ras (270) and adenylyl cyclase (271), which may explain the

low cAMP level found in cells unable to degrade cAMP (*pde1Δ pde2Δ*) and the low basal level found in glucose-grown wild-type cells (246).

I.3.2.4) Downstream elements of the PKA-pathway

The main function of the transient burst in cAMP beyond the basal level is to signal to glucose derepressed cells the presence of glucose in the medium through rapid activation of the PKA (248). Despite the rapid decrease in cAMP levels that follows the primary peak, cells probably retain activated PKA throughout fermentative growth as indirectly measured by its effects on different targets (53). Active PKA presumably phosphorylates a variety of proteins involved notably in glucose metabolism (*e.g.,* glycolysis and gluconeogenesis), storage carbohydrate metabolism (*e.g.,* glycogen and trehalose), cell growth (*e.g.,* Rim15), cell cycle progression and transcription (26). However, *bona fide* targets of PKA are still rare. PKA phosphorylates serine (Ser) and threonine (Thr) residues on proteins containing a PKA consensus phosphorylation site R/KR/KXS/T (where X is any amino acid; R is arginine; K is lysine; S is serine and T is threonine) (33).

I.3.2.4.1) PKA-mediated control of sugar metabolism.

PKA positively controls, probably directly, the activity of the enzymes involved in trehalose and glycogen breakdown such as neutral trehalase (Nth1) (40-42, 272) and glycogen phosphorylase (Gph1) (43). Conversely, it inactivates, most probably indirectly, the activity of enzymes involved in trehalose and glycogen synthesis such as the trehalose synthase subunits, Tps1 and Tps2 (273), and the glycogen synthase (Gsy2) (44, 45). Moreover, neutral trehalase activity is often used as a readout for PKA activity (40). PKA activity also negatively controls transcription of *NTH1*, *GPH1*, *TPS1, TPS2* and *GSY2* genes through their STRE elements. Paradoxically, although their corresponding gene products may have opposing effects on trehalose and glycogen levels, all five genes are induced at the diauxic transition (249, 272, 274-276) (For a review see (193)) (Fig. I.8).

PKA positively regulates the glycolytic flux by phosphorylating and activating the pyruvate kinase (Pyk1, Pyk2) and the 6-phosphofructo-2-kinase (Pfk26) (277-279). Pfk26 produces fructose-2,6-bisphosphate which is itself an activator of the glycolytic key enzyme Pfk1 (247). In contrary, PKA inhibits gluconeogenic enzymes such as fructose-1,6-bisphosphatase (Fbp1) and fructose-2,6-bisphospatase (Fbp26), the enzyme involved in fructose-2,6-bisphosphate degradation (280-282). It has to be mentioned that the PKA pathway is not

required for proper transcriptional response of the corresponding glycolytic and gluconeogenic genes except for *PFK26* (283) (Fig. I.4, Fig. I.8).

I.3.2.4.2) PKA-mediated control of the Rim15 protein kinase

Rim15 (**R**egulator of **Ime**2) was first identified as a stimulator of meiotic gene expression, before being implicated by Reinders *et al.* (1998) in nutrient signal transduction (48). Rim15 is a component of the PKA pathway, which is negatively controlled by PKA-dependent phosphorylation. Actually, this protein is a direct downstream target of PKA and it acts to positively control key aspects of the diauxic transition and of entry into G_0 (48). Consequently, loss of Rim15 is associated with phenotypes characteristics of cells with constitutively high PKA activity. Post-diauxic *rim15Δ* cells are defective in glycogen and trehalose accumulation, in transcriptional derepression of STRE- and PDS element-controlled genes (*e.g., HSP12*, *HSP26* and *SSA3*), in the induction of thermotolerance and in proper entry into G_0 (48). Finally, loss of Rim15 suppresses the growth defect of a *tpk1Δ tpk2Δ* and *tpk3Δ* deletion mutant, indicating that Rim15 acts downstream of PKA to negatively regulate cell growth (48, 51). Conversely, overexpression of *RIM15* partially induces acquisition of G_0 characteristics during exponential growth, and, exacerbates the growth defect of mutants with attenuated PKA activity (*e.g., cdc35* at the permissive temperature) (48).

Rim15 is a distant member of the NDR (**n**uclear **D**bf2-**r**elated) family of Ser/Thr kinases, which is a conserved subclass of the AGC group of kinases containing, among others, PKA and Sch9 (49). NDR members are characterised by the presence of an insert, within their kinase domain (*i.e.*, catalytic domain), between subdomains[3] VII and VIII (49). The function of this domain is not well understood, but in the case of the Ndr1 kinase, it has been shown to contain a nuclear localisation signal (49). Rim15 furthermore possesses an amino-terminal PAS (**P**er-**A**rnt-**S**im) domain, which may be involved in the cis-regulation of Rim15 kinase activity in response to nutrient and stress signals (50), a zinc-finger domain (Zn-finger) and a C-terminal **rec**eiver domain (REC) (3, 50, 284). Rim15 has also five consensus sites for PKA phosphorylation: Ser to Ala replacement in these five consensus sites results in a Rim15 protein which is resistant to PKA inhibition (48).

[3] Kinase subdomain: part of the kinase domain, which contains characterised pattern of conserved amino acid residues.

Genetic data indicate that Rim15 acts upstream of the Msn2 and Msn4, and Gis1 transcription factors to positively regulate STRE- and PDS element-controlled genes ((for more information see section: I.*3.2.4.4.*) (50, 51)). The mechanisms by which Rim15 controls the functional activities of these three transcription factors are still unknown and no *bona fide* targets of Rim15 have been identified yet (Fig. I.8) (for a recent review on Rim15 see: (3)).

I.3.2.4.3) PKA-mediated control of cell growth and cell cycle progression

The growth arrest, which occurs upon nutrient depletion or PKA inactivation, is associated with loss of cyclin synthesis (285). **Cyclin** proteins (Cln) are important elements of cell cycle regulation and are notably involved in regulating passage through START (286, 287). The complex Cln3-Cdc28 kinase is thought to play an important role in transcriptional activation of *CLN1* and *CLN2* at the end of G_1. Cln1 and Cln2 proteins, in association with the cyclin-dependent kinase Cdc28, in turn are thought to trigger the downstream events that allow cells to pass through START to initiate a new cell division cycle (288). Hall *et al.* (1998) have demonstrated that Cln3 quantity and Cln3-Cdc28 kinase activity vary according to the growth medium (being highest on glucose) and may be linked to the intracellular level of cAMP (39). Cln3 may consequently represent a direct or indirect putative PKA target, linking glucose activation to progression through START. Evidence for a direct interaction of Cln3 with PKA, however, is missing (39) (Fig. I.8).

PKA also promotes cell growth by positively regulating transcription of ribosomal protein genes (RPGs), including *RPL16, RPL29* and *RPS13,* possibly through direct or indirect modulation of the transcriptional activators Rap1 and Fhl1 (36-38, 289). Notably, recent data indicate that PKA activates Fhl1, which binds to the promoters of the RPGs, possibly via inhibition of the Yak1 kinase (35, 290). Yak1 inhibition is thought to favour interaction, at the promoter, of Fhl1 with its co-activator Ifh1 (35, 291). As for Rap1, it has been proposed to be required for clearing nucleosomes from a segment of chromatin, permitting notably Fhl1 binding and Ifh1 recruitment (291-293). Because Rap1 constitutively binds DNA, PKA does not modulate RPGs transcription by promoting its recruitment to the promoter (36) (Fig. I.8).

I.3.2.4.4) PKA-mediated control of STRE- and PDS element-controlled genes transcription

The PKA pathway negatively regulates transcription of genes containing in their promoter a STRE element, with the consensus core sequence CCCCT or AGGGG (294), or a PDS element, with the consensus core sequence T(T/A)AGGGAT (14, 36, 295, 296). Notably, while the STRE element mediates transcriptional activation in response to multiple stresses, the PDS element mediates transcriptional activation exclusively in response to nutrient

limitation (51). Their downregulation by PKA occurs through repression of their transcriptional activators Msn2, Msn4 and Gis1 (46, 51, 203, 297).

The two transcription factors Msn2 and Msn4 appear to mediate STRE-controlled gene transcription in response to multiple stresses including nutrient starvation (47, 297, 298). STRE-controlled gene regulation is thought to be achieved by modulating Msn2 and Msn4 intracellular localisation and their subsequent binding to STRE elements (47). During growth on glucose, Msn2 and Msn4 are predominantly cytoplasmic, while carbon starvation or transition through the diauxic phase triggers translocation of Msn2/4 into the nucleus and consecutive STRE-controlled gene transcription (47). Intracellular distribution of Msn2/4 and Msn2/4-mediated transcription are directly modulated by PKA: low PKA activity causes nuclear accumulation and activation of the two transcription factors (47). Interestingly, loss of Msn2 and Msn4 rescues the otherwise fatal growth defect of the *tpk1Δ tpk2Δ tpk3Δ* triple mutant, indicating that the two transcription factors act downstream of PKA to antagonise PKA-dependent growth (249). PKA has been demonstrated to phosphorylate, both *in vitro* and *in vivo,* a domain of Msn2 implicated in its import into the nucleus (*i.e.*, the nuclear localisation signal, NLS) (46, 299). PKA-dependent phosphorylation of Msn2 masks the NLS, thereby preventing Msn2 nuclear localisation and consecutive STRE-controlled gene transcription (46, 300). In addition, PKA may also positively regulate and accelerate nuclear export of Msn2 to the cytoplasm (47) (Fig. I.8).

Gis1 is a transcription factor which positively regulates PDS element-driven and STRE-driven gene expression (51). Gis1 is a possible downstream target of the PKA-regulated Ser/Thr protein kinase Rim15 and mediates a subset of the Rim15-controlled responses (51, 301) (Fig. I.8).

I.3.2.4.4) alternative modes of PKA-mediated transcriptional regulation

PKA not only represses transcriptional activators, but also activates transcriptional repressors of G_0-specific genes such as Sok2 and the Pol(II)-associated complex Srb. Sok2, which is a protein important for regulation of cell growth, has been proposed to counteract Msn2 and Msn4 function under conditions of high PKA activity (250, 302, 303). The Srb complex is required for full repression of genes normally induced during G_0 entry, and it has recently been shown to be positively regulated by PKA (304-307). Indeed, PKA-dependent phosphorylation of Srb9, a member of the Srb complex, increases the activity of the complex and thereby possibly represses induction of the G_0-specifics genes (307).

PKA, may also regulate gene expression by affecting the activity of the transcriptional complex Ccr4-Not, notably by inhibiting the protein kinase Yak1 (308-310). Accordingly, Yak1 phosphorylates Pop2, a subunit of the Ccr4/Not complex. How, Pop2 phosphorylation affects the activity of the Ccr4-Not complex and gene transcription is not fully understood at present, but this event is required for proper cell cycle arrest following carbon source depletion (308) (Fig. I.8).

3.2.4.6) PKA-mediated control of autophagy.

PKA has also recently been shown to negatively regulate macroautophagy a process necessary for long term survival of G_0 cells (87, 311, 312). PKA phosphorylates *in vitro,* and probably inhibits *in vivo*, Atg1/Apg1, a protein which regulates the initial stage of macroautophagy (87, 313) (Fig. I.8).

I.3.2.5) The fermentable growth medium-induced pathway (FGM pathway)

The FGM pathway is a hypothetical pathway which has been proposed on the basis of physiological data (54). This pathway has been suggested to act in parallel to the PKA pathway to control nutrient-regulated PKA activity during fermentative growth as well as to control the switch between fermentative and respiratory growth (133, 184). A protein kinase similar to PKA, Sch9, is the unique component of this pathway.

Yeast cells starved for nitrogen, sulphate or inorganic phosphate (on a glucose-containing medium) arrest growth and enter into the G_0 resting state. Addition of a nitrogen source (amino acids or ammonia) to nitrogen-starved cells, addition of sulphate to sulphate-starved cells or addition of phosphate to phosphate-starved cells triggers (like glucose-induced PKA pathway activation) rapid activation of neutral trehalase and transcriptional induction of RPGs (52, 133, 184, 186, 191). In each of these cases, however, the nutrient-induced effects appear not to be associated with an increase in cAMP signal, yet to depend on the presence of the PKA catalytic subunits (186, 191, 314). This apparent lack of correlation between PKA activity and intracellular cAMP levels can also be observed when fermentative glucose-grown cells are compared to respiratory cells growing on a non-fermentable carbon source (315). The former harbour phenotypic properties indicative of high PKA activity, while the latter harbour phenotypic properties associated with low PKA activity (54). However, the cAMP levels are essentially the same under both growth conditions suggesting that the switch to non-fermentative metabolism is probably not regulated by cAMP (315).

Based on these observations, Thevelein *et al.* (1994) proposed the existence of a cAMP-independent pathway that may directly impinge on PKA or on PKA targets (53, 54, 133). This presumed cAMP-independent pathway was named **f**ermentable **g**rowth **m**edium-induced pathway (FGM pathway) since both, a fermentable carbon source (*e.g.,* glucose) and a complete growth medium, containing all essential nutrients (*e.g.,* nitrogen, sulphur and phosphate) are required for maintenance of a phenotype consistent with high PKA activity (316). In that model, adenylyl cyclase activity would be required just for maintenance of the basal cAMP level necessary for cell growth and for rapid adaptation of post diauxic cells or G_0 cells to high glucose conditions (184). The FGM pathway, which is activated by fermentable sugars together with all other essential nutrients, may be required for sustained PKA activity during exponential growth on glucose (53, 54). The FGM pathway is thought to integrate the availability of the different nutrients present in the growth medium notably via the plasma membrane based nutrient sensors that include: Gap1 (for amino acid sensing) (186), Mep2 (for ammonia sensing) (317) and Pho84 for (phosphate sensing) (191).

The protein kinase Sch9, which is homologous to the mammalian PKB/Akt protein, has been proposed to be the central player of the FGM pathway, because its presence is required for the nitrogen-induced (amino acid) increase in PKA activity (54, 55). However, phosphate activation of PKA targets occurs independently of Sch9, indicating the presence of additional Sch9-independent components (55, 191). Sch9 is a Ser/Thr kinase, which belongs, like PKA, to the AGC kinase family (c**A**MP-, c**G**MP-dependent protein kinases and protein kinase **C**). Given the fact that Sch9 is structurally related to PKA (318), it would not be surprising that both kinase share some targets (318). However, no direct substrate for Sch9 has been identified yet (319). Sch9 genetically interacts with PKA, since Sch9 overexpression suppresses the growth defect of cells with a low PKA phenotype, while, conversely, the slow growth phenotype associated with loss of Sch9 can be suppressed by enhanced PKA activity (318, 319). In addition, *sch9Δ* is synthetically lethal with *gpr1Δ*, *gpa2Δ* or *ras2Δ* mutations, indicating that it probably regulates cell growth in a pathway parallel to the PKA pathway (31, 160). However, the precise relationship between PKA and Sch9 has not been established yet and Sch9 may act either directly on the PKA subunits or on common downstream target(s) (53, 314). Indeed, beyond genetic interactions, no direct interaction between PKA and Sch9 has been shown yet. Finally, interaction of Sch9 with another nutrient regulated pathway, the TOR pathway, is not excluded since the mammalian PKB/Akt protein is a direct downstream target of the mammalian TOR pathway (mTOR) (320, 321).

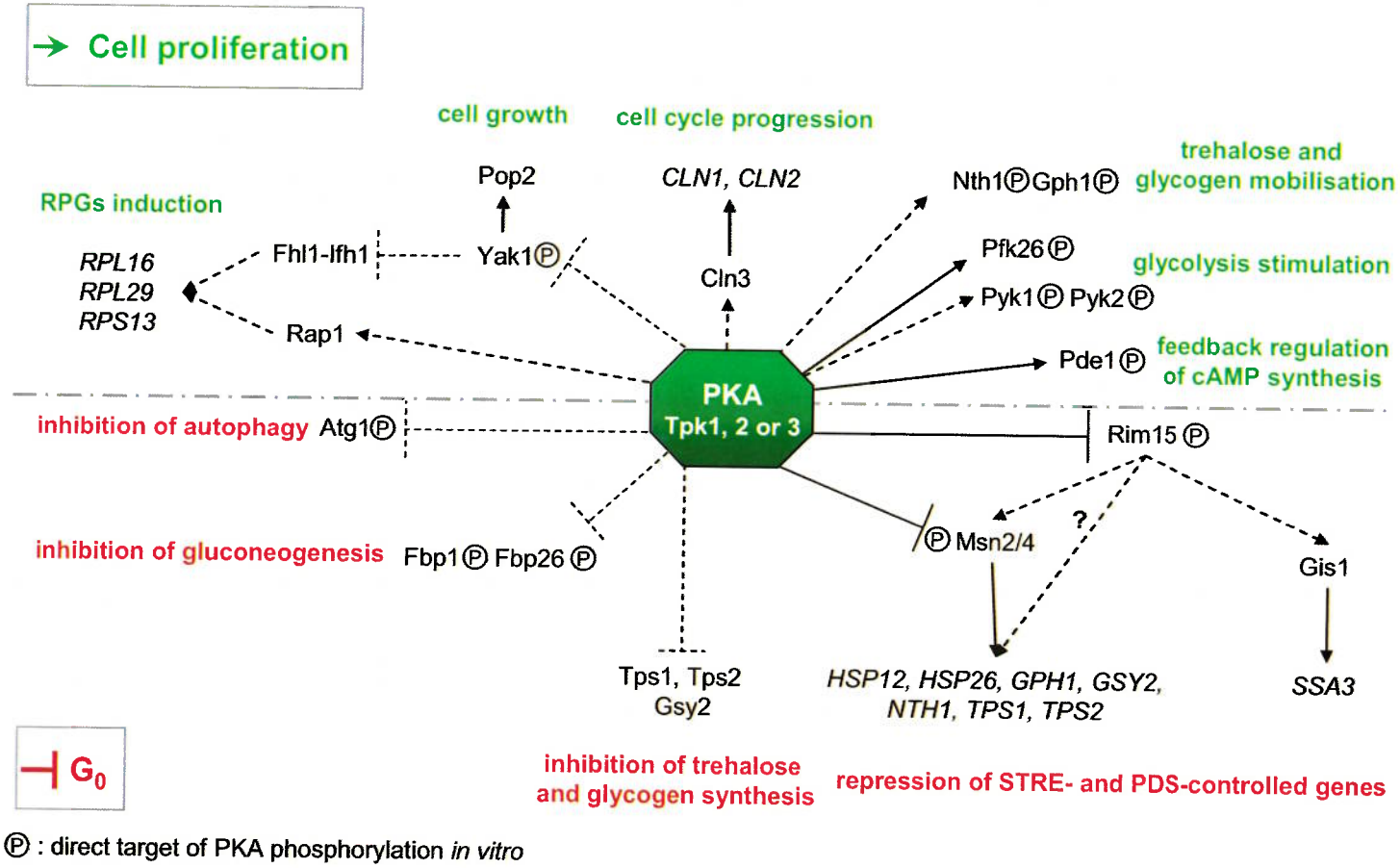


Fig. I.8 Most important downstream targets of the PKA pathway.

The PKA pathway positively regulates cell cycle progression and cell growth (→Cell proliferation) and represses acquisition of the G_0 traits (⊣G_0). Atg1: autophagy related protein; Cln3: G1-cyclin; Fbp1: fructose-1,6-bisphosphatase; Fbp26: fructose-2,6-bisphosphatase; Fhl1: transcriptional activator of RPGs; Gis1: transcription factor binding PDS elements; Gph1: glycogen phosphorylase; Gsy2: glycogen synthase; Ifh1: co-activator of Fhl1; Msn2, Msn4: transcription factors binding STRE elements; Nth1: neutral trehalase; Pde1 low affinity phosphodiesterase; Pfk26: 6-phosphofructo-2-kinase; Pop2/Caf1: subunit of the Ccr4-Not complex phosphorylated by Yak1; Pyk1, Pyk2: pyruvate kinase; Rap1 transcriptional activator of RPGs; Rim15: Ser/Thr kinase required for proper entry into G_0, regulates STRE- and PDS-controlled gene transcription; RPGs: ribosomal protein genes; Tps1, Tps2: subunits of trehalose synthase; Yak1: Ser/Thr kinase required for growth in the absence of PKA; *CLN1, CLN2*: encoding G1-cyclins; *HSP12, HSP26, GPH1, GSY2, NTH1 TPS1, TPS2*, genes containing STRE elements; *SSA3*: gene containing PDS elements; *RPL16, RPL29*: genes encoding protein components of the large ribosomal subunit (60S); *RPS13*: gene encoding protein component of the small (40S) ribosomal subunit.
Arrow: direct activation; Bar: direct inhibition; Dashed arrow: presumable, indirect activation; Dashed bar: presumable, indirect inhibition.

I.3.4) The TOR pathway

The target of rapamycin (TOR) kinase pathway was originally identified in *S. cerevisiae* through the action of a drug with growth inhibitory effects called rapamycin, which targets the large evolutionary conserved Tor kinases (108). The Tor kinase pathway positively regulates cell growth (*i.e.,* increase in cell mass) in response to nutrient-related environmental cues, notably by favouring anabolic processes and antagonising catabolic processes (58, 322, 323). Thus, in a complete growth medium, the TOR pathway is active and yeast cells maintain robust transcription, translation, nutrient import and cell growth. Conversely, when these exponentially growing cells are treated with rapamycin, which specifically inhibits the TOR pathway, they stop growth at G_1 and acquire characteristics of nutrient-deprived G_0 cells. Rapamycin treatment triggers, among others, accumulation of storage carbohydrates (glycogen and trehalose), specific changes in transcription such as up-regulation of starvation-specific transcription (NCR-responsive, RTG-target and STRE-controlled genes), specific changes in amino acid transporter trafficking, down-regulation of general protein synthesis and ribosome biogenesis (Ribi), and up-regulation of macroautophagy (58, 59) (Fig. I.9). The following sections will describe the Tor proteins and their binding partner, give a brief overview of the nutrient-signals which possibly regulate TOR and finally describe the major components downstream of the Tor proteins. The Tor pathway is widely conserved among eukaryotes, and it exists in mammalian cells as the mTOR pathway (6).

Rapamycin
Rapamycin, also known as sirolimus or rapamune, is widely used in medicine. It is a natural secondary metabolite produced and secreted by the soil bacterium found on Easter Island *Streptomyces hygroscopius*. Rapamycin was first developed as an anti-fungal drug directed against the pathogenic yeast *Candida albicans*, but its use was discontinued because of its immunosuppressive effects. It is now currently used as an immunosuppressive drug to prevent the rejection of transplanted organs. Rapamycin treatment blocks growth and proliferation of the mammalian T and B-cells involved in the immune response. More recently, this drug has been used locally to prevent smooth muscle cell proliferation during a phenomenon called restenosis. Moreover, rapamycin derivatives are used as anticancer agents in a variety of malignancies as they delay tumour proliferation. Rapamycin is a macrolide, which means that it is an antibiotic containing a lactone ring produced by a *Streptomyces* species (5-7).

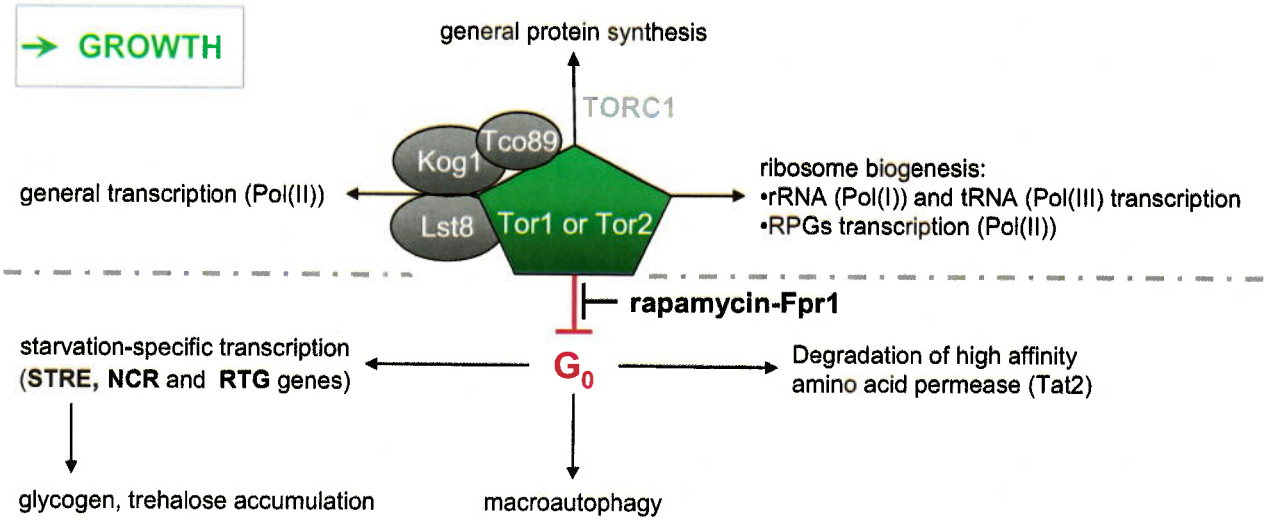


Fig. I.9 TORC1 controls a large and diverse set of growth-related readouts

TORC1 is active in presence of sufficient nutrients and acts to promote cell growth (upper part of the scheme) notably by promoting anabolic processes, such as protein synthesis, and by inhibiting acquisition of G_0 characteristics (lower part of the scheme). Pol(I): RNA polymerase (I); Pol(II): RNA polymerase (II); Pol(III): RNA polymerase (III).

I.3.4.1) The target of Rapamycin proteins Tor1 and Tor2 and the TOR complexes

Targets of the rapamycin drug are the two Tor proteins, Tor1 and Tor2. In fact, rapamycin freely diffuses into cells to form, once in the cytoplasm, a complex with its intracellular receptor Fpr1 (108, 324). This drug-protein complex then binds and inhibits the function of Tor1 and Tor2, thereby promoting the cells entry into a G_0-like state (325). In line with this model, loss of Fpr1 renders cells rapamycin resistant (108). Structurally, Tor1 and Tor2 are members of the phosphatidylinositol 3-kinase-related protein kinase family (PIKK family), which includes a large group of eukaryotic signalling molecules, many of which regulate cell growth, cell cycle progression and DNA damage checkpoints (7, 58, 326). Each member of this family contains a C-terminal kinase domain, and, despite their homology to lipid kinases the Tor proteins function as Ser/Thr kinases (6). Interestingly, the region of the Tor proteins which binds the drug-receptor complex, the **F**pr1-**r**apapamycin **b**inding-domain (FRB), is adjacent to the kinase domain, indicating that rapamycin may act by inhibiting the kinase activity (325). Mutations in the FRB domain of Tor1 and Tor2 (such as *TOR1-1* (Ser1972Ala) and *TOR2-1* (Ser1975Ile) alleles) yield proteins that cannot bind the rapamycin-Fpr1 complex and confer dominant rapamycin resistance (6, 108, 325, 327, 328). Whether this complex effectively inhibits the kinase activity of the Tor proteins is still a matter of debate (329).

TOR proteins have been isolated in two different complexes termed TORC1 for **TOR** **c**omplex **1** and TORC2 for **TOR** **c**omplex **2** (59). TORC1 was proposed to contain Tor1 or Tor2 along with Lst8 (**l**ethal with **s**ec **t**hirteen), Kog1 (**k**ontroller **o**f **g**rowth) and Tco89 (**t**or **c**omplex **o**ne) (59, 60, 94). TORC2 was proposed to contain Tor2 (but not Tor1) in association with Avo1, Avo2, Avo3 (**a**dheres **vo**raciously to Tor2), Bit61 (**bi**nding-partner of **T**or2) and Lst8. These two TOR complexes (TORCs) are functionally distinct: TORC1, is responsible for carrying out the rapamycin-sensitive Tor1 and Tor2 shared functions, while TORC2 is responsible for the Tor2-specific, rapamycin-insensitive, function in cell cycle-dependent polarization of the actin cytoskeleton (58, 59, 330). Thus, rapamycin mediates inhibition of Tor1 and Tor2 in the specific protein context of TORC1 and, apparently, does not affect the integrity of the TORC1 complex (59) (Fig. I.9).

It should be noted that while loss of Tor2, Lst8 or Kog1 causes inviability, loss of Tor1 and Tco89 only renders cells hypersensitive to rapamycin, indicating that the TORC1 pathway is altered but not abolished in the absence of Tor1 or Tco89 (60, 331). This fits with the redundant role of Tor1 and Tor2, in the TORC1 complex. Indeed, in the absence of Tor1, Tor2 can maintain some TORC1 activity, which is sufficient for cell growth in the absence of rapamycin. Since Tor1 is not found in TORC2, the lethality associated with loss of Tor2 cannot be complemented by Tor1 (332). In agreement with the distinct functions of the two complexes, conditional depletion of Tor2, Avo1 or Lst8 (shut off experiments) results in actin depolarisation and growth arrest at various stages of the cell cycle. Conversely, depletion for any essential components of the TORC1 complex, Kog1 or Tor2 and Tor1 together results in a growth arrest in G_1, which is characteristic of cells entering G_0 (59, 332). Interestingly, Lst8 depleted cells resemble in some other aspects (*e.g.,* RTG-target genes transcription) to mutants defective in TORC1 (330). Lst8 has been suggested to be the subunit of the TOR complex 1 that may communicate with downstream effectors of the TORC1 pathway (333) .

The TOR complex 1 seems to be primarily membrane associated (334). Whether it is the plasma membrane, the vacuole or some other intracellular membranous structure is still a matter of debate (60, 94, 328, 334). Recently, Kog1 has been identified at the vacuolar membrane, indicating a potential role of TORC1 at this compartment (60). TORC1 membrane localisation has been proposed to be important for extracellular and intracellular nutrient-signal reception, and hence, for regulation of TORC1 activity (333).

The yeast TORC1 and TORC2 organisation is also conserved in mammalian cells, except for the fact that mammalian cells contain only one single mTOR protein (also referred to as

FRAP/RAFT1/RAPT) that can occur in both TORC1 and TORC2 complexes (322, 335). The mammalian counterpart of Kog1, Raptor, is thought to act as an adaptor that recruits substrates to mTOR while Lst8 is suggested to be an upstream regulator of mTOR (322) (for reviews on mTOR signalling see (7, 57, 320, 322, 336)).

I.3.4.2) Nutrient-regulation of the TORC1 pathway

Because TORC1 disruption or inhibition by rapamycin elicits cellular responses characteristic of nutrient starvation, TORC1 is thought to signal the presence of nutrients to downstream effectors (59). Nitrogen starvation and rapamycin treatment trigger globally similar cellular responses, and it is commonly accepted that nitrogen starvation inhibits TORC1 (68, 71, 95). Additionally, recent microarray analysis showed that many genes, whose transcription is affected by rapamycin treatment, code for proteins that are involved in various aspects of carbon and nitrogen metabolism (61). Accordingly, TORC1 inhibition affects the global pattern of gene expression in a way that is similar to that observed after a shift from a good nitrogen source to a poor nitrogen source and/or after the depletion of glucose (61-63). Rapamycin treatment results, among others, in transcriptional induction of genes required for utilisation of poor carbon and nitrogen sources, indicating that TORC1 may sense the quality of available nitrogen and carbon sources (62, 63, 68). In particular, there is significant correlation between genes regulated by the amino acid glutamine, and genes controlled by TORC1 suggesting that glutamine may be an important indicator of the nutrient status (22, 95). However, glutamine seems to control only a set of the TORC1 readouts indicating that TORC1 must respond to other (yet to be identified) nutrients (6, 95). The exact nature of these nutrients and how these nutrients are sensed by TORC1 remain still unsolved issues (95, 337). Furthermore, it is not clear whether TORC1 senses intracellular or extracellular nutrient levels.

In mammalian cells, mTORC1 is primarily associated with the regulation of protein translation in response to amino acid availability (6, 338-341). In particular, leucine starvation results in rapid dephosphorylation of two direct downstream targets of mTORC1, namely 4E-BP1 and S6K1, both of which are required to sustain general translation (342, 343). The mechanism by which the amino acid status is communicated to mTORC1 is also still a matter of debate (322, 344, 345).

I.3.4.3) TORC1-dependent control of phosphatase complexes

The type 2A phosphatases (PP2A) Pph21 and Pph22, the type 2A-related phosphatase Sit4 (PP2A-related) and Tap42, a phosphatase-associated protein, have been identified in a genetic screen as components of the TORC1 pathway (65). These proteins are implicated in the regulation of many downstream targets of TORC1 and are probably constituents of a major effector branch of the TORC1 pathway (58, 72, 323, 329, 346).

I.3.4.3.1) The PP2A and type PP2A-related phosphatases

PP2A are Ser/Thr phosphatases that can form active trimeric complex composed of one of two catalytic subunits (Pph21 or Pph22), a scaffolding subunit (Tpd3), and one of two regulatory subunits (Cdc55 or Rts1), which determine substrate specificity (347-351). Similarly, the PP2A-related phosphatase Sit4 associates with a family of related proteins termed Saps (**S**it4-**a**ssociated **p**roteins) (352, 353). Association of Sit4 with the Saps promotes cell cycle progression through G_1 (354, 355). In addition to their association in conventional regulatory subunits, Pph21, Pph22 or Sit4 have been found to form dimeric complexes with the phosphatase-associated protein Tap42 (64, 65) (Fig. I.10).

I.3.4.3.2) TORC1-dependent regulation of PP2A and PP2A-related phosphatases

TORC1 signalling pathway positively regulates the Pph21-, Pph22- or Sit4-Tap42 association. Rapamycin treatment, like nutrient starvation, induces dissociation of the dimers (64, 65, 68, 348). Mechanistically, two models that are not mutually exclusive can explain the TORC1-dependent Tap42-PP2As association. In the first model, TORC1 directly phosphorylates Tap42, which increases its binding to Pph21, Pph22 and/or Sit4, whereas rapamycin-induced Tap42 dephosphorylation probably results in its dissociation from the catalytic subunits (64, 65). This model is supported by the finding that Tap42 is phosphorylated *in vitro* by Tor2, and *in vivo* in a TORC1-dependent way (64). In the second model, TORC1 activity indirectly induces the binding of Tap42 to Sit4 by regulating the association of Tap42 with another protein Tip41 (66). In a nutrient-rich medium, TORC1-dependent phosphorylation of Tip41 is thought to cause dissociation of the Tap42-Tip41 complex and consecutive binding of liberated Tap42 to Sit4. Conversely, rapamycin treatment triggers Sit4-dependent Tip41 dephosphorylation, which stimulates the binding between Tip41 and Tap42 (66). Thus, TORC1 might regulate Tap42 both directly and indirectly to control PP2A and Sit4 phosphatase activities (Fig. I.10).

I.3.4.3.3) Downstream signalling via the Tap42 effector branch

Tap42 is thought to signal positively in the TORC1 signalling network to promote cell growth (65). However, the role of Tap42 in phosphatase regulation remains poorly understood. Indeed, contradictory information exists as to whether association of Tap42 with the phosphatases stimulates phosphatases activity toward a particular set of substrates (72, 356) or simply inhibits phosphatase activity (66, 68, 73). To conciliate both models, Wang *et al.* (2003) proposed that the Tap42-phosphatase association was required for targeting phosphatases to their substrates, while rapamycin-induced dissociation of the Tap42-phosphatase complex was required for dephosphorylation of the substrates (357). TORC1-dependent induction of the association between PP2A or PP2A-related phosphatases and Tap42 activates a branch of the TORC1 pathway that is involved in: (i) the induction of translation initiation (65, 69, 70); (ii) the distribution of the amino acid transporters at the cell surface (67); (iii) transcriptional repression of NCR-responsive and STRE-controlled genes (62, 68, 71, 72); and (iv) possibly in repression of RTG-target gene transcription (72, 346). Interestingly, Düvel *et al.* (2003) found that rapamycin-induced transcription of RTG-target gene required Tap42 (72). This result shows that Tap42 plays, during TORC1 inactivation, a positive role on transcription. The Sit4 phosphatase seems to play a major role in the regulation of these TORC1 targets (346) (Fig. I.10).

I.3.4.4) TORC1-dependent control of the nutrient-regulated transcription factors

Under nutrient-rich conditions, TORC1 globally represses starvation-specific transcription (NCR-responsive, RTG-target and STRE-controlled gene expression). This appears to occur in a conserved manner by restricting nuclear import of several nutrient-responsive transcription factors including Gln3, the Rtg1-Rtg3 module, and Msn2/4.

I.3.4.4.1) Regulation of NCR-responsive genes

TORC1 negatively controls NCR-responsive gene transcription trough phosphorylation of the transcription factor Gln3 (61, 62, 68, 73, 93, 358). This phosphorylation event is proposed to promote the association of Gln3 with Ure2, a negative regulator of NCR-sensitive transcription, which may act as a cytoplasmic anchor protein to prevent Gln3 nuclear accumulation (68). An alternative model proposes that Ure2 may act to facilitate Gln3 phosphorylation or to stabilise the phosphorylated form of Gln3, which cannot be transported into the nucleus (73). Rapamycin-induced dephosphorylation of Gln3 triggers (according to the former model) its dissociation from Ure2, and its subsequent entry into the nucleus where it activates NCR-responsive genes (63, 68). Sit4 appears to be the phosphatase responsible

for dephosphorylation of Gln3 (63, 68, 346). However, this model does not fit with all data. For example, rapamycin still induces transcription of some NCR-responsive genes in a *tap42-11* mutant, which has normally lost its ability to respond to rapamycin treatment and to derepress the phosphatases (at the permissive temperature of 24°C) (62, 65) (Fig. I.10).

As mentioned above, rapamycin addition to cells growing in rich media increases expression of NCR-responsive genes (61, 62, 68, 73). Cells starved for ammonia or glutamine, treated with MSX[4], or grown on a poor nitrogen sources (*e.g.,* proline) exhibit the same characteristics (*i.e.*, transcriptional induction of NCR-responsive genes and nuclear localisation of Gln3) (92, 93, 359). Nevertheless, under these different nutrient conditions, the phosphorylation state of Gln3 varies a lot and differs from the phosphorylation state observed upon rapamycin treatment, suggesting that different stimuli, although eliciting globally the same outcome, probably utilise different mechanistic pathways (93, 359). Gat1, the second transcription factor that positively regulates NCR-responsive gene transcription, seems to be globally regulated in the same way as Gln3 (68, 360). It should be mentioned that the NCR-responsive genes not only encode proteins involved in amino acid transport (*e.g.,* Can1, Dur3, Gap1, Mep2, Put4) or in the catabolism of nitrogen compounds (*e.g.,* Dur1,2, Put1,2, Gdh1, Gdh2) but also proteins involved in autophagy (*ATG14*) (18, 361) (Fig. I.6 and Fig. I.10).

I.3.4.4.2) Regulation of RTG-target genes

In the presence of glutamine or glutamate, TORC1 negatively controls RTG-target gene transcription (*e.g., ACO1, CIT1, CIT2, DLD3, IDH1, IDH2, PYC1*) notably by inhibiting translocation of the Rtg1-Rtg3 module from the cytoplasm to the nucleus (22). Mechanistically, TORC1 activity promotes the phosphorylation of Mks1, a negative regulator of RTG-target gene expression (74, 76, 362). Hyperphosphorylated Mks1 interacts with Bmh1/2 to form a complex that inhibits nuclear entry of Rtg1-Rtg3 (76, 363). Conversely, rapamycin treatment, glutamine or glutamate starvation, or MSX treatment, promotes relocation of the Rtg1-Rtg3 module from the cytoplasm to the nucleus where it activates the corresponding target genes (22, 95). These different signals promote dephosphorylation of Mks1 (76-78). This dephosphorylation, results in the preferential attachment of Mks1 to another protein, Rtg2, which inhibits Mks1 and leads to derepression of the Rtg1-Rtg3 module (76-78, 179) (Fig. I.10). As nuclear entry of the Rtg1-Rtg3 module is necessary but

[4] MSX: L-methionine sulfoximine is a glutamine synthetase inhibitor. In fact, it is a glutamate analogue, which inhibits most enzymatic reactions, in which glutamate is used as a substrate including glutamine synthetase. It is utilised to induce intracellular glutamine depletion.

not sufficient for transcriptional activation of the RTG-target genes, additional mechanisms, might be required for activation of the complex (22). Nuclear export of the Rtg1-Rtg3 complex requires the nuclear export protein Msn5 (22). Recently, rapamycin-induced expression of *CIT2,* the prototypical target gene of the RTG pathway, has been shown to be at least partly dependent on the presence of Sit4 or of Pph21 and Pph22, suggesting a role of these phosphatases in Rtg1-Rtg3 regulation (179). This finding still increases complexity of the model.

Rapamycin treatment, in the presence of glucose, generally activates transcription of RTG-target genes (22) (Fig. I.5). Interestingly, RTG-target genes were originally identified as being under positive control of a mitochondria to nucleus pathway, the retrograde response pathway (RTG pathway), which induces RTG-target gene transcription in response to a mitochondrial respiratory deficiency (176). Thus, both TORC1 and retrograde-dependent control of the RTG system converge on the regulation of the heterodimeric transcription factors Rtg1 and Rtg3 (20, 22, 75). Because activation of RTG-target genes via rapamycin treatment is, as for retrograde signalling, strictly dependent on Rtg2, this protein may constitute the point of convergence between the TORC1 and the retrograde system (20, 22, 75) (for a recent review on this topic see (19)). However, Lst8, a component of the TORC1 complex, has recently been shown to negatively regulate RTG-dependent gene transcription, by acting also independently of TORC1 downstream of Rtg2 (20, 59, 333, 364). The signal transmitted by Lst8 is probably integrated at an other point of the RTG pathway upstream of Rtg1-3 (19, 333).

I.3.4.4.2) Regulation of STRE-controlled genes

Rapamycin addition to cells growing on a nutrient-rich medium also triggers induction of STRE-controlled genes, which is notably required for glycogen and trehalose accumulation (68, 73). TORC1 negatively regulates transcription of STRE-controlled genes by preventing nuclear accumulation of the general stress transcription factors Msn2 and Msn4 (46, 47, 68, 88). According to Beck et Hall (1999), TORC1 sequesters Msn2/4 in the cytoplasm by stimulating their binding to the cytoplasmic anchor proteins Bmh1 and Bmh2, via a mechanism independent of the Tap42 and Sit4 effector branch (365). Their model was notably based on the observations that: (i) the overexpression of genes encoding Bmh1 or Bmh2 conferred rapamycin resistance; (ii) the biochemical interaction of Bmh2 with Msn2 or Msn4 was disrupted by rapamycin treatment; and (iii) Msn2 accumulated in the nucleus under growth conditions that triggered disruption of the Bmh2-Msn2 association (68, 71, 366). However, loss of both Bmh1 and Bmh2 (which is lethal except in the Σ background)

does not affect the cytoplasmic localisation of Msn2 on nutrient-rich medium, suggesting that other mechanisms are involved in the regulation of Msn2 and Msn4 localisation (71, 72, 367). In line with this hypothesis, TORC1 has also been involved in regulating nuclear export of Msn2/4 (46). Accordingly, rapamycin treatment promotes nuclear accumulation of a truncated Msn2, which contains the nuclear export sequence (NES), indicating that rapamycin treatment probably blocks nuclear export of Msn2 (46, 71). In line with this reasoning, deletion of the nuclear export factor Msn5 leads constitutive nuclear localisation of Msn2 (46, 368) (Fig. I.10).

A major point of contradiction concerns the involvement of the Tap42 effector branch in Msn2 and STRE-controlled gene activation. Indeed first studies initially set Msn2/4 in a Tap42-independent branch of the TORC1 pathway (68). However, Düvel *et al.* (2003, 2004) recently demonstrated that TORC1-mediated inhibition of STRE-controlled gene induction and nuclear localisation of Msn2 required a wild-type Tap42 protein and probably inactivation of Pph21 and Pph22 (71, 72). Anyway, theses results should be taken with caution as the strain background they used for their studies contains a mutation in *SSD1* (*ssd1-d*), a gene encoding a protein that may influence phosphatase signalling as, in such a mutant, loss of Sit4 is lethal (60, 355). Additionally all experiments were carried out at high temperature (36°C). Thus, except for Gln3-dependent transcription, the contribution of the phosphatases Sit4, Pph21 and Pph22 in transcriptional regulation remains to be clarified (Fig. I.10).

I.3.4.5) TORC1-dependent control of amino acid transporter trafficking

TORC1 not only negatively controls the transcription of nitrogen regulated permeases (via the NCR system), but it also controls the localisation and activity of permeases at the plasma membrane (67, 68). Notably, TORC1 regulates, at the trans Golgi network, their sorting to the plasma membrane or to the vacuole. The following paragraphs will describe in more details regulation of the two amino acid permeases, Tat2 and Gap1.

TORC1 promotes the activity of the constitutive tryptophan permease, Tat2, and of the histidine permease, Hip1 (67, 365). These constitutive/high-affinity amino acid permeases import amino acids for use in protein synthesis and are consequently no longer required when cells stop growth (365). Accordingly, upon rapamycin treatment, Tat2 is removed from the plasma membrane by a mechanism involving its Npi1/Rsp5-dependent ubiquitylation, its consecutive internalisation and degradation within the vacuole (365). Rapamycin treatment also similarly diverts the intracellular pool of Tat2 from the secretory pathway, which targets

transmembrane proteins from the Golgi to the plasma membrane, into the Golgi to vacuole delivery pathway, which triggers degradation of Tat2 (67, 365). Mechanistically, TORC1 is thought to regulate Tat2 via the Ser/Thr kinase Npr1, which is involved in the post-Golgi sorting of the amino acid permeases (4). According to the current model, under nutrient favourable conditions TORC1 keeps Npr1 in a highly phosphorylated and inactive form, preventing Tat2 targeting to the vacuole. Upon rapamycin treatment, Npr1 is rapidly dephosphorylated and activated in a Sit4-dependent way, which promotes vacuolar targeting of Tat2 (66, 67, 369) (Fig. I.10). However, the effect of loss of Npr1 on Tat2 activity has not been tested yet and the way Npr1 regulates permeases sorting is still unsolved.

In contrast to Tat2, the nitrogen-regulated permease Gap1 was initially thought to be negatively regulated by TORC1 (61, 365). This assumption was based on the fact that rapamycin treatment increases the intracellular protein level of Gap1 and on the observation that TORC1-mediated and nitrogen-mediated responses are essentially the same (365). Accordingly, in the presence of good nitrogen sources (*i.e.,* glutamine, glutamate, asparagine), when TORC1 is active, *GAP1* is transcriptionally repressed, Gap1 protein is removed from the plasma membrane via ubiquitin-mediated internalisation, and degraded into the vacuole (365, 370). On poor nitrogen sources such as proline and urea, Npr1 promotes Gap1 activity at the cell surface by stabilising the protein at the plasma membrane (174). Thus, Npr1 apparently regulates Gap1 (stabilisation) inversely from Tat2 (degradation) (67). However, unlike prior expectations (67), rapamycin-treated cells fail to sort Gap1 to the cell surface (333, 369). This failure may be related to a transient increase in the intracellular pool of glutamine and glutamate following rapamycin treatment (possibly caused by derepression of the RTG-target genes) (333). This increase may promote Gap1 sorting to the vacuole (19, 333, 369). Hence, TORC1 may play, instead, an indirect role in the regulation of Gap1 trafficking (370).

I.3.4.6) TORC1-dependent control of protein synthesis

TORC1 positively regulates translation initiation notably by modulating the phosphorylation state and activity of Gcn2 and Eap1 and the stability of the translation initiation factor eIF4G (337).

Gcn2 is a protein kinase which is activated by uncharged tRNAs (90). Activated Gcn2 phosphorylates the translation initiation factor eIF2α on its Ser51. This phosphorylation event drastically decreases the overall translation initiation rate by reducing formation of the ternary

complex (methionyl-tRNA-eIF2-GTP), which is responsible for bringing the methionyl initiator tRNA to the start codon (AUG). TORC1 negatively regulates Gcn2 kinase activity by promoting phosphorylation of its residue Ser577 (371), via a mechanism involving Tap42 and Sit4, possibly Tap42-mediated inhibition of Sit4 (69). Conversely, rapamycin treatment reduces the level of Ser577 phosphorylation, which activates Gcn2, promotes phosphorylation of eIF2α and consequently downregulates general translation initiation (90, 371, 372). Downregulation of general amino acid translation paradoxically promotes starvation-specific translation of the transcription factor Gcn4, which activates transcription of genes encoding enzymes required for amino acid biosynthesis (69, 90, 372-375). Interestingly, Sit4 has been proposed, during growth conditions that sustain elevated TORC1 activity, to be involved in eIF2α dephosphorylation (indeed, loss of Sit4 induces eIF2α hyperphosphorylation). This suggests that the phosphatase plays a role in activating and inhibiting translation initiation (64, 69) (Fig. I.10).

TORC1 is likely to control translation initiation at the level of eIF4E, the cap-binding protein, which is a component of the eIF4F complex (eIF4E-eIF4G-eIF4A) required for recognition of the 5'-cap structure on the mRNA and subsequent ribosome binding (79, 337). Accordingly, TORC1 has been proposed to act positively on translation by stabilising the eIF4E-eIF4G complex: rapamycin treatment effectively causes eIF4G degradation (70). Additionally, TORC1 has been suggested to promote cap-dependent translation initiation by phosphorylating and inhibiting Eap1, a protein, which prevents formation of the translation initiation complex, eIF4F, by competing with eIF4G for binding to eIF4E (6, 79). Although there are no direct evidences of TORC1 mediating regulation of Eap1, its mammalian homolog, 4E-BP, is a direct downstream target of mTORC1 phosphorylation (376). Thus, it would not be surprising to find a similar mechanism in yeast. As a consequence of downregulated cap-dependent protein synthesis, Cln3, a cyclin involved in G_1-progression, decreases in abundance (337, 377). This phenomenon may be at least in part involved in the rapamycin-induced cell cycle arrest in G_1 (6) (Fig. I.10).

I.3.4.7) TORC1-dependent control of ribosome biogenesis

Ribosome biogenesis is an energy- and nutrient-consuming process which is tightly down-regulated upon entry into G_0 in order to avoid any loss of energy (37). Upon rapamycin treatment, cells decrease synthesis of genes encoding components of the translational apparatus such as the RNA and protein subunits of the ribosome. Notably, they downregulate Pol(I)- and Pol(III)- dependent transcription of rRNA and tRNA encoding genes

(61, 378, 379), as well as Pol(II)-dependent transcription of RPGs (57, 61, 322, 380). Conversely, TORC1-mediated transcriptional activation of these genes contributes to maintain an elevated level of protein synthesis that allows efficient cell growth.

TORC1 controls Pol(I) transcription by promoting Rrn3-mediated recruitment of Pol(I) to the ribosomal DNA (rDNA) (80, 381). TORC1 also maintains elevated RPG transcription notably by regulating chromatin remodelling via Esa1 and by modulating the activity of specific transcription factors. In the former case, TORC1 participates in the recruitment of Esa1, the histone H4 acetylase, to the promoters of RPGs (382, 383). Acetylated histones in turn facilitate access of regulatory factors to their appropriate binding sites on promoter and consequently increase transcription (384). In the latter case, TORC1 notably positively regulates the two RPGs-specific transcription factors Fhl1 and Sfp1 (35, 81). TORC1 activates Fhl1 by maintaining Crf1, its co-inhibitor, in the cytoplasm, preventing thereby the association of Crf1 with Fhl1, which localises in the nucleus. The cytoplasmic retention of Crf1 allows Fhl1 to associate in with its co-activator Ifh1 and to mediate transcription of RPGs (35). Upon TORC1 inactivation, Yak1, a protein kinase, which is responsible for *in vivo* and *in vitro* phosphorylation of Crf1, appears to activate Crf1 by promoting its translocation from the cytoplasm to the nucleus (35). TORC1 also activates RPG transcription by maintaining Sfp1, the transcriptional activator of RPGs, in the nucleus (81). The mechanism by which TORC1 controls the activity of all three RNA polymerases in a coordinated manner is not understood yet (Fig. I.10).

I.3.4.8) TORC1-dependent regulation of autophagy

TORC1 has been involved in regulation of autophagy. Autophagy or "self-eating" is defined as the non-selective uptake and degradation of cytoplasm in the vacuole/lysosome and it can occur either as macro- or as microautophagy (for a review see (84)). Macroautophagy, which is induced by nutrient starvation and/or rapamycin treatment, involves the formation of double membrane vesicles in the cytoplasm and their delivery to the vacuole (82, 201, 202). In contrast, microautophagy involves the engulfment of cytosolic compounds directly at the vacuolar surface by invagination, protrusion and/or septation of the vacuolar membrane (202, 385).

I.3.4.8.1) Regulation of macroautophagy

Macroautophagy is a process essential for survival during stationary phase, probably because vacuolar degradation of the cytoplasmic material allows its recycling into macromolecules that will be used later during nutrient starvation (312, 386). TORC1 negatively regulates macroautophagy by preventing association of the Atg1-Atg13-Atg17 protein complex, which initiates the first steps of this process (82, 387). In nutrient-rich medium, TORC1 appears to directly or indirectly cause hyperphosphorylation of Atg13. This prevents or decreases the association of Apg13 with Atg1 and consequently assembly of the autophagic complex (83). Since Apg1-associated proteins are required for its activation, Apg1 kinase is also inactivated.

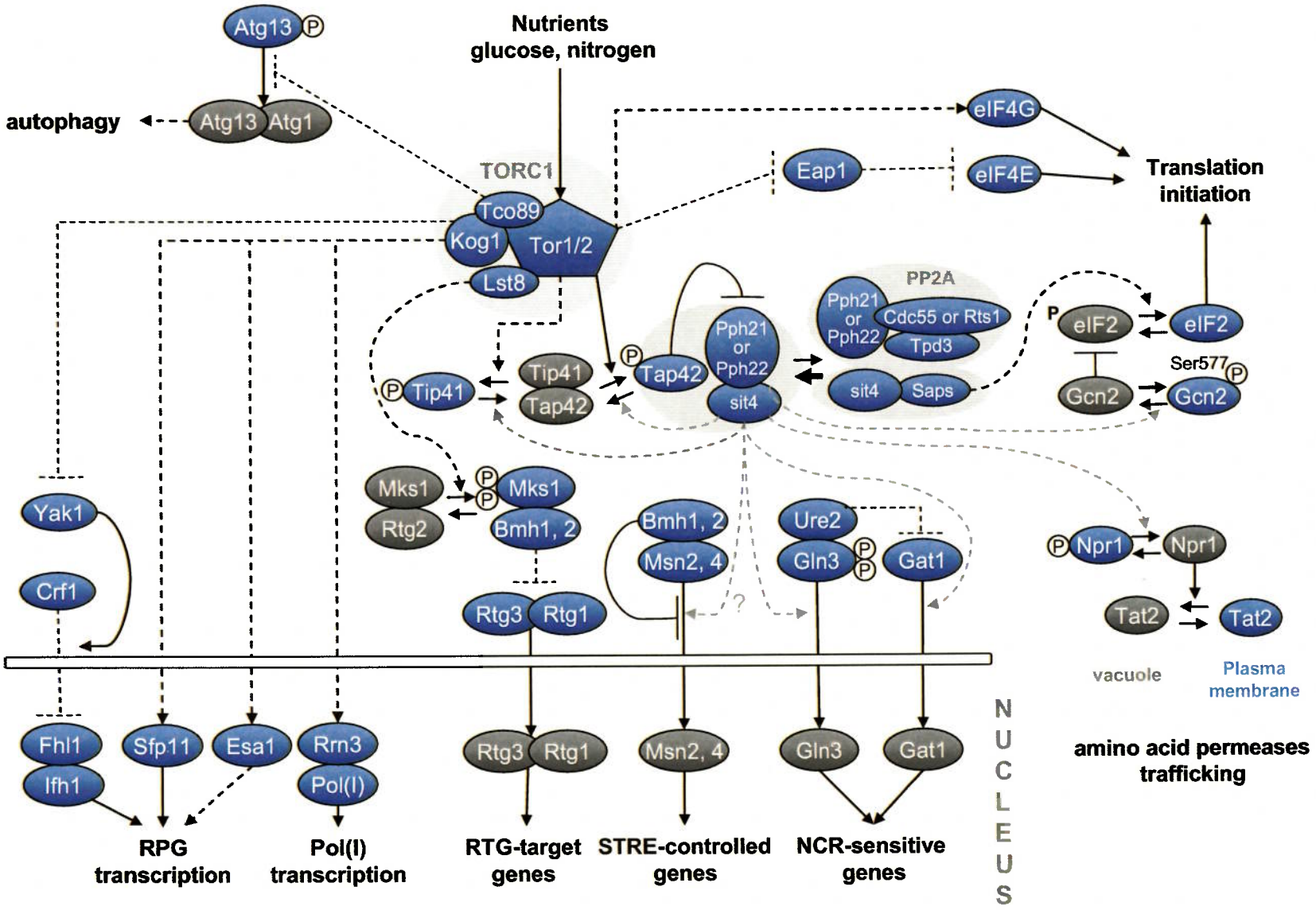
I.3.4.8.2) Regulation of microautophagy

The process of microautophagy can lead to the degradation of soluble components, of non-essential portions of the nucleus (piecemeal microautophagy of the nucleus) or even to the selective uptake of entire organelles such as perroxysomes (micropexophagy) (85, 388, 389). Piecemeal microautophagy of the nucleus is enhanced by nitrogen and glucose starvation as well as by rapamycin treatment (85). In contrary, microautophagy, which is also enhanced by nitrogen starvation, has been shown *in vitro* to be inhibited by rapamycin treatment. More precisely, rapamycin blocks microautophagic vesicles scission (86). Thus, additional studies will be required to determine the exact role of TORC1 in the different forms of microautophagy (Fig. I.10).

Fig. I.10 The TORC1 signalling pathway

Under nutrient-rich conditions, TORC1 regulates the activity of multiple targets. It is generally thought that the interaction of Tap42 with the catalytic subunits of the phosphatases (Sit4, Pph21 and Pph22) inhibits their activity. During exponential growth, an equilibrium exists between Tap42-bound and free phosphatases bound to their regulatory subunits (SAPs for Sit4 and Cdc55 or Rts1 for Pph21 and Pph22). TORC1 promotes the Tap42-phosphatase association by phosphorylating Tap42 (P) and by inhibiting its association with Tip41. Inhibition of the phosphatases prevents transcription of the NCR-responsive genes and possibly (?) of the STRE-controlled genes by favouring the binding of: (i) Gln3, and also possibly Gat1, to Ure2; and (ii) Msn2 and Msn4 to Bmh1 and Bmh2. These interactions allow cytoplasmic retention of the three transcription factors. Tap42-mediated inhibition of Sit4 also prevents dephosphorylation and activation of the Gcn2 kinase and Npr1, a protein involved notably in the sorting of the tryptophan amino acid permease (Tat2) to the vacuole. The Sit4-SAPs complex dephosphorylates and activates the translation initiation factor eIF2. Negative regulation of RTG-targets genes involves TORC1-dependent phosphorylation of Mks1, which promotes its dissociation from Rtg2 and its subsequent interaction with Bmh1 or Bmh2. Mks1 forms with Bmh1/2 an active complex, which inhibits translocation of the Rtg1-Rtg3 module in the nucleus and RTG-target gene transcription. TORC1 positively regulates translation initiation by inhibiting Eap1, which competes with eIF4E for binding to eIF4G in the translation initiation complex, and by stabilising eIF4G. Induction of ribosome biogenesis includes TORC1-mediated activation of ribosomal protein genes (RPGs) and promotion of ribosomal RNA transcription (Pol(I)). TORC1 promotes RPGs transcription: (i) through inhibition of Yak1-mediated nuclear translocation of the Fhl1 co-repressor Crf1, which favours the association of the Fhl1 transcription factor with its co-activator Ifh1; (ii) by maintenance of the Sfp1 transcription factor in the nucleus and; (iii) by maintenance of the Esa1 histone acetylase at the RPG promoters. TORC1 positively regulates the formation of the Pol(I)-Rrn3 complex and, hence, Pol(I) transcription. Finally, TORC1 inhibits macroautophagy by preventing formation of the Atg1-Atg13 complex.

Blue: proteins positively and negatively regulated when TORC1 is active; Grey: proteins positively or negatively regulated when TORC1 is inactive; Arrow: direct activation; Bar: direct inhibition; Dashed arrow: presumable, indirect activation; Dashed bar: presumable indirect inhibition; Grey arrow: dephosphorylation events mediated by phosphatases when TORC1 is inactive.


Atg13 P
Nutrients
glucose, nitrogen
autophagy
Atg13 Atg1
eIF4G
Translation
initiation
TORC1
Eap1
eIF4E
Tco89
Kog1
Tor1/2
Lst8
PP2A
Pph21
or
Pph22
Cdc55 or Rts1
Tpd3
sit4
Saps
P eIF2
eIF2
Ser577 P
Gcn2
Gcn2
Tip41
Tap42
P Tip41
Tap42
P
Pph21
or
Pph22
sit4
Mks1
Rtg2
P P Mks1
Bmh1, 2
Bmh1, 2
Msn2, 4
Ure2
Gln3 P P
Gat1
Yak1
Crf1
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P Npr1
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Plasma
membrane
amino acid permeases
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Fhl1
Ifh1
Sfp11
Esa1
Rrn3
Pol(I)
Rtg3 Rtg1
Msn2, 4
Gln3
Gat1
N
U
C
L
E
U
S
RPG
transcription
Pol(I)
transcription
RTG-target
genes
STRE-controlled
genes
NCR-sensitive
genes
RIBOSOME BIOGENESIS
P TORC1-dependent phosphorylation

I.3.5) Crosstalk between the nutrient-regulated pathways

The crosstalk between different nutrient-signalling pathways assures optimal growth in response to environmental changes. In this context, TORC1 and PKA pathways regulate an overlapping broad set of readouts important for nutrient-controlled cell growth including the transcription of RPGs (36, 38, 380) and STRE-controlled genes (46), and induction of autophagy (87). A common theme is that both pathways control common downstream targets, including Yak1, Msn2/4, and Atg13. The following paragraphs will describe the intersection between these two major nutrient-signalling pathways, the regulation of Gcn2 by TORC1 and amino acid as well as the regulation of Gln3 by nitrogen, TORC1 and Snf1.

I.3.5.1) Coordinate regulation of the Yak1 protein kinase by PKA and TORC1

Both, TORC1 and PKA pathways have been suggested to promote RPG transcription by acting negatively on the protein kinase Yak1. The Yak1 kinase antagonises PKA or TORC1-mediated cell growth, as demonstrated by the fact that loss of Yak1 confers rapamycin resistance and restores growth of a PKA-deficient strain (*tpk1-3Δ*) (88, 309, 310). Yak1 is also a potential direct downstream target of PKA (310, 313), and even if its phosphorylation by PKA has not been directly correlated with decreased Yak1 activity, several data strongly suggest that PKA negatively regulates Yak1 (97, 308-310). Similarly, TORC1 acts upstream of Yak1 and negatively regulates its nuclear localisation (88) as well as its autophosphorylation (35), suggesting that TORC1 negatively controls Yak1 kinase activity. Because Yak1 is required for rapamycin-induced phosphorylation and entry of the RPGs co-repressor Crf1 within the nucleus, and because TORC1 or PKA inhibition also promotes nuclear accumulation of Crf1, it has been suggested that the TORC1 and PKA pathways positively regulate RPGs via coordinate inhibition of Yak1 (35, 335), either through separate or via common inputs (35, 81, 88, 390, 391).

I.3.5.2) Coordinate regulation of Msn2 and Msn4

Both the PKA and the TORC1 pathways negatively regulate transcription of a large number of STRE-controlled genes by affecting Msn2/4 localisation. While PKA prevents Msn2 nuclear import (46, 47, 71, 300), TORC1 promotes the cytoplasmic retention of Msn2, by a mechanism involving binding of the phosphorylated Msn2 to the cytoplasmic anchor protein Bmh2 (68). These observations support a model in which TORC1 and PKA act separately to regulate Msn2. Accordingly, in the complete absence of PKA (in a *tpk1, 2, 3Δ yak1Δ* strain) rapamycin still induces STRE-controlled gene transcription, while constitutive activation of

the cAMP-PKA pathway (in *bcy1Δ* or RAS^{Val19} strain) masks the effects of rapamycin treatment (88, 391).

Msn2 was primarily identified as a multicopy suppressor of a thermosensitive *snf1* (*snf1-ts*) mutant (392). In addition to this genetic interaction, the Snf1 protein kinase negatively regulates Msn2 nuclear localisation at a time where PKA or TORC1-mediated inhibition has been relieved. Indeed, hyperactive Snf1 (*reg1Δ* mutant) specifically prevents the glucose depletion-induced and rapamycin-induced translocation of Msn2 in the nucleus (367). Snf1 has been suggested to act by downregulating nuclear import of Msn2 (393), thus controlling nuclear concentration of Msn2 at the moment when the protein translocates in the nucleus. This mechanism, which should increase also the cytoplasmic pool of Msn2, has been proposed to facilitate Msn2 inactivation when nutrients and glucose are sensed again (367).

I.3.5.3) Coordinate regulation of Atg13

Both, TORC1 and PKA pathways coordinately control early steps of the autophagic process (82, 87). While TORC1-dependent hyperphosphorylation of Atg13 has been shown to prevent formation of the Atg13-Atg1 complex required for macroautophagy, PKA, which phosphorylates Atg13 and Atg1 *in vitro* and Atg1 *in vivo*, acts on Atg1 to negatively control its recruitment to the preautophagosomal structure, the site of autophagosomes formation (83, 313). As a hyperactive PKA pathway ($Ras2^{Val19}$) blocks the rapamycin-induced onset of macroautophagy, TORC1 may feed into the PKA pathway upstream of Ras2. However, PKA and TORC1 could also act independently on different or common targets (87).

I.3.5.4) Coordinate regulation of the Gcn2 protein kinase

TORC1 and amino acids provide separate inputs on the Gcn2 protein kinase to negatively regulate the translation of Gcn4, a transcription factor required for activation of amino acids biosynthetic genes (394). The Gcn2 kinase is able to sense directly the intracellular amino acid availability through its tRNA-binding domain (HisRS) (90). Amino acid limitation leads to increased levels of uncharged tRNAs, which bind to Gcn2, inducing a conformational change that activates its kinase activity (90). Conversely, inhibition of TORC1 increases the affinity of Gcn2 for uncharged tRNA, via Ser577 phosphorylation, allowing Gcn2 to bind uncharged tRNAs even when their levels are very low (*e.g.*, in nutrient-rich conditions) (90).

I.3.5.5) Coordinate regulation of Gln3

Gln3 is regulated by TORC1 and by the quality or the quantity of the nitrogen source in the medium. Whether nitrogen sources (in particular glutamine) signal through or in parallel to TORC1 is still a matter of debate (95, 359). Several studies indicate that carbon levels also control the expression NCR-responsive genes (63, 91, 92). Although the pattern of genes induced upon growth on poor carbon sources differs from the pattern of genes induced during growth on poor nitrogen sources, these studies suggest that there is a crosstalk between the pathways that transmit the corresponding nutrient signals (63). Accordingly, Bertram *et al.* (2002) demonstrated that glucose availability controls Gln3 phosphorylation and subcellular localisation via the Snf1 pathway (91). Indeed, Snf1 can interact with and phosphorylate Gln3 *in vitro*. *In vivo*, glucose starvation in the presence of ammonia as the sole nitrogen source promotes Snf1-mediated hyperphosphorylation and nuclear accumulation of Gln3 as well as Snf1-dependent induction of NCR-responsive genes (91-93). Because Snf1 and TORC1-dependent phosphorylation of Gln3 have opposite effects on localisation of this transcription factor, they are likely to act separately on Gln3, allowing Gln3 to respond to both the nitrogen and glucose signals (91, 93).

When cells are grown on glutamine as the sole nitrogen source, glucose starvation fails to trigger nuclear translocation of Gln3 indicating that the nature of the nitrogen source still dictates whether or not Gln3 is localised in the nucleus (92, 93). Additionally, upon carbon starvation, nuclear translocation of Gln3 is slower and occurs less frequently than upon rapamycin treatment or a shift from good to poor nitrogen sources (92). In order to explain such differences, Cox *et al.* (2002) propose a model in which nuclear import of Gln3 following carbon starvation is an indirect result of the nitrogen starvation brought about by loss of α-ketoglutarate, the carbon skeleton needed for glutamine and glutamate synthesis (92, 93). Accordingly, the slow nuclear translocation of Gln3 during glucose starvation reflects the fact that more time is probably required for α-ketoglutarate to become limiting under this condition (92). These results suggest that Gln3 does not directly integrate the glucose signal but, instead, senses the glucose-derived nitrogen signal from glutamate and glutamine, to properly regulate NCR-responsive genes (91). In line with this model, carbon starvation on glutamine containing medium fails to induce nitrogen starvation and hence translocation of Gln3. Glucose-mediated regulation of Gln3 involves integration of both the Snf1 and the metabolic signals (93).

I.3.6) Concluding remarks

The Snf1, TORC1 and PKA pathways control entry into quiescence in response to different environmental clues. While PKA pathway (directly) and Snf1 (indirectly), integrate glucose signal (5), the signal received by TORC1 is more obscure and may comprise nitrogen and possibly carbon sources (61, 95, 139, 395). However, the PKA pathway, or more precisely PKA targets, can also be activated by nitrogen, sulphur and phosphate signals (184). These signals use a transduction cascade different from the one used by glucose. Thus, this finding supports a model in which individual nutrient signalling cascades are interconnected and may converge on common downstream targets to control common final readouts (390, 391). Accordingly, individual signalling pathways have been placed recently into signalling networks, leading to the identification of converging effector branches that orchestrate the dynamical responses to nutritional cues (336). This is notably true for regulation of NCR-responsive transcription which integrates at least three signals, one from the TORC1 pathway, a nitrogen signal and a glucose signal (via Snf1) (92). These crosstalks make it difficult to establish clearly which signal affects activity of the different nutrient-regulated kinases, unless a direct link can be detected (46, 72).

Finally, the nutrient-regulated signalling pathways, which control G_0 entry, are also expected to play a role in the rapid resumption of proliferative capacity of G_0 cells when the nutritional conditions turn favourable. This is exemplified by the fact that activation of PKA by glucose, ammonia, amino acids or phosphate is required for G_0 nutrient-starved cells to rapidly adapt their metabolism to nutrient-rich conditions (184).

I.4) Outline of this study

The aim of this thesis work was to gain some new insight into the pivotal aspects of the control of cell growth and cell proliferation, by elucidating in more details the mechanisms that govern proper entry into G_0 and exit from G_0. A better knowledge of how cells exit quiescence is indeed expected to be useful to complement the studies done on G_0 entry. This concept is notably based on the assumption that exit from G_0 should imply reverse regulation of the same actors as those implied at the time of the entry in G_0 (8).

The first part of this work, presented in Chapter II, is focused on the study of the regulatory mechanisms lying upstream of the Rim15 protein kinase, which was previously identified as one of the key players that orchestrates coordinated entry into G_0 following nutrient limitation (48, 51). Our investigations led to the identification of two new regulators of Rim15, TORC1 and Sch9, which act in parallel to PKA. Both proteins notably regulate nucleocytoplasmic localisation of Rim15.

The second part of this work, presented in Chapter III and IV, is focused on understanding the mechanisms that trigger cells to exit from G_0. Signals promoting re-entry of G_0 cells into the cell cycle are not completely known but include, for yeast that have experienced carbon starvation the reintroduction of a carbon source (164), or for cells that have experienced a rapamycin-induced growth arrest rapamycin removal. This second condition was used as the starting point of our research on G_0 exit, which was initiated with a genome-wide screen in search for proteins whose loss prevents cells from resuming growth after rapamycin removal.

Chapter II.

TOR and PKA Signaling Pathways Converge on the Protein Kinase Rim15 to Control Entry into G_0

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II.1) Introduction

The nutrient-regulated protein kinase Rim15, which is the object of numerous investigations in our laboratory, is required for acquisition, at the diauxic transition, of a broad range of cellular adaptations necessary for proper entry into G_0 (48). Rim15 is know to function immediately downstream and under direct negative control of the PKA, but regulation of this structurally complex protein is far from being understood (48). Notably, no direct downstream target(s) of Rim15 have been identified yet, although genetic analysis indicated that Gis1 is a possible downstream effector of Rim15 (51). Recent observations revealed that Rim15 is probably downregulated by a PKA-independent signalling pathway (based on unpublished data). Thus, the aim of this research was to identify additional regulator(s) of the Rim15 protein kinase. Given that inactivation of TORC1 and PKA causes a similar phenotype, (*i.e.*, a growth arrest early in G_1 and entry into G_0), and based on the model of the FGM pathway according to which Sch9 could impinge on PKA targets, our interest primarily focused on TORC1 and Sch9 (8, 54). First, we investigated whether Rim15 plays a role in establishing the TORC1-mediated (or Sch9-mediated) G_0 program and subsequently we addressed the underlying mechanisms. Interestingly, this study allowed the discovery of a new control mechanism of Rim15, which is its intracellular compartmentalisation. My main contribution to this paper consisted in the study of the nucleocytoplasmic localisation of Rim15 presented in Fig. 3 and Fig. 4.

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TOR and PKA Signaling Pathways Converge on the Protein Kinase Rim15 to Control Entry into G_0

Short Article

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Summary

The highly conserved Tor kinases (TOR) and the protein kinase A (PKA) pathway regulate cell proliferation in response to growth factors and/or nutrients. In *Saccharomyces cerevisiae*, loss of either TOR or PKA causes cells to arrest growth early in G_1 and to enter G_0 by mechanisms that are poorly understood. Here we demonstrate that the protein kinase Rim15 is required for entry into G_0 following inactivation of TOR and/or PKA. Induction of Rim15-dependent G_0 traits requires two discrete processes, i.e., nuclear accumulation of Rim15, which is negatively regulated both by a Sit4-independent TOR effector branch and the protein kinase B (PKB/Akt) homolog Sch9, and release from PKA-mediated inhibition of its protein kinase activity. Thus, Rim15 integrates signals from at least three nutrient-sensory kinases (TOR, PKA, and Sch9) to properly control entry into G_0, a key developmental process in eukaryotic cells.

Introduction

The highly conserved TOR proteins control growth of proliferating yeast, flies, and mammalian cells in response to growth factors and/or nutrients (Jacinto and Hall, 2003). In yeast, TOR depletion or treatment with rapamycin results in growth arrest that is associated with physiological changes, which are characteristic of stationary phase (G_0) cells (Werner-Washburne et al., 1993). These include G_1 cell cycle arrest, repression of general transcription and mRNA translation, induction of a defined set of stress response genes (e.g., *SSA3*, *HSP26*, and *HSP12*), and synthesis of glycogen and trehalose (Werner-Washburne et al., 1993; Jacinto and Hall, 2003). While TOR controls some readouts via the type 2A (Pph21 and Pph22) or type 2A-related (Sit4) protein phosphatases (PP2As) and their regulator Tap42 (Di Como and Arndt 1996; Jiang and Broach 1999), the effector pathway(s) that controls readouts such as repression of Pol II transcription (and other G_0-associated changes) remain still elusive.

The PKA pathway constitutes another key signaling pathway that controls growth of proliferating yeast in response to nutrients. The mechanism by which PKA controls growth, however, is still an issue of conjecture, and further elucidation of this process will certainly depend on the identification of downstream effectors of PKA. One such effector, the protein kinase Rim15, is required for proper establishment of the G_0 program and is inhibited by PKA-mediated phosphorylation under conditions of nutrient abundance (Reinders et al., 1998). Interestingly, downregulation of PKA following nutrient limitation liberates Rim15 from PKA-inhibition and results in activation of G_0-related changes, which are strikingly similar to the changes observed following rapamycin treatment. These findings suggest a model in which the TOR pathway may converge on a component and/or target of the PKA pathway to control entry into G_0.

Here we show that proper entry into G_0 following TOR inactivation depends on the PKA target Rim15. TOR prevents induction of Rim15-dependent responses via a Sit4-independent mechanism, which alters the phosphorylation status of Rim15 and thereby inhibits nuclear accumulation of Rim15. Moreover, we demonstrate that nucleocytoplasmic distribution of Rim15 is regulated by the yeast protein kinase B (PKB/Akt) homolog Sch9. Thus, Rim15-controlled developmental processes are fine-tuned by integration of signals that are transmitted via at least three key nutrient-sensory kinases, i.e., TOR, PKA, and Sch9.

Results and Discussion

TOR Prevents Induction of Rim15-Dependent G_0 Traits

To investigate whether TOR may feed into the PKA pathway upstream, or at the level of Rim15, we first studied a role of Rim15 in TOR-dependent phenotypes. Wild-type and *rim15*Δ cells were grown in rich medium to early logarithmic phase, treated with rapamycin (0.2 μg ml^{-1}) to inactivate TOR proteins, and assayed for G_1 cell cycle arrest, induction of *SSA3*, *HSP26*, and *HSP12*, and synthesis of glycogen and trehalose. While wild-type cells were blocked in G_1 (Figure 1A), strongly induced *SSA3*, *HSP26*, and *HSP12* transcripts (Figure 1B), and produced high levels of glycogen (Figure 1C) following treatment with rapamycin, *rim15*Δ cells were (despite an early response to rapamycin) defective for proper G_1 arrest (particularly 4 and 6 hr following rapamycin treatment; Figure 1A) and showed no significant increase in the levels of *SSA3*, *HSP26*, and *HSP12* transcripts and of glycogen even after 6–8 hr treatment with rapamycin (Figures 1A–1C). Similarly, a 6 hr rapamycin treatment caused trehalose levels to increase in wild-type cells, but not in the corresponding *rim15*Δ mutant (Figure 1D). Loss of Rim15, however, did not impair the ability of the cells to downregulate translation initiation following TOR inactivation (assayed by polysome profile analysis), nor allow cells to grow on (0.2 μg ml^{-1}) rapa-

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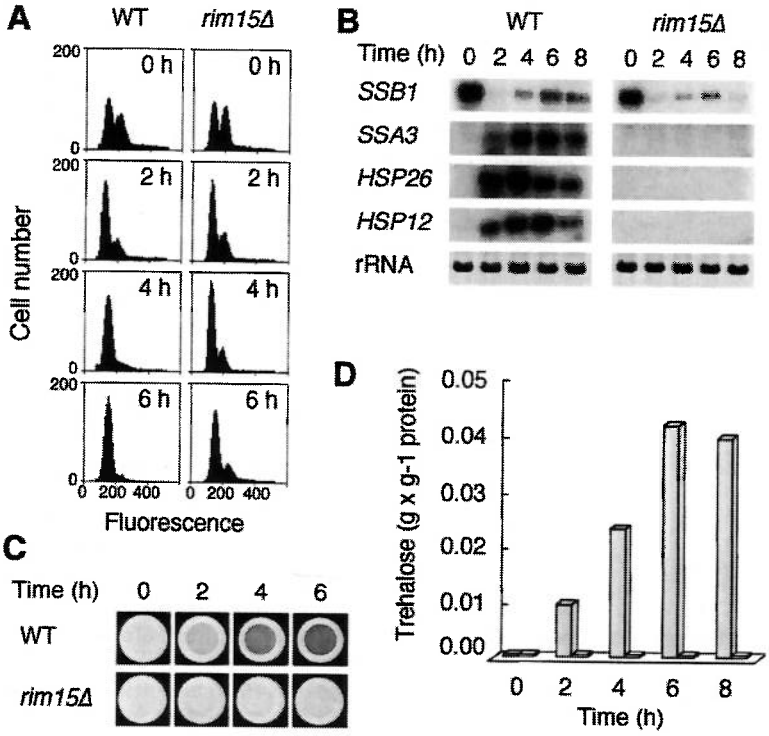

Figure 1. Rim15 Is Required for Entry into G_0 following TOR Inactivation by Rapamycin

(A) DNA content determined by flow cytometry; (B) Northern analysis of indicated genes; (C) glycogen levels (visualized after exposure for 1 min to iodine vapor); and (D) trehalose levels of exponentially growing ($OD_{600} < 0.5$) wild-type (JK9-3da; gray bars) and isogenic *rim15Δ* (IP11; open bars) mutant cells following treatment with rapamycin (0.2 μg ml^{-1}) for the times indicated.

mycin-containing plates (data not shown). Thus, while TOR appears to regulate growth via Rim15-independent mechanisms, it prevents induction of Rim15-dependent G_0 traits.

TOR Controls Rim15 Function via a PKA- and Sit4-Independent Mechanism

Since Rim15 kinase activity is under direct, negative control of PKA (Reinders et al., 1998), we tested whether the rapamycin-induced, Rim15-dependent transcriptional response can be modulated by mutations that affect PKA activity. Interestingly, we found that loss of Ras2, an activator of adenylate cyclase in yeast, caused partial derepression of *HSP12* and *SSA3* and slightly enhanced the rapamycin-induced activation of *SSA3*, *HSP26*, and *HSP12* (Figure 2A). In contrast, constitutive activation of PKA due to loss of the regulatory PKA subunit Bcy1 or following introduction of the dominant active *RAS2^{Val19}* allele (data not shown) almost completely abolished rapamycin-induced induction of all three genes (Figure 2A). The TOR signaling pathway may therefore control expression of these genes by regulating a component of the PKA pathway. In order to examine whether this presumed component functions at the level or upstream of PKA and/or Rim15, we investigated whether the rapamycin-induced transcriptional activation of *SSA3*, *HSP26*, and *HSP12* depends on the presence of PKA and/or Rim15 by using *pka⁻* strains rendered viable through deletion of either *YAK1* or *RIM15* (Reinders et al., 1998; Garrett and Broach, 1989). In the absence of PKA, rapamycin-inducibility of all three genes under study remained high (in the *pka⁻ yak1Δ* strain) and was still strongly dependent on the presence of Rim15 (in the *pka⁻ rim15Δ* strain; Figure 2B). Thus, part of the role of TOR in preventing G_0 entry is mediated by PKA-independent inhibition of Rim15 function.

To determine whether TOR-mediated control of Rim15 requires any of the known PP2A TOR effectors, we investigated whether loss of Sit4 or Pph21 and Pph22 affected basal and/or rapamycin-induced levels of *SSA3, HSP26*, and *HSP12* transcription. We found that loss of Sit4 did not significantly alter the cells' basal or rapamycin-induced *SSA3, HSP26*, and *HSP12* transcript levels (Figure 2C), indicating that TOR regulates Rim15 function via a Sit4-independent pathway. In agreement with this notion, we also found rapamycin-sensitive, Sit4-dependent genes—including Gln3 (e.g., *DAL5* and *PUT1*) as well as Rtg3 target genes (e.g., *PYC1* and *CIT2*; Düvel et al., 2003)—to be regulated largely independently of Rim15 (Figure 2D). Interestingly, loss of both Pph21 and Pph22 strongly enhanced the basal levels of *SSA3, HSP26*, and *HSP12* transcripts in exponentially growing cells, indicating that Pph21 and Pph22 are implicated in inhibition of basal transcription of Rim15-controlled genes (Figure 2E). Irrespective of these high basal transcription levels, however, all three genes remained rapamycin-inducible in the *pph21Δ pph22Δ* double mutant (Figure 2E). Thus, even though the mechanism by which Pph21 and Pph22 control basal transcription levels of *SSA3*, *HSP26*, and *HSP12* remains unknown at present, our results show that rapamycin-induced transcriptional induction of Rim15-controlled genes does not require the known PP2A TOR effectors Pph21 and Pph22 or Sit4. In line with this conclusion, we also found that a semidominant, rapamycin-resistant mutation in the PP2A regulatory Tap42 protein (i.e., *tap42-11*; Di Como and Arndt, 1996) did not prevent induction of *SSA3, HSP26*, and *HSP12* transcription following rapamycin treatment (Figure 2C).

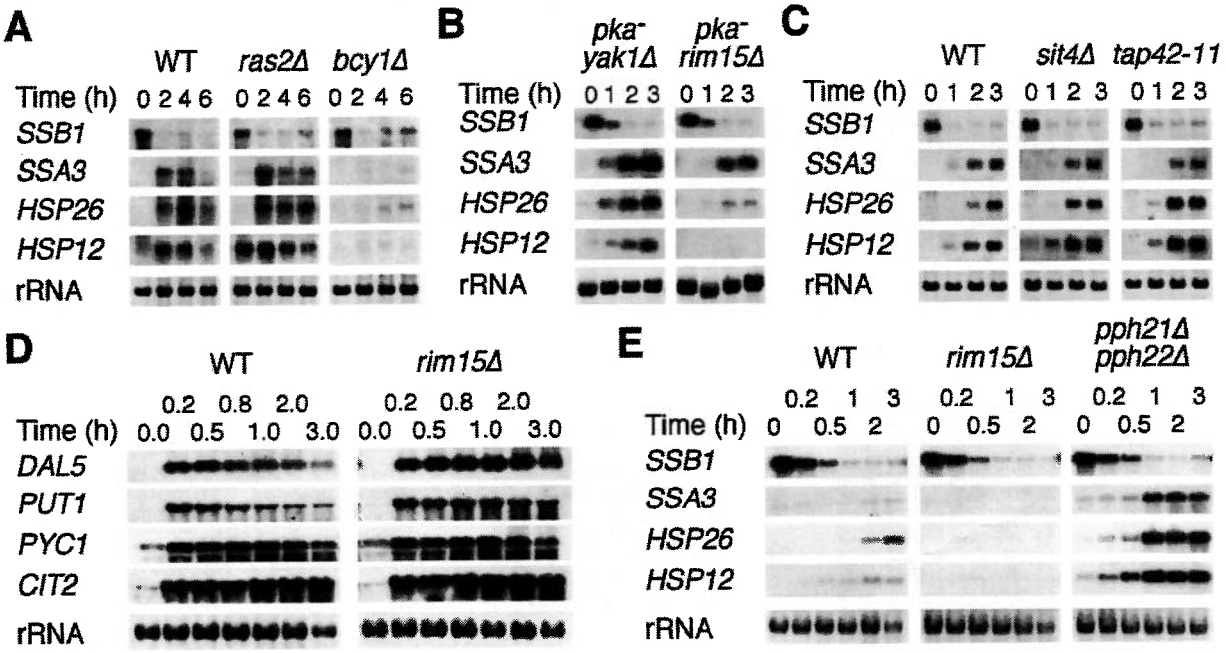

Figure 2. Transcriptional Activation of Rim15-Controlled Genes following TOR Inactivation Does Not Depend on Known PP2A TOR Effectors and Is Negatively Regulated By PKA

SSB1, *SSA3*, *HSP26*, and *HSP12*, or *DAL5*, *PUT1*, *PYC1*, and *CIT2* transcript levels were determined in cells growing exponentially at 30°C (*tap42-11* mutant at 24°C) on rich medium and treated with rapamycin (0.2 μg ml^{-1}) for the times indicated. The decrease in *SSB1* transcript level was used as internal control for rapamycin function. Strains used in (A) were wild-type (SP1), *ras2*Δ (IP16), and *bcy1*Δ (T16-11A); in (B) *pka⁻ yak1*Δ (CDV80-2D) and *pka⁻ rim15*Δ (CDV80-15A); in (C) wild-type (JK9-3da), *sit4*Δ (TS64-1a), and *tap42-11* (TS54-5a); in (D) wild-type (W303-1A) and *rim15*Δ (CDV115); and in (E) wild-type (W303-1A), *rim15*Δ (CDV115), and *pph21*Δ *pph22*Δ (YP0607WH). In order to visualize the strong differences between expression of genes in wild-type and *pph21*Δ *pph22*Δ cells, Northern blots in (E) were all equally exposed, but for a shorter time than in all other experiments. Strains used in individual panels are isogenic.

TOR but Not PKA Regulates Nucleocytoplasmic Distribution of Rim15

To investigate the mechanism by which TOR inactivates Rim15, we transformed various strains with a *GFP-RIM15* plasmid and visualized localization of the corresponding GFP-Rim15 fusion protein in cells treated with rapamycin for various time periods. In untreated cells, GFP-Rim15 was detected predominantly in the cytoplasm and appeared excluded from the nucleus. Within 30 min following rapamycin treatment, however, localization of GFP-Rim15 was predominantly nuclear (Figure 3A), which is consistent with the effects of rapamycin on transcription of Rim15-dependent genes (i.e., measurable induction at 30–45 min following rapamycin treatment; Figure 2E). Moreover, rapamycin-induced nuclear accumulation of GFP-Rim15 was defective in a rapamycin-resistant *TOR1-1* mutant (Helliwell et al., 1994), indicating that the observed effect is due to TOR inactivation. Notably, TOR has been suggested to control subcellular localization of proteins by two different processes, namely prevention of nuclear import (e.g., of Gln3 and Rtg3; Komeili et al., 2000; Crespo et al., 2002) and stimulation of nuclear export (e.g., of Msn2; Görner et al., 2002; Düvel et al., 2003). In the latter case, nuclear appearance of a given protein following TOR inhibition depends largely on its nuclear import rate. The slight delay in rapamycin-induced nuclear appearance of Rim15 (i.e., about 20 min when compared to Gln3 or Rtg3), and possibly also Msn2 (Düvel et al., 2003), could therefore be explained by a model in which inhibition of TOR—rather than activating nuclear import—prevents nuclear export, thus rendering nuclear appearance of Rim15 mainly dependent on a potentially slow import rate.

In order to confirm that TOR controls Rim15 function independently of PKA, we studied rapamycin-induced nuclear translocation of GFP-Rim15 in mutants that are either hyper- or hypoactive for PKA. Accordingly, we found that hyperactivation of PKA (due to expression of *RAS2*^{Val19}) had no significant effect on the rapamycin-induced nuclear translocation of GFP-Rim15 (Figure 3A). For examination of the effects of hypoactive PKA, we used a strain that was rendered responsive to extracellular cAMP by deletion of the genes encoding adenylate cyclase (*CDC35*) and low-affinity phosphodiesterase (*PDE2*). In such a strain, depletion of cAMP (and hence inactivation of PKA) for 30 min (and up to 2 hr) in the presence of rich medium did not cause a change in cytoplasmic localization of GFP-Rim15 (Figure 3B). As a control, we found nuclear import of Msn2-myc9, which is negatively regulated by PKA (Görner et al., 2002), to occur rapidly under the same conditions (Figure 3B). Thus, TOR inactivates Rim15 by preventing its access to or promoting its export from the nucleus via a process that is independent of regulated PKA activity.

TOR-Inhibition Results in Rapid Alteration of the Rim15 Phosphorylation State

How does TOR control nuclear localization of Rim15? GST-Rim15 in total cell extracts from untreated cells

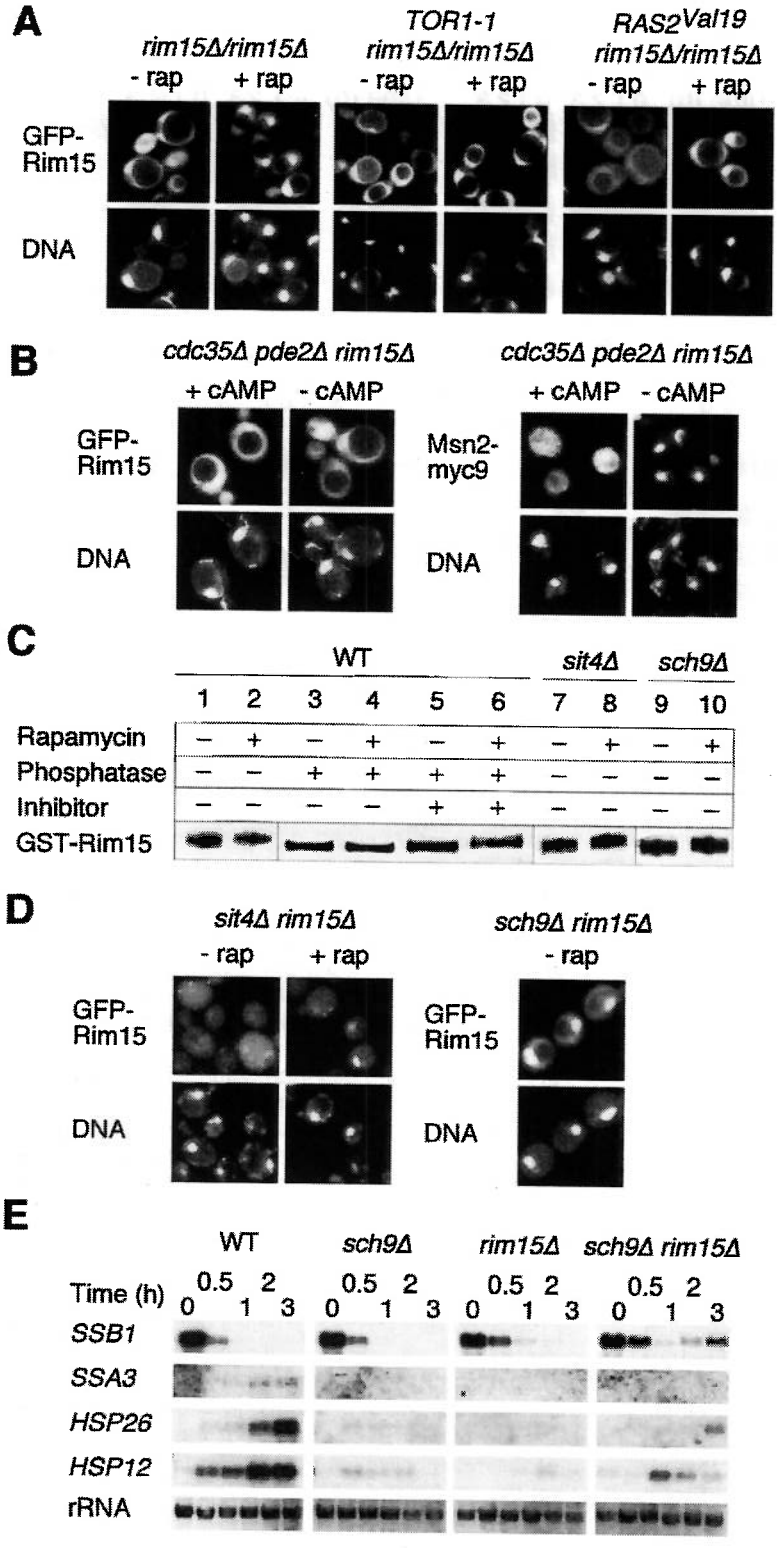

Figure 3. TOR and Sch9 but Not PKA Control Nuclear Localization of Rim15

(A) Localization of GFP-Rim15 in *rim15Δ/rim15Δ* cells (IP37) carrying a control plasmid (YEp213), a plasmid expressing the rapamycin-resistant *TOR1-1* allele, or a plasmid expressing the dominant active *RAS2^{Val19}* allele. Cells were grown on SD-medium, treated for 30 min with rapamycin (+rap) or the drug vehicle alone (−rap), and visualized by fluorescence microscopy. DNA was stained with DAPI.

(B) Localization of GFP-Rim15 and Msn2-myc9 in a *cdc35Δ pde2Δ rim15Δ* triple mutant (CDV177-3C). Cells were grown in SD-medium containing 5 mM cAMP (+) and then shifted for 30 min to the same medium without cAMP (−). Msn2-myc9 was detected by indirect immunofluorescence.

(C) Rim15 is hyperphosphorylated upon rapamycin treatment. GST-Rim15 from *rim15Δ/rim15Δ* (IP37), *sit4Δ* (YPA5H), and *sch9Δ* (TVH301) cells that were untreated (−) or treated (+) for 20 min with rapamycin was detected by immunoblotting. Whole-cell extracts were incubated with alkaline phosphatase (± phosphatase inhibitors).

(D) Localization of GFP-Rim15 in *sit4Δ rim15Δ* (FD6) and *sch9Δ rim15Δ* (RJ201) cells. For details see (A).

(E) *SSB1*, *SSA3*, *HSP26*, and *HSP12* transcript levels were determined in cells growing on rich medium and treated with rapamycin for the times indicated. Strains used were wild-type (W303-1A), *sch9Δ* (TVH301), *rim15Δ* (CDV115), and *sch9Δ rim15Δ* (RJ201). In all experiments, cells were grown exponentially prior to rapamycin treatment (2 μg ml^{-1} for GFP-Rim15 localization studies and 0.2 μg ml^{-1} for all other experiments).

exhibited a higher electrophoretic mobility compared with GST-Rim15 from rapamycin-treated cells (Figure 3C, lanes 1 and 2). Phosphatase treatment of GST-Rim15 prepared from untreated and rapamycin-treated cells converted GST-Rim15 to a similar form (Figure 3C, lanes 3 and 4) that migrated faster than the corresponding controls prepared in the presence of phosphatase inhibitors (Figure 3C, lanes 5 and 6). These results show that exponential-phase Rim15 is phosphorylated in the absence of rapamycin, which is in agreement with our previous findings (Reinders et al., 1998). Moreover, rapamycin treatment induces an additional change in the

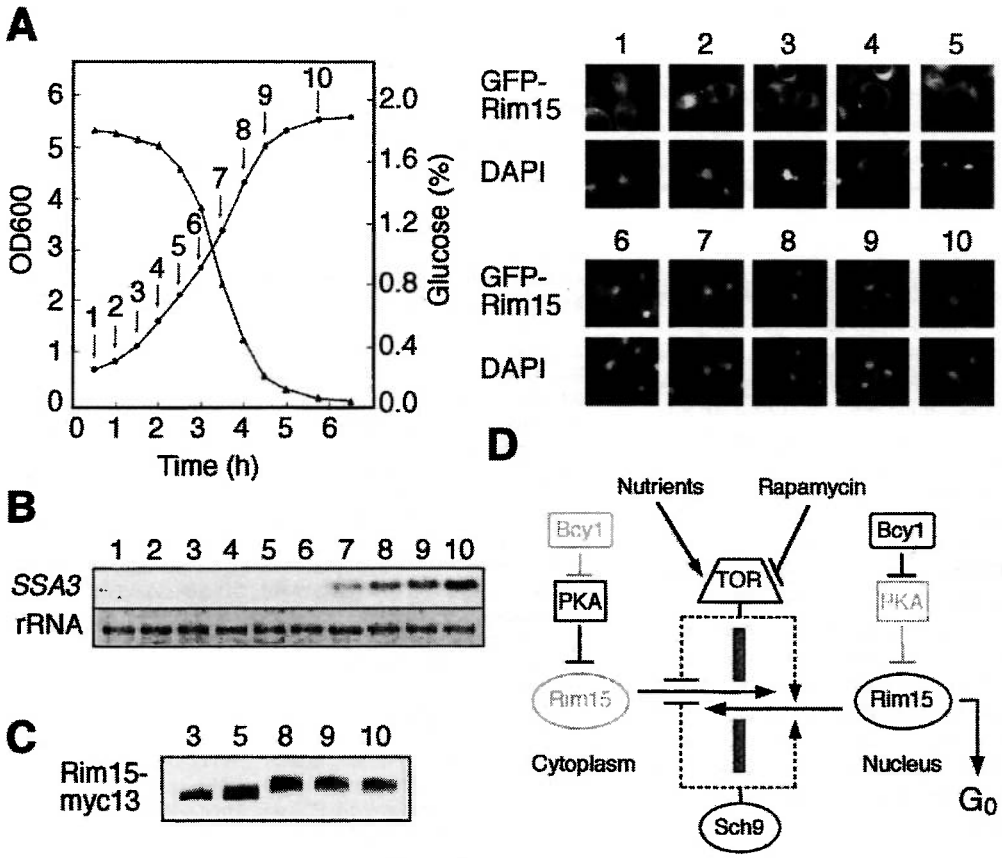

Figure 4. Glucose Limitation Triggers Hyperphosphorylation and Nuclear Accumulation of Rim15, followed by Induction of Rim15-Dependent *SSA3* Transcription

(A) Diploid *rim15Δ/rim15Δ* (IP37) cells carrying a *GFP-RIM15* construct were grown in a batch culture on SD-medium and, at the times indicated by the numbered arrows, samples were withdrawn for glucose assays and OD_{600} determinations (left panel), as well as for GFP-Rim15 localization studies (right panel). Numbers in the right panel refer to the times indicated by the numbered arrows in the left panel.

(B) Glucose-limitation-induced *SSA3* transcript levels in wild-type (KT1960) cells. Numbers refer to the times and corresponding glucose concentrations indicated by the numbered arrows in (A), left panel.

(C) Wild-type strain VW1 expressing Rim15-myc13 was grown in a batch culture and samples were withdrawn for immunodetection of Rim15-myc13 at different time points. Numbers refer to the times and corresponding glucose concentrations indicated by the numbered arrows in (A), left panel.

(D) TOR, via a Sit4-independent mechanism, and Sch9 promote cytoplasmic retention and/or nuclear exclusion of the PKA target Rim15. Arrows and bars denote positive and negative interactions, respectively. Notably, in exponentially growing cells, the cytoplasm hosts low levels of Bcy1 and hence primarily free, active PKA, while the inactive PKA/Bcy1 holoenzyme appears predominantly in the nucleus (Griffioen et al., 2000). Consequently, PKA-mediated inhibition of Rim15 activity is presumably lower in the nucleus than in the cytoplasm. Gray symbols denote low activity levels of the corresponding proteins. TOR and Sch9, according to the simplest interpretation of our results, control Rim15 localization via two different mechanisms. Sch9 may impinge directly or indirectly on Rim15 localization.

phosphorylation state of Rim15, which—based on the lower electrophoretic mobility of Rim15—is likely due to phosphorylation. Interestingly, while rapamycin-induced dephosphorylation of a number of proteins occurs via the Sit4/Tap42 TOR effector branch (e.g., Npr1, Gln3, Rtg3, and Gcn2; Schmidt et al., 1998; Beck and Hall, 1999; Cherkasova and Hinnebusch, 2003; Düvel et al., 2003; Liu et al., 2003), we found rapamycin-induced phosphorylation and nuclear accumulation of Rim15 to be unaffected by the loss of Sit4 (Figure 3C, lanes 7 and 8; Figure 3D). Notably, phosphorylation following rapamycin treatment is not without precedent (e.g., Put3; Saxena et al., 2003), suggesting that TOR may regulate additional proteins via a common, new effector pathway. Taken together, we found that inhibition of the TOR kinases by rapamycin results in both rapid nuclear accumulation of Rim15 and induction of Rim15-dependent transcription. In addition, rapamycin treatment correlates with changes in the phosphorylation state of Rim15, indicating that nucleocytoplasmic transport of Rim15 is likely to be regulated by differential phosphorylation of Rim15.

Nuclear Exclusion of Rim15 Requires the Yeast PKB Homolog Sch9

Recent evidence indicates that the yeast PKB/Akt homolog Sch9 signals the combined presence of glucose and nitrogen and impinges on PKA targets in a pathway parallel to PKA (Crauwels et al., 1997; Lorenz et al., 2000; Fabrizio et al., 2001). In this context, it was reported that life-span extension following both downregulation of PKA (in an adenylate cyclase mutant) or loss of Sch9

depends on the presence of Rim15, suggesting that Rim15 may be negatively regulated by both PKA and Sch9 (Fabrizio et al., 2001). In line with such a model, we found that loss of Sch9—which had no effect on GFP-Rim15 expression levels (data not shown)—resulted in predominantly constitutive nuclear localization of Rim15 (Figure 3D). Thus, Sch9, like TOR, is required for nuclear exclusion and/or cytoplasmic retention of Rim15. Notably, however, since rapamycin-induced phosphorylation of Rim15 appeared to occur even in the absence of Sch9 (Figure 3C, lanes 9 and 10), TOR and Sch9 are likely to regulate Rim15 function via two different mechanisms. Finally, we also studied rapamycin-sensitivity of *SSA3*, *HSP26*, and *HSP12* transcription in wild-type, *rim15Δ*, *sch9Δ*, and *sch9Δ rim15Δ* cells. We found that loss of Sch9 resulted in a defect in rapamycin-induced activation of all three genes, which was independent of the presence or absence of Rim15 (Figure 3E). This rather surprising result indicates that Sch9, while acting as an inhibitor of Rim15 nuclear accumulation, formally also acts (directly or indirectly) as an activator of Rim15-controlled gene expression. In line with this interpretation, Sch9 has been found to be required for downregulation of PKA (Crauwels et al., 1997), an inhibitor of Rim15 protein kinase activity (Reinders et al., 1998). Thus, our ostensibly paradoxical data can be unified in a tantalizing model in which Sch9–in response to the nutritional status—acts a molecular buffer system by regulating the amplitude of Rim15-dependent responses via two opposing mechanisms.

Glucose Limitation Causes Hyperphosphorylation and Nuclear Accumulation of Rim15

We have previously shown that expression driven by the postdiauxic shift (PDS) element, which confers transcriptional activation (e.g., of *SSA3*) following glucose limitation at the diauxic transition, is almost entirely dependent on Rim15 and its presumed target Gis1 (Pedruzzi et al., 2000). Therefore, we tested whether glucose-limitation-induced activation of *SSA3* may be preceded by nuclear accumulation of GFP-Rim15. Indeed, we found that cells grown in a batch culture started to accumulate GFP-Rim15 in their nuclei when 50% of the initial amount of glucose has been consumed (Figure 4A, time point 6), which is consistent with the observed pattern of transcriptional induction of *SSA3* (Figure 4B). Interestingly, we obtained similar results when growing the cells on rich media with only 1% (instead of 2%) initial glucose levels (data not shown), indicating that the underlying regulatory system may (directly or indirectly) sense the kinetics of glucose limitation, rather than the absolute glucose levels. Moreover, since glucose limitation, like rapamycin treatment, caused nuclear accumulation accompanied by hyperphosphorylation of Rim15 (Figure 4C), it is possible that TOR may have a central role in this process (Figure 4D). Thus, together with the recent observation that TOR regulates subcellular localization of proteins in response to glutamine (e.g., Gln3, Rtg3, and Ime1; Crespo et al., 2002; Colomina et al., 2003), our current data lend further support to the idea that TOR may act as a multichannel processor that differentially regulates gene expression in response to specific nutrients.

Experimental Procedures

Strains, Plasmids, and Media

Yeast strains *sit4Δ* (TS64-1a), *tap42-11* (TS54-5a), and their wild-type parent (JK9-3da), and strains *sit4Δ* (YPA5H), *pph21Δ pph22Δ* (YP0607WH), and their wild-type parent (W303-1A) were previously described (Beck and Hall, 1999; Sakumoto et al., 2002). Wild-type strains KT1960 (MATα) and KT1961 (MATa) are *ura3, leu2, his3,* and *trp1*, and congenic to KT1112 (Stuart et al., 1994). Strains AR1-1B, SP1, T16-11A, NB13-1D, SGP406, and PD6517 were also described earlier (Reinders et al., 1998; Pedruzzi et al., 2000; Garrett and Broach, 1989). PCR-based gene deletions (*rim15Δ::kanMX* transformed into JK9-3da, KT1960, and W303-1A to create IP11, IP31, and CDV115, respectively; *ras2Δ::kanMX* transformed into SP1 to create IP16; *sit4Δ::TRP1* transformed into AR1-1B to create FD6; *cdc35Δ::kanMX* and *pde2Δ::TRP1* transformed into CDV147 [KT1960 X KT1961] to create CDV173; *sch9Δ::TRP1* transformed into W303-1A to create TVH301; and *sch9Δ::LEU2* transformed into CDV115 to create RJ201) and tagging of chromosomal *RIM15* (*RIM15-myc13-kanMX* transformed in KT1960 to create VW1) were done as described (Longtine et al., 1998). The homozygous *rim15Δ::kanMX/rim15Δ::kanMX* strain IP37 was created by PCR-based deletion of *RIM15* in CDV147, sporulation of the resulting heterozygous diploid, and mating of appropriate *rim15Δ::kanMX* haploids following tetrad analysis. The isogenic strains CDV80-2D and CDV80-15A are segregants of strain CDV80 that was created by mating S7-7A X S7-5A-derived NB13-1D and SGP406 (Reinders et al., 1998). Strain CDV173-3A (*MATa cdc35Δ::kanMX pde2Δ::TRP1* [pCDV548]) is a segregant of CDV173 that carried a low copy plasmid (*CEN, URA3*; pCDV548) expressing *cdc35-10* (amplified from strain PD6517). Mating of IP31 and CDV173-3A (both of isogenic background), followed by sporulation of the resulting diploid CDV177 yielded CDV177-3C (*MATa cdc35Δ::kanMX pde2Δ::TRP1 rim15Δ::kanMX* [pCDV548]). Growth on plates containing 5-FOA and 5 mM cAMP allowed selection of CDV177-3C that had lost pCDV548.

GFP- and GST-tagged versions of Rim15 were expressed under the control of the *ADH1* and *GAL1* promoters from a low (pFD633) and high (pCDV487) copy number plasmid, respectively. Plasmids YEp213-*RAS2*^{Val19}, pADH1-Msn2-myc9, and pSEY18-*TOR1-1* (pPW2) were described earlier (Broek et al., 1987; Gömer et al., 1998; Helliwell et al., 1994). Strains were grown at 30°C (except the *tap42-11* mutant, which was grown at 24°C) in standard rich medium (YPD) with 2% glucose or synthetic medium with 2% glucose (SD), 4% galactose (SGal), or 2% raffinose (SRaf) as carbon source (Burke et al., 2000). Rapamycin (dissolved in 90% ethanol/10% Tween-20) was added to the media at a final concentration of 2 μg ml^{-1} for GFP-Rim15 localization studies and 0.2 μg ml^{-1} for all other experiments.

Immunoblot Analyses

For immunoblot analyses, cells expressing GST-Rim15 from the *GAL1* promoter were grown to early logarithmic phase in SRaf medium. Expression of GST-Rim15 was induced by growth in SGal for 3 hr, followed by 3 hr in YPD. After that, cells were either treated for 20 min with rapamycin (+rap) or with the drug vehicle alone (−rap). Protein extracts were prepared using a standard postalkaline extraction method (Figure 3C, lanes 1 and 2 and 7–10), or a whole-cell extraction method as described (Figure 3C, lanes 3–6; Reinders et al., 1998). GST-Rim15 fusion proteins from whole-cell extracts were bound to glutathione agarose beads, washed extensively, eluted with 5 mM glutathione, and subjected to standard immunoblot analysis. Dephosphorylation of eluted GST-Rim15 was done by a 15 min incubation at 37° with 1 unit of alkaline phosphatase (Roche Diagnostics). In control reactions, phosphatase inhibitors (10 mM NaF, 10 mM p-nitrophenylphosphate, 10 mM sodiumpyrophosphate, and 10 mM β-glycerophosphate) were added.

Miscellaneous

Northern analyses, trehalose measurements, flow cytometry, and indirect immunofluorescence were performed as described (Reinders et al., 1998; Burke et al., 2000). Glucose was measured using the GOD-PAP kit (HUMAN, GmbH).

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II.3) Conclusion

The TORC1 and PKA pathways regulate cell proliferation in yeast in response to nutrients. Accordingly, rapamycin treatment, loss of TOR or loss of PKA cause cells to arrest growth in early G_1 and to express several physiological characteristics of quiescent cells (G_0). The effector pathway by which TOR inactivation triggers most responses including inhibition of mRNA translation, amino acid import, and cytosolic retention of nutrient responsive transcription factors involves the type 2A protein phosphatase (PP2A) Sit4 and its regulator Tap42 (67-69, 72, 78, 90). This study shows that proper entry into G_0 following TORC1 inactivation depends on the protein kinase PKA target Rim15. TORC1 prevents induction of the Rim15-dependent responses (*i.e.,* glycogen and trehalose accumulation, STRE- and PDS element-controlled gene transcription and proper growth arrest at the diauxic phase) by inhibiting cytosolic to nuclear translocation of Rim15 via a Sit4- and Tap42-independent mechanism. This work also revealed that, similarly to TORC1, the protein kinase Sch9, which acts in a nutrient sensing pathway that controls cell growth, also negatively regulates nuclear localisation of Rim15 (318). Finally, this work led to the finding that Rim15 function is controlled by PKA-dependent inactivation of its kinase activity, and nutrient-limitation or rapamycin-induced nuclear import of the protein. Thus, Rim15 defines a new TORC1 effector branch that controls entry into G_0 and represents a point of convergence of three key nutrient signalling kinases (*i.e.*, the TORC1, Sch9 and PKA).

Chapter III.

The TOR and EGO Protein Complexes Orchestrate Microautophagy in Yeast

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III.1) Introduction

The pathways that regulate entry of cells into G_0 as a result of downregulation of TORC1 and/or of nutrient starvation are understood in considerable details, yet the mechanisms that control maintenance of the G_0 state and the transition from quiescence back to proliferation have received surprisingly little attention thus far. Accordingly, only one gene has been reported to be specifically required for G_0 exit (*GCS1*) (8). Establishment of the G_0 state as a phase distinct from G_1 is mainly based on the fulfilment of two key requirements: the presence of G_0-specific biochemical activities and of proteins that are specifically required for the transition between G_0 and the cell cycle. This study was aimed at identifying genes whose product are required specifically for G_0 exit and, via their description and characterisation, at finding the underlying mechanisms regulating this process. Elucidating these mechanisms is expected to allow a better characterisation of the yeast G_0 state and, because inappropriate exit from G_0 in human cells can cause tumours, possibly lead to a better understanding on how tumour could progress. In a fist approach to find these components, the entire yeast knock out collection (YKO) was screened for mutants with a specific cell proliferation defect following a G_0 arrest artificially induced by rapamycin treatment.

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The TOR and EGO Protein Complexes Orchestrate Microautophagy in Yeast

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Summary

The rapamycin-sensitive TOR signaling pathway in *Saccharomyces cerevisiae* positively controls cell growth in response to nutrient availability. Accordingly, TOR depletion or rapamycin treatment causes regulated entry of cells into a quiescent growth phase. Although this process has been elucidated in considerable detail, the transition from quiescence back to proliferation is poorly understood. Here, we describe the identification of a conserved member of the RagA subfamily of Ras-related GTPases, Gtr2, which acts in a vacuolar membrane-associated protein complex together with Ego1 and Ego3 to ensure proper exit from rapamycin-induced growth arrest. We demonstrate that the EGO complex, in conjunction with TOR, positively regulates microautophagy, thus counterbalancing the massive rapamycin-induced, macroautophagy-mediated membrane influx toward the vacuolar membrane. Moreover, large-scale genetic analyses of the EGO complex confirm the existence of a growth control mechanism originating at the vacuolar membrane and pinpoint the amino acid glutamine as a key metabolite in TOR signaling.

Introduction

The highly conserved target of rapamycin (TOR) proteins control cell proliferation in yeast, flies, and mammalian cells in response to growth factors and/or nutrients (Jacinto and Hall, 2003). *S. cerevisiae* cells express two TOR homologs, Tor1 and Tor2, both of which—when associated with Lst8 and Kog1 in the TORC1 complex—are targets of the therapeutically important, immune-suppressive macrolide rapamycin in complex with the peptidyl-prolyl isomerase Fpr1 (also known as FK506 binding protein [FKBP] in mammals) (Jacinto and Hall, 2003; Loewith et al., 2002). Binding of the rapamycin-FKBP complex to TORC1, a mode of action that is conserved from yeast to human (Hara et al., 2002; Kim et al., 2002), inhibits the activity of the TOR kinases and elicits a number of responses that mimic nutrient starvation, including a decrease of protein synthesis and ribosome biogenesis, specific changes in gene transcription, sorting and turnover of nutrient permeases, induction of autophagy, cell cycle arrest at the G_1/S boundary, and entry into G_0 (Jacinto and Hall, 2003). Downstream of TORC1, many processes are regulated by the type 2A (Pph21 and Pph22) or type 2A-related (Sit4) protein phosphatases (PP2As) and the regulatory proteins Tap42 and Tip41 (Düvel and Broach, 2004). For instance, rapamycin treatment causes activation of the GATA-type transcription factors Gln3 and Gat1 via modulation of Tap42, thereby inducing (among others) the expression of the nitrogen discrimination pathway (NDP) genes, whose products serve to assimilate alternative nitrogen sources (e.g., proline and allantoin) and to synthesize glutamine (Cooper, 2002). In addition, rapamycin-induced, Sit4-dependent dephosphorylation results in activation of the Npr1 protein kinase, which in turn induces degradation of the high-affinity tryptophan permease Tat2 (Schmidt et al., 1998).

TOR has been postulated to act as a multichannel processor that integrates different nutritional signals (Shamji et al., 2000). However, the putative nutrient metabolites that activate TOR signaling still remain elusive, although some evidence exists suggesting that certain amino acids, specifically glutamate and glutamine, may be important nutrients in TOR signaling (Crespo et al., 2002; Komeili et al., 2000; Shamji et al., 2000). Both glutamate and glutamine are key intermediates in nitrogen metabolism that can readily be converted to α-ketoglutarate (for use in TCA cycle) or serve as immediate precursors for the biosynthesis of other amino acids, nucleotides and nitrogen-containing molecules, such as NAD^+ (Magasanik and Kaiser, 2002). Starvation for glutamine results in a phenotype similar to TOR inactivation, inasmuch as it causes nuclear localization and activation of Gln3 and of the heterodimeric transcription factor Rtg1/Rtg3, which in turn activates genes whose products (e.g., mitochondrial and peroxisomal enzymes) are required for biosynthesis and homeostasis of glutamate and glutamine (Butow and Avadhani, 2004; Crespo et al., 2002). Both Rtg1 and Rtg3, as well as the upstream regulatory Rtg2 protein, were originally identified as mediators of a mitochondria-to-nucleus signaling pathway, or retrograde response pathway, that senses the functional state of mitochondria via the level of glutamate and/or glutamine (Butow and Avadhani, 2004). These findings underline the potential importance of glutamine in TOR signaling, although it is not clear whether TOR signaling and retrograde regulation constitute distinct pathways that converge on Rtg1/Rtg3 or whether they are part of the same pathway (Butow and Avadhani, 2004; Crespo et al., 2002; Tate and Cooper, 2003).

An important aspect of cellular fitness under conditions of TOR inactivation and/or nutrient starvation is the capacity to ensure the production of essential proteins, which is achieved in part by downregulation of general (but not specific) protein synthesis. Accordingly, TOR depletion or rapamycin treatment results in the destabilization of the initiation factor eIF4G, inhibition of ribosome biogenesis, and activation of the highly conserved Gcn2 protein kinase via a Tap42-dependent mechanism (Cherkasova and Hinnebusch, 2003; Jacinto and Hall, 2003). Once activated, Gcn2 stimulates phosphorylation of the α subunit of the

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translation initiation factor 2 (eIF2α), thereby slowing the rate of GDP-GTP exchange on eIF2. This process reduces the overall translation initiation rate, yet specifically favors translation of the *GCN4* mRNA, which encodes a transactivator of amino acid biosynthetic genes (Hinnebusch, 1997). Under conditions of TOR depletion, translation of such critical factors as Gcn4 is accomplished by regeneration of internal nutrient pools via macroautophagic recycling of cytoplasmic material (Jacinto and Hall, 2003; Levine and Klionsky, 2004).

The pathways that regulate entry of cells into a quiescent growth phase as a result of downregulation of TOR and/or nutrient starvation are understood in considerable detail, yet the mechanisms that control the transition from quiescence back to proliferation have received surprisingly little attention thus far (Gray et al., 2004). Here, we describe the discovery of a vacuolar membrane associated protein complex, which plays an essential function in exit from rapamycin-induced growth arrest: the EGO complex. We demonstrate that the EGO complex, in conjunction with TOR, positively regulates microautophagy. This process, which counterbalances the massive rapamycin-induced, macroautophagy-mediated membrane influx toward the vacuolar membrane, is essential for resumption of growth following rapamycin treatment.

Results

Exit from Rapamycin-Induced Growth Arrest: A Functional Profile of the Yeast Genome

To identify genes whose products are involved in exit from rapamycin-induced growth arrest, we individually screened (in duplicate) 4857 viable yeast deletion mutants for a defect in resumption of growth following rapamycin treatment (see Experimental Procedures). A total of eight mutants were recovered in this screen: three of these were defective in phospholipid and amino acid metabolism (i.e., *pib2Δ*, *sac3Δ*, and *hom2Δ*), one in molecular chaperone activity (*ydj1Δ*), and one in an as-yet-unidentified cellular process (*ydl172cΔ*). The remaining three mutants (*ykr007wΔ/meh1Δ/ego1Δ*, *gtr2Δ*, and *ybr077cΔ/ego3Δ*) were grouped in a separate class based on our findings that the corresponding gene products act in a common protein complex as assayed by both two-hybrid (Figure 1A) and coprecipitation analyses (Figure 1B). Notably, the *ykr007wΔ/meh1Δ/ego1Δ*, *gtr2Δ*, and *ybr077cΔ/ego3Δ* mutants normally responded to rapamycin treatment by transcriptional induction of a characteristic set of stress response genes (e.g., *HSP12*, *HSP26*, and *GRE1*; Figure 2A), transcriptional repression of *SSB1* (Figure 2A), accumulation of glycogen (Figure 2B), induction of macroautophagy (Figures 2C and 2D), and inhibition of protein synthesis (Figures 2E and 2F). Thus, by several criteria, the three mutants normally enter into the rapamycin-induced growth arrest program. Importantly, despite their defect in recovery from rapamycin treatment (Figure 2G), the *ykr007wΔ/meh1Δ/ego1Δ*, *gtr2Δ*, and *ybr077cΔ/ego3Δ* mutants did not significantly lose viability (as assayed by trypan blue exclusion experiments) during the rapamycin treatment and during at least the first 48 hr following rapamycin release (data not shown). These results, together with our observation that rapamycin-induced inhibition of protein synthesis (as assayed by both incorporation of radioactively labeled amino acids into TCA precipitable material [Figure 2E; and data not shown] and phosphorylation of eIF2α on Ser 51 [Figure 2F]) could be reversed in wild-type cells, but not in the *ykr007wΔ/meh1Δ/ego1Δ*, *gtr2Δ*, and *ybr077cΔ/ego3Δ* mutants, argue strongly that the corresponding three proteins are essential for resumption of growth following rapamycin treatment. For the remainder of the text, we will refer to Ykr007W/Meh1/Ego1 as Ego1, Ybr077C/Ego3 as Ego3, and the protein complex containing Ego1, Gtr2, and Ego3 as the EGO complex.

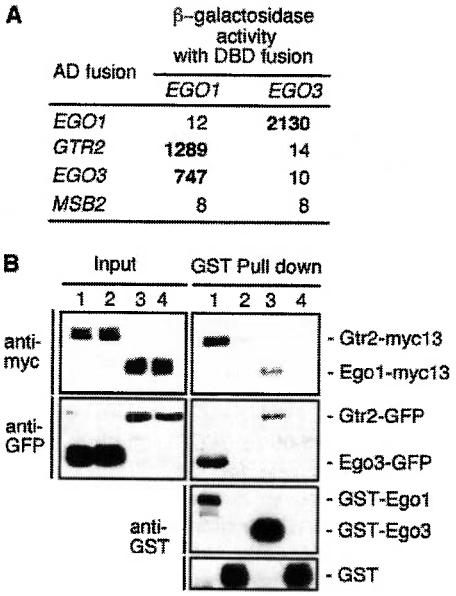

Figure 1. Ego1, Gtr2, and Ego3 Are Constituents of the EGO Protein Complex

(A) Two-hybrid interactions between Ego1, Gtr2, and Ego3. β-Galactosidase activities were measured in three independent isolates of each strain after growth for 16 hr at 30°C in SGal/Raf medium. The average values (in Miller units) are shown. Values that are at least tenfold higher than the corresponding control (*MSB2*) are shown in boldface.

(B) Coprecipitation of Ego1, Gtr2, and Ego3. Strain FD24 expressing genomically tagged Gtr2-myc13 and Ego3-GFP was transformed with pCDV1084, which expresses *GST-EGO1* under the control of the *ADH1* promoter (lane1), or with a control plasmid (pCDV1082; *ADH1-GST*; lane 2). To independently confirm the coprecipitation of the three proteins, strain FD25 expressing genomically tagged Ego1-myc13 and Gtr2-GFP was transformed with pCDV1083, which expresses *GST-EGO3* under the control of the *ADH1* promoter (lane 3), or with a control plasmid (pCDV1082; *ADH1-GST*; lane 4). Cell lysates (Input) and GST pull-down fractions were subjected to PAGE and immunoblots were probed using anti-myc, anti-GFP, or anti-GST antibodies as indicated.

The EGO Complex Harbors a Member of the RagA Subfamily of Ras-Related GTPases

Gtr2 has previously been described as a member of a new G protein family that includes its yeast homolog Gtr1 and the mammalian RagA, -B, -C, and -D (Naka-

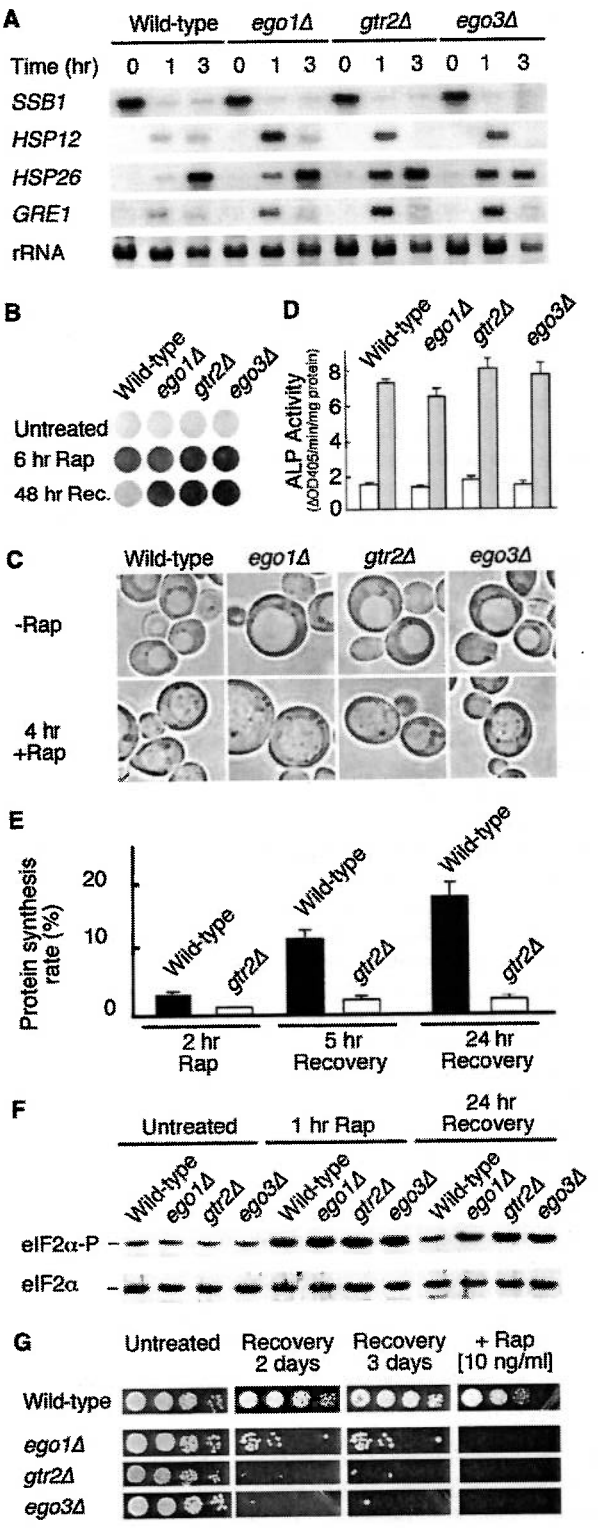

Figure 2. The EGO Complex Is Essential for Exit from, but Not Entry into, the Rapamycin-Induced Growth Arrest Program

(A) Northern analysis of indicated genes; (B) glycogen levels (visualized after exposure for 1 min to iodine vapor); and (C) microscopic analysis of macroautophagy (in the presence of 1 mM PMSF to prevent vacuolar degradation of autophagic bodies) of exponentially growing wild-type (KT1960) and isogenic *ego1Δ* (CDV210), *gtr2Δ* (CDV212), and *ego3Δ* (CDV211) mutant cells following treatment with rapamycin (200 ng/ml) for the times indicated. (D) Wild-type (TS139), *ego1Δ* (CDV284), *gtr2Δ* (CDV286), and *ego3Δ* (CDV285) cells (all expressing the modified alkaline phosphatase form Pho8Δ60) were grown exponentially and treated for 6 hr with rapamycin (200 ng/ml; gray bars) or the drug vehicle alone (white bars) and then subjected to the alkaline phosphatase (autophagy) assay. Data represent mean ± SD (n = 4). (E) Protein synthesis rates were measured in wild-type (KT1960) and *gtr2Δ* (CDV212) mutant cells following a 2 hr rapamycin treatment (200 ng/ml) and after recovery (for either 5 or 24 hr) from a 6 hr rapamycin treatment and expressed in % of the protein synthesis rate in corresponding untreated exponentially growing cells. Protein synthesis rates in exponentially growing wild-type and *gtr2Δ* cells were essentially the same. Data represent mean ± SD (n = 4). (F) Reversal of rapamycin-induced eIF2α phosphorylation (on Ser 51) during recovery from rapamycin treatment requires the presence of Ego1, Gtr2, and Ego3. Wild-type, *ego1Δ*, *gtr2Δ*, and *ego3Δ* cells (same strains as in A–C) were grown and treated as in (E). Whole cell extracts were prepared prior to rapamycin treatment (exponentially growing cells; untreated), after a 1 hr rapamycin treatment, and after recovery for 24 hr from a 6 hr rapamycin treatment. Phosphorylation of Ser 51 in eIF2α was detected by immunoblot analysis with antibodies specific for eIF2α phosphorylated on Ser 51 (eIF2α-P) and compared with the total amount of eIF2α detected with antibodies against eIF2α. (G) Behavior of *ego1Δ*, *gtr2Δ*, and *ego3Δ* mutants with a defect in recovery from rapamycin treatment. Exponentially growing wild-type (BY4741) and isogenic YKO mutant cells (OD_{600} of 1.0) were treated for 6 hr with rapamycin (200 ng/ml), washed twice, resuspended in YPD without rapamycin, and spotted on YPD plates. Spots (4 μl) correspond to serial 10-fold dilutions of equally dense cultures (i.e., the first spot in each column results from a culture with an OD_{600} of 1.0). In control experiments, the same exponentially growing, but untreated cultures were directly spotted on YPD plates (untreated) or on YPD plates containing a subinhibitory concentration of rapamycin (+Rap [10 ng/ml]). Cells were grown on YPD (A–G).

shima et al., 1999; Sekiguchi et al., 2001). While the specific function of these proteins is still unknown, genetic studies have implicated Gtr2 in the control of the Ran/Gsp1-GTPase, which plays a prominent role in nuclear trafficking of macromolecules (Nakashima et al., 1999). To investigate the function of Gtr2, we mutated the conserved Glu (Q) 66 residue to Leu (L), which—in analogy with the paradigmatic Ras mutations that lead to oncogenic transformation (Boguski and McCormick, 1993)—should result in a GTPase-deficient, constitutively GTP bound $Gtr2^{Q66L}$ protein. Interestingly, unlike wild-type Gtr2, the $Gtr2^{Q66L}$ variant did not complement the defect for exit from rapamycin-induced growth arrest of *gtr2Δ* cells (Figure 3A) and caused a defect in recovery from rapamycin treatment and rapamycin hypersensitivity when expressed in wild-type cells (Figures 3A and 3B). This dominant negative phenotype could result from the failure of the presumably GTP-locked $Gtr2^{Q66L}$ protein to effectively release a critical downstream effector and indicates that Gtr2 may function as a small GTPase that becomes essential for growth under conditions of diminished TORC1 activity. In support of this notion, we found that loss of Gtr2, like loss of Ego1 and Ego3, resulted in pronounced rapamycin hypersensitivity (Figures 2G and 3C). Moreover, since overproduction of either Gtr2, or Ego3 conferred significantly enhanced resistance to rapamycin treatment (Figure 3D), our data indicate that the EGO complex functions in the rapamycin-sensitive TORC1 network.

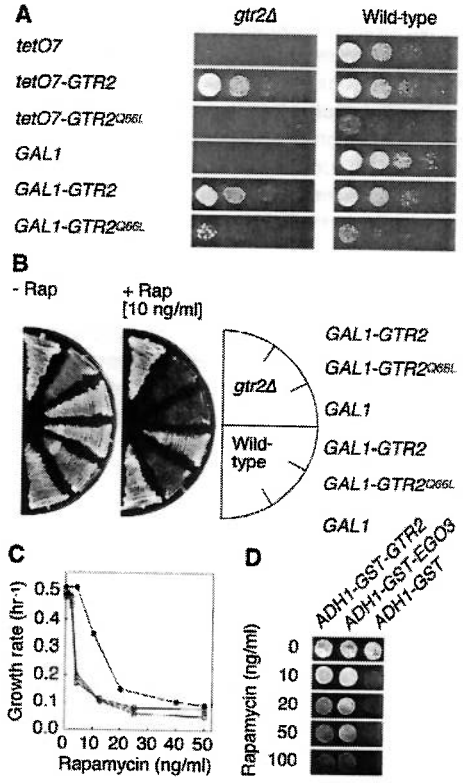

Figure 3. The EGO Complex Functions in the TORC1 Signaling Network

(A and B) Expression of Gtr2^{Q66L} mutant protein results in a dominant-negative rapamycin hypersensitivity phenotype. Expression of the Gtr2^{Q66L} (but not of Gtr2) protein from the *tetO7* or *GAL1* promoter fails to complement the recovery defect following a 6 hr rapamycin treatment in *gtr2Δ* cells (A), strongly reduces the ability of wild-type cells to recover from a 6 hr rapamycin treatment (A), and results in rapamycin hypersensitivity (B). Wild-type (KT1960) and *gtr2Δ* (CDV212) cells bearing *tetO7* (pCM189), *tetO7-GTR2* (pVW1009), *tetO7-GTR2*^{Q66L} (pVW1010), or *GAL1* (YCpIF2) on centromeric plasmids, or else *GAL1-GTR2* (pCDV991) and *GAL1-GTR2*^{Q66L} (pCDV992) integrated at the *LEU2* locus, were pregrown overnight on SD or SGal/Raf to induce expression from either the *tetO7* or *GAL1* promoter, respectively. Subsequently, cells were either subjected to a 6 hr rapamycin treatment, washed, resuspended, and spotted on YPD plates as in Figure 2G (A) or directly streaked on YPD plates containing either no (– Rap), or a subinhibitory amount of rapamycin (+ Rap [10 ng/ml]) (B). Plates were incubated for 2 days at 30°C.

(C) EGO complex mutants are hypersensitive to rapamycin. Growth rates of wild-type (●), *ego1Δ* (□), *gtr2Δ* (○), and *ego3Δ* (△) YKO strains are shown as a function of the rapamycin concentration in the medium (YPD).

(D) Overexpression of either *GTR2* or *EGO3* from the strong constitutive *ADH1* promoter confers enhanced rapamycin resistance. Wild-type cells (CDV147) carrying either the integrative plasmid pCDV1080 (*ADH1-GST-GTR2*), pCDV1083 (*ADH1-GST-EGO3*), or a corresponding control plasmid (pCDV1082; *ADH1-GST*) were grown on SD medium to an equal density (OD_{600} of 2.0), spotted (4 μl) on YPD plates that contained either no—or the indicated amount of—rapamycin, and incubated for 4 days at 30°C.

The EGO Complex Localizes to the Vacuolar Membrane

Based on our biochemical and genetic results, we anticipated that the localization of Ego1, Gtr2, and Ego3 should overlap in the cell. To test this prediction, the chromosomal copies of *EGO1*, *GTR2*, and *EGO3* were fused at their 3′-encoding ends with the green fluorescent protein (GFP)-expressing tag. All three GFP fusion constructs were expressed under their own promoters (Figure 4A, left panel) and functioned normally (as assayed by their competence to promote exit from rapamycin-induced growth arrest; Figure 4A, right panel). Interestingly, all three fusion proteins predominantly localized to the limiting membrane of the vacuole, which was confirmed by their colocalization with the lipophilic vacuolar membrane dye FM4-64 (Vida and Emr, 1995) (Figure 4B). Consistent with their observed localization to the vacuolar membrane, Ego1-GFP and Ego3-GFP cosedimented largely with the vacuolar alkaline phosphatase (ALP) in the low-speed pellet fraction (Figure 4C). A large part of Gtr2-GFP, however, was lost from this fraction and solubilized readily into the supernatant, indicating that Gtr2 is more weakly associated to the vacuolar membrane than Ego1 and Ego3. (Notably, the levels of Ego1-GFP, Gtr2-GFP, and Ego3-GFP did not dramatically change during the first 3 hr of rapamycin treatment; Figure 4D).

EGO and Rapamycin-Sensitive TOR Complexes Regulate Microautophagy

The vacuoles of EGO mutants, like the ones in wild-type cells, appeared normal during exponential growth and steadily increased in size in response to rapamycin treatment (Figure 5A). This phenotype results from the massive influx of membranous material toward the vacuolar membrane due to the continuous fusion of the outer membranes of macroautophagosomes (Levine and Klionsky, 2004). Accordingly, 3–6 hr following rapamycin treatment, most mutant and wild-type cells harbored almost exclusively one single, enlarged vacuole (Figure 5A). Intriguingly, however, while this process was completely reversed in wild-type cells upon release of the rapamycin block, the vacuoles in *ego1Δ*, *gtr2Δ*, and *ego3Δ* mutants continued to increase in size during the 24 hr recovery period on medium without rapamycin, indicating persistent macroautophagy in these cells (Figure 5A). Therefore, the EGO complex plays a critical role in counterbalancing the influx of membranous material toward the vacuolar membrane.

Current evidence suggests that membrane efflux from the vacuolar membrane can occur by either of two fundamentally different processes, namely microautophagy and retrograde traffic out of the vacuole (Bryant et al., 1998; Dove et al., 2004; Müller et al., 2000). To determine whether the EGO complex mediates microautophagy, we expressed Gtr2 conditionally during the recovery phase from rapamycin treatment. Interestingly, following an induction period of 8 hr on galactose-containing medium, we detected in *gtr2Δ* cells carrying the *GAL1-GTR2* construct, but not in control cells (or in cells expressing *GAL1-GTR2*^{Q66L}; data not shown), the simultaneous establishment of vacuolar membrane invaginations (sometimes occurring concurrently at different sites) leading to the formation and release of small vesicles into the lumen of the vacuole (Figure 5B; similar results were obtained when *GAL1-EGO1* and *GAL1-EGO3* were conditionally expressed

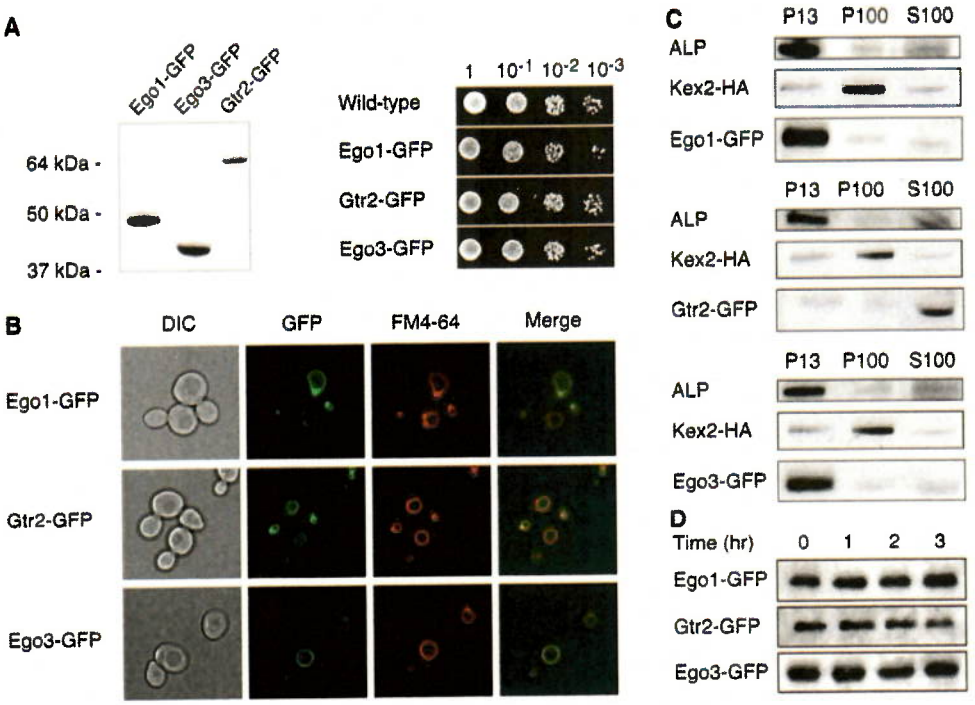

Figure 4. The EGO Complex Localizes to the Limiting Membrane of the Vacuole

(A) Wild-type cells (KT1961) carrying chromosomally tagged *EGO1-GFP* (CDV213), *EGO3-GFP* (CDV214), and *GTR2-GFP* (CDV215) express proteins of 48.2, 46.4, and 66.6 kDa (left panel), respectively, which function normally in permitting exit from rapamycin-induced growth arrest (right panel). For details, see Figure 2G.

(B) Ego1-GFP, Gtr2-GFP, and Ego3-GFP localize to the limiting membrane of the vacuole. Cells (same as in [A]) were labeled with the vacuolar membrane fluorescent dye FM4-64 and the localization of FM4-64 and Ego1-GFP, Gtr2-GFP, and Ego3-GFP was compared by fluorescence microscopy. DIC, differential-interference contrast.

(C) Subcellular fractionation of cells (same as in [A]) expressing Ego1-GFP (upper panel), Gtr2-GFP (panel in the middle), and Ego3-GFP (lower panel), as well as Kex2-HA (pSN222). Exponentially growing cells were made into spheroplasts, lysed by osmotic shock (Dove et al., 2004), and sedimented sequentially to give a 13,000 × g pellet (P13), a 100,000 × g pellet (P100), and a supernatant (S100). Fractions were separated by SDS-PAGE and analyzed by immunoblot analyses using anti-ALP, anti-HA, and anti-GFP antibodies. Kex2-HA and ALP serve as Golgi and vacuolar membrane markers, respectively.

(D) Levels of Ego1-GFP, Gtr2-GFP, and Ego3-GFP following rapamycin treatment (200 ng/ml) for the times indicated were analyzed by immunoblot analysis using anti-GFP antibodies. Equal amounts of total protein were loaded in each lane. Strains (same as in [A]) were grown on YPD medium.

during the recovery phase from rapamycin treatment in corresponding *ego1Δ* and *ego3Δ* mutants, respectively; Figure 5C). These vesicles, which could be released in less than 90 s (Figure 5D), moved freely around the vacuolar lumen indicating that they had fully separated from the vacuolar membrane. Since the formation of these vesicles, which was also observed in wild-type cells recovering from rapamycin treatment (Figure 5B), meets the classical definition of microautophagy, we conclude that the EGO complex is required for the induction of microautophagy during recovery from rapamycin treatment. We could not observe (neither in wild-type nor in *ego* mutant cells) microautophagic vesicle formation during the treatment with rapamycin, and therefore further conclude that TORC1 positively controls microautophagy. This conclusion is supported by the recent finding that rapamycin specifically inhibits microautophagic scission of vesicles into the vacuolar lumen as assayed in a cell-free microautophagy assay (Kunz et al., 2004). Finally, wild-type cells overproducing Gtr2 or Ego3, but not control cells, were competent to form microautophagic vesicles even in the presence of 50 ng/ml rapamycin (Figure 5E), indicating that the EGO complex acts downstream and/or in parallel of TORC1 to control microautophagy.

We also tested whether the phenotype of an EGO complex mutant may be due to a failure of membrane recycling via the retrograde traffic out of the vacuole. To this end, we determined in both wild-type and *gtr2Δ* cells the localization of an ALP variant (RS-ALP) with an engineered Golgi-retrieval sequence (FXFXD) at its N-terminal region (Bryant et al., 1998). RS-ALP exhibited only the punctate staining pattern that is characteristic of localization to the trans-Golgi network irrespective of either rapamycin treatment or the presence or absence of Gtr2 (Figure 6A). In addition, Golgi membrane-containing cell fractions of exponentially growing wild-type and *gtr2Δ* cells and of corresponding rapamycin treated cells (6 hr) contained both pro-RS-ALP, as well as the proteolytically matured mRS-ALP (data not shown). Thus, the ratio between RS-ALP influx to and retrieval from the vacuolar membrane does not sig-

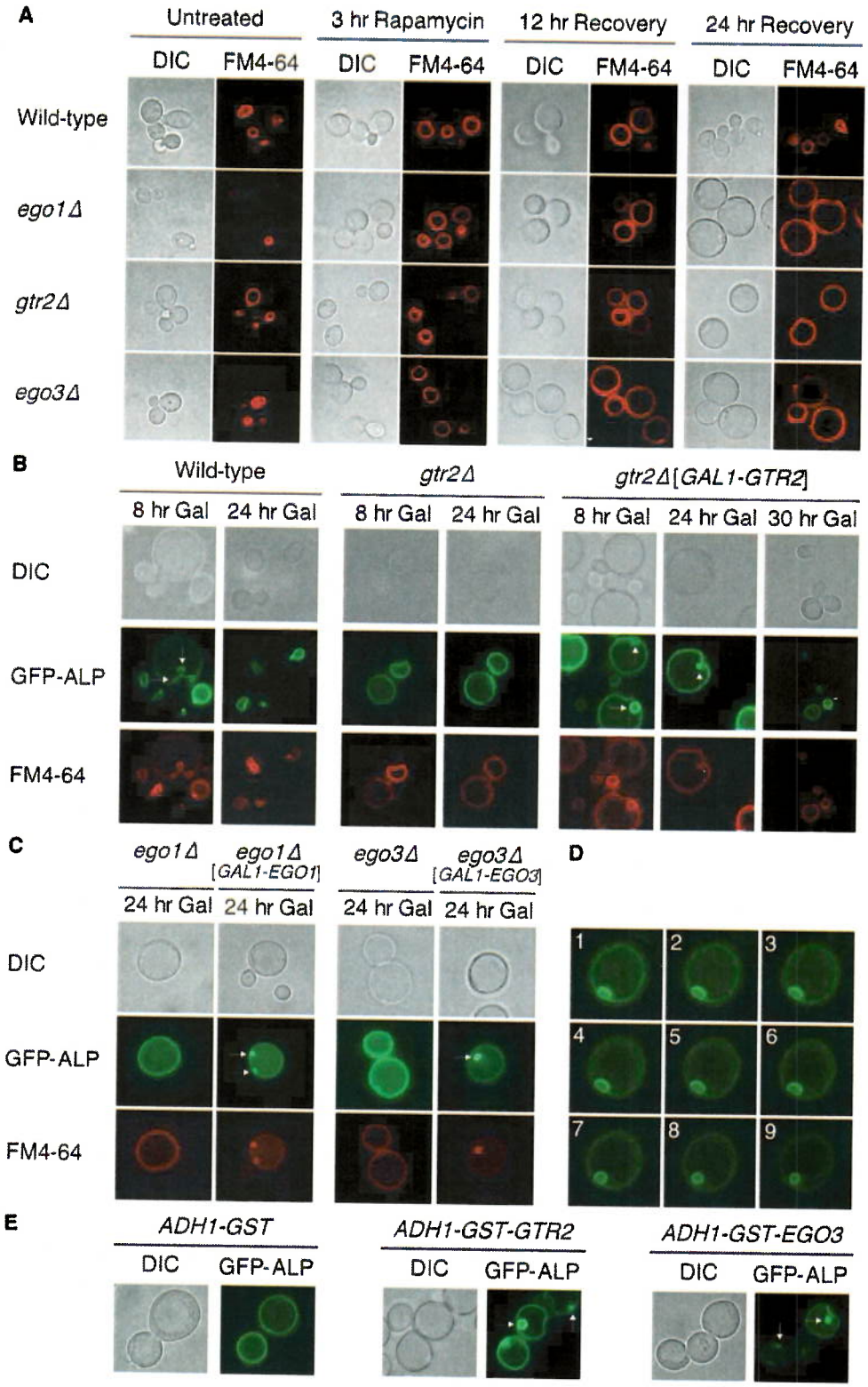

Figure 5. The EGO Complex Triggers Microautophagy during Recovery from Rapamycin Treatment

(A) EGO complex mutants display a single, dramatically enlarged vacuole following release from the rapamycin block. Wild-type (KT1960), *ego1Δ* (CDV210), *gtr2Δ* (CDV212), and *ego3Δ* (CDV211) cells growing exponentially on YPD were treated for 6 hr with rapamycin (200 ng/ml), washed twice, and resuspended in YPD. Cells were labeled with the fluorescent dye FM4-64 before rapamycin treatment (untreated) or following 3 hr of rapamycin treatment, and after 12 and 24 hr of recovery from the rapamycin treatment. For details, see Figure 4B.

(B) Conditional expression of *GTR2* during the recovery phase from rapamycin treatment restores microautophagy. Wild-type (KT1960) and

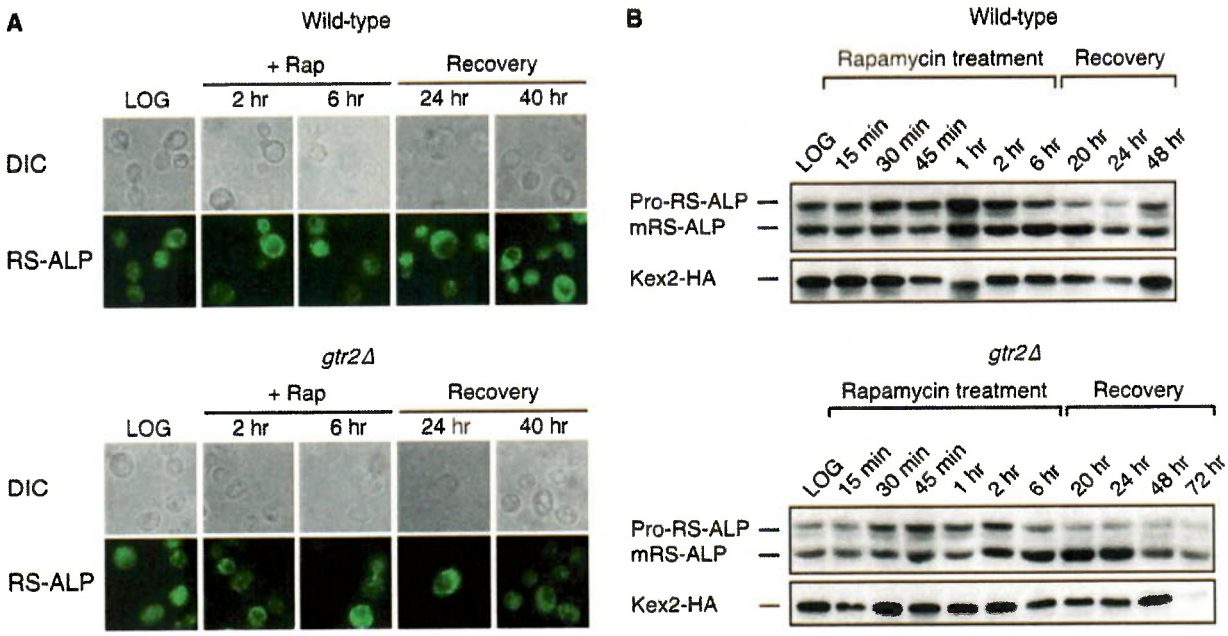

Figure 6. Gtr2 Is Not Required for Retrograde Traffic out of the Vacuole

(A and B) Localization (A) and processing (B) of RS-ALP during exposure to and following recovery from rapamycin treatment. Wild-type (CDV266-1D) and *gtr2Δ* (CDV266-22A) strains bearing plasmids expressing RS-ALP (pSN97) and HA-tagged Kex2 (pSN222) were grown on SD to exponential growth phase (LOG), subjected to a 6 hr rapamycin treatment, washed, and allowed to recover in SD without rapamycin for the indicated times. Localization of RS-ALP was visualized by indirect immunofluorescence (using anti-ALP antibodies) and DIC microscopy (A). Processing of RS-ALP was analyzed by immunoblot analysis using anti-ALP (and anti-HA for visualizing the Golgi membrane marker Kex2-HA; [B]).

nificantly change as a result of TORC1 inhibition and/or loss of Gtr2. During the rapamycin treatment (compare 2 hr versus 6 hr time point; Figure 6B) and during the first 20 hr of recovery from rapamycin treatment, almost the entire pool of preexisting pro-RS-ALP gradually underwent maturation irrespective of the presence or absence of Gtr2 (Figure 6B; notably, general protein synthesis resumes in wild-type cells, but not in *gtr2Δ* cells, after 24 hr of recovery; Figures 2E and 2F). Thus, both influx of pro-RS-ALP to and retrieval of mRS-ALP from the vacuole may operate normally in the presence or absence of TORC1 and/or EGO complex function. Consequently, retrograde traffic out of the vacuole is neither sufficient nor necessary for the recovery from rapamycin-induced growth arrest. This conclusion is supported by our finding that mutants blocking this pathway (e.g., *vac7, vac14*, and *svp1* [Bryant et al., 1998; Dove et al., 2004]) normally resume cell proliferation following release from rapamycin arrest (data not shown).

Large-Scale Genetic Network Analyses of the EGO Complex Identify Glutamine as a Key Metabolite in TOR Signaling

To extend our understanding of EGO complex function, we performed a synthetic genetic array analysis (SGA; see Experimental Procedures). As expected on the basis of the rapamycin-hypersensitivity of *gtr2Δ* cells, *tor1Δ* was found to be synthetically sick when combined with *gtr2Δ* (Figure 7A). In addition, either *rtg2Δ* or *rtg3Δ* mutations, both of which cause serious defects in glutamate/glutamine homeostasis (Butow and Avadhani, 2004), resulted in a synthetic lethal phenotype when combined with *gtr2Δ* that could be reversed by either introduction of a Gtr2-expressing plasmid, or supplementing the medium with glutamate (Figure 7B).

gtr2Δ (CDV212) cells without (*gtr2Δ*) and with the integrative *GAL1-GTR2* (pCDV991) plasmid (*gtr2Δ* [*GAL1-GTR2*]) were grown to exponential phase on SD medium, treated for 6 hr with rapamycin (200 ng/ml), washed twice, and resuspended in SGal/Raf (Gal). To visualize the boundaries of the vacuolar membrane, all strains additionally expressed GFP-ALP (from plasmid pRS426-GFP-ALP). Cells were labeled with the fluorescent dye FM4-64 (after the indicated recovery times on SGal/Raf), and the localization of FM4-64 and GFP-ALP was compared by fluorescence microscopy. White arrows indicate some sites of microautophagic vesicle formation.

(C) Same experiment as in (B) using *ego1Δ* (CDV210 ± *GAL1-EGO1* [pCDV1086]) and *ego3Δ* (CDV211 ± *GAL1-EGO3* [pCDV1085]) mutant cells.

(D) Budding of a microautophagic vesicle into the lumen of the vacuole. The sequence of pictures (covering 90 s in total) was taken from a *gtr2Δ* [*GAL1-GTR2*] cell (see [B] for details) 24 hr following release from rapamycin-containing medium into SGal/Raf. GFP-ALP was visualized as in (B).

(E) Wild-type (CDV147; pRS426-GFP-ALP) cells overexpressing *GTR2* (pCDV1080; *ADH1-GST-GTR2*), or *EGO3* (pCDV1083; *ADH1-GST-EGO3*) from the strong constitutive *ADH1* promoter, but not control cells (carrying pCDV1082; *ADH1-GST*), form microautophagic vesicles in the presence of 50 ng/ml rapamycin. GFP-ALP was visualized as in (B), following a 6 hr rapamycin treatment.

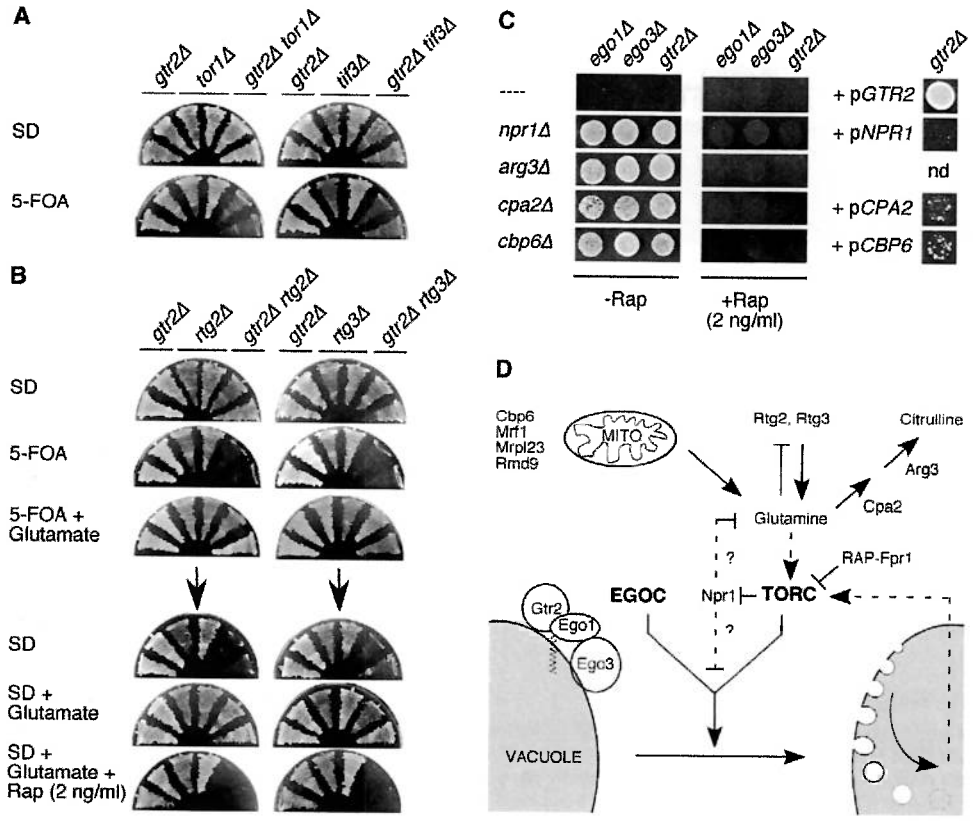

Figure 7. Global Mapping of the *gtr2Δ* Genetic Interaction Network

(A and B) SGA analysis using a *gtr2Δ* strain (CDV209) revealed synthetic sick or lethal interactions with *tor1Δ* and *tif3Δ* (A) or *rtg2Δ* and *rtg3Δ* (B), respectively. To verify the synthetic genetic interactions, original YKO mutants were backcrossed to the *gtr2Δ* strain (CDV209) and the corresponding diploids were transformed with the centromeric YCplac33-*GTR2* plasmid, sporulated, dissected on YPD medium, and streaked on SD plates, SD plates containing 5′-FOA (to select for cells that have lost the *URA3*-containing YCplac33-*GTR2* plasmid) (Boeke et al., 1987), or SD plates containing 5′-FOA plus glutamate (0.2%). The synthetic sick phenotype of the *gtr2Δ tif3Δ* double mutant was also reversed by the expression of Tif3 from a complementing plasmid (data not shown). The relevant genotypes are indicated. In (B), cells growing on plates containing 5′-FOA plus glutamate (0.2%) were subsequently replica-plated on SD, SD-glutamate (0.2%), and SD-glutamate (0.2%) plus rapamycin (2 ng/ml) plates.

(C) Suppressor mutations isolated from the *gtr2Δ* SGA double mutant collection. Original YKO mutants were backcrossed with *ego1Δ* (CDV207), *gtr2Δ* (CDV209), or *ego3Δ* (CDV208) mutants. Spores carrying both mutations were selected and assayed for recovery from rapamycin treatment as in Figure 2G except that recovery was additionally tested on YPD plates containing 2 ng/ml rapamycin. To verify that the suppressive effect is due to the loss of function mutation, selected double mutants were complemented with a plasmid harboring the corresponding wild-type gene and assayed (following growth on SD) for recovery from rapamycin treatment as in Figure 2G.

(D) Schematic pathway depicting the regulation of microautophagy by the TOR and EGO complexes. Arrows and bars denote positive and negative interactions, respectively. Interactions may be direct or indirect as further detailed in the text. Notably, even though we favor the possibility that the Rtg1/2/3-dependent retrograde response pathway acts upstream of TOR (with a presumed positive feedback control mechanism of TOR on glutamine levels; see also Butow and Avadhani, 2004), we presently cannot distinguish it from a model in which retrograde regulation of Rtg1/2/3 in response to decreased glutamate/glutamine levels is via TOR (Crespo et al., 2002). Finally, even though our genetic data favor a model in which glutamine acts as a key nutrient-signaling molecule upstream of TOR, alternative models in which the various suppressor mutations may impinge on TOR via a glutamine-independent mechanism(s) cannot be excluded at present.

Notably, the observed effect of glutamate was strongly dependent on TORC1 function, since it was entirely abolished in the presence of very low, subinhibitory levels of rapamycin (i.e., 2 ng/ml; Figure 7B). These findings indicate that glutamate/glutamine may act upstream of TORC1 and are in accord with the suggestion that the TOR pathway may sense glutamine, among other unknown nutrient compounds (Crespo et al., 2002). This model is further corroborated by the finding of several mutations (isolated in a genome-wide screen for *gtr2Δ* suppressors; see Experimental Procedures) that suppressed the defect in exit from rapamycin-induced growth arrest of all three EGO mutants (Figures 7C and 7D). These mutations include (1) *cbp6Δ*, *mrf1Δ*, *mrpl23Δ*, and *rmd9Δ*, all of which exhibit severe mitochondrial defects and hence are expected to induce robust Rtg2/3-mediated upregulation of glutamate/glutamine synthesis (Butow and Avadhani, 2004); (2) *cpa2Δ* and *arg3Δ*, which lack carbamoyl-phosphate synthase and ornithine carbamoyl transferase, respectively, that normally remove glutamine by converting it in sequential enzymatic reactions to citrulline; and (3) *npr1Δ*, which causes defects in degradation of amino acid permeases (Schmidt et al., 1998), and which has

recently been reported to slightly increase glutamine levels (Crespo et al., 2004) (an effect that may be more pronounced following rapamycin treatment). Importantly, the suppressive effects of the isolated mutations depended on a fully functional TORC1 complex, as illustrated by the fact that the presence of very low, subinhibitory levels of rapamycin (i.e., 2 ng/ml) during the recovery phase (a concentration that does not prevent the recovery from rapamycin treatment of wild-type cells; data not shown) completely abolished the observed suppressive effects (Figure 7C). Taken together, our large-scale genetic analyses converge on glutamine as a key molecule that may act in the TOR signaling pathway upstream of TORC1 (Figure 7D).

Discussion

In a first attempt to unravel the mechanisms that control exit from quiescence, we sought to identify those yeast mutants that are specifically defective in exit from rapamycin-induced growth arrest. Here, we focused our efforts on the characterization of three mutants, namely *ego1Δ*, *gtr2Δ*, and *ego3Δ*, and showed that they are likely defective in the same (EGO) protein complex. Several observations support this conclusion. First, all three mutants exhibit the same defects in both resumption of growth following rapamycin treatment and growth in the presence of subinhibitory rapamycin levels. Second, these defects can be reversed in all three mutants by the same suppressor mutations (including *npr1Δ*, *arg3Δ*, *cpa2Δ*, and *cbp6Δ*). Finally, and most compellingly, the three corresponding gene products colocalize to the vacuolar membrane and interact which each other as assayed by both two-hybrid and classical biochemical coprecipitation experiments.

How is the EGO complex localized to the vacuolar membrane? First, Ego1 is likely to be anchored to the vacuolar membrane via its N-terminal myristoyl group (Ashrafi et al., 1998). Second, Ego3 has a predicted membrane-spanning domain (at position 120–143 of its amino acid sequence; see *S. cerevisiae* genome database, http://www.yeastgenome.org/) and may bind PtdIns(3,5)P_2 (Zhu et al., 2001). From these and our own data, we propose the following topology of the EGO complex: (1) Gtr2, which lacks the classical C-terminal lipid modification site that is characteristic to members of the Ras-related GTPase family (Boguski and McCormick, 1993; Nakashima et al., 1999), interacts with Ego1 at the cytoplasmic side of the vacuolar membrane; (2) Ego1 is associated with the vacuolar membrane through its covalently attached N-terminal myristoyl group (and its interaction with Ego3); and (3) Ego3 is anchored in the vacuolar membrane via its presumed membrane-spanning and lipid binding domains (and its interaction with Ego1; Figure 7D). In support of this topology model, we observed that loss of individual components of the EGO complex did not significantly affect the localization of the other two, except for loss of Ego1 that resulted in a partial redistribution of Gtr2-GFP to the cytoplasm (data not shown).

Our current data indicate an essential role of the EGO complex in activation of microautophagy following release from a rapamycin block, a process that is specifically required to counterbalance the massive membrane influx toward the vacuolar membrane resulting from rapamycin-induced macroautophagy. The observation that EGO complex function becomes essential for growth when TORC1 activity is reduced (i.e., in the presence of subinhibitory rapamycin levels or in the absence of Tor1) substantiates this notion. An interesting consequence of this genetic interaction is that mutations, which positively or negatively modulate TORC1 activity, are expected to either alleviate or aggravate, respectively, the defects exhibited by EGO complex mutants. To identify potential regulators of TORC1, we therefore performed a genome-wide screen for mutations that either suppressed the *gtr2Δ* phenotype, or, conversely, resulted in a synthetic lethal/sick phenotype when combined with *gtr2Δ*. As schematically illustrated in Figure 7D, these studies identified a regulatory network of proteins that converges on the amino acid glutamine as a key metabolite in TOR signaling. These finding are in line with those of a previous report (Crespo et al., 2002). Nevertheless, we would like to emphasize that our genetic data allow at present no definite exclusion of alternative models in which the various suppressor mutations may impinge on TORC1 via a glutamine-independent mechanism(s).

An important finding of our present study is that diverse readouts, which are negatively controlled by TORC1 (e.g., glycogen accumulation, phosphorylation of eIF2α on Ser 51, and macroautophagy; Figures 2B and 2F; and data not shown), depended on the presence of Ego1, Gtr2, and Ego3 during recovery from rapamycin treatment for resetting to their initial status. These data suggest the presence of an EGO complex-mediated positive feedback loop that impinges on TOR itself, which is in line with our finding that overproduction of Gtr2 or Ego3 enhances rapamycin resistance. In further support of this suggestion, a recent study identified Ybr077C/Nir1/Ego3 as the target of an engineered molecule that suppresses the effects of rapamycin, possibly by causing a gain of function of Ybr077C/Nir1/Ego3 (Huang et al., 2004). Importantly, the transcript profile of exponentially growing *ybr077cΔ/nir1Δ/ego3Δ* cells was reported to be strikingly similar to the one of rapamycin-treated wild-type cells, further substantiating a role for Ego3 in TOR activation (Huang et al., 2004).

A formal prediction inferred from our model that the EGO complex activates TOR activity is the existence of mutations that are simultaneously synthetically lethal/sick with either loss of EGO complex function or reduced TOR activity. Interestingly, in this context, our SGA analysis recovered the *tif3Δ* mutant, which meets both of these requirements, i.e., it is hypersensitive to rapamycin (Parsons et al., 2004) and synthetically sick in combination with *gtr2Δ* (Figure 7A). Tif3, the yeast eIF4B homolog, is thought to stimulate eIF4A helicase activity to enhance unwinding of inhibitory secondary structures in the 5′ untranslated region of mRNAs. In mammalian cells, eIF4B has been identified as an indirect target of mTOR and suggested to be particularly important for the translation of mRNAs encoding proteins implicated in cell cycle progression (Raught et al., 2004). These data not only indicate that Tif3 may play a critical role in cell proliferation control under condi-

tions of starvation and/or low TOR activity but also further support the notion that the EGO complex positively acts on TOR.

How could the EGO complex-mediated microautophagy be coupled to the control of TOR activity? We speculate that microautophagy reestablishes a balance in the distribution of membranes in the entire endomembranous system and, in parallel, promotes a concentration of the vacuolar amino acid reservoir. Because TOR is localized to distinct membrane-associated protein complexes (Kunz et al., 2000; Wedaman et al., 2003), it is ideally placed to integrate information on the general dynamics of membrane fluxes in the cell. Alternatively, TOR could be implicated in communicating the status of the internal vacuolar nutrient pool. This may be accomplished via Kog1 (the yeast homolog of the mTOR binding partner raptor), which is localized at the vacuolar membrane (http://yeastgfp.ucsf.edu) and which may regulate TOR activity in analogy to the situation in animal cells (Kim et al., 2002), possibly in response to the vacuolar amino acids level. In this context, it is intriguing that Sch9, the closest yeast homolog of the mTOR upstream regulatory protein kinase B (PKB/Akt), has recently been localized to the vacuolar membrane as well (Jorgensen et al., 2004). Taken together, while the detailed molecular mechanism by which the EGO complex may regulate TOR awaits further elucidation, our study demonstrates the existence of a new growth control mechanism that originates at the vacuolar membrane.

Experimental Procedures

Strains, Media, and Genetic Techniques

Wild-type strains KT1960, KT1961, CDV147, and EGY48 and the *pho8Δ60* strain TS139 were described earlier (Gyuris et al., 1993; Pedruzzi et al., 2003; Schmelzle et al., 2004). The yeast knockout collection (YKO) wild-type strains BY4741 and Y2922 (MATα *mfa1Δ::MFA1pr-HIS3 can1Δ his3Δ1 ura3Δ0 lys2Δ0*) were provided by C. Boone (Giaever et al., 2002; Tong et al., 2001). PCR-based gene deletions (*ykr007w/ego1::natMX4*, *gtr2::natMX4*, and *ybr077c/ego3::natMX4* either transformed into Y2922 to create CDV207, CDV209, and CDV208, respectively, or transformed into KT1960 to create CDV210, CDV212, and CDV211, respectively, or transformed into TS139 to create CDV284, CDV286, and CDV285, respectively) and tagging of chromosomal genes (*YKR007W/EGO1-GFP-TRP1*, *GTR2-GFP-TRP1*, and *YBR077C/EGO3-GFP-TRP1* transformed into KT1961 to create CDV213, CDV215, and CDV214, respectively, and *YKR007W/EGO1-myc13-kanMX6* and *GTR2-myc13-kanMX6* transformed into KT1961 to create FD19 and FD21, respectively) were done as described (Longtine et al., 1998). Diploid strains FD24 (*ego1::natMX4/EGO1 GTR2-myc13-kanMX6/GTR2 EGO3-GFP-TRP1/EGO3*) and FD25 (*EGO1-myc13-kanMX6/EGO1 GTR2-GFP-TRP1/GTR2 ego3::natMX4/EGO3*) were constructed by a series of combinatorial mating and sporulation among the isogenic strains CDV210, CDV214, and FD21, and CDV211, CDV215, and FD19, respectively. CDV266-1D (*pho8Δ::kanMX*) and CDV266-22A (*gtr2Δ::natMX4 pho8Δ::kanMX*) are segregants of CDV266, which was created by mating CDV209 and the YKO strain *pho8::ΔkanMX4* (Giaever et al., 2002). Strains were grown at 30°C in standard rich medium (YPD) with 2% glucose or synthetic defined medium (SD) complemented with the appropriate nutrients for plasmid maintenance and either 2% glucose or 2% galactose and 1% raffinose (SGal/Raf) as carbon source (Guthrie and Fink, 1991). Standard genetic manipulations were used (Guthrie and Fink, 1991). Gene deletions were confirmed by phenotypic analyses and/or PCR with gene-specific primers.

Plasmids

Full-length Ykr007W/Ego1, Gtr2, Gtr2^{Q66L} and Ybr077C/Ego3 were expressed under the control of the *GAL1* promoter from integrative plasmids pCDV1086, pCDV991, pCDV992, and pCDV1085, respectively; N-terminal, GST-tagged versions of full-length Ykr007W/Ego1, Gtr2, and Ybr077C/Ego3 were expressed under the control of the *ADH1* promoter from integrative plasmids pCDV1084, pCDV1080, and pCDV1083, respectively; and full-length Gtr2 and Gtr2^{Q66L} were expressed under the control of the *tetO7* promoter from plasmid pVW1009 and pVW1010, respectively. GST was expressed under the control of the *ADH1* promoter from the low copy number plasmid pCDV1082 (*ADH1-GST*). Control plasmids pCM189, YCpIF2, and YCp*ADH1* were described earlier (Garí et al., 1997; Reinders et al., 1998). To fuse the various genes to the LexA DNA binding domain (DBD) and/or to the activation domain (AD) coding sequences in plasmids pEG202 and pJG4-5 (Gyuris et al., 1993), respectively, *YKR007W/EGO1*, *GTR2*, *YBR077C/EGO3*, and *MSB2* full-length coding sequences were cloned at the polylinker of pJG4-5 and/or pEG202 to yield pJG4-5-*EGO1*, pJG4-5-*GTR2*, pJG4-5-*EGO3*, pJG4-5-*MSB2*, pEG202-*EGO1*, and pEG202-*EGO3*. Gtr2, Cpa2, and Cbp6 were expressed under the control of their own promoters from plasmids YCplac33-*GTR2*, -*CPA2*, and -*CBP6*, respectively. Plasmids expressing HA-tagged Npr1 (pAS103), HA-tagged Kex2 (pSN222), RS-ALP (pSN97), and GFP-ALP (pRS426-GFP-ALP) were described previously (Cowles et al., 1997; Nothwehr et al., 1995; Schmidt et al., 1998).

Isolation of EGO Mutants

To identify genes whose products are involved in exit from rapamycin-induced growth arrest, we screened (in duplicate) 4857 viable yeast deletion mutants by arraying strains on YPD plates, replica plating on rapamycin-containing (200 ng/ml) YPD plates followed by incubation for 24 hr at 30°C, and subsequent replica plating on YPD plates (without rapamycin). Of the originally 58 mutants for which both clones assayed exhibited a defect in resumption of growth on the final plates, eight could be confirmed by serial dilution spot assays (using a cut-off value of 100-fold lower colony formation efficiency than wild-type cells; Figure 2G; and data not shown). Notably, all eight mutants were hypersensitive to rapamycin (Figures 2G and 3C; and data not shown).

Immunoblot Analyses and GST Pull-Down

Standard procedures were used for yeast cell extract preparation and immunoblotting (Pedruzzi et al., 2003). Dr. T. Dever kindly provided polyclonal antibodies against eIF2α. Phosphospecific anti-eIF2α[pS^{51}], monoclonal anti-GFP (JL-8), monoclonal anti-HA (HA.11), monoclonal anti-myc (9B11), polyclonal anti-GST, anti-ALP (1D3), and Alexa Fluor® 488 secondary antibodies were purchased from Biosource (Camarillo, CA), BD Biosciences (Palo Alto, TX), Covance (Berkeley, CA), Cell Signaling (Beverly, MA), Covance (Berkeley, CA), Invitrogen, and Molecular Probes (Eugene, OR), respectively. For coprecipitation experiments, GST-tagged Ego1 and Ego3 were purified (using glutathione sepharose beads) from clarified extracts (prepared as in [Lenssen et al., 2005]) of strains FD24 and FD25, respectively, which express the corresponding GST-tagged proteins from integrative plasmids. Bound proteins were eluted with sample buffer (5 min, 95°C) and subjected to standard immunoblot analysis for detection of coprecipitated proteins.

Synthetic Genetic Array Analysis

Synthetic genetic array (SGA) analysis was performed as described (Tong et al., 2001), using a *gtr2Δ* mutant (CDV209) as the bait strain. In total, four double-deletion combinations were found that resulted in a synthetic growth defect and fulfilled the following two criteria: (1) the corresponding YKO single mutant strains (i.e., *tor1Δ*, *tif3Δ*, *rtg2Δ*, and *rtg3Δ*) also exhibited a synthetic growth defect when combined with the dominant negative Gtr2^{Q66L} (expressed from pCDV992), and (2) the double-deletion combinations could be complemented as indicated in Figures 7A and 7B. The double mutant collection was also screened for suppressors of the *gtr2Δ*-dependent defect in exit from rapamycin-induced growth arrest. In total, 8 suppressors could be confirmed by the ability of the corresponding YKO single mutants (i.e., *fpr1Δ*, *cbp6Δ*, *mrf1Δ*,

mrpl23Δ, *rmd9Δ*, *npr1Δ*, *cpa2Δ*, and *arg3Δ*) to suppress the dominant negative effect of $Gtr2^{Q66L}$ (expressed from pCDV992) with respect to exit from rapamycin-induced growth arrest. The isolation of the FKBP-deficient *fpr1Δ* mutant validated our suppressor screen.

Miscellaneous

Vacuole membranes were visualized in vivo by labeling cells with the fluorescent dye FM4-64 (Molecular Probes) and cells were observed by fluorescent microscopy (Vida and Emr, 1995). Indirect immunofluorescence, two-hybrid, and Northern blot were performed as described (Guthrie and Fink, 1991; Lenssen et al., 2005; Pedruzzi et al., 2003). Progression of autophagy was analyzed by the increase of alkaline phosphatase activity in cells expressing Pho8Δ60, a cytosolic proform of the alkaline phosphatase (Noda et al., 1995). Protein synthesis was measured by the incorporation of [^{35}S] Pro-Mix (Amersham Biosciences) into TCA-precipitable material (De Virgilio et al., 1991).

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III.3) Conclusion

Rapamycin treatment arrests cell growth by shutting down the TORC1 pathway in a reversible way, at least in our strain background (336). This inhibition of TORC1 by rapamycin is conserved from yeast to human and mimics nitrogen/or nutrient starvation. The study presented here identifies mutants that are unable to exit from a rapamycin-induced G_0 block. We focused on the biological, biochemical and genetic characterisation of three specific mutants, namely *ego1Δ*, *gtr2Δ* and *ego3Δ*. The three mutants are likely to be defective in the same EGO protein complex (for **e**xit from a rapamycin-induced **gro**wth arrest), which is localised at the vacuolar outer membrane. Gtr2 is a member of the mammalian RragA subfamily of the Ras-related small GTPase and acts together with Ego1 and Ego3 to positively regulate, in conjunction with TORC1, microautophagy. This process, which counterbalances the massive membrane influx brought about by induction of macroautophagy at the onset of rapamycin treatment, appears to be essential for resumption of growth following rapamycin removal. Thus, it represents a novel growth control mechanism that originates at the vacuolar membrane. Large-scale genetic analysis of the EGO complex allowed us also to pinpoint glutamine as a key molecule that may act in the TORC1 pathway possibly upstream of TORC1.

Chapter IV.

Further Characterisation of the EGO Complex via the Study of Protein-Protein Interactions

IV.1) Introduction

The previous chapter described the identification and characterisation of the exit from G_0 complex (EGO), composed of Ego1, Gtr2 and Ego3. Based essentially on genetic data, our model predicts that the EGO complex may act to positively regulate TORC1 activity during recovery from a rapamycin treatment. In order to get more information concerning the EGO complex and the mechanisms by which it regulates cell growth, interactions between the EGO complex and the TORC1 pathway were studied further, notably via phenotypic characterisation of the *tco89* and *tor1* mutants, encoding the two non-essential members of TORC1 (59, 60). We also decided to undertake a genome-wide two-hybrid screen with each members of the EGO complex as a bait. The two-hybrid system is a method that allows the identification of *in vivo* protein-protein interactions through reconstitution, with two proteins, of the activity of the yeast Gal4 transcription factor, which is then used to activate reporter genes such as *lacZ, HIS3* or *ADE2*. Expression of these genes is then revealed by β-galactosidase assay or growth on selective medium without adenine or histidine (396). An array, comprising nearly all *S. cerevisiae* full length proteins fused to the Gal4 activation domain was used in order to identify potential direct upstream and downstream targets as well as potential new components of the EGO complex. To find the GTPase activating protein (GAP) and the GTP exchange factor (GEF) of the small GTPases of the complex, the putative constitutive GTP-bound and GDP-bound mutants were also used to screen the genome-wide yeast two-hybrid array (397). Identification of proteins, which specifically bind to the GTP- or GDP-bound form may also provide information concerning the function of each form and provide clues to explain the dominant negative phenotype of the presumably GTPase deficient protein $Gtr2^{Q66L}$.

This chapter will depict results of the two-hybrid interactions found in the different two-hybrid screens, which among others allowed the identification of a supplemental member of the EGO complex, Gtr1. In addition, we identified Nvj1 as an interactor with Ego1 or Gtr2. Nvj1, is a critical protein for piecemeal microautophagy of the nucleus (PMN), indicating that the EGO complex may also play a role in this form of microautophagy. We also found preliminary evidence indicative of a direct interaction between the EGO complex and TORC1.

IV.2) Material and methods

IV.2.1) Yeast strains construction

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. Strains PJ69-4a and α contain the reporter genes *ADE2* and *HIS3* under control of the *GAL2* and *GAL1* promoters, respectively. Mutants in the BY4741 background have been picked up from the yeast knockout collection (YKO) provided by C. Boone (331, 398). Strains FD27, FD29 and FD37 were created with PCR-based gene deletions cassettes (*tco89::kanMX*, *tor1::kanMX*, and *grt1::natMX*) that were transformed in KT1960 as previously described (399). Prof. R. Loewith kindly provided strains expressing the genomically tagged Tco89 and Kog1-GFP.

Table I

Strains	Relevant genotype	Source
PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(114)
PJ69-4α	*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(114)
EGY48	*MATa his3 trp1 ura3 LEU2::pLexAop6-LEU2* pSH18-34	R. Brent
BY4741	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 yfg::kanMX*	C. Boone
KT1960	*MATα ura3-52 leu2 his3 trp1*	K. Tatchell
FD27	*MATα ura3-52 leu2 his3 trp1 tco89::kanMX*	This study
FD29	*MATα ura3-52 leu2 his3 trp1 tor1::kanMX*	This study
FD32	*MATα ura3-52 leu2 his3 trp1 gtr1::natMX*	This study

IV.2.2) Media and growth conditions

Strains were grown at 30°C in standard rich medium (YPD) with 2% glucose or synthetic dextrose medium (SD) supplemented with the appropriate nutrients for plasmid maintenance, ammonium sulphate (0.67 g/l) and either glucose 2% or galactose 2%. For rapamycin treatment, the drug (stock solution 1 mg/ml in EtOH 90% tween 10%) was added to a final concentration of 200 ng/ml. For the two-hybrid screen, SD minus leucine and YPD media were supplemented with adenine, and plates SD minus histidine were supplemented with appropriate concentrations of 3-amino-1, 2, 4-triazole (3-AT) from stock solution (3M solved in water) (Gibco).

IV.2.3) Plasmid construction

The bait plasmids used in the two-hybrid screen were built as follow: *EGO1, EGO3,* and *GTR2* were amplified from genomic DNA, digested with *NcoI-XhoI*, *EcoRI-XhoI* and *EcoRI-XhoI*, respectively, and cloned into the *NcoI-SalI* or *EcoRI-SalI* sites of pOBD2 (96) in frame with a sequence encoding the Gal4 DNA-binding domain. *GTR1* was cut with *EcoRI-NcoI* from pJG4-5-*GTR1* and cloned directly into pOBD2, while $gtr1^{Q65L}$ (GTP-bound), $gtr1^{S20L}$ (GDP-bound), $gtr2^{Q66L}$ (GTP-bound) and $gtr2^{S23L}$ (GDP-bound) were PCR-amplified from the plasmid templates YpIF2-$gtr1^{Q65L}$, YpIF2-$gtr1^{S20L}$, YpIF2-$gtr2^{Q66L}$ and YpIF2-$gtr2^{S23L}$, respectively. $gtr1^{Q65L}$ and $gtr1^{S20L}$ were cloned at the *EcoRI-NcoI* sites of pOBD2 and $gtr2^{Q66L}$ and $gtr2^{S23L}$ were digested with *EcoRI-XhoI* and cloned at the *EcoRI-SalI* sites of pOBD2. For more information about the *gtr2* and/or the *gtr1* variants see Chapter III and reference (397). For the two-hybrid assay, *TCO89* was amplified from a genomic template, digested with *BamHI-NotI* and cloned *BamHI-NotI* in the polylinker of the pJG4-5+PL (400) and pEG202 (401) vectors, in frame with the sequence containing the activation domain (AD) or the DNA-binding domain (DBD), respectively. The construct with *CDC3* was described previously (402). The plasmid expressing *GTR1* under the endogenous promoter (pRS416-*GTR1*) was a gift from Joerg Urban and mock vector was YCplac111 (403). The plasmid expressing GFP-ALP (pRS426-GFP-ALP) has been previously described (404). All plasmid constructs were confirmed by sequencing.

IV.2.4) Two-hybrid screening of protein interactions

This two-hybrid screen relies in concomitant expression, in a cell, of a protein fused with the Gal4 DNA-binding domain (DBD) (bait) and a protein fused with the Gal4 transcriptional activation domain (AD) (prey). If both proteins interact in the nucleus they reconstitute the Gal4 transcription factor and can activate the reporter genes of the PJ69-4a and α strains (Table I) allowing the cells to grow on a media lacking adenine or histidine (396). In this study, the screen for interacting proteins was done using a genome-wide array consisting of approximately ~6000 yeast haploid strains, each containing a plasmid (pOAD) with an ORF fused to the Gal4 AD (405). The bait plasmids pOBD2-*EGO1*, pOBD2-*EGO3*, pOBD2-*GTR1*, pOBD2-$gtr1^{Q65L}$, pOBD2-$gtr1^{S20L}$, pOBD2-*GTR2*, pOBD2-$gtr2^{Q66L}$ and pOBD2-$gtr2^{S23L}$ were transformed into yeast strain PJ69-4a according to the lithium acetate method (406). Baits in PJ69-4a were tested for self-activation by plating onto SD minus tryptophan, leucine and histidine or onto SD minus tryptophan, leucine and adenine. Due to basal *HIS3* expression, the self-activation test on histidine selection was done in presence of increasing

concentrations (10 to 100 mM) of 3-AT, a competitive inhibitor of the *HIS3* gene product. Having ensured, from the absence of appreciable growth, the baits did not auto-activate the reporters, the baits in PJ69-4a were mated to the yeast array (format: 16-plate, each with 384-well) containing the AD-proteins fusions in strain PJ69-4α (prey) (396). All replications and inoculations were carried out using the 384-pin replicator of a Biomek 2000 Laboratory Automation Workstation, with movements programmed using the BioWorks Version software (Beckman). After mating, PJ69-4 diploids were selected by plating onto SD minus leucine and tryptophan. Screening for protein-protein interactions was done by the pinning of these diploids onto SD lacking leucine, tryptophan, and histidine and supplemented with concentrations of 3-AT of 10 mM (Gtr1, $Gtr1^{S20L}$), 25 mM ($Gtr1^{Q65L}$, $Gtr2^{S23L}$) and 100 mM (Ego1, Ego3, Gtr2, $Gtr2^{Q66L}$). Positive colonies, which grew on either one of the selection medium were identified by their position in the array. Growth was scored after 4, 7, 10, 16 and 20 days at 30°C. For further description of plasmid and yeast strains see also the following website: (http://depts.washington.edu/sfields/protocols/protocols.html).

Preys that yielded colonies in several independent screens carried out with unrelated baits (*i.e.*, Ade2, Fob1, Gal4, Yth1 on adenine selection, and Atr1 and His3 on histidine selection) were not taken into account and considered as bait-unspecific false-positive. Notably, the preys Ade2 and His3 directly complement the *ade2* and *his3* mutations of the PJ69 strain, without needing to activate the reporter genes. Gal4 is the transcription factor, which is reconstituted by the two-hybrid interactions and can activate transcription of the reporter genes on its own. Transcriptions factors (including Cin5, Gal1, Mot3, Yap6 and Yap7) can bind DNA and activate genes on their own, and were consequently considered as false-positives.

IV.2.5) Two-hybrid assay

Quantitative β-galactosidase assays were performed as described previously (407) with reporter plasmid pSH18-34. For the assays, we used strain EGY48 bearing the *lacZ* reporter plasmid pSH18-34 that had been co-transformed with a pEG202-based plasmid and a pJG4-5-based plasmid.

IV.2.6) FM4-64 staining

N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64, Molecular Probes) was added to a final concentration of 8 µM, 75 min before

microscopy (stock solution 16 mM in DMSO). After 15 min of incubation at 30°C, cells were washed, resuspended in SD or YPD and incubated for 1 hr on a rotating wheel in order to allow proper integration of the dye to the vacuolar membrane. Cells were then washed with and resuspended in PBS 2% glucose and observed by fluorescent microscopy (408).

IV.3) Results

IV.3.1) Identification of a new component of the EGO complex: Gtr1

The genome-wide two-hybrid screens were first carried out with Ego1, Gtr2 and Ego3 fused to the Gal4 DNA-binding domain (DBD). The baits were individually crossed with the array collection containing one representative of each of the nearly ~6000 AD-fused proteins. Protein-protein interactions were tested for activation of the *GAL1-HIS3* and *GAL2-ADE2* reporter genes, by looking for clones able to grow on histidine and adenine selections, respectively. Use of both selections was expected to minimise the pool of false-positives (409). These screens identified interactions between Gtr2 or Ego1 and the small GTP binding protein Gtr1 (Fig. IV.1 A). These interactions were considered to as very strong, since colonies were detected after 2 or 7 days of growth on adenine or histidine selections, respectively. They seemed to be even stronger than the Gtr2-Ego1 interaction, if one referred to the size of the colony (Fig. IV.1 A) and, since identified on both selections, they were likely to be true-positives. This finding was not surprising as both interactions had previously and independently been reported in the literature (410, 411). Interactions between Ego1 or Gtr2 and Gtr1 were also confirmed in an independent liquid two-hybrid assay (data not shown). *gtr1Δ* mutants displayed an ego phenotype[5] as demonstrated by the fact that they were unable to resume growth following a rapamycin-induced growth arrest. This defect could be rescued by introducing a plasmid expressing *GTR1* from the strong TEF promoter (Fig. IV.1 B). Since in their global analysis of protein localisation Huh *et al.* (2003) identified Gtr1 at the vacuolar membrane, Gtr1 might indeed belong to the EGO complex (412).

[5] ego phenotype: the inability of cells to resume growth following a rapamycin-induced growth arrest.

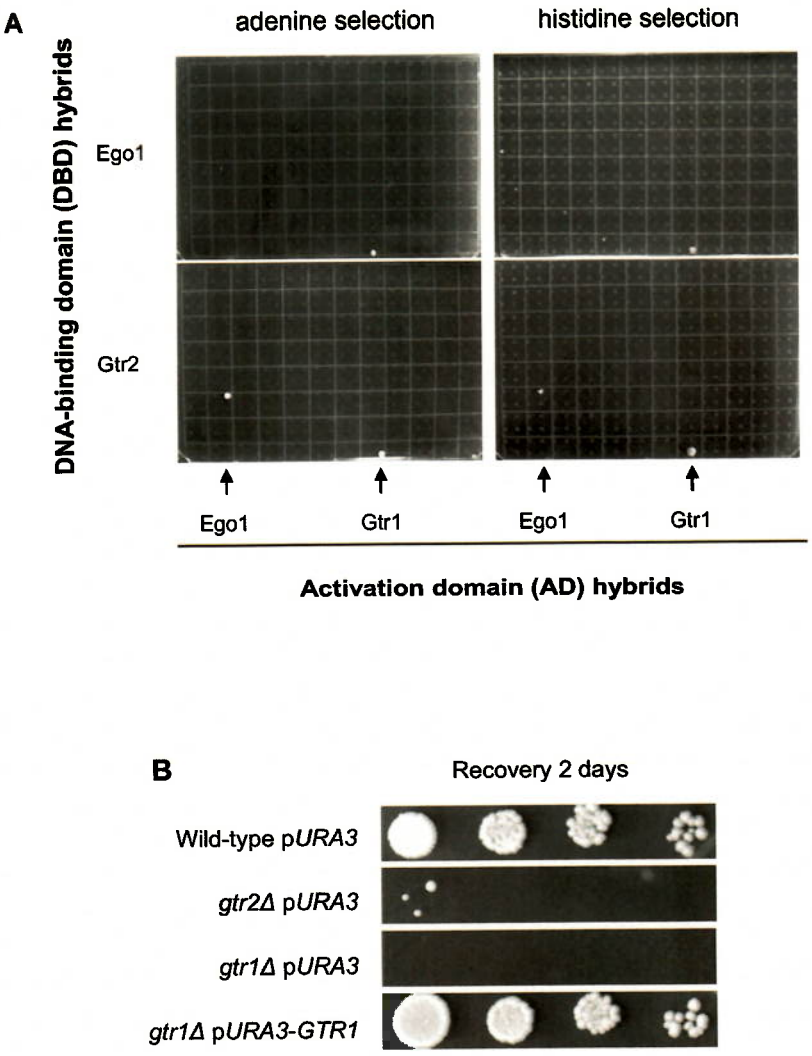


Fig. IV.1 Identification, via two-hybrid screening, of a new member of the EGO complex.

(**A**) Positive interactions between Ego1 or Gtr2 and Gtr1. Diploid cells were plated on SD minus adenine (adenine selection) and on SD minus histidine supplemented with 100mM of 3-AT. Pictures were taken at 2 days (left) and 7 days (right) for adenine or histidine selection, respectively. White spot: positive clone. (**B**) Loss of Gtr1 results in an ego phenotype. Exponentially growing wild-type cells (KT1960) and isogenic mutants carrying the empty control *URA3* plasmid or *pGTR1* (pRS416-*GTR1*) were treated for 6 hr with rapamycin in a liquid SD minus uracil medium, washed with water, spotted on YPD plates and incubated for two days at 30°C. Spots (4µl) correspond to serial 10 fold dilutions of equally dense cultures (OD_{600} of 0.5).

IV.3.2) Screen for interaction of the EGO complex proteins using a genome-wide protein array

Five additional two-hybrid screens were undertaken using Gtr1 wild-type, $Gtr1^{S20L}$ and $Gtr2^{S23L}$, the two variants predicted to be GDP-locked and $Gtr1^{Q65L}$ and $Gtr2^{Q66L}$, the two

variants predicted to be GTP-locked as baits (397). Taking the eight screens together, a total of 106 interactions was found (excluding the false-positive described in the material and methods section). Among them, 26, all mediating strong interactions, appeared on both adenine and histidine selection and included members of the EGO complex (Fig. IV.2 A). 46 appeared exclusively on adenine selection and 34 interactions appeared only on histidine selection (Fig. IV.2 B,C). As two-hybrid screens give a great number of false-positive clones (114, 409), proteins interacting with either one member of the EGO complex were then classified in eight distinct groups in order to easily identify those that were the more likely to represent physiological interactions.

Whatever the prey group is, most relevant interactions were expected to appear on adenine and histidine selection (Fig. IV.2 A). However, Fpr3 and Gal83, which allow growth on both selections, are unlikely to interact physiologically with the EGO complex, inasmuch as they localise within the nucleus. Conversely, although still uncharacterised, Ybr053c, Ybr138c and Yml081w may be of interest for our future studies. Interactions between members of the EGO complex are drawn in dark green. The group in light green includes proteins, which seem functionally unrelated to the EGO complex, but whose absence renders cell growth hypersensitive to rapamycin treatment, like loss of any member of the EGO complex (indicating that they may signal positively in the EGO pathway). The group in brown encloses interactions with membrane transporters (which localise at the plasma or vacuolar membrane), the yellow group interactions with components of the intramembranous system (*i.e.*, ER, Golgi endosome and vacuole), and the dark blue group interactions with nuclear membrane proteins. The group "others", represented in purple, includes several proteins involved in cellular functions, which are apparently unrelated to the function of the EGO complex and were considered to be less likely to be true interacting proteins. Similarly, highlighted in light blue, nuclear proteins are unlikely, under physiological conditions, to interact with members of the EGO complex at the vacuolar membrane. Finally, uncharacterised proteins or dubious proteins are depicted in grey.

A **Adenine and histidine selection**

AD domain hybrid: Protein name	DBD domain hybrid: Ego3	Ego1	Gtr1	Gtr1^{Q65L}	Gtr1^{S20L}	Gtr2	Gtr2^{Q66L}	Gtr2^{S23L}	Function
Ego1	s		s	s		s	s		exit from G_0
Ego3		s							exit from G_0
Gtr1		s				s	s	s	exit from G_0
Fpr3		s				s	s	s	unknown
Ptp1		s				s			phosphatase of Fpr3
Gal83						s	s		Snf1 complex subunit
Ybr053c		s	s	s	s				unknown
Ybr138c						s	s		unknown
Yml081w						s	s		unknown

B **Adenine selection**

AD domain hybrid: Protein name	DBD domain hybrid: Ego3	Ego1	Gtr1	Gtr1^{Q65L}	Gtr1^{S20L}	Gtr2	Gtr2^{Q66L}	Gtr2^{S23L}	Function
Ego1								m	exit from G_0
Fmo1			m						protein folding in the ER
Bet4				m			m	m	geranylgeranylation (ER)
Csg2						s	s		mannosylation (ER)
Ste14		s					s		carboxyl methylation
Vma8			s		s		s	s	vacuolar acidification
Sss1				w					protein translocation in the ER
Ysn1				m					unknown
Ypt1							w		small GTPase; ER to Golgi transport
Msc1				w					unknown
Fen1								w	sphyngolipid biosynthesis
Pho87								s	Pi transport, plasma membrane
Yol092w					w				unknown, vacuolar membrane
Yol162w					w				unknown
Zrt1			m					m	Zinc transport; plasma membrane
Frs1								w	phenylalanyl tRNA synthesis
Mtq2					w				methyl transferase
Nma2								w	NAD biosynthesis
Cdc20						s	s		ubiquitylation
Ubc9				w					sumoylation
Yor004w		s							unknown
Gsp2								w	small GTPase; nuclear organisation
Met18								w	Pol(II) transcription
Pgd1								s	Pol(II) transcription
Slx4		s							DNA replication
Spt20			m						histone acetylation
Tid3				w					chromosome segregation
Ybr206w					m				unknown
Ybr277c			m						unknown
Ycr049c			m						unknown
Yhr177w				m					unknown
Yjr038c							s		unknown
Ymr111c				w					unknown
Yol036w								m	unknown
Yor237w								s	unknown
Ypl071c								w	unknown
Vts1					m				unknown

C

Histidine selection

DBD domain hybrid

AD domain hybrid

Protein name	Ego3	Ego1	Gtr1	Gtr1^{Q65L}	Gtr1^{S20L}	Gtr2	Gtr2^{Q66L}	Gtr2^{S23L}	Function
Mon1						m	m		transport to the vacuole
Pep8							s		endosome to golgi trafficking
Vps69							w		protein targeting to the vacuole
Ycl073c		m				m	m		unknown
Ybr241c					w				unknown
Asm4						m	m		member of the nuclear pore complex
Nvj1		s					s		piecemeal nuclear microautophagy
Erg10		m							mevalonate biosynthesis
Hul4				w					ubiquitylation
Img1			w						mitochondrial protein synthesis
Ysc84							w		actin organisation
Sen2		s							tRNA splicing
Sol3		m							pentose phosphate pathway
Ser2						m			serine and glycine biosynthesis
Hht2					s				chromosome assembly
Ixr1		w							DNA repair
Rlf2			w						chromosome assembly
Urb1					w				rRNA processing
Yer051w		s							histone demethylase
Ybr241c					w				unknown
Yjl075c	s								unknown
Yil176c		m							unknown
Ymr279c		s					s		unknown
Yor080w		s							unknown
Yor364w			w						unknown
Ygr268c					w				unknown
Ypl229w						m	m		unknown

Fig. IV.2 Two-hybrid interactions of the EGO complex subunits.

Two-hybrid-screens against the activation domain array of *S. cerevisiae* proteins (AD domain hybrid) were performed with Ego3, Ego1, Gtr1, Gtr1^{Q65L}, Gtr1^{S20L}, Gtr2, Gtr2^{Q66L} and Gtr2^{S23L} proteins as baits (DBD fusion), as described in material and methods. **A**: interactions detected on adenine and histidine selection; **B**: interactions detected solely on adenine selection; **C**: interactions detected solely on histidine selection. Colours determining the different categories are listed below. **s**: strong interaction, colony appears between day 1-7; **m**: mediocre interaction, colony appears between day 7-14; **w**: weak interaction, colony appears between day 15-20. AD: activation domain; DBD: DNA-binding domain.

- Members of the Ego complex
- Loss of the protein confers rapamycin sensitivity
- Membrane transporters
- Intramembranous system (ER/Golgi/Vacuole)
- Nuclear membrane proteins
- Others
- Nuclear proteins
- Putative protein/ protein of unknown function

Among the most promising interacting proteins which we considered to be physiologically relevant, we found Mon1, Yol092w and Nvj1, which had all been previously reported to be present at the vacuolar membrane (388, 412, 413). Mon1, which interacted with Gtr2 and $Gtr2^{Q66L}$, is implicated in bringing membranous material to the vacuole. It is indeed involved in vacuolar fusion of autophagosomes and of endosomes with the vacuolar membrane (414, 415). Accordingly, loss of Mon1 confers a small vacuolar phenotype as opposed to the big vacuolar phenotype found in *ego* mutants (416). *YOL092W,* and *NVJ1*, which encode transmembrane proteins, appeared also as particularly interesting. Yol092w, which interacted with $Gtr1^{S20L}$, is a vacuolar transmembrane protein that belongs to the Ers1 transporter family of the human cystinosin (417). Ers1 genetically interacts with Ego1, which is indicative of a potential link between the EGO complex and members of this family of transporters (411). $Gtr1^{S20L}$ bound also Ybr241c, a transporter with homology to the plasma membrane glucose transporters (Snf3, Rgt2), which is localised at the vacuolar membrane, and whose function is still uncharacterised (412). Finally, Nvj1 which interacted with Gtr2 and Ego1 is involved in the nucleus-vacuole junctions during the microautophagic process called piecemeal microautophagy of the nucleus (418, 419).

Proteins, which specifically interacted with the dominant negative protein, $Gtr2^{Q66L}$, were further tested for a role in exit from rapamycin-induced growth arrest. The underlying idea being that the GTP-bound form of Gtr2 ($Gtr2^{Q66L}$) may be able to bind, but not release its target proteins. This could inhibit the function of the corresponding proteins and hence impair EGO signalling. Accordingly, deletion of the non-essential genes *YJR038C*, *PEP8*, *VPS69*, *NVJ1*, *YSC84* or *YMR279C* could have been expected, like overexpression of $Gtr2^{Q66L}$, to prevent cells from exiting a rapamycin-induced growth arrest. However, we found that none of these proteins was required for the exit from a rapamycin-induced growth arrest (data not shown). This suggests that the phenotype conferred by $Gtr2^{Q66L}$ is due to either simultaneous sequestration/inactivation of several proteins, or that it is due to constitutive activation of one or several target(s) that negatively control exit from the rapamycin-induced growth arrest. In line with the later hypothesis, Mon1, which interacted with $Gtr2^{Q66L}$, promotes fusion of membranous material to the vacuolar membrane, an event that, if constitutively induced by the $Gtr2^{Q66L}$ variant, might prevent vacuolar size reduction and proper exit from G_0 (415). Accordingly, loss of Mon1 did not impair cells from exiting the rapamycin-induced G_0 state (data not shown). It will therefore be interesting to test whether loss of Mon1 suppresses the ego phenotype of $Gtr2^{Q66L}$ or any one of the other *ego* mutations.

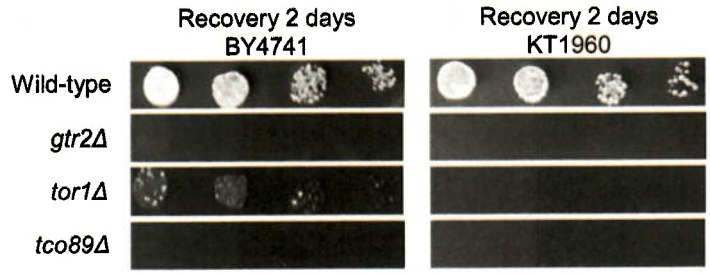
IV.3.3) Does the EGO complex act at the TORC1 level?

One prediction of the model presented in Chapter III is that the EGO complex may act to stimulate TORC1 activity during recovery from rapamycin treatment. In such a hypothesis, loss of Tco89 or Tor1, which partly impairs TORC1 signalling, should prevent full activation of the TORC1 pathway during recovery and consequently confer an ego phenotype (60). We found indeed that loss of Tco89 or Tor1 impaired cells to recover from a transient rapamycin-induced growth arrest (Fig. IV.3 A). While loss of Tco89 conferred, during recovery, a growth defect similar to that observed in *gtr2Δ* mutants, loss of Tor1 allowed partial recovery at least in the YKO background (BY4741). Additionally, after rapamycin removal, *tco89Δ* and *tor1Δ* cells also harboured the big vacuolar phenotype typical of EGO complex mutants (Fig. IV.3 B). In line with the fact that loss of Tor1 allowed partial recovery, we could observe microautophagic vesicles (arrow) within *tor1Δ* mutants. Interestingly, Tco89 and Kog1 localised mainly to the vacuolar membrane, a faint signal also appearing to the plasma membrane. Their vacuolar localisation was unaffected by 1 hr and 6 hr rapamycin treatment, despite a decrease in the GFP signal following rapamycin treatment caused by downregulation of translation initiation (Fig. IV.3 C and data not shown). This localisation pattern of Tco89 fits with the data previously obtained by Reinke *et al.* (2004) via immunogold electron microscopy (60). These results indicate that members of the TOR complex 1 are in close vicinity to members of the EGO complex. Thus, both complexes may actually interact with each other rather directly. The two-hybrid assay done with members of the TORC1 and EGO complex tends to confirm this hypothesis as it revealed a significant interaction between Tco89 and Ego1, although not as strong than between the *bona fide* members of the EGO complex (Fig. IV.3 D).

Fig. IV.3 The EGO complex is linked to the TOR complex 1

(**A**) Loss of the two non-essential components of TORC1, Tco89 or Tor1 impairs the cells to exit from a rapamycin-induced growth arrest. Exponentially growing wild-type cells (BY4741 or KT1960) and their isogenic YKO or KT mutants (OD_{600} of 0.5) were treated with rapamycin for 6 hr, washed once in water and spotted (4µl) on YPD plates. Spots correspond to serial 10-fold dilution of equally dense culture: first spots on the right result from an OD_{600} culture of 1. (**B**) Tco89 interacts with Ego1. β-galactosidase activities were measured in triplicate after growth for 20 hr at 30°C in SGal/Raf medium supplemented with leucine. The average values (in Miller units) are shown. Values that are at least ten fold higher than the corresponding control are shown in bold. AD: activation domain, DBD: DNA-binding domain. (**C**) *tor1Δ* (FD29) or *tco89Δ* (FD27) mutants displayed a dramatically enlarged vacuole following release from the rapamycin block. Wild-type (KT1960), *gtr2Δ* (CDV212), *tor1Δ* (FD29) and *tco89Δ* (FD27) cells growing exponentially on SD minus uracil (Untreated) were treated for 6 hr with rapamycin (Rapamycin 6 hr), washed twice, resuspended in the same medium without rapamycin and incubated for 42 hr at 30°C (Recovery). To visualise boundaries of the vacuolar membrane cells were labelled with the fluorescent dye FM4-64 and additionally expressed *GFP-ALP* (from plasmid pRS426-*GFP-ALP*), which encodes the vacuolar membrane protein alkaline phosphatase fused to GFP. The arrow shows a microautophagic vesicle. (**D**) Kog1 and Tco89 localise to the plasma membrane and the limiting membrane of the vacuole. Exponentially growing wild-type cells, carrying chromosomally tagged *KOG1-GFP* or *TCO89-GFP* were treated or not with rapamycin and visualised with fluorescent microscopy.

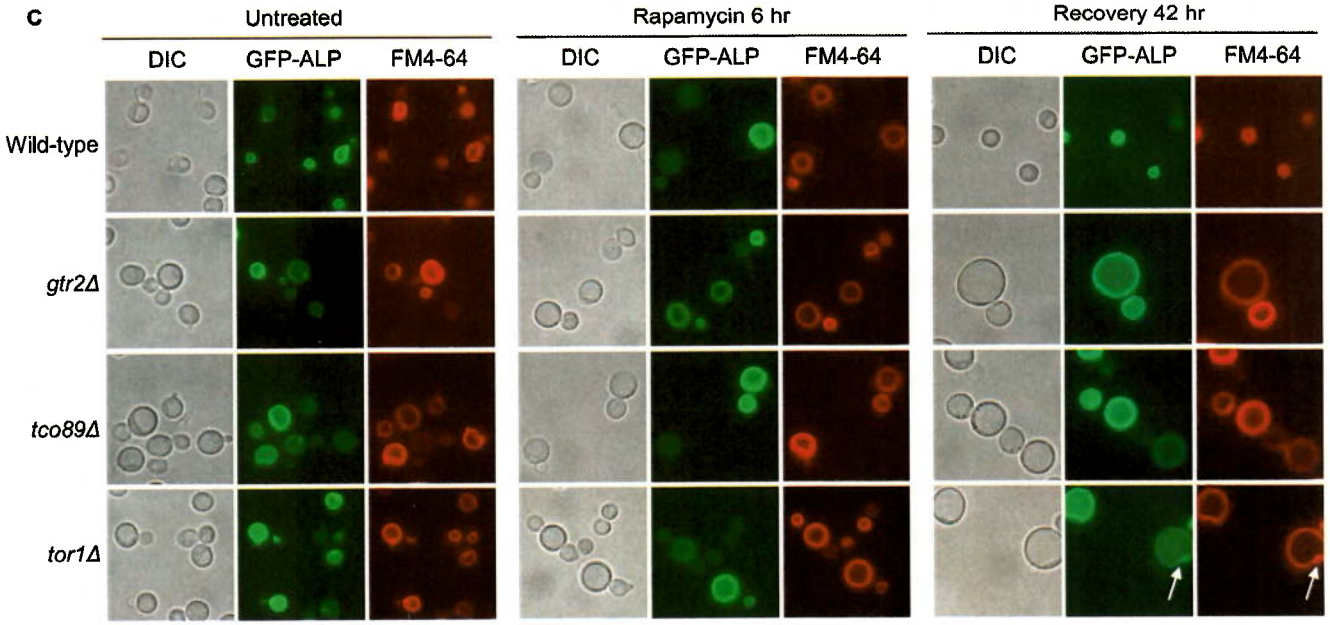
A



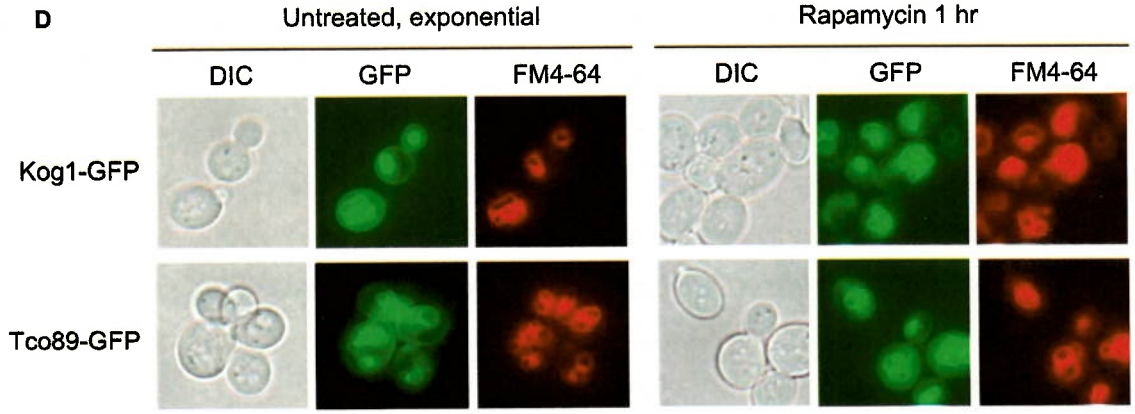
B

	B-galactosidase activity with DBD fusion		
AD fusion	*CDC3*	*EGO3*	*EGO1*
CDC3		10	19
EGO3	3		**2515**
EGO1	4	**3064**	
GTR2	3	8	**2047**
TCO89	1	5	**467**

C



D



IV.4) Discussion

This chapter describes our recent advances concerning our knowledge of the EGO complex, collected notably, through study of the protein-protein interactions via two-hybrid analysis. With this method, we identified the putative small GTPase Gtr1 as a strong binding partner of Ego1 and Gtr2. Based on our results and on present information available on Gtr1 (*i.e.*, it resides at the vacuolar membrane, Co-immunoprecipitates with Ego1, and its loss causes both a slow growth phenotype (SGD data) and rapamycin hypersensitivity (411, 412, 420)), Gtr1 is likely an additional new member of the EGO complex. It is still necessary to confirm that Gtr1 is a true member of the EGO complex, notably by validating the two-hybrid interactions found with Gtr2 and Ego1 using Co-immunoprecipitation (Co-IP) experiment, by testing the rapamycin sensitivity of *gtr1Δ* mutants and by checking additional G_0 readouts including the vacuolar phenotype of *gtr1Δ* mutants and the capacity of Gtr1 to restore microautophagy. It will be also interesting to characterise the phenotype of the possible GTP- and GDP-bound variants of Gtr1. Indeed, according to a previous study, the $Gtr1^{S20L}$ variant, similarly to $GTR2^{Q66L}$ is likely to result in a dominant negative form of the protein (397). It should be mentioned that we failed to detect *gtr1Δ* in the screen for *ego* mutants because the YKO *gtr1Δ* is apparently not correctly deleted in *GTR1*.

The different two-hybrid screens yielded several proteins among which three, Yol092w, Nvj1 and Mon1, are particularly interesting because they all have a function in relation with the vacuolar compartment and potentially related to the function of the EGO complex. Yol092w belongs to a transporter family which comprises Ers1. Ers1 localises at the vacuolar membrane and has a genetic link with Ego1 (411). The human homolog of Ers1, namely cystinosin, is involved in the transport of cystine from the vacuole to the cytoplasm, where cystine is further reduced into the amino acid cysteine (411, 417). If such a mechanism is conserved, this interaction provides a link between the EGO complex and vacuolar amino acids transport. One may speculate that the EGO complex use Yol092w to sense the vacuolar amino acids concentrations or to promote amino acid export from the vacuole for refeeding in the cytoplasm. The possibility that Gtr2 may regulate another microautophagic process, named piecemeal microautophagy of the nucleus, is particularly interesting and will require further investigations (388, 419). However, interactions between EGO members and these three proteins will have first to be confirmed in a second two-hybrid assay and possibly via Co-IP experiments. Their corresponding mutants will then be tested for ego phenotype or for suppression of the ego phenotype. If positive, further studies should focus on the role played by these proteins in EGO signalling.

Unfortunately, the screens undertaken failed to fish the expected GAP and GEF of Gtr1 and Gtr2. Anyway, utilisation of the predicted GTP- and GDP-bound conformations of Gtr1 and Gtr2, as expected, greatly enlarged the number of proteins potentially interacting with the two putative small GTPases. It is clear also that the two-hybrid collection bears a certain amount of errors (clones expressing pAD-*GTR2* and pAD-*EGO3* failed to reveal their strong positive interaction with Ego1) and the screen is therefore not fully saturated. As envisaged, the two-hybrid screen also revealed many potential false-positive clones which, from their localisation or their function have very few chances to physiologically bind the EGO complex (409). Nevertheless, it would be interesting to reproduce independently the two-hybrid interactions between EGO complex members and the proteins of unknown function, as in this case, the relevance of the interaction cannot be established.

Finally, the most promising finding of this chapter concerns the close interaction between TORC1 and the EGO complex, strongly suggesting that both complexes act in the same pathway. Several observations support a rather direct link between the EGO complex and TORC1. First, we found that Ego1 directly interacts with Tco89. Secondly, in line with previously published results, we observed that the TORC1 components Tco89 and Kog1, like the EGO complex, localise to the limiting membrane of the vacuole (60, 412). Thirdly, loss of Tco89 or Tor1 caused a phenotype similar to the phenotype caused by loss of EGO complex components. Our preliminary results have to be confirmed notably through Co-IP of Tco89 with the different members of the EGO complex and by epistasis analysis between the EGO complex and TORC1. These should address the role of both complexes for various known TORC1 readouts and for the newly identified potential TORC1-controlled process of microautophagy

Chapter V.

Concluding Discussion and Perspectives

V.1) General discussion

The study of the mechanisms controlling entry into, survival in and exit from G_0 is the object of numerous investigations in particular because, as mentioned in the introduction, this subject could end in many outlets, notably in biomedical fields as various as research for fight against cancer, slowing down aging and fighting pathogenic microorganisms (8, 89). Despite progress made, the underlying mechanisms are poorly understood and still under intense study.

In this thesis work, two different approaches have been used that aimed at elucidating the processes that regulate cell growth and cell proliferation in response to nutrients. In a first approach, presented in Chapter III, the study focused on the understanding on how cells properly enter into G_0. The protein kinase Rim15 is crucial in this response. In this context, I found that nutrient signals not only regulate Rim15 kinase activity, but also its intracellular localisation. This work also demonstrated that Rim15 can integrate signals from TORC1 and Sch9, in addition to PKA, and hence represents a central hub that integrates nutritional signals, including glucose and nitrogen signals, to control G_0 entry. In a second approach, used in Chapter III and IV, studies were focused on experiments aimed to understand the mechanisms governing the exit of cells from a G_0 state, which was artificially induced by rapamycin treatment. This second approach led to the identification of a new protein complex, the EGO complex, which localises to the vacuolar membrane. The EGO complex is linked to the TORC1 pathway and is required for exit from a rapamycin-induced G_0-like state. Taken together our studies point toward a role of the TORC1 complex in two distinct processes (which take place in G_1) that are regulation of entry into and exit from quiescence.

V.1.1) Gaining new insights into the mechanisms regulating G_0 entry: integration of signals from three nutrient-regulated pathways by the Rim15 protein kinase

When we first initiated this work, only limiting information was available on the crosstalks existing between the PKA and the TORC1 pathways (46, 421). Our study demonstrates that TORC1 and PKA act separately to negatively regulate Rim15. Particularly interesting, our finding that TORC1, Sch9 and nutrient limitation negatively regulate cytoplasmic to nuclear translocation of Rim15 provides a powerful readout to check whether a particular nutrient impinges on Rim15. This tool was particularly useful in depicting upstream regulators of Rim15. In this part of my thesis work, I will discuss the results obtained in Chapter II, in light of recent studies undertaken in our laboratory a well as in the context of recently published work in the literature.

V.1.1.1) TORC1 regulates nucleocytoplasmic localisation of Rim15

Rim15 has previously been shown to be important for cells to be able to acquire, upon nutrient limitation, a set of physiological characteristics necessary for proper entry into G_0 (48). Results presented in Chapter II demonstrate that, similarly to what happens at the diauxic transition, acquisition of some of the G_0 traits induced upon TORC1 inhibition are strongly dependent on the presence of Rim15. Thus, like PKA (48), TORC1 is involved in the negative regulation of Rim15. Rapamycin treatment also correlates with the appearance of a hyperphosphorylated form of Rim15, likely to reflect autophosphorylation of the kinase, and hence indicative of Rim15 activation (98). Since TORC1 inhibition increases Rim15 kinase activity and induces Rim15-controlled readouts, this suggests that the kinase function of Rim15 is critically involved in expression of the G_0 traits (98).

Unlike PKA, TORC1 appears to regulate negatively Rim15 by preventing its access to the nucleus. A recent study showed that TORC1 similarly prevents nuclear accumulation of two other nutrient-regulated kinases associated with the PKA pathway, namely Yak1 and Tpk1 (88). Yak1 is a protein kinase, which is probably, like Rim15, negatively regulated by PKA, while Tpk1 is one of the three catalytic subunits of the yeast PKA (239, 249, 309, 422). This common regulation suggests that intracellular compartmentalisation could be a conserved mechanism by which TORC1 not only controls activity of the transcription factors, but also the activity of protein kinases, specifically those that are also implicated in the PKA pathway (35, 68, 81). This could make sense in view of the fact that Bcy1, the inhibitory subunit of Tpks, is mostly localised in the nucleus (during exponential growth). Accordingly, the nucleus could constitute a potentially low PKA environment, which favours activation of targets

negatively regulated by PKA (97, 243). Thus, rapamycin-induced nuclear accumulation of Rim15 and of Yak1 may allow these proteins to escape PKA-mediated inhibition in the cytoplasm.

Tap42 and its associated protein phosphatases mediate many of the TORC1 functions in yeast (68, 69, 72, 423). Our results demonstrate that TORC1 regulates Rim15-dependent transcription independent of Tap42 and its associated protein phosphatases (*i.e.*, Pph21 and Pph22 or Sit4). Similarly, Rim15 subcellular localisation appears to be controlled independently of Sit4. Thus, Rim15 likely constitutes a new TORC1 effector branch. According to Beck *et al.* (1999) The two transcription factors Msn2 and Msn4 are also regulated via a Tap42-independent branch of the TORC1 pathway (68). Since Msn2 mediates part of the Rim15-dependent transcriptional response, it makes sense to find both, the protein kinase and the transcription factors, in a Tap42-independent effector branch of the TORC1 pathway (50). This could indicate a common and hence coordinate mode of regulation. However, results of Beck *et al.* (1999) were recently called into question by Düvel *et al.* (2003), who claim that rapamycin-induced expression of STRE-controlled genes is dependent on a Tap42 function (68, 72). In further studies, this group also demonstrated the requirement of Pph21 and Pph22 for rapamycin-induced nuclear localisation of Msn2 (68, 71). In fact, the studies from the Hall and Broach groups use of different properties of specific *TAP42* alleles (*i.e., tap42-11*, *tap42-106,* or *tap42-109*). Additionally, their experiments were carried out in different strain backgrounds, in which the primary response of Msn2 to rapamycin is apparently different, making direct comparison obviously difficult (65, 110). As long as the multiple roles played by the phosphatases and Tap42 are not entirely understood, it will remain difficult to reconcile these contradictory results (348).

V.1.1.2) Sch9 also regulates nucleocytoplasmic localisation of Rim15

Sch9 is a protein kinase that is related structurally and functionally to PKA. As a member of the FGM pathway, Sch9 has been proposed to act independently of cAMP to control the phenotypic characteristics of an activated PKA pathway in response to the presence of nitrogen and glucose in the medium (54, 55, 191, 318). Surprisingly, our study reveals that Sch9 negatively regulates the nuclear localisation of Rim15, a readout, which is also controlled by TORC1 but not by PKA. This finding indicates that Sch9 acts upstream of Rim15 and likely impinges on the protein independently from the PKA pathway. This conclusion is in line with that of Roosen *et al.* (2005), who put Sch9 and PKA in two parallel pathways, based notably on the finding that Sch9 controls some growth regulatory functions

independently from PKA (*i.e.*, Sch9 improves growth on glycerol independently of PKA) (319). Although TORC1 and Sch9 both prevent Rim15 nuclear localisation, they may provide separate inputs on Rim15 since, in cells deleted for *SCH9*, Rim15 phosphorylation remains sensitive to rapamycin treatment. In this context, Roosen *et al.* (2005) also prefer a model in which TORC1 and Sch9 act in two separate pathways to control STRE-controlled gene transcription, a notion that is based notably on their observation that TORC1, but not Sch9, regulates Msn2 localisation (319).

Despite the constitutive nuclear localisation of Rim15, *sch9Δ* mutants are unable to trigger transcriptional activation of *HSP12, SSA3* and *HSP26* even in the presence of rapamycin. This is somewhat unexpected, since loss of Sch9, like rapamycin treatment, was predicted to allow Rim15 to accumulate in the presumably low PKA nuclear environment, a response which was a priori, envisaged to promote Rim15 activity. In view of this same paradox, Roosen *et al.* (2005) suggested that Sch9, while acting as a negative regulator of Rim15, could act independently of Rim15 to positively regulate PDS element-controlled gene transcription, possibly, through Sch9-mediated positive regulation of Gis1 (319). Hence, loss of Sch9 would downregulate Gis1 and consequently alter PDS element- and also possibly STRE-controlled gene transcription (provided Gis1 has overlapping functions with Msn2 and Msn4) (319). Additionally, in their study, Msn2/4 appeared to be cytoplasmic and inactive in *sch9Δ* mutants. Thus, in such a mutant, the nuclear pool of Rim15 even if activated would be unable to properly induce transcription of the STRE-controlled genes since one of its indirect downstream targets is missing.

Nevertheless, these explanations fail to account for the lack of Rim15-dependent transcriptional response observed in *sch9Δ* mutant following rapamycin treatment. Indeed, according to the study of Roosen *et al.* (2005). TORC1 inhibition should promote nuclear translocation of Msn2 independently of Sch9 and hence activate at least STRE-controlled genes (46, 68). On the other side, several observations tend to indicate that deletion of *SCH9*, causes elevated PKA activity (55). Whatever is the cause, this increased PKA activity is expected to reinforce the negative signal of PKA on Rim15, possibly also within the nucleus. Thus, according to this model, the nuclearly localised Rim15 protein is kept inactive due to the negative control of PKA and cannot induce STRE- and PDS element-controlled transcription even after rapamycin treatment. In line with such a model, the transcriptional pattern of *SCH9* deleted cells strikingly resembles that of cells with a hyperactive PKA pathway (*bcy1Δ*, $Ras2^{Val19}$), in which rapamycin fails to induce transcription of *HSP12*, *SSA3* and *HAP26* despite localisation of Rim15 in the nucleus (88). Several independent studies

further confirmed that hyperactivation of the PKA pathway blocks rapamycin-induced responses including, in addition to STRE-driven gene transcription, glycogen accumulation or translocation of Msn2 in the nucleus (88, 391).

In fact, the high PKA phenotype of *sch9Δ* mutants observed in our work and in several other independent studies could be caused by the presence of additional mutation(s) in this mutant (55, 319). Indeed, according to recent observations done in the R. Loewith group and in our group, it seems that the slow growing *sch9Δ* mutants easily pick up suppressors, which likely activate the PKA pathway. Thus, it is possible that the *sch9Δ* mutant used for the Northern blot presented in our publication has acquired such a mutation. It is therefore difficult to interpret this data and the experiment will have to be repeated, with *bona fide sch9Δ* mutant cells (which can be identified by their elevated glycogen level during exponential growth). Because Rim15 nucleocytoplasmic localisation is regulated independently of the PKA pathway, *sch9Δ* suppressor mutations that presumably hyperactivate PKA should not have an impact on Rim15 localisation.

The mechanisms by which Sch9 regulates localisation of Rim15 are not determined yet. However, recent analysis of the primary sequence of Rim15, revealed in its kinase insert three putative consensus phosphorylation sites for Sch9, which actually fit with the consensus motif for PKB/Akt phosphorylation (RXRXXS/T). This suggests that Rim15 may possibly be a direct target of Sch9 phosphorylation (3, 49, 424). Interestingly these sites also overlap with low stringency Bmh1 and Bmh2 binding sites (according to scansite) leading to the hypothesis that Sch9 could directly phosphorylate Rim15 and promote its binding to Bmh1 and Bmh2. Because 14-3-3 proteins have already been involved in nuclear exclusion of Yak1 or in the cytoplasmic retention of Msn2, it would then not be surprising to find them implicated in regulation of Rim15 subcellular localisation (68, 425, 426).

V.1.1.3) Does TORC1 act upstream of Sch9?

In mammalian cells, the Pkb/Akt protein kinase, which is notably involved in cell proliferation and cell survival, acts in insulin signalling upstream of the rapamycin-sensitive mTORC1 pathway and downstream of the rapamycin-insensitive mTORC2 pathway (320). Because the TORC1 pathway is quite conserved, this raises the question whether Sch9, the yeast Pkb/Akt homolog, previously suggested to feed into the PKA pathway (54), could instead feed into or act in the TORC1 pathway similarly to what has been described in mammalian cells. In support of such a model, both TORC1 and Sch9 similarly regulate Rim15

localisation. Additionally, Sch9 has been shown to share common downstream targets with TORC1. For instance, Sch9 activates transcription of RPGs and of genes required for ribosome assembly, which are also positively regulated by the TORC1 and the PKA pathways (390, 427). However, in yeast Sch9 seems to act downstream of TORC1. Indeed, recently, a study demonstrated that rapamycin treatment promotes degradation of Sch9 and causes its dephosphorylation (427). In the same study, Sch9 has been identified as being localised to the vacuolar membrane. Since components of the TOR complex one were found in association with membranous structures (including the vacuole), it is possible that Sch9 directly interacts with TORC1 for its regulation (427). Thus, in such a model, the FGM pathway would simply be a TORC1 effector branch that impinges on PKA targets.

The model of Sch9 being downstream of TORC1 fits with a possible role of the PKB/Akt homolog in the direct phosphorylation of Rim15. However, the phosphorylation pattern of Rim15 is still responsive to rapamycin treatment in the absence of Sch9, supporting our model in which TORC1 and Sch9 act on Rim15 via separate inputs. The hypothesis of Sch9 being downstream of TORC1 could nevertheless be reconciled in a scheme where TORC1 provides two separate inputs on Rim15, one through Sch9, to regulate the localisation of the protein and the second, independent of Sch9, to modulate the phosphorylation state of Rim15.

V.1.1.4) The Pho80/Pho85 module also regulates nucleocytoplasmic localisation of Rim15

Recent findings by Wanke *et al.* (2005) allowed us to understand mechanistically the way Rim15 localisation is controlled in response to phosphate starvation (98). This study focused on Pho85 a protein previously found to directly interact with Rim15 in a systematic study of protein complexes in *S. cerevisiae* (428). The Pho85 protein is a cyclin-dependent kinase (CDK), which can associate with ten different cyclin and assumes particular functions according to the cyclin it binds (429). Pho85 forms, in association with Pho80, a nutrient-regulated complex, which is inactivated by low levels of inorganic phosphate and may consequently link Rim15 function to the phosphate metabolism (430).

The model proposed by Wanke *et al.* (2005) establishes that the nuclear Pho80-Pho85 complex acts, under high phosphate conditions, upstream of Rim15 to promote its cytoplasmic retention by the 14-3-3 proteins Bmh1/2 (98, 425, 431). Notably, Pho85-dependent phosphorylation in the Rim15 kinase insert of the residue Thr1075, which is part of a consensus 14-3-3 binding motif, promotes binding of that domain of Rim15 to Bmh2

(432). Their study demonstrates that Bmh1/2 are of major importance to maintain Rim15 in the cytoplasm and the model point toward an important role of the kinase insert in the mechanism of cytoplasmic retention (98). Conversely, in low phosphate conditions Thr1075 is dephosphorylated and Rim15 accumulates in the nucleus (98). The Pho80-Pho85 module seems to operate in a pathway parallel to TORC1 based on the synergistic effects of phosphate starvation and TORC1 inactivation (98).

V.1.1.5) Control of nucleocytoplasmic localisation of Rim15: a model

The sum of the data obtained by the different studies undertaken on the regulation of Rim15 makes it possible to establish a model on how Rim15 nucleocytoplasmic localisation is regulated (Fig. V.1).

Rim15 localisation is regulated by at least tree nutrient regulated kinases, Pho85, Sch9 and TORC1 (98). Pho85 transmits information on phosphate availability, while the nutrient information transmitted by TORC1 and Sch9 is less well defined, but probably includes glucose and nitrogen availability (possibly for TORC1 glutamine availability) (6, 55, 61). Accordingly, Rim15 translocates into the nucleus upon phosphate starvation or, during mid-exponential phase, when half of the glucose has been consumed in the growth media. In the latter case, the exact nutrient signal that triggers Rim15 translocation remains elusive and probably involves integration of several other clues than simply glucose. Indeed, unpublished data demonstrated that neither sudden glucose depletion, nor artificial induction of the diauxic phase (by incubating the cells in a mixture containing 0.02% of glucose and 2% ethanol supplemented with all other essential nutrients) were able by themselves to induce nuclear translocation of Rim15. Interestingly, the time course of this entry corresponds to the onset of glycogen biosynthesis and of SSA3 transcription, two cellular traits controlled by Rim15 (48, 194). Thus, Rim15 most probably senses the general nutrient level or the gradual changes that occur during exponential growth in the nutrient status (*e.g.*, glucose) or in the level of (an)other metabolite(s).

Rapamycin- or nutrient-induced nuclear accumulation of Rim15 correlates with acquisition of the Rim15-dependents G_0 readouts. As already observed for transcription factors, the localisation of Rim15 in the nucleus is probably useful to bring the protein kinase in close proximity of its putative nuclear target(s), to mediate Gis1 and Msn2-dependent transcription, and to potentially relieve Rim15 from PKA inhibition provided the nucleus is a low PKA environment (97, 243, 433). Because rapamycin-induced hyperphosphorylation of Rim15

correlates with nuclear accumulation of the protein, it was tempting to speculate that phosphorylation is the mechanism by which Rim15 translocates from the cytoplasm to the nucleus upon TORC1 inactivation. However, while Rim15 is in the nucleus of *sch9Δ* mutants and of rapamycin-treated cells, it is hyperphosphorylated only in the second case, indicating that, as in the case of Gln3, there is no clear correlation between the general phosphorylation state of Rim15 and its nuclear transport (359). This is not surprising as Rim15 contains multiple phosphorylation sites. If a specific phosphorylation or dephosphorylation event indeed regulates Rim15 localisation, it is likely not to be detected by simple gel-shift experiments.

Mechanistically, Pho85 negatively regulates nuclear accumulation of Rim15 through cytoplasmic retention (434). The Pho80-Pho85 complex maintains Rim15 in the cytoplasm in part by discrete phosphorylation of its kinase insert (Thr1075) and subsequent binding of the phosphorylated residue to Bmh2 (98). Preliminary data indicate that TORC1 activity is required for maintaining the Thr1075 residue phosphorylated, probably by keeping a phosphatase inactive (98). In addition, Sch9 may phosphorylate *in vitro* the kinase insert of Rim15 which contains supplemental possible 14-3-3 binding sites (98). These observations suggest that the three kinase use similar mechanisms (*i.e.*, phosphorylation and cytoplasmic retention via Bmh1/2) to regulate Rim15 localisation. Finding that Sch9 phosphorylates a 14-3-3 binding site in the kinase insert different from the one targeted by Pho80-Pho85 would not be surprising, inasmuch as phosphorylation of the Thr1075 residue accounts only for a part of the cytoplasmic retention mediated by the kinase insert (98). Additionally, PKB/Akt, the mammalian homolog of Sch9, has been shown to phosphorylate and consecutively mediate the binding of three of its targets (FKHRL1, BAD and FKHR) to 14-3-3 proteins (435, 436). This reinforces our predictive model and indicates that the way PKB kinases regulates some of their downstream targets might be conserved among eukaryotic cells. In contrary to the Pho80-Pho85 complex, which is supposed to modify Rim15 in the nucleus, Sch9 and TORC1 most probably act in the cytoplasm (94, 98, 427, 431).

Nuclear export and import of proteins occurs through the nuclear pore complex and is regulated by exportins and importins, respectively (for reviews see (437, 438)). These proteins, also named karyopherins, act as transport receptors that bind and mediate the translocation of cargoes. Screening a collection of viable exportin mutants (transformed with the *GFP-RIM15* construct) for cells with constitutive nuclear localisation of Rim15, allowed us to identify Msn5 as the potential Rim15 exportin (98). This result is particularly relevant as Msn5 is also involved in the nuclear export of several transcription factors that are negatively

regulated by TORC1 or Pho85 (*e.g.*, Msn2/4, Rtg1/3, and Pho4) (46, 431, 433, 439). Nucleocytoplasmic localisation of proteins depends on a balance between import and export processes (440). Because inhibition of nuclear export (in the *msn5Δ* mutant) during exponential growth can trap Rim15 in the nucleus, the mechanisms of cytoplasmic retention probably decrease but do not completely abolish nuclear import of the protein. This finding implicates that, during exponential growth, a small quantity of Rim15 keeps shuttling in and out of the nucleus. Interestingly, the kinase activity of Rim15 has been shown to facilitate nuclear export (98). In fact, facilitating nuclear export of the kinase active Rim15 could constitute a mechanism of defence used by the cells to prevent that a too large amount of the active protein accumulates and transmits excessive signal in the nucleus. Indeed, excessive nuclear signalling could be, as mentioned in the Wanke *et al.* (2005) study, potentially toxic for cells (at least when Rim15 is overexpressed) (98). It is also possible, as suggested for Snf1-mediated regulation of Msn2, that rapid export of the active form of Rim15 in the cytoplasm allows rapid inactivation of the protein kinase by PKA and TORC1 when nutrients are again abundant (367).

The mechanisms used by cells to control the localisation of Rim15 resemble those used to control the localisation of Msn2. Indeed, both protein are retained in the cytoplasm via Bmh1 and Bmh2 and exported via Msn5, and in both cases nuclear accumulation of the proteins is negatively controlled by TORC1 via an effector branch independent of Sit4 (46, 68, 71). Thus, Msn2 and Rim15 share common regulatory mechanisms, which could be important for coordination of the STRE-controlled gene transcription at the diauxic transition (50). However, in contrary to Rim15, nuclear import and export of Msn2 is also negatively regulated by PKA (46, 47).

V.1.1.6) PKA regulates Rim15 activity independently of TORC1

Results obtained in Chapter II led to the conclusion that the TORC1 and PKA pathways act separately to inhibit Rim15. This conclusion was principally based on the following observations: (i) TORC1, but not PKA, regulates Rim15 localisation; (ii) decreased PKA activity and TORC1 inactivation have additive effects with respect to the induction of *SSA3*, *HSP26* and *HSP12* expression; (iii) hyperactive PKA can apparently keep Rim15 inactive independently of TORC1, since it maintains repression on Rim15 upon TORC1 inhibition despite nuclear accumulation of Rim15; (iv) rapamycin-induced transcription of *SSA3*, *HSP26* and *HSP12* depends on the presence of Rim15 but not of PKA (in *tpk1, 2, 3Δ yak1Δ* strain). In other words, the latter result also indicates that in the absence of PKA, Rim15 is

still kept inactive by TORC1-dependent cytoplasmic retention. In line with these data, the study of Zurita-Martinez *et al.* (2005) also came to the general conclusion that TORC1 and PKA constitute parallel pathways that regulate common downstream targets (391). Their results are essentially based on the comparison of rapamycin-mediated responses obtained in the presence or absence of PKA. They notably demonstrate that TORC1 inactivation and loss of PKA have cumulative effects on glycogen accumulation and on STRE-controlled gene transcription (*SOD2*). Finally, based on the fact that rapamycin treatment does not affect intracellular cAMP synthesis, they also discarded the possibility that TORC1 acts as an upstream regulatory component of the PKA pathway (391). Similarly, Marion *et al.* (2004) demonstrated that both, TORC1 and PKA, negatively regulate nuclear exclusion of the transcription factor Sfp1, at least in part via two separate pathways. This model was based on the observation that in the absence of PKA, Sfp1 still localises in the nucleus and properly translocates into the cytoplasm upon rapamycin treatment (81).

In contrast to the study mentioned above, Schmelzle *et al.* (2004) concluded from their experiments that TORC1 feeds into the cAMP-PKA signalling pathway to regulate among others STRE-controlled gene transcription (88). Their conclusions are essentially based on the following observations: (i) constitutive activation of the PKA pathway (*RAS2*^{Val19} or *bcy1Δ* mutants) blocks several rapamycin-induced G_0 responses (*e.g.,* glycogen accumulation, STRE-controlled gene transcription and RPGs downregulation); (ii) constitutive activation of the PKA pathway confers rapamycin resistance, indicating that PKA acts downstream of TORC1; and (iii) TORC1 negatively regulates Tpk1 nuclear localisation and hence should act upstream of PKA. However, whether this change in Tpk1 localisation has an impact on PKA activity is not known yet. Although the results they obtained fit globally with our results, their entire study exclusively relies on phenotypical observations of mutant cells with a hyperactive PKA pathway. Responses following TORC1 inactivation were not assessed in the absence of PKA (88). The simple fact that hyperactive PKA prevents part of the rapamycin-induced readouts, or confers rapamycin resistance does not allow to discriminate between a model in which TORC1 and PKA act in parallel on common downstream targets, or a model in which TORC1 regulates PKA. In fact, these results simply demonstrate that TORC1 is not downstream of PKA.

In principle, the studies of Schmelzle *et al.* (2004) and of Zurita-Martinez *et al.* (2005) come to different conclusions, while essentially based on similar results (88, 391). Notably, these opposite interpretations must not be mutually exclusive and both models may to some extend reflect true cellular mechanisms. At present, however, the single strong evidence of

TORC1 being upstream of the PKA pathway relies on the results that TORC1 regulates Tpk1 localisation (88). Conversely, there is strong evidence for TORC1 and PKA pathways providing separate inputs to regulate Msn2 nuclear localisation (see Chapter I) (46). Accordingly, TORC1 promotes nuclear export while PKA nuclear import of Msn2, two processes involving modifications of different part of the protein (88). Clearly, discrimination between the different models requires further studies, notably also aimed at understanding mechanistically how TORC1 and PKA impinge on common targets.

V.1.1.7) Rim15 orchestrates G_0 entry: a comprehensive model

The Rim15 protein regulates proper entry into G_0 (48, 51). Induction of the Rim15-dependent G_0 traits is assumed to require: (i) nuclear accumulation of Rim15, which is negatively regulated by a Sit4-independent TORC1 effector branch, Sch9 and Pho85, and (ii) release from PKA-mediated inhibition (98). PKA directly phosphorylates and inhibits the kinase activity of Rim15, while TORC1 probably indirectly inhibits Rim15 kinase activity by preventing translocation of the protein into the presumably low PKA nuclear environment (48). Accordingly, to acquire Rim15-dependent G_0 characteristics cells have to be relieved from TORC1 inhibition, which promotes nuclear entry of Rim15 and from PKA inhibition. Constitutive activation of the PKA pathway (in *bcy1Δ, RAS2*^{Val19} strain) may prevent rapamycin-induced transcription, probably because PKA-mediated inhibition can occur in the nucleus. Conversely, in cells with constitutively low PKA activity (*ras2Δ*), full (or even enhanced) transcriptional derepression of Rim15-controlled genes occurs only after TORC1 inactivation and consequently after cytoplasmic to nucleus transfer of Rim15. Sch9 probably acts to inhibit Rim15 nucleocytoplasmic localisation. Since TORC1-dependent regulation of Rim15-dependent G_0 readouts occurs independently of the Pph21 and Pph22 phosphatases, this suggests that subcellular localisation of Rim15 may also be regulated independently of the Pph21 and Pph22 phosphatases. However, due to the low GFP signal in the *pph21Δ* p*ph22Δ* double mutants we were unable to confirm this hypothesis. Finally, acquisition of the Rim15-dependent phenotypes observed at the onset of the diauxic transition are probably caused by simultaneous downregulation of several regulators of Rim15, including the PKA and the TORC1 pathways.

Rim15 integrates signals from at least four nutrient-sensory kinases (Pho85, PKA, Sch9 and TORC1) to control G_0 entry via its presumed downstream targets Msn2, Msn4 and Gis1 (50, 51). Rim15 also similarly positively controls another readout which is extension of chronological life span (*i.e.*, the long term survival of a population of non-dividing cells) (56).

Chronological life span extension is caused in part by transcriptional induction of *SOD2*, a gene whose products is involved in antioxidant response (441). Its transcriptional induction is strongly dependent on the presence of Rim15 (50, 442). Interestingly, Rim15 is required to mediate the extension of chronological life span observed in *sch9Δ* mutants or in mutants with reduced PKA activity (*cyr1::mTn*) (56, 257, 442). Thus, downregulation of PKA or loss of Sch9 may activate Rim15 and its presumed downstream targets Msn2/4, and Gis1 to induce transcription of *SOD2* and thereby increase longevity in G_0 (50). Recently, TORC1 downregulation has also been involved in chronological life span extension (443), suggesting that activation of Rim15 is also involved in this phenotype. This supports our finding that Rim15 constitutes a point of convergence between the major nutrient-regulated kinases PKA, TORC1 and Sch9 (Fig. V.1). Thus Rim15, by connecting these different nutrient signalling pathways, seems to play a central role in regulating important processes involved in yeast proliferation and aging. Interestingly, as reduced PKA activity is required to increase longevity, the *sch9Δ* mutants used in these studies probably do not have a hyperactive PKA pathway. If the high PKA phenotype observed in *sch9Δ* mutants is caused by suppressors, it might be possible that the strains used in the aging experiments acquire suppressors less frequently than our strains, or that the experimental conditions used for aging studies allow for selection of *bona fide sch9Δ* mutants.

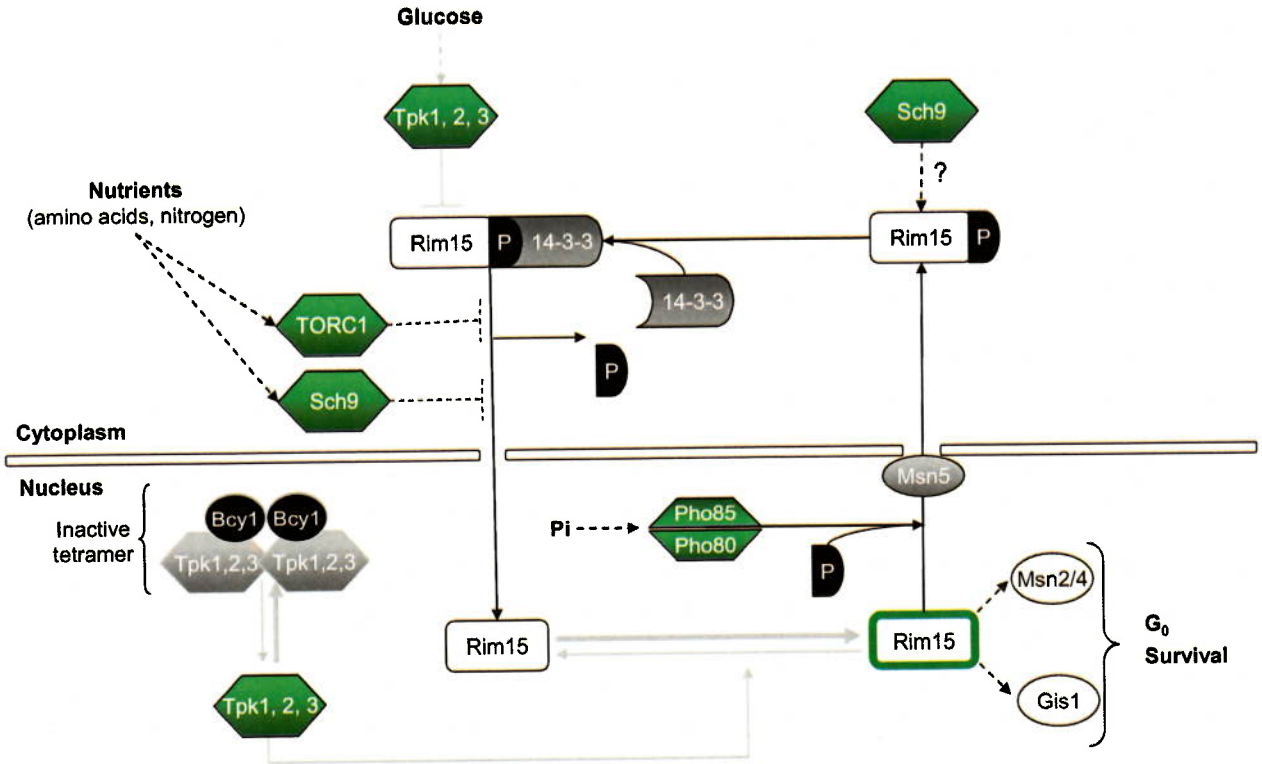


Fig. V.1. Model for the nucleocytoplasmic regulation of Rim15 by nutrient-sensory kinases

When nutrients (glucose, nitrogen, amino acids and phosphate) are abundant, Rim15 localises in the cytoplasm, where it is anchored through its binding to the 14-3-3 proteins Bmh1 and Bmh2. TORC1 keeps Rim15 in the cytoplasm, notably by preventing dephosphorylation of Thr1075, while Sch9 negatively regulates nuclear entry of Rim15 by a still unidentified mechanism. High phosphate levels promote phosphorylation of Thr1075 in Rim15 (P) by the Pho80-Pho85 complex and positively control cytoplasmic retention of Rim15. Upon nutrient limitation or TORC1 inactivation, Rim15 translocates into the nucleus, where its activation is likely facilitated due to the low level of catalytically active PKA (Tpk1, 2, 3) in the nuclear environment. Active Rim15 (green) regulates entry into G_0 and cell survival into G_0. Export of Rim15 out of the nucleus requires the nuclear exportin Msn5. Adapted from (3).

Green: activated nutrient-sensory kinases; Arrow: direct activation; Bar direct inhibition; Dashed arrow: presumable, indirect activation; Dashed bar: presumable, indirect inhibition; Double arrows: equilibrium. Black arrow/bar: regulation of Rim15 localisation; Grey arrow/bar: regulation of Rim15 kinase activity.

V.1.1.8) Rim15: future issues to be addressed

Despite of the results obtained in this study, the way Rim15 controls mechanistically entry into G_0 is still not fully understood. Indeed, while the domain architecture of Rim15 is fairly well characterised and several regulators of Rim15 are known (50, 98), the way TORC1 and Sch9 impinge on Rim15 has still to be determined, direct downstream targets of Rim15 have still to be found, and the precise mechanisms regulating nucleocytoplasmic localisation of Rim15 have to be further characterised.

5.1.1.8.1) Identification of the mechanisms used by the nutrient-sensitive kinases to regulate Rim15

It would be interesting to clarify the mechanisms by which Rim15 integrates the signals from TORC1 and Sch9 in order to establish whether TORC1 regulates Rim15 through or in parallel to Sch9. Notably, it should be studied whether TORC1 and/or Sch9 can directly phosphorylate Rim15 *in vitro*, which would confirm the convergence of both pathways on Rim15 (kinase assay). In fact, direct phosphorylation of Rim15 by Sch9 could be expected to occur based on the consensus Sch9 phosphorylation sites found in the sequence of the Rim15 kinase insert. If this first experiment works, the corresponding phosphorylated residues may then be identified by mass spectrometry. Mutation of these residues into alanine (which mimics a constitutive dephosphorylated state) or into aspartate (which mimics a constitutive phosphorylated state) will permit to address *in vivo* the functional role of these residues. Finally, production of phosphospecific antibodies is expected to provide a supplemental tool to study *in vivo* Sch9 and/or TORC1-mediated phosphorylation on Rim15. In parallel, it would be interesting to determine in two-hybrid assays and Co-IP experiments whether Rim15 full length, or functional parts of Rim15, could interact with Sch9 or TORC1 components. Mapping the sites of interaction on a particular domain of Rim15, might also provide information on the mechanisms by which the two kinases inhibit Rim15.

V.1.1.8.2) Identification of downstream effectors of Rim15

A major progress in understanding the function of Rim15 would be the identification of *bona fide* physiological targets of the kinase. Direct interactions between Rim15 and its presumed downstream targets (Msn2, Msn4 and Gis1) have not been detected yet. Recent studies identified Ume6, a protein involved in transcriptional induction of early meiotic genes, as being a direct target of Rim15 *in vitro,* but this result has still to be confirmed *in vivo* (444). Although some potential candidates of the Rim15 kinase have been already identified in a high-throughput *in vitro* kinase assay, these studies will have to be confirmed both *in vitro* and *in vivo* (445). Initiation of two-hybrid screens with functional parts of Rim15, may also

allow identification potential target proteins and supplemental regulators of Rim15. These interactions will then have to be confirmed notably *in vitro* by Co-IP experiments and, depending on the protein found, tested for their ability to be *in vitro* and *in vivo* targets of Rim15 phosphorylation. The requirement of the identified downstream protein for acquisition of any Rim15-dependent readout should specify Rim15 downstream branches.

Factors specifically involved in the control of cell proliferation might also be identified in a screen for high-dosage suppressors of the growth defect on galactose conferred by conditional overexpression of Rim15 (*pGAL1-RIM15*) in the *tpk1, 2, 3Δ rim15Δ* mutant. This should identify products which are downregulated by Rim15, and which positively control cell proliferation. In term of genetic screen, it has also to be noticed that a recent study identified a set of genes, which when deleted enhanced longevity (443). Thus, it would also be interesting to test whether these mutations can suppress the short-lived phenotype of *rim15Δ* mutants or vice-versa. Such a screen could identity upstream regulators or downstream targets of Rim15.

V.1.1.8.3) Further characterisation of nucleocytoplasmic localisation of Rim15

Cytoplasmic retention, involving binding of the 14-3-3 proteins Bmh1/2 to specific phosphorylated sites of the kinase insert prevents, at least in part, nuclear accumulation of Rim15 during exponential growth. However, mechanisms triggering Rim15 nuclear accumulation are still incompletely understood. Notably, the identity of the receptor responsible for Rim15 nuclear import is still not known. Based on the hypothesis that a block in Rim15 import into the nucleus will prevent interaction of the kinase with its presumed downstream targets, cells deleted for the Rim15 importin are expected to be deficient in acquiring the rapamycin-induced Rim15-dependent G_0 readouts. Based on this reasoning, a collection of importin mutants has already been tested in order to detect cells unable to induce transcription of *HSP12*, *HSP26* and *SSA3* following rapamycin treatment. Unfortunately, this method failed to pick up potential candidates, indicating either that import of Rim15 is carried out redundantly by two or more factors, or by an essential importin. Accordingly, conditional mutants (ts) of genes encoding the essential importins should be tested for Rim15 localisation at both permissive and restrictive temperature. Since heat shock causes, by itself, induction of the three previously mentioned genes, the temperature shift long should be performed at least 2 hr before rapamycin treatment, which should allow dissociation of the transient effects of the heat shock from those caused by rapamycin treatment (72).

Rim15 does not contain a classical consensus sequence for nuclear export, NES (which consists in clusters of hydrophobic amino acids), or for nuclear export, NLS (which consists in clusters of basic amino acids). Mapping the minimal region of Rim15 required to interact with the importin (once known) should allow identification of the NLS (using two-hybrid assays and Co-IP experiments with different C-terminal and N-terminal truncated parts of Rim15). Similarly mapping the minimal region required for interaction with Msn5, should allow identification of Rim15 NES. Finding the minimal NLS or NES may provide useful tools to study the kinetics of nuclear import and export of the protein and may provide supplemental mechanistic information on how Rim15 localisation is regulated.

V.1.2) A novel growth control mechanism originates at the vacuolar membrane

A main finding of this thesis is that microautophagy, which takes place at the vacuolar membrane, is likely crucial for cells to resume proliferation following their release from a rapamycin-induced growth arrest. This mechanism requires the function of a complex named EGO (for **e**xit from a rapamycin-induced **g**ro**w**th arrest). Interestingly, recent findings indicate that the function of the EGO complex may be broader and that it is not only required for G_0 exit. In the following sections, the results presented in Chapter III and IV will be briefly summarised and discussed in the context of the latest available data.

V.1.2.1) Identification and composition of the EGO complex

Rapamycin treatment promotes entry of exponentially growing cells into a G_0-like state, which mimics nutrient starvation. Although often considered as toxic, the effects of growth inhibitory concentration of rapamycin are reversible in our strain backgrounds (BY4741 and KT), since wild-type cells, upon removal of rapamycin, can resume growth (89, 446). This capacity to resume growth upon rapamycin removal was the basis for a genetic screen during which we looked for genes whose products are required for G_0 exit following release from a rapamycin treatment. This method led to the identification of Ego1, Gtr2 and Ego3. Several data already linked these three proteins together (412, 447) and our results confirmed that the three proteins form a complex. This is exemplified by the fact that Ego3, which does not directly interact with Gtr2 in two-hybrid assays, can pull down Gtr2 probably via Ego1 in Co-precipitation experiments. All three proteins are also functionally related and loss of Ego1, Gtr2 or Ego3 confers the same global phenotype, *i.e.*, it renders cells unable to reactivate processes that are positively regulated by TORC1 (following rapamycin removal). As an example, *ego* mutants have a large vacuole and high levels of glycogen during recovery from a rapamycin treatment, while wild-type cells rapidly resume growth diminish vacuolar size and degrade glycogen under the same conditions. It is worth mentioning that the putative small GTPase Gtr1 is highly homologous to Gtr2 (and to the mammalian RragA protein) and interacts in our studies, but also in several independent studies, with members of the EGO complex (397, 411, 448, 449). The nucleotide-bound state of the two putative small GTPases has also recently been proposed to regulate the activity of the EGO complex with Gtr1 acting upstream of Gtr2 (448).

We found the EGO complex at the vacuolar membrane based on *in vivo* microscopy and subcellular fractionation experiments. These results also indicated that Gtr2 is probably weakly associated with the vacuolar membrane. This observation fits with the fact that Gtr2

lacks the lipid modification domain that is responsible for membrane targeting of the other members of the Ras-related protein family (397). Localisation of the EGO proteins at the vacuolar membrane is in line with several studies and is consistent with the finding that Ego3 is able to bind *in vitro* the phosphatidyl-inositol-(3,4)-bisphosphate ($PI(3,4)P_2$), a phospholipid, which is specifically synthesised at the vacuolar membrane (412, 450, 451). Ego1 possesses a N-terminal sequence for myristoylation which is required for vacuolar membrane attachment (411). Moreover Gao *et al.* (2006) detected in the N-terminal part of Ego1 two putative sites for palmitoylation, which seem to be also involved in its membrane attachment (448). Since Ego1 interacts with all members of the EGO complex and is able to bind vacuolar membrane, it might constitute the core subunit of the complex, which confers activity and membrane attachment for other members. Nevertheless, Ego1 is not essential for vacuolar membrane localisation of Gtr2 and Ego3 (unpublished results).

V.1.2.2) Role of the EGO complex: control of microautophagy and/or piecemeal microautophagy?

The EGO complex positively controls microautophagy. Accordingly, during recovery from a rapamycin treatment, microautophagic vesicles are specifically observed in wild-type cells but not in *ego* mutants (385). This mechanism, which consists in the engulfment of the vacuolar membrane into the vacuolar lumen, seems particularly relevant to reduce the size of the dramatically enlarged vacuole of cells that have been treated with rapamycin. Importantly, under these conditions, retrograde trafficking (*i.e.,* vesicular trafficking that goes from the vacuole to the endosome and the Golgi (452, 453)), which seems to be functional in *ego* mutants, is apparently not sufficient to allow vacuolar size reduction and G_0 exit. According to our *in vivo* data and those found *in vitro* by Kunz *et al.* (2004), TORC1 positively regulates microautophagy, while it is known to negatively control macroautophagy. This fits with previous findings that both events can occur independently (86, 419). However, macro- and microautophagy are indirectly linked. Indeed, according to Müller *et al.* (2000) macroautophagy provides the excess of membrane required to form microautophagic tubes (385). In Chapter III, we speculate that microautophagy stimulates TORC1 activity by promoting an overall redistribution of membranes, which may be sensed by the membrane-bound TORC1 (94, 334). Alternatively, microautophagy could also simply promote redistribution of the TORC1 complex to different intracellular membranous systems, where it is then activated. The vacuolar compartment, in addition to its digestive role, also functions as a storage compartment for amino acids (*e.g.,* arginine and glutamine) (454, 455). Thus, microautophagy may also increase the intravacuolar concentration of amino acids, which could act as a nutritional signal to reactivate cell growth via TORC1.

Microautophagy of soluble components is still a poorly defined process, which can be characterised through *in vitro* experiments on isolated vacuoles and morphologic observations (385). So far, no proteins have been identified that are implicated in microautophagy. This makes it difficult to establish whether the EGO complex directly controls this process. Interestingly, piecemeal microautophagy of the nucleus, which is topologically related to microautophagy, follows apparently different mechanisms and is better defined (456). It is initiated by formation of nucleus to vacuole junctions, mediated by two transmembrane proteins Vac8 (vacuole) and Nvj1 (nucleus) (85, 388). The subsequent invagination of the vacuolar membrane and extrusion of the nuclear membrane into the vacuole allow internalisation and degradation of a piece of the nucleus within the vacuolar lumen (388). Ego1 and Gtr2 possibly interact with Nvj1, suggesting that the EGO complex may play a direct role in the regulation of piecemeal microautophagy. In contrast to microautophagy, however, piecemeal microautophagy of the nucleus is induced following TORC1 inhibition. Thus, although both events respond to TORC1 they seem to be regulated in opposite ways (419). A possible model would be that TORC1 inactivation releases the EGO complex from the site of microautophagy, allowing the complex to mediate a second function in the regulation of piecemeal nuclear microautophagy. The EGO complex could consequently be involved in two mutually exclusive processes, one mediated during TORC1 activation and the other mediated upon TORC1 inactivation (85). The possible dual role of the EGO complex could constitute an elegant way to separate two antagonistically regulated processes. Clearly, these issues will have to be addressed in more details in the near future.

V.1.2.3) Does the EGO complex act in parallel or upstream of TORC1?

A broad range of genetic data clearly established that EGO complex may act in parallel to or in the TORC1 pathway to positively regulate cell growth. Accordingly: (i) Ego3 binds SMIR4, a compound that inhibits the anti-proliferative effects of rapamycin (457); (ii) *ego* mutants are rapamycin hypersensitive, while overexpression of EGO complex members confers rapamycin resistance; (iii) loss of Tor1 decreases TORC1 signalling and confers a growth defect when combined with loss of Gtr2 or Ego3 (457); and (iv) loss of Tif3, which is the yeast homolog of the mammalian direct downstream target of mTORC1, eIF4B, severely impairs growth of a *gtr2Δ* mutant (337, 458, 459).

Based on the additive effects of TORC1 downregulation and loss of EGO complex members, both pathways may act in parallel. However, because induction of TORC1-activated phenotypes (after release from a rapamycin treatment) is dependent on the presence of

Ego1, Gtr2 and Ego3, the EGO complex may also directly or indirectly impinge on TORC1. In line with such a model, we found that the TORC1 subunit Tco89 interacted with Ego1 in two-hybrid assays. Moreover, Tco89 and EGO complex both co-localise at the vacuolar membrane, and loss of Tco89 results in a similar phenotype as loss of EGO complex components (60). Thus, both complexes may in fact function in the same pathway. In such a model, the EGO complex may be upstream of TORC1 and TORC1 may, as a downstream event, control microautophagy. Notably, Kog1, another TORC1 subunit, also localises at the vacuolar membrane (412).

While genetic data tend to indicate that glutamate and glutamine are important for reactivation of the TORC1 pathway in the absence of a functional EGO complex, the exact nature of the signal required for TORC1 reactivation upon rapamycin removal is still unsolved. Provided that the EGO complex acts upstream of TORC1, its localisation close to the vacuolar reservoir may indicate that it is itself implicated in sensing vacuolar nutrients (454, 460, 461). This speculation is reinforced by our newly identified interaction of Gtr1 with a hypothetical transmembrane amino acid transporter (*i.e.*, Yol092w) which resides in the vacuolar membrane and which may relay a vacuolar amino acid signal to Gtr1. (417). Thus, in an intriguing model, the EGO complex would directly relay a vacuolar amino acid signal to TORC1, a process that could be essential for TORC1 reactivation. In line with the possible role of the EGO complex in nutrient sensing, Huang *et al.* (2004) found that in a "glucose only" condition (*i.e.*, starvation medium to which glucose is added), the *ego3Δ* mutant is less susceptible to cell death than wild-type cells (457). According to this scheme, detection of the change in a critical vacuolar nutrient(s) may require intravacuolar nutrient sensing or export of this nutrient (likely an amino acid) into the cytoplasm. In the latter case, the EGO complex may sense either the increased cytoplasmic concentration of this nutrient or the increased flux through a critical membrane transporter. In a speculative model extracted from these observations, the EGO complex-TORC1 interaction may be required for amino acids to mediate reactivation of the TORC1 pathway. In other words, nutrient or amino acid signal would be sensed by the EGO complex and transmitted to TORC1. The small GTPases, Gtr1 and Gtr2 could regulate the dynamic interaction between the EGO complex and TORC1 (Fig. V.2).

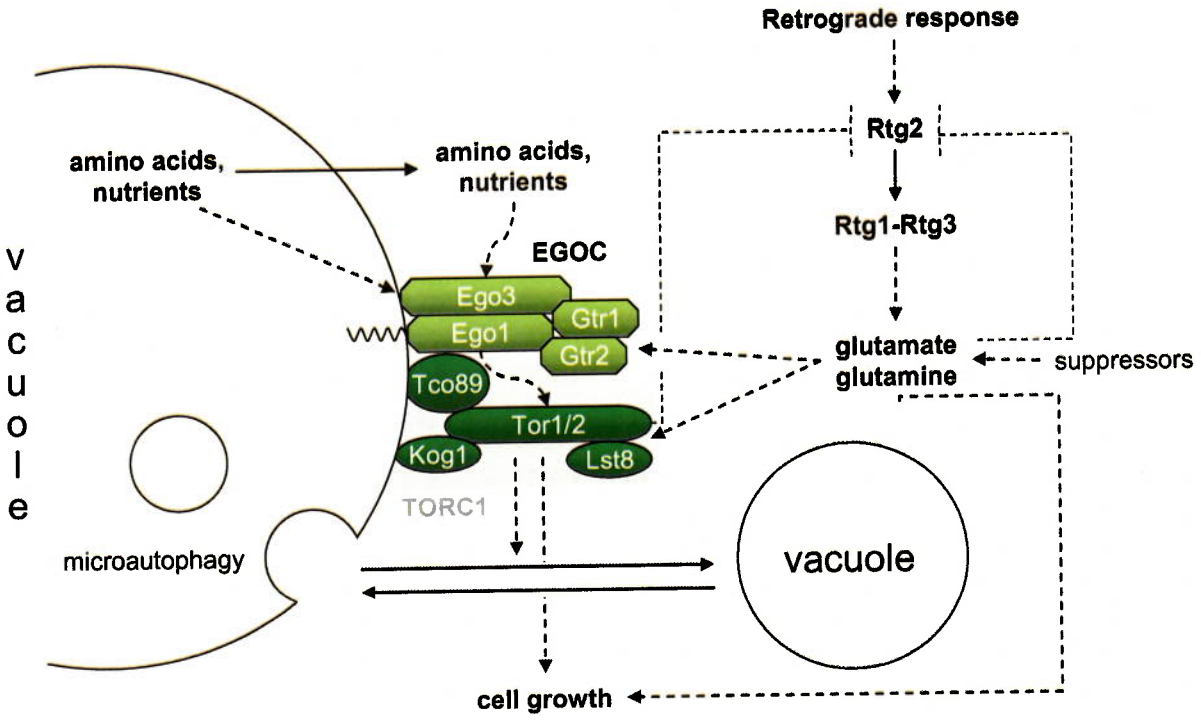


Fig. V.2. New model depicting the possible regulation of microautophagy by TORC1 and the EGO complex

During recovery from a rapamycin-induced growth arrest, the EGO complex (consisting of Ego1, Ego3, Gtr1 and Gtr2) is proposed to act upstream of TORC1. In this model, the EGO complex (EGOC) senses (cytoplasmic or vacuolar) amino acids (or other nutrients) and transmits a positive signal to TORC1 to activate microautophagy and thereby participate in vacuolar size reduction. The retrograde response pathway is drawn upstream of TORC1, but based on complex feedback loops it also often appears downstream of TORC1. In line with the model of Chapter III glutamate and glutamine are the key nutrients in EGO complex and TORC1 complex signalling. It can not be excluded at this point that glutamine-independent mechanism could also occur. Based on the current knowledge, glutamine signals positively in the TORC1 pathway, either upstream or downstream of TORC1. Dashed arrow: indirect or putative positive interaction; Dashed bar: indirect or putative negative interactions.

V.1.2.4) TORC1 and EGO complexes, a key role for permease sorting?

A recent study from Gao *et al.* (2006) identified a new complex, the GSE complex (for **G**TPase-containing complex required for Gap1 **s**orting in the **e**ndosome), which is virtually identical to the EGO complex, except it contains an additional member, Ltv1 (448). As indicated by its name the complex appears to be required to sustain transport activity of the general amino acid permease (Gap1) at the cell surface during growth on poor nitrogen conditions (448). Part of the results presented in this publication are based on the study of Gap1 trafficking in a *vps27Δ* mutant, which is notably blocked in the transport of proteins from the endosome to the vacuole (462). This mutant harbours an enlarged endosomal

compartment, which accumulates proteins "en route" to the vacuole (463). Thus, under good nitrogen conditions (glutamine or asparagine medium), Gap1 (expressed from an *ADH1* promoter), instead of being targeted to the vacuole, accumulates in the abnormal compartment of the *vps27Δ* mutant. When these *vps27Δ* cells are shifted to a poor nitrogen source (ammonia in this work), Gap1 accumulates in an EGO complex-dependent manner at the cell surface (464). Kaiser *et al.* (2006) further found that the secretory transport from the Golgi to the cell surface, which is the main "route" taken by proteins to reach the plasma membrane, is not impaired in *gse/ego* mutants (448). Based on these data, they propose a model in which the EGO/GSE complex is required to specifically retrieve Gap1 (via a hypothetical trafficking pathway) from the endosome to the plasma membrane. In line with such a model, they also show that the EGO/GSE complex, at variance with the published literature, localises to the endosome (412, 450, 451). Moreover, they claim that Gap1 interacts with Gtr2 but do not consistently show all the appropriate controls in the corresponding Co-IP experiment (Fig. V.3).

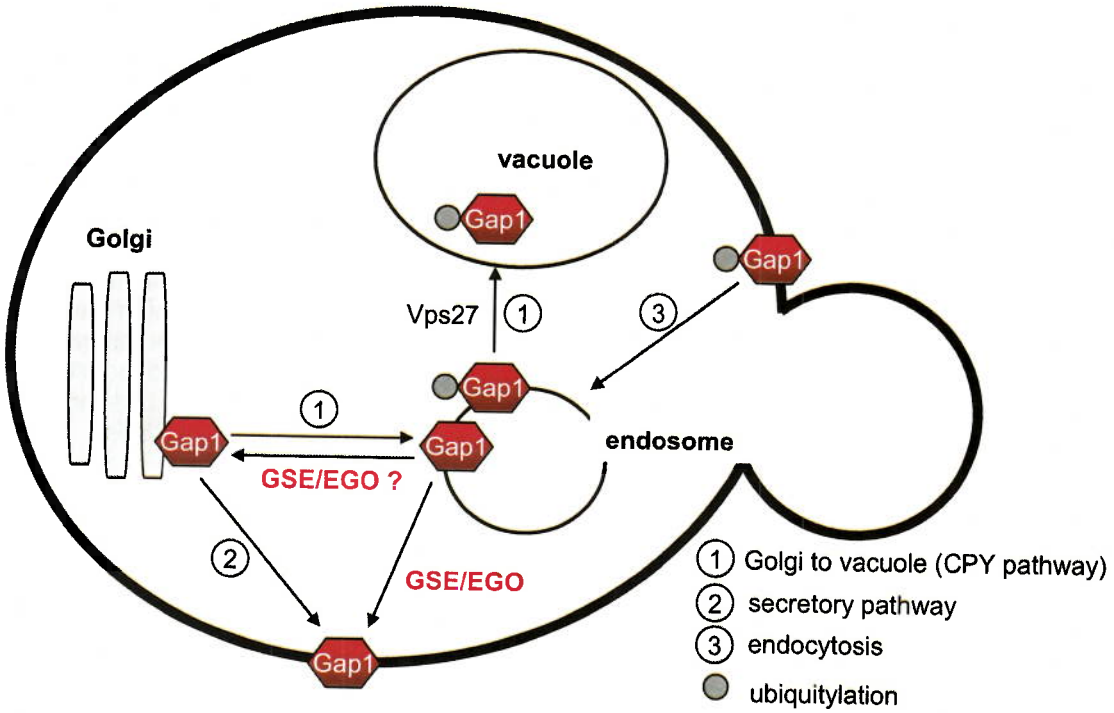


Fig. V.3 Model for GSE/EGO complex regulation according to Gao *et al.* (2006)

①: Under nitrogen good conditions, the general amino acid permease, Gap1, is ubiquitylated and transported to the vacuole for degradation. This pathway can be blocked in a *vps27* mutant causing Gap1 to accumulate in the endosome. ②: When nitrogen is limiting, Gap1 is mobilised and delivered to the plasma membrane either directly from the endosome to plasma membrane or indirectly from the endosome to Golgi and the plasma membrane through the action of the GSE/EGO complex.

Whether the GSE/EGO complex is directly involved in Gap1 sorting from the endosome to the cell surface is far from clear at present. For instance, the localisation of the GSE/EGO complex with Gap1 at the endosomal compartment, does not fit with our and several other published results, in which the same complex localises at the vacuolar membrane (412, 448, 450, 451). Notably, Gao *et al.* (2006) performed their GSE/EGO complex localisation studies in a *vps27Δ* mutant (448). It is therefore not surprising to find EGO complex members in the enlarged endosomal structure of these mutants, instead of being at vacuolar compartment. In a *vps27Δ* mutant, Gse/Ego proteins can probably simply not reach the vacuole, due to deficient endosome to vacuole transportation (448) (Fig. V.3). Thus, in a normal setting, Ego proteins have not even a chance to come into contact with Gap1 in wild-type cells (*i.e.*, Ego proteins localise at the vacuolar membrane, while Gap1 is sent to the vacuolar lumen) (464). In fact, we argue that the defect of *gse/ego* mutants to sort Gap1 to the cell surface (in wild-type and *vps27Δ* mutants) may be an indirect consequence of their decreased TORC1 signalling pathway. Indeed, wild-type cells treated with subinhibitory concentrations of rapamycin or *lst8-1* mutants, which are rapamycin hypersensitive, have like *gse/ego* mutants a constitutively low level of Gap1-mediated amino acid transport activity and Gap1 expression at the cell surface (333, 369, 370). In line with this model, *lst8-1* mutant and GSE/EGO complex mutants have several traits in common, such as their hypersensitivity to rapamycin and, at least for *ego3Δ* and *lst8-1* mutants, a constitutive derepression of some of the Gln3-controlled genes. It is therefore not surprising to find that Gap1 trafficking is apparently regulated similarly in both mutants (333, 457).

Treating wild-type cells with an inhibitory concentration of rapamycin does not promote Gap1 sorting to the plasma membrane (369). This situation is somehow logical as, in contrary to nitrogen-starved cells, rapamycin-treated cells stop growth on nutrient-rich medium and do not truly experience a situation of nitrogen-starvation (at least at the beginning of the treatment) (21, 341). Based on that observation, it is highly improbable that the defect in Gap1 sorting to the plasma membrane accounts for the failure of *ego* mutants to exit from a rapamycin-induced growth arrest inasmuch as wild-type cells, which have the same phenotype, can exit the growth arrest. Our study suggests nevertheless that inappropriate localisation of amino acid permeases, may contribute to the ego phenotype. For instance, loss of Npr1 suppresses the defect of the tree *ego* mutants in exit from a rapamycin-induced growth arrest, indicating that Npr1 is required expression of the ego phenotype. Npr1 is dephosphorylated and probably activated by rapamycin treatment or nitrogen starvation, and is presumably needed for sorting the constitutive amino acid permease Tat2 to the vacuole (67). Thus, a decrease in TORC1 signalling caused by loss of the EGO complex may

increase Npr1 activity and, as a consequence, affect permease sorting which could in turn change intracellular amino acid concentration. Conversely, loss of Npr1, which slightly increases glutamine concentration, may re-establish at the cell surface the distribution of amino acid permeases that is probably lost in the *ego* mutants (465). Loss of Npr1 is also expected to prevent rapamycin-mediated vacuolar targeting of Tat2 and possibly of other constitutive amino acid permeases (although their Npr1-mediated regulation has not been demonstrated yet), an effect which may also account for suppression of the ego phenotype (67).

The molecular mechanisms through which Npr1 affects the activity of transport proteins remain a mystery. Accordingly, Npr1 was primarily identified as an essential component required for stabilisation at the plasma membrane of the inducible amino acid permeases, including Gap1 (174, 466). However, in rapamycin-treated cells, Npr1 is apparently activated but Gap1 fails to be detected at the cell surface and is instead targeted to the vacuole, indicating that a mechanism different from Npr1 can also control Gap1 sorting. Clearly, understanding how Npr1 controls sorting of permeases and how this relates to TORC1 activity is one of the most challenging task to be addressed in the near future. Because the amino acid level (in particular glutamine and asparagine levels) greatly affects permease sorting, answer may ultimately only be obtained by detailed analyses of the distribution of amino acids in the cytoplasm and the vacuole in wild-type, *npr1Δ* and *ego* mutants subjected to different nutrient conditions (369).

V.1.2.5) EGO complex and chronological life span extension.

Interestingly, *ego* mutants (*ego1Δ*, *gtr1Δ*, *gtr2Δ* and *ego3Δ*) were recently identified in a genome-wide analysis of yeast deletion mutants, which exhibit increased chronological life span (443). Since downregulation of TORC1 also increases the chronological lifespan, this result supports our view that *ego* mutants have a reduced TORC1 pathway. Notably, because the screen performed in this study consists in testing nutrient-starved G_0 cells for their ability to reform colonies on YPD, the finding that the *ego* mutants were long-lived implicates also that they could exit from the G_0 state induced by nutrient starvation. Thus, it appears that the EGO complex is specifically required for cells to exit from a rapamycin-induced G_0 state.

In this context, rapamycin treatment and nutrient starvation differently regulate microautophagy. Indeed, microautophagy is apparently induced following nitrogen starvation,

but not upon rapamycin treatment (86). On the other hand both growth conditions (*i.e.*, rapamycin or nitrogen starvation) induce macroautophagy and, hence, influx of membranes to the vacuolar compartment (82, 467). It seems that, upon nitrogen starvation, both microautophagy and macroautophagy occur at the same time allowing probably the vacuole to keep a constant size (385), while upon rapamycin treatment, both mechanisms may be dissociated. This is also supported by the fact that rapamycin can block *in vitro* the microautophagy induced by nitrogen starvation, leading to the typical grossly enlarged vacuole. Whether microautophagy observed in nitrogen-starved cells occurs because TORC1 is not completely downregulated, or whether nitrogen starvation activates other pathway(s) that impinge on microautophagy is not known. This difference may account for the specific requirement of the EGO complex for exiting from a rapamycin-induced G_0 state but not from nutrient starvation.

However, in the study of Powers and colleagues (2006), the "quiescent" cells were obtained growing the different yeast mutants to saturation in a synthetic medium (instead of YPD) (443). Under such growth conditions cells, although arrested, are known to retain a relatively high metabolism and therefore loose relatively quickly viability. Thus, these cells may not represent true quiescent cells (8). Whether the EGO complex is necessary to exit from G_0 brought about by nutrient starvation remains an open question. It could be that rapamycin treatment is simply much more efficient in inducing a G_0 state than nutrient limitation (*i.e.*, under nutrient limitation conditions, cells may just take much more time to be in a true G_0 state). This is also in line with a recent publication from Werner-Washburn group, which demonstrates that at the onset of G_0 the cell culture is a mix quiescent and non-quiescent arrested cells (12). The non-quiescent cells found in this culture could account for the ability of *ego* mutants to exit from a G_0 state induced by nutrient starvation.

V.1.2.6) EGO complex: concluding remarks and future issues to be addressed

This thesis work led to the identification of a new complex, the EGO complex, which may constitute the first upstream regulator of TORC1 identified in yeast cells. Such an attractive speculative model, however, will have to be addressed experimentally in the near future.

The interaction between the EGO complex and TORC1 has still to be assessed in more details. Co-precipitation experiments should confirm the physical and biochemical interaction between Tco89 and Ego1. If possible one should use a strain expressing all EGO complex members differently tagged (GST, Myc, HA and GFP) as well as Tco89 fused to a TAP tag,

in order to test whether Tco89 can pull down the whole EGO complex. In parallel, individual interactions between members of the EGO and TORC1 complex may also be tested in two-hybrid assays. Once the Ego1-Tco89 binding may be established, it will be interesting to study whether this interaction could be regulated by growth conditions such as rapamycin treatment, nitrogen starvation and whether the GTP/GDP load of Gtr1/Gtr2 may affect this interaction. Thus, Co-precipitation experiments could be repeated with cells treated with rapamycin or starved for nitrogen, or with cells expressing the dominant negative form of *GTR2* (*gtr2*^{Q66L}) or of *GTR1* (*gtr1*^{S20L}) (448). Our hypothesis being that these two alleles may prevent EGO complex signalling through disruption of the TORC1-EGO complex interaction. Finally, localisation of GFP-tagged Tco89 or Kog1 should be determined in different *ego* mutants to assess whether the EGO complex is required for the localisation of TORC1 at the vacuolar membrane.

Our speculative model maps the EGO complex upstream of TORC1. To reinforce this assumption, additional TORC1-controlled readouts should be first assessed in *ego* mutants during recovery from the rapamycin-induced G_0 block (*e.g.*, transcriptional induction of RPGs and transcriptional repression of the RTG-target gene *CIT2*). Additionally, to firmly establish the ego phenotype of the *tco89* and *tor1* mutants, degradation of glycogen and the translation rate (through dephosphorylation of eIF2α) will be tested in these two mutants during recovery from a rapamycin-induced growth arrest. The more phenotypes *ego* mutants have in common with *tco89* or *tor1* mutants, the more likely they will be in the same pathway. Hence, the sensitivity of *ego* mutants and of the non-essential *tor1* mutant to rapamycin and to wortmannin may be tested. Wortmannin is an inhibitor of the mammalian phosphatidylinositol 3-kinases (PI3K), which like rapamycin inhibits mTORC1 (320, 420, 468). Importantly, *ego* mutations should not be additive with the *tco89* mutations. Test of epistasis could also be performed. For instance, one could use the property of SMIR4 which forms a complex with Ego3 to confer rapamycin resistance (457). If loss of Tco89 alters the capacity of SMIR4 to confer rapamycin resistance, this may indicate that Tco89 is downstream of EGO (or at least of Ego3).

The nature of the presumed signal necessary for reactivation of TORC1 and transmitted by the EGO complex is still obscure. The localisation of EGO at the vacuolar membrane and the preliminary data, showing a possible interaction of Gtr1 with a protein that is likely a vacuolar transporter of amino acids points toward a role of vacuolar amino acids. To assess this hypothesis the interaction between Gtr1 and Yol092w will primarily have to be confirmed, possibly using a yeast membrane two-hybrid system (469). The yeast proteins homologous

to Yol092w (*i.e.*, Ybr147w, Ydr352w, Ydr090c and Ers1) as well as the established vacuolar amino acid transporters may also be tested for their interaction with members of the EGO complex (417, 455). Further characterisation of these transporters may then reveal the identity of the amino acids that are transported from one compartment to another. The transporters of the vacuolar compartment are involved in the import and export of amino acids (454). Among them, Avt3, Avt4 and Avt6 are particularly interesting as they are involved in the export of glutamine from the vacuole to the cytoplasm, an activity that could be required to replenish the cytoplasm with amino acid during recovery from rapamycin treatment (or nutrient starvation) (455). Thus, one may test whether loss of these vacuolar transporters affect the cells ability to recover from a rapamycin treatment or their sensitivity toward rapamycin.

An interesting question is whether the EGO complex is conserved among eukaryotic cells. Mammalian homologs of Gtr1, RragA and RragB, and of Gtr2, RragC and RragD, exist although their function is not clearly defined (99). Accordingly, it will be interesting to test whether expression of *RRAGA*, *RRAGB, RRAGC* or *RRAGD* can complement the ego phenotype of *gtr1Δ* and *gtr2Δ* mutants, respectively. It would be also interesting to perform a yeast two-hybrid array using a mammalian cDNA library as a prey, and RragA, RragB, RragC or RragD as baits in order to find the members of the putative mammalian complex (notably we could find proteins homologous to Ego1 or Ego3 in higher organisms). The potential target(s) will then be tested in yeast *ego1Δ* and *ego3Δ* mutants for their ability to complement the ego phenotype.

Finding a mammalian equivalent to the EGO complex, which is potentially upstream of TORC1, is particularly interesting since inhibition or downregulation of the mTORC1 pathway in human cells has several biomedical implications. Rapamycin is commonly used to prevent host rejection of transplanted organs and TORC1 may also be a critical target for anticancer therapy (336). Accordingly, mTORC1 is a downstream effector of the PI3K pathway which is inappropriately activated in many human cancers (470). Therefore, the rapamycin analogues (CCI-779, RAD001, AP23576) are currently undergoing clinical trials to test their efficiency as anti-cancer agents. Interestingly, these drugs have already been reported to have significant anti-tumour effects in certain patients. Unfortunately, rapamycin seems to increase, in some cases, malignancy of cells. This effect can be explained by the fact that the downstream effector of mTORC1, the S6 kinase 1 (S6K1), in addition to promote protein synthesis, has a feedback inhibitory effect on the PI3K-Akt pathway. Thus, rapamycin treatment may indirectly increase PI3K signalling. Definitely, more information is required on how the mTORC1

pathway is regulated and on how rapamycin impinges on TORC1, in order to determine whether rapamycin can be efficiently used in anti-cancer therapies. In this context, our study of the EGO complex in yeast may help depicting the complex molecular mechanism of TORC1 signalling and ultimately contribute to our understanding, and eventually treatment, of cancer in higher eukaryotes (for a review on mTORC and cancer see (471)).

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