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MINIREVIEW

The Suppressor of Killer of prune, a unique glutathione S-transferase

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Abstract The *prune-Killer of prune* conditional dominant, lethal interaction in Drosophila was identified in the 1950s, but its mechanism remains unknown. We undertook a genetic screen for suppressors of this lethal interaction and identified a gene we named, *Suppressor of Killer of prune Su(Kpn)*. Su(Kpn) is a unique protein with four N-terminal FLYWCH zinc-finger domains, an acidic domain and a C-terminal glutathione S-transferase (GST) domain. The GST domain of Su(Kpn) is of particular interest because GSTs are usually independent of other protein domains. While GSTs are generally thought of as detoxifying enzymes, they are also associated with cellular toxicity. We predict that the GST domain of the Su(Kpn) creates a toxic product in *prune-Killer of prune* flies that is lethal. The substrate of the Su(Kpn) remains unknown.

Keywords Prune · AWD · Killer of prune · Suppressor of Killer of prune · Glutathione S-transferase · FLYWCH · Zinc binding

The *prune-Killer of prune* lethal interaction in Drosophila

In the 1950s A.H. Sturtevant made a surprising discovery. While experimenting on the Drosophila eye color mutant *prune (pn)*, he identified a conditional lethal genetic interaction between *pn* and a second gene he named *Killer of prune (Kpn)*. Sturtevant mated females homozygous for *pn* on their X-chromosome with a lab stock of males, believed to be wild-type. He expected to recover males with prune colored eyes and females heterozygous for *pn* with wild-type, brick-red eyes. However, he recovered no males from the cross. Through subsequent genetic experiments he determined the lab stock was actually homozygous for a dominant mutation in a gene he named *Kpn*. The *Kpn* stock had no visible phenotype associated with this mutation, and only when both the *pn* and *Kpn* genes are present in combination are they lethal. If the stock had been heterozygous for *Kpn*, Sturtevant would have recovered prune males, albeit at half the expected ratio, and it is unlikely he would have suspected this lethal interaction. Further, if *Kpn* was not dominant, the lethal *pn;Kpn* interaction would have gone unnoticed. Thus the *pn;Kpn* lethal interaction was found to be both conditional on both genes being mutant and dominant because a single copy of *Kpn* was capable of mediating the lethality (Sturtevant, 1956). While Sturtevant was able to map the *Kpn* gene to the third chromosome, his work was done prior to the modern molecular age, and the identity of the *Kpn* gene would remain a mystery for almost 30 years.

In the 1980s in a screen for genes that affect the development of the imaginal discs, the tissue that specifies the adult structures during Drosophila metamorphosis, the *abnormal wing discs, (awd)* gene was identified (Dearolf et al., 1988). It was subsequently discovered *Kpn* was a specific mutation, P97S, in the *awd* gene (Biggs et al., 1988; Timmons et al., 1995). *Kpn* was renamed awd^{Kpn}.

The wild-type *awd* gene encodes a nucleotide diphosphate kinase (NDPK) (Biggs et al., 1988; Xu et al., 1996). NDPK activity is required for synthesis of non-adenine containing nucleoside triphosphate from nucleoside diphosphate and ATP. The *awd* gene encodes a single transcript of a 17 KDa subunit of a 100 KDa homo-hexameric protein (Timmons and Shearn, 2000; Dearolf et al., 1988). AWD is homologous to mammalian NM-23, a metastasis suppressor gene that

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also has NDPK activity (Steeg et al., 1988, Rosengard et al., 1989). While mammals have many isoforms of NM-23 with NDPK activity (reviewed, Lacombe et al., 2000), in flies nearly all of the NDPK activity comes from AWD. *Awd^{null}* flies are lethal. Thus, while other NDPKs may exist in the fly, they do not adequately compensate for AWD when it is absent. However, flies also have much higher levels of AWD than necessary for viability. Transgenic rescue of *awd^{null}* flies requires only 5% of the wild-type AWD levels to restore viability (Xu et al., 1996).

The *awd^{Kpn}* mutation has no appreciable phenotype. The P97S change in AWD^{Kpn} (Timmons et al., 1995) does not affect the hexameric conformation of AWD^{Kpn}, nor accumulation of the protein. However, it does reduce NDPK activity of AWD^{Kpn} to about 30% of its wild-type levels (Lascu et al., 1992, Timmons et al., 1995). However, even reduced levels of AWD^{Kpn} NDPK activity are sufficient to rescue an *awd^{null}* fly. In *pn* mutants, AWD^{Kpn} accumulation and specific activity are not further reduced (Timmons et al., 1995). Therefore, the *pn;awd^{Kpn}* lethality is not due to inappropriate AWD^{Kpn} levels or further diminished NDPK activity in *pn* flies.

Because of the lethal interaction between the *pn;awd^{Kpn}* genes, it is tempting to speculate there is a physical interaction between PN and AWD^{Kpn}. However, in Drosophila, this has proven not to be true. In yeast-two hybrid assays using *pn, awd*, or *awd^{Kpn}*, no physical interaction was detected between PN:AWD or PN:AWD^{Kpn} (Timmons unpublished data). Further, using antibodies generated to PN, co-immunoprecipitation from relevant larval extracts did not demonstrate a physical interaction between these proteins (Timmons, unpublished data). In contrast, Zollo and colleagues, studying the human homologs of PN and AWD have reported a physical interaction h-PN and NM23 (Reymond et al., 1999; D'Angelo et al., 2004). Because we have been unable to identify a physical interaction between PN and AWD/AWD^{Kpn} by any biochemical means, we conclude this physical interaction does not exist in flies.

To explain the *pn;awd^{Kpn}* lethality we propose the following model. We hypothesize that loss of function in the *pn* gene results in accumulation of a molecule that, while non-toxic in *pn* mutants, can be converted into a lethal toxin in *awd^{Kpn}* mutants. While AWD^{Kpn} remains functional as an NDPK, because of the P97S mutation in its binding region, we believe it functions as a more promiscuous enzyme (Lascu et al., 1992). Thus, the accumulated molecule in *pn* mutants is a substrate for AWD^{Kpn}. The non-toxic substrate produced in *pn* mutants, once metabolized by AWD^{Kpn} is toxic and results in larval lethality of the *pn;awd^{Kpn}* flies. In hopes of understanding the *pn*;awd^{Kpn} interaction, we undertook an EMS screen to recover suppressors of the *pn;awd^{Kpn}* phenotype.

Identification of the Suppressor of Killer of prune

In Drosophila, chemical mutagens, such as EMS (ethyl methane sulfonate), are effective for inducing global genetic mutations. This approach was taken to identify suppressors the of *pn;awd^{Kpn}* lethal interaction (Provost et al., 2006). To identify suppressors we mutagenized *pn* males and crossed them to attached-X *awd^{Kpn}* homozygous females. Because the females are attached-X, the X-chromosome is inherited through the father and all males generated from this cross will be *pn*. If a suppressor mutant was generated by EMS in the *pn* male, a viable male *pn;awd^{Kpn}* fly would be recovered from the cross. However, this screen failed and no *bona fide* suppressors were recovered from 248,000 flies (Provost et al., 2006).

We reasoned the screen had failed because of the high levels of AWD^{Kpn} in the flies. Because flies have much more AWD than is required for viability, it was possible high levels of endogenous AWD*Kpn* were overwhelming the assay and the suppressor phenotype could not be recovered. Therefore, the screen was redesigned using a transgenic *awd^{Kpn}* fly, which has lower levels of AWD^{Kpn}. These flies were generated on an *awd^{null}* background so the only AWD present was transgenic AWD^{Kpn}. Although the *awd^{Kpn}* transgenic fly had lower levels of AWD^{Kpn}, the transgene was capable of both rescue of the *awd^{null}* and mediated *pn;awd^{Kpn}* lethality. On the transgenic background the screen for suppressors was successful and three suppressor mutants were obtained. These suppressors were named Su(Kpn)^1, Su(Kpn)^2, and Su(Kpn)^3. These three suppressor mutants were allelic, lethal in *trans*-heterozygous combination with each other and are loss-of-function mutations. They were also strong mutants, two of which would subsequently be identified as null alleles containing premature stop codons. As suggested by the failure of the first screen, the suppressors were able to suppress the *pn* and transgenic *awd^{Kpn}* lethality, but not *the pn* and endogenous *awd^{Kpn}* lethality (Provost et al., 2006).

Because we now had three *Su(Kpn)* alleles, we designed a non-complementation screen to identify a wider range of mutants. Using this strategy, 16 more alleles were identified. Because these candidates had been identified by complementation, it was possible we had generated mutants that were lethal with regard to viability, but not able to suppress the *pn;awd^{Kpn}* lethality. We tested these 16 alleles and found that 15 were able to suppress the *pn;awd^{Kpn}* lethality. As expected, some of the alleles were weaker suppressors than *Su(Kpn)^1*. We now had a large collection of mutant alleles with which to evaluate the function of the Su(Kpn) and it's role in *pn;awd^{Kpn}* lethality (Provost et al., 2006).

Through extensive genetic mapping techniques, the *Su(Kpn)* was narrowed to two candidate genes in the region of 3R:84C6. Ultimately, through PCR and direct

sequencing, CG10065 was identified as the *Su(Kpn)*. From the 19 mutant alleles generated in the EMS study, 17 were positively identified as containing mutations in CG10065. Of the two remaining alleles, analysis of the coding regions and intron/exon splice sites indicated they were wild-type. The reason for the mutant phenotype in these two samples is unclear at this time. The 17 mutations we positively identified include point mutations, small deletions and frame shifts, and premature stop codons. All the weak mutations with respect to viability are point mutations, while the strong mutations include point mutations, deletions and premature stop codons. In contrast to the nearly complete set of mutants found in our analysis of CG10065, sequence analysis of 8 of these alleles for a neighboring gene, CG2656, were all wild-type. The finding that a neighboring gene was wild-type underscores the specificity of the screen for mutants in the *Su(Kpn)*.

Su(Kpn), a unique glutathione S-transferase containing FLYWCH zinc-finger protein

When we identified CG10065 as the *Su(Kpn)*, it had recently been reported and named by another group as dGFZF, a glutathione S-transferase containing FLYWCH zinc finger protein (Dai et al., 2004) based on its constituent protein domains. This protein had been serendipitously discovered in a screen for proteins that interacted with Drosophila p53 (dp53). Dai et al. produced a dp53-GST fusion protein bound to glutathione-agarose beads to pull down interacting proteins from Drosophila S2 lysate. A major band appeared on SDS-PAGE analysis of these dp53 pull downs, and it was identified by mass spec analysis as an unnamed Drosophila protein, CG10065. It was subsequently shown that CG10065 has no interaction with dp53, but rather, because it contained an endogenous GST domain, bound the glutathione-agarose beads used in the assay.

In characterizing CG10065, Dai et al. demonstrated a single 4 Kb transcript encoding a 140 KDa protein. They defined three distinct domains of the protein based on homology searches: four FLYWCH domains, the glutathione S-transferase domain, and an acidic domain (Dai et al., 2004). The structure of this protein is quite unique because the FLYWCH domains are endogenously fused to a GST, a domain which generally exists independently.

The Su(Kpn) FLYWCH domains

The four FLYWCH domains occur throughout the N-terminal portion of Su(Kpn). The conserved sequence for FLYWCH is F/Y-X_n-L-X_n-F/Y-X_n-WXCX_{6-12}-CX_{17-22}HXH, where X indicates any amino acid (Buchner et al., 2000). The FLYWCH domain was originally identified in the Drosophila *modifier of mdg4 (mod[mdg4])* gene. *Mod(mdg4)* encodes a family of proteins by alternate splicing that function as chromatin insulator proteins (Krauss and Dorn, 2004). Because of their cysteine-histidine rich character, the FLYWCH consensus sequence is predicted to be a zinc-finger motif (Buchner et al., 2000). However, the FLYWCH domain is far less common than other zinc-finger domains. In Drosophila, zinc finger domains are present in 2% of genes (Rubin et al., 2000). By contrast, Drosophila FLYWCH containing proteins number only thirteen entries in public databases (www.ebi.ac.uk). FLYWCH domains occur both as single and repeated domains within proteins. In Drosophila, Su(Kpn) has the most repeats of FLYWCH, while other Drosophila proteins with the FLYWCH domain only contain a single motif (www.ebi.ac.uk).

The FLYWCH domain has not been fully characterized in mod(mgd4) proteins, but has begun to be studied in an unrelated C. elegans protein, PEB1. PEB1 has an N-terminal DNA binding domain unrelated to other known DNA binding domains, but containing a FLYWCH consensus sequence. The function of PEB1 is unknown, but PEB1 contains an NLS sequence and localizes to the nucleus where it is believed to be involved in transcriptional regulation of yet unidentified genes. Importantly, while PEB1 FLYWCH is capable of binding DNA directly, it did not demonstrate any affinity for binding metal ions (Beaster-Jones and Okkema, 2004). Thus, in the context of PEB1, the FLYWCH domain does not appear to be functioning as a zinc binding motif. The ability of the FLYWCH sequence in mod(mgd4) to bind metal ions was not tested directly, but inferred from sequence data (Krauss and Dorn, 2004). Therefore, it is unclear whether FLYWCH actually binds zinc. Potentially the role of the FLYWCH domain is dependent upon the context of the other domains in the protein, or has a distinct and undiscovered function.

The role of the FLYWCH domains in the Su(Kpn) remains to be determined. Zinc finger motifs have been shown to bind DNA, RNA and mediate protein-protein interactions (Laity et al., 2001). Assuming it is a zinc finger, it is unlikely to be interacting with DNA. In their analysis, Dai et al. generated an antibody to Su(Kpn) and demonstrated cytoplasmic localization in Drosophila S2 cells. Su(Kpn) has no NLS signal and, thus, it is unlikely, Su(Kpn) interacts with DNA. It has not been excluded that the Su(Kpn) interacts with RNA, but we favor the idea that the Su(Kpn) is involved in protein-protein interactions. The presence of an endogenous glutathione S-transferase domain supports a cytosolic, rather than nuclear, localization of Su(Kpn), as well.

The Su(Kpn) GST domain

GSTs are a large superfamily of proteins. Cytosolic GSTs are the most common and well-characterized group of GSTs, although both microsomal and mitochondrial GSTs exist as well (Hayes et al., 2004). GSTs are enzymes that catalyze the addition of reduced glutathione to endogenous or xenobiotic electrophilic compounds. Some GSTs are also capable of adding glutathione to products of oxidative stress, such as peroxides, in a process termed glutathione peroxidation (Hayes et al., 2004). A monomer of GST contains both a binding site for reduced glutathione, the G site, and a substrate-binding site, the H-site. The G-site is generally well conserved amongst GSTs, while, because of the diverse substrates they utilize, the H-site is more plastic (reviewed Oakley, 2005).

Functionally, GSTs must dimerize to be active enzymes. GSTs homo dimerize or heterodimerize within their class. In Drosophila, there are six classes of GSTs, three of which are characterized. The delta and epsilon classes are the largest with ten members each, and they are insect specific (Sawicki et al., 2003). They have roles implicated in resistance to insecticide (Ranson et al., 2001). The dGST-Sigma represents another smaller class of GST. GST-Sigma is an interesting enzyme containing a short N-terminal hydrophobic region that associates with the thin filament of troponin in the indirect flight muscles (Clayton et al., 1998). Because of the amount of cellular respiration in the indirect flight muscles, it has been suggested that a high amount of oxidative stress concentrates in this tissue. The association with GST-Sigma to the indirect flight muscles results in a localized pool of GST functioning to protect this tissue against oxidative damage (Clayton et al., 1998). Thus, although GSTs are highly redundant in the genome, it is possible for them to also be quite specialized with respect to function.

While GSTs predominantly exist as independent proteins, there are examples of GST domains associated with other protein motifs. For example, intracellular chloride ion channels (CLICs) have a domain that adopts a GST fold and contains a G site. However, no enzymatic transferase activity has been identified (reviewed, Oakley, 2005). In the era of genomics, more proteins are being identified with predicted GST domains. It is not clear the GST domain is functioning as a transferase and/or peroxidase in these proteins. Other roles for the GST domain may emerge as protein structure and function are characterized (reviewed, Oakley, 2005).

We were interested in classifying the GST domain of the Su(Kpn). Originally, the Su(Kpn) GST domain was classified as a delta class enzyme by cluster analysis (Ranson et al., 2001). Delta class GSTs are insect specific, which is true of the Su(Kpn) as well. Blast searches of genome databases demonstrate there is some homology between the GST domain of Su(Kpn) and GSTs of other species, but we have not identified a full-length protein bearing any homology to Su(Kpn), either in insect or vertebrate databases (unpublished observation). When we compared the amino acid sequence of the Su(Kpn) GST domain to the Drosophila delta and epsilon classes of enzymes, we found the Su(Kpn) was 31% similar to delta class enzymes and 29% similar to epsilon class enzymes. In contrast, delta and epsilon enzymes averaged 69% and 62% amino acid similarity within their class respectively. Compared to GST-Sigma, the Su(Kpn) was only 9% similar, suggesting it is quite different from this enzyme (unpublished data, Vector NTI software). Thus, because the Su(Kpn) does not demonstrate strong similarity to either GST delta or epsilon enzymes and because of its unusual protein structure, including the FLYWCH and acidic domains, we conclude the Su(Kpn) GST domain represents its own class of GST. Because GSTs are understood to compensate for one another when impaired (Hayes, 2005), it is unlikely the Su(Kpn) GST would have been identified in our screen if it were not special. If another GST was able to compensate for the Su(Kpn), the mutations generated by the EMS would not have suppressed the *pn;awd*^{Kpn} lethality and no *Su(Kpn)* alleles would have been recovered. This further underscores the unique nature of the Su(Kpn).

While bioinformatic approaches suggest the C-terminal domain of the Su(Kpn) is a GST, actual enzymatic activity had not yet been demonstrated. The GST domain of Su(Kpn) has previously been shown to bind reduced glutathione (Dai et al., 2004). However, CLIC proteins also bind reduced glutathione, but do not have transferase activity. To test the enzymatic transferase activity of Su(Kpn) we used the 2,4-dichlorodinitrobenzene (CDNB) assay. CDNB serves as a near universal substrate for GST activity (Hayes et al., 2004). We demonstrated in a CDNB assay the full-length Su(Kpn) does not have GST activity. However, when the GST domain alone is expressed, it has GST activity in a CDNB assay (unpublished data). We conclude the N-terminal portion of the protein is inhibiting the GST activity *in vitro*. We have not evaluated other proteins that may interact with the Su(Kpn) *in vivo*. If Su(Kpn) participates in protein:protein interactions, these binding partners may function to modulate GST activity. Additionally, post-translational modifications may also play a role in regulating Su(Kpn) GST activity. Because the FLYWCH domain may be a zinc finger (Krauss and Dorn, 2004), it is possible zinc ion binding regulates Su(Kpn) as well. Additionally, GST activity may be inhibited because the N-terminal domains are preventing GST dimerization required for its activity. This situation would be eliminated when we expressed the GST domain alone *in vitro*. Future studies will be needed to evaluate these possibilities.

Generally, GSTs are thought of as detoxifying enzymes that metabolize electrophiles and reactive oxygen species

generated by aerobic respiration. Addition of glutathione (GSH) to electrophilic and hydrophobic substrates, either native or xenobiotic, by GST typically results in increased solubility of GSH conjugated molecules and their excretion from the cell (Hayes et al., 2004). However, examples exist in which addition of GSH to substrates renders them cytotoxic. This cytotoxicity results from the formation of unstable thiols that are converted to alkylating agents or stable, but toxic metabolites (Hayes et al., 2004; Pickett and Lu, 1989).

The Su(Kpn) acidic domain

The remaining domain defined by Dai et al. is an acidic domain located between the last FLYWCH domain and the GST domain. This 49 amino acid domain has 46% acidic residues. The role of this domain is unknown. In other systems, acidic domains have specific roles in protein-protein interactions. The amino acids aspartate and glutamate are known to participate in stable intermolecular bridges, for example, between integrins and ECM proteins (Lee et al., 1995; Parker et al., 2005). We did not recover any mutant alleles specific to the acidic domain in our analysis of the Su(Kpn). This may indicate that the acidic domain is dispensable for Su(Kpn) function.

The *Su(Kpn)* mutants

We found point mutations, deletions and premature stop codons in our Su(Kpn) mutants. All the mutations we identified were within the coding region of the gene. This suggests protein function, rather than transcription, is important in mediating the *pn*; awd^{Kpn} lethality. The N-terminal point mutants were within the first 169 amino acids, which includes, the first full FLYWCH domain and beginning of the second. Mutants that were recovered for the remaining FLYWCH domains were deletions that caused a frame-shift and premature stop codons. Thus, a hot-spot may exist within the N-terminal of the Su(Kpn) that is essential for its function. Of particular interest was residue R169. This residue was mutated in three of our alleles, $Su(Kpn)^1$(R169C), AP434 (R169H) (Provost et al., 2006) and AP320, (P4S and R169H) (unpublished data). All three of these alleles are strong alleles, both in terms of survival and suppression of the *pn;*awd^{Kpn} lethality. We suggest that R169 is a critical residue for Su(Kpn) function and future studies will be aimed at understanding its role in both normal Su(Kpn) function and suppression of *pn;*awd^{Kpn} lethality.

Point mutations were also recovered in the GST domain. BX123 (Y882N) (Provost et al., 2006) is a mutation in a phosphorylatable residue. Whether this tyrosine is phosphorylated and how this affects Su(Kpn) function is unknown. Structure modeling comparing the GST domain of Su(Kpn) to other known GST structures indicates a second point mutant, CL1027 (P821L) (Provost et al., 2006) is within the predicted GSH binding domain. CL1027 is a weak allele, therefore, we suggest it may disrupt, but not eliminate, GSH binding. Further analysis will be required to understand the role of the particular point mutants in protein function.

The role of the Su(Kpn) in normal development

The role of the Su(Kpn) in normal development is of interest. We have shown the Su(Kpn) plays a role in the development of imaginal discs. The original suppressor mutants, $Su(Kpn)^1$, $Su(Kpn)^2$, and $Su(Kpn)^3$ do not have imaginal discs. We have not evaluated the remaining mutants for the presence of discs, but suspect because the strong alleles are larval lethal, they also will have defects in their imaginal discs. Amongst our weaker alleles we have recovered several temperature sensitive mutants. At a permissive temperature, trans-heterozygotes between $Su(Kpn)^1$ (R169C) or $Su(Kpn)^2$ (W47stop) and CU338 (L27F) are viable as adults. When shifted to a higher, non-permissive temperature these trans-heterozygotes are no longer viable. In both cases we found the larvae must complete their third larval instar at the permissive temperature to be adult viable at the non-permissive temperature. The reciprocal is true as well. Larvae raised at the non-permissive temperature are only recovered as adult trans-heterozygotes at the permissive temperature if they are shifted during larval development. We attribute these results to a defect in imaginal disc development during morphogenesis (unpublished data).

Other evidence for a role of Su(Kpn) in disc development comes from *in vivo* culturing experiments. Because $Su(Kpn)^1$, $Su(Kpn)^2$ and $Su(Kpn)^3$ do not have imaginal discs, to assay whether this phenomenon is disc autonomous, the anterior half of homozygous mutant larvae were transplanted into the abdomens of wild-type female hosts. After an appropriate incubation period the hosts were sacrificed and the imaginal discs of the cultured embryo identified. The Su(Kpn) homozygous mutants were unable to develop imaginal discs in the wild-type host, while their heterozygous sibling embryos did form discs (Provost et al., 2006). This suggests the Su(Kpn) is essential for the disc and this requirement is disc autonomous. That is, surrounding wild-type host tissue is not capable of providing factors necessary to allow the disc to overcome the homozygous mutation for Su(Kpn) and survive in the host. Thus, we believe Su(Kpn) plays a role in normal imaginal disc development and is involved in a cell signaling.

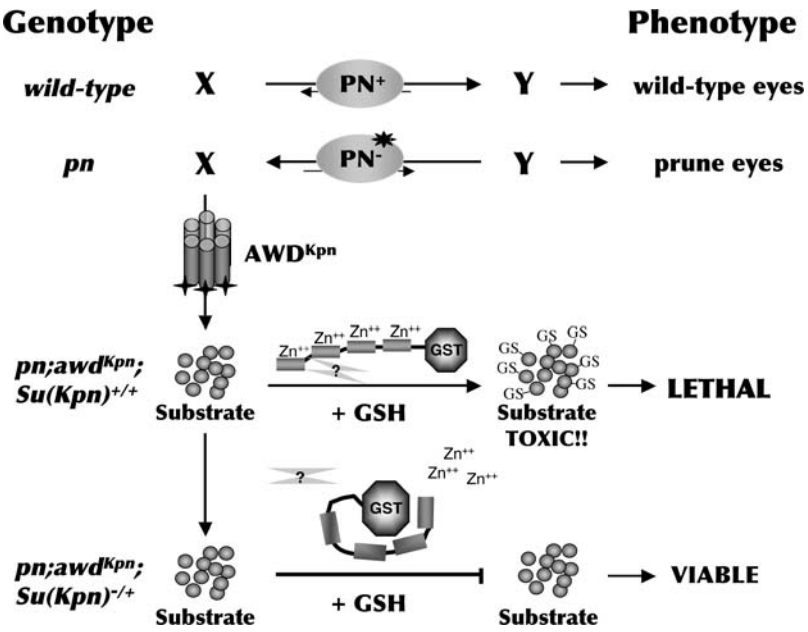

Fig. 1 In wild-type flies, the PN enzyme converts substrate X to product Y resulting in low levels of X and high levels of Y. This results in a wild-type brick red eye phenotype. In *pn* mutants, the PN^{-} enzyme is unable to convert substrate X to product Y and X accumulates at high levels compared to Y. The resulting flies have brownish-purple prune eyes. In flies mutant for *pn* and awd^{Kpn} and wild-type for *Su(Kpn)*, X becomes a substrate for AWD^{Kpn}. This substrate is further modified by the conjugation of reduced glutathione by Su(Kpn). The regulation of the Su(Kpn) by post-translational modifications, its binding of metal ions and/or protein:protein interactions are suspected. The conjugation of reduced glutathione, GSH, to the substrate is toxic and lethal to the flies. In flies mutant for *pn* and awd^{Kpn} and mutant for *Su(Kpn)*, the accumulated substrate is not conjugated to GSH because mutations in the N-terminal or GST domain prevent this activity. The unconjugated substrate is no longer toxic and the flies are viable. Phenotypically, these flies have prune colored eyes

The role of the Su(Kpn) in *pn;awd^{Kpn}* lethality

As previously noted, GSTs are generally thought of as protective against xenobiotic and toxic native compounds by conjugating target substrates with reduced glutathione increasing their cellular export. Conversely, GSTs can create toxic compounds when addition of reduced glutathione to a substrate creates a stable, toxic molecule (Pickett and Lu, 1989; Hayes et al., 2004). We believe the role of the Su(Kpn) in mediating the *pn;awd^{Kpn}* lethality is the latter.

In our model (Provost et al., 2006), the presence of mutant *pn* and awd^{Kpn} in the same fly create a substrate that is not present in either *pn* or awd^{Kpn} mutants individually. It is this compound that is the substrate for the Su(Kpn) GST activity. The Su(Kpn) adds glutathione to the substrate and this glutathione conjugated compound accumulates to lethal levels. This model depends upon the Su(Kpn) having GST activity and that the addition of glutathione to the *pn;awd^{Kpn}* generated substrate is toxic. Our recent *in vitro* data suggest that the GST domain of Su(Kpn) is capable of glutathione transferase activity, but in the presence of the N-terminal FLYWCH and acidic domains, this activity is inhibited. Therefore, we hypothesize an adaptor protein, post-translational modification event or requirement for biochemical cofactors, such as metal ions, regulate the Su(Kpn) with respect to its GST activity. Thus, we have amended our model to include the possibility of other regulatory events that play an important role in the *in vivo* function of the Su(Kpn) (Fig. 1).

As previously mentioned, genomic bioinformatic data suggest the GST protein motif is not exclusively limited to classical GST molecules. If there is an uncoupling between GST structure and function, this suggests the GST motif has been selected not for its enzymatic activity, but another function, perhaps dimerization. The activity of the GST motif in CDNB transferase assays would be a remnant of its evolutionary heritage, rather than its function in multidomain proteins (reviewed, Oakley, 2005). Such a scenario would explain why no GST activity is observed for the full-length protein *in vitro*, but when the GST domain is expressed independently it is active. *In vivo* analysis will be necessary to determine the role of the Su(Kpn) GST domain.

If future studies establish the *in vivo* function of the Su(Kpn) is not associated with GST activity, this would significantly change our model of Su(Kpn) mediated lethality in the *pn;awd^{Kpn}* interaction. Because we recovered mutants in the GST domain, we would hypothesize, regardless of GST activity, the GST has an important function in the Su(Kpn). A key matter which remains unresolved is the identity of the molecule that accumulates in *pn;awd^{Kpn}* larvae which serves as a substrate for the Su(Kpn). Identification of this substrate would be profoundly important in understanding the mechanism of the *pn;awd^{Kpn}* lethal interaction. It is hoped

future studies of Su(Kpn) will provide an answer to this question.

Su(Kpn) was tested for GST activity in a 4-HNE (4-hydroxynonenal) assay. Compared to the previously tested universal substrate CDNB, 4-HNE acts as a substrate for only a few select GSTs. GSTs that utilize 4-HNE have been implicated in both oxidative stress and cellular signaling. Neither the full-length Su(Kpn) nor its GST domain expressed alone were active in a 4-HNE assay *in vitro* (Shearn and Zimniak labs, unpublished data).

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