Mi-2 chromatin remodeling factor functions in sensory organ development through proneural gene repression in *Drosophila*

Yasutoyo Yamasaki and Yasuyoshi Nishida*

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan.

*Author to whom all correspondence should be addressed: Laboratory of Developmental Biology, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan

Phone: 81-052-789-2508

Fax: 81-052-789-2511

E-mail: nishida@bio.nagoya-u.ac.jp

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Key words: NuRD complex, chromatin remodeling factor, sensory organ development, proneural gene, macrochaete.

Abstract

Mi-2, the central component of the nucleosome remodeling and histone deacetylation (NuRD) complex, is known as an SNF2-type ATP-dependent nucleosome remodeling factor. No morphological mutant phenotype of *Drosophila Mi-2* (*dMi-2*) had been reported previously, however, we found that rare escapers develop into adult flies showing an extra bristle phenotype. The *dMi-2* enhanced the phenotype of ac^{Hw49c}, which is a dominant gain-of-function allele of *achaete* (*ac*) and produces extra bristles. Consistent with these observations, the *ac*-expressing proneural clusters were expanded, and extra sensory organ precursors (SOPs) were formed in the *dMi-2* mutant wing discs. Immunostaining of polytene chromosome showed that dMi-2 binds to the *ac* locus, and dMi-2 and acetylated hisotones distribute on polytene chromosomes in a mutually exclusive manner. The chromatin immunoprecipitation assay of the wing imaginal disc also demonstrated a binding of dMi-2 on the *ac* locus. These results suggest that the *Drosophila* Mi-2/NuRD complex functions in neuronal

differentiation through the repression of proneural gene expression by chromatin remodeling and histone deacetylation.

Introduction

Epigenetic transcriptional regulation by chromatin remodeling complexes plays pivotal roles during development (for a review, see Müller & Leutz 2001). The nucleosome remodeling and deacetylation (NuRD) complex is one of the major chromatin remodelers, which are postulated to act as transcriptional co-repressors (for reviews, see Ahringer 2000; Bowen *et al.* 2004). Mi-2 is a central component of the NuRD complex, and is an SNF2-type nucleosome remodeling ATPase. The complex also contains histone deacetylase Rpd3 and metylated DNA-binding protein MBD2/3.

A *Drosophila* homolog of Mi-2, dMi-2, has been originally identified by a physical association with Hunchback, which represses transcription of homeotic

(HOX) genes and delimits HOX expression domains (Kehle *et al.* 1998). dMi-2 has been proposed to be involved in the maintenance of the HOX repression domains. dMi-2 also binds to DNA replication-related element (DRE) binding factor (DREF), a transcriptional activator, and attenuates the transcription activity of DREF (Hirose *et al.* 2002). A physical association of dMi-2 with Tramtrack69 (Ttk69) has also been reported, and it has been proposed that Ttk69 represses some target genes by remodeling chromatin structure through recruitment of the NuRD complex (Murawsky *et al.* 2001). *In vitro* biochemical analyses have shown that dMi-2 actually has nucloeosome binding and mobilization activities (Brehm *et al.* 2000; Bouazoune *et al*. 2002).

Several loss-of-function mutants for *dMi-2* have been reported, and they arrest development at first or second instar larval stages (Kehle *et al.* 1998). This late lethal phase would depend on a large amount of the transcripts being derived maternally and zygotically in embryos (Kettak *et al.* 2002).

On epidermal tissues of an adult fly, a great number of external mechanosensory organs are distributed stereotypically, and the underlying

mechanisms of their differentiation and development have been described in detail (for reviews, see Modolell & Campuzano 1998; Simpson *et al.* 1999; Calleja *et al.* 2002). Expression of the proneural genes of the *achaete* (*ac*)-*scute* (*sc*) complex (*AS-C*) is pivotal in proneural cluster determination and sensory organ (SO) differentiation. Loss of proneural gene expression causes loss of SOs. However, an excess expression of the proneural genes or loss-of-function mutations of the repressor genes cause expansion of the proneural clusters and extra SO formation. Ttk69 cooperates to prevent inappropriate neural development outside of proneural clusters in wing discs, and its overexpression represses *ac* transcription (Badenhorst *et al.* 2002).

In this study we show that loss of *dMi-2* activity causes the formation of supernumerary sensory bristles and an expansion of the Achaete (Ac)-expressing proneural clusters in the wing disc. We also demonstrate that dMi-2 binds to the *ac* locus, and the binding of dMi-2 on chromatin correlates with histone deacetylation. These findings suggest that the Mi-2/NuRD complex

directly regulates *ac* expression as a transcription repressor to regulate the sensory organ development in *Drosophila.*

Materials and methods

Fly stocks

Fly stocks were maintained at 25ºC on standard medium. The Canton-S, Oregon-R or *white*^{1118} were used as the wild-type strain. The *dMi-2*^{4}, *dMi-2*^{5} alleles and *arm-dMi-2* transgenic flies (Kehle *et al.* 1998) were kindly supplied by J. Müller. *UAS-HA-dMi-2* transgenic flies (Hirose *et al*. 2002) were gifts from F. Hirose. The *UAS-HA-ttk69* transgenic fly was gifted from Z-.C. Lai. The *dMi-2*^{L1243}*, Df(3L)BSC1, ac*^{4}*, ac*^{Hw49c}*, neur*^{A101}, *rpd*^{304556} and *da-GAL4* strains were obtained from the Bloomington Stock Center. The GAL4 enhancer trap

strain *NP3126* was obtained from the *Drosophila* Genetic Resource Center (DGRC) of Kyoto Institute of Technology, Japan.

Histochemistry

X-gal staining and immunostainings of the imaginal discs and the polytene chromosomes were performed according to the protocols described in *Drosophila* Protocols (Sullivan *et al.* 2000). Immunostaining was performed using the following primary antibodies: anti-Ac mouse antibody (1:10 dilution; Developmental Studies Hybridoma Bank (DSHB)); anti-dMi-2 rat antibody (1:200 dilution; Hirose *et al.* 2002); anti-Ttk69 rabbit antibody (1:100 dilution; Jordi Bernués), Y11 anti-HA rabbit antibody (1:100 dilution; Santa Cruz); anti-acetylated Histone H3 and antiacetylated Histone H4 rabbit antibodies (1:100 dilution; Upstate). Anti-rat Cy3- (Jackson ImmunoResearch), anti-mouse Alexa488-, and anti-rabbit Alexa568- (Molecular probes) conjugated antibodies

were used at 1:200 dilution. The preparations were viewed under an LSM510 laser-scanning confocal microscope (Carl Zeiss).

Chromatin immunoprecipitation assay

We basically followed a protocol described in the manual of the Chromatin Immunoprecipitation Assay Kit (Upstate). Two hundreds or more pairs of wing discs were dissected in PBS, and the samples were fixed with 2% paraformaldehyde in PBS for 15 minutes. Then, the samples were sonicated in an SDS lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% SDS and protease inhibitors) with the VP-55 sonicator (Taitec, Japan) at power 5 for eight pulses of 10 seconds sonications with 20 seconds intervals. After dilution with ChIP dilution buffer, Y11 anti-HA rabbit antibody (Santa Cruz) and protein A-sepharose were mixed for immunoprecipitation. Proteins were digested and extracted with PCR purification kit (QIAGEN). The oligonucleotide primers used

for PCR were: for DNApol-α180 (a negative control),

5’-GTGATCCACTGCAATTAAAAAGCTGAAGCTCG and

5’-CACGGTGATTCAGACATTGCAACTGGC; for *ac-p1*,

5’-CTGTATACCACAGGACACGCT and 5’-GATCGATCGATCTCTCCGGAA; for

ac-p2, 5’-AGTCATCTTCGGCCTTTAATGGAC and

5’-CTTGTGCTGCCAGTTGGAGA; for *ac-p3*, 5’-GTTGCGATAAAAGCACACTG

and 5’-ATGACAACGAGTTCGAGGAT; and for *ac-p4*,

5’-AAGCTGTCCTGTAATCGAGG and 5’-TGTGAAAGGGACATCACAGG.

Results

dMi-2 *mutants show an extra sensory organ phenotype*

Most *dMi-2* mutants die during the first or second instar larval stages (Kehle *et al*.

1998), but we found that approximately 0.1% of the *dMi-2* null mutants survived

and became adults. The escapers showed an extra sensory organ phenotype on the epidermis of the thorax. On the mesothoracic notum, a number of ectopic mechanosensory bristles were formed without any significant positional specificity (Fig. 1B, C). The ectopic rows of microchaetae were also formed between the normal rows, and the spacing of each rows was disturbed less strongly than in the Notch (N) pathway mutants, such as *Delta*^{7} (*Dl*^{7}) or *scabrous*^{1} (*sca*^{1}). Defects of ocellar bristles on the head capsule were also observed (Fig. 1E, F). In addition, extra sensilla companiformia, one type of mechanosensory organ, were formed on the third longitudinal vein and the anterior cross vein of the wing (Fig. 1G, H). These phenotypes were found in several *dMi-2* mutant alleles and the allele combinations, including the P-element insertional alleles, *NP3126* and *l*(*3*)*L1243* (Fig. 1A; see below). However, the other organs, including the compound eyes and legs, appear to be normal (Fig. 1E, F; and data not shown).

To confirm whether the phenotypes are caused by the *dMi-2* mutations, we carried out a rescue experiment using the GAL4/UAS system (Brand and

Perrimon 1993). As *arm-dMi-2* and *HA-dMi-2* driven by *hs-GAL4* failed to rescue the lethality of the *dMi-2* mutants (Kehle *et al*. 1998; Y. Yamasaki unpublished results), we searched the databases for GAL4 enhancer trap lines to express *UAS-dMi-2* in the native pattern. As a result, we found one enhancer trap line, *NP3126*, in which *P{GawB}* is inserted within the fourth intron of the *dELL* gene, encoding a RNA polymerase II elongation factor. The *dELL* gene is nested in the first large intron of the *dMi-2* gene (Fig. 1A). *NP3126* failed to complement with the known *dMi-2* point mutations, indicating that it is a new allele of *dMi-2* and is expected to express GAL4 under the native *dMi-2* transcription pattern. The transheterozygotes between the known alleles of *dMi-2* and *NP3126* were rescued by HA-dMi-2 driven by GAL4 in *NP3126.* In these flies, the extra bristle phenotype was also recovered (Fig. 1D). According to these results, we concluded that *dMi-2* is the responsible gene for the extra SO phenotype and is involved in sensory organ development.

dMi-2 interacts with an ac *gain-of-function mutation*

During *Drosophila* bristle development, the proneural gene *ac* is induced by the prepattern information at the late third instar larval stage, and this is a prerequisite for neuronal differentiation. Extra bristles are formed when proneural clusters are enlarged by hyperexpression of *AS-C* (for reviews, see Modolell & Campuzano 1998; Simpson *et al.* 1999; Calleja *et al.* 2002).

The extra bristle phenotype of the *dMi-2* mutants is similar to those of the *ac* gain-of-function mutants. Thus, we tested the genetic interaction of *dMi-2* with an *ac* gain-of-function allele, ac^{Hw49c} (Table 1). ac^{Hw49c} phenotype is repressed by a hypomorphic allele ac^{4}, and *dMi-2* mutants have no dominant phenotype on macrochaetae. The severity of the dominant ac^{Hw49c} phenotype was increased when flies were also heterozygous for the *dMi-2* alleles. However, the *arm-dMi-2* or *HA-dMi-2* driven by GAL4 in *NP3126* reduced the enhancement. The heterozygosity for *dMi-2* also enhanced the dominant mutants for *extramacrochaet* (*emc*), an Ac antagonist, and for $E(spl)^{R1}$, a transcription

repressor (data not shown). These genetic interactions suggested that *dMi-2* is involved in the regulation of *AS-C*.

Proneural clusters are expanded in dMi-2 mutant wing discs

We next observed the proneural clusters in the wing discs of the *dMi-2* mutants. The proneural clusters were visualized by immunostaining with an anti-Ac monoclonal antibody (Fig. 2A-C). In the *dMi-2* mutant wing discs, the Ac-expressing proneural clusters were significantly expanded as compared to the control (Fig. 2A, B). Consistent with the observations of the expanded proneural clusters, ectopic SOPs were detected in the *dMi-2* mutant discs by using *lacZ* in $neur^{A101}$ (Fig. 2D, E). These results demonstrate that *dMi-2* is an upstream repressor of *ac* and contributes in the proneural specification.

Khattak *et al.* (2002) indicated that no transcript of *dMi-2* was detected during the third instar larval stage. However, the *dMi-2* mutant phenotype

described above contradict their observation, and suggests that dMi-2 protein is present at this stage and functions in the repression of *AS-C*. To clarify this point, we stained the wing discs with an anti-dMi-2 antibody. To our surprise, dMi-2 was ubiquitously distributed throughout the wing disc cells including the Ac-expressing cells and SOPs (Fig. 2F-H).

dMi-2 binds to the ac *locus*

dMi-2 is present uniformly in the wing discs, while the mutant phenotype was restricted to SOPs. Therefore dMi-2 must be recruited by other transcription factor(s) to the target genes and function to remodel the chromosome regions. It has been reported that dMi-2 and Ttk69 interact physically with their C-terminal domains (Murawsky et al. 2001). Furthermore, clusters of the consensus Ttk69-binding sites (AGGAY motifs) distribute in the *AS-C* locus, including downstream of *ac* and upstream of *asence* (*ase*) (Badenhorst *et al.* 2002).

Moreover, Badenhorst *et al.* (2002) have shown that a Ttk69 construct that lacks the dMi-2-interacting domain reduces its neuronal repression activity. Thus, Ttk69 is a reasonable general recruiter of dMi-2.

We examined the co-localization of dMi-2 and Ttk69 on polytene chromosomes. As previously reported (Murawsky *et al*. 2001), dMi-2 co-localized with Ttk69 at many discrete sites (Fig. 3A-C). dMi-2 and Ttk69 co-localized at the *AS-C* locus (Fig. 3D-F).

To investigate whether dMi-2 binds to the *ac* locus directly, we tried the chromatin immunprecipitation (ChIP) assay. Samples were prepared from HA-dMi-2 overexpressed or wild-type wing discs and an anti-HA antibody was used for immunoprecipitation. Promoter region of DNA pol α180 was used as a negative control. A DNA fragment containing the putative Ttk69-binding motifs (Fig. 4. p1) and a fragment of near it (Fig. 4. p2) were amplified when the HA-dMi-2 was expressed. Distal regions of the putative Ttk69-binding sites (Fig. 4. p3 and p4) were not amplified even though HA-dMi-2 was expressed. This indicates that dMi-2 binds to the Ttk69-binding region *in vivo*.

dMi-2 binding to chromosomes and histone deacetylation

The NuRD complex contains a histone deacetylase Rpd3, and its recruitment to chromatin can be expected to lead to histone deacetylation. dMi-2 and acetylated histone H3 were mapped on polytene chromosomes by a double-label immunofluorescence microscopy (Fig. 5A-C). dMi-2 and acetylated histone H3 are located in a mutually exclusive pattern. Staining with anti-acetylataed histone H4 also showed no overlap with dMi-2 (Fig. 5D). These results and the ChIP results suggest histone deacetylation-mediated transcriptional repression of *AS-C* by the NuRD complex containing dMi-2 and Ttk69.

Discussion

dMi-2 *is involved in proneural differentiation*

In this article we demonstrated that the *dMi-2* mutants showed an extra sensory organ phenotype. In the *dMi-2* mutant wing discs, Achaete-expressing proneural clusters in the wing imaginal discs were enlarged, and extra SOPs were generated. The ChIP assay indicated that dMi-2 binds to the *ac* locus. We think that the mutant phenotype results from the reduced transciptional repression of the proneural genes. The mutually-exclusive distribution pattens of dMi-2 and acetylated histones on polytene chromosomes support the idea that the dMi-2 binding to chromosomes induces histone deacetylation and transcriptional repression. According to these findings, we propose an idea that dMi-2 functions in the sensory organ differentiation through the *ac* transcriptional repression as a component of the NuRD complex.

dMi-2 *expression and its putative redundancy*

arm-dMi-2 and *hs-GAL4* driven *HA-dMi-2* failed to rescue the *dMi-2* phenotypes (Kehle *et al*. 1998; Y. Yamasaki unpublished results). This may be due to an insufficient expression of dMi-2 transgenes by these drivers, as HA-dMi-2 induced by *NP3126* could rescue dMi-2 mutant phenotypes at 25^{o}C, but it failed to rescue at 17^{o}C. Or it may be possible that the *dMi-2* transcription is regulated in some spatially or temporarily restricted manners.

Distribution of dMi-2 at a large number of sites on polyten chromosomes suggests the presence of multiple target genes. A mosaic analysis has shown that the *dMi-2* mutant cells cannot survive (Kehle *et al*. 1998), and the lethality in the null mutants occurs at the first or second instar larval stages, suggesting a pleiotoropy of the phenotypes. However, about 0.1% of the *dMi-2* null mutants can develop into adults showing a rather simple extra bristle phenotype. This may suggest the existence of a redundant gene of *dMi-2*. *Chd3*, a possible

candidate, shows a primary structure highly similar to dMi-2 (FlyBase). A double mutation analysis would be required to clarify this point.

It would be surprising for two P insertion mutations, *NP3126* and *l*(*3*)*L1243*, to demonstrate the only *dMi-2*-specific phenotypes. A P insertion into the first intron of *dELL* is reportedly lethal, while the lethality can be prevented by a *dELL* transgene (Eissenberg *et al*. 2002). The former insertions would not affect *dELL*.

dMi-2 recruited on chromosomes by other factors

Two recruiting mechanisms of NuRD complexes have been proposed; a targeting mediated via protein-protein interaction between a component of the complex and a DNA-bound transcription factor, and a direct binding of the MBD-related protein to methylated DNAs (for reviews, see Ahringer 2000; Bowen *et al.* 2004). dMi-2 distributed ubiquitously even in the cells in the proneural clusters or SOPs in the wing disc, but the mutants showed a

neuronal-specific phenotype. Furthermore, a dMi-2 over-expression caused no abnormality on the morphology, viability and fertility of the flies. These results may suggest that dMi-2 must be recruited on some loci by other factors. We think a candidate for dMi-2 recruiter would be Ttk69.

Ttk69 expression is excluded from the proneural clusters, and Ttk69 interacts physically with dMi-2 (Badenhorst *et al.* 2002). There are several predicted Ttk69 binding motifs on *AS-C*, and Ttk69 over-expression represses the *ac* transcription. Furthermore, a construct of Ttk69 lacking dMi-2 binding domain reduced its ability for neuronal repression (Badenhorst *et al.* 2002). The result of the ChIP assay and co-localization of dMi-2 with Ttk69 at the *AS-C* locus may indicate that Ttk69 is a recruiter of dMi-2. Further studies on dMi-2 recruitment mechanisms may give the answer to the tissue- or stage-specific functions of dMi-2.

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Legends to figures

Fig. 1. The *dMi-2* gene structure, P-element insertions and *dMi-2* mutant phenotypes. (A) *dMi-2* (black boxes) has a large first intron containing the gene for a transcription elongation factor, *dELL* (white boxes). P-element insertions (triangles) in this intron are indicated with triangles. *dMi-2*^{4} and *dMi-2*^{5} are EMS-induced mutants (Kehle *et al*. 1998). P{GawB} insertion line, *NP3126*, was used as a *dMi-2* mutant allele and as a GAL4 driver. (B-D) Dorsal views of thorax of adult flies. Anterior is up. (B) Bristles on the notum in wild-type. (C) a *dMi-2*^{5}*/Df(3L)BSC1* fly. *dMi-2* mutant adults form extra sensory organs. Arrows indicate ectopically formed macrochaetae (large bristles). Number of microchaetae (small bristles) on the notum was also increased and their arrays were slightly disturbed. (D) *UAS-HA-dMi-2*/+; *dMi-2*^{4}*/NP3126*. *dMi-2* transgene induced by *NP3126* rescued the lethality and the adult phenotypes. (E, F) Ocelli and ocellar bristles on dorsal side of the head. In the *dMi-2* mutant, ocellar bristle defects were seen, but the arrays of ocelli and ommatidia are

normal. Bristles on the compound eyes are also normal in the mutants (data not shown). (G, H) Sensilla campaniformia (s.c.; arrows) on wing veins. (G) In the wild-type wing, three s.c. develop on the third longitudinal vein, and one on the anterior cross vein. (H) An extra s.c. (inset) was formed on the *dMi-2* mutant wing, and the distribution was disturbed.

Fig. 2. Proneural clusters and sensory organ precursors in the wing discs. (A-C) Proneural clusters (PCs) in the notum region of wing imaginal discs. PCs were visualized with fluorescent immunostaining for the Achaete (Ac) proneural protein. (A) Wild-type. (B) In the *dMi-2* wing disc, clusters of Ac positive cells are expanded. (C) *Hw49c*, a known Ac hyper-expressing mutant, for a comparison. (D, E) Staining of sensory organ precursor cells (SOPs) in the wing discs. SOPs were detected by using enhancer trap line $neur^{A101}$. (E) Black arrows indicate ectopic SOPs in the *dMi-2* mutant disc. (F-H) dMi-2 protein and PCs/SOPs. (F) dMi-2 was detected ubiquitously in whole wing disc, and overlapped with PCs and SOPs. (F, G) dMi-2 and Ac were detected by

immunofluorescent staining of the wild-type discs. (H) dMi-2 and β -galactosidase were double stained in *neur*^{A101}*/TM6C* larvae.

Fig. 3. dMi-2 co-localizes with Ttk69 and binds to the *ac* locus. (A-F) Binding sites of dMi-2 (green) and Ttk69 (magenta) on polytene chromosomes are highly overlapped. (D-F) dMi-2 and Ttk69 appear to localize on the *AS-C* locus (arrow).

Fig. 4. dMi-2 binds to the Ttk69 putative binding motifs at the *ac* locus. Chromatin immunoprecipitation indicates a direct binding of dMi-2 to the *AS-C* locus. The promoter region of DNA polymerase α 180 was used as a negative control. Black box indicates *achaete* gene, and dashed line shows the cluster of Ttk69-binding motifs at the 3' terminal region of *ac*.

Fig. 5. Distributions of dMi-2 and acetylated histones on polytene chromosomes. (A-C) Distrubutions of HA-dMi-2 (green) and acetylated histone

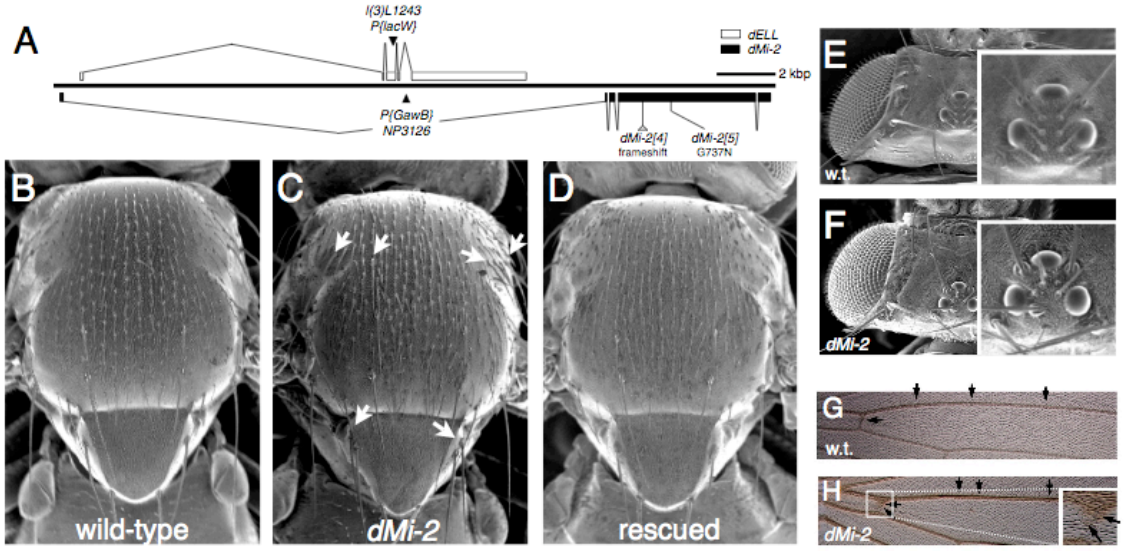
H3 (AcH3; magenta) are mutually exclusive. Arrowheads point to the tip of X chromosome where the *AS-C* locus locates. (D) Acetylated histone H4 (AcH4; magenta) also distributes in a mutually exclusive pattern against dMi-2 (green).

Table 1. Genetic interactions of *dMi-2* with an extra bristle-producing mutation, ac^{Hw49c}.

Genotype	Mean no. of extra bristles* ±SE	N**
ac^{Hw49c} /+; *+/+*	2.57±0.15	133
ac^{Hw49c} /+; *dMi-2*^{4}/+	5.45±0.23	102
ac^{Hw49c} /+; *NP3126*/+	4.81±0.27	85
ac^{Hw49c} /*arm-dMi-2*; *dMi-2*^{4}/+	2.99±0.24	86
ac^{Hw49c} /*UAS-dMi-2*; *NP3126*/+	3.29±0.24	58
ac^{Hw49c} /ac^{4}	1.07±0.16	41
+/+; *dMi-2*^{4}/+	0.05±0.02	128
+/+; *NP3126*/+	0.08±0.03	98

* Total number of extra bristles/numbers of the mesothoracic nota.

** Numbers of nota counted.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dELL
dMi-2
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NP3126
dMi-2[4]
frameshift
dMi-2[5]
G737N
B
wild-type
C
dMi-2
D
rescued
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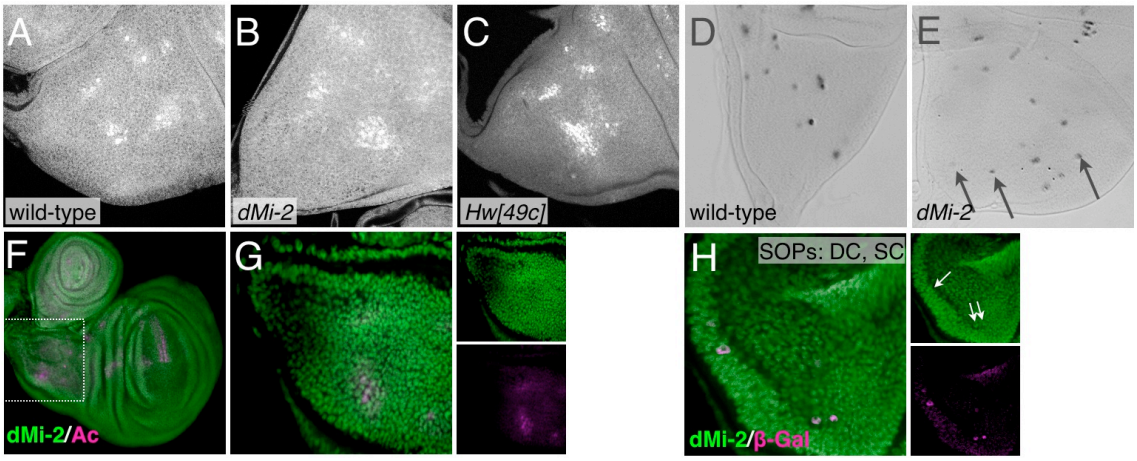
w.t.
F
dMi-2
G
w.t.
H
dMi-2

Fig. 1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wild-type
B
dMi-2
C
Hw[49c]
D
wild-type
E
dMi-2
F
dMi-2/Ac
G
H
SOPs: DC, SC
dMi-2/β-Gal

Fig. 2

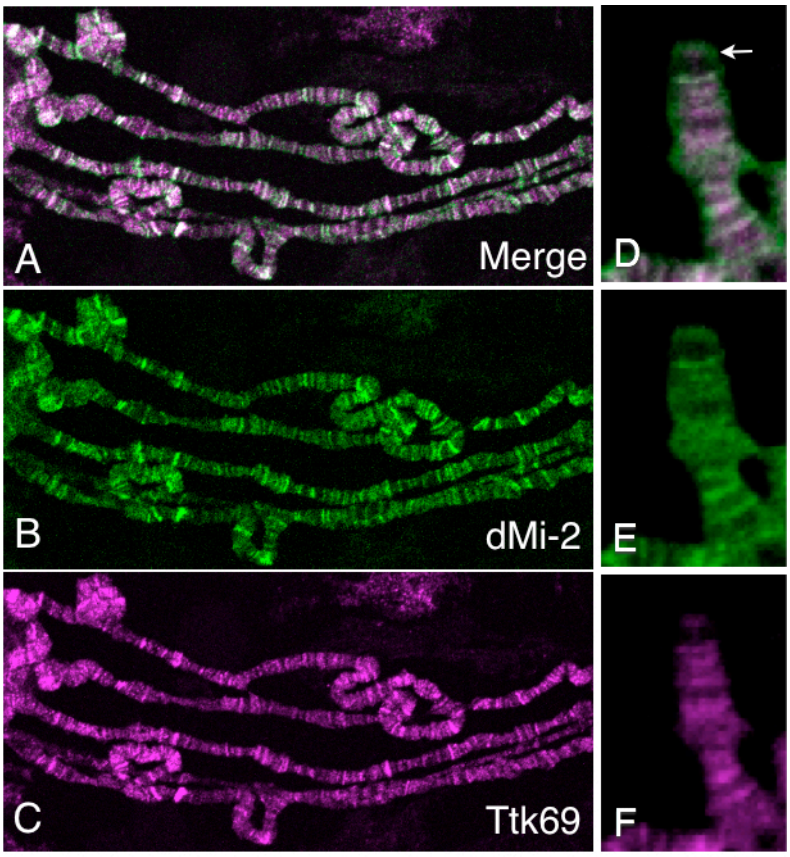
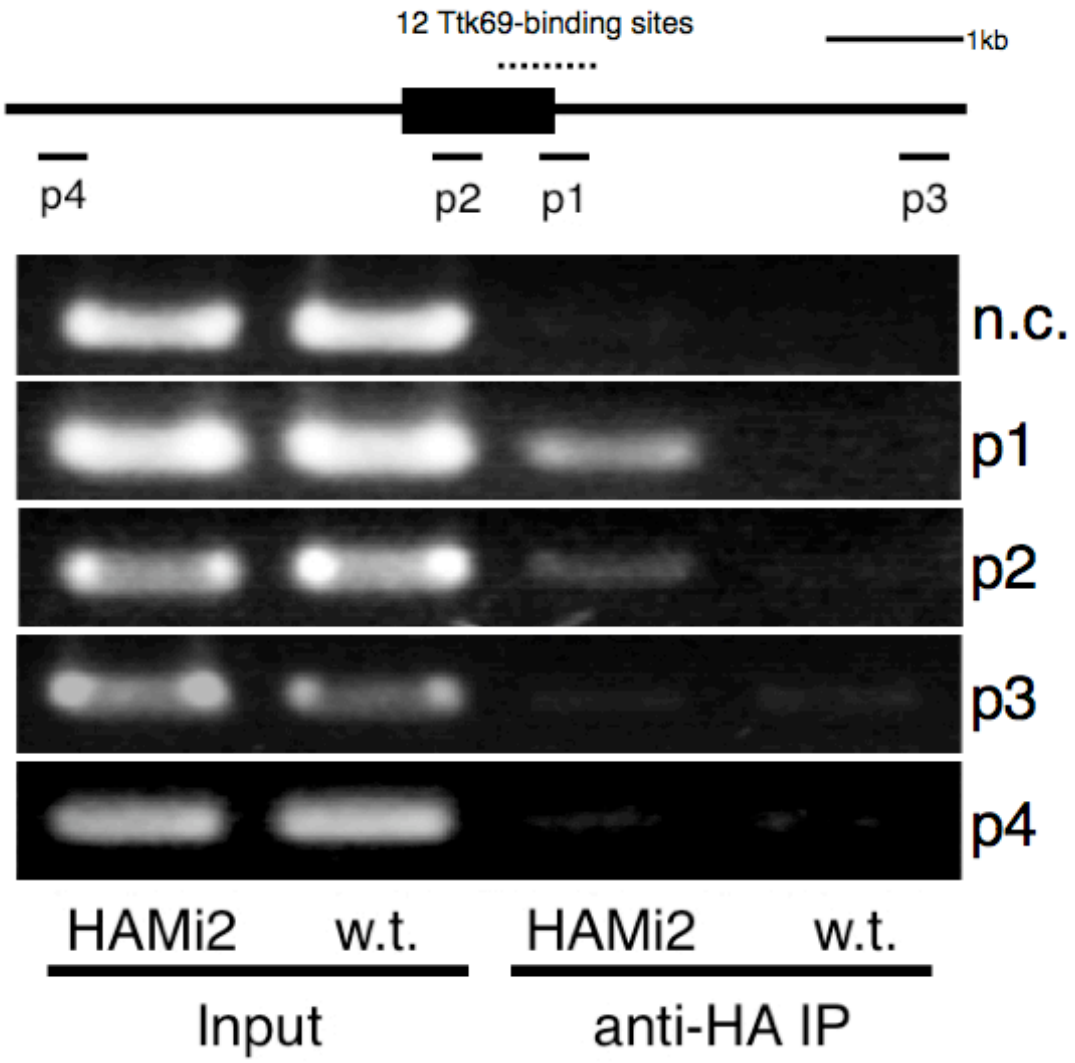


Fig. 3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p1
p3
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p1
p2
p3
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HAMi2
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anti-HA IP

Fig. 4

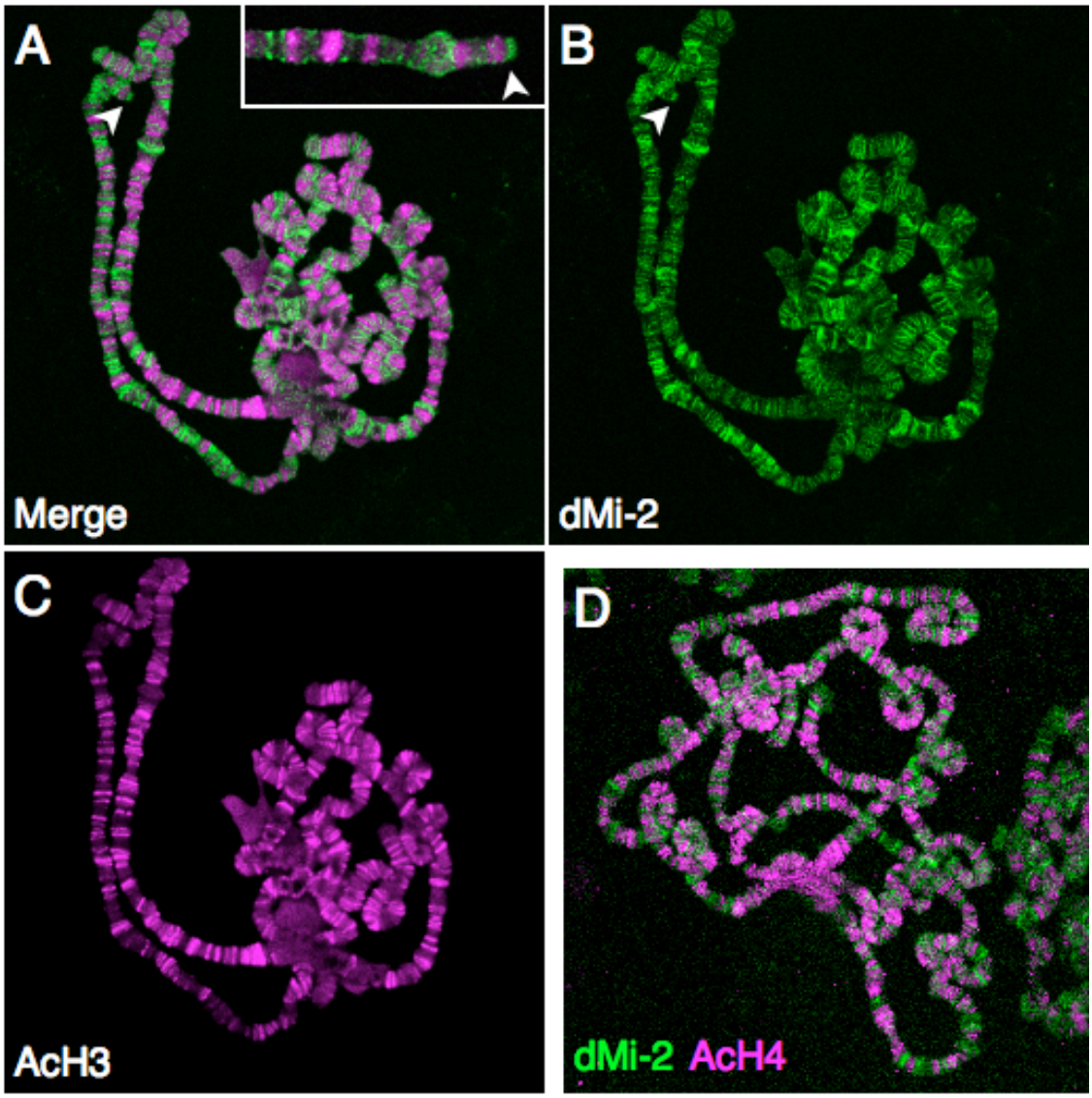

Fig. 5