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The Drosophila _domino_ SWI2/SNF2 Gene Product Represses Cell Death in _Drosophila melanogaster_

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**ABSTRACT** The Drosophila _domino_ locus encodes DNA-dependent ATPases of the SWI2/SNF2 class. This class of chromatin remodeler is associated with an array of cellular activities encompassing transcription, replication, repair and recombination. Moreover, _domino_ was observed initially to maintain a repressive chromatin state via genetic interaction studies with homeotic genes. Although _domino_ mutations were also characterized with a cell death phenotype, its association with a death pathway has not been investigated. Here we have used targeted RNA interference to depress _domino_ function in the wing. Resultant wing damage phenotypes were found to be enhanced through overexpression of pro-apoptotic loci, and suppressed through loss of function of these loci. Loss of wing margin and blade tissue was correlated with activation of the effector Caspase Dcp-1, a marker for apoptosis. The affected wing regions also exhibited lower levels of the DIAP1 protein, an inhibitor of apoptosis. The lower level of DIAP1 protein was not correlated with an effect on the activity of a DIAP1 gene transgenic reporter (thread-LacZ), suggesting that loss of DIAP1 occurred post transcriptionally. In some cases excessive cell proliferation within the targeted tissue, measured through BrdU incorporation, was also observed. Finally, we used a transgenic reporter construct to monitor the chromatin state upstream of the proapoptotic _reaper_ locus. In genotypes exhibiting targeted _domino_ loss and wing phenotypes, we observed increased reporter activity only in the affected areas. These data support the conclusion that _domino_ normally functions to maintain pro-apoptotic genes in a repressed state.

The Drosophila _domino_ (_dom_) locus was identified during an enhancer trap screen for _P_ element-mediated disruptions of hematopoiesis (Braun et al. 1997). Homozygous _dom_ larvae were observed to be deficient in hemocytes and exhibited lymph glands that contained necrotic and melanized cells. The substantial cell death of differentiating hemocytes is associated with defective host defense against septic injury, when _dom_ is combined with other immune system mutations (Braun et al. 1998). Aberrant phenotypes of _dom_ mutant larvae were also noted in several other proliferating tissues, including imaginal discs, brain and germline (Braun et al. 1997, Ruhf et al. 2001). Moreover, mutant clones of strong _dom_ alleles are not recovered, even in the genetic background of a _Minute_ mutation (Ruhf et al. 2001), demonstrating that _dom_ function is essential for cell viability. The _dom_ gene sequence predicts two major products of the SWI2/SNF2 class of DNA-dependent ATPase, implicating Dom proteins in chromatin modification/nucleosome remodeling (Ruhf et al. 2001). Consistent with this idea, Dom protein is associated with the Tip60 acetyltransferase complex and functions in histone exchange (Kusch et al. 2004, Lu et al. 2007, Börner and Becker 2016); moreover genetic analysis showed that _dom_ synergizes with numerous other loci that encode chromatin-associated proteins (Ellis et al. 2015). Previous characterizations of _dom_ alleles also revealed a repressive role on homeotic genes (Ruhf et al. 2001) and E2F targets; the latter indicating that _dom_ may function to restrict cell proliferation (Lu et al. 2007). Additional functions associated with _dom_ include stem cell maintenance and renewal (Xi and Xie 2005, Morillo Prado et al. 2013, Yan et al. 2014, Börner and Becker 2016) and regulation of telomere capping (Rong 2008). Genetic
interaction analyses have also linked Dom to Notch signaling (Hall et al. 2004, Eisenberg et al. 2005, Gause et al. 2006, Kwon et al. 2013). The apparent roles of Dom in both gene repression and activation are predicted by its participation in the Tip60 complex (Gause et al. 2006, Schirling et al. 2010).

We previously reported a wing phenotype modifier screen designed to expand the gene network contributing to Dom function (Kwon et al. 2013). A prominent group of modifiers identified in this screen includes loci that regulate growth, proliferation and autophagy. Notably, we found that multiple genotypes containing down-regulated autophagy loci exhibited enhanced Dom phenotypes. Given the well-described cross-talk and mutual inhibition between autophagy and cell death (Marinó et al. 2014), these results may reflect a predominant role of Dom in cell viability and restriction of cell death (Braun et al. 1997, Ruhi et al. 2001). Consistent with this idea, loss of Dom function in ovaries is associated with germline cell loss and apoptosis (Yan et al. 2014), and Dom can also synergize with other mutations to increase cell death (Ellis et al. 2013). Here we have investigated the relationship between loss of Dom function and apoptotic cell death. Using a set of UAS-regulated Dom RNAi strains and wing Gal4 drivers we found that targeted expression of Dom RNAi leads to potent activation of Dcp-1, a marker for induction of apoptosis, as well as depression in the levels of DIAP1, an apoptosis inhibitor. We also observe significant genetic interactions between strains showing Dom RNAi-mediated phenotypes and strains carrying altered dosages of cell death associated products. Additionally, using a genetic construct that reports the epigenetic state of proapoptotic loci, we determined that loss of Dom activity leads to derepression of the reporter. Finally, loss of Dom function was associated with excess cell proliferation, as measured through incorporation of BrdU. These results support roles for Dom as a pleiotropic regulator, that can block both apoptosis and cell proliferation.

**Materials and Methods**

**Drosophila strains**

Strains were obtained from the following labs: C96-GAL4 (G. Boulianne, Toronto), UAS-Hid (H. Ryoo, NYU Medical Center), IURER{ubi-DsRed}E225 (L. Zhou, University of Florida), C96-domR was described previously (Kwon et al. 2013).

The following strains were obtained from Bloomington Stock Center (BL# in parentheses):

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nick Ratio</th>
<th>N</th>
<th>C96-domR Control</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>1.0</td>
<td>&gt;145</td>
<td>NE</td>
<td>1000</td>
</tr>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>0.9</td>
<td>220</td>
<td>NE</td>
<td>518</td>
</tr>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>0.14</td>
<td>350</td>
<td>NE</td>
<td>659</td>
</tr>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>1.0</td>
<td>337</td>
<td>NE</td>
<td>530</td>
</tr>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>1.0</td>
<td>449</td>
<td>NE</td>
<td>204</td>
</tr>
<tr>
<td>w1118; P[w+; +mC]=UAS-prp.C14</td>
<td>1.0</td>
<td>130</td>
<td>NE</td>
<td>155</td>
</tr>
<tr>
<td>Df[3L]ED225</td>
<td>0.58</td>
<td>104</td>
<td>NE</td>
<td>110</td>
</tr>
</tbody>
</table>

The C96-domR strain was tested for phenotypic modification when combined with genotypes carrying gain or loss of function in cell death loci. Tester genotypes were scored as transheterozygotes with the C96-domR chromosome. w1118 control crosses were included for each test and used to calculate the nick ratio for each set of crosses. In the table we express the percent of nicked wings as the ratio of experimental %/control %. Ratios greater than 1.0 represent enhancement, and ratios less than 1.0 represent suppression. A minimum of 146 control wings were scored for each C96-domR experimental cross. Using a Chi square test all viable crosses produced phenotypic modifications that were highly significant (P < 0.001) except for Df[3L]ED225 (P = 0.02) which was significant. Phenotypes were observed in C96-Gal4 control crosses for only two tester strains. UAS-Reaper did not produce viable offspring with either the control or experimental crosses.

**Antibody staining of third instar larval wing discs**

Imaginal wing discs were dissected in 1X phosphate buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde, and washed 3 times (1X PBS) prior to being permeabilized with 0.3% Triton X-100 in PBS (PBST) for 20 min, and washed once more in 1X PBS (Moberg et al. 2005). The discs were then incubated overnight, at 4°C, with 10% normal goat serum (NGS) and primary antibody in 0.1% PBST. Subsequently, the discs were washed 5 times (0.1% PBST) and then incubated overnight, at 4°C, with NGS and secondary antibody in 0.1% PBST. After the discs were washed 5 more times, they were incubated overnight in n-propyl gallate in glycerol at 4°C, and prepared for confocal microscopy. Confocal images were gathered with a Zeiss LSM710 confocal microscope and imaged using the identical optical settings. Images are merged projections. Images were assembled with Photoshop software (Adobe). Primary antibodies include mouse anti-β-Gal (1:1000, Promega); mouse anti-BrdU (1:50, Becton Dickinson); rabbit anti-cleaved Dcp-1 (1:100; Cell Signaling); mouse anti-DIAP1 (1:50; DSHB); rabbit anti-GFP and mouse anti-BrdU (1:50, Becton Dickinson).
Strains available upon request. The authors acknowledge the Bloomington TRiP for providing reagents and data.

Reagent and Data Availability

Strains available upon request. The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures.

RESULTS

Localized Down regulation of dom in the wing elicits both cell death and hyperproliferation

The recombinant chromosome strain C96-domR contains a wing margin Gal4 driver (C96) and UAS-RNAi transgenes directed against a sequence common to all dom transcripts. The C96-domR chromosome produces a dominant, and partially-penetrant wing nicking phenotype that is enhanced by various dom alleles, and suppressed by overexpression of a wild type version of dom RNA (Kwon et al. 2013). Figure 1 (A-D) shows the wing nicking phenotypes of C96-domR heterozygotes and homozygotes along with C96-Gal4 controls. The homozygous phenotype is severe and completely penetrant, with significant loss of the anterior and posterior wing margins, and some blade material (Figure 1D). Imaginal wing discs from these strains were stained with cleaved Dcp-1 antibody to detect apoptosis. The control strains show occasional areas of staining throughout the disc (Figure 1E-F), whereas C96-domR heterozygous and homozygous discs show significantly higher levels of staining across the margin (Figure 1G-H), within the domain of C96-Gal4 expression (Figure 1E inset and Helms et al. 1999). We validated these effects with additional RNAi strains from the Bloomington TRiP collection targeting dom sequences in four different regions of the transcripts. When C96-Gal4 was used to drive these hairpin constructs very strong wing margin defects were produced in heterozygotes (Figure 1I-J); utilizing En-Gal4 we observed massive loss of posterior wing compartment material in heterozygotes (Figure 1K-L). Wing discs from each of these constructs show wing mounts from following strains: C96-Gal4/w1118 and C96-Gal4/C96-Gal4 (panels A-B); C96-domR/w1118 and C96-domR/C96-domR (panels C-D). Extent of margin loss reflects dose of C96-domR. Wing discs from these strains were stained with antibody to cleaved Dcp-1 protein to measure cell death: C96-Gal4/w1118 and C96-Gal4/C96-Gal4 (panels E-F); C96-domR/w1118 and C96-domR/C96-domR (panels G-H). Cell death levels match wing margin nicking in adult wings. The inset in panel E shows the domain of C96-Gal4 activity across the dorsal-ventral wing margin (arrow), as reported by yellow color UAS-GFP. Four additional dom TRiP RNAi constructs were driven by either C96-Gal4 (panels I, M: BL 38385 and panels J, N: BL 41674) or En-Gal4 (panels K, O: BL 40914 and panels L, P: BL 55917). Three of these constructs (38385, 41674 and 40914) target both major dom A and B form transcripts (Ruhf et al. 2001), whereas the 55917 strain targets only the B transcript. The region of adult wing loss again reflects areas undergoing cell death. Panels Q-T show wing mounts from C96-domR outcrossed to UAS-P35 (panel Q), UAS-Hid (panel R), UAS-Warts (panel S), and a UAS-TRIP RNAi strain targeting Reaper (panel T). Suppression and enhancement is consistent with the cell death phenotype of C96-domR wings (also see Table 1).
these crosses were stained with cleaved Dcp-1 antibody, revealing high levels of staining in the regions of Gal4 activity (Figure 1M-P).

We extended these data by testing for genetic modifications of the C96-domR heterozygous phenotype through altered dosage of the products of cell death loci. C96-domR flies were outcrossed to strains carrying UAS-regulated components of the cell death pathway and the wings were scored for penetrance of nicking vs. crosses to control w1118 flies (Table 1). We observed that coexpression of inhibitors of apoptosis, P35 and DIAP1, strongly suppressed wing nicks, whereas coexpression of two pathway components, Hid and Warts (Bergmann 2010) enhanced. Furthermore, loss of function for reaper via coexpression of reaper RNAi led to significant nick suppression, as did chromosomal deletions which eliminate multiple cell death pathway loci (Df(3L)H99 and Df(3L)ED225). Representative wings exhibiting enhanced and suppressed C96-domR phenotypes are shown in Figure 1 panels Q-T. These results support the contention that dom wing phenotypes derive from elevated levels of apoptosis.

As proapoptotic activity is regulated by activity of the DIAP1 protein (Lee et al. 2011), we assayed DIAP1 levels in discs with localized depressions of dom function. Normally DIAP1 protein accumulates widely in wing discs with marked accumulation along the dorsoventral margin (Ryoo et al. 2002). Figure 2 shows DIAP1 staining in control C96-Gal4 discs and C96-Gal4 driving dom RNAi. In contrast to the controls, there is a marked reduction of DIAP1 along the wing margin (panels A and B). Moreover, when En-Gal4 is used to drive dom RNAi expression we observe reduced DIAP1 staining within posterior relative to anterior regions of wing discs (Figure 2, panels C-D). Therefore, the elevated levels of apoptosis within regions of discs depressed in dom function (Figure 1) is correlated with down regulation of the cell death inhibitor DIAP1. The effect on DIAP1 levels does not appear to be at the level of transcription. Utilizing a thread-LacZ reporter (th-LacZ) reflecting transcription of the diap1/th locus, we do not observe lower levels of activity along wing margins expressing dom RNAi (Figure 2 I, J).

In a cell culture based screen for regulators of E2F targets, dom was identified as an E2F repressor; further, dom mutation was found to interact genetically with strains showing excessive or diminished cell proliferation in eye tissue (Lu et al. 2007). We investigated the effects of dom RNAi expression on cell proliferation, measured through incorporation of BrdU (Moberg et al. 2005). When dom RNAi was driven by C96-Gal4 we could not detect significant effects on BrdU incorporation relative to the control discs (Figure 2 E-F). However, En-Gal4 driving dom RNAi led to significant increases in BrdU incorporation within the posterior compartment of the wing disc (Figure 2 G-H). Additionally, hyperproliferation of cells within the posterior compartment of these discs can be manifested as misshapen discs, for example, Figure 2H. Therefore, depression in dom function can be correlated with elevated levels of cell proliferation.

Expression of dom RNAi alters the epigenetic state of IRER near proapoptotic loci

Expression of the proapoptotic genes reaper, sickle and hid has been shown to be regulated by an irradiation-responsive enhancer region (IRER) located upstream of reaper (Zhang et al. 2008). Whereas early embryos have been shown to undergo apoptosis in response to irradiation, later embryos transition to a state that is not responsive. This developmental transition is mediated by epigenetic silencing of the

Figure 2

Further effects of dom RNAi expression on cell death pathway and proliferation. Wing discs from the following strains were stained with antibody to DIAP1 protein: C96-Gal4/w1118 and C96-Gal4/dom TRiP RNAi BL 41674 (panels A-B). Arrows in A and B show dorsal-ventral wing margin area that is enlarged in the insets, highlighting diminished stain across the margin in C96-Gal4/dom TRiP discs. En-Gal4/w1118 and En-Gal4/dom TRiP RNAi BL 40914 (panels C-D). Arrows in C and D point to posterior compartment of wing disc, the region of En-Gal4 expression (data not shown). Depression of DIAP1 stain is evident in posterior compartment of wing disc, including the dorsal-ventral margin in En-Gal4/dom TRiP discs. Wing discs from the same strains described above were also stained for incorporation of BrdU (Moberg et al. 2005): C96-Gal4/w1118 and C96-Gal4/dom TRiP RNAi BL 41674 (panels E-F). We could not detect significant differences in BrdU incorporation between these discs. En-Gal4/w1118 and En-Gal4/dom TRiP RNAi BL 40914 (panels G-H). Posterior compartment of wing disc oriented rightward, as in panels C and D. Examination of BrdU incorporation is evident in posterior compartment of wing discs in En-Gal4/dom TRiP discs, reflecting excess cell proliferation. Wing discs of the genotype C96-Gal4 + th-LacZ (panel I) and C96-Gal4 + th-LacZ + dom TRiP RNAi BL 41674 (panel J) were stained with antibodies to β-Gal to monitor activity of the DIAP1 (th) locus. No depression in activity was evident in discs expressing dom RNAi (J) vs. the control discs (I).
IRER. The state of IRER accessibility can be monitored in the IRER(ubi-DsRed) strain (Zhang et al. 2015). This strain contains a ubiquitin-DsRed reporter that was inserted into IRER via homologous recombination. In this strain, the open or closed chromatin structure of IRER is reflected by the expression of ubiquitin-DsRed (Zhang et al. 2015). We investigated the effect of dom RNAi expression in wing disc on the levels of the ubiquitin-DsRed reporter (Figure 3). UAS-P35 was included in the genotype to prolong the survival of cells that initiate the apoptosis pathway (Hay et al. 1994) and thereby preserve the reporter signal. Control discs containing En-Gal4, UAS-GFP, UAS-P35 and IRER(ubi-DsRed) show variegated, but overall low levels of ubiquitin-DsRed activity (Figure 3, panel A). This variegated expression in controls matches the original observations of Zhang et al. (2015). Moreover, we found that the misshapen nature of discs associated with domIR expression enhanced the irregularity of the IRER signal. In contrast, discs containing En-Gal4 driving UAS-P35 and either of two dom RNAi constructs in a IRER(ubi-DsRed) genetic background exhibit high levels of IRER(ubi-DsRed) activity in the GFP-positive posterior compartment: the region of En-Gal4 expression (Figure 3, panels B-D). Therefore, depression of dom function appears to modify the chromatin structure proximal to proapoptotic genes, potentially allowing higher levels of expression.

DISCUSSION

The initial characterization of a dom mutation implicated the locus in cell death regulation as homozgous dom larvae contained necrotic lymph glands and a deficiency in hemocytes (Braun et al. 1997). Loss of dom function subsequently was associated with apoptosis in the germline (Yan et al. 2014). Here we have shown that RNAi-mediated depressions in dom function lead to activation of apoptosis in the wing disc, as measured through staining for cleaved Dcp-1 and resultant adult wing phenotypes (Figure 1). Current models of cell death regulation propose that DIAP1 binds and inhibits the activity of the cleaved effector Caspase Dcp-1. This inhibition appears to be overcome by elevated levels of the RHG proteins (Reaper, Hid and Grim), which bind DIAP1 and lead to its degradation (Bergmann 2010). Consistent with these models, the dom wing phenotype is sensitive to the dosage of several proapoptotic gene products as well as the inhibitors P35 (Hay et al. 1994) and DIAP1 (Table I). Further, we found that dom RNAi expression leads to depression in the levels of DIAP1 protein, without a detectable effect on the levels of diap1 gene (th) transcription, measured with a th-LacZ reporter (Figure 2). A prediction of this model, not yet tested, is that dom IR-induced depressions in DIAP1 levels would be suppressed via loss of function for RHG loci.

Given these data, along with the classic description of dom as a genetic repressor (Ruhl et al. 2001, Lu et al. 2007) a reasonable explanation for the effects of dom RNAi invokes derepression of RHG loci. To address this possibility, we assayed the expression of a ubiquitin-DsRed reporter, resident within an irradiation-responsive enhancer region (IRER) of the proapoptotic RHG loci (Zhang et al. 2008). We found that dom RNAi expression in wing discs strongly increased the level of expression of ubiquitin-DsRed relative to control discs (Figure 3). Therefore, the loss of dom function likely alters the chromatin state surrounding the RHG loci, leading to their elevated expression. The consequent increase in amounts of RHG proteins would likely launch apoptosis.

Dom has also been linked to regulation of cell proliferation. Lu et al. (2007) found that Dom associates with E2F at promoters and contributes to a repressed state at loci involved in cell proliferation. Genetic interaction studies also implicate dom in proliferation (Lu et al. 2007, Kwon et al. 2013, Ellis et al. 2015). We tested the prediction that loss of dom function can lead to excess proliferation by measuring BrdU incorporation. We observed that En-Gal4 directed expression of dom RNAi in the posterior compartment of wing discs led to higher levels of BrdU incorporation (Figure 2). The increased BrdU levels were also associated with enlarged and misshapen posterior regions of the discs, presumably due to the excess number of cells. The adult phenotype derived from wing discs of this genotype shows massive loss of posterior compartment material (Figure 1), indicating that cell death ultimately masks the hyperproliferation effect. Importantly, these data do not necessarily implicate dom directly in genetic regulation of hyperproliferation. There are multiple lines of evidence linking apoptosis to a compensatory proliferation response in damaged tissues (Fogarty and Bergmann 2017). In any case, the phenotype derived from loss of dom function is pleiotropic, consistent with its broad range of genetic interactions with other regulatory proteins (Ellis et al. 2015).

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