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Genetic Modifier Screens to Identify Components of a Redox-Regulated Cell Adhesion and Migration Pathway

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Abstract

Under normal physiological conditions, cells use oxidants, particularly H₂O₂, for signal transduction during processes such as proliferation and migration. Though recent progress has been made in determining the precise role H₂O₂ plays in these processes, many gaps still remain. To further understand this, we describe the use of a dominant enhancer screen to identify novel components of a redox-regulated cell migration and adhesion pathway in *Drosophila melanogaster*. Here, we discuss our methodology and progress as well as the benefits and limitations of applying such an approach to study redox-regulated pathways. Depending on the nature of these pathways, unbiased genetic modifier screens may prove a productive way to identify novel redox-regulated signaling components.

Keywords

Drosophila; Peroxiredoxin; Hydrogen peroxide; Embryo; Migration; Adhesion

1. Introduction

Oxidants are generated within organisms as unwanted by-products of aerobic respiration, but they also function as signaling molecules in physiological processes such as proliferation, cell migration and adhesion (Finkel, 2011; Flohe, 2010; Janssen-Heininger *et al.*, 2008; Rhee *et al.*, 2000). These physiological functions are carried out by mild oxidants, such as H₂O₂, in a compartmentalized and coordinated fashion through the reversible oxidation of target proteins (Finkel, 2011; Flohe, 2010; Janssen-Heininger *et al.*, 2008; Rhee *et al.*, 2000). Though much progress has been made identifying such protein targets and the regulatory components of redox signaling pathways, significant gaps in our knowledge still remain. Most efforts have focused on proteomic methods to identify redox sensitive proteins that may be pathway components (Chouchani *et al.*, 2011; Thamsen and Jakob, 2011). Here we describe an alternative approach, in which a specialized type of genetic screen, a dominant modifier screen (Simon *et al.*, 1991; St Johnston, 2002), is used to elucidate

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redox-regulated pathway components. Dominant modifier screens have been used extensively to identify new components of phosphorylation based signaling pathways (Jorgensen and Mango, 2002; St Johnston, 2002) and could potentially yield novel and hitherto unsuspected components when applied to H₂O₂-regulated processes.

To illustrate how such an approach would work in practice, we discuss an *in vivo* modifier screen currently being conducted to identify unrecognized components of a redox-regulated cell adhesion and migration pathway. For this screen, we use a *Drosophila melanogaster* fly strain harboring a mutation in a gene encoding a H₂O₂-degrading peroxiredoxin called *jafrac1*. Mutations in this peroxiredoxin gene cause defects in the adhesion of a particular cell type, germ cells, during their migration into *Drosophila* embryos. We then introduce second-site mutations and test their ability to modify the germ cell migration phenotype. We expect the genes identified from this screen to encode proteins that impact on novel components of this pathway. Before discussing modifier screens further, we first briefly describe germ cell migration, and the mutant phenotype we are trying to better understand.

2. Mutations in a *D. melanogaster* gene Encoding a Peroxiredoxin Cause Germ Cell Adhesion and Migration Defects

Regulated adhesion between cells and their environment is critical for normal cell migration. To study this process *in vivo*, we use *Drosophila* germ cell migration as a model (Richardson and Lehmann, 2010). Germ cells initially form at the posterior pole of the embryo (Figure 12.1A), and as the embryo develops, they are carried along into the embryo proper (Figure 12.1B). Maintaining the appropriate contacts between germ cells and the rest of the embryo is critical during this stage of migration (DeGennaro *et al.*, 2011).

Through a screen, we previously identified mutations in *jafrac1* that caused germ cells to fail to be internalized into the embryo during their migration, leaving some germ cells trailing on the outside of the embryo (Figure 12.1C and D) (DeGennaro *et al.*, 2011). *jafrac1* encodes a homolog of the human cytosolic typical 2-cysteine peroxiredoxin II, which acts as a peroxidase catalyzing the reduction of H₂O₂ and alkyl hydroperoxides (Kang *et al.*, 1998; Lee *et al.*, 2009; Rodriguez *et al.*, 2000). Further experimentation showed that in embryos from *jafrac1* mutant mothers, germ cells lose adherence to the embryo during their internalization due to a disruption in the cell-cell adhesion molecule DE-cadherin (DeGennaro *et al.*, 2011; Harris and Tepass, 2010). Indeed, mutations in the gene *shotgun* (*shg*), which encodes DE-cadherin, cause similar germ cell internalization defects (DeGennaro *et al.*, 2011). As Jafrac1 is a peroxidase, we wanted to know whether increased H₂O₂ levels were responsible for the germ cell adhesion defect. Treating embryos with H₂O₂ caused a decrease in the protein levels of DE-cadherin suggesting that Jafrac1 might regulate germ cell adhesion by controlling H₂O₂ levels (DeGennaro *et al.*, 2011). Given that *jafrac1* mutants have a distinct cell migration phenotype that is caused at least in part by redox changes such as those brought about by H₂O₂, we decided to harness the power of *Drosophila* genetics and conduct an unbiased modifier screen.

3. Dominant Modifier Screens

Modifier screens have been successfully used to identify missing components of genetic pathways (Jorgensen and Mango, 2002; Rubin *et al.*, 1997; St Johnston, 2002). Such a screen normally begins with a strain of defined genetic composition and robust phenotypic defect. Second-site mutations are then introduced into this strain and assayed to determine if they enhance (worsen) or suppress (ameliorate) the starting phenotype. Second-site mutations that modify the phenotype may be in genes that fall within the same pathway that is perturbed in the starting strain. Secondary screens must then be conducted to confirm whether the modifying gene acts in the same pathway, a parallel one, or nonspecifically influences the process (Jorgensen and Mango, 2002; Karim *et al.*, 1996; Rubin *et al.*, 1997; St Johnston, 2002).

A particularly useful type of modifier screen is a sensitized screen for dominant modifiers. This screen is based on the premise that while a 50% reduction in the wild-type levels of a protein is most often sufficient for normal function (there are very few loci that have observable haploid phenotypes (Deutschbauer *et al.*, 2005; Lindsley *et al.*, 1972)), when a particular process is already partially disrupted by another mutation this 50% reduction might no longer suffice (Rubin *et al.*, 1997; St Johnston, 2002). It has been shown that for most, but not all, genes, the level of expression of a locus is proportional to the gene copy number (Malone *et al.*, 2012; Springer *et al.*, 2010). Therefore, if mutating one copy of a gene modifies a phenotype in a genetic background where a pathway of interest is already partially perturbed (sensitized), then this gene is likely to be involved, directly or otherwise, in the pathway. Rubin *et al.* carried out some of the first and most successful screens of this type, identifying several signal-transduction components downstream of the receptor tyrosine kinase, Sevenless (Rubin *et al.*, 1997; Simon *et al.*, 1991).

This approach has two important advantages. First, recessive lethal mutations can be isolated since they are screened in a heterozygous state. Second, unlike most screens which require at least two generations (F2 and F3 screens) to isolate homozygous mutants, the progeny of the mutagenized flies can be screened directly (F1 generation) because they need not be made homozygous. This increases the throughput of the screen by an order of magnitude (St Johnston, 2002). Given these advantages we decided to use this approach to search for new components of the *jafrac1* pathway.

4. Conducting a Dominant Modifier Screen to Identify Missing Components of a Redox-Regulated Germ Cell Migration Pathway

In the following sections, we discuss the methods and experiments done to identify components of the *jafrac1*-regulated germ cell migration pathway. We also discuss alternative ways of screening where applicable.

4.1 *D. melanogaster* strains

The following strains were used in this study:

- y^1 *P{SUPor-P}Jafrac1^{KG05372}* (referred to as *jafrac1^{null}*)

- *jafracI^{null}; P{hs-hid, w⁺} wg^{Sp-1}/CyO* (*P{hs-hid, w⁺} wg^{Sp-1}* is referred to as *Sp hs-hid*)
- *PBac{PB}JafracI^{f08066}* (is referred to as *jafracI^{hypo}*)
- *jafracI^{hypo}; hs-hid/CyO*
- *w¹¹¹⁸; Df(2R)BSC814/SM6a* (*Df(2R)BSC814* is referred to as *shg^{null}*)
- Deficiencies spanning 2L and 2R from the Bloomington deficiency kit (Cook *et al.*, 2012; Parks *et al.*, 2004)
- Deficiencies
- *pr¹ cad² P{neoFRT}40A/CyO* (*pr¹ cad² P{neoFRT}40A* is referred to as *cad²*)
- *y1 w67c23; P{EPgy2}PompEY06518/CyO* (*P{EPgy2}PompEY06518* is referred to as *pomp^{EY}*)
- *w*; Df(2L)vari48EP Pomp48EP vari48EP/CyO* (*Df(2L)vari48EP Pomp48EP vari48EP* is referred to as *vari^{48EP}*)
- *y¹ w*; Mi{MIC}CG9328^{MI02953}/SM6a* (*Mi{MIC}CG9328^{MI02953}* is referred to as *CG9328^{MI}*)
- *y¹; P{SUPor-P}CG9328^{KG09432}; ry⁵⁰⁶* (*P{SUPor-P}CG9328^{KG09432}* is referred to as *CG9328^{KG09}*)
- *y¹; P{SUPor-P}CG9328^{KG02184}/CyO; ry⁵⁰⁶* (*P{SUPor-P}CG9328^{KG02184}* is referred to as *CG9328^{KG02}*)
- *w¹¹¹⁸; Mi{ET1}CG33322^{MB07355}* (*Mi{ET1}CG33322^{MB07355}* is referred to as *CG33322^{MB}*)

All strains used in this study were obtained from the Bloomington *Drosophila* Stock Center at Indiana University except for the *jafracI^{hypo}* strain, which was from Exelixis at Harvard Medical School (Thibault *et al.*, 2004).

4.2 D. melanogaster culture and husbandry

Flies were maintained at 25°C and 60% humidity on media (3.6% (v/v) molasses (LabScientific Inc.), 0.532% (w/v) agar (MoorAgar Inc.), 3.6% (w/v) cornmeal (LabScientific Inc.), 1.488% (w/v) yeast (LabScientific Inc.), 0.112% (w/v) Tegosept® (Sigma), 1.12% (v/v) ethanol (Fisher Scientific), 0.38% (v/v) propionic acid (Fisher Scientific)) in polystyrene vials. Virgin females were used for all crosses conducted for this study. Since female flies do not mate within the first 8 h of adulthood, virgin females can be obtained by collecting flies within 8 h of their eclosion (emergence from their pupal case). For each cross, approximately 20 virgin females were mated with 10 males in polystyrene vials containing 20–24 ml of culture media. Flies were transferred to fresh vials every 1 or 2 days for up to 10 days.

4.3 Choosing a suitable genetic background

In order to attempt a dominant modifier screen, an appropriate genetic background must first be identified. The most important consideration before starting such a screen is that the phenotype and the methods used to measure it are robust and consistent.

If one is interested in finding genes that enhance a phenotype, then a sensitized background must be identified, where the expression of the gene or the activity of its product is substantially altered, but a weak or absent phenotype is exhibited. For example, we identified a loss-of-function allele of *jafrac1* (called *jafrac1^{hypo}*) that has greatly reduced RNA expression (Figure 12.2A), but exhibits largely normal germ cell migration when homozygous or in combination with a null allele of *jafrac1* (Figure 12.2B). To demonstrate that this background was sensitized enough to be dominantly modified we crossed a null allele of *shg* (the gene that encodes DE-cadherin), which has previously been shown to interact with *jafrac1* (DeGennaro *et al.*, 2011). The *shg* null allele substantially dominantly enhanced the germ cell migration defect, demonstrating that this background is suitable for screening (Figure 12.2B).

If a stronger loss-of-function mutation is used, one can screen for dominant suppressors, or if an intermediate is chosen potentially both enhancers and suppressors can be screened for. Whether one chooses to screen for enhancers, suppressors, or both depends on what one is hoping to find and on the phenotype being investigated. Typically, many genes can enhance a mutant phenotype, while only mutations in a few key regulators suppress (Jorgensen and Mango, 2002; Karim *et al.*, 1996).

In addition to using loss-of-function mutations, sensitized backgrounds can be generated with neomorphic alleles, RNA interference (RNAi), or gene overexpression. If dominant mutations or transgenes are used to create a sensitized background, the number of crosses needed to perform the screen might be reduced, as they need not be made homozygous.

4.4 Inducing second-site mutations

Second-site mutations and alterations can be introduced in multiple ways. The way in which one chooses to induce second-site mutations will depend largely on whether the phenotype is amenable to high-throughput analysis. In *D. melanogaster*, there are three principle ways in which this is commonly done, each with its own benefits and limitations.

The first uses X-rays or chemical mutagens, most commonly ethyl methanesulfonate (EMS), which primarily induces single-base changes (Lewis and Bacher, 1968; St Johnston, 2002). A major benefit of these types of mutagens is that mutations can be induced directly in the sensitized strain and thus the progeny (F1 generation) of the mutagenized parent can be screened. This obviates the need to individually cross each second-site mutation one-by-one into the sensitized background (see, e.g., Simon *et al.*, 1991). However, F1 screens can only be conducted for phenotypes that can be measured without killing the animal because if the animal is killed there will be no material left to map the modifier mutation. A major limitation to using X-rays or chemical mutagens is that mapping mutations induced by these agents is labor-intensive and difficult (Bokel, 2008). New genome sequence-based approaches might alleviate this somewhat (Bokel, 2008).

In the second method, second-site mutations are instead introduced into the sensitized background using engineered transposons, which when inserted into the genome frequently interfere directly with gene expression and function (Bellen *et al.*, 2011). A major advantage of this method is that once a modifier is found, the gene mutated within it can be easily and rapidly identified by PCR using unique sequences of the transposon. New insertions can be generated by mobilizing a transposable element within the sensitized background (Mathieu *et al.*, 2007). The utility of this approach may be limited, however, because transposons tend to be very inefficient mutagens and often exhibit considerable biases when integrating into the genome (Mathieu *et al.*, 2007). Though possibly more labor-intensive, strains containing transposons of known location can instead be crossed one-by-one into the sensitized background (see, e.g., Karim *et al.*, 1996). To facilitate this, the *Drosophila* Gene Disruption Project has generated a public collection of mutant strains, each containing a single unique transposon insertion of known location, which covers nearly two-thirds of all annotated protein-coding genes (Bellen *et al.*, 2011; Bellen *et al.*, 2004; Spradling *et al.*, 1999). Many of these transposons contain upstream activating sequences (UAS) of yeast which can be used to transcriptionally activate an endogenous gene next to the insertion site (Beinert *et al.*, 2004; Bellen *et al.*, 2004; Rorth, 1996; Staudt *et al.*, 2005; Thibault *et al.*, 2004). This permits gain-of-function modifier screens in which the effect of misexpressing a gene can be tested (see, e.g., Gregory *et al.*, 2007). A major advantage of this approach is that redundant genes can be identified.

Third, large deletions (or deficiencies) that remove hundreds of genes at the same time can be used to identify modifiers. As deficiencies are equivalent to null mutations in all of the genes that are deleted, this type of deficiency screen provides a rapid way to identify regions of the genome that contain potential enhancers or suppressors (St Johnston, 2002). This approach is particularly useful if it is difficult or time consuming to screen the phenotype of interest and allows for rapid survey of the entire genome. To facilitate deficiency screens the Bloomington stock center has assembled a collection of deficiencies that cover 98.4% of annotated euchromatic genes (Cook *et al.*, 2012; Parks *et al.*, 2004). Recently, microRNAs (miRNAs) have been used as an alternative means to deficiencies to identify modifiers (Szuplewski *et al.*, 2012). miRNA overexpression can cause simultaneous reduction of the expression levels of hundreds of genes concurrently. One advantage to using miRNAs to induce second-site alterations is that they may allow access to genes not covered by the deficiency kit (Szuplewski *et al.*, 2012). However, this approach is limited in that miRNAs do not target all protein coding genes with equal efficiency, and identification of biologically significant targets can be problematic (Szuplewski *et al.*, 2012).

Although chemical mutagens, transposons and deficiencies are most commonly used, second-site alterations can be induced in other ways, for example using RNAi. As with transposons, a major advantage of using RNAi is that the gene affected is already known, providing no other off-target genes are being silenced. Publically available collections of transgenic flies containing conditionally expressible RNAi constructs have been generated by the Transgenic RNAi Project at Harvard Medical School (Ni *et al.*, 2008; Ni *et al.*, 2011) and the Vienna *Drosophila* RNAi Center (which covers 88% of *Drosophila* protein-coding genes) (Dietzl *et al.*, 2007). A benefit and a limitation to using RNAi is that the target gene

expression will in most cases be reduced by more than 50% of wildtype levels. Although removing one copy of a gene rarely leads to observable phenotypes, greater reductions in gene expression may prevent scoring the phenotype being measured, for example, due to lethality. In some instances, this can be avoided using tissue specific or heat-shock inducible drivers or generating clones. Genes that are less dose-sensitive are more likely to be identified using this method.

Lastly, instead of searching for genes that modify a particular phenotype, a similar approach can be used to identify small molecule modifiers (Gonsalves *et al.*, 2011; Lawal *et al.*, 2012). Most recently, sensitized *Drosophila* strains have been used to screen for neuropsychiatric drugs (Lawal *et al.*, 2012). Such screens could potentially be used to identify small molecules that modify redox-regulated pathways.

4.5 Crossing schemes

If second-site mutations are induced directly in the sensitized background, then crossing schemes such as those used in the original enhancer screens might be appropriate (Simon *et al.*, 1991; St Johnston, 2002). In our case, however, because we used deficiencies, it was necessary to first cross the deficiencies into the sensitized *jafrac1* background as outlined in Figure 12.3.

We screened deficiencies on the second chromosome that are maintained in trans with a special type of chromosome called a balancer chromosome. Balancer chromosomes have three important features. First and most importantly, they contain multiple inversions, which prevent proper pairing with the homologous chromosome and suppress recovery of viable progeny after recombination. As a consequence, the deficiency-bearing chromosome remains stable through the crossing scheme. Second, balancers contain easily visualized dominant markers so that they can be followed in living flies one generation to the next. For example, all flies with the second chromosome balancer, CyO, have curly wings. And last, most balancer chromosomes are homozygous-lethal. These features allow us to follow the balancer, and by its absence the deficiencies, so as to ensure they are faithfully crossed into the sensitized background.

To efficiently bring a set of individual deficiencies into the *jafrac1* sensitized mutant background, we first crossed deficiency strains with a stock homozygous for the *jafrac1^{hypo}* loss-of-function mutation. This *jafrac1^{hypo}* strain also contained one copy of the balancer CyO in trans with a chromosome that bears a transgene expressing the cell death-promoting gene *hid* under control of the heat shock promoter (*hs-hid*) (Grether *et al.*, 1995; Moore *et al.*, 1998). By simply heat shocking the progeny of a cross, all flies inheriting the *hs-hid* chromosome will die. This “trick” eliminates the need for sorting correct genotypes in the next generation. This process was then repeated by crossing virgin females containing the null allele of *jafrac1^{null}* to *jafrac1^{hypo}* males containing one copy of the deficiency and one copy of CyO. Germ cell migration was subsequently assayed in embryos from *jafrac1* mutant flies containing one copy of a 2nd chromosome deficiency.

4.6 Assaying germ cell migration

To assay germ cell migration, germ cells were visualized in fixed embryos using an antibody that detects the germ cell specific protein, Vasa (McDonald and Montell, 2005; Richardson and Lehmann, 2010). Briefly, 50–100 female and male adult flies were placed in a cage (a perforated 125 ml plastic beaker). Yeast paste was dabbed on a 60-mm diameter egg collection plate (2.1% (w/v) BactoAgar (Fisher Scientific), 2.4% (w/v) granulated sugar (Domino®), 23.7% (v/v) apple juice (Hansens's Natural), 0.07% (w/v) Tegosept® (Sigma), 0.7% (v/v) ethanol (Fisher Scientific)), which was placed on the mouth of the beaker and affixed with a piece of tape to prevent flies from escaping. The caged flies were then incubated overnight at 25°C. The following morning the embryo-laden plate was removed from the cage, and the outer impermeable layer of the eggshell, the chorion, was removed by incubating the eggs in 50% Chlorox® bleach solution for 2 min. The bleach was then washed away with water, and the embryos were permeabilized and fixed in 5% formaldehyde, 50% heptane, and 45% water in a glass scintillation vial with constant agitation for 40 min. Lastly, the inner impermeable layer of the eggshell, the vitelline membrane, was removed by hand using a needle (Rothwell and Sullivan, 2007). The vitelline membrane can also be removed osmotically by methanol shock (Rothwell and Sullivan, 2007).

To visualize the germ cells, the fixed embryos were incubated in blocking buffer (phosphate-buffered saline solution containing 0.1% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin) for 1 h at 23°C. The embryos were then probed with rabbit Vasa antiserum (Moore *et al.*, 1998) diluted in blocking buffer (1:5000) and incubated overnight at 4°C. The next day the embryos were washed with blocking buffer three times (30 min incubation per wash), and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (A11034, Invitrogen) for 2 h at 23°C. Embryos were washed as before in PBS to remove the secondary antibody, mounted in VectaShield mounting medium (H-1000, Vector Labs) on glass slides and covered with a 20 × 40 mm glass slip. Germ cells were imaged using an Axiovert 200M fluorescent microscope (Zeiss) equipped with a X-cite 120 mercury arc lamp (EFXO). The average number of germ cells left outside the embryo was determined in embryos which had already gastrulated and were approximately 4–11 h after egg laying (AEL) old corresponding to stages 9–14, respectively (Campos-Ortega and Hartenstein, 1985).

4.7 Identifying enhancers

To identify enhancers of *jafrac1* we screened deficiencies covering the second chromosome. Of the deficiencies screened, two, Df(2L)ED1315 and Df(2R)BSC595, significantly enhanced the *jafrac1* phenotype (Figure 12.4). In both cases, we observed a substantial and a significant increase in the number of germ cells that failed to be internalized and were subsequently left on the outside of the embryo. If we had induced second-site mutations with a mutagen, such as EMS, then the next step would be to identify the mutated enhancer gene by standard procedures (Blumenstiel *et al.*, 2009; Bokel, 2008). However, because we used deficiencies, it was necessary to conduct a secondary screen to identify the causative genes within the enhancing deficiencies.

To do this with Df(2L)ED1315, we repeated the process described above with smaller deficiencies spanning the locus deleted by Df(2L)ED1315. From this analysis, we were able to further map the enhancer gene to an eight gene region because the smaller Df(2L)Exel7079 enhanced while Df(2L)ED1378 did not (Figure 12.5A). Since according to publically available temporal expression data (modENCODE (Graveley *et al.*, 2011)) only five of these genes are expressed in the embryo, we tested whether mutations in any of these five genes enhanced the *jafrac1* phenotype. Mutations in one of the genes in this region, *caudal*, enhanced to a similar degree as the Df(2L)ED1315, identifying *caudal* as an enhancer of *jafrac1*.

Further experiments are currently underway to identify the enhancer within Df(2R)BSC595, the other deficiency we identified that enhances the *jafrac1* phenotype. Using a number of smaller deficiencies we have been able to map the enhancer to a small region containing 13 genes, three of which are homologous to mammalian peroxiredoxin VI. Further work is underway to determine the precise enhancer gene(s).

A difficult aspect to using deficiencies as modifiers is identifying the causative modifying gene within the deficiency. Although it is possible to narrow candidates down using overlapping deficiencies, loss-of-function alleles and validated RNAi constructs, there will still be instances where tools are not available to test all putative modifier genes within a deficiency. As the collections of deficiencies and number of mutations in genes improve, however, this should become less and less of a problem. A further possible issue with using deficiencies to identify modifiers is the potential for synthetic effects. For example, if both a suppressor and an enhancer are contained within one deficiency, when assayed in a sensitized background these modifiers might not be identified because they might cancel each other out. On the flip side, if a gene is duplicated and multiple copies have redundant functions, deficiencies might be the one way to identify the gene as a modifier when all copies are included in the deficiency. For example, if the three peroxiredoxin VI homologs contained within Df(2R)BSC595 are indeed enhancing the *jafrac1* phenotype and have completely overlapping functions, it seem unlikely these genes would have been found using other methods (see also mis-expression screens above). Lastly, although there are limitations to using deficiencies in modifier screens, deficiencies can always be used as a quick means of assessing the number of potential target genes for a more extensive modifier screen using other methods (St Johnston, 2002).

5. Limitations to Dominant Modifiers Screens

Modifier screens are an unbiased way to uncover components in a pathway. However, there are several reasons why one might not expect to isolate mutants in all critical genes within a pathway using dominant modifier screens.

First, although interacting genes that have a direct role in the pathway can be identified, many mutants may be identified that modify the phenotype for less obvious or direct reasons (Simon *et al.*, 1991; St Johnston, 2002). It is therefore necessary to conduct secondary screens to understand the nature of the genetic interaction. For example, some mutants might influence the phenotype by varying the expression of the allele used to create the sensitized

background, whereas others might do so by perturbing unrelated or indirect processes (St Johnston, 2002). *caudal*, the enhancer of *jafrac1* we identified, might be an example of the latter. *caudal* encodes a homeodomain transcription factor that is involved in the formation of the hindgut in embryos (Wu and Lengyel, 1998). Since germ cells are directly apposed to the gut primordium during their internalization, it would not be surprising if perturbations in hindgut formation caused defects in germ cell internalization (Wu and Lengyel, 1998). Indeed, in *caudal* mutants, the hindgut (and presumably germ cells) fails to internalize (Wu and Lengyel, 1998). Therefore, while mutations in *caudal* dominantly enhance *jafrac1* mutants, the two genes are probably not directly acting within the same pathway.

Second, not all components of a pathway are dose-sensitive. As the premise of the screen is that a 50% reduction in the wild-type levels of a critical protein will be sufficient to alter the phenotype, only those proteins that meet this criterion will be found. For example, if a pathway component is in vast excess or only a small percentage of its activity is required for normal function regardless of the background, then a far greater than 50% reduction will be necessary to visibly alter the phenotype. In this case, only dominant negative or constitutive active alleles, which are relatively rare and impossible to uncover using deficiencies, may dominantly modify.

Last, mutations identified in modifier screens may not have phenotypes in the absence of the sensitized background (St Johnston, 2002). Unfortunately, enhancement or suppression of a phenotype of interest does not guarantee that a gene plays an indispensable role in the process being studied.

6. Concluding Remarks

The use of modifier screens to elucidate components of redox-regulated pathways has not, to our knowledge, been widely employed. Whether this strategy will prove fruitful depends largely on the nature of these pathways and types of modifier screens conducted. If redox signaling pathways are organized around central nodes or regulators in a manner akin to phosphorylation cascades, then modifier screens, particularly suppressor screens, should be a viable unbiased way of identifying novel components of these pathways. However, if these pathways are organized considerably differently, then the utility of such screens might be limited. Here we have described an enhancer screen to identify components of a redox-regulated cell adhesion and migration pathway in *Drosophila*. As proof-of-principle, we demonstrated that genetic enhancers of the phenotype could be identified. Only once further screening is concluded, will we know the true utility of this approach, and one hopes in the process novel components of a redox-regulated germ cell migration pathway will be discovered.

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Figure 12.1.

The peroxiredoxin, Jafrac1 is required for germ cell internalization during embryogenesis. (A and B) Images of representative wild type embryos (*w¹¹¹⁸*) approximately 2.5 h (A), and 4.5 h (B) AEL. (C and D) Images of representative embryos from *jafrac1*^{null} homozygous mutant mothers approximately 2.5 h (C), and 4.5 h (D) AEL. Embryos were stained for Vasa protein to mark germ cells (brown). Embryos are oriented with the posterior to the right, anterior to the left, dorsal up, and ventral down.

Figure 12.2.

A weak loss-of-function allele of *jafrac1* can be used to screen for enhancers. (A) The levels of *jafrac1* RNA are significantly reduced in embryos from *jafrac1^{hypo}* mothers when compared to a heterozygous control as measured by RT-PCR. (B) Germ cells in embryos from wildtype (*w¹¹¹⁸*), *jafrac1* transheterozygotes or *shg* heterozygotes rarely fail to internalize and as such are not often observed on the outside of the embryo. However, when one copy of *shg* is removed from embryos from *jafrac1* transheterozygous mutant mothers, the number of germ cells that fail to internalize increases. Data are the means $\pm$ standard error (S.E.). *jf1*, *jafrac1*.

Figure 12.3.

Schematic of the crosses carried out to identify dominant enhancers of the *jafrac1* germ adhesion defect. A stock homozygous for the *jafrac1^{hypo}* loss-of-function mutation that also contained one copy of *hs-hid* in trans to the balancer *CyO* was generated. Next, each deficiency line was crossed to females from this stock. Approximately five days later, when the embryos had developed into 3rd instar larvae, they were heat shocked by immersion of the vials for 2 h in a 37°C water bath. This induced the expression of the lethal gene *hid*, which caused all larvae that had inherited it to die. Any larvae that had inherited two copies of the balancer also died (because it is homozygous-lethal) leaving alive only flies with one copy of the deficiencies and one copy of the *CyO* chromosome. This process was then repeated by crossing virgin females containing the null allele of *jafrac1^{null}* to *jafrac1^{hypo}* males containing one copy of the deficiency and one copy of *CyO*. Df, deficiency.

Figure 12.4.

Deficiencies Df(2L)ED1315 and Df(2R)BSC595 significantly increase germ cell adhesion defects in *jafrac1* mutant embryos. Germ cell migration is normal in the deficiencies lines (white boxes). However, deficiencies Df(2L)ED1315 and Df(2R)BSC595 cause significant increases in the germ cell adhesion defects when *jafrac1* levels are reduced (black boxes). Data are the means $\pm$ S.E.

Figure 12.5.

Null mutations in *caudal* dominantly enhance germ cell adhesion defects in *jafrac1* mutants.

(A) A map of the deficiencies available on 2L between 20,100 and 20,900 kb.

Df(2L)Exel6046, and Df(2L)ED1378 did not modify the *jafrac1* phenotype. Df(2L)ED1315 and Df(2L)Exel7079 significantly enhanced germ cell migration defects in *jafrac1* mutants uncovering an 8 gene region within which the enhancer gene must lie. (B) The effect of various alleles (EMS and P-transposon-induced) on germ cell migration in a *jafrac1* sensitized background. One copy of a null mutation in the gene *caudal*, *cad*² (Macdonald and Struhl, 1986), significantly increases the number of germ cells that fail to internalize in a *jafrac1* hypomorphic mutant. Mutations in other genes in this region have no significant effect of germ cell migration. Data are the means $\pm$ S.E.