dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression

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The ATP-dependent chromatin remodeller Mi-2 functions as a transcriptional repressor and contributes to the suppression of cell fates during development in several model organisms. Mi-2 is the ATPase subunit of the conserved Nucleosome Remodeling and Deacetylation (NuRD) complex, and transcriptional repression by Mi-2 is thought to be dependent on its associated histone deacetylase. Here, we have purified a novel dMi-2 complex from *Drosophila* that is distinct from dNuRD. dMec (dMEP-1 complex) is composed of dMi-2 and dMEP-1. dMec is a nucleosome-stimulated ATPase that is expressed in embryos, larval tissues and adult flies. Surprisingly, dMec is far more abundant than dNuRD and constitutes the major dMi-2-containing complex. Both dNuRD and dMec associate with proneural genes of the achaete–scute complex. However, despite lacking a histone deacetylase subunit, only dMec contributes to the repression of proneural genes. These results reveal an unexpected complexity in the composition and function of Mi-2 complexes.

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Introduction

ATP-dependent chromatin remodelling and histone modification are central processes underlying the dynamic regulation of chromatin structure. A classic example of a multisubunit protein complex combining ATP-dependent chromatin remodelling and histone modification activities is the Nucleosome Remodeling and Deacetylation (NuRD) complex. NuRD complexes have been purified from several vertebrates, and NuRD subunits are highly conserved in metazoans (Bowen *et al*, 2004). They include an Mi-2 nucleosome remodelling ATPase, one or two type I histone deacetylases, members of the *metastasis-associated* (MTA) and p66

protein families, the histone-binding proteins RbAp46 and RbAp48, and a subunit containing a *methylated DNA-binding domain* (MBD). NuRD complexes likely also exist in invertebrates. Mi-2 (dMi-2), MTA (dMTA), p66 (dp66), HDAC1 (dRPD3), RbAp48 (CAF1p55) and MBD (dMBD2/3) all have a homologue in *Drosophila*, many of which interact (Nan *et al*, 1998; Brehm *et al*, 2000; Ballestar *et al*, 2001; Marhold *et al*, 2004; Kon *et al*, 2005). However, dMi-2-containing complexes have so far not been biochemically purified from *Drosophila*.

Recombinant Mi-2 is sufficient to remodel nucleosomes *in vitro* (Brehm *et al*, 2000; Wang and Zhang, 2001; Bouazoune *et al*, 2002; Bouazoune and Brehm, 2005). The additional subunits present in NuRD complexes are believed to contribute to targeting of NuRD to its sites of action and to regulate its activity. NuRD is recruited to chromatin by interaction with methylated DNA or DNA-bound transcription factors, including BCL-6, FOG-1/GATA-1, Ikaros, Aiolos, Hunchback and *Tramtrack 69* (TTK69) (Kehle *et al*, 1998; Kim *et al*, 1999; Murawsky *et al*, 2001; Hong *et al*, 2005; Jaye *et al*, 2007; Sridharan and Smale, 2007). In addition, NuRD binds the N-terminus of histone H3 *in vitro*, and binding is disrupted by methylation of lysine residue number 4 (H3K4me3) (Zegerman *et al*, 2002).

Transcriptional repression of NuRD is thought to depend on its associated histone deacetylase activity. It has been proposed that the ATP-dependent chromatin remodelling activity provided by Mi-2 makes histone tails accessible for deacetylation by the HDAC subunits (Xue *et al*, 1998; Zhang *et al*, 1998).

Mi-2 is an important regulator of cell fate during development (Fujita *et al*, 2003, 2004; Kon *et al*, 2005). During lymphocyte development, NuRD cooperates with BCL6 to repress plasma cell-specific genes and so favours differentiation into B cells (Fujita *et al*, 2004). In *Drosophila*, dMi-2 and Hunchback cooperate in the repression of homeotic genes during embryogenesis (Kehle *et al*, 1998). In addition, dMi-2 cooperates with *Tramtrack 69* in the repression of neuronal cell fate, and it has been proposed that the dNuRD complex represses proneural genes (Murawsky *et al*, 2001; Yamasaki and Nishida, 2006).

In this study, we have biochemically purified dMi-2-associated proteins from *Drosophila*. We have discovered a novel, stable two-subunit dMi-2 complex containing the dMEP-1 protein. We have termed this complex dMec (*Drosophila* MEP-1-containing complex). dMi-2 and dMEP-1 are coexpressed during development and colocalise in embryonic nuclei. dMec is distinct from dNuRD and, surprisingly, constitutes the major dMi-2-containing complex in *Drosophila*. Recombinant dMec displays nucleosome-stimulated ATPase activity. dMec and dNuRD co-occupy promoters of proneural genes of the *achaete–scute complex* (AS-C). However, despite the lack of an associated histone deacetylase, only dMec contributes to their repression. These results reveal an unexpected complexity in the composition and function of Mi-2 complexes.

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Results

Purification of dMec

We fractionated nuclear extracts from Kc cells by ion exchange chromatography to purify dMi-2-associated proteins (Figure 1A). The peak dMi-2 fraction eluting off the third column (Resource Q) was subjected to immunoaffinity purification using a monoclonal dMi-2 antibody (Murawska *et al*, 2008). This resulted in the copurification of two polypeptides, which migrated with apparent molecular weights of 220 and 170 kDa, respectively, during SDS-PAGE (Figure 1B, lane 2). Peptide mass fingerprint analysis identified these two polypeptides as dMi-2 and dMEP-1 (encoded by *CG1244*), respectively (Supplementary Table 1). Proteins that were present in substoichiometric amounts also gave rise to tryptic peptides derived from dMi-2 and dMEP-1 and most likely represent degradation products. We generated a dMEP-1-specific monoclonal antibody to confirm the interaction of both proteins (see Materials and methods). Both dMi-2 and dMEP-1 copurified when anti-dMEP-1 antibody was used in the immunoaffinity purification step (Figure 1B, lane 1). We term this novel dMi-2 complex *dMec*.

We were surprised that no putative dNuRD subunits were associated with dMi-2 in our purification. We compared gel filtration profiles of crude and fractionated extracts to determine whether dNuRD subunits or other dMi-2-associated proteins had been lost during ion exchange fractionation (Figure 1C). Dissociation of dMi-2-associated proteins would be expected to result in a shift of the dMi-2 elution profile towards fractions corresponding to smaller molecular weights. However, when crude and fractionated extracts were applied to a Superose 6 column, in both cases dMi-2 and

dMEP-1 coeluted and peaked in fraction 22. We probed Superose 6 fractions from crude nuclear extract with antibodies directed against dMec and dNuRD subunits to determine whether both complexes are separated by gel filtration (Supplementary Figure 1). Elution peaks of dMi-2 (fraction 21), dMEP-1 (fraction 21) and the dNuRD-specific subunit dp66 (fractions 19 and 21) were largely overlapping. We also subjected recombinant dMec (see below) to gel filtration chromatography (Supplementary Figure 2). dMi-2 and dMEP-1 of recombinant dMec exhibited the same elution profile as native dMec. Taken together, these analyses suggest that dMec is present in nuclear extracts of Kc cells, and that no subunits have been lost during fractionation. Furthermore, despite their different molecular masses, dNuRD and dMec have very similar gel filtration properties.

dMec is a nucleosome-stimulated ATPase

We reconstituted dMec by coinfecting Sf9 cells with recombinant baculoviruses expressing dMi-2 and FLAG-tagged dMEP-1. Recombinant dMec was purified by anti-FLAG immunoaffinity purification. Both subunits were recovered in stoichiometric amounts, confirming a strong interaction of dMi-2 and dMEP-1 (Figure 2A). We then subjected recombinant dMec to ATPase assays (Figure 2B). Recombinant dMec displayed a basal ATPase activity that was not significantly increased in the presence of DNA. However, addition of nucleosomes resulted in a robust stimulation of ATPase activity. This property of dMec is shared by the dNuRD complex and suggests that dMec has ATP-dependent nucleosome remodeling activity (Brehm *et al*, 2000).

Figure 1 Purification of dMec. **(A)** Schematic representation of *dMEP-1*-containing complex (*dMec*) purification procedure. **(B)** The Resource Q dMi-2 peak fraction was immunopurified using anti-dMEP-1 and anti-dMi-2 antibodies, respectively, as indicated. Bound material was eluted with appropriate peptides and subjected to SDS-PAGE and colloidal Coomassie staining. Polypeptides identified by peptide mass fingerprinting are marked with solid black circles. Substoichiometric proteins were identified as degradation products of dMi-2 or dMEP-1 (Supplementary Table 1). SDS-PAGE size markers are shown on the right. dMEP-1 complex (*dMec*) subunits are indicated by arrows on the left. MW, molecular weight markers; IN, input. **(C)** Crude nuclear extracts of Kc cells (upper panel) and the Resource Q dMi-2 peak containing fraction (lower panel) were subjected to Superose 6 gel filtration. Fractions were analysed by western blot using specific antibodies as indicated on the right. Fraction numbers are denoted on top, size standards above. IN, input.

Figure 2 dMec is a nucleosome-stimulated ATPase. **(A)** Reconstitution of recombinant dMec. Extracts derived from baculovirus infected Sf9 cells expressing FLAG-tagged dMEP-1 (dMEP-1F) and untagged dMi-2 were immunopurified with anti-FLAG antibodies and eluted with FLAG peptide. The first two eluates (E1, E2) were subjected to SDS-PAGE and Coomassie staining. The position of dMi-2 and dMEP-1F are indicated on the left, and molecular weight markers are indicated on the right. **(B)** dMec has nucleosome-stimulated ATPase activity *in vitro*. dMec was incubated in the absence or presence of 100 ng DNA or nucleosomes (Nucl.) and 32 P-ATP as indicated. Percentage of hydrolysed ATP was determined by thin layer chromatography and quantified by phosphorimager analysis. Three independent experiments were performed using two different protein preparations; standard deviations are indicated by error bars. **(C)** Schematic representation of dMEP-1 mutants used (top panel). Zinc-finger domains are indicated by black bars, and a C-terminal FLAG-tag is indicated by a grey bar. Extracts derived from Sf9 cells expressing untagged dMi-2 and a FLAG-tagged dMEP-1 mutant (Δ C-dMEP-1, Δ N-dMEP-1 and Δ N Δ Zn-dMEP-1), as indicated on the top, were immunoprecipitated with anti-FLAG antibodies and analysed by western blot using anti-dMi-2 and anti-FLAG mouse antibodies, as indicated on the right. The calculated molecular mass of each mutant is given in brackets. Molecular weight markers are shown on the left. IN, input; IP, immunoprecipitate.

The dMi-2-binding domain of dMEP-1 resides in the N-terminus

dMEP-1 contains seven zinc fingers in its C-terminal half. The zinc fingers are arranged in two groups of three and four, respectively, and are separated by a glutamine-rich region (Figure 2C). The N-terminal half of dMEP-1 does not contain recognizable domains. We created three recombinant baculoviruses expressing FLAG-tagged dMEP-1 deletion mutants. Sf9 cells were coinfecting with baculoviruses expressing a dMEP-1 deletion mutant and untagged dMi-2, respectively. Extracts were then purified by anti-FLAG immunoprecipitation, and copurification of dMi-2 was assessed by western blot (Figure 2C). In the absence of dMEP-1 coexpression, dMi-2 was not detected in the immunoprecipitate (lane 2). Coexpression of a dMEP-1 fragment encompassing the N-terminal half of the protein (aa 1–679) resulted in efficient copurification of dMi-2 (lane 4). By contrast, coexpression of C-terminal dMEP-1 fragments containing all seven zinc fingers (aa 680–1152) or the three zinc fingers closest to the

C-terminus (aa 770–1152) did not result in the copurification of detectable levels of dMi-2 (lanes 6 and 8). We conclude that the zinc fingers are dispensable for dMi-2 binding, and that the dMi-2 interaction domain resides within the N-terminal half of dMEP-1.

dMec expression in embryos

We subjected extracts from staged embryos to western blot analysis to determine the temporal expression of dMec subunits during embryogenesis. dMi-2 and dMEP-1 displayed remarkably similar expression profiles. Both dMi-2 and dMEP-1 were present in early embryos; their levels increased during the first 9 h of development and sharply decreased thereafter (Figure 3A). We have previously shown by immunofluorescence staining that dMi-2 is expressed in the nuclei of early embryos and the syncytial blastoderm stage (Murawska *et al*, 2008). Likewise, we found dMEP-1 to be expressed in the nuclei of early embryos, including the pole cell nuclei and the syncytial blastoderm stage (Figure 3B,

Figure 3 dMec expression in embryos. **(A)** Protein extracts were prepared from embryos at different developmental stages as indicated (lanes 1–4) and analysed by western blotting using anti-dMEP-1 (top panels), anti-dMi-2 (middle panels) and anti-tubulin (bottom panels) antibodies. Molecular weights are indicated on the left. **(B)** Confocal micrographs of immunofluorescence-stained embryos. Top panel: early embryo; second to fourth panels: syncytial blastoderm stage embryos; third panel: interphase nuclei; bottom panel: metaphase nuclei. Embryos were stained with DAPI (first column) and anti-dMEP-1 antibody (second column, green). The third column shows overlays of DAPI and dMEP-1 stainings. Pole cells are indicated by arrows. Scale bars in the two top panels: 100 μ m; scale bars in the two bottom panels: 10 μ m. **(C)** Confocal micrographs of immunofluorescence-stained syncytial blastoderm embryos. Embryos were stained with DAPI (first column), anti-dMi-2 antibody (second column, red) and anti-dMEP-1 antibody (third column, green). The fourth column shows an overlay of dMi-2 and dMEP-1 signals. The second panel shows an enlargement of the micrographs in the first panel. Scale bars: 5 μ m. **(D)** Protein extracts were prepared from embryos at the developmental stages indicated and analysed by western blotting after immunoprecipitation with the indicated antibodies. Beads, antibody was omitted from precipitation; AED, after egg deposition.

upper panels). As we have previously observed for dMi-2, dMEP-1 staining was nuclear during interphase but became diffuse when the nuclei underwent mitosis (Figure 3B, lower panels; Murawska *et al* (2008)). This

indicates that both subunits of dMec lose interaction with chromatin during metaphase. Within nuclei, both dMi-2 and dMEP-1 were detected in partially overlapping speckles (Figure 3C).

The close temporal and spatial coexpression of dMi-2 and dMEP-1 during embryogenesis suggests that the dMec complex exists in embryos. To test this, we subjected extracts from staged embryos to coimmunoprecipitation using dMi-2- and dMEP-1-specific antibodies, respectively (Figure 3D). dMi-2 and dMEP-1 were efficiently coimmunoprecipitated from extracts of all stages, where expression of these proteins could be detected. These results strongly suggest that dMi-2 and dMEP-1 form a complex in *Drosophila* embryos.

dMec expression in larvae and adult flies

Although the overall levels of dMi-2 and dMEP-1 sharply decrease 9 h after egg deposition (Figure 3A), coexpression and interaction of both proteins can be detected in several larval tissues, including brains and wing imaginal discs (data not shown). Analysis of extracts from adult flies revealed that dMEP-1 is more strongly expressed in female flies compared with male flies (Figure 4A). We have previously shown that the same is true for dMi-2 (Murawska *et al*, 2008). Immunofluorescence analysis of ovaries showed that dMi-2 and dMEP-1 are strongly expressed in nurse cells and the germinal vesicle of the oocyte (Figure 4B). This provides a potential explanation for the differential expression of dMi-2 and dMEP-1 in both sexes. Coimmunoprecipitation experiments from ovary extracts confirmed that dMi-2 and dMEP-1 form a complex in this tissue (Figure 4C). These results suggest that both dMi-2 and dMEP-1 are expressed in ovaries and maternally contributed to the oocyte.

dMec is the major dMi-2-containing complex in *Drosophila*

Our purification of dMi-2-associated proteins from nuclear extracts did not result in the copurification of putative *Drosophila* NuRD subunits (Figure 1). Probing the eluates of the initial fractionation steps (Q-Sepharose FF, Biorex 70) with anti-dMi-2 and -dMEP-1 antibodies demonstrated that both proteins cofractionated quantitatively (Supplementary

Figure 4). To determine the fate of dNuRD subunits during fractionation, we used antibodies directed against the histone deacetylase dRPD3 and dp66. Western blotting of fractions eluting from the final Resource Q column revealed that the bulk of dMi-2 coeluted with dMEP-1 (Figure 5A, fraction 20). dRPD3 and dp66 peaked in an earlier fraction that contained only little dMi-2 but no detectable dMEP-1 (fraction 18). It is notable that the dRPD3/dp66 and the dMi-2/dMEP-1 peaks are only separated by two fractions. Indeed, prolonged exposure of western blots allowed the detection of dRPD3 in the dMi-2/dMEP-1 peak fraction (Supplementary Figure 3, lane 1). However, affinity purification of dMec from this fraction using either dMi-2 or dMEP-1 antibodies, according to the scheme shown in Figure 1A, did not result in copurification of dRPD3 (lanes 2 and 3). This shows that the low levels of dRPD3 that are present in the dMi-2/dMEP-1 peak fraction are not associated with dMec. These findings suggest that dNuRD and dMec are distinct complexes, the bulk of dMi-2 resides in dMec and only a minor fraction of dMi-2 is present in dNuRD.

To estimate the amount of dMi-2 associated with dMEP-1 in nuclear extracts derived from embryos, we performed serial immunodepletion experiments (Figure 5B). Successive depletion of extract with dMEP-1-specific antibody resulted in efficient codepletion of dMi-2 (lanes 5–7). Depletion with dMEP-1 antibody removed as much dMi-2 from the extract as depletion with dMi-2 antibody (compare lanes 2–4 with lanes 5–7). We performed this serial immunodepletion also with nuclear extracts from cell lines and obtained the same results (data not shown). These findings support the view that dMec is the major dMi-2-containing complex in *Drosophila*.

We used coimmunoprecipitations to further characterise the relation between dNuRD and dMec in nuclear extracts (Figure 5C). Antibodies directed against dp66 and dRPD3 coprecipitated dp66, dRPD3 and dMi-2 but not dMEP-1 (lanes 3 and 4). Anti-dMEP-1 antibody coprecipitated dMi-2 but not

Figure 4 dMec expression in ovaries. (A) Proteins extracts were immunoprecipitated with rat anti-dMEP-1 antibody and immunoprecipitates were subjected to western blotting using rabbit anti-dMEP-1 antibody. (B) Confocal micrographs of immunofluorescence-stained *Drosophila* ovaries. Ovaries were stained with DAPI (left panel), anti-dMi-2 antibody (second panel, red) and anti-dMEP-1 antibody (third panel, green). The fourth panel shows an overlay of dMi-2 and dMEP-1 staining. The germinal vesicle of the oocyte is indicated by an arrow. Scale bar: 50 μ m. (C) Protein extracts from *Drosophila* ovaries were immunoprecipitated with anti-dMEP-1 antibody and subjected to western blot analysis using anti-dMi-2 (top panel) and anti-dMEP-1 (bottom panel) antibodies. Beads, antibody was omitted from precipitation.

Figure 5 dMec is the major dMi-2-containing complex in *Drosophila*. (A) Kc cell nuclear extract was fractionated as shown in Figure 1A, and Resource Q column eluates were analysed by western blot using specific antibodies, as indicated on the right. Fraction numbers are shown on top. IN, input. (B) Serial immunodepletion experiments with anti-dMi-2 antibody (α-dMi-2 IP) or anti-dMEP-1 antibody (α-dMEP-1 IP) from *Drosophila* embryo nuclear extract. Supernatants from subsequent immunodepletions (s1, s2, s3) were subjected to western blotting using antibodies, as shown on the left. (C) Kc cell nuclear extract was immunoprecipitated with anti-dp66, -dRPD3, -dMi-2 and -dMEP-1 antibodies, as indicated on top. Immunoprecipitates were analysed by western blotting using antibodies indicated on the left. IP, immunoprecipitate; Beads, antibody was omitted from the precipitation.

the dNuRD subunits dp66 and dRPD3 (lane 6). Anti-dMi-2 antibody coprecipitated dMEP-1, dp66 and dRPD3 (lane 5). We conclude that dNuRD and dMec are distinct complexes that do not physically associate in soluble nuclear extracts.

dMec binds to proneural genes within the AS-C locus

dMi-2 and the transcriptional repressor Tramtrack 69 (TTK69) cooperate in the suppression of neuronal cell fate during development (Murawsky *et al*, 2001; Yamasaki and Nishida, 2006). TTK69 represses proneural genes of the AS-C in non-neuronal cells (Badenhorst *et al*, 2002). We used chromatin immunoprecipitation (ChIP) followed by end-point PCR to assess the association of dNuRD and dMec subunits with the four proneural genes of AS-C (*achaete* (*ac*), *scute* (*sc*), *lethal-of-scute* (*l'sc*) and *asense* (*ase*)) (Figure 6A). We also investigated binding to *pepsinogen-like* (*pcl*), a gene

that resides in the AS-C locus but has no function in neurogenesis (Badenhorst *et al*, 2002). dMi-2 and dp66 immunoprecipitates were enriched for promoter fragments of the four proneural genes as well as the *pcl* gene (lanes 2 and 4). By contrast, no binding of dMi-2 or dp66 to the unrelated *hsp70* promoter was detected (last panel). In addition, no association of dMi-2 and dp66 with a region located 1.3 kb downstream of the *ac* transcriptional start site (*ac-p1*) was detected.

dMEP-1 immunoprecipitates were enriched for promoter fragments derived from the four proneural genes *ac*, *sc*, *l'sc* and *ase* (lane 3). In contrast to dMi-2 and dp66, dMEP-1 did not show significant association with the *pcl* promoter. Furthermore, we failed to detect an interaction of dMEP-1 with the *hsp70* promoter and the region 1.3 kb downstream of the *ac* transcriptional start site.

Taken together, the analysis of ChIP by end-point PCR indicated a preferential association of dNuRD and dMec, with promoter sequences within the AS-C locus.

To refine this analysis, we also measured chromatin binding by ChIP followed by quantitative PCR (qPCR; Figure 6A). This allowed the detection of dMi-2, dMEP-1 and dp66 binding to control regions such as sequences downstream of transcriptional start sites that were not detected by end-point PCR (*sc-p2*; compare with anti-GST signals). Again, promoters of proneural genes displayed stronger association of dMi-2, dMEP-1 and dp66. In addition, preferential binding of dMi-2 and dp66 to the *pcl* promoter over the control region was detected. By contrast, the dMEP-1 association with the *pcl* promoter was not significantly greater than the dMEP-1 association with the control region.

In summary, ChIP followed by end-point or qPCR suggests a preferential association of dNuRD and dMec with gene promoters of the AS-C locus.

dMec contributes to repression of proneural genes

We depleted dMi-2, dMEP-1 and dp66 from SL2 cells by RNA interference (RNAi) to assess the contribution of dNuRD and dMec to transcriptional repression of the AS-C locus (Figure 6B). We noted that the depletion of dMi-2 led to a decrease of dMEP-1 levels and *vice versa* (lanes 3 and 6). This suggests that the stability of both proteins depends on their mutual association.

We used qRT-PCR to determine changes in gene expression following depletion of dMi-2, dMEP-1 and dp66. Transcription of the four proneural genes of the AS-C locus was increased from two- to four-fold following depletion of dMEP-1 or dMi-2 (Figure 6C). Depletion of dMi-2 and dMEP-1 affected gene transcription to similar extents, and codepletion of both proteins did not result in an additional increase of expression (Supplementary Figure 5). Reduction of dp66 levels did not result in significant changes in transcription of the four proneural genes. These results are consistent with the hypothesis that dMec—but not dNuRD—contributes to gene repression even though both complexes are bound to proneural gene promoters.

Transcription of the *pcl* gene was not significantly affected by depletion of dMi-2, dMEP-1 or dp66. This result confirms that depletion of the three factors did not result in an unspecific general increase in transcription. As was the case with the proneural genes, the presence of dNuRD at the *pcl*

Figure 6 dMec contributes to the repression of AS-C genes. **(A)** Top panel: schematic representation of the AS-C locus. Position of amplicons is denoted on top, genes are indicated by black boxes, the direction of transcription is shown with arrows (modified from Badenhors *et al*, 2002). Middle panels: *Drosophila* embryos were subjected to ChIP analysis using anti-dMi-2-specific, anti-dMEP-1-specific, anti-dp66-specific and anti-GST-specific (NS Ab, used as control antibody) antibodies, as shown on top. Specific primers were used to amplify sequences derived from the AS-C locus and the *hsp70* gene by end-point PCR, as indicated on the right. Bottom panels: Chromatin immunoprecipitated DNA was analysed in triplicate by qPCR with primers for promoter sequences of genes from the AS-C locus (from left to right: *ac*, *sc*, *l'sc*, *pcl*, *ase*) and an indicated control region (*sc*-p2). Mean values are expressed as percentage of input (% input). **(B)** Extracts from SL2 cells treated with different double-stranded RNAs (dsRNA), as indicated on top, were analysed by western blot using specific antibodies, as indicated on the left. Mock: untreated cells. **(C)** Total RNA isolated from SL2 cells shown in (B) (as indicated on the left) was analysed by qRT-PCR for expression levels of genes from the AS-C locus (from left to right: *ac*, *sc*, *l'sc*, *pcl*, *ase*). RNA levels determined for untreated samples (Mock) were set to 1. **(D)** Total RNA isolated from SL2 cells treated for 18 h with 1 μ M TSA (dissolved in DMSO) or control cells treated with DMSO, as indicated at the bottom, was analysed by qRT-PCR for expression levels of genes from the AS-C locus (from left to right: *l'sc*, *pcl*) and of the *CG8399* gene as a positive control for the effectiveness of the TSA treatment. RNA levels determined for DMSO treated samples (DMSO) were set to 1.

Figure 6 Continued.

promoter does not seem to make a major contribution to gene regulation.

HDAC inhibition does not lead to derepression of transcription

The lack of a histone deacetylase implies that dMec uses an HDAC-independent repression mechanism. To test this hypothesis, we treated SL2 cells with the histone deacetylase inhibitor trichostatin A (TSA) and measured changes in gene transcription by qPCR (Figure 6D). *CG8399* was used as a positive control, as this gene has been previously shown to be derepressed upon TSA treatment (Taylor-Harding *et al*, 2004). Indeed, transcription of *CG8399* was induced two-fold by TSA. By contrast, no derepression of the *AS-C* locus genes *l'* and *pcl* was detected. Both genes responded to TSA treatment with a decrease in transcription.

This result supports the notions that dNuRD does not have an important function in repressing proneural genes and that dMec uses an HDAC-independent repression mechanism.

Discussion

dMec is a novel dMi-2-containing complex

Mi-2 has been shown to reside within NuRD complexes and, although interactions between Mi-2 and other proteins have been reported, there has been no indication so far that Mi-2 also exists in other stable and abundant remodelling complexes (Bowen *et al*, 2004). Here, we provide evidence for a novel dMi-2-containing complex, dMec, which we have purified to apparent homogeneity from nuclear extracts of *Drosophila melanogaster*. dMec consists of two subunits: dMEP-1 and dMi-2. Unlike NuRD, dMec does not contain a histone deacetylase activity.

Previous studies had suggested that *Caenorhabditis elegans* MEP-1 binds both to an Mi-2 homologue (LET-418) and to an HDAC1 homologue (HDA-1) (Unhavaithaya *et al*, 2002). It was therefore hypothesised that MEP-1 associates with the NuRD complex in *C. elegans*. In contrast, our immunoprecipitations, depletions and chromatographic fractionations strongly suggest that dMec and dNuRD or dRPD3 do not physically associate in solution. Given that the dNuRD subunit dp66 and dMEP-1 co-occupy a number of promoters in the *AS-C* locus, it remains possible that dMec and dNuRD or dRPD3 interact when bound to chromatin. In addition, we cannot rule out that dMec and dNuRD or dRPD3 associate in contexts or cell types not investigated in this study. It is clear, however, that dMEP-1 is not a stable subunit of dNuRD. Rather, it forms a distinct complex with dMi-2 in nuclear extracts derived from *Drosophila* cell lines.

The tight physical association of dMi-2 and dMEP-1 is mirrored by their virtually identical temporal and spatial coexpression profiles during embryogenesis. The subunits of the dMec complex are detectable in early stages of embryogenesis before the onset of zygotic gene expression. The robust expression of dMi-2 and dMEP-1 in ovaries suggests that dMec is maternally contributed. dMi-2 and dMEP-1 levels increase during the first 9 h of embryogenesis and sharply decline thereafter. This suggests an important role of dMec during embryogenesis. Although the levels of dMec subunits in larvae are much lower than in embryos, we have detected robust interactions between dMi-2 and dMEP-1 in

extracts derived from larval tissues including larval brains and wing imaginal discs (data not shown).

dMec represses proneural genes

We were surprised to find that the bulk of dMi-2 resides in dMec and only a minor fraction is associated with dNuRD. This raises the possibility that phenotypes that have been observed in dMi-2 mutant flies are not a consequence of a defective dNuRD complex, but a result of a lack of dMec activity. Genetic analysis has demonstrated that dMi-2 and TTK69 cooperate to repress differentiation into neuronal cells during development of the *Drosophila* nervous system. TTK69 achieves this in part by repressing the proneural genes of the *AS-C* (Murawsky *et al*, 2001; Badenhorst *et al*, 2002). Yamasaki and Nishida have recently shown that dMi-2 and TTK69 colocalise to the *AS-C* locus on polytene chromosomes and that an ectopically expressed HA-tagged dMi-2 protein associates with the *ac* gene in wing imaginal discs (Yamasaki and Nishida, 2006). These experiments did not distinguish between dNuRD and dMec. Therefore, we asked whether endogenous dMec and/or dNuRD are binding to and are involved in repression of proneural genes. Our ChIP experiments show that both complexes localise to an overlapping set of genes within this locus. However, whereas dNuRD binds to promoters of proneural genes as well as the promoter of the unrelated *pcl* gene, dMec appears to bind preferentially to the proneural genes. We have recently identified dMi-2 and dMEP-1 in a screen for corepressors involved in SUMOylation-mediated transcriptional repression (Stielow *et al*, 2008). Both proteins can bind to the SUMO-group *in vitro* (Stielow *et al*, 2008). Given that TTK69 is SUMOylated *in vivo* and that SUMOylation is important for TTK69 function, it is conceivable that dMec is recruited to the *AS-C* locus by interacting with DNA-bound, SUMOylated TTK69 (Bajpe *et al*, 2008).

We note that the analysis of ChIP by quantitative PCR has also revealed an association of dMi-2, dMEP-1 and dp66 with non-promoter regions. It is conceivable that this is a reflection of a general, non-targeted association of these factors with chromatin. It is interesting to note that such a 'constitutive' association with chromatin has been demonstrated for mammalian Mi-2 (Li *et al*, 2002).

dNuRD is considered to contribute to transcriptional repression through its associated histone deacetylase activity. dMec lacks a histone deacetylase subunit. Nevertheless, depletion of dMEP-1 results in derepression of proneural genes of the *AS-C* locus. This suggests that dMec actively contributes to the repression of proneural genes in an HDAC-independent manner. Several findings suggest that dNuRD, although present at the promoters, does not have a significant function in maintaining proneural gene repression. First, depletion of dMi-2, which is expected to affect both dMec and dNuRD, does not result in stronger derepression than dMEP-1 depletion. Second, codepletion of dMEP-1 and dMi-2 does not produce stronger derepression effects than depletion of dMEP-1 alone. Third, reducing the levels of the dNuRD subunit dp66 does not alter proneural gene transcription. Fourth, TSA, which is expected to inhibit the dNuRD-associated HDAC activity, does not derepress transcription of proneural genes. Taken together, our findings suggest that dMec represses transcription in an HDAC-independent manner, although it remains possible that other (TSA-insensitive)

HDAC-containing complexes are involved. Consistent with this view, SUMOylation-mediated transcriptional repression depends on dMi-2 and dMEP-1 but does not require histone deacetylases and is insensitive to TSA (Stielow *et al*, 2008). It is likely that the ATP-dependent nucleosome remodelling activity of dMec is involved in transcriptional repression. In agreement with this hypothesis, dMec displays nucleosome-stimulated ATPase activity *in vitro*.

The identification of dMec raises the question of whether mammalian cells also contain Mi-2 complexes in addition to NuRD. On the basis of amino acid sequence comparison, it may be inferred that there is no obvious homologue of dMEP-1 encoded in mammalian genomes. Moreover, the region of dMEP-1 responsible for dMi-2 binding does not contain easily recognizable structure or sequence motifs. However, the region of dMi-2 that is bound by dMEP-1 is highly conserved in mammalian Mi-2 homologues (Klinker, Kunert and Brehm, unpublished data). This conservation of the dMEP-1-binding region suggests that mammalian cells might contain dMi-2-binding proteins with functional similarity to dMEP-1.

Several chromatin remodelling complexes can share a common ATPase subunit (Eberharter and Becker, 2004). ISWI ATPases are present in NoRC, ACF, CHRAC and NURF. Brahma is a component of both BAP and PBAP. The functional characteristics of each complex are determined by which subunits the ATPase is associated with. Our discovery of dMec extends this theme to the CHD family of ATP-dependent nucleosome remodellers and uncovers an unexpected complexity in the composition and function of Mi-2 complexes.

In the future, it will be important to determine the molecular mechanisms of transcriptional repression by dMec and dNuRD, to identify their respective sets of target genes, to elucidate their modes of recruitment and to analyse the relative contributions of dMec and dNuRD to neural development *in vivo*.

Materials and methods

Cell culture and HDAC-inhibitor assay

D. melanogaster SL2 and Kc cells were cultured at 25°C in Schneider's insect medium (Gibco) supplemented with 10% fetal bovine serum. SL2 cells (7×10^6) were plated into 60-mm dishes. After washing with PBS, fresh medium with 1 μ M TSA (Sigma, dissolved in DMSO) was added. Samples were harvested 18 h post-inhibitor treatment for total RNA isolation. Control cells were treated with DMSO (Carl Roth). *RP49* transcription, which was not affected by TSA treatment, was used as reference for normalisation.

Antibodies

Previously described antibodies include rabbit polyclonal anti-dMi-2 (Brehm *et al*, 2000), anti-dRPD3 (Brehm *et al*, 2000), anti-dp66 (Kon *et al*, 2005) and rat monoclonal anti-dMi-2 #4D8 (Murawska *et al*, 2008). For the generation of monoclonal dMEP-1 antibody, Lou/C rats were immunised with a dMEP-1-specific KLH-coupled peptide (Peptide Specialty Laboratories, Heidelberg, Germany: PSHKRQRSSTPSGMGC). dMEP-1-specific polyclonal antiserum was raised by standard immunisation of rabbits, with a mixture of three KLH-coupled peptides (Peptide Specialty Laboratories, Heidelberg, Germany: PSHKRQRSSTPSGMGC, CKMRQR-APQPPKQNIIVRNPA and CPASKPRITNMESHVID). Details are available upon request. Antibody specificity was verified by western blotting of extracts of cells treated with double-stranded RNA (dsRNA) directed against dMEP-1 (see Figure 6B).

Immunostaining of *Drosophila* embryos and ovaries

Immunostaining was performed as described previously (Rhyu *et al*, 1994). Briefly, wild-type Oregon R embryos were collected, washed, dechorionated, fixed and permeabilised. Fixed tissue was incubated with primary antibodies overnight at 4°C followed by incubation with secondary antibodies. After mounting, embryos were analysed by confocal laser scanning microscopy (TCS SP2, Leica Microsystems). For immunofluorescence staining, the following primary antibodies were used: polyclonal rabbit anti-dMEP-1 antibody (1:200), concentrated monoclonal rat anti-dMi-2 antibody (1:2). *Drosophila* ovaries were dissected from well-fed adult flies in PBS, ovarioles were separated, fixed for 15 min at room temperature in a 1:1 fixation solution of 5% paraformaldehyde and *n*-heptane, followed by treatment as used in embryo stainings. DNA was stained with DAPI (1 ng/ml, Molecular Probes).

Preparation of nuclear and whole cell extracts

Preparations of extracts from cultured *Drosophila* embryonic cells, *Drosophila* embryos and various stages of *Drosophila* development have been described elsewhere (Brehm *et al*, 2000; Murawska *et al*, 2008).

Fractionation of *Drosophila* Kc cell extracts by gel filtration

Crude nuclear extract from *Drosophila* Kc167 cells was applied to a Superose 6 gel filtration column (HR 10/30, GE Healthcare) and resolved in 10 mM Hepes pH 7.6, 300 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA and 10% glycerol on an Äkta purifier system (GE Healthcare) according to the manufacturer's instructions. Fractions were subjected to SDS-PAGE and processed for western analysis as described previously (Murawska *et al*, 2008).

Purification of dMi-2/dMEP-1 complex (dMec)

Nuclear extract was prepared from Kc cells as described above and dialysed against 2 l of buffer Q₁₀₀ (10% glycerol, 20 mM Tris pH 8.0, 100 mM KCl, 1 mM MgCl₂, 1 mM DTT, 0.2 mM PMSF, 1 mM sodium meta-bisulphite) for 4 h at 4°C. After centrifugation in a SS-34 rotor (15 min, 8000 r.p.m.), the supernatant was applied to 120 ml Q-Sepharose Fast Flow anion exchange resin (GE Healthcare) with Q₁₀₀ as running buffer. Proteins were eluted with Q₄₅₀ (see Q₁₀₀; 450 mM KCl) buffer and protein-containing eluates were combined. Presence of dMi-2 in the 450-mM KCl eluate was confirmed by western blot. The 450-mM eluate was dialysed against 4 l of buffer B₁₀₀ (10% glycerol, 25 mM Hepes pH 7.6, 100 mM KCl, 1 mM MgCl₂, 1 mM DTT, 0.2 mM PMSF, 1 mM sodium meta-bisulphite) for 4 h. The sample was then applied to a 25-ml Bio-Rex 70 cation exchange resin (Bio-Rad Laboratories) at a flow rate of 1 ml/min with B₁₀₀ as running buffer. Proteins were eluted by stepwise increasing the KCl concentration in the running buffer to 250, 500 and finally 1000 mM. dMi-2 eluted with the 500-mM fraction. The 500-mM eluate was dialysed against 4 l of buffer Q₁₀₀ for 4 h. The sample was applied to a 5-ml Resource Q anion exchange column (GE Healthcare) at 0.5 ml/min with Q₁₀₀ as running buffer. The column was resolved with a linear gradient from 100 to 500 mM KCl over 25 column volumes. Fractions of 3 ml were collected and fractions containing dMi-2 were identified by western blotting and pooled. dMi-2 was eluted in a single peak at a KCl concentration of approximately 370 mM. Pooled fractions were used for immunoprecipitation with anti-dMEP-1 or anti-dMi-2 antibody, respectively, and dMEP-1 complex (dMec) was eluted by adding the appropriate peptide (see Antibodies section). Eluted samples were analysed by western blot and Colloidal Coomassie (Invitrogen) staining. Bands were excised and analysed by peptide mass fingerprinting (Zentrum für Proteinanalytik, Adolf-Butenandt-Institut, Munich, Germany).

Immunoprecipitations and western blot analysis

Immunoprecipitations were carried out in PBS. Antisera (1 μ l of polyclonal antibody or 50 μ l of monoclonal antibody) were added to 50 μ l of nuclear extract and incubated for 3 h at 4°C. Protein G beads (GE Healthcare) were added and incubation was continued for 1 h. Protein G-antibody complexes were collected by centrifugation, washed three times in 1 ml of PBS, and resuspended in loading buffer for SDS-PAGE. For western blot analysis, immunoprecipitates and column fractions were separated by SDS-PAGE, transferred to PVDF membranes (Millipore) and probed with the relevant antibodies (mouse anti-FLAG antibody diluted 1:4000 from Sigma; polyclonal rabbit anti-dMEP-1 antibody 1:5000).

Expression and purification of recombinant proteins

dMEP-1 cDNA (BDGP clone RE60032) was used for generation of full-length FLAG-tagged dMEP-1 by PCR (primer sequences are available upon request). For baculovirus expression, the PCR product was cloned into pVL1392 (Invitrogen) and the construct was verified by sequencing. Recombinant dMi-2, baculovirus production and protein purification procedures have been described previously (Brehm *et al*, 2000).

Histone octamer purification, nucleosome assembly and ATPase assay

Histone octamers were isolated from 100 g dechorionated embryos as described elsewhere (Brehm *et al*, 2000). Polynucleosomes were assembled and ATPase assays were performed as described previously (Murawska *et al*, 2008).

ChIP

ChIPs were performed on chromatin prepared from 0 to 12 h AED mixed-sex Oregon-R wild-type embryos. ChIP was performed as described previously, using a CsCl-free protocol (Schwartz *et al*, 2005). Embryos were fixed in 4% formaldehyde at 18–20°C for 15 min. Previously described antibodies include rabbit polyclonal anti-dp66 (2 µl/IP; Kon *et al*, 2005) and rat monoclonal anti-GST antibody (200 µl/IP; Scharf *et al*, 2009). Monoclonal rat antibodies against dMi-2 and dMEP-1 (200 µl/IP) are described in this study. Following immunoprecipitation and reversal of cross-links, eluted DNA was purified with QIAquick columns (Qiagen) and subjected to gene-specific end-point PCR with the following primers: ac-p1for 5'-CTGTATACACAGGACACGCT-3', ac-p1rev 5'-GATCGATCGATCTC TCCGGA-3'; ac-p2for 5'-GCACGCGACAGGCCAG-G-3', ac-p2rev 5'-GTCCATTAAGGCCGAAGATGACT-3'; sc-p1for 5'-GAGCCCTCAC TCAGATACC-3', sc-p1rev 5'-CGTTTGACACCTTACACAAG-3'; sc-p2for 5'-CGACCATCAATTCGGCAACG-3', sc-p2rev 5'-CCAAAGTAGA CACACTGCGC-3'; l'sc-p1for 5'-GTAGGAATAGAGGCACCCAC-3', l'sc-p1rev 5'-GGTGATGCTGATGTTGCAGC-3'; pcl-p1for 5'-GGCAAGT TATCGCAGTGAGC-3', pcl-p1rev 5'-CTTTGTGGGACAACACAATG C-3'; ase-p1for 5'-GGCAAGGAACAGTTCTTCTAG-3', ase-p1rev 5'-G ATCCTTCCTCTCAGGTAGTTTC-3'; ase-p2for 5'-GGCAAGGAACAGT TCCTTCTAG-3', ase-p2rev 5'-CGTCCTTTGTGCTCTAGA-GG-3'; hsp70for 5'-TGCCAGAAAGAAAAGTTCGAGAAA-3', hsp70rev 5'-GACAGAGTGAGAGAGCAATAGTACAGAGA-3', or amplified by qPCR, which was performed using SybrGreen (Abgene) and the Mx3000P real-time detection system (Stratagene). Amplifications were performed in triplicate, and mean values were expressed as percentage input and compared with immunoprecipitations using

anti-GST antibody. Standard deviation was calculated from the triplicates, and error bars are indicated accordingly.

RNA interference in SL2 cells

dMEP-1, dMi-2 and dp66 dsRNAs were prepared as described previously using primers according to GenomeRNAi database (<http://www.dkfz.de/signaling2/rnai/>; Stielow *et al* (2008), primer sequences available upon request) and MEGAscript T7 High Yield Transcription Kit (Ambion) according to the manufacturer's instructions. 1×10^6 SL2 cells were incubated with 15 µg of dsRNA in serum-free medium for 40 min at 25°C. Complete medium with serum was added, and the cells were incubated for 4–5 days at 25°C. Knock-down was verified by western blot.

qRT-PCR

Total RNA from SL2 cells was isolated using the peqGold total RNA kit (PeqLab). One µg of RNA was applied to RT by incubation with 0.5 µg of oligo(dT)17 primer and 100 U of M-MLV reverse transcriptase (Invitrogen). cDNA was analysed by qPCR, which was performed using SybrGreen (ABgene) and the Mx3000P real-time detection system (Stratagene). Primers sequences are available upon request. All amplifications were performed in triplicates. Triplicate mean values were calculated according to the $\Delta\Delta CT$ quantification method (Pfaffl, 2001) using *GAPDH1* transcription as reference for normalisation. Standard deviation was calculated from triplicates, error bars are indicated accordingly. Relative mRNA levels in control SL2 cells were set to 1 and the other values were expressed relative to this.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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