# The Ras recruitment system, a novel approach to the study of protein–protein interactions

Yehoshua C. Broder*, Sigal Katz* and Ami Aronheim

The yeast two-hybrid system represents one of the most efficient approaches currently available for identifying and characterizing protein–protein interactions [1–4]. Although very powerful, this procedure exhibits several problems and inherent limitations [5]. A new system, the Sos recruitment system (SRS), was developed recently [6] based on a different readout from that of the two-hybrid system [6–8]. SRS overcomes several of the limitations of the two-hybrid system and thus serves as an attractive alternative for studying protein–protein interactions between known and novel proteins. Nevertheless, we encountered a number of problems using SRS and so have developed an improved protein recruitment system, designated the Ras recruitment system (RRS), based on the absolute requirement that Ras be localized to the plasma membrane for its function [9,10]. Ras membrane localization and activation can be achieved through interaction between two hybrid proteins. We have demonstrated the effectiveness of the novel RRS system using five different known protein–protein interactions and have identified two previously unknown protein–protein interactions through a library screening protocol. The first interaction (detailed here) is between JDP2, a member of the basic leucine zipper (bZIP) family, and C/EBPγ, a member of the CCAAT/enhancer-binding protein (C/EBP) family. The second interaction is between the p21-activated protein kinase Pak65 and a small G protein (described in the accompanying paper by Aronheim *et al.* [11]). The RRS system significantly extends the usefulness of the previously described SRS system and overcomes several of its limitations.

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## Results and discussion

### Rationale for development of the Ras recruitment system
RRS is based on the strict requirement that Ras be localized to the plasma membrane for it to function [9,10]. This localization is normally achieved via attachment of a farnesyl moiety to a consensus sequence in the Ras carboxy-terminal domain known as the CAAX box [10]. In the yeast temperature-sensitive *cdc25-2* strain, the yeast Ras (yRas) is rendered inactive at the restrictive temperature (36°C) due to lack of a functional Cdc25 guanyl nucleotide exchange factor [7]. The expression of mammalian Ras lacking its CAAX box in *cdc25-2* yeast is not expected to activate the adenylyl cyclase pathway. On the basis of this, we tested whether Ras membrane localization can be achieved through protein–protein interaction and result in complementation of the *cdc25-2* mutation. For this, a protein of interest (X) is fused to a cytoplasmic Ras mutant (the 'bait'), and a protein partner is fused to a membrane localization signal such as a myristoylation sequence (the 'prey'). A protein–protein interaction between the bait and prey would be expected to result in Ras membrane localization and complementation of the *cdc25-2* mutation (Figure 1).

### Interactions between known protein pairs
A plasmid encoding an activated Ras lacking its carboxy-terminal CAAX box [Ras(61)ΔF] was generated by PCR and was used to fuse different bait proteins. To test the feasibility of the RRS system, the bait plasmids were cotransfected into *cdc25-2* yeast either with an empty expression plasmid as a control or with plasmids encoding known protein partners and tested for their ability to complement the *cdc25-2* mutation. Interactions were tested between the following proteins: the phosphatidylinositol-3-phosphate kinase subunits p110 and p85 [12]; c-Jun and c-Fos [13]; hSos and Grb2 [14]; Pak65 [15,16] and Src homology 3 (SH3)-containing proteins [14,17]; and Pak65 and different Rac1 mutants [18,19] (Figure 2a–e). The *cdc25-2* yeast transformants expressing different combinations of bait and prey proteins were tested for their ability to grow at 36°C (Figure 2). In all cases tested, only transformants expressing both the bait fusion and the corresponding known membrane-anchored prey were able to grow at 36°C. Negative controls, such as those expressing the bait protein alone or with the predicted protein partner lacking the membrane-targeting signals, were growth arrested at 36°C. These results demonstrate that Ras membrane localization, via protein–protein interactions, is sufficient for activation of its downstream effector, adenylyl cyclase.
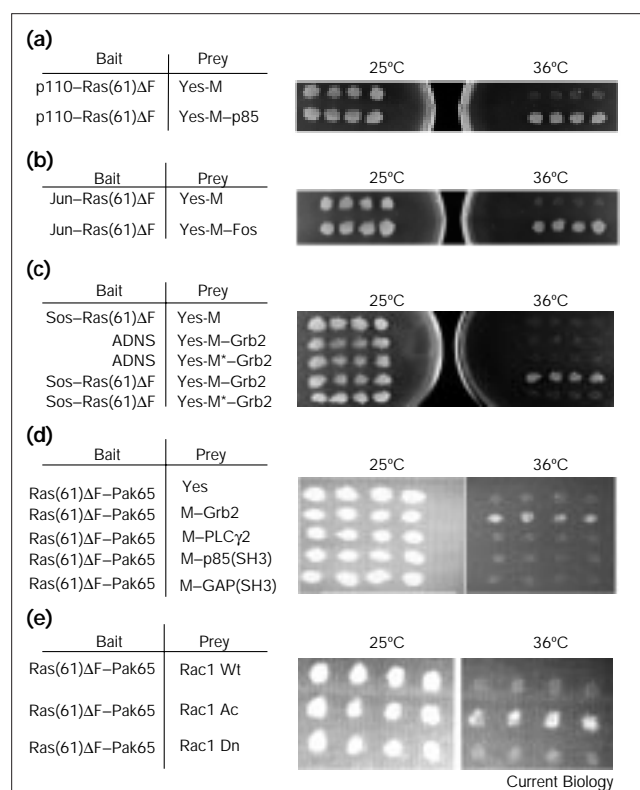
**Figure 1**
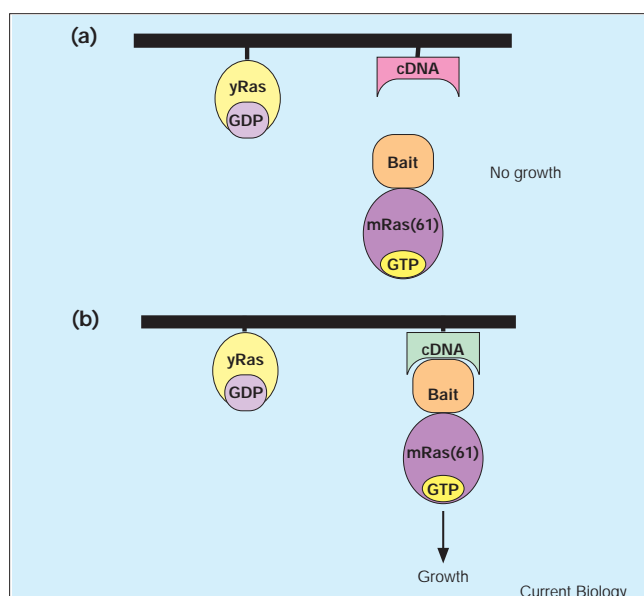


Schematic representation of the RRS system. (a) Expression of a cytoplasmic mammalian activated Ras (devoid of its CAAX box; mRas(61)) fused to protein X (the 'bait') in *cdc25-2* cells is unable to promote cell growth at the restrictive temperature (36°C). The yeast Ras (yRas) is present in the GDP-inactive form due to the absence of a functional exchange factor Cdc25 at the restrictive temperature. (b) Upon interaction between the bait protein and a membrane-localized protein partner, Ras will be localized to the plasma membrane and will be able to confer cell growth at the restrictive temperature. The protein partner for the bait could be a known protein partner, a putative protein partner ('prey'), or a protein encoded by a cDNA in a library screen – as shown here.

**Figure 2**



Complementation of the temperature-sensitive *cdc25-2* mutation through protein–protein interactions using the RRS system. Growth is seen for combinations of plasmids indicating (a) p110–p85 interaction, (b) Jun–Fos interaction, (c) hSos–Grb2 interaction, (d) Pak65–Grb2 interaction, and (e) Pak65–Rac1 interaction. The *cdc25-2* cells were transformed with the indicated plasmids and plated on glucose minimal medium supplemented with the appropriate amino acids and bases. Four independent transformants were grown at 25°C, replica-plated onto appropriately supplemented galactose minimal plates and grown at the restrictive temperature, 36°C. The shaded patches of the control transformants correspond to growth-arrested cells. The appearance of these cells in the figure may vary due to different exposure times. Constructs used are described in Materials and methods; myristoylation or myristoylation-defective membrane localization sequences are indicated by M and M* respectively.

## RRS can be used to identify novel protein partners

We also investigated whether RRS could be used to identify previously unknown protein–protein interactions in a screening approach. We fused Ras(61)ΔF to JDP2, a member of the bZIP family that has been shown to interact with c-Jun and repress transcription from AP-1-dependent reporters [6], and used the resulting fusion protein, Ras(61)ΔF–JDP2, to screen a rat pituitary cDNA expression library fused to membrane-localization sequence [6]. About 500,000 independent transformants were screened for interaction with the Ras(61)ΔF–JDP2 bait in the presence of a plasmid encoding mammalian GTPase-activating protein (GAP) [20,21]. Four clones were isolated that exhibited efficient growth at 36°C only when grown under galactose-inducing conditions, which allows a high level of expression of the library plasmid. Plasmids derived from these yeast clones were cotransfected with either the original bait, Ras(61)ΔF–JDP2, or a nonspecific bait, Ras(61)ΔF–Pak. Three clones interacted specifically with JDP2 and one (clone #1) exhibited efficient growth with both baits (Figure 3a). Sequence analysis of clone #1 showed it to encode a Sos homologue. Membrane-bound Sos is

expected to activate the yeast Ras and represents a possible false positive in this assay. Clone #3 did not show significant sequence homology with known genes, but the two other clones (#5 and #8) represented cDNA encoding C/EBPγ [22]. To further demonstrate the association between JDP2 and C/EBPγ *in vitro*, we mixed reticulocyte lysates programmed with either JDP2 or members of the C/EBP family γ and β and tested for their ability to bind a radio-labelled Jun–TRE DNA probe in an electrophoretic mobility shift assay. While JDP2-containing lysate showed a unique binding to the Jun–TRE sequence, the addition of C/EBPγ lysate, surprisingly, resulted in a faster-migrating band, in a dose-dependent manner (Figure 3b, lanes 2–6). No such heterodimerization was observed when C/EBPβ

**Figure 3**

(a) Isolation using RRS of a previously unknown protein–protein interaction with JDP2 as bait. The Ras(61)ΔF–JDP2 bait was used to screen a rat pituitary cDNA library in a *cdc25-2* yeast strain. Four colonies exhibited efficient growth at 36°C only when grown on medium containing galactose. Plasmid DNA was extracted from these colonies and reintroduced into *cdc25-2* cells with either the specific bait Ras(61)ΔF–JDP2 or a nonspecific bait, Ras(61)ΔF–Pak. Cells were grown at 25°C or 36°C. (b) Functional association between JDP2 and C/EBPγ. Electrophoretic mobility shift assay was used to test the association between JDP2 and C/EBPγ. Reticulocyte lysate was programmed with either JDP2 (JDP2 lysate is indicated by +), C/EBPγ or C/EBPβ (indicated by γ or β). A maximal volume of 7 μl reticulocyte lysate was used for each binding reaction (20 μl). Unprogrammed reticulocyte lysate was used to adjust the final lysate volume whenever necessary. JDP2 (1 μl) lysate was used either alone (lane 2) or with different volumes (2, 4, 6 μl) of C/EBPγ (lanes 4,5,6, respectively). For C/EBPβ lysate, 6 μl was used (lanes 7,8). The indicated lysates were mixed at room temperature for 10 min prior to the addition of the $^{32}$P-labelled annealed Jun–TRE oligonucleotide. Competition was performed by addition of 50-fold excess of unlabeled annealed oligonucleotide of either specific competitor, Jun–TRE (sp; lane 9), or nonspecific competitor, C/EBP/Chop annealed oligonucleotide (ns; lane 10), together with the $^{32}$P-labelled Jun–TRE probe. The JDP2 homodimer and JDP2/C/EBPγ heterodimer are indicated.



was mixed with JDP2 (Figure 3b, lane 8). Neither C/EBP alone showed binding to the Jun–TRE sequence (Figure 3b, lanes 3,7). The binding specificity was confirmed using homologous Jun–TRE as a specific competitor or a nonspecific annealed oligonucleotide (Figure 3b, lanes 9,10). These data strongly suggest that JDP2 associates specifically with C/EBPγ and the complex formed is able to bind the Jun–TRE sequence. The interaction between JDP2 and C/EBPγ is the first demonstration of a functional interaction between members of the bZIP and C/EBP transcription factor families. As both JDP2 and C/EBPγ are considered dominant-negative components within their family [6,23], it would be of great interest to explore the transcriptional role resulting from their association.

The data presented demonstrate that RRS is a bone fide method for detecting interactions between known or unknown protein partners. Although RRS can be used for analyses of protein interactions for a wide range of proteins (Figure 2), it will not be suitable for studying interactions with an integral membrane protein or a protein that interacts with a membrane component. On the other hand, RRS can be used to map the domain responsible for the membrane association of such a protein.

RRS combines the advantages of the SRS system with a solution to several of its limitations. For example, protein baits such as Pak65 (data not shown) that produce a positive signal in the absence of an interacting partner and therefore could not be used with the SRS system showed lower background in the RRS system and could be used to detect protein–protein interactions. In addition, RRS efficiently eliminates the isolation of Ras false positives (though not Sos false positives) and therefore represents a more efficient system for isolating novel interacting proteins. Finally, the Ras protein is relatively small, thereby overcoming several technical limitations encountered with SRS.

## Materials and methods

*Bait plasmids and constructs*
The ADNS expression plasmid was used to express the bait proteins under the control of the alcohol dehydrogenase constitutive promoter [24]. For Ras plasmid (Ras(61)ΔF) construction, Ras-specific oligonucleotides were designed to generate a Ras fragment corresponding

to amino acids 2–185. The RasL61 plasmid was used as a DNA template. The Ras DNA fragment was designed to contain 5′-AAG CTT CCC GGG ACC ATG to provide *Hind*III, *Sma*I and *Nco*I restriction sites to allow amino-terminal fusion (nucleotide triplets indicate the coding frame). The Ras carboxy-terminal end was designed to contain either 5′-TGG ATC CTC or 5′-GGA ATT CTC to provide either a *Bam*HI (Yes–Ras(61)ΔF-*Bam*HI) or *Eco*RI (Yes–Ras(61)ΔF-*Eco*RI) restriction sites, respectively, to allow carboxy-terminal fusion. The following proteins were fused to Ras(61)ΔF: p110β amino acids 31–150; c-Jun DNA-binding domain amino acids 249–331; hSos carboxy-terminal domain amino acids 1066–1333; and Pak65 regulatory domain amino acids 3–215. The DNA fragments of p110, Jun, and hSos were generated by PCR and fused to Ras(L61)ΔF by *Hind*III–*Sma*I restriction. The JDP2 cDNA fragment was fused to the Ras carboxy-terminal domain using *Eco*RI–*Xho*I. Pak65 cDNA was fused to the Ras carboxy-terminal domain using *Bam*HI digestion.

### Prey plasmids and constructs
All prey expression vectors are based on the Yes2 expression plasmid (Invitrogen Ltd). pYes2 was used to express prey proteins designed under the control of the Gal1 promoter. Yes-M, Yes-M–p85, Yes-M–Fos are as described [6], Yes-M–Grb2 and Yes-M*–Grb2 encode full-length Grb2 fused to either myristoylation (M) or myristoylated-defective (M*) sequence [8]. PLCγ2 amino acids 405–1252, p85 amino acids 2–84 and mGAP amino acids 262–345 were fused to the v-Src myristoylation signal. The Rac1 constructs used were: wild-type Rac1 (Rac1 Wt); constitutively active Rac1 Q61L (Rac1 Ac); and dominant-negative Rac1 T17N (Rac1 Dn) inserted in *Bam*HI–*Eco*RI sites in pYes2.

### Yeast growth and manipulations
Conventional yeast transfection and manipulation protocols were used as described [6].

### Library screening
The library used has been described previously [6]. Library screening was performed stepwise. First, plasmids encoding the bait and mGAP [21] were cotransfected into *cdc25-2* cells plated on glucose minimal medium lacking leucine and tryptophan. Subsequently, 3 ml culture was grown in liquid at 25°C overnight and used to inoculate 200 ml medium for an additional 12 hr. To allow high transformation efficiency, cells were pelleted and resuspended in 200 ml YPD medium for 3–5 hr at 25°C prior to transfection with 20–40 μg of library plasmid DNA. Cells were plated on about twenty 100 mm plates resulting in 5,000–10,000 transformants/plate. Transformants were incubated for 4 days at 25°C followed by replica plating onto galactose plates incubated at 36°C for 2–4 days. Transformants exhibiting efficient growth on galactose plates at 36°C were isolated and grown on glucose minimal medium for 2 days at 25°C followed by replica plating onto both glucose and galactose plates, incubated at 36°C.

### Electrophoretic mobility shift assay
Rabbit reticulocyte lysate (TNT Quick Coupled Transcription/Translation System Promega Ltd) was used for *in vitro* transcription translation of pCDNA–JDP2 (pCDNA Invitrogen Inc.), Bs–C/EBPγ and Bs–C/EBPβ constructs were made according to the manufacturer's instructions. Mobility shift analysis was performed as described in [6]. The Jun–TRE oligonucleotide sequence is 5′-TTCGGGGTGACAT-CATGGGCTAT and the C/EBP/CHOP oligonucleotide sequence is 5′-AGATCTGGCTGCAACCCCCCCTCGAGCCAGATC.

### Supplementary material
A screening flow chart for RRS and diagrams of plasmids used in this work are published with this article on the internet.

## Acknowledgements

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# Supplementary material

# The Ras recruitment system, a novel approach to the study of protein–protein interactions

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**Figure S1**
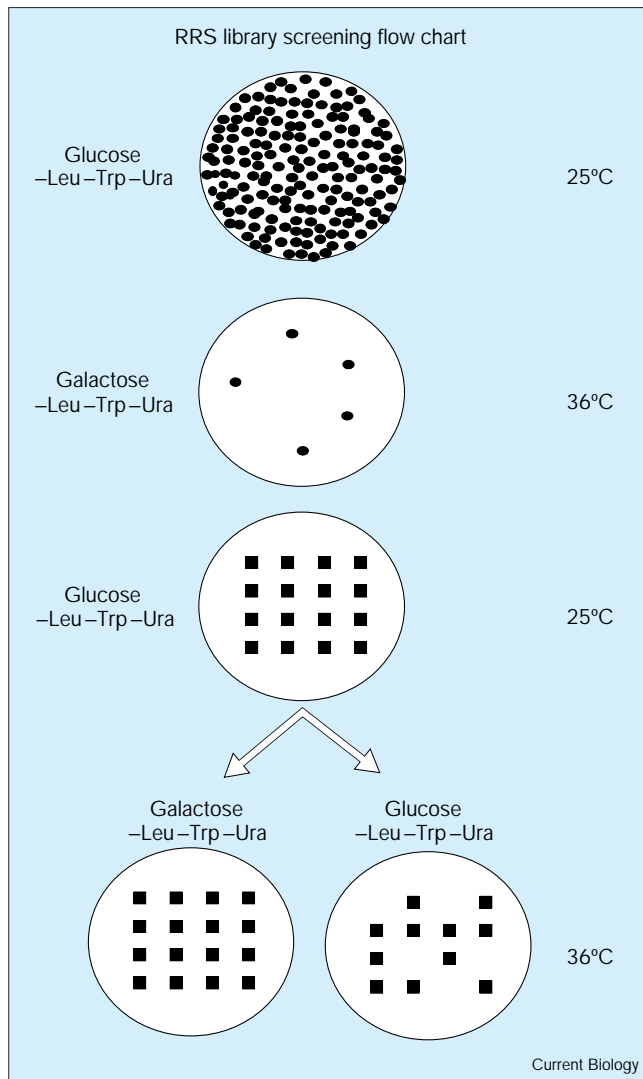


RRS screening flow chart. Temperature-sensitive *cdc25-2* yeast cells transformed with bait and GAP expression plasmids are transfected with a cDNA expression library plasmid. Cells are plated on glucose minimal medium lacking tryptophan, uracyl and leucine and incubated at the permissive temperature 25°C. Transformants are allowed to grow for 4–5 days at 25°C before replica plating to galactose-containing plates incubated at the restrictive temperature 36°C for 3–4 days. Clones that exhibit efficient growth at 36°C are isolated and streaked onto a glucose-containing plate grown for an additional 2 days at 25°C. Subsequently, the isolated clones are tested for their galactose-dependent growth at 36°C. Clones exhibiting efficient growth on galactose-containing medium but slower growth on glucose-containing medium are considered promising candidates and are further analyzed.

**Figure S2**

**(A2)**

BamHI    / NotI

Ras (600bp)

HindIII/SmaI/NcoI

CYC1 terminator    ColE1

GAL1

2μ

pYesRas(61) ΔF-BamHI (6.5KB)

URA3

Amp ʳ

3´-BamHI
TGG ATC CTC
W    I    L

HindIII        SmaI        NcoI
5´-AAG CTT CCC GGG ACC ATG GAA TAT AAG CTG GTG GTG
M    E    Y    K    L    V    V

**(A1)**

BamHI    NotI

Ras (600bp)

HindIII/SmaI/NcoI

ADH terminator        BamHI

ADH promoter

BamHI

2μ

ADNS-Ras(61) ΔF (9KB)

LEU2

Amp ʳ

3´-BamHI
TGG ATC CTC
W    I    L

Hind3        SmaI        NcoI
5´-AAG CTT CCC GGG ACC ATG GAA TAT AAG    CTG GTG GTG
M    E    Y    K    L    V    V

**(A3)**

EcoRI /    NotI

Ras (600bp)

HindIII/SmaI/NcoI

CYC1 terminator    ColE1

GAL1

2μ

pYesRas(61) ΔF-EcoRI (6.5KB)

URA3

Amp ʳ

XhoI
GCA G
A    R

ATC    ACA CTG GCG GCC GCT CGA
I    T    L    A    A    A

GA ATT CTG CAG ATA TCC
I    L    Q    S

EcoRI

HindIII        SmaI        NcoI
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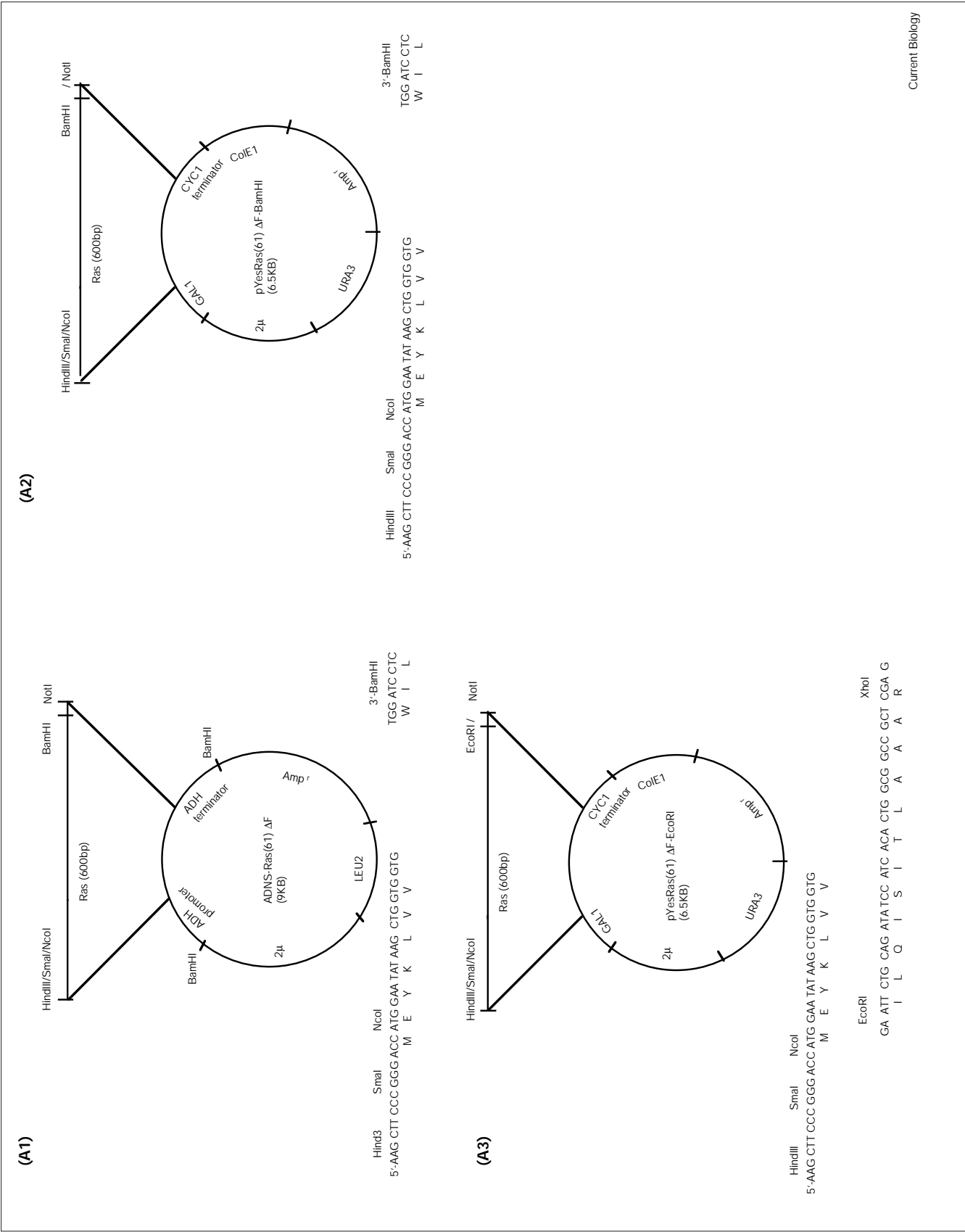M    E    Y    K    L    V    V

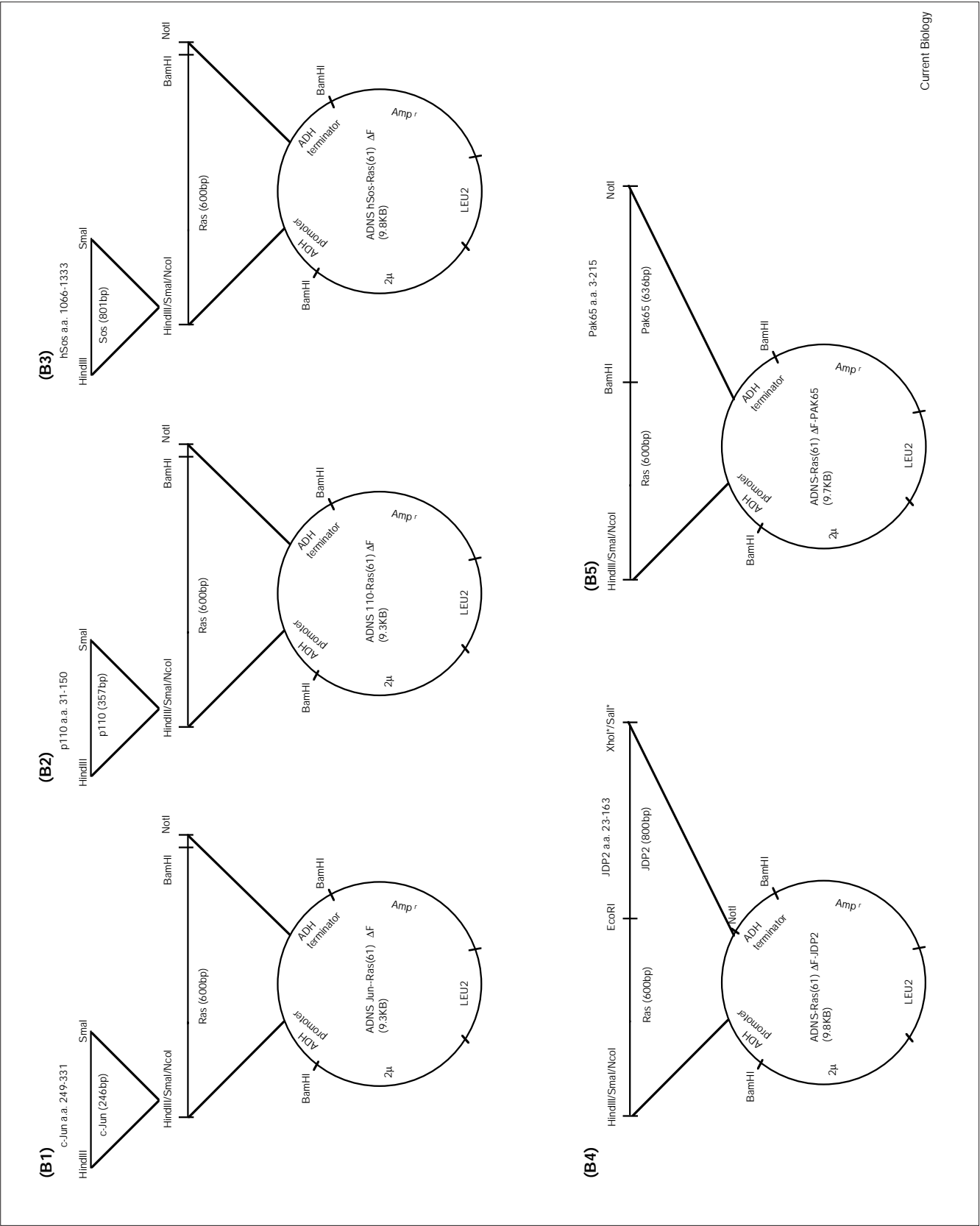Current Biology

**Figure S2 (continued)**

## Figure S2

Ras-based expression vectors. Plasmids A1–A3 are the basic vectors used for the fusion of different bait-encoding cDNAs. ADNS–Ras(61)ΔF (A1) is an ADNS-derived plasmid [23] and is used for fusion of baits to the amino-terminal region of Ras using the HindIII–SmaI unique restriction sites. The following baits were fused to A1 at the amino-terminal domain: ADNS–Jun–Ras(61)ΔF (B1), ADNS–p110–Ras(61)ΔF (B2), ADNS–hSos–Ras(61)ΔF (B3). Yes–Ras(61)ΔF-BamHI (A2) and Yes–Ras(61)ΔF-EcoRI (A3) are based on the commercially available Yes2 expression plasmid (Invitrogen Inc.). These plasmids are used for fusion of baits to the carboxy-terminal region of Ras. This can be done by first introducing the gene of interest into the Yes2–Ras(61)ΔF plasmid and then transferring the fusion gene to ADNS by HindIII–NotI restriction (as for plasmid B5). Alternatively, a three-fragment ligation is possible using the Ras(61)ΔF DNA fragment cut with HindIII and either EcoRI (A3) or BamHI (A2) and the gene of interest restricted with the corresponding 5′ EcoRI or BamHI and a 3′ NotI or XhoI. The latter was performed for plasmid B4. Note that the EcoRI frame of plasmid A3 was designed to fit the EcoRI frame of clones derived from the library (C2).

## Figure S3

Yes2-based expression vectors. Plasmids C1–C2 are the basic vectors for fusion of different prey-encoding cDNAs. All prey-encoding plasmids are based on the Yes2 expression plasmid (Invitrogen Inc.). Yes-M-polyA is used to fuse prey-encoding cDNAs using the BglII site. In this manner, the following cDNAs were fused in frame with the v-Src myristoylation sequence: c-Fos (D1), p85 (D2), Grb2 (D3), GAP(SH3) (D4) and p85(SH3) (D5). Yes-MΔpolyA (C2) was designed to fuse specific cDNA or cDNA libraries to the v-Src myristoylation sequence using the EcoRI–XhoI restriction. To do so, the BglII site in the C1 plasmid was fused with the BamHI site. This made possible the use of the downstream restriction sites from the Yes2 polylinker. The PLCγ2 cDNA was fused in this manner (D6) as well as the rat pituitary cDNA library.

**Figure S3**

**(C2)**

BglII* BamHI*

Myristoylation

HindIII

pYesM ΔpolyA (6KB)

CYC1 terminator — ColE1 — Amp^r — URA3 — 2μ — GAL1

HindIII
AAG CTT TCTAGACC ATG GGG AGT AGC AAG AGC AAG CCT
            NcoI
                 M   G   S   S   K   S   K   P
            Sma    BglII*BamHI*
AAG GAC CCC AGC CAG CGC CGG CCC GGG AGA TCC  ACT AGT AAC GGC CGC CAG TGT
 K   D   P   S   Q   R   R   P   G   R   S   T   S   N   G   R   Q   C
      EcoRI                             XhoI
GCT GGA ATT CTG CAG ATA TCC ATC ACA CTG GCG GCC GCT CGA G
 A   G   I   L   Q   I   S   I   T   L   A   A   A   R

**(C1)**

BamHI

SV40-PolyA

BglII

Myrist.

HindIII

pYesMpolyA (6.7KB)

CYC1 terminator — ColE1 — Amp^r — URA3 — 2μ — GAL1

HindIII
AAG CTT TCTAGACC ATG GGG AGT AGC AAG AGC AAG CCT
            NcoI
                 M   G   S   S   K   S   K   P
            Sma    BglII
AAG GAC CCC AGC CAG CGC CGG CCC GGG AGA TCT
 K   D   P   S   Q   R   R   P   G   R

**Figure S3 (continued)**



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