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DEVELOPMENT AND DISEASE

Drosophila necrotic mutations mirror disease-associated variants of human serpins

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SUMMARY

Polymerization of members of the serpin superfamily underlies diseases as diverse as cirrhosis, angioedema, thrombosis and dementia. The *Drosophila* serpin Necrotic controls the innate immune response and is homologous to human α_1-antitrypsin. We show that *necrotic* mutations that are identical to the Z-deficiency variant of α_1-antitrypsin form urea-stable polymers in vivo. These *necrotic* mutations are temperature sensitive, which is in keeping with the temperature-dependent polymerization of serpins in vitro and the role of childhood fevers in exacerbating liver disease in Z α-antitrypsin deficiency. In addition, we identify two *nec* mutations homologous to an antithrombin point mutation that is responsible for neonatal thrombosis. Transgenic flies carrying an S>F amino-acid substitution equivalent to that found in Siiyama-variant antitrypsin (*nec*$^{S>F.UAS}$) fail to complement *nec*-null mutations and demonstrate a dominant temperature-dependent inactivation of the wild-type *nec* allele. Taken together, these data establish *Drosophila* as a powerful system to study serpin polymerization in vivo.

Key words: Necrotic, Serpin, Polymer, Z-variant α_1-antitrypsin, Conformational disease, *Drosophila*

INTRODUCTION

The serpin (serine proteinase inhibitor) superfamily includes antithrombin, α_1-antitrypsin and PAI-1, which control the coagulation, inflammation and fibrinolytic pathways, respectively. Serpins have a unique method of inhibition that involves a conformational change of the protein (Huntington et al., 2000). This transition is essential for the mechanism of serpin inhibition but it renders serpins susceptible to mutations that affect conformational stability (Carrell and Lomas, 2002) and lead to polymer formation (Lomas et al., 1992; Dafforn et al., 1999; Huntington et al., 1999; Sivasothy et al., 2000). The most well characterized of these conditions is deficiency of the human serpin α_1-antitrypsin.

α_1-Antitrypsin is synthesized in the liver and is the most abundant circulating proteinase inhibitor in humans. Most individuals carry the normal M allele but 1 in 25 northern Europeans are heterozygous for the Z variant (Blanco et al., 2001). The Z mutation (Glu^{342}→Lys) favors the spontaneous formation of polymers between the reactive center loop of one molecule and β-sheet A of another (Lomas et al., 1992; Dafforn et al., 1999; Sivasothy et al., 2000). These polymers are retained within hepatocytes to form inclusion bodies that are associated with neonatal hepatitis (Sveger, 1976), cirrhosis (Sveger, 1988) and hepatocellular carcinoma (Eriksson et al., 1986). In addition, the lack of circulating α_1-antitrypsin predisposes to early-onset emphysema, by failing to protect the lungs against proteolytic attack (Eriksson, 1965). Thus, α_1-antitrypsin deficiency results in two clear phenotypes. The toxic effect of polymer accumulation causes cirrhosis while the consequent lack of inhibitory activity results in emphysema.

Polymerization also underlies deficiency of antithrombin, C1-inhibitor and α_1-antichymotrypsin, which are associated with thrombosis (Bruce et al., 1994), angio-edema (Aulak et al., 1993; Eldering et al., 1995) and emphysema (Gooptu et al., 2000), respectively, and the accumulation of mutant neuroserpin protein within the brain, which causes an inclusion body dementia (Davis et al., 1999; Belorgey et al., 2002).

Fig. 1. Dorsal thoracic region of *nec* null mutant fly (nec^{2}/nec^{19}). Within a few hours of eclosion, adult flies develop black cuticular patches that are associated with cellular necrosis of the underlying epithelial cells (Green et al., 2000). Necrotic patches are randomly distributed over most of the body surface, but occur preferentially at the proximal leg joints (arrows). Distal leg segments have been dissected away.

The *necrotic* (*nec*) gene in *Drosophila melanogaster* is one of a cluster of serpin transcripts at 43A on the second chromosome (Green et al., 2000). Loss-of-function *nec* mutants hatch as weak adults that develop black melanized spots on the body and leg joints (Fig. 1) and die within a few days of eclosion. In addition to the visible phenotype, the Toll-mediated immune response to fungal infections is constitutively activated in *nec* mutants (Levashina et al., 1999). The Nec protein consists of a serpin core, which has sequence homology with α_1-antitrypsin, and a polyglutamine-rich N-terminal extension of 79 amino acids that is not found in other serpins (Green et al., 2000). Nec protein is synthesized in the fat body, the insect equivalent of the liver, and secreted into the hemolymph.

We characterize *necrotic* mutations with amino acid substitutions equivalent to that found in the human Z-variant α-antitrypsin. These mutants survive longer than *necrotic* null flies and retain inactive polymers of Nec. Moreover, the mutants are sensitive to increases in temperature: this is in keeping with the temperature-dependent formation of serpin polymers in vitro and the role of childhood fevers in exacerbating liver disease in Z-variant α-antitrypsin carriers (Lomas et al., 1992). To test the similarities in the functional constraints between the Nec and α_1-antitrypsin serpins further, we engineered the $Ser^{131}\rightarrow$Phe transition in the Nec protein ($Nec^{S>F}$) to be equivalent to that responsible for the most extreme polymerogenic α_1-antitrypsin variant, α_1-antitrypsin-Siiyama [$Ser^{53}\rightarrow$Phe (Lomas et al., 1993)]. We found that overexpression of $Nec^{S>F}$ failed to complement the genetic lesion in *nec*-null mutant flies and, furthermore, produced a temperature-dependent dominant phenotype in a nec^{+} genetic background. The striking parallels between the behavior of Nec and human serpins establishes *Drosophila* as a powerful in vivo system with which to both study polymerization and test therapeutic agents for human disease.

MATERIALS AND METHODS

Fly strains

The *nec* alleles used in this study were generated by EMS and X-rays. Transgenic *nec* mutations were constructed using a *nec* cDNA (Green et al., 2000) and PCR-based site-directed mutagenesis. Both wild-type (nec^{UAS}) and mutant ($nec^{1.UAS}$, $nec^{9.UAS}$ and $nec^{S>F.UAS}$) strains were expressed using the *Gal4/UAS* system (Brand and Perrimon, 1993). The *Gal4-Act5c* driver strain gives constitutive high-level expression of transgenic *UAS* strains, whereas *Gal4-Yp* drives high levels of *UAS* gene expression that are restricted to the fat body of adult female flies [Yolk protein driver *P{Yp-Gal4.G}* (Georgel et al., 2001)].

DNA sequencing

PCR fragments containing the whole of the *nec* transcript were isolated from the genomic DNA of each mutant using the primers 3′-TGTGATCGACACGGAATCCCA-5′ and 3′-CTCTTCCAATCGC-CGTATAGC-5′. Both strands of each fragment were sequenced using oligonucleotide primers (Sigma-Genosys) and the ABI Bigdye Terminator Cycle Sequencing Kit (Perkin Elmer).

Survival times

Flies (n=50) of each mutant or wild-type (Oregon-R) strain, heterozygous with the null mutation nec^{2}, were scored for survival at 18°C, 25°C and 29°C. Survival of 50 transgenic flies overexpressing mutant or wild-type Nec protein, in a nec^{+} genetic background, were scored at 18°C, 25°C and 29°C. The log rank test was used for statistical analysis of results.

Protein analysis

Protein was extracted by homogenizing whole flies in 100 mM Tris (pH 8), 5 mM EDTA, 50 mM NaCl and treating with general use protease and phosphatase inhibitor cocktail (Sigma). Following centrifugation, the supernatant was removed and loaded on a polyacrylamide gel. Protein was detected by western blotting using a rabbit anti-nec antibody and a goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody. Protein bands were detected by chemiluminescence. M and Z α_1-antitrypsins were purified from the plasma of homozygotes as described previously (Lomas et al., 1993) and detected directly by Coomassie staining of gels.

Native and transverse-urea-gradient polyacrylamide gel electrophoresis

Native and transverse-urea-gradient (TUG) PAGE were carried out using 10% and 8% (w/v) polyacrylamide gels, respectively (Lomas et al., 1995a).

RESULTS

nec mutations mirror human disease-associated serpin variants

Fourteen mutant strains carrying point mutations in *nec* were isolated in Cambridge and Strasbourg (by D.G. and J.-M.R.), in addition to mutants isolated by P. Heitzler (Heitzler et al., 1993). Sequencing of these *nec* alleles identified a range of mutations, including both stop codons and single amino acid substitutions (Table 1). Of particular interest was a mutation that occurred on two occasions (nec^{9} and nec^{20}) within our collection of alleles. This mutation ($Glu^{421}\rightarrow$Lys) is the same as that found in the Z allele of human α_1-antitrypsin ($Glu^{342}\rightarrow$Lys), as shown on the molecular model in Fig. 2. The $Glu^{342}\rightarrow$Lys substitution in Z α_1-antitrypsin is at the hinge region of the serpin (Stein and Carrell, 1995), at the junction of β-sheet A and the base of the reactive center loop (Fig. 2A). This mutation perturbs the structure of the serpin molecule such that the reactive center loop of one molecule inserts into β-sheet A of another to initiate polymerization (Fig. 2B) (Lomas et al., 1992). It is these Z-α_1-antitrypsin polymers that

Table 1. Molecular lesions in *nec* alleles

Allele	Amino acid change	Position in secondary structure	Human variant pathology
nec^{1}	ΔIle^{118} and Ile^{119}	α-helix A	No equivalent
nec^{7}	Gly^{466}→Ser	β-strand 5B	Thrombosis
nec^{9}	Glu^{421}→Lys	β-strand 5A	Liver disease, emphysema
nec^{10}	Met^{327}→Lys	β-strand 3B	No equivalent
nec^{16}	Gly^{423}→Ser	P15 of reactive site loop	No equivalent
nec^{20}	Glu^{421}→Lys	β-strand 5A	Liver disease, emphysema
nec^{22}	Gly^{466}→Ser	β-strand 5B	Thrombosis
nec^{2}	Glu^{37}→Stop	N-terminal extension	No equivalent
nec^{19}	Glu^{32}→Stop	N-terminal extension	No equivalent

accumulate in hepatocytes thereby causing 'neomorphic function' liver disease and 'hypomorphic function' lung disease.

Similarly, Nec^{E421K} protein gives a fly mutant phenotype characteristic of lack of Nec activity in the hemolymph. Remarkably, a second amino acid substitution, Gly^{466}→Ser (*Nec*^{G466S}), also occurred twice within the 14 *nec* point mutations (*nec*^{7} and *nec*^{22}). This lesion is analogous to a mutation of antithrombin that is associated with polymer formation, loss of inhibitory function and thrombosis [equivalent to the Gly^{424}→Arg substitution in antithrombin (Jochmans et al., 1994)].

The *nec*^{1} mutation results in the deletion of two isoleucine residues at positions 118 and 119 in the α-helix A of the serpin. The internal face of the α-helix A forms part of the protein core, with the residues from this face interdigitating with those at the back of the β-sheet A. Any perturbation of these residues will lead to a destabilization of the β-sheet A and is likely to lead to polymerization. A number of clinically relevant mutations have been found in the α-helix A (Stein and Carrell, 1995), and it is likely that the deletion of the two residues observed in the *nec*^{1} mutation would represent an extreme case of α-helix A disruption.

nec mutants form urea-stable serpin polymers

The properties of *nec*^{9} (Glu^{421}→Lys) and *nec*^{1} [an extreme hypomorphic mutation (Δ Ile^{118}, Ile^{119}) with slight residual Nec activity] were analyzed alongside the wild-type protein using non-denaturing and transverse-urea-gradient (TUG) gels. A progressive reduction in the native protein was seen from wild type through *nec*^{9} to *nec*^{1} (Fig. 3A). A corresponding progressive increase in the higher molecular mass species was seen in these mutants. These higher molecular mass bands were resistant to unfolding in 8 M urea (Fig. 3B), which is characteristic of serpin polymers. The resolution of only a single higher molecular mass band in samples from *nec*^{9} and *nec*^{1} flies suggests that polymer formation is halted at a low-order oligomer stage. Similar behavior is shown by human polymeric variants, such as the Mmalton variant of α_1-antitrypsin (Lomas et al., 1995b) (Δ Phe^{52}) and the Rouen VI variant (Asn^{187}→Asp) of antithrombin (Bruce et al., 1994), as well as the trimeric form of Hsp47 (Dafforn et al., 2001). Note, however, that the clear Z-α_1-antitrypsin polymer ladder represents Coomassie-stained purified protein, whereas the Nec data are from immunoblotted TUG gels of whole-fly protein extracts. Under these conditions, the failure to detect high-order Nec polymer ladders may reflect the increased background staining or post-translational modification (e.g. glycosylation) of Nec. Despite these caveats, the critical feature that the Nec^{9}, Nec^{1} and Z α_1-antitrypsin TUG gels have in common is the lack of a S→R serpin transition, which is clearly shown by the sigmoidal form in the wild-type Nec and antitrypsin TUG gels.

The rate of polymerization of α_1-antitrypsin variants is temperature dependent in vitro (Lomas et al., 1992; Lomas et al., 1993). It has thus been suggested that childhood fevers might exacerbate liver disease in individuals with Z α_1-antitrypsin (Lomas et al., 1992), although the secretion of Z α_1-antitrypsin from cultured cell lines does not support this hypothesis (Burrows et al., 2000). The temperature dependence of serpin polymerization was assessed in vivo by the survival of *nec* mutant fly strains. Null mutations of *nec* cause adult flies to die rapidly after eclosion (Green et al., 2000) but hypomorphic *nec* mutants survive for several days to a week at 25°C (Fig. 4B). We tested survival of each *nec* allele, heterozygous with a deletion of the *nec* chromosomal region, at a range of defined temperatures. When the culture temperature was reduced from 25°C to 18°C, the relative survival rate of different

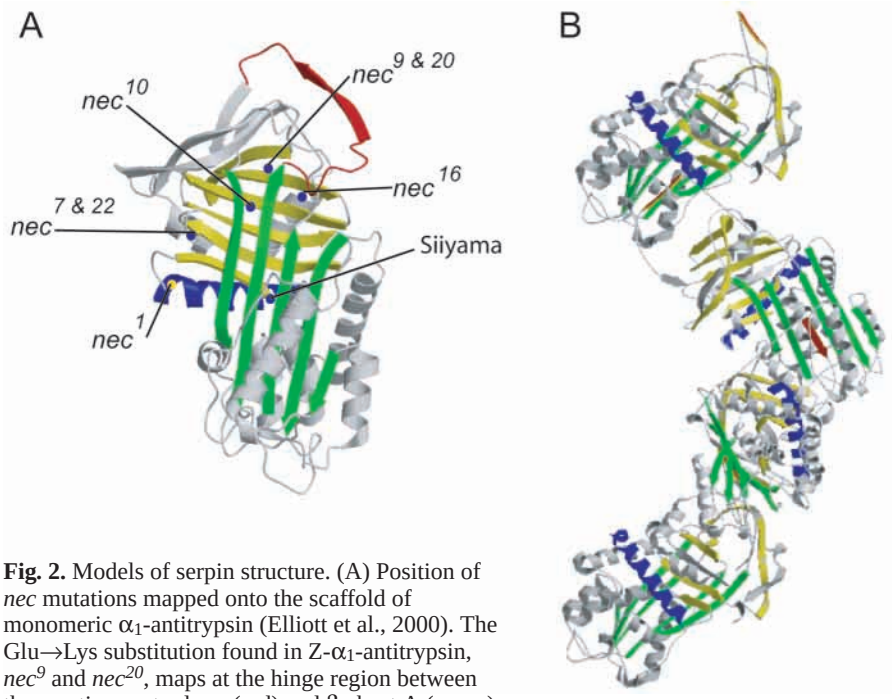

Fig. 2. Models of serpin structure. (A) Position of *nec* mutations mapped onto the scaffold of monomeric α_1-antitrypsin (Elliott et al., 2000). The Glu→Lys substitution found in Z-α_1-antitrypsin, *nec*^{9} and *nec*^{20}, maps at the hinge region between the reactive center loop (red) and β-sheet A (green). β-sheet B is colored yellow and α-helix A is blue. Nec$^{S>F}$ carries the Ser^{131}→Phe substitution homologous to that of α_1-antitrypsin-Siiyama, Ser^{53}→Phe. (B) Loop-sheet polymer of Z-α_1-antitrypsin and Nec^{9}.

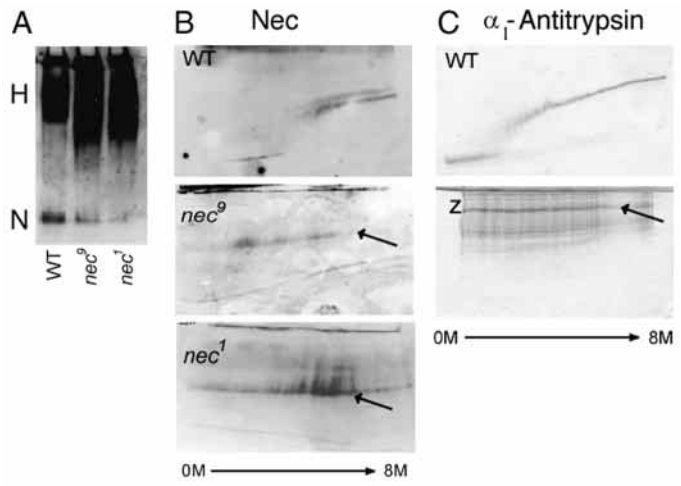

Fig. 3. Conformational stability of mutant serpins. (A) 7.5-15.0% (w/v) non-denaturing PAGE of cell extracts from flies carrying the E421K (*nec*^{9}) mutation show reduced levels of native-like protein, N, and an increase in levels of higher molecular mass species, H. The more extreme phenotype of *nec*^{1} is associated with a further reduction in native protein. (B) 7.5% (w/v) TUG PAGE demonstrates that the higher molecular mass species (arrows) observed in both *nec*^{9} and *nec*^{1} are insensitive to denaturation in 8M urea. This behavior is characteristic of loop-sheet polymers such as those observed in the livers of individuals with Z-α_1-antitrypsin deficiency. These stabilized polymers are not observed in the wild-type flies. (C) The profile of monomeric α_1-antitrypsin and polymerized Z-α_1-antitrypsin are shown for comparison, using purified proteins stained with Coomassie. The left of each gel represents 0 M urea and the right 8 M urea.

alleles was not affected. An increase to 29°C, however, had a significant effect: *nec*^{9} flies had a shorter survival time relative to control flies than that seen at 25°C (P=0.0068). Moreover, *nec*^{1} flies had a shorter survival time than *nec*^{9} flies (Fig. 4A-C). The more severe phenotype of the *nec*^{1} flies correlates with the thicker polymer band and the reduced monomer band seen in these mutants. These results provide clear evidence that higher temperatures reduce survival times, and that the probable mechanism is by increasing the rate of polymer formation.

To test directly the toxicity of mutant Nec proteins, we overexpressed putative polymeric mutant proteins in a *nec*$^{+}$ genetic background (Fig. 4D-G). In addition to *nec*$^{9.UAS}$ and *nec*$^{1.UAS}$, we recovered a *nec*$^{S>F.UAS}$ strain (carrying the amino acid substitution homologous to that found in the extreme polymeric Siiyama variant of α_1-antitrypsin, Ser^{53}→Phe). Consistent with our previous results, Nec^{1} overexpression strongly reduced viability, whereas Nec^{9} overexpression resulted in a moderate reduction. Overexpressing wild-type Nec protein also weakly reduced viability compared with +/*nec*^{2} flies (Fig. 4D-F). An unexpected result, however, is

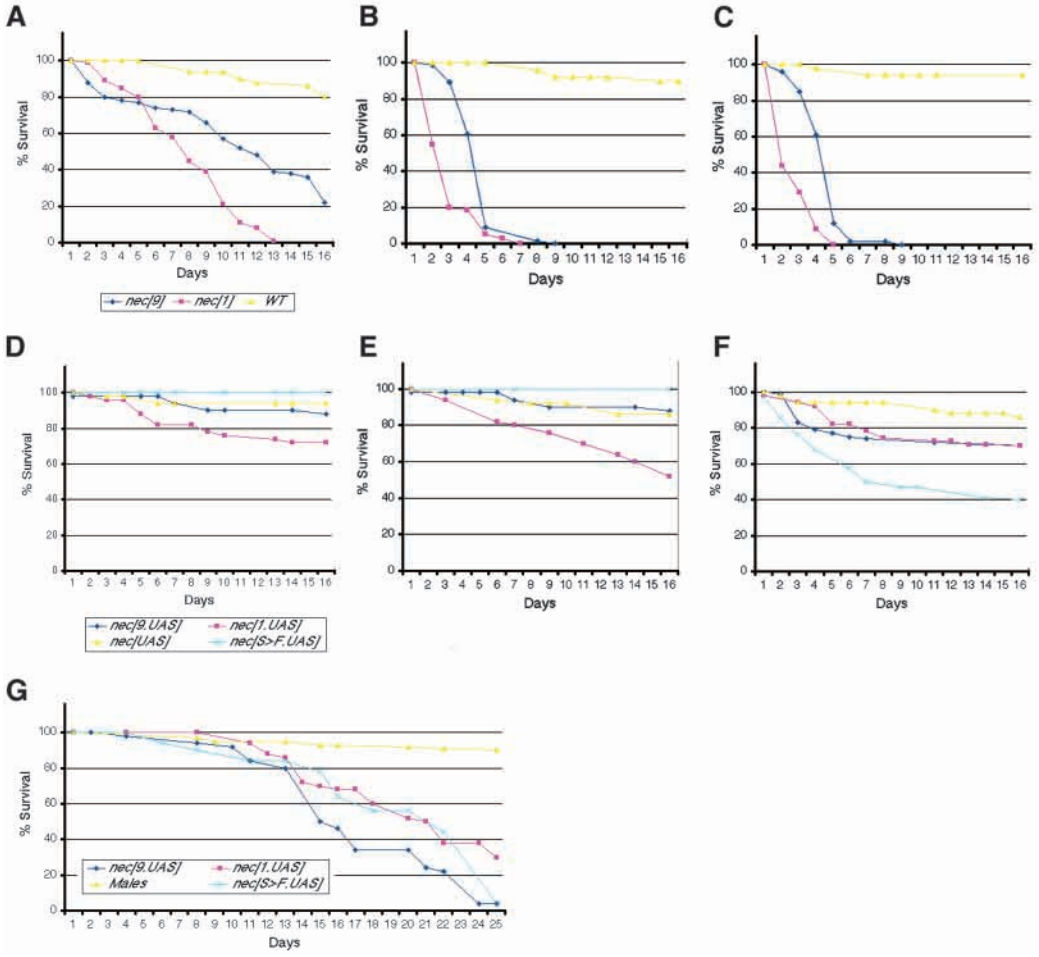

Fig. 4. (A-C) Viability of *nec*^{9}, *nec*^{1} and wild-type (WT) flies at 18°C (A), 25°C (B) and 29°C (C). The survival of *nec*^{9} flies, homologous to Z-variant α_1-antitrypsin, is greatly reduced at 25°C and 29°C compared with that of wild-type flies. *nec*^{1} flies have shorter survival times than *nec*^{9}, but survive about twice as long as complete null alleles (*nec*^{2}/*nec*^{19}, data not shown). (D-F) Viability of transgenic flies overexpressing wild-type or mutant Nec in a *nec*$^{+}$ genetic background (*Gal4-Act5c/+; nec*^{UAS}/+) at 18°C (D), 25°C (E) and 29°C (F). (G) Viability of transgenic females overexpressing mutant Nec at 29ºC in the fat body (*Gal4-Yp/+; nec*^{UAS}/+). Sibling males for these genotypes, in which the Yp promotor is inactive, were all healthy and the combined data for all three strains has been plotted as a control.

that, at all three culture temperatures, *Gal4-Act5c/+; nec$^{1.UAS}$/+* flies show a moderate *nec* phenotype, despite carrying a wild-type *nec* allele. The Nec$^{S>F}$ protein appears to be inactive at 18°C and 25°C, with the viability of *nec$^{S>F.UAS}$* flies being comparable with *+/nec^{2}* (compare Fig. 4D,E with 4A,B) and greater than *nec^{UAS}* controls. The Nec$^{S>F}$ protein fails to complement lack of wild-type Nec in a *nec*-null background and *nec^{2}/nec^{19}; nec$^{S>F.UAS}$/Gal4-Act5c* flies retain an extreme Nec phenotype. However, Nec$^{S>F}$ behaves like Nec^{1} at 29°C in a *nec^{+}* background, and *Gal4-Act5c/+; nec$^{S>F.UAS}$/+* flies develop a moderate Nec phenotype that is associated with strongly reduced viability. The major site of toxicity of the mutant Nec proteins is probably in the fat body, the normal site of Nec synthesis. The female-specific fat body driver *Gal4-Yp* reduces viability of transgenic *nec$^{9.UAS}$*, *nec$^{1.UAS}$* and *nec$^{S>F.UAS}$* females compared with sibling males (Fig. 4G).

DISCUSSION

Taken together, our findings identify a class of mutants in the *Drosophila* Nec serpin that undergo temperature-dependent polymerization and demonstrate, for the first time, an associated temperature-dependent mortality. In human serpins, clinically important mutations cluster in regions critical for the conformational changes essential to the normal function of the protein. We have found that *nec* mutations in *Drosophila* map to these same critical regions, underlining their significance in protein function. Furthermore, some *nec* mutations cause amino acid substitutions identical to those responsible for disease in humans. In particular, both Nec^{E421K} and Nec^{G466S} have arisen twice independently, proving these residues to be critical for conformational stability. In addition, engineering an amino acid substitution in Nec homologous to that found in the extreme polymeric Siiyama variant of α_1-antitrypsin produces an inactive serpin that becomes a dominant-negative-mutant form at higher temperatures. It seems likely that the mechanism of inactivation of the wild-type Nec serpin in flies overexpressing Nec^{1} or Nec$^{S>F}$ is that the polymeric serpin recruits sufficient wild-type Nec to give a deficiency of Nec in the hemolymph. Such heteropolymerization has been observed for the S, I, and Z variants of α_1-antitrypsin (Mahadeva et al., 1999).

The genetic analysis of serpins in model organisms has been hampered by the broad substrate specificity of most serpin molecules. As a consequence, loss-of-function serpin mutations rarely produce mutant phenotypes in mice and the target proteases remain inhibited by the activity of related serpins. The tight specificity of Nec for its substrate protease and the lack of functionally redundant Nec-like serpin activities in *Drosophila* provides us with a unique opportunity for genetic analysis of serpin function. The Nec mutant phenotype will enable the development of a model to facilitate the study of serpin polymerization in vivo and to test therapeutic agents for human disease.

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