

Construction of minimally-sheltered knockout mutants of *Neurospora crassa*

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I have been developing strains which I think will make it possible to produce "minimally-sheltered knockouts" of essential genes routinely. They could equally well be called "self-adjusting knockdowns" of essential genes. Such minimally-sheltered knockouts could give results in microarray analysis that would be less subject to artifact than results with heterokaryons or with homokaryons sheltered with an inducible wild-type allele of the gene in question.

The method addresses two questions about each cloned gene, henceforth and collectively called "your favorite gene" (*yfg*). *yfg* should be one which does *not* pass successfully through a cross as a hygromycin-resistant knockout.

(1) Is *yfg* a dispensable gene?

(2) If *yfg* is found to be essential, what is the "terminal phenotype" of the *yfg*^Δ knockout mutation? By "terminal phenotype," I mean that of the heterokaryon between *yfg*^Δ and a partial diploid, *yfg*^{+/Δ}, in which the partial diploid is the minimum proportion that allows somewhat suboptimal growth

THE DILEMMA POSED BY SLOW-GROWING OR SUB-VITAL KNOCKOUT MUTATIONS VS. LETHAL KNOCKOUT MUTATIONS

When a knockout cassette containing the flanking sequences of *yfg* is used to transform wild-type, the result is almost always a heterokaryon containing both transformed and untransformed nuclei. If the cassette contained a resistance determinant like *hygR* between those flanking sequences, the *hygR* transformants will almost always be heterokaryons between transformed and untransformed nuclei.

Dispensable genes. If *yfg* is dispensible, and potential transformant homokaryons are not at a disadvantage compared to the heterokaryon, homokaryons can be isolated in various ways. The current method of making homokaryotic knockout mutants involves outcrossing and isolating hygromycin-resistant segregants. It is likely, however, that there will be a class of *yfg* mutants that do not give rise to viable ascospores, but are dispensable in the sense that they would be viable as vegetative cultures if these could be obtained.

Essential genes. If the *yfg*^Δ mutations are lethal, of course, they cannot be isolated, but the heterokaryon can be maintained indefinitely under selection with hygromycin. However, nothing can be said for sure about the relation of phenotype and limitation of YFG product. One cannot assume that any limitation of growth that occurs is due to a suboptimal amount of YFG. In addition, the necessary presence of hygromycin, an inhibitor of protein synthesis, can have major effects on the phenotype that have nothing to do with YFG.

Slow-growing knockout types. Finally, if *yfg*^Δ mutations are sub-vital, homokaryons would be, by definition, viable, but it might be very difficult to isolate them if they are always greatly outgrown by heterokaryons. A *yfg* in this class could easily be misclassified as essential. As with heterokaryons involving essential genes, the phenotypes given by growth of slow-growing knockout types on hygromycin could also be misleading.

USE OF A VERY UNSTABLE PARTIAL DIPLOID TO SHELTER A LETHAL KNOCKOUT AND EXAMINATION OF THE LIMITING PHENOTYPE

A translocation mutant with remarkable properties has been studied by Perkins et al. (see pp. 349-350, Perkins 1997) and by Metzenberg and Chia (Metzenberg and Chia 1979). This translocation, called T(I → V)AR190, moves almost the entire right arm of LG I to the end of the nucleolus organizer on the left arm of LG V. Among the many genes that have become attached to the nucleolus organizer on LG V is *his-3*⁺, which is very near the new junction, and *nic-2*, *cyh-1*, and *al-1*.

When T(I → V)AR190 is crossed to wild type, 1/4 of the progeny ascospores emerge as each of the two parental types, 1/4 are inviable because they lack almost the entire right arm of LG I, and 1/4 are partial diploids that carry two copies of this arm. (For the moment, we will ignore crossingover.) The partial diploids break down extremely rapidly and become 100% euploid and

homokaryotic in the vegetative phase unless they carry some allele on the ectopic segment necessary for survival because it is absent from the canonical segment. This selective marker may be the wild-type allele of a dispensable nutritional marker, but it can also be the wild-type allele of an essential gene (See Figure). It is invariably the ectopic segment that is lost. When such an unstable partial diploid carries the wild-type selectable marker, e.g., for prototrophy on the ectopic segment, it quickly and spontaneously becomes a balanced, somewhat suboptimally-growing heterokaryon between residual partial diploid nuclei (2-5%, depending on genotype) and normal-sequence nuclei (98-95%). It was seen in past experiments that if the canonical LG I is also carrying an albino mutation and the ectopic segment carries its wild-type allele, the balanced heterokaryon has a very pale orange color -- almost albino, a visual indicator of this imbalance in favor of the canonical segment.

THE TYPICAL EXPERIMENT

The typical experiment applies if *yfg* is anywhere in the genome except on LG IR appreciably distal to *his-3*, or on LG IVR linked to *mus-51*. (These exceptions will be dealt with later.) *his-3; mus-51 a* (FGSC #9538) - (normal sequence) is transformed to hygromycin resistance with the *yfg* knockout cassette (but obviously will still require histidine, and they must be held under selection with hygromycin). This strain carries *mus-51* so that only true knockouts will be generated, as described by Inoue and his coworkers (Ninomia *et al.* 2004). Meanwhile, *mus-52; T(I → V)AR190 his-3 nic-2 cyh-1 al-1 A* (FGSC #9541) is transformed to histidine independence with *phs-3^{trunc} yfg⁺*, plated onto minimal medium plus nicotinamide (2 ug/ml), and allowed to conidiate. (The inclusion of the *mus-52* mutation can be expected to boost the chances of integration at *his-3* from the usual 50-60% to nearly 100%.) There is probably no absolute need to isolate the homokaryon, but it seems advisable to do so. If the histidine-independent transformant is isolated as a homokaryon, it is advantageous to re-form a heterokaryon by co-inoculating it with the sterile helper strain *a^{ml} ad-3B cyh-1* (FGSC #4564). This increases the fertility of the subsequent cross. The two transformant strains are then mated; the translocation strain, or its heterokaryon with the sterile helper, is used as the female parent, and after it has started to produce protoperithecia, the female lawn is overlaid with a water suspension of conidia from the male (knockout) strain, which had been grown to conidiation on histidine plus hygromycin. Ascospores are allowed to ripen and are plated onto minimal medium with hygromycin. Only the desired partial diploids should grow out to give macroscopic colonies. These will arise at about three days at 33C. (Microscopic colonies will arise in considerably higher frequency, but they stop growing and die before they become visible to the naked eye.) Spores originating from untransformed cells of *T(I → V)AR190 his-3 nic-2 cyh-1 al-1*, of course, do not grow. Euploid *T(I → V)AR190 his-3 nic-2 cyh-1 al-1* ascospores that have been transformed to histidine independence do not grow because they will still require nicotinamide. Parental strains *his-3; yfg⁺* do not grow because they will still require histidine. *his-3 yfg^Δ* does not grow because it requires histidine and is inviable even on histidine medium. (See Figure). The *his-3⁺ yfg⁺ nic-2 cyh-1 al-1 / his-3 nic-2⁺ cyh-1⁺ al-1⁺; yfg^Δ* hygromycin-resistant heterozygotes, which will spontaneously have become a heterokaryon with euploid *his-3 nic-2⁺ cyh-1⁺ al-1⁺; yfg^Δ* nuclei, are then picked to minimal medium without hygromycin. They can be maintained and their phenotypes studied in detail without having to take into account the unknown effects of hygromycin. If *yfg* is actually dispensable for vegetative growth and the partial diploids are then transferred to histidine-supplemented medium, they will rapidly lose their ectopic segment and become pure, euploid histidine auxotrophs. This will be true even if null mutants are severely stunted in growth. If *yfg* is essential, this will be evident because the partial diploid will test out as prototrophic after transfer back to minimal medium, even after a period of growth on histidine.

Although the *mus* mutations are not known to produce a visible phenotype or one that alters microarray findings, it seems desirable to have the final prototrophic partial diploid strain be wild type at both *mus-51* and *mus-52*. If this is desired, it is essential that the cross in which the partial diploid is produced be *mus-51 (::Bar)* in one parent and *mus-52 (::Bar)* in the other. A handful of prototrophs are screened for resistance to Bar (Basta, Ignite, glufosinate at 400 ug/ml) and one that is sensitive to this antimetabolite is chosen as being free of both *mus* mutations.

THE CASE IN WHICH *yfg* IS ON LINKAGE GROUP IVR

The original knockout mutation should be made in *his-3; mus-52 a* (FGSC #9539), and the hygR transformant should be crossed to *mus-51; T(I → V)AR190 his-3 nic-2 cyh-1 al-1 A* (FGSC #9540). All other manipulations are the same as above.

THE CASE IN WHICH *yfg* IS ON LINKAGE GROUP IR DISTAL TO *his-3*.

It will be clear that there is already an endogenous wild-type copy of *yfg* on LG IR, and at first glance, it would appear that there is no need to place another one at the *his-3* locus. The problem is the increased risk that the partial diploid will undergo a mitotic crossover that will introduce a *yfg⁺* copy onto the normal sequence chromosome in place of the knockout. If this happens, it will defeat the purpose of the experiment. In the "typical" experiment in which the *yfg⁺* is at the *his-3* locus, we can expect destructive mitotic crossovers to be rare because the distance between the *T(I → V)AR190* breakpoint and the *his-3* locus is very short. However, if one tries to take advantage of an endogenous wild-type copy that is substantially to the right of *his-3*, the risk of such

a crossover becomes greater as a function of distance. In general, these undesired crossovers should be recognizable simply by their faster growth, and also by plating a sample to histidine + nicotinamide and observing albino sectors.

The "safe" procedure is, in effect, to move *yfg*⁺ from its endogenous location to the *his-3* locus. This can be done in two steps. First, *mus-52*; T(I → V)AR190 *his-3 nic-2 cyh-1 al-1* A(FGSC #9541) should be transformed to histidine independence with *phs-3*^{trunc} *yfg*⁺ in the usual way. Then the endogenous *yfg*⁺ should be transformed to hygromycin resistance with the same knockout cassette as was used to make the (heterokaryotic) normal sequence knockout mutant.

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