AMPK protein interaction analyses by yeast two-hybrid

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Running title: Y2H interaction analyses

Abstract:

Mammalian AMP-activated protein kinase (AMPK) is a Ser/Thr protein kinase that acts as a crucial energy sensor in the cell. Since AMPK plays a key role in a multitude of different pathways in the cell, major efforts have been concentrated to elucidate its signaling network, mainly by the identification of AMPK downstream targets. In this chapter we describe a yeast two-hybrid method for the direct evaluation of the interaction between an AMPK subunit and putative substrates.

Key Words: AMPK, bait plasmid, prey plasmid, β-galactosidase, yeast two-hybrid.

1. Introduction

Mammalian AMP-activated protein kinase (AMPK) is a Ser/Thr protein kinase that acts as a crucial energy sensor in the cell. It is activated by nutritional and other kind of stress conditions and once activated it stimulates metabolic pathways that produce energy (catabolic pathways) whereas inhibits those that consume energy (anabolic pathways) in order to maintain energy status. In mammalian cells, AMPK is a heterotrimer composed of three different subunits: a catalytic subunit which harbors the Ser/Thr protein kinase activity (AMPKα, with two isoforms α1 and α2), a nucleotide binding regulatory subunit (AMPKγ, with three isoforms γ1, γ2 and γ3, which differ in the N-terminal extension) and a scaffolding subunit where both AMPKα and AMPKγ subunits interact (AMPKβ, with two isoforms β1 and β2) ([1], [2], [3]).

AMPK function has been implicated in multiple metabolic pathways. It interacts with a great variety of different substrates leading to short-term (i.e. regulation of the activity of

defined enzymes by direct phosphorylation) and long-term effects (i.e. regulation of the transcriptional activity of several transcription factors) ([1], [2], [3]). Since AMPK plays a key role in a multitude of different pathways in the cell, major efforts have been concentrated to elucidate its signaling network, mainly by the identification of AMPK downstream targets. Several physical protein-protein interaction techniques (multidimensional substrate-screen, affinity purification, etc.) have been reported in the literature ([4], [5], [6], [7], [8]). Alternatively, genetic techniques such as yeast two-hybrid have also been used to identify putative AMPK interactors ([9], [10], [11], [12]). In this chapter we will describe a yeast two-hybrid method for the direct evaluation of the interaction between an AMPK subunit and putative substrates ([13], [14], [15], [16]), and the possibility to express a third component in the assay (yeast triple-hybrid) that could modify the initial interaction between AMPK subunit and its putative substrate [11] (**Fig. 1**). The effect of this third component could allow, stabilize, regulate or even inhibit the interaction between the bait and the prey proteins.

2. Materials

2.1. Plasmids

The ORFs corresponding to any of the AMPK subunits have to be cloned into appropriate bait yeast two-hybrid vectors containing a DNA-binding domain, such as pBTM116 [17], which carries a *TRP1* selection marker and produces a fusion protein with LexA at the N-terminus of the AMPK subunit (LexA-AMPK subunit; bait plasmid) (See **Notes 1 and 2**). The ORF corresponding to the putative AMPK interactor has to be cloned into Gal4-Activating-Domain containing plasmids, such as pACT2 [18] or pGADT7 (Clontech), which carry a *LEU2* selection marker and produce a fusion protein with GAD at the N-

terminus of the protein (GAD-protein; prey plasmid) (see **Note 3**). If a third protein needs to be present in the two-hybrid system to regulate the interaction between the bait and the prey, the corresponding ORF has to be cloned into compatible yeast plasmids such as pWS93 [19], which carries an *URA3* selection marker and produces a fusion protein with an HA epitope at the N-terminus of the third protein. In this case, the additional plasmid has to be introduced into the cells containing already the bait and prey plasmids, and the new transformants selected on SC+2% glucose medium lacking tryptophan, leucine and uracil. As control, the same transformation should be conducted using an empty plasmid (see **Note 4**).

2.2. Yeast and bacterial strains

1. When the bait plasmid contains a LexA module, a yeast strain carrying *lexA* operators regulating the expression of reporter genes has to be used in the two-hybrid assay. We use the *Saccharomyces cerevisiae* THY-AP4 strain (*MATa, ura3, leu2, lexA::lacZ::trp1, lexA::HIS3, lexA::ADE2*) [20]. This strain requires the complementation of the culture medium with uracil, leucine, tryptophan, histidine and adenine, for growth (see **Note 5**). An advantage of this strain is that it carries the yeast biosynthetic gene *HIS3* under the control of the *lexA* operator, so that the interaction between AMPK subunits and putative partners can be carried out both by nutritional selection for histidine prototrophy and by an assay for β-galactosidase activity (which results from the expression of the *lacZ* gene under the control of *lexA* operator). To maintain the strain, cells are grown in complete YPD medium (see below).

2. Bacterial strain: *Escherichia coli KC8* (*pyrF::Tn5, leuB600, trpC-9830, hisB463*).

2.3. Culture media

1. Complete YPD medium plates [21]: 2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) agar, adjusted to pH 6.0.

2. Synthetic complete (SC)+2% glucose medium plates [21]: To prepare 400 ml of medium, autoclave 340 ml of water containing 8 g agar. Cool down the medium to 55 ºC , and add 20 ml of 40% (w/v) glucose solution and 40 ml of a 10x solution of YNB + amino acid mix [6.7 g of yeast nitrogen base without amino acids plus 0.95 g of an amino acid mix (1.25 g arginine, 1.25 g methionine, 1.88 g tyrosine, 1.88 g isoleucine, 1.88 g lysine, 3.13 g phenylalanine, 6.25g glutamic acid, 6.25 g aspartic acid, 9.38 g valine, 25 g serine and 25 g threonine) in 100 ml]. When necessary, 4 ml of each of the following stock solutions should be added to 400 ml of SC medium: 10 mg/ml leucine, 10 mg/ml tryptophan, 10 mg/ml histidine, 2.5 mg/ml uracil, 2.5 mg/ml adenine.

3. M9 minimal medium plates for *E. coli KC8* cells: To prepare 400 ml of medium, autoclave 315 ml of water containing 8 g agar. Cool down the medium to 55 ºC , and add 40 ml of a 10x solution of M9 salts (sterile filtered 128 g/l $Na_2HPO_4.12\ H_20$, 30 g/l KH_2PO_4, 5 g/l NaCl, 10 g/l NH_4Cl), 5.5 ml of 1 M NaOH, 4 ml of 40% (w/v) glucose, 40 ml of 10x amino acid mix (9.5 g/l amino acid mix, as above), 400 µl of 100 mg/ml ampicillin, 400 µl of 1M thiamine-HCl, 800 µl of 1 M $MgSO_4$ and 40 µl of 1 M $CaCl_2$. Then add 4 ml each of sterile filtered 10 mg/ml histidine, 10 mg/ml tryptophan and 2.5 mg/ml uracil.

4. LB+Amp plates: 1% (w/v) NaCl, 1% (w/v) peptone, 0.5% (w/v) yeast extract, 2% (w/v) agar, adjusted to pH: 7.5. Autoclave and cool down the medium to 55 ºC, and add 1 ml/l of 100 mg/ml ampicillin.

2.4. Yeast transformation materials

1. TE-LiAc solution: sterile 0.1 M lithium acetate in Tris-EDTA buffer (TE, 10 mM Tris-HCl pH 8.0, 1 mM EDTA).

2. PEG-TE-LiAc solution: sterile 40% (w/v) polyethylene glycol 3350 in TE-LiAc solution.

3. Salmon sperm DNA: sterile denaturized 10 mg/ml salmon sperm DNA.

4. Extraction buffer for plasmid recovery: 10 mM Tris-HCl pH: 8.0, 100 mM NaCl, 1 mM EDTA, 2% (v/v) Triton X-100, 1% (w/v) SDS.

5. Acid-washed glass beads 425-600 μm diameter.

6. Phenol-Chloroform solution: 50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol.

2.5. Qualitative β-galactosidase assay

1. Z-Buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol. Weigh 16.1 g of $Na_2HPO_4.7H_2O$, 5.5 g of $NaH_2PO_4.H_2O$, 0.75 g of KCl, 0.25 g of $MgSO_4.7H_2O$, and add 2.7 ml 2-mercaptoethanol, in water up to one liter of solution. Adjust pH to 7.0. 2-mercaptoethanol should be added extemporaneously.

2. X-Gal stock solution: 100 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethylformamide.

2.6. Quantitative β-galactosidase assay

1. SDS: 0.1% (w/v) sodium dodecyl sulfate (SDS).

2. Chloroform.

3. ONPG: 4 mg/ml o-nitrophenylgalactopyranoside (ONPG).

4. Na_2CO_3: 1 M Na_2CO_3.

2.7. Other materials

1. Sterile flat tooth-picks.

2. Glass rod 0.3 cm diameter.

3. 90 mm wide nitrocellulose (0.45 μm) circular filters.

4. Regular filter paper.

5. 90 mm wide circular 3MM chromatography paper.

3. Methods

3.1. Transformation of yeast cells with bait and prey plasmids and selection of putative positive transformants

1. THY-AP4 yeast cells are transformed with a combination of bait and prey plasmids using the lithium acetate method [22]. Remove 50 ml of yeast cells growing exponentially in YPD liquid medium (A_{600} 0.5) and spin them down at 4,000 x g for 5 min.

2. Resuspend the pellet in 5 ml of sterile TE-LiAc solution and spin down the samples again at 4,000 x g for 5 min. Then, resuspend the cells in 0.5 ml of TE-LiAc solution.

3. To 50 µl of cell suspension, add 2 µl of carrier DNA (denaturized 10 mg/ml salmon sperm DNA), 2-3 µl of each plasmid (containing 100 ng each) and 300 µl of PEG-TE-LiAc solution. Mix carefully and incubate the mixture at 30ºC for 30 min.

4. Then, incubate the samples at 42ºC for 15 min.

5. Spin down the samples at 4,000 x g for 5 min and resuspend the cell pellet in 1 ml of water (by pipetting up and down with a blue tip). Spin down the cells at 4,000 x g for 5 min and resuspend them in 200 µl of water.

6. Spread 100 µl of each transformation onto SC+2% glucose plates lacking tryptophan and leucine.

7. Individual colonies should appear around 36-48 hours of growth at 30ºC. With the help of a sterile tooth-pick, recover the cells from a single colony and spread them in a line (0.5 cm long) of two consecutive plates of SC+2% glucose lacking tryptophan and leucine. For each combination of bait and prey plasmids around 8-10 colonies should be analyzed. As sometimes colonies of different size appear in the plates, we normally make lines of colonies of different sizes to conduct qualitative β-galactosidase assays. Placing a form with marked squares (grid form) at the bottom of the plate helps identifying the position of the different lines **(Fig. 2A)**. Allow the cells to grow for 36-48 h at 30ºC **(Fig. 2B)**. One set of plates will be used in the qualitative β-galactosidase assay whereas the second one will be used as master plate to inoculate cells in the quantitative β-galactosidase assay.

As controls, combinations of bait with an empty prey plasmid and of empty bait with the corresponding prey plasmid are also introduced into the yeast cells. In addition we recommend carrying out an additional transformation with plasmids that give a positive interaction as control.

3.2. Qualitative β-galactosidase assay

The technique consists basically in transferring colonies to a nitrocellulose membrane and then carrying a β-galactosidase assay in the colonies that are on the membrane [23]. It has several steps:

1. Lay a 90 mm wide nitrocellulose circular filter onto one of the plates with the yeast growth lines and allow it to wet completely. With a glass rod press the filter to make sure it contacts with the cell cultures of the plate. With a needle, drill holes in the membrane and plate medium in order to orientate the culture lines

2. Lift the nitrocellulose filter off of the plate carefully to avoid smearing the colonies and place it with the colonies side up on top of a regular filter paper. Place the filters at -80ºC for at least 2 hours.

3. Remove the nitrocellulose filters from the freezer and, in a fume hood, place them with the cells side up in a petri dish containing a 90 mm wide circular 3MM chromatography paper, soaked with 3 ml of Z buffer containing 1 mg/ml X-Gal. Seal the plates with parafilm to avoid the nasty odor of 2-mercaptoethanol and incubate the filters at 30ºC for no more than 2 hours. If there is an interaction between the bait and prey fusion proteins, the expression of the *lacZ* gene will be activated, resulting in the synthesis of β-galactosidase. This enzyme will act on the X-gal substrate releasing a blue color that will remain in the cells (**Fig. 3A**). The time at which the blue color appears and its intensity is a qualitative reflection of the intensity of the interaction between the bait and prey proteins. For this reason we recommend checking the color of the colonies after periods of 30 min. By comparison with the positive and negative control, one can estimate qualitatively the

strength of the interaction (**Fig. 3A**) (see **Note 6**). After two hours of incubation at 30ºC, remove the nitrocellulose filters and let them dry in the fume hood, to keep them as records of the experiment.

3.3. Qualitative growth assay in plates lacking histidine

An alternative method to assess qualitatively the interaction is by growing the transformants in a culture medium lacking tryptophan (to maintain the bait plasmid), leucine (to maintain the prey plasmid) and histidine (to select for two-hybrid interaction). If there is an interaction between AMPK subunit and the putative interactor, the transcription of the *HIS3* gene will be activated resulting in allowing the growth of the transformants in this selective medium. Colonies of the corresponding transformants are spread on SC+2% glucose plates lacking tryptophan, leucine and histidine and incubated at 30ºC for 24-48 h (**Fig. 3B**).

3.4. Quantitative β-galactosidase assay

1.- From the master plate with the yeast growth lines, inoculate cells in 5 ml of liquid SC+2% glucose medium lacking tryptophan and leucine. Inoculate at least 6 individual colonies from each combination of bait and prey plasmids. When they are growing at the exponential phase (A_{600} between 0.3 and 0.8) collect 0.5 OD of cells (i.e., 1 ml of a culture at A_{600} 0.5) in a small glass tube. Spin down the cells for 5 min at 4,000 x g and discard the supernatant.

2. Resuspend the cell pellet in 1ml of Z buffer. In parallel carry one tube with 1 ml Z of buffer alone (negative control). Add 25 μl of 0.1% SDS and 25 μl of chloroform. Vortex

the tubes for 15 seconds and leave them warming at 30ºC in a water bath. This treatment opens some holes in the yeast surface and allows the contact of the β-galactosidase enzyme with its substrate.

3. In a time dependent way, add 0.2 ml of ONPG solution and incubate at 30ºC in a water bath. If the transformants express the *lacZ* gene, the β-galactosidase will act on the ONPG substrate releasing o-nitrophenol which gives a yellow color. As soon as a yellow color appears in the tubes, we recommend stopping the reaction and annotating the time.

4. Stop the reaction at a defined period of time (max. 2 hours) with 0.5 ml of 1 M Na_2CO_3. Spin down for 5 min to remove cell debris. Transfer the supernatant to a clean tube and measure the yellow absorbance at 420 nm. In our hands, the color is only stable during the first 15 min after stopping the reaction, so the measurement of the absorbance should be performed during this period of time.

5. The units of β-galactosidase activity (Miller Units) are defined as value of A_{420} x 2000/ time of the reaction in minutes [24]. A regular two-hybrid assay should contain the values of the controls with the empty plasmids and the values of the interaction between the AMPK subunit and the putative interactor (**Fig. 4**) **(**see **Note 7)**.

6. In order to validate the results of the two-hybrid analysis it is necessary to check the correct expression of the fusion proteins (**Fig. 5**) (see **Note 8**).

3.5. Screening of yeast two-hybrid libraries for new interactors.

The yeast two-hybrid system allows the screening of a cDNA library using a LexA-AMPK subunit as bait. Different cDNA libraries from different tissues exist in the market and distinct protocols can be used to perform the screening, but we still carry out the one based

on the co-transformation of the yeast TAT7 or THY-AP4 strains with a pBTM116-AMPK subunit plasmid and a commercial cDNA library in a pGAD-based vector (Clontech) ([10], [11]). We carry out the following steps:

1. Transform the yeast strain (i.e., THY-AP4) with the bait plasmid (i.e., pBTM-AMPKα2) and different amounts of the selected cDNA library (based in pACT2; prey plasmid). Follow the steps described in section 3.1 (steps 1 to 6). In this way we will determine the best ratio between bait and prey plasmids to obtain the highest number of transformants.

2. Once the best ratio of the combination of bait and cDNA library plasmid is determined, repeat the transformation under these conditions, but select the transformants in SC+2% glucose plates lacking tryptophan, leucine and histidine. Only transformants that are able to activate the expression of the *HIS3* gene will be recovered (see **Note 9**). Spread also one aliquot of the transformed cells in SC+2% glucose plates lacking only tryptophan and leucine to assess the total number of transformants. Repeat this step as many times as necessary to cover at least a total of 500,000 independent transformants.

3. Pick up the putative positive colonies that have grown up in the absence of tryptophan, leucine and histidine and screen them for β-galactosidase activity using the qualitative method described in section 3.2.

4. Recover the corresponding library plasmid in those transformants that give a clear positive β-galactosidase reaction. To do this, grow the corresponding transformants in SC+2% glucose medium lacking only leucine, since the prey plasmid contains a *LEU2* selection marker. At the exponential phase (A_{600} 0.5) remove 5 ml of culture and spin it down at 4,000 x g for 5 min. Resuspend the pellet in 1 ml of Tris-EDTA (TE) buffer and spin the cells down again. Resuspend the pellet in 200 μl of extraction buffer. Add 0.3 g of

acid-washed glass beads and 200 µl of phenol-chloroform solution. Vortex at full speed for 2 min and spin down the suspension at 4,000 x g for 5 min. Finally, transfer the aqueous phase to a clean tube.

5. Transform the *Escherichia coli KC8* strain with 10 µl of samples obtained above. Plate the bacteria first on LB+Amp plates. Colonies will appear after 24-48 hours of incubation at 37ºC. This step improves the recovery of colonies carrying the prey plasmid. Transfer colonies to M9 minimal medium plates lacking leucine. Incubate bacteria at 37ºC for 24-48 hours until colonies appear. Obtain the prey plasmid from cultures of these colonies by standard bacterial miniprep methods. Make sure that the recovered plasmids are prey plasmids, i.e., by enzyme restriction digestion.

6. These putative positive plasmids are rechecked for two-hybrid interaction with empty pBTM116 and the LexA-AMPK subunit plasmids. Only those plasmids that do not have self-activating properties and maintain the interaction with the corresponding AMPK subunit are sequenced, and the sequences characterized by BLAST analysis [26]. The strength of the interaction is quantified by measuring the β-galactosidase activity in these selected transformants (see above). In this way, a collection of putative AMPK interactors is defined (see **Note 10**) [10], [11].

4. Notes

1. The yeast two-hybrid is based on the reconstruction of a transcription factor that has to go to the nucleus to exert its function **(Fig. 1)**. Therefore the yeast two-hybrid system only works for soluble proteins that must be transported to the nucleus. Thus, the assay is not valid for membrane proteins or for proteins that aggregate in the cytosol. The original

method was based on the reconstruction of the Gal4 transcription factor, placing the Gal4-DNA Binding Domain (GBD) in the bait plasmid and the Gal4-Activating Domain (GAD) in the prey plasmid [27]. Later on, the Gal4-GBD was substituted by the bacterial LexA repressor ([17], [28]). In our hands, the use of LexA-based system has some advantages on the GBD-based one: i.e, it produces less false positives since LexA is an heterologous protein that does not interact with yeast proteins, the strength of the interaction is normally higher when using a LexA-based bait plasmid in comparison to the GBD-based one and, in addition, there are good anti-LexA antibodies in the market that can be used to test the production and quality of the bait fusion protein (unfortunately no good commercial antibody for Gal4-GBD is in the market yet).

2. There are alternative vectors in the market that produce LexA-fusion proteins both with LexA at the N-terminus or as a C-terminal fusion. The choice depends on the stability of the fusion protein and on the preservation of the function of the AMPK subunit. In our hands, N-terminal fusions of LexA give better results than C-terminal fusion proteins.

3. The main difference between the available GAD-based plasmids is the type of multicloning site present in them. Both pACT2 and pGADT7 contain an HA-epitope between the GAD and the corresponding ORF which can be used to detect the production and quality of the prey fusion proteins (GAD-HA-prey fusion protein). AMPK subunits should be also subcloned into these plasmids to confirm the interaction with the putative substrate. In this way both directions of the two-hybrid assay are covered [i) bait-AMPK subunit and prey-interactor; ii) bait-interactor and prey-AMPK subunit], since sometimes one direction gives stronger results that the other. The GAD-based constructs become

completely necessary when a particular AMPK subunit shows signs of self-activating properties when fused to LexA (see **Note 9**).

4. Since the yeast strain contains four usable different auxotrophies (*trp1, leu2, ura3* and *his3*), up to two additional plasmids carrying the selection markers *URA3* and *HIS3* can be introduced in the cells to regulate the interaction mediated by the bait (*TRP1*) and prey (*LEU2*) plasmids. In **Figure 4**, it is shown that the expression of a third protein (protein F) improves the interaction between AMPKα2 and AMPKβ1.

5. Alternative yeast strain can be used in the yeast two-hybrid assay. For example CTY10.5d *(MATa ade2 his3 leu2 trp1 gal4 gal80 URA3::lexAop-lacZ)* or TAT7 *(MATa ade2 his3 leu2 trp1 gal4 gal80 LYS2::lexAop-HIS3 URA3::lexAop-lacZ)* ([11], [25]). However, in our hands the THY-AP4 strain gives better performance that the other ones.

6. In the yeast two-hybrid assay it is essential to analyze if the bait construct by itself has self-activating properties. In other words, if the bait plasmid by itself is able to activate the transcription of the reporter genes. In the case of AMPK, bait plasmids such as LexA-AMPKα1, LexA-AMPKα2, LexA-AMPKβ2, LexA-AMPKγ1 and LexA-AMPKγ2 give negative results on self-activation (**Fig. 3A**). However, LexA-AMPKβ1 has a weak self-activating performance (**Fig. 3A**) and LexA-AMPKγ3 has a strong self-activating profile [13]. In the case of weak self-activating properties, the regular measurement of the β-galactosidase activity is still a good way to measure the interaction with a prey, provided this interaction gives higher enzymatic activity than the bait alone. In the case of strong

self-activating properties, probably the amount of β-galactosidase is too high to allow the detection of differences in the activity when a prey protein is present. In these cases, moving the ORF of the AMPK subunit into the prey plasmid and placing the protein of interest into the bait plasmid may help. Alternatively, the ORF of the self-activating subunit can be shortened either from the N- or the C-terminus to obtain a construct devoid of self-activating properties [11].

7. The yeast two-hybrid system is very versatile and allows checking whether the interaction between two components changes depending on the growth environmental conditions. For example, by growing the cells in high glucose (4%) and shifting them to low glucose (0.05%), the two-hybrid system allows knowing whether the interaction changes under glucose starvation conditions. The effect of alternative changes in growth media conditions or the effect of the presence of different kind of stress conditions (i.e., heat shock, salt stress, etc.) on the strength of the interaction can easily be detected by this technique.

8. It is essential to check the expression of the bait and prey proteins to understand the results of the two-hybrid technique. With this aim, we obtain yeast extracts and analyze them by SDS-PAGE and immunoblotting ([11], [25]). To check the expression of the bait fusion proteins, we normally use either commercial anti-LexA antibodies or the corresponding anti-AMPK subunit antibodies. Sometimes there is no detectable two-hybrid interaction but this is due to poor expression of the proteins being involved. In this case, we

recommend repeating the two-hybrid with proteins with different tags or with truncated forms of the proteins, if the full length protein is unstable.

9. In the case of weak self-activating properties of the bait or to enhance the stringency of the screening, the enzymatic activity encoded by the *HIS3* reporter gene may be partially inhibited by increasing concentrations of 3-aminotriazole (3-ATZ) (from 5 mM to 10 mM) in the culture medium. In this way, only transformants with a strong interaction between the bait and the prey will grow in the selective medium (SC+2%glucose -Trp, -Leu, -His + 3-ATZ).

10. Several proteins appear continuously in unrelated two-hybrid screenings. Golemis and co-workers created a list of false positive proteins for classic yeast two-hybrid analysis [28]. In brief, they are heat shock, ribosomal and mitochondria proteins; proteasome subunits, elongation factors, etc. The collection of positive interactors obtained after the AMPK-based two-hybrid screening should be compared with this list to validate the possible candidates.

Acknowledgements

This study was supported by grants from the Spanish Ministry of Education and Science (SAF2014-54604-C3-1-R) and Generalitat Valenciana (PrometeoII/2014/029) to P.S.

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Figure Captions

Fig. 1: Schematic drawing of the yeast two-hybrid system. The technique is based on the reconstitution of a transcription factor [a DNA binding module (LexA) and a transcription factor activating domain (GAD)] when a bait protein (i.e. AMPK subunit) interacts with a prey protein (putative AMPK interactor). This leads to the expression of a reporter gene (i.e. *lacZ*, encoding the β-galactosidase enzyme). The interaction between the bait and the prey proteins can be modulated by the expression of a third protein, which could be necessary for the interaction, could improve it by stabilizing the interaction, could regulate it (by i.e. by introducing post-translational modifications) or could inhibit the interaction.

Fig. 2: Growth of the selected transformants on SC+2% glucose lacking tryptophan and leucine. A) Example of a grid form that can be used to identify the position of the different lines of growth. B) Example of a plate after growing the transformants at 30ºC for 48 h.

Fig. 3: Qualitative assays for two-hybrid interaction. A) Qualitative β-galactosidase assay. Transformants containing different combinations of plasmids were grown in SC+2% glucose plates lacking tryptophan and leucine. When the growth in the form of lines was evident, plates were subjected to the qualitative β-galactosidase assay described in Methods. Blue colonies are indicative of a two-hybrid interaction. This filter shows the assay of transformants containing an empty prey plasmid (pGADT7) and either LexA-AMPKα1, LexA-AMPKα2, LexA-AMPKβ1, LexA-AMPKβ2 or LexA-AMPKγ1. As it can be observed, the expression of LexA-AMPKβ1 has weak self-activating properties

(pale blue color of the corresponding colonies in comparison to the pale brown color present in cells containing LexA-AMPKβ2). The bottom row shows the assay of transformants containing LexA-AMPKα2 and GAD-AMPKβ1, which shows a clear two-hybrid interaction (blue colonies). B) Qualitative growth assay in plates lacking histidine. Selected transformants were grown in SC+2% glucose medium lacking tryptophan and leucine and in SC+2% glucose medium lacking tryptophan, leucine and histidine. Only transformants showing an interaction between the bait and the prey proteins will allow the transcription of the *HIS3* gene resulting in allowing the growth of the transformants in the selective medium lacking histidine. Three colonies of independent transformants containing different combination of plasmids were grown in the culture media indicated above: A: pBTM116 (empty) and pGADT7-PSMD11; B: pBTM-AMPKα2 and pGADT7-PSMD11; C: pBTM-AMPKα2 and pGADT7 (empty); pBTM-AMPKγ1 and pGADT7-PSMD11. Only AMPKα2 but not AMPKγ1 is able to interact with PSMD11, a non-ATPase subunit of the proteasome [11].

Fig. 4: Quantitative β-galactosidase assay of the interaction between LexA-AMPKα2 and GAD-AMPKβ1. In the nomenclature (i.e., α2/F/β1), the first position corresponds to transformants expressing the bait (0, means empty pBTM116 vector; α2, means LexA-AMPKα2), the second position corresponds to a third protein being expressed (0, means empty pWS93 vector; F, means pWS93-F) and the third position corresponds to the prey (0, means empty pGADT7 vector; β1, means pGADT7-AMPKβ1). The strength of the interaction is a direct correlation of the levels of β-galactosidase activity (values are means of at least six transformants per condition; bars indicate standard deviation). As observed,

the expression of protein F improves the interaction between LexA-AMPKα2 and GADT7-AMPKβ1 (** $p<0.01$).

Fig. 5: Western blot analysis of selected transformants. Transformants containing different combination of plasmids were analyzed by SDS-PAGE and immunoblotting using anti-LexA and anti-HA antibodies. In this case, all transformants contained the prey plasmid pGADT7-PSMD11 (expressing a non-ATPase subunit of the proteasome [11]) and different bait plasmids expressing LexA-AMPKα1, LexA-AMPKα2, LexA-AMPKβ1, LexA-AMPKβ2 or the empty plasmid pBTM116 (ϕ). Molecular size markers are indicated. The empty pBTM116 plasmid produces LexA protein (27 kDa) but it is not shown in the picture. The protein bands of lower molecular size in the lanes of LexA-AMPKα1 and LexA-AMPKα2 are probably due to degradation products of the full length forms. The position of the full length GAD-HA-PSMD11 protein is indicated with an arrow.

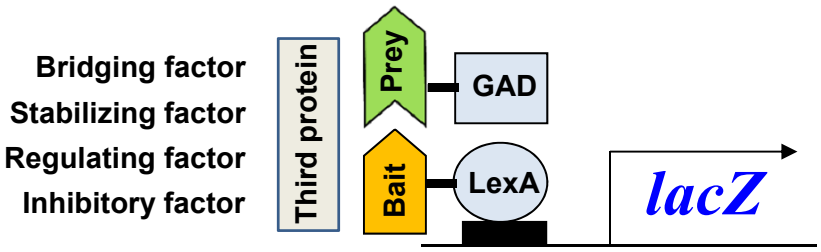
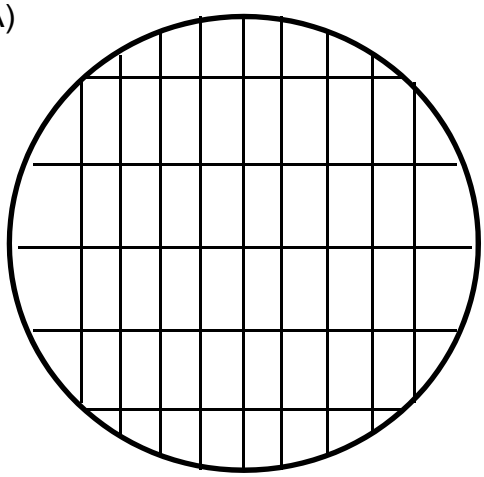
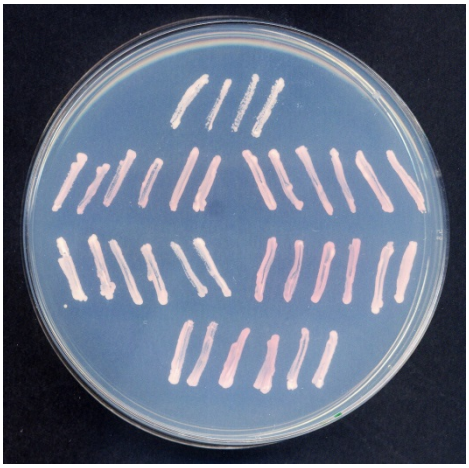

Bridging factor
Stabilizing factor
Regulating factor
Inhibitory factor
Third protein
Prey
GAD
Bait
LexA
lacZ

Figure 1

A)



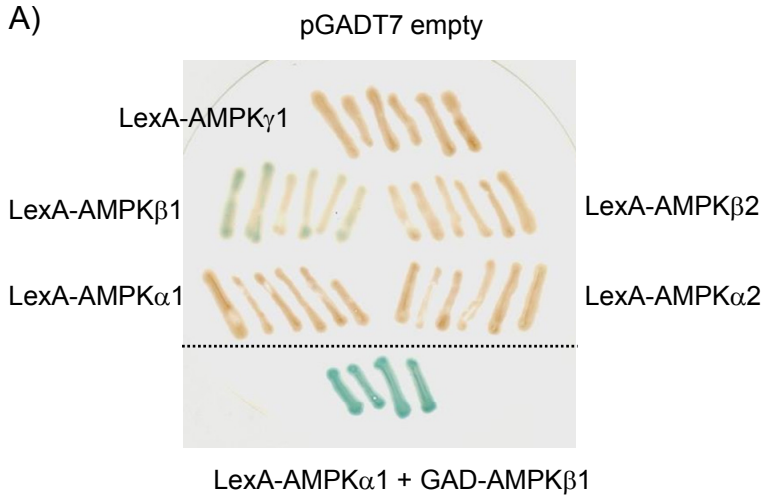
B)



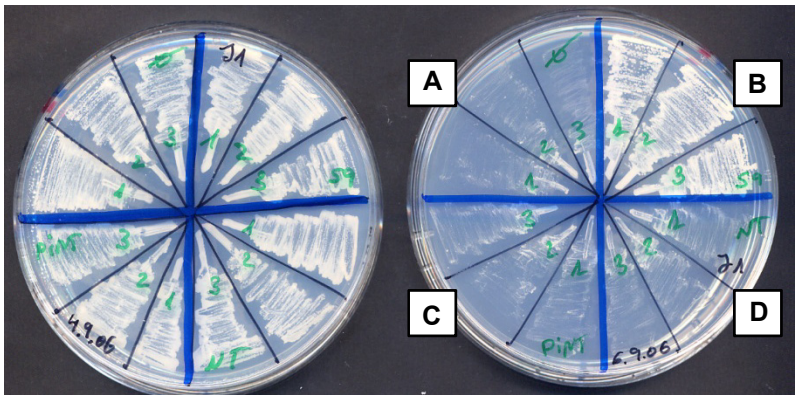
SC+2% glucose -Trp -Leu

Figure 2

A)



B)



SC+2%Glu -Trp -Leu SC+2%Glu -Trp -Leu -His

Figure 3

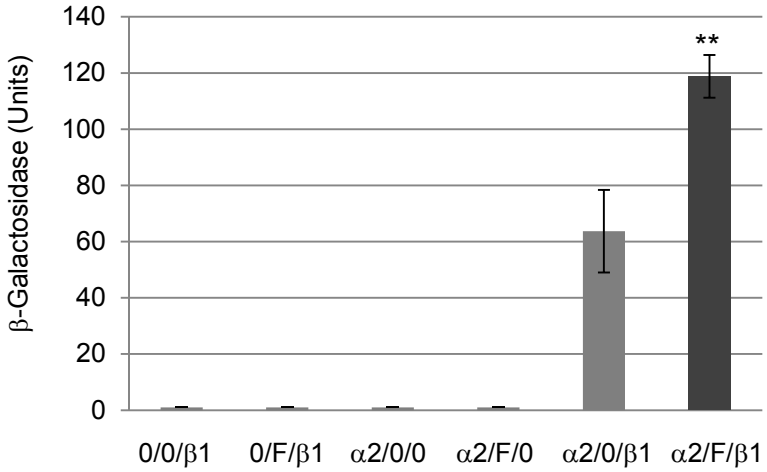

β-Galactosidase (Units)
140
120
100
80
60
40
20
0
**
0/0/β1
0/F/β1
α2/0/0
α2/F/0
α2/0/β1
α2/F/β1

Figure 4

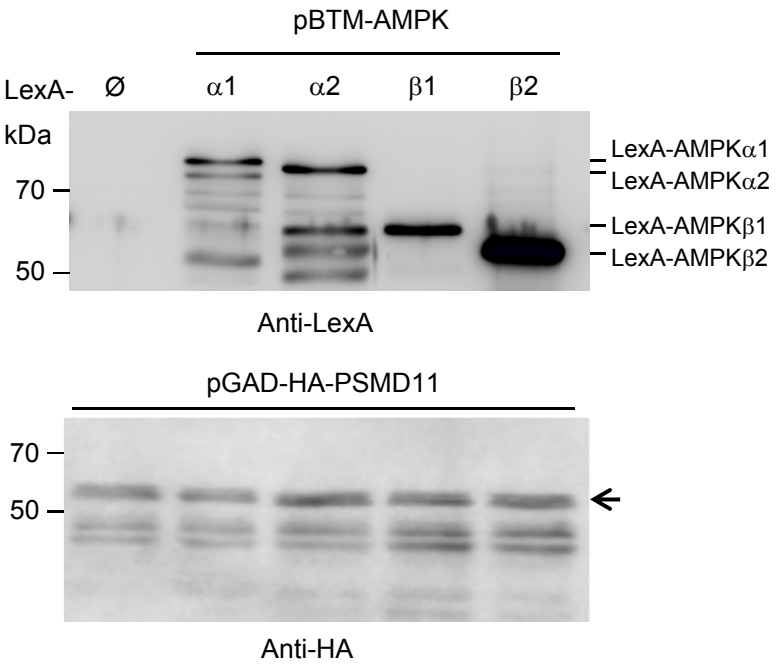

pBTM-AMPK
LexA-
Ø
α1
α2
β1
β2
kDa
70
50
LexA-AMPKα1
LexA-AMPKα2
LexA-AMPKβ1
LexA-AMPKβ2
Anti-LexA
pGAD-HA-PSMD11
70
50
Anti-HA

Figure 5