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Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators

(interaction trap/two-hybrid/protein networks/interaction matrix)

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ABSTRACT **We characterized interactions between *Drosophila melanogaster* cell cycle regulatory proteins by a yeast interaction-mating technique. The results were displayed as two-dimensional matrices that revealed individual binary interactions between proteins. Each protein (Cdi, cyclin-dependent kinase interactor) interacted with a distinct spectrum of cyclin-dependent kinases (Cdk) from *Drosophila* and other organisms. Some Cdis interacted with other Cdis, indicating that these proteins may form trimeric complexes that include Cdks. Similar analysis of interaction matrices may be generally useful in detecting other multiprotein complexes and in establishing connectivity between individual complex members. Moreover, such analysis may also help assign function to newly identified proteins, identify domains involved in protein–protein interactions, and aid the dissection of genetic regulatory networks.**

Many of the proteins that govern cell cycle decisions in higher eukaryotes ultimately affect the activity of cyclin-dependent kinases (Cdks) (1–4). The activity of these kinases is required for progression through specific phases of the cell cycle. Cdk activity depends on association of the kinases with positive regulatory proteins called cyclins (5), including the D-type and E-type cyclins active during the G_1 phase of the cell cycle. In addition to cyclins, other proteins also act on the Cdks (6–9); these proteins may control Cdk activity in response to extracellular and intracellular signals that control cell proliferation—for example, during development. An understanding of this regulatory circuitry will ultimately require the identification of the entire set of Cdk modulatory proteins, characterization of their function, and their placement into ordered genetic pathways.

Recently, many cell cycle regulators have been identified by using yeast two-hybrid systems like the interaction trap (10–13). In two-hybrid systems (14), two proteins are expressed in yeast: one (the "bait") contains a DNA-binding moiety; the other ("activation tagged" or "prey") contains a transcription activation domain. If the two proteins interact, the complex activates transcription of a reporter gene that contains a binding site for the DNA-binding domain of the bait. The interaction trap (10) uses *Escherichia coli* LexA repressor as the DNA-binding moiety and two different reporter genes, *LEU2* and *lacZ*, each of which contains upstream LexA operators. Proteins that may interact with the bait, such as those encoded by members of cDNA libraries, are fused to an activation domain and expressed conditionally under the control of the yeast *GAL1* promoter. Yeasts that contain proteins that associate with the bait are selected because they grow in the absence of leucine due to activation of the *LEU2* reporter and because they form blue colonies on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) medium due to activation of the *lacZ* reporter.

We have used the interaction trap to isolate, from an embryonic *Drosophila melanogaster* library, seven cDNAs that encode proteins that interact with two *Drosophila* Cdks, DmCdc2 and DmCdc2c (15) (Table 1; unpublished data); we call these proteins cyclin-dependent kinase interactors (Cdis). Here we characterize specific associations of these proteins by a yeast interaction-mating technique.

Interaction mating relies on the fact that haploid yeast have two different mating types, *MAT***a** and *MATα*, which fuse to form diploids (17). In interaction mating, the bait and activation-tagged proteins are expressed in different haploid strains and are brought together by mating. By this means, large numbers of individual protein–protein interactions can be tested, and the results of these tests can be displayed as two-dimensional arrays (interaction matrices). We made a collection of strains, each of which expressed a different bait, and mated them with test strains that contained different activation-tagged Cdi. Examination of the resulting interaction matrices showed that each Cdi associates specifically with a distinct spectrum of Cdks, and that some Cdis may form trimeric complexes with *Drosophila* Cdks. The results suggest a number of applications of this method to genetic characterization of larger sets of proteins.

MATERIALS AND METHODS

Yeast Manipulations. We used standard microbiological techniques and media (18, 19). Media designations are as follows: YPD is YP (yeast extract plus peptone) medium with 2% glucose. Minimal dropout media (18) are designated by the component that is left out (e.g., −ura −his −trp −leu medium lacks uracil, histidine, tryptophan, and leucine). Each minimal dropout medium contains either 2% glucose (Glu) or 2% galactose plus 1% raffinose (Gal). X-Gal minimal drop-out plates contained X-Gal and phosphate buffer at pH 7.0 (18). DNA was introduced into yeast as in the protocol of Gietz *et al.* (20).

Yeast Strains and Plasmids. RFY206 (*Mat***a** *his3Δ200 leu2-3 lys2Δ201 ura3-52 trp1Δ::hisG*) (R.L.F., unpublished data) was made from yeast strain L4035 (a derivative of S288C, provided by B. Ruskin and G. Fink, Whitehead Institute). EGY48 [*Matα his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2* (10); J. Estojak, E. Golemis, and R.B., unpublished data] personal communication] contains a chromosomal *LEU2* gene (*3Lexop-LEU2*) with its upstream regulatory elements replaced with three high-affinity LexA operators.

pSH18-34, the *lacZ* reporter plasmid (S. Hanes and R.B., unpublished data), is a pLR1Δ1 (21) derivative similar to *lacZ* reporters previously described (22–24); it contains the yeast 2-μm replication origin, the *URA3* gene, and a *GAL1–lacZ*

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Abbreviations: Cdk(s), cyclin-dependent kinase(s); Cdi(s), cyclin-dependent kinase interactor(s); X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

Table 1. Cdis used in this study

Cdi	Function (from sequence)	Isolated with*
Cdi2	Cks†	DmCdc2
Cdi3	D-type cyclin	DmCdc2, DmCdc2c
Cdi4	Unknown	DmCdc2c
Cdi5	Cyclin	DmCdc2c
Cdi7	E-type cyclin‡	DmCdc2
Cdi11	Unknown	DmCdc2c
Cdi12	Unknown	DmCdc2c

Cdi were isolated from a *Drosophila* embryonic cDNA library in interactor hunts using the indicated *Drosophila* Cdk (unpublished data).

*Based on amino acid sequence similarity, DmCdc2 is a likely homolog of human Cdc2, and DmCdc2c may be a homolog of human Cdk2 (15).

†Human or *S. cerevisiae* Cks (Suc1 in *Sc. pombe*) are small proteins of unknown function that associate tightly with Cdk.

‡Cdi7 is almost identical to *Drosophila* cyclin E-type II isolated by Richardson *et al.* (16).

fusion with the *GAL1* upstream regulatory elements replaced with four *colE1* LexA operators. Activation-tagged proteins were conditionally expressed from the *GAL1* promoter on plasmid pJG4-5 (10), which contains the yeast 2-μm origin and *TRP1* gene. pRF4-5-Cdi2, -Cdi3, -Cdi4, -Cdi5, -Cdi7, -Cdi11, and -Cdi12 are pJG4-5 derivatives that express *Drosophila* cDNAs isolated in *Drosophila* Cdk interactor hunts and are described elsewhere (Table 1; unpublished data). LexA fusions (baits) were expressed from the *ADH1* promoter on one of the bait expression vectors, pLexA (1-202)+PL (25) or pEG202 (10); both contain the yeast 2-μm origin and *HIS3* gene. Bait plasmids expressing LexA fused to human Cdc2, Cdk2, Cdk3, and Cdk4; *Saccharomyces cerevisiae* Cdc28; and *Drosophila* Cdc2 and Cdc2c have been described (10). Cdi2, Cdi3, Cdi5, and Cdi11 bait expression plasmids were made by inserting *Eco*RI/*Xho* I fragments from the respective pRF4-5-Cdi plasmids into the backbone of pLexA (1-202)+PL cut with *Eco*RI and *Sal* I. The bicoid derivative bait (DmBcdΔC) contains amino acids 2–160 of *Drosophila* Bicoid fused to LexA. The "no LexA" control vector, pRFHM0, was made by deleting the *Sph* I/*Sph* I fragment that contains part of the *ADH1* promoter and terminator and all of the LexA coding region from pLexA(1-202)+PL. The *Drosophila* Raf protein kinase bait was expressed from a pEG202-derivative provided by J. Duffy and N. Perrimon, Harvard Medical School.

Interaction Mating. Interaction mating uses many of the components of the original interaction trap (10). Fig. 1 shows a typical experiment. Bait strains are RFY206 derivatives (*MAT***a**) that contain a *LexAop-lacZ* reporter plasmid (pSH18-34) and different bait plasmids. The other strains are EGY48 derivatives (*MATα*) that contain plasmids that express different activation-tagged proteins. We streaked bait strains in horizontal rows on a Glu −ura −his plate and EGY48 derivatives in vertical columns on a Glu −trp plate. We replica plated these two sets of streaks onto YPD plates, where strains mated and formed diploids at the intersections of the horizontal and vertical streaks. We used the YPD plates as master plates to make four replica indicator plates: 1, Glu −ura −his −trp X-Gal (Glu X-Gal); 2, Gal −ura −his −trp X-Gal (Gal X-Gal); 3, Glu −ura −his −trp −leu (Glu −leu); 4, Gal −ura −his −trp −leu (Gal −leu). All four plates lacked nutrients required for growth of each of the parental haploid strains. On the Gal plates, synthesis of the activation-tagged proteins is induced. On the −leu plates, diploids grow only if they contain an activation-tagged protein that interacts with the bait (growth on Gal −leu only) or if the bait itself activates transcription (growth on both Glu −leu and Gal

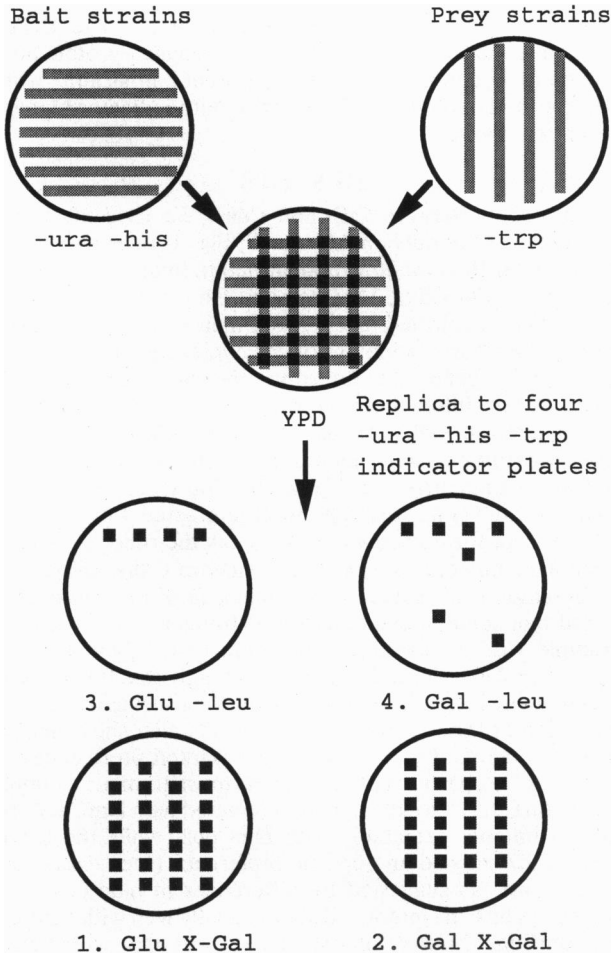

FIG. 1. Typical interaction mating. Haploid bait strains are streaked onto Glu −ura −his plates in horizontal lines. Bait strains are RFY206 derivatives that harbor *lacZ* reporter pSH18-34 (*URA3*$^+$), and each contains a different bait expressing plasmid (*HIS3*$^+$). Prey strains, haploid EGY48 derivatives, are streaked onto Glu −trp plates in vertical lines. In addition to the *3Lexop-LEU2* reporter, each prey strain contains a different pJG4-5 derivative (*TRP1*$^+$) capable of expressing an activation-tagged protein on galactose medium. The −trp plates and the −ura −his plates are sequentially pressed onto the same replica velvet and each imprint is lifted with a YPD plate. The YPD master plate is incubated for 12–20 hr at 30°C, during which time diploids form at the intersections of the two strains. The YPD master plates are then replica plated to four indicator plates. Neither of the parental strains is able to grow on the indicator plates. All of the diploids grow on the X-Gal plates (plates 1 and 2). On the −leu plates (plates 3 and 4) diploids grow only if the *LexAop-LEU2* gene is activated. On the two Glu plates (plates 1 and 3) the activation-tagged proteins are not expressed and only diploids that contain a bait that activates the reporters will grow (−leu, plate 3) or turn blue (X-Gal, plate 1), as represented by the bait strain in the top row. On the Gal plates (plates 2 and 4) the activation-tagged proteins are expressed and, if they interact with the bait, will allow the diploids to grow on the −leu plate (plate 4) and may also cause them to turn blue on the X-Gal plate (plate 2).

−leu; see, for example, LexA-Cdi2, Fig. 3). We scored interaction by the presence of galactose-dependent Leu$^+$ diploids. On the X-Gal plates, all diploids grow and those that express *lacZ* turn blue. In some instances, differences in the level of *lacZ* expression may reflect the relative strength of the interactions. This is most likely to be true of differences between β-galactosidase levels caused by interactions of a given activation-tagged protein with highly related baits that are expressed at similar levels and occupy LexA operators to similar extents. For tests against a larger number of baits, we have also used (data not shown) a variant of this procedure,

in which one plate contained a lawn of a single EGY48 derivative that expressed an activation-tagged protein and a second plate contained a grid of different bait strains, which we replica plated onto YPD and then onto indicator plates as described above.

RESULTS

Interactions Between Cdis and Cdks. We used interaction mating (see *Materials and Methods*; Fig. 1) to characterize a set of seven *Drosophila* Cdi identified in hunts with either of two *Drosophila* Cdks, DmCdc2 and DmCdc2c (15), as baits (Table 1) (unpublished data). From their sequences, some of the Cdi are clearly homologs of cell cycle regulators, like Cks (26, 27) and cyclins (28, 29), from other organisms (Table 1). Fig. 2 shows interactions between the *Drosophila* Cdi and several different Cdks. As indicated by Gal-dependent growth on −leu medium, each Cdi interacted with a distinct spectrum of Cdks (summarized in Table 2). For example, the Cks homolog Cdi2 (Fig. 2*A*, column 2) interacted with all of the Cdks except for human Cdk4, whereas the other Cdis interacted with only one or a smaller number of Cdks. Differences in the amount of galactose-dependent *lacZ* expression suggested that some interactions were stronger than others. For example, Cdi2 appeared to interact most strongly with human Cdk3 (Fig. 2*A*, column 2 row 5), so strongly that the normally undetectable level of its expression on glucose was detected by activation of the reporters. These results also show that the cyclins (Cdi3, Cdi5, and Cdi7) have marked preferences for particular Cdk partners (Fig. 2). In the most dramatic example, the cyclin Cdi5 ("novel" cyclin) interacted with DmCdc2c but did not interact detectably with DmCdc2, while the E-type cyclin Cdi7 showed an opposite preference (see *Discussion*). By contrast, as suggested by differences in blue color, the D-type cyclin Cdi3 interacted about equally well with DmCdc2 and DmCdc2c, but it interacted most strongly with human Cdk4, raising the possibility that a relevant partner of Cdi3 in *Drosophila* may be a Cdk4 homolog (see *Discussion*).

Interactions Between Cdis. Some of the Cdis interacted with other Cdis (Fig. 3, summarized in Table 2). For example, the Cdi5 bait interacted with activation-tagged Cdi2, and the Cdi11 bait interacted with activation-tagged Cdi3 and Cdi4. This result is consistent with the idea that these proteins may form ternary complexes. Formation of ternary complexes with Cdk is expected for Cdi2 and Cdi5, Cks, and cyclin homologs, respectively (Table 1); these proteins are known to simultaneously bind Cdk (30). However, our results also suggest that Cdi11 may form two other ternary complexes: one that contains DmCdc2c and the D-type cyclin Cdi3 and another that contains DmCdc2c and the protein Cdi4 ("novel" protein). The fact that, in the first case, Cdi11 interacts with two known cell cycle regulators, a cyclin and a kinase, is consistent with the idea that it functions in cell cycle regulation. Moreover, the fact that Cdi11 has a unique sequence suggests that it may confer a different function on the cyclin–kinase pair it interacts with (Cdi3–DmCdc2c) than Cdi2 confers on the cyclin–kinase pair it recognizes (Cdi5–DmCdc2c). The Cdi did not interact with human Max, *Drosophila* Raf kinase, the N-terminal part of *Drosophila* Bicoid (Fig. 3), or with 60 other baits (data not shown). This result extends previous findings in haploids (unpublished data) and further confirms that the observed interactions of the Cdis are specific.

Table 2. Summary of interactions

	Cdi2	Cdi3	Cdi4	Cdi5	Cdi7
DmCdc2	+	+	−	−	+
DmCdc2c	+	+	+	+	−
HsCdc2	+	−	−	−	−
HsCdk2	±	−	−	−	−
HsCdk3	+	+	+	−	−
HsCdk4	−	+	−	−	−
ScCdc28	+	+	−	+	−
Cdi5	+	−	−	−	−
Cdi11	−	+	+	−	−

Data are from Figs. 2 and 3.

DISCUSSION

We characterized a set of Cdis proteins from embryonic *Drosophila melanogaster* that interact with *Drosophila* Cdks, by a yeast interaction-mating technique that extends

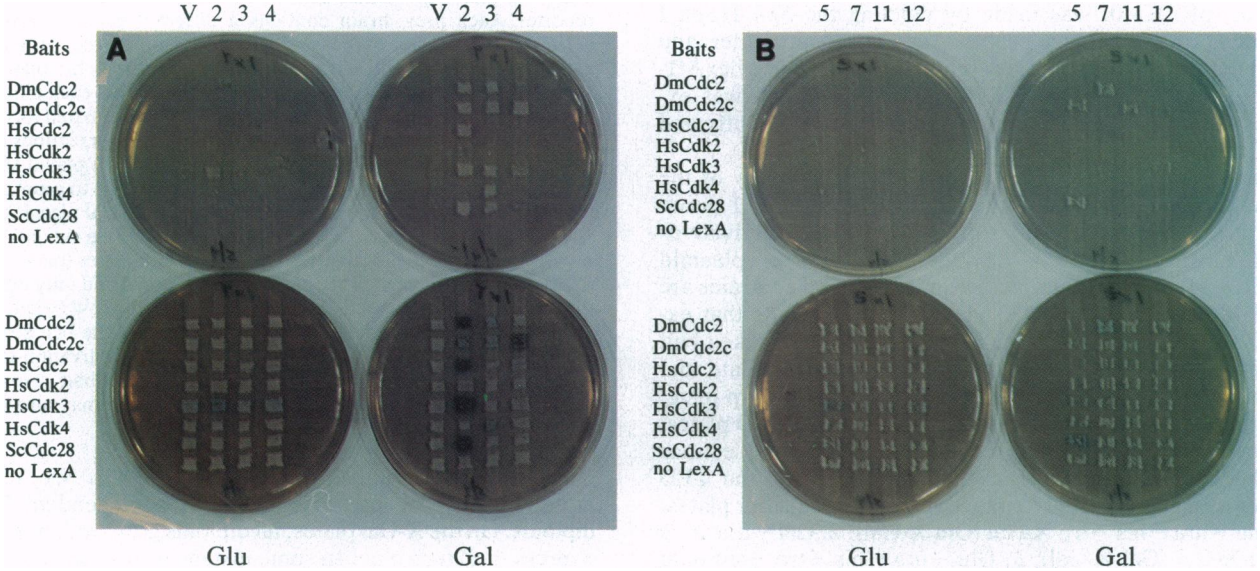

FIG. 2. Interaction of Cdis with Cdc2 kinases. Two YPD master plates from two separate matings (as in Fig. 1) were each replica plated to four indicator plates. In both cases, bait strains contained bait plasmids that expressed no LexA (no LexA) or LexA fusions to *Drosophila* Cdc2 and Cdc2c (−DmCdc2, −DmCdc2c); human Cdc2, Cdk2, Cdk3, and Cdk4 (−HsCdc2, −HsCdk2, −HsCdk3, −HsCdk4); and *S. cerevisiae* Cdc28 (−ScCdc28). (*A*) Bait strains were mated with EGY48 derivatives that contained the pJG4-5 vector (column V) or pJG4-5-Cdi2, −Cdi3, or −Cdi4 (columns 2–4). (*B*) Bait strains were mated with EGY48 derivatives that contained pJG4-5-Cdi5, −Cdi7, −Cdi11, or −Cdi12 cDNA inserts (columns 5, 7, 11, and 12). Indicator plates were incubated at 30°C for 2 days. Top two plates are −ura −his −trp −leu and contain either glucose (Glu) or galactose plus raffinose (Gal). Bottom two plates are −ura −his −trp X-Gal plates with Glu or Gal.

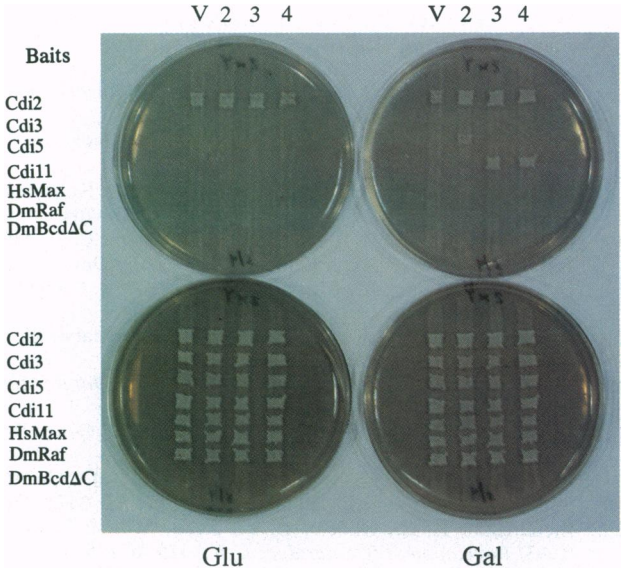

FIG. 3. Interaction between Cdis. Bait strains were mated as in Fig. 1 with EGY48 derivatives that contained pJG4-5 vector (column V) or pJG4-5-Cdi2, −Cdi3, or −Cdi4 (columns 2–4) (note, this is the same plate of EGY48 derivatives used in Fig. 2*A*) and the YPD plate was replica plated to the four indicator plates shown. Bait strains contained bait plasmids that expressed LexA fusions to *Drosophila* Cdi2, Cdi3, Cdi5, and Cdi11 (−Cdi2, −Cdi3, −Cdi5, −Cdi11); human Max (−HsMax); amino acids 2–160 of *Drosophila* Bicoid (−DmBcdΔC); or *Drosophila* Raf (−DmRaf). As in Fig. 2, top two plates lack leucine and bottom two plates contain X-Gal. Cdi2 bait activated transcription of the *Lexop-LEU2* reporter, allowing growth on Glu −leu and Gal −leu plates.

the interaction trap (a yeast two-hybrid method). We mated yeast that contained activation-tagged Cdis with a large number of strains that each expressed a different bait protein to generate individual diploid strains that tested individual protein–protein interactions. We displayed this pattern of interactions in interaction matrices, two-dimensional arrays of diploid strains on appropriate indicator plates. We scored interaction between the Cdi and the baits by accessing growth of the resulting diploid yeast in the absence of leucine, due to activation of the *LEU2* reporter. We estimated the relative strengths of interactions by observing activation of the *lacZ* reporter.

Inspection of the interaction matrices (Figs. 2 and 3) showed that the Cdis showed distinct spectra of interaction with different Cdks and other Cdis (Table 2). Further examination of these data showed several results. First, Cdi2, the Cks homolog, interacted with all of the Cdks except for human Cdk4, suggesting that it interacts with conserved structural elements of these related proteins. Second, Cdi5, the novel cyclin, interacted with DmCdc2c and not DmCdc2, while Cdi7, the E-type cyclin, interacted with DmCdc2 but not DmCdc2c, suggesting that such differences in interaction affinity may help explain the observed preference of cyclins for different Cdk partners. Third, Cdi3, the D-type cyclin, interacted with both *Drosophila* Cdk, *S. cerevisiae* Cdc28, and human Cdk3 but appeared to interact most strongly with human Cdk4 (Fig. 3). This suggests that, as in human cells where cyclin D forms active complexes with Cdk4 (1), a relevant partner of cyclin D in *Drosophila* may be a hitherto unidentified Cdk4 homolog. Finally, Cdi2 (Cks homolog) interacted with Cdi5 (novel cyclin), and Cdi11 (novel protein) interacted with Cdi3 (D-type cyclin) and Cdi4 (a second novel protein) (Fig. 3), suggesting that these proteins may function in different multiprotein complexes.

Our results suggest a number of uses for interaction mating. Some of these are consequences of the fact that, by obviating the need to introduce DNA into bait strains by transformation, the technique simplifies established interaction trap experiments. First, multiple hunts can now be conducted by collecting a very large number of cells transformed with interaction library members, freezing the collection, and mating thawed aliquots with individual bait strains (ref. 31; see also ref. 32). Second, specificity of candidate interactors can be rapidly determined by testing interaction with a large number of different baits.

More interestingly, by greatly increasing the number of individual interactions that can be tested, interaction mating enables new sorts of experiments to be conducted. For example, enough potential interactions can be surveyed to give a reasonable chance of detecting particular binary protein–protein interactions that may signal multiprotein complexes. We found, for example, that two *Drosophila* Cdk interacting proteins, Cdi2 and Cdi5, interacted with each other. This indicates that Cdi2, Cdi5, and the kinase DmCdc2c all have a surface for interaction with the other two proteins or for interaction with a common yeast protein acting as a bridging molecule (e.g., Cdc28). In either case, this result suggests that these proteins may form a trimeric complex. Note that there are two special cases in which these sorts of individual binary interactions would not indicate the existence of a higher-order protein complex: (*i*) if each of the interacting proteins possesses one, and only one, surface for interaction with the other two proteins; or (*ii*) if formation of a binary complex causes an allosteric change in both proteins that precludes their interaction with a third. Since trimeric complexes have been demonstrated *in vitro* for *Schizosaccharomyces pombe* homologs of Cdi2, Cdi5, and DmCdc2c [Suc1, Cdc13, and Cdc2, respectively (30)], the interaction mating data almost certainly indicate that they form a similar ternary complex. We obtained similar patterns of interactions with DmCdc2c, Cdi3, and Cdi11 and with DmCdc2c, Cdi4, and Cdi11, suggesting that these two sets of proteins may also form trimeric complexes. Along with standard two-hybrid methods (33, 34), such analysis may be generally useful for establishing connectivity between proteins in known multiprotein complexes and in sets of proteins known to interact genetically.

Similarly, analysis of interaction matrices may extend one form of classical suppressor genetics. Consider the fact that two similar proteins, the cyclins Cdi5 and Cdi7, show contrasting interaction specificity for two other proteins of similar sequence, DmCdc2 and DmCdc2c. This finding is formally similar to the extreme allele specificity sometimes found for second site suppressors in classical genetics [for example, between the *cheC* and *cheZ* gene products (35)], which is a strong genetic indication of direct protein–protein interaction. We propose that observation of strong and reciprocal interaction specificity between sets of proteins of related sequence in interaction mating experiments constitutes equivalent genetic evidence for direct protein–protein interaction. Moreover, we note that such findings open the way to construction of appropriate "amino acid swap" mutations to change specificity and further delineate the residues involved in the contact.

Interaction mating can also identify previously unsuspected interactions of potential biological significance. For example, the *Drosophila* D-type cyclin, Cdi3, interacts with another protein, Cdi11, which interacts specifically with *Drosophila* Cdk (Fig. 3) but whose sequence so far does not allow prediction of its function. Cdi11 may thus exemplify a previously unknown type of protein that modulates activity of cyclin/Cdk complexes. The cumulative probability that a biologically relevant but previously unsuspected interaction will be revealed by this approach increases with the number of potential interactions tested. Identification of unsuspected interactions may be generally useful in suggesting protein

function and may help position proteins within genetic and biochemical pathways. The ability to estimate relative interaction affinities using the *lacZ* reporters may add an additional dimension to the interaction matrices and may aid such analysis. Such analyses may also help identify loci affected in genetic diseases for which allelic variants of one gene are already implicated. Affected individuals who lack lesions in the first gene may have mutations in other genes involved in the process; one criterion for identification of such genes is to identify those encoding proteins whose interaction with the first protein is affected by mutations in the first protein associated with the disease state.

Even absent a biological connection between interacting proteins, structural information about the contact may be derived from consideration of the protein sequences. If, for example, Cdi3 had been isolated by some other method and tested for interaction with the 60 bait strains we used, we would have learned that Cdi3 frequently makes contacts with serine/threonine kinases (Fig. 2), suggesting that Cdi3 contacts common sequence or structural elements in these proteins. The larger the panel of baits, the more it constitutes a representative protein surface space that can be searched for such interaction motifs.

Since there are now hundreds of strains that contain different bait proteins, and since their number is increasing, the power of interaction mating to reveal connections between proteins should expand. Display of this information in interaction matrices and its analysis by computational techniques should allow analysis of the topology of the underlying protein network [reminiscent of the protein linkage maps envisioned by Bartel *et al.* (36)], which may aid the assignment of protein function and the identification of genetic pathways. It is thus possible that analysis of interaction matrices may aid efforts, such as genome characterization, in which tentative hypotheses about newly identified genes are useful.

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